STUDIES ON OXIDATIVE METABOLISM IN BIOLOGICAL SYSTEMS

A collection of research papers with
Introduction and Discussion submitted

by

Alan Marlow Snoswell, Ph.D. (Adel.)
(Department of Animal Sciences)

for the Degree of Doctor of Science
University of Adelaide

September 1983.

LIST OF CONTENTS

		Page No.
	STATEMENT	*i*
	ACKNOWLEDGEMENTS	*ii*
	INTRODUCTION	*iii*
	SUMMARY	*iv*
	DELINIATION OF MY CONTRIBUTION TO EACH RESEARCH PAPER	1
SECTION 1:	Papers related to the mechanism of oxidative phosphorylation in animal mitochondria	1
SECTION 2:	Papers related to oxidative systems in *Escherichia coli K.12*	2
SECTION 3:	Papers related to acetate and oxidative metabolism in animal systems	4
SECTION 4:	Papers related to carnitine and fatty acid oxidation in animal systems - mainly ruminant	5
SECTION 5:	Papers related to choline and fat metabolism in ruminant animals	12
	DISCUSSION OF THE SIGNIFICANCE OF THE MAIN FINDINGS RECORDED IN THE SUBMITTED PAPERS	14
SECTION 1:	Papers related to the mechanism of oxidative phosphorylation in animal mitochondria	14
SECTION 2:	Papers related to oxidative systems in *Escherichia coli K.12*	17
SECTION 3:	Papers related to acetate metabolism and oxidation in animal systems	18
SECTION 4:	Papers related to carnitine and fatty acid oxidation in animal systems - mainly ruminant	21
SECTION 5:	Papers related to choline and fat metabolism in ruminant animals	26
	BIBLIOGRAPHY	28

STATEMENT

The papers submitted here have not been submitted for any Degree in this or any other University.

A.M. SNOSWELL
September 1983.

ACKNOWLEDGEMENTS

I am indebted to the many students and colleagues who have worked with me over the years in the Physiological Chemistry Department of the University of Amsterdam (1960-61), the Biochemistry Department of the University of New South Wales (1961-64) and in the sub-Department of Veterinary Biochemistry of the University of Melbourne (1965-68). Also to colleagues in the Biochemistry Department of the University of Bristol, U.K. and the Biochemistry Department of the University of Cambridge, U.K., together with those in the Biochemistry Department of the ARC Institutes of Animal Physiology, Cambridge, U.K. and Research on Animal Diseases, Compton, U.K., where I spent study leave periods in both 1971 and 1977 respectively.

However, I am particularly indebted to my colleagues and students at the Waite Agricultural Research Institute of The University of Adelaide who have supported and encouraged me with their friendly enthusiasm and loyalty since July 1968. I would like in particular to acknowledge the help of my research assistant for the last 15 years, Mr. Richard Fishlock, who has always faithfully supported me and has particularly helped me with this submission.

Finally, the research work over the years would not have been possible without the devoted and loving support of my wife, Marie, and my four sons.

INTRODUCTION

The 35 research papers presented represent the majority of papers prepared for publication out of my research work between the years 1961 to 1983.

The work covered in the studies on oxidative metabolism in biological systems is presented in five areas:

1) Papers related to the mechanism of oxidative phosphorylation in animal mitochondria (papers 1 to 6).

2) Papers related to oxidative systems in *Escherichia coli K.12* (papers 7-9).

3) Papers related to acetate metabolism and oxidation in animal systems (papers 10 to 13).

4) Papers related to carnitine and fatty acid oxidation in animal systems - mainly ruminant (papers 14 to 32) i.e. by far the largest section

and finally

5) Papers related to choline and fat metabolism in ruminant animal (sheep) (papers 33 to 35).

In the sections that follow under each of these five areas I have endeavoured to set out the contribution to knowledge made by each paper, referring, where possible, to subsequent references to these papers by other workers, especially those in review articles or books. Finally I have indicated in each section the role I personally contributed to the work in each paper, especially in those papers (the majority) which have more than one author.

I have not attempted to provide an extensive background for the reader in each area as most of this is adequately covered in the papers themselves. Nor are the papers necessarily presented in chronological order of their publication in each area but are arranged in a manner, so far as is possible, to present a flow and development of my ideas and thinking over the years.

SUMMARY

Thirtyfive published papers are presented in five sections. Papers in section 1 are related to the mechanism of oxidation phosphorylation in animal mitochondria. The first five papers deal with the phenomenon of reversed electron transport. These detailed investigations showed for the first time that energy other than "high-energy phosphate compounds" produced in the terminal region of the respiratory chain could be used in the reduction of NAD^+. The sixth paper was one of the first published to provide experimental evidence to support the chemiosmotic theory of oxidative phosphorylation, which later became generally accepted.

Section 2 contains papers related to oxidative systems in *Escherichia coli*. These papers were some of the first to report the use of bacterial mutant strains in investigating the role of the newly-discovered compound, ubiquinone, in respiration. Ubiquinone was found to be a respiratory chain carrier in malate oxidation and probably functioned at more than one site in the respiratory chain.

The third section deals with acetate metabolism and oxidation in animal systems. Evidence is presented that in lactating ewes a significant amount of 'endogenous' acetate is produced by the liver, probably as a result of the incomplete oxidation of fatty acids. In contrast, important work with perfused rat livers (paper 11) showed that rat liver only produced acetate when the incoming concentration was below 0.25 μmole/ml but above that concentration acetate was an important hepatic substrate, in contrast to the ruminant situation. There was a direct competition between acetate and lactate for lipogenesis and oxidation. The concentration of acetate in portal blood of rats and pigs was similar to that in ruminants but with a 40% fractional extraction by the liver.

The main group of papers (section 4) are related to carnitine and fatty acid oxidation in animals, particularly ruminants. The main findings were that carnitine was actually produced by the liver in quite large amounts in severe stress states, such as diabetes and pregnancy toxaemia. This increased production of carnitine appears possible

because of reduced creatine synthesis. Carnitine played an important physiological role in buffering the tissue coenzyme A pool with very substantial amounts of acetyl carnitine accumulating, particularly in muscle tissues, in times of metabolic stress. Carnitine accumulated very rapidly in muscle tissues of young lambs following birth and this carnitine appeared to be derived from the milk. This transfer was diminished in cold stress. Carnitine found in the epidydymis of the male animal is apparently transferred from the blood stream in an active process and is concentrated some 2000-fold. Carnitine concentration of the luminal fluid rises rapidly in distal output epididymis where the sperm become mobile.

The final paper in this section reports the first case of a human patient maintained on long-term total parenteral nutrition being treated with intravenous L-carnitine to correct induced carnitine deficiency and general muscular weakness.

The final section presents papers on choline and fat metabolism in ruminant animals. One paper shows that the fatty liver condition that occurs in pregnancy toxaemia in sheep results in a significant shift of polyunsaturated fatty acid from phospholipids to triglycerides. These changes in turn probably affect cellular membranes and liver function. The last paper presents evidence that the bulk of choline in the sheep appears to be produced in extra hepatic tissues and that there is extensive retention and recycling of bile choline, which is in contrast to the situation in non-ruminant animals.

DELINIATION OF MY CONTRIBUTION TO EACH RESEARCH PAPER

SECTION 1

Papers related to the mechanism of oxidative phosphorylation in animal mitochondria

Paper No.1 **The mechanism of the reduction of mitochondrial DPN+ coupled with the oxidation of succinate**

A. M. SNOSWELL

This was the first paper I produced as a post-doctoral worker in Professor Slater's laboratory in Amsterdam. Professor Slater suggested the problem but I executed the research and wrote the paper. This was a preliminary version of the full paper (No.3) but was published quickly to coincide with the 5th International Congress of Biochemistry held in Moscow in July 1961, where I presented some of the data verbally in the discussion in a Symposium on Oxidative Phosphorylation.

Paper No.2 **The mechanism of the reduction of mitochondrial diphosphopyridine nucleotide by succinate in rabbit-heart sarcosomes**

E. C. SLATER J. M. TAGER A. M. SNOSWELL

This paper was produced with Professor Slater and another new post-doctoral student, Dr. Joseph Tager. Professor Slater and I combined to carry out the experiments in Figure I but the other experiments were carried out by Dr. Tager. We each contributed to writing the paper.

Paper No.3 THE REDUCTION OF DIPHOSPHOPYRIDINE NUCLEOTIDE OF RABBIT-HEART SARCOSOMES BY SUCCINATE

A. M. SNOSWELL

This was the main paper on my work in Amsterdam and I was the sole author. I received some guidance in both the experimental work and in the writing from Professor Slater.

Paper No.4

SYNTHESIS OF GLUTAMATE FROM α-OXOGLUTARATE AND AMMONIA IN RAT-LIVER MITOCHONDRIA

IV. REDUCTION OF NICOTINAMIDE NUCLEOTIDE COUPLED WITH THE AEROBIC OXIDATION OF TETRAMETHYL-*p*-PHENYLENEDIAMINE

J. M. TAGER, J. L. HOWLAND, E. C. SLATER AND A. M. SNOSWELL

The data tabulated in 3 of the 8 tables presented was the result of research work which I conducted at the University of New South Wales. Work recorded in the other tables was conducted by Drs. Tager and Howland in Amsterdam. Professor Slater combined all the data together and wrote the paper and we all contributed to the Discussion and concepts in the paper.

Paper No.5

Energy requirements for the reduction of mitochondrial NAD^+ by succinate: Externally added ATP as an energy source in aerobic systems

A. M. SNOSWELL

This was solely my own work carried out in the University of New South Wales.

Paper No.6

Respiration-Dependent Proton Movements in Rat Liver Mitochondria

Alan M. Snoswell

This was solely my own work carried out in the University of Melbourne. The concepts and approach to experimental work were developed entirely on my own as there was virtually no one in Melbourne at that time with whom I could discuss such work. I sent this paper to the then new journal, *BIOCHEMISTRY*, as one could nominate referees and I nominated Professor Britton Chance. The only significant change he recommended was a reduction in the discussion of the paper.

SECTION 2

Papers related to oxidative systems in *Escherichia coli K.12*

THE USE OF A UBIQUINONE-DEFICIENT MUTANT IN THE STUDY OF MALATE OXIDATION IN *ESCHERICHIA COLI*

Paper No.7

G. B. COX, A. M. SNOSWELL AND F. GIBSON

This paper arose out of an approach to me by Professor Frank

Gibson, then Professor of Microbiology in the University of Melbourne. Professor Gibson and his group had isolated a series of mutants of *E. coli K.12* which they had used to elucidate biochemical pathways for synthesis of a number of aromatic compounds. One of these compounds was ubiquinone, which had been suggested a year or so earlier, i.e. 1965, as component of the respiratory chain in a number of systems. Professor Gibson felt his mutants might be useful in elucidating the role of ubiquinone in the respiratory system of *E. coli* but he had little expertise in this area. I had had considerable experience in respiratory studies and in oxidative phosphorylation so we combined activities. At that stage Graham Cox was a Ph.D. student with Professor Gibson and they prepared the particles from the mutants in the Microbiology Department of the University of Melbourne and brought them across to me in the sub-Department of Veterinary Biochemistry and I carried out the respiratory studies in my oxygen electrode system. I also measured the reduction of ubiquinone in the particles. We all combined in writing the paper.

Paper No.8 **Piericidin A and inhibition of respiratory chain activity in *Escherichia coli* K12**

A. M. Snoswell G. B. Cox

The work in this paper was largely carried out on my initiative with Graham Cox, who had then completed his Ph.D, and had moved to the Australian National University with Professor Gibson, and who provided the particle preparations from the *E. coli*-K-12 mutants.

Paper No.9 **The Function of Ubiquinone in *Escherichia coli***

G. B. COX, N. A. NEWTON, F. GIBSON, A. M. SNOSWELL AND J. A. HAMILTON

The bulk of the research work in this paper was carried out by Professor Gibson and colleagues in the Australian National University. I contributed the data in Table 7 by working on particle preparations which were airfreighted from Canberra to me in Adelaide. I also made some minor contributions to the discussion and the writing of the paper.

SECTION 3

Papers related to acetate and oxidative metabolism in animal systems

Paper No.10 **Production of Endogenous Acetate by the Liver in Lactating Ewes**

N. D. Costa , G. H. McIntosh and A. M. Snoswell

The work in this paper was essentially carried out at my initiative and under direction. Mr. Nick Costa was a Ph.D. student and the work was a progression from work on the possible enzymic mechanism of acetate formation outlined in papers 21 and 22. In this paper the other co-author, Dr. Graham McIntosh, was a veterinary surgeon in CSIRO who carried out the surgery inserting the indwelling arterial and portal and hepatic venous cannulae in the sheep. He also supervised the measurement of hepatic blood flows. I prepared the manuscript of the paper.

Paper No.11

METABOLIC EFFECTS OF ACETATE IN PERFUSED RAT LIVER

STUDIES ON KETOGENESIS, GLUCOSE OUTPUT, LACTATE UPTAKE AND LIPOGENESIS

ALAN M. SNOSWELL , RODNEY P. TRIMBLE , RICHARD C. FISHLOCK , GERALD B. STORER and DAVID L. TOPPING

This is the major paper in the acetate section and one for which we received over 400 reprint requests. The work was carried out at my initiative and suggestion.

Dr. David Topping, a senior research scientist, in the CSIRO Division of Human Nutrition had a very useful recirculating rat-liver perfusion system using whole blood. His was one of the very few laboratories in the world using such a physiological preparation. I persuaded Dr. Topping to modify this system to a non-recirculating system in order to study acetate metabolism, as it was known from the work of others that the liver both produced and utilized acetate. This collaborative work showed us the importance of acetate metabolism in non-ruminant systems, something we had never considered,and 'sparked off' a series of papers on aspects of acetate metabolism in which we collaborated.

In regard to the other co-authors in this paper, Mr.Rodney Trimble and Mr. Gerald Storer are experimental officers in CSIRO in Dr. Topping's group and they looked after the liver perfusion system and assayed a number of the metabolites. Mr. Richard Fishlock is my

technical officer and assisted me with the acetate and lactate assays.

Paper No.12

CARBOXYHAEMOGLOBIN INHIBITS THE METABOLISM OF ETHANOL
BY PERFUSED RAT LIVER

David L. Topping, Richard C. Fishlock, Rodney P. Trimble,
Gerald B. Storer, and Alan M. Snoswell

The same co-authors as in the previous paper. These experiments were largely carried out under Dr. David Topping's initiative and direction, although I had inputs regarding the role of acetate and its measurement. I combined with Dr. Topping in writing the paper.

Paper No.13

DAILY VARIATIONS IN THE CONCENTRATIONS OF VOLATILE FATTY ACIDS
IN THE SPLANCHNIC BLOOD VESSELS OF RATS
FED DIETS HIGH IN PECTIN AND BRAN

R.J. Illman, R.P. Trimble, A.M. Snoswell, and D.L. Topping

Co-authors similar to the previous papers but with the addition of Mr. Rick Illman an experimental officer in Dr. David Topping's group who carried out the gas chromatographic assays of the volatile fatty acids. This paper was largely carried out on Dr. Topping's initiative but again following my stimulus with an interest in acetate metabolism. I assisted Dr.Topping in preparing the manuscript.

SECTION 4

Papers related to carnitine and fatty acid oxidation in animal systems - mainly ruminant

Paper No.14

Ketone Body and Fatty Acid Metabolism in Sheep Tissues
3-HYDROXYBUTYRATE DEHYDROGENASE, A CYTOPLASMIC ENZYME IN
SHEEP LIVER AND KIDNEY

PATRICIA P. KOUNDAKJIAN AND A. M. SNOSWELL

The work recorded in this paper was essentially carried out at my initiative and under my supervision. The other co-author, Mrs. Patricia Koundakjian, was a Ph.D. student working under my supervision and this was her first paper so that although she actually wrote the paper I played a very large part in preparing the manuscript.

Paper No.15 **Aspects of Carnitine Ester Metabolism in Sheep Liver**

A. M. SNOSWELL AND G. D. HENDERSON

The work in this paper was very much carried out under my direction and supervision. The other co-author, Mr. Graham Henderson had been an Honours student for part of the preceeding year before going into the Army for National Service training. I prepared the manuscript.

Paper No.16

Relationships between Carnitine and Coenzyme A Esters in Tissues of Normal and Alloxan-Diabetic Sheep

A. M. SNOSWELL and PATRICIA P. KOUNDAKJIAN

This was very much my own work for although my co-author Mrs. Patricia Koundakjian was a Ph.D. student working under my supervision I personally conducted a significant part of research work in the University of Bristol while on study leave in 1971. This was because the manuscript as first submitted required extensive revision and extra experimental work.

Paper No.17 **3-Hydroxy Acid Dehydrogenases in Sheep Tissues**

PATRICIA P. KOUNDAKJIAN and A. M. SNOSWELL

Again the co-authors were myself and my Ph.D. student Mrs. Patricia Koundakjian. The work was essentially carried out by Mrs. Koundakjian under my supervision as the result of a suggestion I personally received from Dr. Derek Williamson in Oxford, following the publishing of our first paper - No.14. Mrs. Koundakjian prepared the manuscript with relatively little guidance from me.

Paper No.18 **Effects of a Glucocorticoid on the Concentrations of CoA and Carnitine Esters and on Redox State in Bovine Liver**

G. David BAIRD, Raymond J. HEITZMAN, and Alan M. SNOSWELL

This paper was the result of collaborative research with Drs. David Baird and Ray Heitzman who were Senior Research Scientists in the ARC Institute at Compton, U.K. During my study leave period at the University of Bristol in 1971 I visited their laboratory and we discussed the collaborative work. I subequently took samples back to Bristol for carnitine and coenzyme A assays. We each contributed in preparing the manuscript.

Paper No.19 **The Liver as the Site of Carnitine Biosynthesis in Sheep with Alloxan-induced Diabetes**

A. M. Snoswell and G. H. McIntosh

The work in this paper was initiated and largely executed by me. My co-author Dr. Graham McIntosh was the veterinary surgeon in CSIRO with whom I also collaborated on acetate metabolism (see paper 10). Again he prepared and maintained the surgically cannulated sheep. I prepared the manuscript with some assistance from Dr. McIntosh.

Paper No.20

Carnitine secretion into milk of ruminants

A. M. SNOSWELL AND J. L. LINZELL

This work was the direct result of collaborative research between myself and the late Dr. Jim Linzell, then Head of the Physiology Department at the ARC Institute of Animal Physiology at Cambridge. Dr. Linzell was a world authority on lactation and it was during my visit to his laboratory late in 1971 that I stimulated his interest in carnitine secretion in milk. As a result of that visit I took samples back to the University of Bristol, where I was based while on study leave, for carnitine assays. The results were so stimulating that Dr. Linzell arranged for several more lots of samples to be airfreighted out to me in Adelaide, Australia, over the next year for assay. Then, in mid-1973, I spent 3 days in his laboratory while en route to the 9th International Congress of Biochemistry in Stockholm. I received a grant from the Wellcome Trust to support this work and took the collected samples back to Australia with me. Other samples I collected at the Dairy Research Farms at Werribee in Victoria and also at our own experimental station at Mortlock, S.A. In mid-1974 Dr. Linzell came to Sydney, Australia, for the 3rd International Meeting on Ruminant Physiology. At that meeting we worked together on the manuscript of this paper. This was an exciting piece of collaborative work which was finally published in 1975. I felt privileged to have been one of the last research workers to have worked with Dr. Linzell for he died suddenly some months later.

Paper No.21 **Enzymic Hydrolysis of Acetylcarnitine in Liver from Rats, Sheep and Cows**

N. D. COSTA and A. M. SNOSWELL

The work described in this paper was very much carried out at my initiative and under my supervision by Mr. Nick Costa during the first year of his Ph.D. work.

Paper No.22 **Acetyl-Coenzyme A Hydrolase, an Artifact?**

THE CONVERSION OF ACETYL-COENZYME A INTO ACETATE BY THE COMBINED ACTION OF CARNITINE ACETYLTRANSFERASE AND ACETYLCARNITINE HYDROLASE

N. D. COSTA and A. M. SNOSWELL

The same comments as for paper 21 apply.

Paper No.23 **Deacylation of Acetyl-Coenzyme A and Acetylcarnitine by Liver Preparations**

ALAN M. SNOSWELL* and PHILIP K. TUBBS

The work described in this paper was all executed by me personally while on study leave in Dr. Phillip Tubb's laboratory in Cambridge in 1977. Dr. Tubbs provided the facilities and we wrote the manuscript together.

Paper No.24

Interrelationships between acetylation and the disposal of acetyl groups in the livers of dairy cows

ALAN M. SNOSWELL, NICHOLAS D. COSTA, JOCK G. McLEAN

G. DAVID BAIRD, MICHAEL A. LOMAX[2] AND HERBERT W. SYMONDS

This paper was prepared on my initiative. During the latter part of 1977 while I was on study leave at the ARC Institute of Animal Physiology at Cambridge I also visited the ARC Institute at Compton to visit my old colleague and friend Dr. David Baird. During that visit I suggested to Dr. Baird that we could combine work which we had done on dairy cows some years earlier with some of their work to make a useful publication. I agreed to initiate this project and put together, in manuscript form, data which myself and Mr. Nick Costa, my Ph.D. student, had obtained in collaboration with a former Ph.D. student of mine, Dr. Jock McLean. Dr. McLean was then Lecturer in Veterinary Biochemistry in the University of Melbourne. He arranged for us to take liver biopsies from

cows in the herd at the State Research Farm at Werribee in Victoria. Mr. Nick Costa and I travelled to Melbourne for the sampling and processing. The other English authors in this paper were Mr. Michael Lomax, then a Ph.D. student working with Dr. David Baird, who carried out most of the assays in England, and Dr. Herbert Symonds, the Veterinary Surgeon who prepared the surgically cannulated dairy cows at Compton.

Paper No.25

Effect of Cold Exposure on Mammary Gland Uptake of Fat Precursors and Secretion of Milk Fat and Carnitine in the Goat

E. M. THOMSON, A. M. SNOSWELL, P. L. CLARKE and G. E. THOMPSON

The collaborative work recorded in this paper was initiated during my visit to the Hannah Research Institute in Ayr in Scotland while I was on study leave during the latter part of 1977. I persuaded Dr. Gordon Thompson, Senior Research Scientist, that studies on carnitine would significantly extend their work on the effects of cold stress on lipid metabolism in goats. Samples were subsequently sent to me at the ARC Institute of Animal Physiology, Cambridge for carnitine assays. The bulk of the work recorded in this paper on other aspects of lipid metabolism was carried out by Mr. E. Thomson and Mr. P. Clarke, a Ph.D. student and technician, respectively, working with Dr. Thompson at the Hannah Institute. Dr. Thompson prepared the manuscript with inputs from me.

Paper No.26

The concentration of carnitine in the luminal fluid of the testis and epididymis of the rat and some other mammals

B. T. Hinton, A. M. Snoswell and B. P. Setchell

The work in this paper was undertaken while I was on study leave at the ARC Institute of Animal Physiology, Cambridge in the latter half of 1977. Mr. Barry Hinton was working on a Ph.D. project under the supervision of Dr. Brian Setchell. They were studying epididymal function and were anxious to extend this work into the carnitine area and I arrived in the laboratory at the opportune time. I personally adapted the carnitine assay to accomodate the tiny samples of epididymal fluid obtained by micropuncture. I taught Mr. Hinton the technique and he was able to complete subsequent assays. We all combined in preparing the manuscript.

Paper No.27

KINETICS OF CARNITINE UPTAKE BY RAT EPIDIDYMAL CELLS

Androgen-dependence and lack of stereospecificity

M.J. JAMES, D.E. BROOKS and A.M. SNOSWELL

This paper arose out of research in a joint ARGC project in which myself and Dr. David Brooks, who is an expert in the male reproductive system, were joint investigators. Mr. Michael James was a research assistant we employed on the project. We all combined in preparing the manuscript.

Paper No.28

CARNITINE ESTERS AND CARNITINE ACYLTRANSFERASE ACTIVITY IN NORMAL AND ALLOXAN-DIABETIC SHEEP

R. C. Fishlock A. M. Snoswell K. Valkner and L. L. Bieber

This paper arose out of an approach to me by Dr. Loran Bieber from Michigan State University, U.S.A. He had read some of our earlier papers and suggested some joint work on diabetic sheep. Mr. Richard Fishlock, my Technical Officer, and I ran the experiments with the diabetic sheep here in Adelaide to my design. Tissue samples were obtained, partly processed and then freeze-dried and airfreighted to Michigan for gas chromatographic assay by Dr. Bieber and his assistant, Miss Kim Valkner. We carried out all the enzyme assays and I wrote the manuscript which was sent to Dr. Bieber in Michigan before submission.

Paper No.29

CARNITINE AND METABOLISM IN RUMINANT ANIMALS

A. M. Snoswell G. D. Henderson

This is the major paper in the carnitine area and is essentially a review of most of our own work plus the work of others in the ruminant area. It is an extended version of an invited paper which I presented verbally at the international symposium on Carnitine Metabolism held in Dallas, Texas in April 1979. The proceedings of the symposium were published in book form a year later. I prepared the manuscript and included my former Ph.D. student, Mr. Graham Henderson, as a co-author as I included some results which he had obtained and which had not previously been published.

Paper No.30

CARNITINE AND CREATINE CONTENT OF TISSUES OF NORMAL AND ALLOXAN-DIABETIC SHEEP AND RATS

GRAHAM D. HENDERSON, GANG-PING XUE and ALAN M. SNOSWELL

All the work in this paper was carried out on my initiative and under my supervision. Mr. Graham Henderson, a former Ph.D. student of mine returned to do the work as an unpaid postdoctoral student when the employment situation was very difficult. Mr. Gang-Ping Xue is a Chinese student currently working in my laboratory undergoing research training. Although he only carried out a very small proportion of the research work recorded I gave him the task of writing the manuscript which he completed very well, but of course with a good deal of guidance.

Paper No.31

ACUTE EFFECTS OF GLUCAGON ON FATTY ACID METABOLISM AND ENZYMES OF GLYCEROLIPID SYNTHESIS IN PERFUSED RAT LIVER

E. David Saggerson, Alan M. Snoswell, Rodney P. Trimble, Richard J. Illman and David L. Topping

This paper was essentially a collaborative piece of work between Dr. David Saggerson of University College London, Dr. David Topping of CSIRO Division of Human Nutrition and myself. We had arranged for Dr. Saggerson to spend one month working in the CSIRO Division here in Adelaide prior to the Specialist Conference on 'Lipid Metabolism in Health and Disease' held here in Adelaide in May 1981. I was the chairman of the Organizing Committee for that meeting and Dr. David Topping was the Secretary and Dr.David Saggerson was one of our 13 invited overseas speakers. I personally carried out all the assays recorded in one of the two tables in the paper. All three senior authors contributed in writing the manuscript. The other two co-authors on the paper, Mr. Rodney Trimble and Mr. Richard Ilman, were experimental officers in Dr.Topping's group who assisted in the experimental procedures.

Paper No.32

Carnitine Deficiency with Hyperbilirubinemia, Generalized Skeletal Muscle Weakness and Reactive Hypoglycemia in a Patient on Long-term Total Parenteral Nutrition: Treatment with Intravenous L-Carnitine

L. I. G. WORTHLEY, R. C. FISHLOCK, AND A. M. SNOSWELL

The work in this paper arose out of an initial approach to me by Dr. Lindsay Worthley, Senior Staff Specialist in Intensive Care at the Royal Adelaide Hospital. He approached me as an 'expert' in the carnitine area to enlist my help with patients on long-term total parenteral

nutrition. I designed the experimental protocols and Dr. Worthley supervized their clinical execution. We both combined in preparing the manuscript. Mr. Richard Fishlock, the other co-author, was my technical officer who carried out most of the actual carnitine assays.

SECTION 5

Papers related to choline and fat metabolism in ruminant animals

Paper No.33 The Low Availability of Dietary Choline for the Nutrition of the Sheep

A. R. NEILL, D. W. GRIME, A. M. SNOSWELL, A. J. NORTHROP, D. B. LINDSAY and R. M. C. DAWSON

The work reported in this paper was carried out while I was on study leave at the ARC Institute of Animal Physiology, Babraham, Cambridge in the latter part of 1977. It was part of an ongoing study on choline metabolism conducted by the research group leader, Dr. R.M.C. Dawson, Head of the Biochemistry Department. I had a number of interests in choline metabolism from our previous work in Adelaide and I arrived at Babraham at a most opportune time. A considerable part of the work reported in this paper had already been conducted by Dr. A. Neill and Dr. Dawson when I arrived.

I proceeded to get a sensitive enzymic assay for choline going as this was a vital part of the balance studies on choline metabolism. Dr. D. Lindsay and Mr. A. Nortrop, his technician, carried out the whole animal isotopic studies and the other co-author, Mr. D. Grime, was the technician who assisted Dr. Niell and myself with assays. Dr. Dawson prepared the manuscript with inputs from all the senior authors.

Paper No.34 STUDIES OF LIVER LIPIDS IN NORMAL, ALLOXAN-DIABETIC AND PREGNANCY-TOXAEMIC SHEEP

GRAHAM D. HENDERSON, LEANNA C. READ and ALAN M. SNOSWELL

This piece of research work was undertaken on my initiative and under my supervision. Some of the experimental work was carried out by Mr. G. Henderson, a Ph.D. student who had worked with me some years earlier. The other co-author Miss L. Read was an Honours student who had worked under my supervision some years earlier. I prepared the manuscript myself and personally carried out some additional experiments.

Paper No.35 UPTAKE AND OUTPUT OF VARIOUS FORMS OF CHOLINE BY ORGANS OF THE CONSCIOUS CHRONICALLY CATHETERISED SHEEP

Brenton S. Robinson, Alan M. Snoswell, William B. Runciman and Richard N. Upton

This final paper was again very much a result of my own initiative. The experimental work was undertaken mainly by Mr. B. Robinson a Ph.D. student working under my supervision. However he needed a good deal of close guidance, both in the work and in writing of the manuscript. The work was carried out as part of a collaborative project with Dr. B. Runciman and Mr. M. Upton, his Masters Degree student, in the Department of Anaesthesia and Intensive Care at Flinders University. They prepared the chronically cannulated sheep for their drug studies and also provided access to the sheep for our work. I arranged the supply of sheep from the Waite Institute and particularly the ability to select Merino wethers with A-group haemoglobin (nearest to the human type) from our large flock of Merinos at our Martindale Station. Dr. Runciman suggested a number of additional stimulating experimental approaches during this work.

DISCUSSION OF THE SIGNIFICANCE OF THE MAIN FINDINGS RECORDED IN THE SUBMITTED PAPERS

SECTION 1

Papers related to the mechanism of oxidative phosphorylation in animal mitochondria

The first five papers in this section may be treated collectively as they all deal with the phenomenon of reversed electron transport in animal mitochondria.

It was CHANCE (1956) who first made the observation that mitochondrial NAD^+ (or DPN^+ as it was then called) was reduced to a greater extent during succinate oxidation, which involved a flavo-protein dehydrogenase, than during the oxidation of NAD^+-linked substrates. This rather paradoxical observation was later suggested by CHANCE and HOLLUNGER (1961) to be due to succinate oxidation providing energy necessary to reverse electron flow in the respiratory chain leading to the reduction of mitrochondrial NAD^+ by reduced flavo-protein, also produced during succinate oxidation. This rather radical view (for that time) was strongly challenged by the KREB'S group (e.g. KREBS, EGGLESTON and D'ALESSANDRO, 1961) who suggested a 'switch' or 'malate theory'. They suggested that the rapid flow of electrons from succinate via the cytochrome system to oxygen effectively blocked the oxidation of NADH (or DPNH), at the same time producing malate which reduced the NAD^+.

When I first arrived in Amsterdam, in the early 1960s, as a young postdoctoral worker, Professor Slater suggested this reduction of NAD^+ by succinate was a 'hot problem' and required investigation by an independant laboratory and the first five papers in this section were addressed to this problem.

The first paper was a preliminary paper and the main points established were that the reduction of NAD^+ by succinate was significantly inhibited by amytal, a respiratory chain inhibitor at the flavo-protein level, thus ruling out the 'switch' mechanism and the reduction was not inhibited by oligomycin, a inhibitor of phosphorylation reactions. These obsevations were greatly extended in the third paper, which was the main paper to arise out of this work in Amsterdam. The data recorded in this paper also clearly showed that the reduction of mitochondrial NAD^+ by succinate was

not due to the presence of oxidizable endogenous substrate and proceeded even in the absence of inorganic phosphate. This last observation was particularly critical as combined with the lack of inhibition by oligomycin clearly indicated that the reversed electron transport did not actually require the formation of ATP or a 'high energy' phosphorus compound. In a later review by SLATER (1966) it is acknowledged that ERNSTER (1961) and myself independently established this important point.

The reduction of mitochondrial NAD^+ by succinate was linked to reduction of α-ketoglutarate and ammonia to glutamate in the presence of arsenite (to prevent α-ketoglutarate oxidation) in papers 2 and 4. Also in paper 4 we established that the oxidation of tetramethyl-p-phenylenediamine (which donated electrons at the cytochrome c level of the respiratory chain) also led to a reduction of mitrochondrial NAD^+ by reversed electron transport.

This series of papers, together with those from other laboratories, convinced most people that reversed electron transport was a real phenomenon. Professor KREBS remained sceptical for some years until GRIFFITHS and ROBERTON (1966) in his own laboratory showed that due to the stereo-specificity of the labelling of NAD^+ reduced by succinate the NAD^+ was reduced by flavo-protein dehydrogenase and <u>not</u> an NAD^+-linked dehydrogenase. This finally proved conclusively that the early workers, such as CHANCE, ERNSTER and myself as reviewed by BRODIE and GUTNICK (1972), had been correct in ascribing the reduction of NAD^+ by succinate to reversed electron transport. Possibly, in retrospect, one of the main reasons why the phenomenon of reversed electron transport in the mitochondrial respiratory chain was not accepted by everyone was that there did not seem to be any clear physiological reason why the process should occur. ALEEM, LEES and NICHOLAS (1963) were the first to show that in chemoautotrophic bacteria NAD^+ was reduced by reversed electron transport and there was a very good physiological reason for this as NADH for reductive syntheses was in short supply and the normal redox gradients did not produce NADH. Also, SLATER and TAGER (1963) emphasized the role of reversed electron transport in providing energy and reducing equivalents for the synthesis of glutamate in animal mitochondria, and in that review refer extensively to my own contributions in establishing reversed electron transport a recognized phenomenom. Very recently BERRY, GRIVELL & WALLACE (1983) have suggested that the reduction of mitrochondrial NAD^+ by reversed electron transport during the oxidation of fatty acids plays an important physiological role in the 'calorigenic effect' produced by fatty acids.

The last paper (paper 6) in the section deals with a rather different area of oxidative phosphorylation. In the early 1960s MITCHELL (1963) suggested that the separation of protons and hydroxyl ions on opposite sides of the mitochondrial membrane during respiration provided the driving potential for the synthesis of ATP. However, there was virtually no experimental evidence to support this view at that time. In paper 6 I reported some of the first data which clearly showed that in a lightly buffered medium protons were released from mitochondria during respiration and that this proton release was dependent on respiration i.e. it was inhibited by antimycin or cyanide or the absence of oxygen, or by aging of the mitochondria. Uncoupling reagents substantially reduced the ratio of the rate of proton extrusion to the rate of electron transport.

While this work was in progress MITCHELL and MOYLE (1965) produced the first of their ingenious experimental results showing a fall in pH in anaerobic mitochondrial suspensions following the admission of small amounts of oxygen. Nevertheless, paper 6, was one of the first to clearly demonstrate respiration linked proton extrusion as an essential part of respiration linked phosphorylation. ROBERTSON (1968), in his book, *'Protons, Electrons, Phosphorylation and Active Transport'*, devotes three pages to discussing the importance of results in paper 6. He particularly makes the point that the experiments with ferricyanide in the presence of cyanide were 'interesting as a model'; ferricyanide accepts electrons at the cytochrome c level. ROBERTSON makes the point that 'if hydrogen and hydroxyl ions are completely separated by the mitochondrial membrane as suggested by the MITCHELL hypothesis, the rate of proton extrusion would be equivalent to the rate of electron transport'. This is just what I reported in paper 6 in the presence of ferricyanide (and cyanide to block oxygen uptake). Thus this paper provided some of the first experimental evidence for the MITCHELL 'chemi-osmotic' theory of oxidative phosphorylation. Quite some years later, this theory became generally accepted as the most probable mechanism of oxidative phosphorylation, in mitochondria, chloroplasts and respiratory systems generally.

SECTION 2

Papers related to oxidative systems in *Escherichia coli - K12*

At the time of the first paper (paper No.7) of this section there had already been a good deal of discussion about the role of ubiquinone in respiratory systems in animal mitochondria (see MORTON, 1965) but not very much on the role in bacterial systems. The development of mutants blocked in the pathway for ubiquinone biosynthesis by COX, GIBSON and PITTARD (1968) provided ideal tools for studying ubiquinone function in respiratory systems, as Professor GIBSON outlined in his Leeuwenhoek Lecture to the Royal Society in 1981 (GIBSON, 1982).

In paper 7, using mutants of *E. coli - K12*, we clearly established that ubiquinone had an important role in malate oxidation in this organism and that dicoumarol, thought to be a vitamin K antagonist, inhibited respiration in particles lacking vitamin K but containing ubiquinone.

The second paper in this section, paper 8, was a short paper devoted to the role of Piericidin A, an antibiotic inhibitor, on respiratory chain activity in *E. coli - K12*. The data presented in the paper showed that in the respiratory pathways that involved ubiquinone, such as the malate oxidative pathway, Piericidin A inhibits at or near the ubiquinone site and that Piericidin inhibition was specifically reversed by added ubiquinone-2.

The final paper in the Section, paper 9, is the main paper of the Section dealing with ubiquinone function in *E. coli - K12* and although I only contributed a small part in this paper I had been involved in much of the initial work on ubiquinone function. The main additions to our knowledge brought out in this paper were that from studies of the steady state reduction of other respiratory chain components, in addition to ubiquinone itself, it was suggested that ubiquinone functioned at more than one site. We finally proposed that ubisemiquinone, complexed to an electron carrier, functioned in at least two positions in the respiratory chain in *E. coli - K12*. Our definition of the role of ubiquinone as the natural electron acceptor in the malate oxidative system was acknowledged in the review by CRANE and SUN (1972).

SECTION 3

Papers related to acetate metabolism and oxidation in animal systems

The first paper in the section, paper 10 on 'the production of endogenous acetate by the liver of lactating ewes' was important because it was the first paper that clearly demonstrated the production of 'endogenous' acetate by a tissue in the whole conscious animal. The concept of 'endogenous acetate' was established in the early 1960s when workers such as ANNISON and WHITE (1962) showed, on the basis of acetate entry studies, that in addition to exogenous acetate produced by rumen fermentation there was up to 25% of the total acetate entry due to 'endogenous' acetate. It was not known in which tissue this endogenous acetate arose. In our experiments, described in paper 10, we clearly showed that in surgically cannulated sheep the liver produced 0.75 mmol/min of acetate in lactating animals. We also clearly showed that the liver had sufficient enzymic capacity to hydrolyse acetyl CoA to acetate *via* carnitine acetyl-transferase and acetyl-carnitine hydrolase to account for the rate of acetate production.

This rate of acetate production by the liver was very considerable i.e. 56 mmol/hr in the whole animal or just over half the total acetate production. We also showed that there was a significant simultaneous uptake of free fatty acids by the liver and the acetate production accounted for 70% of the fatty acids taken up. The correlation coefficient between the uptake of fatty acids and acetate production was 0.83 ($P<0.01$). These results strongly implied that acetate was produced in the liver of the lactating animals from fatty acids. We found significant utilization of acetate by the mammary glands, as evidenced by significant difference between acetate concentration in the arterial blood and that in the milk vein, although that was not included in the paper.

Taken together these results clearly suggested that this endogenous acetate production by the liver was due to the inability of the liver to completely oxidize fatty acids (a point we had earlier shown - see paper 14) and that acetate, like ketone bodies, could be looked on as a product of incomplete fatty acid oxidation. In this case probably produced because of the needs of the mammary glands for acetate for oxidation and fatty acid synthesis. We subsequently demonstrated that this endogenous acetate production by the liver did not occur in non-lactating ewes

(unpublished results) as had been shown by BERGMAN and WOLF (1971) some years earlier. BAIRD, SYMONDS and ASH (1974) in, in preliminary note, had demonstrated significant production of acetate by the liver in lactating cows.

In those days, i.e. the mid-1970s, we knew little about what regulates acetate production in the liver and that is why paper No.11 is such an important paper, possibly one of the most important is the series of papers submitted. There has been little time to judge the impact of this paper on other workers as it only appeared some 9 months ago although we have had over 400 reprint requests for the paper.

It is well known that acetate plays a central role in energy metabolism in ruminant animals but it was generally considered as being relatively unimportant in non-ruminants. This is probably because the concentration of acetate in peripheral venous blood in these animals and in humans is relatively low compared to ruminants (e.g. see BALLARD, 1972). In paper 10 we clearly showed that in the rat, using a non-recirculating perfusion system with whole blood, that the liver only produces acetate when the incoming acetate concentration is below 0.25 µmol/ml (which agreed very closely with a figure reported by BUCKLEY and WILLIAMSON (1977) for whole animals). Above that concentration acetate became an important hepatic substrate with a 40% fractional extraction, which is as high as for substrates like fructose. This is in direct contrast to the ruminant situation where the liver does not utilize acetate. Further, in paper 10, we clearly showed that acetate uptake was influenced by blood lactate concentration, for raised lactate concentrations depressed acetate uptake. We also showed that there was a direct competition between acetate and lactate as substrates for lipogensis.

Paper 12 was in part an extension of these studies for here we were examining the effect of carboxyhaemoglobin on ethanol utilization in perfused rat-liver. Acetate is a major end product of ethanol metabolism and accounted for 32% of the ethanol used in the normal state and 60% in the presence of carboxyhaemoglobin. The latter compound significantly depressed ethanol utilization by the liver, which has implications for heavy smokers who drink alcohol! Carboxyhaemoglobin also significantly increased the output of lactate by the livers using ethanol, again demonstrating an important metabolic relationship between acetate and lactate metabolism.

Acetate, in rats at least has been shown to be derived from microbial fermentation in the large intestine (BUCKLEY and WILLIAMSON, 1977) and is absorbed in the portal blood. We have found concentrations in the portal blood of 2-3 mM (unpublished results) which is the same as that found in ruminants. In paper 13 we showed that the acetate production in the large intestine, and to a much lesser extent that of the other major volatile fatty acids, propionate and butyrate, is dependent on the type of fibre eaten. More digestible fibres such as pectin, lead to a greater production of acetate. This finding obviously has important implications in regard to fibre type in managing a number of disease conditions in man such as diabetes, obesity and colonic cancer.

These findings for acetate metabolism not only apply to a non-ruminant animal, such as the rat, but we have recently demonstrated the same phenomenon in the pig,TOPPING *ET AL.*, (1983), which is an omnivore much closer to man in it's metabolism.

SECTION 4

Papers related to carnitine and fatty acid oxidation in animal systems - mainly ruminant

The first paper in this section, paper No.14, dealing with ketone body and fatty acid metabolism in sheep tissues established a number of important points. Firstly, free fatty acids were oxidized very poorly by sheep liver mitochondria and only the fatty acyl-esters of L-carnitine were oxidized at significant rates and acetoacetate accounted for 63% of the oxygen uptake with palmitoyl-L-carnitine as the substrate. No 3-hydroxybutyrate was formed in contrast to the situation with rat-liver mitochondria and the activity of 3-hydroxybutyrate dehydrogenase was very low in sheep liver and what activity was present appeared to be cytoplasmic. The virtual absence of this dehydrogenase in ruminant liver was noted by VanDAM and MEYER (1971) in their review when discussing the β-hydroxybutyrate cycle in animal mitochondria.

Papers 15 and 16 may be grouped together as they both deal with carnitine esters in sheep tissues. The data in the first paper showed that not only did the acetyl-carnitine content of sheep liver increase markedly on fasting but the total carnitine content rose some five-fold. These observations were extended to the diabetic state in paper 16 where the total carnitine content of liver rose 6-fold. This latter observation has been referred to in over 30 published papers by other workers (Citation Index Survey) and has been referred to in a number of reviews e.g. VanGOLDE and Van Den BERGH (1977), McGARRY and FOSTER (1980) and BREMER (1977). The two former reviews make the point that this adaption in the carnitine content of liver points to a regulatory role for carnitine in controlling the rate of fatty acid oxidation at the rate limiting step i.e. the carnitine palmitoyl-transferase step (see review by McGARRY and FOSTER (1980) for a full discussion).

However, it should be clearly pointed out that the changes we observed in the carnitine content of sheep liver as reported in papers 15 and 16 are very considerably greater than those reported by other workers in the rat and point to a more important regulatory role of carnitine in the sheep, as we emphasized in later papers.

Other important findings revealed in paper 15, and particularly 16, are that the changes in acetyl-carnitine content appeared to be a physiological method of allowing buffering of the tissue coenzyme-A pool *via* the

carnitine acetyl-transferase reaction. We showed that in both liver and muscle tissue, under a variety of physiological and pathological conditions, this enzyme was sufficiently active to allow equilibration of the reaction.

Acetyl-L-carnitine + Co A $\rightleftarrows$ acetyl Co A + L-carnitine. This was shown by the fact that the mass action ratios measured on freeze-clamped tissue samples gave values close to the apparent equilibrium constant for the isolated enzyme reaction. Although PEARSON and TUBBS (1967) first suggested this buffering role of carnitine acetyl-transferase of relieving 'acetyl-pressure' on the vital Co-A system, on the basis of their studies with perfused rat hearts, it was not until these studies of ours that the full physiological significance of this role was realized. We found very little total Co-A in sheep muscle tissue i.e. approx. 6 nmol/g wet weight, but very high concentrations of carnitine 12.9 µmol/g wet weight. This allows for enormous 'acetyl buffering' capacity *via* the carnitine acetyl-transferase reaction and as MUNN (1974) refers to in his book on 'Mitochondria' we have shown that in a 50kg sheep as much as 6g of acetyl groups can be stored as acetyl carnitine.

Paper 17 is just a short paper which takes up a suggestion made to me privately that the 'soluble' 3-hydroxy-butyrate dehydrogenase activity we had earlier reported in sheep tissues may be due to a L-3-hydroxy acid dehydrogenase and points out the complicated interpretation of earlier data.

Paper 18 deals with changes in Co-A and carnitine, both free and esterified, in the liver of dairy cows following treatment with Voren, a glucocorticoid drug. The results showed that the antiketogenic action of Voren was apparent in increasing the ratio of free Co-A/acetyl Co-A but in the dairy cow liver the carnitine system did not appear to play an important buffering role, as we had seen in sheep liver.

Paper 19 was an important paper in that it clearly demonstrated that the liver was the organ responsible for the extra production of carnitine seen in the diabetic state in the sheep. Due to the very high concentrations of carnitine in muscle tissues of the sheep it was possible that carnitine was mobilized from muscle tissue and passed to the liver, *via* the blood, in diabetes. This paper clearly established that was not so and was the first paper published to clearly demonstrate carnitine biosynthesis in the liver in an intact animal. This paper has been quoted

in at least 14 papers by other workers (Citation Index Survey).

Paper 20 was also an important paper in that it clearly demonstrated that the carnitine secreted into the milk of cows and goats was taken up from the blood as it passed through the mammary glands and was not synthesized within the glands. Also ewes' milk contained quite a high concentration of carnitine with respect to that found in the milk of dairy cows and goats.

Papers 21 and 22 dealt with the mechanism of acetyl-Co·A breakdown *via* carnitine acetyl-transferase and suggested an acetyl-carnitine hydrolase. However, it was not until we reported detailed attempts to isolate the latter enzyme that this activity was shown to be due to acetyl-Co·A deacylase plus carnitine acetyl-transferase. This retraction, also reported in the *Biochemical Journal* (paper 23), unfortunately does not seem to have been noticed by other workers to the same extent as the original papers (papers 21 and 22).

In paper 24 we again report our researches dealing with the dairy cow in a further combined study with the group at the ARC Institute at Compton, U.K. We reported that in lactating cows there was significant fall in the hepatic ratio of [free Co·A]/[acetyl-Co·A] to 0.11 from 0.59 in non-lactating cows and a significant fall in total carnitine in the lactating cows. There was also a significant increase in the hepatic output of both acetate and ketone bodies in the lactating cows and the rate of enzymic degradation of acetyl-Co·A *via* carnitine acetyl-transferase was sufficient to account for the rate of acetate release. This paper is closely allied to those presented in Section 3 and once again demonstrates the release of acetate from the liver as an additional product to ketone bodies, probably arising from acetyl-Co·A derived from the incomplete oxidation of fatty acids.

The effects of cold exposure on the secretion of milk fat constituents in the goat was reported in paper 25 in a combined study with a group and the Hannah Institute in Ayr, Scotland. In regard to my contributions on the carnitine side we demonstrated a decreased secretion of carnitine into the milk on cold exposure. As carnitine in milk is a vital nutrient for the newborn this finding has important consequences for newborn animals exposed to cold.

The epididymis of the male animal has the highest concentrations of carnitine in any known animal tissue. In paper 26, in a study at the ARC Institute at Babraham, U.K., we report the carnitine content of luminal fluid collected by micropuncture from the epididymis of various species. The fluid was collected along various parts of the epididymal tract and the concentration of free L-carnitine was shown to rise from <1 mM in testicular fluid from the rat to 53 mM in fluid from the caudal epididymis, 2000 times greater than that in blood plasma. A high concentration of carnitine was found in the luminal fluid from the region of the distal caput epididymis, at about where the sperm became motile. Thus, this and other studies, suggest an important relationship between carnitine concentration and sperm motility. This is an area of considerable interest in reproductive physiology and this paper has been cited by some 27 other workers in the field (Citation Index Survey). Paper 27 also deals with carnitine and the epididymis and reports that uptake of L-carnitine by isolated epididymal cells from the rat was found to be androgen dependent.

In a combined study with Dr. Loran Bieber of Michigan State University, U.S.A. we reported, in paper 28, that in diabetic sheep that, in addition to acetyl carnitine being the main esterified form of carnitine in both liver and skeletal muscle, there were significant increases in both propionyl-carnitine and the branched chain derivatives isobutyryl- and iso-valeryl- carnitines in the diabetic state. Thus pointing to further roles of carnitine in propionate and branched-chain amino acid metabolism in the sheep.

Paper 29 is the main paper in this Section as it is essentially a review article summarizing all our own work, plus that of others, on the role of carnitine in ruminant animals. A number of our important findings on carnitine metabolism not previously presented are also contained in this review. Emphasis is made of the rapid increase in the carnitine content of the muscle of the new-born lamb following birth. The 15-fold increase in the first week is very much more dramatic than that reported in any other species and points to need of the new-born animal to receive carnitine from the milk as a vital nutrient. Also in this review the very dramatic increases in hepatic carnitine content seen in sheep in severe diabetes (28-fold) and severe cases of pregnancy toxaemia (40-fold) are reported. These are very much greater than the

2-4 fold increases reported in any other species and particularly emphasize the important metabolic role of carnitine in ruminant animals. In this review we also report the linear relationship between hepatic carnitine and hepatic fat content in the sheep up to about 1gm fat/g dry weight of fat free tissue, suggesting that carnitine biosynthesis in the liver increases in an attempt to increase fatty acid oxidation in response to incoming fat. Above this point carnitine biosynthesis appears to run out of control and carnitine concentration increases in the blood and spills out into the urine. Paper 30 examines this point in more detail and suggests this increased hepatic carnitine biosynthesis can only take place in the sheep, in contrast to the rat, because methyl groups are diverted from hepatic creatine synthesis. Creatine (or creatinine) being the normal main methyl excretion product in animals with 80% of the methyl output being in this form.

The effects of glucagon on fatty acid metabolism in perfused rat liver are described in paper 31. This work, in collaboration with Dr. David Topping's group in the CSIRO Division of Human Nutrition, showed that glucagon stimulated both mitochondrial and peroxisomal fatty-acid oxidation. The stimulation of peroxisomal fatty-acid oxidation was the first recorded effect of a hormone on peroxisomal metabolism and was our contribution to the work.

The final paper in this Section (paper 32) deals with induced carnitine deficiency in a human patient on long term total parenteral nutrition. It records the first documented case of the successful treatment of such a patient by intravenous infusion of L-carnitine. Other recorded cases have involved oral carnitine administration but L-carnitine has not yet been cleared for intravenous use. We received special permission from the Australian Department of Health for treatment of this case after we produced evidence for the efficacy of L-carnitine administration.

SECTION 5

Papers related to choline and fat metabolism in ruminant animals

Data dealing with choline nutrition in sheep is presented in paper 33. This is essentially the work of a group in the Biochemistry Department of the ARC Institute of Animal Physiology at Babraham of which I was member for a time in 1977. The paper shows that choline of plant origin is largely degraded in the rumen of the sheep and converted to trimethylamine and methane and this is the main reason why the sheep only obtained about 20-25mg of choline from the diet per day. This is 50 times less than the minimum required to prevent pathological lesions and death in other species. The sheep oxidized [1,2 ^{14}C]-choline to CO_2 at a rate considerably less than the rat and this could help explain the minimal requirement for dietary choline in the sheep.

Paper 34 is quite an important paper, especially from a practical animal husbandry viewpoint. It deals with the changes in liver lipids in alloxan-diabetes and in pregnancy toxaemia. The livers become very fatty under these conditions and the triacylglycerol content increased 15-25 fold. More importantly there was a change in the fatty acid composition of both the triacylglycerols and phospholipids. The triacylglycerols in the two disease states contained more polyunsaturated fatty acids at the expense of the phospholipids, which normally contain high amounts of these fatty acids. Also the synthesis of phosphatidylcholine by methylation was impaired in the disease states. As the phospholipids are vital components of intracellular membranes these changes in the composition of phospholipids toward more saturated fatty acids may explain, in part, the degenerative changes in membrane and subcellular organelle structure observed in the diabetic sheep by TAYLOR, WALLACE and KEECH (1971). These changes probably explain why there is no effective treatment for pregnancy toxaemia in ewes beyond the very early stages. The ruminant liver appears to have a very limited capacity to oxidize the vast amounts of fatty acids that come to the liver and this inflow causes subsequent changes in the composition of stored triaylglycerols and more critically of the phospholipids vital for membrane structure. The latter changes in turn probably lead to the breakdown of liver cell structure and function. Thus, the critical message to farmers is to prevent their ewes from becoming overfat in the early stages of pregnancy as this causes major problems in the final stages of pregnancy when food is often scarce and pregnancy toxaemia is prevalent.

The final paper in the Section, paper 35, deals with the uptake and output of choline by organs of the conscious sheep. The main findings were that tissues of the upper and lower body, drained by the venae cavae, supply the bulk (about 82%) of the free choline in body of the sheep. This suggests the sheep synthesizes substantial amounts of choline in extra hepatic tissues. Also there is evidence for the substantial reabsorption of choline from the alimentary tract suggesting extensive capacity for retention and recycling of bile choline. These observations help explain the low requirement of sheep for dietary choline in contrast to non-ruminant animals.

BIBLIOGRAPHY

ALEEM, M.I.H., LEES, H. and NICHOLAS, D.J.D. (1963). *Nature* 200: 759-761

BERRY, M.N., GRIVELL, A.R. and WALLACE, P.G. (1983). *Eur. J. Biochem.* 131;

BREMER, J. (1977). *Trends in Biochem. Sci.* 2: 207.

BRODIE, A.F. and GUTNICK, D.L. (1972). In: *'Electron and Coupled Energy Transfer in Biological Systems'*. Eds. T.E. King and M. Klingenberg, Marcel Dekker Inc., New York. Vol. Part B, p. 662.

CHANCE, B. (1956). In: O.H. Gaebler, *'Enzymes : Units of Biological Structure and Function'*. Academic Press Inc., New York, p.454

CHANCE, B. and HOLLUNGER, G. (1957). *Federation Proc.* 16: 163. also *Nature (1960)* 185: 666.

COX, G.B., GIBSON, F. and PITTARD, J. (1968). *J. Bact.* 95: 1591-1598.

CRANE, F.L. and SUN, F.F. (1972). In: *'Electron and Coupled Energy Transfer in Biological Systems'* Eds. T.E. King and M. Klingenberg, Marcel Dekker Inc., New York, Vol.1. Part B. p. 530.

ERNSTER, L. (1961). IUB/IUBS Symp. on Biological Structure and Function, Stockholm, Vol.2. Academic Press, New York, p. 139.

GIBSON, F. (1982). *Proc. R. Soc. Lond.* B. 215: 1-18.

GRIFFITHS, D.E. and ROBERTON, A.M. (1966). *Biochim. Biophys. Acta,* 113: 13-26.

KREBS, H.A., EGGLESTON, L.V. and D'ALESSANDRO, A. (1961). *Biochem. J.* 78: 95.

McGARRY, J.D. and FOSTER, D.W. (1980). *Ann. Rev. Biochem.* 49: 406.

MITCHELL, P. (1963). *Symp. Biochem. Soc.* 22: 142.

MITCHELL, P. and MOYLE, J. (1965). *Nature* 208: 147.

MORTON, R.A. (1965). *'Biochemistry of Quinones'* Academic Press, London.

MUNN, E.A. (1974). *'The Structure of Mitochondria'* Academic Press, New York. p.319.

ROBERTSON, R.N. (1968). *'Protons, Electrons, Phosphorylation and Active Transport'*. Cambridge Univ. Press, Cambridge. p. 27-29.

SLATER, E.C. (1966). In: *'Oxidative Phosphorylation contained in Comprehensive Biochemistry, 14 (Biochemical/Oxidations).'* Eds. M. Florkin and E.H. Stoltz, Elsevier Publ. Co. Amsterdam. p. 327-396.

SLATER, E.C. and TAGER, J.M. (1963). In: *'Provision of Reducing Power for Glutamate Synthesis contained in Energy-linked Functions in Mitochondria'*. Ed. B. Chance, Academic Press, New York. p. 97-113.

TAYLOR, P.H., WALLACE, J.C. and KEECH, D.B. (1971). *Biochim. Biophys. Acta.* 237: 179-191.

Van DAM, K. and MEYER, A.J. (1971). *An. Rev. Biochem.* 40: 117-118.

Van GOLDE, L.M.G. and Van Den BERGH, S.G. (1977). In: *'Lipid Metabolism in Mammals'*. Ed. F. Snyder, Plenum Press, New York, Vol.1, p. 43.

The mechanism of the reduction of mitochondrial diphosphopyridine nucleotide by succinate in rabbit-heart sarcosomes

The observation that mitochondrial DPN^+ is reduced by succinate to a greater extent than by DPN-linked substrates was first reported by CHANCE[1]. CHANCE AND HOLLUNGER[2] later suggested that the oxidation of succinate provided energy necessary to reverse electron flow in the respiratory chain leading to the subsequent reduction of DPN^+ by reduced flavoprotein also produced by the oxidation of succinate (*cf.* KREBS[3]). This "reversal" theory was supported by KLINGENBERG, SLENCZKA AND RITT[4] and has recently been further substantiated experimentally by CHANCE AND HOLLUNGER[5]. However, the group of workers led by BARTLEY[6] and KREBS[7,8] favour a different mechanism to explain the reduction of mitochondrial DPN^+ by succinate. They suggest that the rapid flow of electrons from succinate via the cytochrome system to oxygen effectively blocks the oxidation of DPNH, at the same time producing malate which reduces the DPN^+. This mechanism may be referred to as the "malate theory". SLATER[9] has suggested that the energy derived from succinate oxidation may be used to activate endogenous substrates (presumably fatty acids) which could then reduce DPN^+.

TABLE I

THE REDUCTION OF THE DPN^+ OF RABBIT-HEART SARCOSOMES BY ENDOGENOUS SUBSTRATE AND BY SUCCINATE

In the aerobic experiments the reaction mixture contained 40 m*M* succinate, 4–8 mg sarcosomal protein, 0.22 *M* sucrose and 0.01 *M* EDTA (pH 7.4) in a total volume of 1.25 ml. The reaction mixture was incubated at 0° for 90 sec with vigorous agitation. (Experiments with an oxygen electrode showed that the suspension did not become anaerobic under these conditions.) The anaerobic experiments were carried out in Thunberg tubes under nitrogen. The reaction mixture contained 2–4 mg sarcosomal protein and the same sucrose–EDTA solution as above in a volume of 0.5 ml. DPN^+ and DPNH were estimated fluorimetrically by the procedure described by PURVIS[12] slightly modified by SLATER *et al.*[13].

Addition	*No. of expts.*	$\frac{DPNH}{DPN^+ + DPNH}$	
		Anaerobic system, endogenous substrate	*Aerobic system with succinate*
None	11	0.46–0.86** Mean 0.66	0.58–0.86 Mean 0.76 (0.17*)
None	2	0.70	0.79 (0.24)
Oligomycin (1.9 µg/mg protein)	2	0.81	0.81 (0.23)
None	2	0.58	0.72 (0.09)
1 m*M* arsenite	2	0.17	0.75 (0.11)

* The figures in brackets refer to the degree of reduction in the absence of substrate.
** In 4 out of the 11 experiments the degree of reduction produced by endogenous substrate was the same as produced by succinate.

The experiments reported here with rabbit-heart sarcosomes (mitochondria) indicate that endogenous substrate, under anaerobic conditions, is capable of reducing sarcosomal DPN^+ to the same extent as succinate under aerobic conditions (Table I). The addition of succinate under anaerobic conditions did not give any further reduction.

Table I also indicates that the addition of oligomycin to sarcosomal preparations which did not give "full" reduction of DPN^+, compared with succinate, restored this "full" reduction. Oligomycin, the specific inhibitor of oxidative phosphorylation reactions first introduced by LARDY, JOHNSON AND MCMURRAY[10], inhibits ATPase and prevents the formation of ATP from "high-energy" intermediates in the series of phosphorylation reactions associated with the respiratory chain[11]. Thus in the experiments mentioned above the degree of reduction produced by endogenous substrate was apparently dependent on the supply of "high-energy" compounds, the accumulation of which would be favoured by addition of oligomycin.

Arsenite (1 m*M*) did not affect the reduction of sarcosomal DPN^+ by succinate (Table I) but substantially decreased the reduction by endogenous substrate under anaerobic conditions. Thus it is most unlikely that endogenous substrate was responsible for the reduction of DPN^+ in the experiments with succinate.

The "malate" theory requires that oxaloacetate produced by the oxidation of malate derived from succinate should be removed efficiently in order to establish an equilibrium in the malic dehydrogenase reaction favourable for DPN^+ reduction. This may be achieved by the efficient operation of the Krebs Cycle or by the condensation of oxaloacetate and acetyl-CoA to give malonyl-CoA as suggested by SLATER AND HÜLSMANN[14]. Acetyl-CoA could be produced from endogenous fatty acids activated by ATP produced during succinate oxidation.

The following experiments with rabbit-heart sarcosomes indicate that the reduction of sarcosomal DPN^+ indeed requires the utilisation of "high-energy" compounds but not necessarily ATP: The reduction of DPN^+ by succinate was completely inhibited by antimycin A or 2,4-dinitrophenol, and to the extent of 83 % by Amytal (Table II). These results agree with those of CHANCE AND HOLLUNGER[2,5] with rat-liver and guinea-pig-kidney mitochondria. On the other hand, oligomycin did not inhibit the reduction (*cf.* ERNSTER[15]). Acetate inhibited the reduction approx. 60 %, and this inhibition was partially prevented by oligomycin; pyruvate had no effect. The inhi-

TABLE II

THE EFFECT OF VARIOUS COMPOUNDS ON THE REDUCTION OF SARCOSOMAL DPN^+ BY SUCCINATE

Experimental conditions were the same as in Table I.

Compound	*% inhibition of the degree of reduction of DPN^+ by succinate*
0.1 m*M* 2,4-dinitrophenol	100
Oligomycin (up to 6.8 μg/mg protein)	nil
Antimycin A (more than 1.2 μg/mg protein)	100
3.4 m*M* Amytal	83
40 m*M* pyruvate	nil
40 m*M* acetate	56
40 m*M* acetate plus oligomycin (1.8 μg/mg protein)	32

bition by acetate is presumably due to removal of "high-energy" compounds through reaction with ATP, since the inhibition was partially prevented by oligomycin. The acetyl-CoA formed cannot be responsible for this inhibition, since pyruvate which would also form acetyl-CoA was without effect.

These results cannot be reconciled with the "malate theory". According to this theory, acetate and pyruvate, if they are to have any effect, should stimulate DPN^+ reduction, since the acetyl-CoA formed would assist the removal of oxaloacetate. Further, as CHANCE AND HOLLUNGER[5] have stressed, this theory does not explain the inhibition by Amytal or the requirement for "high-energy" compounds which is clearly indicated by the results in Table II.

Thus the only mechanism which can adequately explain how succinate reduces sarcosomal DPN^+ in these experiments is one which involves an energy-linked reversal of electron flow in the respiratory chain.

However, it is possible that in other systems mitochondrial DPN^+ may be reduced by succinate by different mechanisms.

The author is indebted to Professor E. C. SLATER and Dr. J. M. TAGER for their helpful advice and criticism during this work.

This investigation was supported by grants from the Australian Dairy Produce Board, and the life Insurance Medical Research Fund.

Laboratory of Physiological Chemistry, A. M. SNOSWELL
University of Amsterdam (The Netherlands)

[1] B. CHANCE, in O. H. GAEBLER, *Enzymes: Units of Biological Structure and Function*, Academic Press, Inc., New York, 1956, p. 454.
[2] B. CHANCE AND G. HOLLUNGER, *Federation Proc.*, 16 (1957) 163; *Nature*, 185 (1960) 666.
[3] H. A. KREBS, *Bull. Johns Hopkins Hosp.*, 95 (1954) 34.
[4] M. KLINGENBERG, W. SLENCZKA AND E. RITT, *Biochem. Z.*, 332 (1959) 47.
[5] B. CHANCE AND G. HOLLUNGER, *J. Biol. Chem.*, 236 (1961) 1534, 1555, 1562, 1577.
[6] L. M. BIRT AND W. BARTLEY, *Biochem. J.*, 76 (1960) 427.
[7] R. G. KULKA, H. A. KREBS AND L. V. EGGLESTON, *Biochem. J.*, 78 (1961) 95.
[8] H. A. KREBS, L. V. EGGLESTON AND A. D'ALESSANDRO, *Biochem. J.*, 79 (1961) 537.
[9] E. C. SLATER, unpublished discussion at the *IUB/IUBS Symp. on Biological Structure and Function*, Stockholm, 1960.
[10] H. A. LARDY, D. JOHNSON AND W. C. MCMURRAY, *Arch. Biochem. Biophys.*, 78 (1958) 587.
[11] F. HUIJING AND E. C. SLATER, *J. Biochem. (Tokyo)*, 49 (1961) 493.
[12] J. L. PURVIS, *Biochim. Biophys. Acta*, 38 (1960) 435.
[13] E. C. SLATER, M. J. BAILIE AND J. BOUMAN, in *Proc. IUB/IUBS Symp. on Biological Structure and Function*, Vol. 2, Stockholm, Academic Press, Inc., 1960, p. 207.
[14] E. C. SLATER AND W. C. HÜLSMANN, *Proc. Natl. Acad. Sci. U.S.*, in the press.
[15] L. ERNSTER, *in Proc. IUB/IUBS Symp. on Biological Structure and Function*, Vol. 2, Stockholm, Academie Press, Inc., 1960, in the press.

Received July 21st, 1961

Printed in The Netherlands

The mechanism of the reduction of mitochondrial DPN^+ coupled with the oxidation of succinate

There is now considerable evidence[1–5] in support of CHANCE's original suggestion[6] that the reduction of mitochondrial DPN^+ brought about by the addition of succinate to aerobic mitochondria in State 4 (*i.e.* rate of respiration limited by ADP concentration) is due to a reversal of the respiratory chain.

We have studied this reaction in rat-liver mitochondria by coupling it to the synthesis of glutamate in the presence of α-ketoglutarate and NH_3, with arsenite added to prevent the oxidation of α-ketoglutarate. Aspartate was found as well as glutamate; it must have been synthesised from malate (derived from succinate) according to Reactions 1 and 2.

$$\text{malate} + \text{DPN}^+ \leftrightarrows \text{oxaloacetate} + \text{DPNH} + \text{H}^+ \qquad (1)$$

$$\text{glutamate} + \text{oxaloacetate} \leftrightarrows \alpha\text{-ketoglutarate} + \text{aspartate} \qquad (2)$$

Since in State 4 the oxidation of DPNH by oxygen through the respiratory chain is almost completely inhibited, the DPNH formed in Reaction 1 must be oxidized by α-ketoglutarate and NH_3 (Reaction 3).

$$\text{DPNH} + \alpha\text{-ketoglutarate} + \text{NH}_3 + \text{H}^+ \leftrightarrows \text{DPN}^+ + \text{glutamate} \qquad (3)$$

Reaction 4, the sum of Reactions 1–3,

$$\text{malate} + \text{NH}_3 \rightleftharpoons \text{aspartate} \qquad (4)$$

describes the synthesis of aspartate and is a measure of the amount of DPN^+ reduced by malate. The extent of reduction of DPN^+ by succinate is given by the amount of glutamate synthesised.

Table I shows that the synthesis of glutamate was increased by oligomycin (*cf.* ERNSTER[4]) and inhibited by dinitrophenol, antimycin, Amytal or malonate. The remainder of the experiments described in this paper were carried out in the presence of oligomycin.

Oligomycin inhibits the formation of ATP from a dinitrophenol-sensitive high-energy intermediate of oxidative phosphorylation[9,10]. The considerable stimulation by oligomycin of the synthesis of glutamate and the inhibition by dinitrophenol (even in the presence of oligomycin which inhibits the dinitrophenol-induced ATPase[9]) shows clearly that the dinitrophenol-sensitive intermediate (or another intermediate in equilibrium with it) is involved in the reduction of DPN^+ by succinate. The utilization of the energy of a high-energy intermediate implies that it will be split. Thus, the reduction of DPN^+ would be expected to "uncouple" the system and to relieve the inhibition of respiration by oligomycin, in much the same way as dinitrophenol (*cf.* ERNSTER[4]). Fig. 1 shows, indeed, that the addition of α-ketoglutarate and NH_3, which will promote the succinate-linked reduction of DPN^+ by removing DPNH,

TABLE I

EFFECT OF INHIBITORS ON GLUTAMATE SYNTHESIS COUPLED TO SUCCINATE OXIDATION

Reaction mixture (1.0 ml) contained: 15 m*M* KCl, 2 m*M* EDTA, 50 m*M* Tris–HCl, 20 m*M* KH_2PO_4–K_2HPO_4, 5 m*M* $MgCl_2$, 20 m*M* NH_4Cl, 10 m*M* (Expts. 71 and 78) or 20 m*M* (Expts. 90 and 104) α-ketoglutarate, 60 m*M* succinate, 0.1 m*M* ADP, 1 m*M* arsenite, 25 m*M* sucrose and 3.9–5.5 mg mitochondrial protein. The pH was 7.5. Reaction carried out in Warburg vessels at 25°, with KOH in centre well. Reaction time, 15 min (Expts. 71 and 78) or 20 min (Expts. 90 and 104). Glutamate was determined with glutamate decarboxylase[7], and aspartate by the method of PFLEIDERER *et al.*[8].

Expt.	*Addition*	*ΔGlu (μmoles)*	*ΔAsp (μmoles)*
71	none	0.94	0.29
	oligomycin (1.6 μg/mg protein)	2.84	1.61
	dinitrophenol (50 μ*M*)	0.35	0.13
	oligomycin + dinitrophenol	0.29	0.17
78	none	0.69	0.24
	oligomycin (1.5 μg/mg protein)	3.24	1.44
	Amytal (2 m*M*)	0.69	0.38
	Amytal + oligomycin	0.54	0.90
90	oligomycin (1.1 μg/mg protein)	6.39	3.09
	oligomycin + malonate (20 m*M*)	0.43	0.09
104	oligomycin (2.5 μg/mg protein)	4.22	2.42
	oligomycin + antimycin (0.5 μg/mg protein)	0.49	0

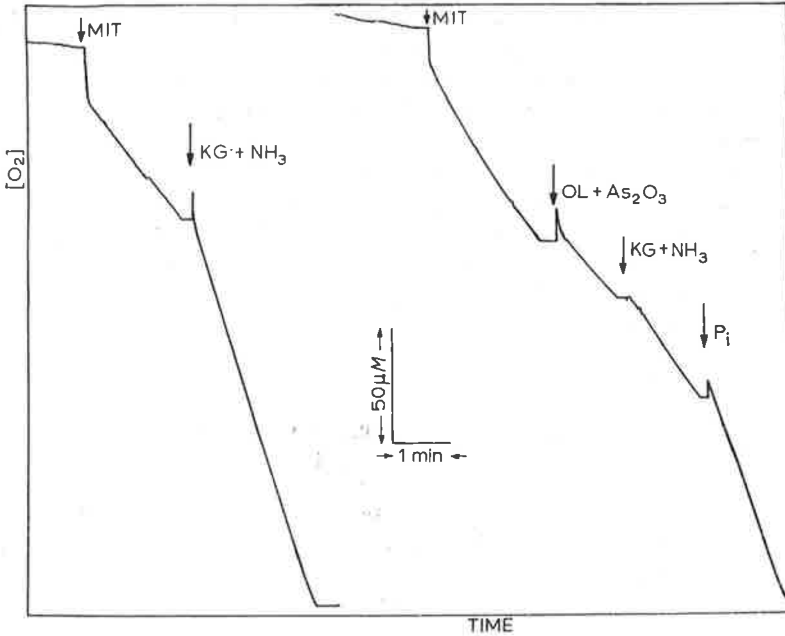


Fig. 1. Effect of α-ketoglutarate + NH_3 on succinate oxidation. Tracings of G.M.E. Oxygraph records. Mitochondria (4.3 mg protein) added to solution containing: 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris-HCl, 0.1 mM ADP, 10 mM glutamate, 20 mM glucose, hexokinase, 30 mM succinate and (left-hand trace only) 1 mM arsenite, 10 µg oligomycin and 5 mM P_i. Additions made as indicated by the arrows. Abbreviations: MIT, mitochondria; KG + NH_3, 18 µmoles α-ketoglutarate + 18 µmoles NH_4Cl; OL + As_2O_3, 10 µg oligomycin + 2 µmoles arsenite; P_i, 10 µmoles P_i. Final volume, 2.0 ml; pH, 7.5; temperature, 25°.

stimulates the O_2 uptake, but only in the presence of inorganic phosphate. Other experiments showed that phosphate or arsenate is also required for the synthesis of glutamate under these conditions.

Table II shows some aspects of the stoicheiometry. The following points are worthy of attention: (i) in the absence of oligomycin, the addition of α-ketoglutarate and NH_3 led to a definite lowering of the P:O ratio, confirming that the synthesis of glutamate brings about an uncoupling of oxidative phosphorylation; (ii) the increased

TABLE II

STOICHEIOMETRY OF OXYGEN UPTAKE, PHOSPHORYLATION AND GLUTAMATE SYNTHESIS

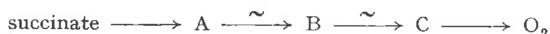
Experimental conditions as in Table I, with addition of 20 mM glucose and hexokinase. Reaction time, 20 min. Esterified phosphate was determined by Method I of SLATER[11].

Oligomycin (µg/mg protein)	*α-Keto-glutarate (mM)*	*NH_3 (mM)*	*ΔO (µatoms)*	*Δest. P (µmoles)*	*ΔGlu (µmoles)*	*ΔAsp (µmoles)*	*P:O*	*Glu:O*
0	0	0	10.1	14.4	0	0.04	1.43	0
0.2	0	0	3.9	1.1	—	—	0.28	—
2.1	0	0	4.6	0	—	—	0	—
21.0	0	0	5.2	0	—	—	0	—
0	20	20	9.6	10.6	2.36	2.20	1.11	0.25
0.2	20	20	6.8	0.8	4.84	2.82	0.12	0.71
2.1	20	20	7.0	0	4.33	2.84	0	0.62
21.0	20	20	6.5	0	4.30	2.18	0	0.66

oxygen uptake brought about by the addition of α-ketoglutarate + NH_3 in the presence of oligomycin was not associated with any phosphorylation; (iii) the ratio glutamate:O in the presence of oligomycin was about one-half the P:O ratio measured in the absence of oligomycin, α-ketoglutarate and NH_3. (The mean glutamate:O ratio in 11 experiments was 0.73.)

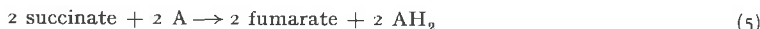
This last-mentioned finding strongly suggests that only one of the two dinitrophenol-sensitive intermediates formed during the oxidation of succinate is involved in the reduction of DPN^+. Phosphate (or arsenate) presumably in some way uncouples the other phosphorylation step. This must be unrelated to the normal phosphorylation reaction, since no phosphorylation was found.

Formulating the succinate chain thus



$$\text{succinate} \longrightarrow \text{A} \xrightarrow{\sim} \text{B} \xrightarrow{\sim} \text{C} \longrightarrow O_2$$

we propose the mechanism given in Reactions 5–12 to describe the reduction of mitochondrial DPN^+ coupled with the oxidation of succinate.



$$2 \text{ succinate} + 2 \text{ A} \rightarrow 2 \text{ fumarate} + 2 \text{ AH}_2 \quad (5)$$

$$AH_2 + B + I \leftrightharpoons A \sim I + BH_2 \quad (6)$$

$$A \sim I + H_2O \rightarrow A + I \quad (7)$$

$$BH_2 + C + I' \leftrightharpoons B \sim I' + CH_2 \quad (8)$$

$$CH_2 + O \rightarrow C + H_2O \quad (9)$$

$$B \sim I' + DPN^+ + AH_2 \leftrightharpoons B + I' + DPNH + A + H^+ \quad (10)$$

$$DPNH + H^+ + \alpha\text{-ketoglutarate} + NH_3 \leftrightharpoons DPN^+ + \text{glutamate} \quad (11)$$

$$\textit{Sum: } 2 \text{ succinate} + O + \alpha\text{-ketoglutarate} + NH_3 \rightarrow 2 \text{ fumarate} + \text{glutamate} \quad (12)$$

Reaction 7 is promoted by phosphate or arsenate.

Our conclusions differ in some respects from those made by ERNSTER[4] from experiments on the succinate-induced reduction of acetoacetate. These differences will be discussed in detail elsewhere.

This work was supported in part by grants from the Life Insurance Medical Research Fund and the Australian Dairy Produce Board.

Laboratory of Physiological Chemistry, University of Amsterdam, Amsterdam (The Netherlands)

E. C. SLATER
J. M. TAGER
A. M. SNOSWELL

1 B. CHANCE AND G. HOLLUNGER, *J. Biol. Chem.*, 236 (1961) 1534.
2 M. KLINGENBERG AND W. SLENCZKA, *Biochem. Z.*, 331 (1959) 486.
3 L. ERNSTER, *I.U.B./I.U.B.S. Symp. on Biological Structure and Function*, Stockholm, 1960, Vol. 2, Academic Press, Inc., New York, 1961, p. 139.
4 L. ERNSTER, *Symp. on Intracellular Respiration: Phosphorylating and Non-phosphorylating Reactions, Proc. 5th Intern. Congr. Biochem.*, Moscow, 1961, Vol. 5, Pergamon Press, Inc., London, in the press.
5 A. M. SNOSWELL, *Biochim. Biophys. Acta*, 52 (1961) 216.
6 B. CHANCE AND G. HOLLUNGER, *Federation Proc.*, 16 (1957) 163.
7 E. F. GALE, *Biochem. J.*, 39 (1945) 46.
8 G. PFLEIDERER, W. GRÜBER AND TH. WIELAND, *Biochem. Z.*, 326 (1955) 446.
9 H. A. LARDY, D. JOHNSON AND W. C. MCMURRAY, *Arch. Biochem. Biophys.*, 78 (1958) 587.
10 F. HUIJING AND E. C. SLATER, *J. Biochem. (Japan)*, 49 (1961) 493.
11 E. C. SLATER, *Biochem. J.*, 59 (1955) 392.

Received November 3rd, 1961

Reprinted from
Biochimica et Biophysica Acta
Elsevier Publishing Company
Amsterdam
Printed in The Netherlands

THE REDUCTION OF DIPHOSPHOPYRIDINE NUCLEOTIDE OF RABBIT-HEART SARCOSOMES BY SUCCINATE

A. M. SNOSWELL*

Laboratory of Physiological Chemistry, University of Amsterdam (The Netherlands)

(Received November 10th, 1961)

SUMMARY

1. The DPN+ of rabbit-heart sarcosomes is reduced by succinate to a greater extent than by DPN-linked substrates.

2. The reduction was inhibited by 2,4-dinitrophenol and arsenate but not by oligomycin. Preincubation of the sarcosomes with oligomycin prevented the inhibition by arsenate. The reduction was also inhibited by antimycin, Amytal and cyanide.

3. The reduction was inhibited by acetate, β-hydroxybutyrate, malate and α-ketoglutarate but the latter two compounds also inhibited succinate oxidation. Pyruvate did not inhibit the reduction and the inhibition by acetate was partially reversed by oligomycin.

4. Endogenous substrate was capable of reducing the sarcosomal DPN+ under anaerobic conditions to the same extent as succinate under aerobic conditions. Since the former reaction was inhibited by arsenite, whereas the latter was not, it can be concluded that endogenous substrate is not the source of reducing equivalents in the reduction of DPN+ by succinate.

5. Sarcosomes depleted of inorganic phosphate still reduced DPN+ in the presence of succinate.

6. The results of these experiments can only be explained by a mechanism involving an energy-linked reversal of electron flow in the respiratory chain. Some details of this mechanism are discussed.

INTRODUCTION

CHANCE[1] first reported that mitochondrial DPN+ is reduced by succinate to a greater extent than by DPN-linked substrates. This report was subsequently confirmed by other workers[2-4]. CHANCE AND HOLLUNGER[5] suggested that this reduction of mitochondrial DPN+ by succinate was due to a reversal of electron flow in the respiratory chain and that the oxidation of succinate provided the energy necessary for this reversal. This "reversal" theory was supported by KLINGENBERG AND SLENCZKA[2]. KLINGENBERG AND BÜCHER[6] showed that glycerol 1-phosphate reduced the DPN+ of flight-muscle mitochondria to approximately the same extent as succinate. This observation provided further valuable support for the "reversal" theory since in the

* Present address: Biochemistry Department, University of New South Wales, Sydney (Australia).

oxidation of glycerol 1-phosphate, as well as in succinate oxidation, reducing equivalents enter the respiratory chain at the flavoprotein level. The mechanism of the succinate-induced reversal of electron transport has recently been the subject of much experimental work in several laboratories[7–10].

The "reversal" theory was first challenged by BIRT AND BARTLEY[4] and later by KREBS and co-workers[11, 12]. These workers suggested that the rapid flow of electrons from succinate to oxygen via the cytochrome system effectively blocks the oxidation of DPNH, at the same time producing malate which reduces the DPN^+. This theory has been referred to as the "switch" mechanism by CHANCE AND HOLLUNGER[10] but may be more conveniently termed the "malate" theory as malate is a key compound in this mechanism. KREBS[13] has recently re-emphasized the "malate" theory in opposition to the "reversal" theory to explain the reduction of mitochondrial DPN^+ by succinate.

Other workers have also questioned the "reversal" theory. PACKER[14] suggested that succinyl-CoA may be involved in the reaction and SLATER[15] suggested that energy derived from succinate oxidation may be used to activate endogenous substrates (presumably fatty acids) which could then reduce DPN^+.

In view of these conflicting theories it appeared that further experimental work was needed to resolve the mechanism of the reduction of mitochondrial DPN^+ by succinate. SLATER, BAILIE AND BOUMAN[16] had found that succinate produced a substantial reduction of DPN^+ in rabbit-heart sarcosomes and that this reduction was more striking than in rat-liver mitochondria (as the latter contain over 50% of the (DPN^+ + DPNH) as DPNH when isolated). Thus rabbit-heart sarcosomes were used in this work.

The results presented in this paper can only be explained successfully by a mechanism in which the reduction of mitochondrial DPN^+ by succinate involves an energy-linked reversal of electron flow in the respiratory chain as originally proposed by CHANCE AND HOLLUNGER[5].

A preliminary report of this work has been published[17].

METHODS

Sarcosomes

Rabbit-heart sarcosomes were prepared in 0.22 *M* sucrose + 0.01 *M* EDTA (pH 7.4) according to the method of CLELAND AND SLATER[18] except that the heart muscle was initially homogenized in a Potter–Elvehjem homogenizer instead of by grinding with quartz sand.

Aerobic incubations of the sarcosomes with succinate and other substrates were carried out for 90 sec at 0° in a medium containing 0.22 *M* sucrose and 0.01 *M* EDTA (pH 7.4) in a total volume of 1.25 ml. The incubation mixture was agitated vigorously. Experiments with an oxygen electrode indicated that without shaking the suspension became anaerobic after 4.5 min, *i.e.* three times the normal incubation period. Thus it is certain that the suspension did not become anaerobic under the test conditions.

Anaerobic incubations were carried out in Thunberg tubes under nitrogen (freed from oxygen by passage through alkaline pyrogallol); otherwise the conditions were essentially the same as in the aerobic incubations except that the volume was only 0.5 ml. Oxygen uptakes were measured in an Oxygraph, manufactured by Gibson

Medical Electronics, Middleton, Wisc. (U.S.A.). The experiments were carried out at 20° in a medium containing 0.22 *M* sucrose and 0.01 *M* EDTA (pH 7.4) in a volume of 2 ml.

Analytical methods

DPN+ and DPNH were estimated fluorimetrically according to the method of PURVIS[19] slightly modified as described by BORST AND COLPA-BOONSTRA[20]. Protein was determined by the biuret method as described by CLELAND AND SLATER[18] using egg albumin as a standard.

MATERIALS

Yeast hexokinase was prepared according to DARROW AND COLOWICK[21] omitting the final crystallization step. DPNH was prepared from DPN+ (Sigma Chemical Co.) using yeast alcohol dehydrogenase (Boehringer) according to the procedure of SLATER[22]. Antimycin was kindly donated by the Kyowa Fermentation Company and oligomycin by Dr. J. LINKS (this preparation consisted mostly of oligomycin B) and by the Upjohn Chemical Company (this preparation contained a mixture of oligomycin A and B). The nucleotides were supplied by the Sigma Chemical Co., sodium succinate by Boehringer and the other chemicals by British Drug Houses.

RESULTS

Before any studies could be made on the reduction of mitochondrial DPN+ by various substrates it was necessary to confirm that the method used for the estimation of DPN+ and DPNH was reliable. The method used was essentially the fluorimetric method of PURVIS[19] who reported good recoveries of added DPN+ and DPNH. However, in view of the claims by PURVIS[19,23] of an "extra" form of DPN+ in mitochondria which could not be detected by other workers[2,4,24] it was suggested[2] that this method under-estimated the DPNH. Table I shows the recovery of DPNH added to rabbit-heart sarcosomes which had been incubated under various conditions.

TABLE I

RECOVERY OF DPNH ADDED TO RABBIT-HEART SARCOSOMES UNDER VARIOUS CONDITIONS

The DPNH solution was added to 2.8 ml of boiling 0.125 *M* Na_2CO_3 immediately prior to the addition of the sarcosomes.

Preparation	*DPNH in sarcosomes* (mμmoles/ml)	*DPNH added* (mμmoles/ml)	*Total DPNH recovered* (mμmoles/ml)	*Added DPNH recovered* (mμmoles/ml)	*% Recovery*
Fresh sarcosomes	4.8	12.8	17.5	12.7	99
Fresh sarcosomes	5.4	18.8	23.6	18.2	97
Sarcosomes incubated with succinate*	8.9	18.8	28.3	19.4	102
P_i-depleted sarcosomes incubated with oligomycin and succinate**	12.7	15.7	26.8	14.1	89

* 4–8 mg sarcosomal protein were incubated with 40 m*M* succinate, 0.22 *M* sucrose and 0.01 *M* EDTA (pH 7.4) in a volume of 1.25 ml for 90 sec at 0°.

** P_i-depleted sarcosomes were prepared as described in Table X. These sarcosomes were then incubated for a further 2 min at room temperature with oligomycin (1.2 μg/mg protein) before incubation at 0° with succinate.

The recoveries ranged from 89 to 102%. The recovery of DPN^+ was also virtually 100% in all cases.

Several workers have reported that mitochondrial DPN^+ is reduced to a greater extent by succinate than by DPN-linked substrates[2,4,5]. The results shown in Table II indicate that this is also true for rabbit-heart sarcosomes; succinate produced the greatest degree of reduction, followed by malate + glutamate, and α-ketoglutarate. Reduction by α-ketoglutarate is probably due to succinate formed from the α-ketoglutarate, since the reduction was much less with 40 m*M* α-ketoglutarate, which inhibited succinate oxidation quite extensively (see Table VII). Table II also indicates that 4 m*M* succinate was as effective as 40 m*M* succinate in reducing sarcosomal DPN^+. This is in contrast to the findings of KREBS *et al.*[12] who reported that high concentrations of succinate are necessary for maximal reduction.

TABLE II

THE REDUCTION OF THE DPN^+ OF RABBIT-HEART SARCOSOMES BY VARIOUS SUBSTRATES

4–8 mg sarcosomal protein were incubated with substrate, 0.22 *M* sucrose and 0.01 *M* EDTA (pH 7.4) in a total volume of 1.25 ml for 90 sec at 0°.

Expt.	*Substrate added*	*DPN⁺* (*µmoles/g protein*)	*DPNH* (*µmoles/g protein*)	*DPN⁺ + DPNH* (*µmoles/g protein*)	$\frac{DPNH}{DPN^+ + DPNH}$
36 S	None	6.1	0.7	6.8	0.10
	40 m*M* pyruvate	6.1	1.1	7.2	0.15
	40 m*M* malate	6.7	1.2	7.9	0.15
	40 m*M* succinate	1.7	3.0	4.7	0.64
37 S	None	5.0	0.4	5.4	0.07
	40 m*M* acetate	4.8	0.8	5.6	0.14
	40 m*M* succinate	1.5	3.6	5.1	0.71
33 S	None	6.5	1.4	7.9	0.18
	40 m*M* α-ketoglutarate	6.7	1.7	8.4	0.20
	40 m*M* glutamate	5.2	1.9	7.1	0.27
	40 m*M* succinate	1.1	4.1	5.2	0.79
38 S	None	6.2	0.6	6.8	0.09
	20 m*M* glutamate + 20 m*M* malate	3.8	3.6	7.4	0.49
	40 m*M* succinate	1.3	3.7	5.0	0.74
39 S	None	4.9	0.9	5.8	0.16
	4 m*M* α-ketoglutarate	2.9	2.8	5.7	0.49
	4 m*M* succinate	1.0	4.2	5.2	0.81

A more detailed analysis of the reduction of sarcosomal DPN^+ by succinate is shown in Table III. The DPN^+ and DPNH contents of the sarcosomes showed considerable variation but on the average 22% of the (DPN^+ + DPNH) was present as DPNH, the degree of reduction increasing to 80% after incubation with succinate. Sarcosomes prepared between July and September, 1961, failed to show this high degree of reduction when incubated with succinate despite precautions to prepare the sarcosomes under the best possible conditions. The reduction of DPN^+ by succinate in these preparations could not be increased by adding ATP. Sarcosomes prepared in October, 1961, again showed a high degree of reduction when incubated with succinate.

The reduction of sarcosomal DPN^+ by succinate was inhibited by the respiratory

TABLE III

THE REDUCTION OF DPN^+ OF RABBIT-HEART SARCOSOMES BY SUCCINATE

The experimental conditions are the same as in Table II, with 40 m*M* succinate as substrate. The values given are means ± the standard deviation with the range in brackets.

Type of sarcosomes	*No. of preparations**	*Experimental conditions*	*DPN^+* (μmoles/g protein)	*DPNH* (μmoles/g protein)	*DPN^+ + DPNH* (μmoles/g protein)	$\frac{DPNH}{DPN^+ + DPNH}$
Rabbit-heart sarcosomes,	44	Fresh	4.5 ± 1.3	1.3 ± 0.6	5.8 ± 1.4	0.22 ± 0.11
prepared from			(2.2–7.4)	(0.2–2.6)	(3.2–9.8)	(0.05–0.48)
Nov. 1960 to June 1961	44	Incubated	1.0 ± 0.4	3.8 ± 1.1	4.8 ± 1.3	0.80 ± 0.07
		with succinate	(0.1–2.0)	(1.9–7.6)	(2.3–9.1)	(0.64–0.97)
Rabbit-heart sarcosomes,	6	Fresh	7.1 ± 1.3	0.6 ± 0.3	7.7 ± 1.4	0.09 ± 0.05
prepared from			(5.4–8.1)	(0.3–1.0)	(5.9–9.3)	(0.05–0.16)
July 1961 to September 1961	6	Incubated	4.3 ± 0.6	2.2 ± 0.7	6.4 ± 1.0	0.33 ± 0.08
		with succinate	(3.1–4.9)	(0.8–2.9)	(5.5–7.3)	(0.16–0.44)

* In 44 out of the 50 preparations (or 88 %) the DPN^+ + DPNH after succinate incubation was less than in the fresh sarcosomes. The difference ranged between —0.7 to 2.7 μmoles/g protein with a mean of 1.0 μmole/g protein and a standard error of ± 0.1.

TABLE IV

THE EFFECT OF RESPIRATORY INHIBITORS ON THE REDUCTION OF SARCOSOMAL DPN^+ BY SUCCINATE

The reaction mixture contained 40 mM succinate, 0.22 M sucrose, 0.01 M EDTA (pH 7.4), inhibitor and 4–8 mg sarcosomal protein (except experiments with Amytal where the protein range was 3–25 mg) in a total volume of 1.25 ml. The reaction time was 90 sec at 0°. Oligomycin was pre-incubated with the sarcosomes for 2 min at room temperature before the addition of succinate.

Inhibitor	*Concentration*	*Percent inhibition**
Antimycin	0.62 μg/mg protein	0
Antimycin	0.86 μg/mg protein	39
Antimycin	1.25 μg/mg protein	95
Amytal	0.09 μmole/mg protein (2 mM)	18
Amytal	0.24 μmole/mg protein (2 mM)	50
Amytal	0.61 μmole/mg protein (2 mM)	76
Amytal	0.80 μmole/mg protein (3.4 mM)	83
KCN	1 mM	36
KCN	10 mM	74–100
2,4-dinitrophenol	0.1 mM	100
2,4-dinitrophenol + Amytal	0.1 mM 2 mM	100
Oligomycin	0.13–6.8 μg/mg protein	0
Mg^{2+}	7 mM	0

* The method of calculating the degree of inhibition may be illustrated with the example of antimycin at 0.86 μg/mg protein—line 2 of the Table. $\frac{DPNH}{DPN^+ + DPNH}$ in absence of substrate = 0.24 and with succinate 0.80. Therefore, the reduction produced by succinate = 0.56. $\frac{DPNH}{DPN^+ + DPNH}$ in presence of antimycin = 0.20 and in presence of succinate + antimycin = 0.54. Therefore, the reduction produced by succinate + antimycin = 0.54 — 0.20 = 0.34 or 0.34/0.56 × 100 = 61 % of that produced by succinate alone, *i.e.* antimycin inhibited the succinate reduction by 39 %.

TABLE V

THE EFFECT OF ARSENATE AND OLIGOMYCIN ON THE REDUCTION OF SARCOSOMAL DPN^+ BY SUCCINATE

Experimental conditions as in Table IV.

Sarcosomes	*Additions*	*Expt. 1* DPN^+ (μmoles/g protein)	*Expt. 1* DPNH (μmoles/g protein)	*Expt. 1* $\frac{DPNH}{DPN^+ + DPNH}$	*Expt. 2* DPN^+ (μmoles/g protein)	*Expt. 2* DPNH (μmoles/g protein)	*Expt. 2* $\frac{DPNH}{DPN^+ + DPNH}$
Normal	None	5.1	2.6	0.34	8.5	0.8	0.09
Normal	40 mM succinate	0.8	5.1	0.86	4.9	2.2	0.31
Normal	40 mM succinate + 24 mM arsenate	6.1	2.7	0.31	10.2	0.4	0.04
Pre-treated*	40 mM succinate + 24 mM arsenate	2.3	4.7	0.68	5.7	2.3	0.29
Normal	40 mM succinate + oligomycin (1.2 μg/mg protein) + 24 mM arsenate	3.4	2.7	0.51	9.8	1.0	0.09

* These sarcosomes were pre-incubated for 2 min at room temperature with oligomycin (1.2 μg/mg protein).

inhibitors antimycin, Amytal and cyanide (Table IV). Similar results were observed by CHANCE AND HOLLUNGER[5,10] with rat-liver and guinea-pig-kidney mitochondria. Mg^{2+} did not inhibit the reduction (contrast CHANCE AND HOLLUNGER[10]). The inhibition by Amytal was dependent on the amount of the inhibitor per milligram protein, rather than purely on the concentration in the reaction medium. This result was rather unexpected as 2 mM Amytal is usually considered sufficient to inhibit respiration[25]. The reduction of sarcosomal DPN+ by succinate was also inhibited by uncoupling agents such as 2,4-dinitrophenol (see refs. 5, 10) and arsenate (see ref. 26). The inhibition by 2,4-dinitrophenol was not relieved by Amytal (contrast KREBS[13]). However, oligomycin did not inhibit the reduction even when pre-incubated with the sarcosomes or when added in concentrations up to 6.8 μg/mg protein (see ref. 27). The inhibition by arsenate could be largely prevented by pre-incubation of the sarcosomes with oligomycin but not by the simultaneous addition of oligomycin as shown in Table V. These results indicate that oligomycin must be pre-incubated with the sarcosomes under the conditions of these experiments in order to be effective. However, no lag period could be observed in the inhibition of glutamate oxidation by oligomycin with these rabbit-heart sarcosomes in State 3 (terminology of CHANCE AND WILLIAMS[28]) as measured by the oxygen electrode.

TABLE VI

THE INHIBITION OF THE REDUCTION OF SARCOSOMAL DPN+ BY SUCCINATE BY ADDITIONAL SUBSTRATES

Experimental conditions as in Table IV with the additional substrate included in the total volume of 1.25 ml.

Additional substrate	*Percent inhibition**
40 mM pyruvate	0
40 mM glutamate	5
20 mM malate + 20 mM glutamate	3
40 mM β-hydroxybutyrate	23
40 mM malate	37
4 mM α-ketoglutarate	15
40 mM α-ketoglutarate	61
40 mM acetate	56
40 mM acetate + oligomycin** (1.8 μg/mg protein)	32

* The degree of inhibition was calculated as in Table IV.

** The sarcosomes were pre-incubated with the oligomycin for 2 min at room temperature before the succinate incubation.

The addition of other substrates substantially decreased the degree of reduction of sarcosomal DPN+ by succinate in some instances as shown in Table VI. Pyruvate, glutamate and glutamate + malate had virtually no effect, but β-hydroxybutyrate, acetate, malate and α-ketoglutarate inhibited the reaction. The inhibition by malate and α-ketoglutarate could possibly be explained by the fact that both these compounds inhibit succinate oxidation, as shown in Table VII. The inhibition by α-ketoglutarate was much less at lower concentrations. The inhibition by acetate and by β-hydroxybutyrate could conceivably be due to the removal of either CoA or ATP, both of which are required for the activation of these compounds, as neither compound inhibited succinate oxidation (Table VII). However, it is unlikely that the inhibition is due to removal of CoA since pyruvate, which also requires CoA for oxidation, did

TABLE VII

THE RATE OF OXYGEN UPTAKE OF RABBIT-HEART SARCOSOMES IN THE PRESENCE OF VARIOUS SUBSTRATES

Oxygen uptake was determined with an oxygen electrode at 20°. The reaction vessel contained substrate, 2.0–2.5 mg sarcosomal protein, 0.22 M sucrose and 0.01 M EDTA (pH 7.4) in a volume of 2 ml.

Substrate	Q_{O_2}*
Endogenous	5
40 mM malate	10
40 mM α-ketoglutarate	11
40 mM α-ketoglutarate + (P_i, ADP, glucose–hexokinase)**	61
40 mM β-hydroxybutyrate	15
40 mM glutamate	15
40 mM glutamate + (P_i, ADP, glucose–hexokinase)**	72
40 mM succinate	99
40 mM succinate + (P_i, ADP, glucose–hexokinase)**	99
40 mM succinate + 40 mM acetate	99
40 mM succinate + 40 mM β-hydroxybutyrate	99
40 mM succinate + 40 mM malate	69
40 mM succinate + 40 mM α-ketoglutarate	62
40 mM succinate + 4 mM α-ketoglutarate	85

* μl O_2/mg protein/h.
** 10 mM P_i, 0.3 mM ADP, 20 mM glucose and 150 units of hexokinase.

TABLE VIII

REDUCTION OF SARCOSOMAL DPN^+ BY SUCCINATE AND BY ENDOGENOUS SUBSTRATE

The aerobic incubations with succinate were carried out as in Table IV. The anaerobic experiments were carried out in Thunberg tubes under nitrogen. The reaction mixture contained 2–4 mg sarcosomal protein, 0.22 M sucrose and 0.01 M EDTA (pH 7.4) in a volume of 0.5 ml. The values given are the means of 11 experiments.

	DPN^+	*DPNH*	*DPN^++DPNH*	*DPNH / (DPN^++DPNH)*
	(μmoles/g protein)			
Fresh sarcosomes	4.5	1.0	5.5	0.18
Sarcosomes incubated aerobically with 40 mM succinate	0.8	3.8	4.6	0.83
Sarcosomes incubated anaerobically	1.7	3.7	5.4	0.69*

* In 4 out of the 11 experiments the degree of reduction produced by endogenous substrate was the same as that produced by succinate.

not inhibit the reduction. It is much more likely that acetate (and probably β-hydroxybutyrate) inhibited by removing ATP (and thereby lowering the concentration of other high-energy compounds in equilibrium with ATP) since the inhibition was partially reversed by oligomycin. Oligomycin would prevent the formation of ATP from intermediate high-energy compounds[29].

SLATER[15] has suggested that endogenous substrate (such as fatty acids) may actually supply the reducing equivalents involved in the reduction of mitochondrial DPN^+ by succinate. Recent experiments by BODE AND KLINGENBERG, reported by KLINGENBERG[30], have shown that caproate can reduce DPN^+ in rat-liver and rat-heart mitochondria. Table VIII shows that the DPN^+ of rabbit-heart sarcosomes

was substantially reduced by endogenous substrate under anaerobic conditions (*i.e.* conditions where no reoxidation of DPNH formed could occur), the degree of reduction being comparable to that obtained with succinate under aerobic conditions. Incubation of the sarcosomes with succinate under anaerobic conditions failed to produce any greater reduction than under aerobic conditions.

Since endogenous substrate, under anaerobic conditions, can reduce the sarcosomal DPN+ to the same extent as succinate (Table VIII) it would be expected that the use of the respiratory inhibitors antimycin and cyanide would also lead to substantial reduction of the DPN+ by endogenous substrate even if succinate oxidation were inhibited by these reagents. However, the experiments summarized in Table IV gave no indication of this. Addition of antimycin to the sarcosomes failed to induce any reduction of sarcosomal DPN+ by endogenous substrate but these same rabbit-heart sarcosomes also have a substantial antimycin-resistant respiration (with a Q_{O_2} of 13.5). This antimycin-resistant respiration would be sufficient to allow a rapid oxidation of any DPNH formed, even in the presence of antimycin. In the presence of 10 mM cyanide endogenous substrate gave $\frac{\text{DPNH}}{\text{DPN}^+ + \text{DPNH}}$ ratios as high as 0.50, in contrast to 0.23 found in the absence of cyanide. The ratio in the presence of succinate + 10 mM cyanide was also 0.50. While this figure is not as high as produced by anaerobic conditions (*i.e.* 0.69, see Table VIII) it indicates that endogenous substrate can substantially reduce sarcosomal DPN+ in the presence of cyanide. Possibly the reaction time of 90 sec was too short to allow "full" reduction.

The reduction of sarcosomal DPN+ by endogenous substrate, like the reduction by succinate, was inhibited by malonate and 2,4-dinitrophenol (Table IX). The inhibition by 2,4-dinitrophenol was probably due to the hydrolysis of high-energy intermediates leading to a removal of ATP which is necessary for the activation of fatty acids (which form the main part of the endogenous substrate). The inhibition by malonate is also probably due to the removal of ATP which is used to convert malonate to malonyl-CoA in the presence of CoA and the appropriate activating

TABLE IX

THE EFFECT OF INHIBITORS ON THE REDUCTION OF SARCOSOMAL DPN+ BY SUCCINATE AND BY ENDOGENOUS SUBSTRATE

Conditions for the aerobic system as in Table IV and for the anaerobic system as in Table VIII. Values given are the means of the two experiments.

Addition	*DPNH / (DPN+ + DPNH)*	
	Anaerobic system, endogenous substrate	*Aerobic system with succinate*
None	0.64	0.77 (0.05)*
4 mM malonate	0.28	0.10 (0.05)
None	0.69	0.80 (0.22)
c.5 mM 2,4-dinitrophenol	0.11	0.04 (0.04)
None	0.70	0.79 (0.24)
Oligomycin (1.9 μg/mg protein)	0.81	0.81 (0.23)
None	0.58	0.72 (0.09)
1 mM arsenite	0.17	0.75 (0.11)

* The values in brackets refer to the degree of reduction in the absence of substrate.

enzyme. HÜLSMANN[31] has demonstrated this latter reaction in rabbit-heart sarcosomes. The results shown in Table IX also show that oligomycin did not inhibit the reduction by endogenous substrate. In fact, in preparations which did not give "full" reduction of DPN^+ by endogenous substrate, compared with succinate, oligomycin restored this "full" reduction.

The addition of arsenite substantially inhibited the reduction by endogenous substrate but did not inhibit the reduction by succinate indicating that endogenous substrate was not responsible for the reduction of DPN^+ during succinate oxidation.

The fact that the reduction of sarcosomal DPN^+ by succinate is inhibited by uncoupling agents such as 2,4-dinitrophenol and arsenate (Table IV) indicates that this reduction is energy dependent. Most energy-transfer processes involve phosphate compounds and thus it was of interest to determine whether this reduction also utilized phosphate compounds. Sarcosomes depleted of their "reactive" inorganic phosphate by prior incubation with glucose, hexokinase, Mg^{2+} and ADP still effectively reduced DPN^+ in the presence of succinate (Table X). In fact succinate reduced sarcosomal DPN^+ in these P_i-depleted sarcosomes better than when P_i was also added. The oligomycin prevented the P_i from bringing about a conversion from State 4 to State 3 (as defined by CHANCE AND WILLIAMS[28]; *cf.* lines 4 and 5 Table X). The results shown in Table X also indicate that the degree of reduction of sarcosomal DPN^+ by succinate is the same in State 3 as in State 1 (*i.e.* in the absence of added substrate).

TABLE X

THE EFFECT OF PHOSPHATE DEPLETION ON THE REDUCTION OF SARCOSOMAL DPN^+ BY SUCCINATE

The sarcosomes were depleted of P_i by incubating them for 15 min at room temperature with 20 mM glucose, 6 mM $MgCl_2$, 0.1 mM ADP and 150 units of hexokinase. The oxidation of endogenous substrate appeared sufficiently fast to deplete the sarcosomes of P_i in this time without adding additional substrate. The incubations with succinate at 0° for 90 sec were carried out as in Table IV. The values given are the means of two experiments carried out in September, 1961 (see Table III).

Experimental conditions	*DPN⁺* (*µmoles/g protein*)	*DPNH* (*µmoles/g protein*)	*DPN⁺+DPNH* (*µmoles/g protein*)	*DPN⁺/(DPN⁺+DPNH)*
Fresh sarcosomes	8.9	0.9	9.8	0.09
Fresh sarcosomes + oligomycin* + 40 mM succinate	4.5	2.7	7.2	0.36
P_i-depleted sarcosomes + oligomycin* + 40 mM succinate	3.6	2.4	6.0	0.40
P_i-depleted sarcosomes + oligomycin* + 50 mM P_i + 40 mM succinate	4.6	1.9	6.5	0.29
P_i-depleted sarcosomes + 50 mM P_i + 40 mM succinate	7.0	0.6	7.6	0.08

* Oligomycin (1.2 µg/mg protein) was incubated with the sarcosomes for 2 min at room temperature immediately after the incubation to deplete the sarcosomes of P_i, and immediately before the addition of the succinate.

DISCUSSION

The recoveries of DPN^+ and DPNH obtained with rabbit-heart sarcosomes under various conditions indicate that the method of estimation used in these experiments (*i.e.* that of PURVIS[19] slightly modified as described by BORST AND COLPA-BOONSTRA[20]) is quite reliable. The statement by PRESSMAN[32] that good recoveries of DPNH cannot be obtained in the presence of sarcosomes does not apply in these experiments.

Table III shows that the (DPN+ + DPNH) content of sarcosomes is significantly lowered by aerobic incubation with succinate. This difference suggests the formation of "extra" DPN+ in the presence of succinate. PURVIS[23] (see also SLATER AND HÜLSMANN[33]) has presented evidence that mitochondria contain a DPN+ compound, termed "extra" DPN+, in addition to DPN+ and DPNH and has suggested that it was this "extra" DPN+ which was produced when mitochondria were incubated with succinate, rather than DPNH as proposed by CHANCE AND HOLLUNGER[5]. However, SLATER, BAILIE AND BOUMAN[16], using the same methods as PURVIS[19], found that most of the DPN+ was converted to DPNH after incubation of mitochondria with succinate, although some "extra" DPN+ was formed with sarcosomes. The results presented in this paper are thus in agreement with those of SLATER *et al.*[16].

Sarcosomes which had been prepared during the summer months and which were depleted of inorganic phosphate prior to incubation with succinate showed a highly significant decrease in the amount of (DPN+ + DPNH) compared with fresh sarcosomes (*cf.* lines 1 and 3 in Table X). Similar results were reported by SLATER *et al.*[16] and these would appear to be the most suitable conditions for demonstrating the formation of Purvis' "extra" DPN+.

The experiments reported here indicate that rabbit-heart sarcosomes contain sufficient endogenous substrate to produce a rapid and substantial reduction of DPN+ under anaerobic conditions and that the degree of reduction can be the same as that produced by succinate aerobically. However, whereas arsenite substantially inhibited the reduction of DPN+ by endogenous substrate it did not inhibit the reduction by succinate. Thus endogenous substrate does not supply the reducing equivalents for the reduction of DPN+ during succinate oxidation (*cf.* CHANCE AND HOLLUNGER[10]). KLINGENBERG AND SCHOLLMEYER[8] had eliminated the participation of endogenous substrate in the reduction of mitochondrial DPN+ by glycerol 1-phosphate by the use of malonate and had suggested, by analogy, that endogenous substrate was not involved in the reduction of DPN+ by succinate.

The results presented in this paper provide further information concerning the reduction of mitochondrial DPN+ by succinate. Three lines of evidence indicate that the DPN+ is reduced by a reversal of the respiratory chain as proposed by CHANCE AND HOLLUNGER[5] rather than according to the "malate" theory.

1. The reduction of mitochondrial DPN+ by succinate in the present experiments is clearly dependent on the utilization of high-energy compounds as it is completely inhibited by 2,4-dinitrophenol and arsenate. These results are in agreement with those of several groups of workers using different systems[2,5,7,10]. According to the "malate" theory the reduction of mitochondrial DPN+ by succinate is not energy dependent. KREBS[13] has pointed out that the inhibition by uncoupling agents such as 2,4-dinitrophenol is not, by itself, sufficient proof of an energy requirement as these compounds also stimulate respiration, thereby increasing the rate of oxidation of DPNH. He reported[13] that the addition of Amytal, which inhibits the oxidation of DPNH[25], prevented the inhibition of the reduction of acetoacetate (used as a measure of DPN+ reduction) by 2,4-dinitrophenol in his system. However, in the experiments reported here 2,4-dinitrophenol completely inhibited the reduction of DPN+ even in the presence of Amytal. Further, the rabbit-heart sarcosomes used in these experiments show no difference in respiratory rate with succinate in the presence or absence of P_i, ADP, glucose and hexokinase (Table VII). Despite this lack of respiratory

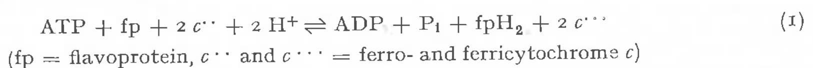
control DPN^+ is not reduced by succinate in State 3 unless oligomycin is present (Table X). This then is a clear indication of an energy requirement and can not be explained by changes in respiratory rate. Also despite the lack of respiratory control with succinate these rabbit-heart sarcosomes showed a substantial reduction of DPN^+ by succinate due to a reversal of electron transport. KLINGENBERG[30] has suggested that mitochondria must exhibit respiratory control if they are to show this reversal phenomenon but the experiments reported here indicate that this is not necessarily true.

2. The reduction of sarcosomal DPN^+ is inhibited by Amytal, which is in agreement with the findings of CHANCE AND HOLLUNGER[5,10] and ERNSTER[34], and is in accord with the "reversal" theory. However, one of the main points in favour of the "malate" theory, as recently re-emphasized by KREBS[13], is that the system with sheep-heart homogenate studied by KREBS *et al.*[12] is not inhibited by Amytal. Serious doubts have now been cast on the relevance of these results for the mitochondrial system by the recent experiments of ERNSTER[34], who showed that the addition of the liver supernatant fraction to an isolated mitochondrial system completely removed the Amytal sensitivity of the succinate-linked acetoacetate reduction of the isolated mitochondria. The acetoacetate reduction, which was used as a measure of DPNH formation, was even slightly stimulated thus duplicating the behaviour of the whole homogenate as reported by KREBS *et al.*[12].

3. An efficient removal of oxaloacetate is implied by the "malate" theory in order to establish an equilibrium in the malate dehydrogenase reaction favourable for DPN^+ reduction. Thus, according to this theory the addition of pyruvate and acetate would be expected to stimulate, rather than inhibit, the reduction of DPN^+ by succinate since acetyl-CoA formed from these compounds would assist the removal of oxaloacetate (*cf.* SLATER AND HÜLSMANN[35]). However, acetate markedly inhibited the reduction of sarcosomal DPN^+ by succinate, apparently by the removal of high-energy compounds by the formation of ATP since the inhibition was partially reversed by oligomycin. Acetyl-CoA formed during the reaction can not be responsible for this inhibition since pyruvate, which would also produce acetyl-CoA, was without effect.

A further piece of evidence against the "malate" theory was recently reported by CHANCE[36]. He found that the ATP-stimulated reduction of DPN^+ by succinate in pigeon-heart sarcosomes was not inhibited by the presence of D-malate (a specific inhibitor of the malate dehydrogenase reaction) indicating that malate was not involved in the reaction.

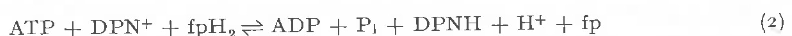
Thus the reduction of mitochondrial DPN^+ by succinate can only be explained successfully by the "reversal" theory. It is interesting to note that an energy-linked reversal of electron transport was proposed as early as 1952 by DAVIES AND KREBS[37], on purely theoretical grounds, to explain the secretion of H^+ ions in gastric mucosa. They proposed the reaction:



$$ATP + fp + 2\,c^{\cdot\cdot} + 2\,H^+ \rightleftharpoons ADP + P_i + fpH_2 + 2\,c^{\cdot\cdot\cdot} \quad (1)$$

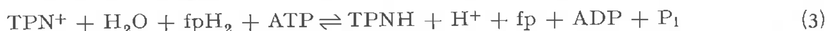
(fp = flavoprotein, $c^{\cdot\cdot}$ and $c^{\cdot\cdot\cdot}$ = ferro- and ferricytochrome c)

SLATER[38] also suggested that an energy-linked reversal of electron transport might be possible in the pyridine nucleotide–flavoprotein region, *i.e.*:



$$ATP + DPN^+ + fpH_2 \rightleftharpoons ADP + P_i + DPNH + H^+ + fp \quad (2)$$

In 1954 KREBS[39] provided the first experimental indication that such an energy-linked reversal of electron transport was possible. He showed that 2,4-dinitrophenol inhibited the aerobic formation of malate from pyruvate and carbon dioxide in pigeon-liver homogenates by approximately 90% and suggested that the 2,4-dinitrophenol had prevented the formation of ATP and that ATP shifted the equilibrium of the reaction involving TPN^+ and TPNH in favour of TPNH. In other words ATP had induced a reversal of electron transport according to Reaction 3:



$$TPN^+ + H_2O + fpH_2 + ATP \rightleftharpoons TPNH + H^+ + fp + ADP + P_i \quad (3)$$

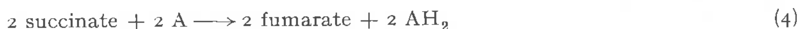
It is now worthwhile to consider the "reversal" theory for the reduction of mitochondrial DPN^+ by succinate in more detail. The reduction requires energy as indicated by the fact that it is inhibited by 2,4-dinitrophenol and arsenate. The reduction is not inhibited by oligomycin and the results shown in Table V indicate that oligomycin prevents the inhibition by arsenate. Therefore, in view of the site of action of oligomycin (see LARDY *et al.*[40] and HUIJING AND SLATER[41]) it would appear that the high-energy intermediate used in the reduction is not a phosphorylated compound[42]. This suggestion is further supported by the fact that sarcosomes depleted of inorganic phosphate still reduced DPN^+ in the presence of succinate and oligomycin. The results shown in Table X also indicate that oligomycin was necessary to preserve the high-energy intermediate by preventing its conversion to ATP in presence of inorganic phosphate and ADP. A similar result was recently reported by AZZONE *et al.*[43] with the succinate-linked acetoacetate reduction system in liver mitochondria but was misinterpreted by these workers as indicating that a high-energy phosphate compound was involved in the reduction of mitochondrial DPN^+ by succinate. In fact ERNSTER[34] subsequently reported that their system had no phosphate requirement.

CHANCE[9] reported that in pigeon-heart mitochondria, which require added ATP to reduce DPN^+ in the presence of succinate, oligomycin inhibited the reduction induced by added ATP. However, the reduction was only inhibited approx. 60% indicating that there was some non-phosphorylated high-energy intermediate produced during succinate oxidation that stimulated the reduction of DPN^+ without the participation of added ATP[44]. A stimulation by oligomycin of the succinate-linked reduction of α-ketoglutarate (in the presence of NH_3) has been demonstrated in this laboratory[45] and by ERNSTER[46].

A possible mechanism for the reduction of mitochondrial DPN^+ by succinate has been proposed previously[47]. The oxidation of succinate is visualized as involving three respiratory carriers and two phosphorylation steps. The latter are denoted by ~ signs in the following sequence:

$$\text{Succinate} \longrightarrow A \overset{\sim}{\longrightarrow} B \overset{\sim}{\longrightarrow} C \longrightarrow O_2$$

Then the mechanism is as follows:



$$2 \text{ succinate} + 2\,A \longrightarrow 2 \text{ fumarate} + 2\,AH_2 \quad (4)$$

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2 \quad (5)$$

$$A \sim I + H_2O \longrightarrow A + I \quad (6)$$

$$BH_2 + C + I' \rightleftharpoons B \sim I' + CH_2 \quad (7)$$

$$CH_2 + O \longrightarrow C + H_2O \quad (8)$$

$$B \sim I' + DPN^+ + AH_2 \rightleftharpoons B + I' + DPNH + H^+ + A \quad (9)$$

$$\text{Sum} \quad 2 \text{ succinate} + O + DPN^+ \longrightarrow 2 \text{ fumarate} + DPNH + H^+$$

The first reaction involves the reduction of carrier A which could be the flavoprotein, succinate dehydrogenase, and it is the reduced carrier (AH_2) which provides the reducing equivalents (although not necessarily directly) for the reduction of DPN^+ (Reaction 9). A second molecule of AH_2 is oxidized to give BH_2 and subsequently B ~ I' which is the intermediate high-energy compound used in the reduction of DPN^+. The oxidation of AH_2 is normally associated with a phosphorylation step but in the rabbit-heart sarcosomes used in this work this phosphorylation step is only loosely coupled so that A ~ I reacts with water (Reaction 6). In the mechanism previously proposed for the succinate-linked reduction of α-ketoglutarate in rat-liver mitochondria[47] P_i (or As_i) is required for the hydrolysis of A ~ I (in a reaction unrelated to the normal action of phosphate)*.

The detailed mechanism of Reaction (9) is a matter of considerable interest.

ACKNOWLEDGEMENTS

The author is indebted to Professor E. C. SLATER and Dr. J. M. TAGER for valuable discussions and criticism during this work.

The investigations were supported by grants from the Australian Dairy Produce Board and from the Life Insurance Medical Research Fund.

REFERENCES

1 B. CHANCE, in O. H. GAEBLER, *Enzymes: Units in Biological Structure and Function*, Academic Press, Inc., New York, 1956, p. 447.
2 M. KLINGENBERG AND W. SLENCZKA, *Biochem. Z.*, 331 (1959) 486.
3 M. KLINGENBERG, W. SLENCZKA AND E. RITT, *Biochem. Z.*, 332 (1959) 47.
4 L. M. BIRT AND W. BARTLEY, *Biochem. J.*, 76 (1960) 427.
5 B. CHANCE AND G. HOLLUNGER, *Federation Proc.*, 16 (1957) 163; *Nature*, 185 (1960) 666.
6 M. KLINGENBERG AND T. BÜCHER, *Biochem. Z.*, 334 (1961) 1.
7 G. F. AZZONE, L. ERNSTER AND M. KLINGENBERG, *Nature*, 188 (1960) 552.
8 M. KLINGENBERG AND P. SCHOLLMEYER, *Biochem. Z.*, 333 (1960) 335.
9 B. CHANCE, *J. Biol. Chem.*, 236 (1961) 1544, 1569.
10 B. CHANCE AND G. HOLLUNGER, *J. Biol. Chem.*, 236 (1961) 1534, 1555, 1562, 1577.
11 R. G. KULKA, H. A. KREBS AND L. V. EGGLESTON, *Biochem. J.*, 78 (1961) 95.
12 H. A. KREBS, L. V. EGGLESTON AND D'ALESSANDRO, *Biochem. J.*, 79 (1961) 537.
13 H. A. KREBS, *Biochem. J.*, 80 (1961) 225.
14 L. PACKER, *Discussion at the I.U.B./I.U.B.S. Symp. on Biological Structure and Function, Stockholm, 1960*, unpublished.
15 E. C. SLATER, *Discussion at the I.U.B./I.U.B.S. Symp. on Biological Structure and Function, Stockholm, 1960*, unpublished.
16 E. C. SLATER, M. J. BAILIE AND J. BOUMAN, *Proc. I.U.B./I.U.B.S. Symp. on Biological Structure and Function, Stockholm*, Vol. 2, Academic Press, Inc., New York, 1960, p. 207.
17 A. M. SNOSWELL, *Biochim. Biophys. Acta*, 52 (1961) 216.
18 K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
19 J. L. PURVIS, *Biochim. Biophys. Acta*, 38 (1960) 435.
20 P. BORST AND J. P. COLPA-BOONSTRA, *Biochim. Biophys. Acta*, 56 (1962) 216.
21 R. A. DARROW AND S. P. COLOWICK, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Academic Press, Inc., New York, Vol. V, 1961, p. 226.
22 E. C. SLATER, *Biochem. J.*, 53 (1953) 157.
23 J. L. PURVIS, *Nature*, 182 (1958) 711.
24 K. H. BAESSLER AND B. C. PRESSMAN, *Federation Proc.*, 18 (1959) 184.
25 L. ERNSTER, O. JALLING, H. LOW AND O. LINDBERG, *Exptl. Cell Research Suppl.*, 3 (1955) 124.
26 G. F. AZZONE AND L. ERNSTER, *Nature*, 187 (1960) 65.

* Addendum added on proof (March 31st, 1962). See, however, TAGER *et al.*[48].

[27] L. Ernster, *Proc. I.U.B./I.U.B.S. Symp. on Biological Structure and Function, Stockholm,* Vol. 2, Academic Press, Inc., New York, 1960, p. 139.
[28] B. Chance and G. R. Williams, *J. Biol. Chem.*, 217 (1955) 409.
[29] E. C. Slater, *Proc. 5th Intern. Congr. Biochem., Moscow, 1961,* Vol. V, Pergamon Press, London, in the press.
[30] M. Klingenberg, *Proc. 5th Intern. Congr. Biochem., Moscow, 1961,* Vol. V, Pergamon Press, London, in the press.
[31] W. C. Hülsmann, *Biochim. Biophys. Acta,* 58 (1962) 417.
[32] B. C. Pressman, *Proc. 5th Intern. Congr. Biochem., Moscow, 1961,* Vol. V, Pergamon Press, London, in the press.
[33] E. C. Slater and W. C. Hülsmann, *Regulation of Cell Metabolism,* Ciba Found. Symp., 1959, p. 58.
[34] L. Ernster, *Proc. 5th Intern. Congr. Biochem., Moscow, 1961,* Vol. V, Pergamon Press, London, in the press.
[35] E. C. Slater and W. C. Hülsmann, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1109.
[36] B. Chance, *Proc. 5th Intern. Congr. Biochem., Moscow, 1961,* Vol. V, Pergamon Press, London, in the press.
[37] R. E. Davies and H. A. Krebs, *Biochem. Soc. Symposia (Cambridge Engl.)*, 8 (1952) 77.
[38] E. C. Slater, *Ann. Rev. Biochem.*, 22 (1953) 17.
[39] H. A. Krebs, *Bull. Johns Hopkins Hosp.*, 95 (1954) 34.
[40] H. A. Lardy, D. Johnson and W. C. McMurray, *Arch. Biochem. Biophys.*, 78 (1958) 587.
[41] F. Huijing and E. C. Slater, *J. Biochem. (Tokyo)*, 49 (1961) 493.
[42] A. M. Snoswell, *Proc. 5th Intern. Congr. Biochem., Moscow, 1961,* Vol. V, Pergamon Press, London, in the press.
[43] G. F. Azzone, L. Ernster and E. C. Weinbach, *Abstr. Commun. 5th Intern. Congr. Biochem., Moscow, 1961,* Pergamon Press, London, p. 459.
[44] B. Chance, *Proc. 5th Intern. Congr. Biochem., Moscow, 1961,* Vol. V, Pergamon Press, London, in the press.
[45] E. C. Slater, *Proc. 5th Intern. Congr. Biochem., Moscow, 1961,* Vol. V, Pergamon Press, London, in the press.
[46] L. Ernster, *Proc. 5th Intern. Congr. Biochem., Moscow, 1961,* Vol. V, Pergamon Press, London, in the press.
[47] E. C. Slater, J. M. Tager and A. M. Snoswell, *Biochim. Biophys. Acta,* 56 (1962) 177.
[48] J. M. Tager, J. L. Howland and E. C. Slater, *Biochim. Biophys. Acta,* 58 (1962) 616.

Reprinted from
Biochimica et Biophysica Acta
Elsevier Publishing Company
Amsterdam
Printed in The Netherlands

SYNTHESIS OF GLUTAMATE FROM α-OXOGLUTARATE AND AMMONIA IN RAT-LIVER MITOCHONDRIA

IV. REDUCTION OF NICOTINAMIDE NUCLEOTIDE COUPLED WITH THE AEROBIC OXIDATION OF TETRAMETHYL-*p*-PHENYLENEDIAMINE

J. M. TAGER, J. L. HOWLAND, E. C. SLATER AND A. M. SNOSWELL

Laboratory of Physiological Chemistry, University of Amsterdam, Amsterdam (The Netherlands)
and Biochemistry Department, University of New South Wales, Sydney (Australia)

(Received February 8th, 1963)

SUMMARY

1. The synthesis of glutamate from α-oxoglutarate and ammonia in rat-liver mitochondria has been studied with succinate or malate as hydrogen donor and with the aerobic oxidation of tetramethyl-*p*-phenylenediamine, in the presence of antimycin to inhibit the oxidation of succinate or malate, as a source of energy.

2. Tetramethyl-*p*-phenylenediamine itself is a relatively inefficient hydrogen donor for glutamate synthesis in rat-liver mitochondria.

3. The synthesis of glutamate in the succinate–antimycin–tetramethyl-*p*-phenylenediamine system is inhibited by Amytal or dinitrophenol, requires inorganic phosphate and is not significantly affected by oligomycin. The addition of α-oxoglutarate (+ ammonia) in the presence of oligomycin stimulates oxygen uptake slightly.

4. It is concluded that the synthesis of glutamate in the succinate–antimycin–tetramethyl-*p*-phenylenediamine system represents an energy-linked reversal of the respiratory chain.

5. The energy necessary for the reduction of α-oxoglutarate (+ ammonia) by malate can also be generated during the aerobic oxidation of tetramethyl-*p*-phenylenediamine. In this case, Amytal can be used instead of antimycin to inhibit the aerobic oxidation of malate.

6. In rabbit-heart sarcosomes in the presence of oxygen, mitochondrial NAD^+ is reduced to a greater extent by tetramethyl-*p*-phenylenediamine than by succinate. The reduction of mitochondrial NAD^+ coupled with the aerobic oxidation of tetramethyl-*p*-phenylenediamine is inhibited by azide, antimycin, Amytal, dinitrophenol, arsenate and phosphate + phosphate acceptor, and is unaffected by oligomycin, arsenite and malonate. Oligomycin prevents the inhibition by arsenate or by phosphate + phosphate acceptor. In the presence of antimycin, mitochondrial NAD^+ becomes reduced when succinate and tetramethyl-*p*-phenylenediamine are added together .

7. The pathway of hydrogen transfer from tetramethyl-*p*-phenylenediamine to NAD^+ is discussed.

8. It is concluded that high-energy intermediates of oxidative phosphorylation

Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine.

generated in the cytochrome oxidase region can probably react at the cytochrome *b*–cytochrome *c* and NAD+–flavoprotein couples.

INTRODUCTION

In the first three papers of this series[1-3], it was shown that high-energy intermediates of oxidative phosphorylation are necessary for the synthesis of glutamate from α-oxoglutarate and ammonia in rat-liver mitochondria when either succinate or malate is the hydrogen donor. With succinate, energy is required in stoicheiometric amounts to reverse the respiratory chain between flavoprotein and NAD+ (refs. 2, 4). In contrast, high-energy intermediates are required in less than stoicheiometric amounts for the transfer of hydrogens from malate to α-oxoglutarate + ammonia[3,4]. The high-energy intermediates can be generated during the aerobic oxidation of the hydrogen donor via the respiratory chain, even when oligomycin is present to block the formation of ATP[1-3].

Since there are two energy-conservation sites in the respiratory chain between succinate and O_2, it is clearly desirable to determine whether both or only one can provide the energy for glutamate synthesis. The report by JACOBS[5] that P:O ratios approaching 1 for the cytochrome oxidase step can be obtained by using TMPD to reduce cytochrome *c* suggested a direct method of testing whether high-energy intermediates generated in the cytochrome oxidase region could be used for glutamate synthesis. By using TMPD as oxidizable substrate in the presence of antimycin and succinate, the energy-generating reaction is separated from the reduction of NAD+. The hydrogen atoms for the synthesis of glutamate from α-oxoglutarate + ammonia are provided by succinate and energy by the cytochrome oxidase step of the respiratory chain.

A preliminary account of studies of this system has appeared[6]. A similar system in which NAD+ reduction was measured directly instead of coupling the reaction with the synthesis of glutamate has been used by PACKER AND DENTON[7]. TMPD has also been used, in the absence of antimycin, as a donor of reducing equivalents for the energy-linked reversal of electron transfer from cytochrome *c* to NAD+ (refs. 8–10) and to ubiquinone[11] in the respiratory chain. That the respiratory chain could be reversed over the whole span NADH–cytochrome *c* was first shown by CHANCE AND FUGMANN[12], using ATP as source of energy.

In this paper, it is shown that the high-energy intermediates necessary for the synthesis of glutamate from α-oxoglutarate and ammonia in rat-liver mitochondria when either succinate or malate is the hydrogen donor can be generated during the aerobic oxidation of TMPD. In the absence of antimycin, TMPD can also provide reducing equivalents for the synthesis of glutamate, although relatively inefficiently. On the other hand, NAD+ in rabbit-heart sarcosomes is readily reduced by TMPD.

MATERIALS AND METHODS

Experiments with rat-liver mitochondria

The methods, materials and experimental procedure employed are described in the first paper of the series[1]. The reaction mixture contained 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer, 0.1 mM ADP, 25 mM sucrose (derived

from the mitochondrial suspension) and the additions indicated in the legends to the tables in a final volume of 1 ml. The final pH was 7.5 and the reaction temperature was 25°.

Experiments with rabbit-heart sarcosomes

The methods and experimental procedure employed have been described by SNOSWELL[13].

Incubations of the sarcosomes were carried out at 0° for 90 sec. The reaction mixture contained 0.22 M sucrose, 0.01 M EDTA (pH 7.4), sarcosomes and the additions indicated in the legends to the tables in a final volume of 1.25 ml. The mixture was agitated vigorously in order to ensure that the suspension did not become anaerobic during the incubation.

RESULTS

Glutamate synthesis coupled with the oxidation of TMPD in rat-liver mitochondria

TMPD as hydrogen donor. In all the experiments with rat-liver mitochondria, arsenite was added to prevent the oxidation of α-oxoglutarate. In the two experiments shown in Table I, only 0.1 µmole glutamate was synthesized when no hydrogen donor was added, and 0.5–0.7 µmole when TMPD (kept in the reduced state by ascorbate) was present. Antimycin inhibited glutamate synthesis in one experiment,

TABLE I

SYNTHESIS OF GLUTAMATE COUPLED TO THE AEROBIC OXIDATION OF SUCCINATE AND OF TMPD IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 1 mM arsenite, 2% ethanol, 10 µg oligomycin, 20 mM potassium phosphate buffer and 5.4 mg (Expt. 131) or 4.7 mg (Expt. 132) mitochondrial protein. Reaction time, 30 min.

Additions	Expt. 131			Expt. 132		
	Δ O (µatoms)	Δ Glutamate (µmoles)	Δ Aspartate (µmoles)	Δ O (µatoms)	Δ Glutamate (µmoles)	Δ Aspartate (µmoles)
None	0.8	0.12	0	0.6	0.13	0
Antimycin (4 µg)	0.9	0.13	0	—	—	—
Succinate (60 mM)	11.6	7.64	4.66	9.8	4.49	1.02
Succinate + antimycin	0.8	0.58	0	0.5	0.22	0.15
TMPD (0.3 mM) + ascorbate (20 mM)	14.1	0.68	0	15.1	0.46	0
TMPD + ascorbate + antimycin	18.4	0.22	0	13.5	0.46	0
Succinate + antimycin + TMPD + ascorbate	15.3	2.02	0.05	13.7	1.41	0.23

as would be expected if TMPD were the hydrogen donor, but was without effect in the other (Table I, line 6; see also Table IV). Amytal inhibited slightly (Table IV).

Succinate as hydrogen donor. Table I shows that when the aerobic oxidation of succinate was blocked by antimycin, glutamate synthesis was also inhibited (*cf.* ref. 2). In the presence of antimycin the amount of glutamate found when

succinate and TMPD were added together considerably exceeded the sum of the amounts found when the succinate and TMPD were added separately*. In Table II, it can be seen that the synthesis of glutamate in the succinate–antimycin–TMPD system is inhibited by Amytal or dinitrophenol, but is not significantly affected by oligomycin. In the experiments of Tables I and II, the amounts of aspartate found

TABLE II

EFFECT OF INHIBITORS ON GLUTAMATE SYNTHESIS COUPLED WITH THE AEROBIC OXIDATION OF TMPD IN THE PRESENCE OF SUCCINATE AND ANTIMYCIN IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM potassium phosphate buffer, 1 mM arsenite, 20 mM ascorbate, 0.3 mM TMPD, 60 mM succinate, 4 µg (Expt. 125), 3.3 µg (Expt. 133) or 2 µg (Expt. 134) antimycin, 2% ethanol, and 5.7 mg (Expt. 125), 4.4 mg (Expt. 133) or 6.0 mg (Expt. 134) mitochondrial protein. Reaction time, 30 min (Expt. 125) or 20 min (Expts. 133 and 134).

Expt.	Additions	Δ O (µatoms)	Δ Glutamate (µmoles)	Δ Aspartate (µmoles)
125	None	14.2	2.07	0.28
	Amytal (2 mM)	16.9	0.61	0
	Dinitrophenol (50 µM)	16.4	0.30	0
133	None	11.6	2.02	0.46
	Amytal (2 mM)	10.5	0.34	0
	Dinitrophenol (50 µM)	10.7	0.16	0.01
	Oligomycin (2.3 µg/mg protein)	10.6	1.94	0.50
	Oligomycin + Amytal	9.3	0.25	0.04
	Oligomycin + dinitrophenol	12.3	0.55	0.04
134	None	11.0	3.37	—
	Oligomycin (1.7 µg/mg protein)	9.7	3.70	—

represent the contribution of malate (derived from succinate) as hydrogen donor for the synthesis of amino acids from α-oxoglutarate and ammonia[1].

In the first paper of this series[1], it was shown that inorganic phosphate (or arsenate) was necessary for the synthesis of glutamate and aspartate coupled to the

TABLE III

EFFECT OF INORGANIC PHOSPHATE ON THE SYNTHESIS OF GLUTAMATE IN THE SUCCINATE–ANTIMYCIN–TMPD SYSTEM

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 60 mM succinate, 0.3 mM TMPD, 2–4 kg antimycin, 15–20 mM ascorbate, 1 mM arsenite, 2% ethanol, 10 µg oligomycin, and 5.1–6.4 mg mitochondrial protein. Reaction time, 20 min (Expt. 118) or 30 min.

Expt.	Δ Glutamate (µmoles)		Δ Aspartate (µmoles)	
	No P_i	20 mM P_i	No P_i	20 mM P_i
117	2.8	4.3	0.6	1.5
118	1.3	1.9	—	—
124	2.0	2.4	0	0.1
129	2.0	4.2	0.2	1.2

* Similar results were obtained when 2-heptyl-4-hydroxyquinoline-*N*-oxide (kindly provided by Dr. J. LIGHTBOWN) was used instead of antimycin to inhibit the respiratory chain in the cytochrome *b* region.

aerobic oxidation of succinate. The same is true of the succinate–antimycin–TMPD system (Table III), although quantitatively the effects of phosphate were less in this system.

Malate as hydrogen donor. The results of two experiments with malate as hydrogen donor for the synthesis of amino acids from α-oxoglutarate and ammonia are presented in Table IV. Antimycin or Amytal inhibited the synthesis of amino acids markedly (*cf.* ref. 3). This inhibition by either antimycin or Amytal could be overcome by adding TMPD.

TABLE IV

SYNTHESIS OF GLUTAMATE + ASPARTATE COUPLED WITH THE AEROBIC OXIDATION OF TMPD IN THE PRESENCE OF MALATE + ANTIMYCIN OR MALATE + AMYTAL IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM potassium phosphate buffer, 5 mM glutamate, 1 mM arsenite, 2% (Expt. 160) or 1% (Expt. 169) ethanol, 10 μg oligomycin and 4.5 mg (Expt. 160) or 7.1 mg (Expt. 169) mitochondrial protein. Reaction time, 20 min.

Expt.	*Substrate*	*Δ O (μatoms)*	*Δ Glutamate (μmole)*	*Δ Aspartate (μmoles)*	*Δ (Glutamate + aspartate) (μmoles)*
160	Malate (20 mM)	1.0	0.50	3.68	4.18
	Malate + antimycin (1 μg)	0.4	0.61	0.92	1.53
	TMPD (0.1 mM) + ascorbate (20 mM)	3.6	0.48	0.15	0.63
	TMPD + ascorbate + antimycin	4.0	0.50	0.08	0.58
	Malate + antimycin + TMPD + ascorbate	3.8	0.76	2.49	3.25
169	Malate (20 mM)	1.6	−0.32	5.77	5.45
	Malate + Amytal (2 mM)	0.1	0.56	0.22	0.78
	TMPD (0.1 mM) + ascorbate (20 mM)	3.7	0.78	0.14	0.92
	TMPD + ascorbate + Amytal	3.8	0.54	0.24	0.78
	Malate + Amytal + TMPD + ascorbate	4.8	0.96	4.88	5.84

The reduction of mitochondrial NAD^+ coupled with the oxidation of TMPD in rabbit-heart sarcosomes

In Table V the results of experiments are summarized in which the extent of reduction of NAD^+ was measured in fresh sarcosomes and after incubation with TMPD or with succinate. Both substrates brought about the reduction of NAD^+, but the extent of reduction was appreciably greater with TMPD.

The effect of inhibitors and of phosphate acceptor on the reduction of NAD^+ coupled with the aerobic oxidation of TMPD is shown in Table VI. The reduction was completely inhibited by azide, antimycin, dinitrophenol and arsenate and strongly inhibited by Amytal and phosphate acceptor. Oligomycin alone had no effect but partially prevented the inhibition by arsenate and by phosphate + phosphate acceptor. Table VI also shows that the reduction of NAD^+ coupled with the oxidation of TMPD was not inhibited by arsenite or malonate.

Table VII shows that, in the presence of antimycin, which inhibits the reduction of NAD^+ coupled with the aerobic oxidation of either succinate or TMPD, an extensive reduction of NAD^+ could be obtained when succinate and TMPD were added together. The reduction of NAD^+ that occurred in the presence of antimycin, succinate and TMPD was stimulated by oligomycin.

TABLE V

THE REDUCTION OF NAD^+ COUPLED WITH THE AEROBIC OXIDATION OF TMPD AND OF SUCCINATE IN RABBIT-HEART SARCOSOMES

The reaction mixture contained, in addition to the basic components, 4–8 mg sarcosomal protein and, where indicated, 0.24 mM TMPD + 12 mM ascorbate, or 40 mM succinate. The values given are the mean's with the range in brackets.

Experimental conditions	*No. of Expts.*	*NAD^+ (µmoles/g protein)*	*NADH (µmoles/g protein)*	*NAD^+ + NADH (µmoles/g protein)*	$\frac{NADH}{NAD^+ + NADH}$
Fresh sarcosomes	10	4.5 (3.2–7.7)	2.9 (1.8–4.6)	7.4 (5.7–9.5)	0.39 (0.19–0.55)
Incubated with TMPD	10	0.7 (0.5–1.4)	6.4 (4.8–8.1)	7.1 (5.4–8.6)	0.91 (0.81–0.98)
Incubated with succinate	4	1.2 (0.8–1.7)	5.1 (3.9–6.4)	6.3 (5.0–7.7)	0.80 (0.73–0.88)

TABLE VI

EFFECT OF INHIBITORS AND OF PHOSPHATE ACCEPTOR ON THE REDUCTION OF NAD^+ COUPLED WITH THE AEROBIC OXIDATION OF TMPD IN RABBIT-HEART SARCOSOMES

The reaction mixture contained, in addition to the basic components, 0.24 mM TMPD, 12 mM ascorbate and 4–8 mg sarcosomal protein. The reaction time was 90 sec at 0°. When oligomycin was used, the sarcosomes were preincubated with the inhibitor for 2 min at room temperature before the addition of TMPD + ascorbate.

Additions	*Concentration*	*Inhibition* (%)*
Azide	4 mM	100
Antimycin	3.4 µg/mg protein	100
Amytal	0.4 µmole/mg protein (2 mM)	49
Amytal	1.3 µmole/mg protein (4 mM)	83
Malonate	4 mM	0
Arsenite	1 mM	0
2,4-Dinitrophenol	0.1 mM	100
2,4-Dinitrophenol + Amytal	0.1 mM, 4 mM	100
Oligomycin	4.3 µg/mg protein	0
Arsenate	24 mM	100
Arsenate + oligomycin	24 mM, 3.9 µg/mg protein	35
ADP, P_i, glucose, hexokinase	0.1 mM, 20 mM, 20 mM and 150 units	79
ADP, P_i, glucose, hexokinase + oligomycin	0.1 mM, 20 mM, 20 mM, 150 units and 3.9 µg/mg protein	26

* The method of calculating the degree of inhibition may be illustrated with the example of Amytal as shown in line 4. $\frac{NADH}{NAD^+ + NADH}$ in the absence of substrate = 0.32 and with TMPD = 0.93. Therefore the reduction by TMPD = 0.61. $\frac{NADH}{NAD^+ + NADH}$ in the presence of Amytal = 0.30 and in the presence of TMPD plus Amytal = 0.40. Therefore the reduction produced by TMPD + Amytal = 0.40 − 0.30 = 0.10, or $\frac{0.10}{0.61} \times 100 = 17\%$ of that produced by TMPD alone, *i.e.* Amytal inhibited the TMPD reduction by 83%.

TABLE VII

REDUCTION OF NAD^+ BY TMPD AND BY SUCCINATE IN RABBIT-HEART SARCOSOMES AND THE EFFECT OF ANTIMYCIN

Experimental conditions as in Table V. Each value is the mean of two determinations.

Additions	$\frac{NADH}{NAD^+ + NADH}$
None	0.29
Antimycin (5.0 µg/mg protein)	0.24
Succinate (40 mM)	0.77
Succinate + antimycin	0.23
TMPD (0.24 mM) + ascorbate (12 mM)	0.88
TMPD + ascorbate + antimycin	0.24
Succinate + TMPD + ascorbate + antimycin	0.70
Succinate + TMPD + ascorbate + antimycin + + oligomycin (3.2 µg/mg protein)	0.79

DISCUSSION

The synthesis of glutamate from α-oxoglutarate and ammonia in rat-liver mitochondria with succinate as hydrogen donor can take place in the presence of antimycin by coupling the reduction with the aerobic oxidation of TMPD. The properties of the succinate–antimycin–TMPD system are similar to those of the system in which glutamate synthesis is coupled with the aerobic oxidation of succinate[1,2]. In both systems, the synthesis of glutamate is inhibited by dinitrophenol and Amytal, and the addition of α-oxoglutarate (+ ammonia) in the presence of phosphate acceptor and absence of oligomycin leads to a lowering of the P:O ratio, and to an increase in the oxygen uptake in the presence of oligomycin[4,6]. However, the increased O_2 uptake is smaller with the succinate–antimycin–TMPD system than that observed in the succinate system[2]. These results indicate that the transfer of hydrogens from succinate to α-oxoglutarate + ammonia in the succinate–antimycin–TMPD system represents an energy-linked reversal of the respiratory chain[14].

There is one quantitative difference between the two systems. In the succinate system, the synthesis of glutamate is greatly stimulated by oligomycin even in the absence of phosphate acceptor[2], while in the succinate–antimycin–TMPD system, oligomycin has only a slight effect. The reason for the lack of an effect of oligomycin in the latter system may be that the maximal synthesis of glutamate is limited by other factors, for instance by the presence of antimycin. It has been shown that antimycin at high concentrations has an inhibitory effect on the synthesis of glutamate with succinate as hydrogen donor and ATP as energy source which is not obviously related to its inhibitory action on electron transport in the cytochrome *b* region of the respiratory chain[2]. Table VIII shows that this inhibition is found in the succinate–antimycin–TMPD system as well. The requirement for phosphate (Table III) was also less marked in the succinate–antimycin–TMPD system than in the succinate system.

TABLE VIII

EFFECT OF ANTIMYCIN CONCENTRATION ON THE SYNTHESIS OF GLUTAMATE WITH SUCCINATE AS HYDROGEN DONOR IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 1 mM arsenite, 60 mM succinate, 2 mM potassium phosphate buffer, 1 % ethanol and 6.7 mg mitochondrial protein. Reaction time, 20 min. (Expt. 251).

Additions	*Antimycin (μg/mg protein)*	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*
ATP (10 mM)	0.07	0.7	1.85	0.49
ATP	0.75	0.6	1.07	0.21
TMPD (0.1 mM) + ascorbate (20 mM)	0.07	8.6	3.43	1.27
TMPD + ascorbate	0.75	8.6	2.45	0.37

These results and those of PACKER[7–9] and LÖW AND VALLIN[10] provide direct evidence that high-energy intermediates of oxidative phosphorylation generated in the cytochrome oxidase region can react at the NAD+–flavoprotein couple and bring about a reversal of the respiratory chain.

In the experiments with rabbit-heart sarcosomes, it was found that the re-

duction of NAD^+ coupled with the aerobic oxidation of TMPD was inhibited by azide, antimycin, Amytal, dinitrophenol and (in the absence of oligomycin) by arsenate, and was not affected by arsenite, malonate or oligomycin. The lack of inhibition by arsenite or malonate shows that endogenous substrate does not play a role (*cf.* ref. 13). These results show that TMPD can provide reducing equivalents for an energy-linked reversal of the respiratory chain from cytochrome *c* to NAD^+ (*cf.* refs. 9, 12, 15 and 16). The pathway followed by the reducing equivalents from TMPD is shown in Fig. 1. Ubiquinone, which is also reduced by TMPD in a reaction

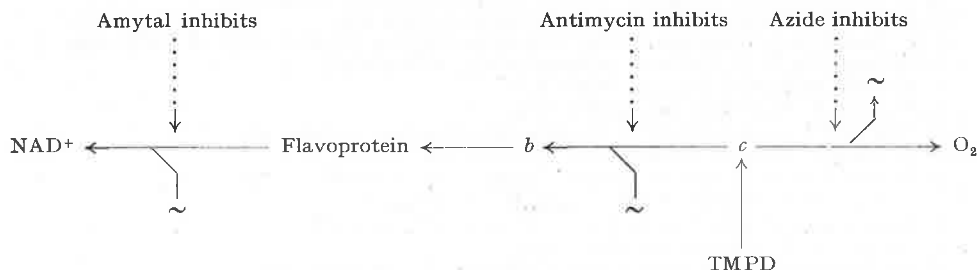


Fig. 1. Pathway for the reduction of NAD^+ by TMPD.

coupled to the oxidation of TMPD[11], may lie between cytochrome *b* and the flavoprotein. Since the reduction of NAD^+ by TMPD is inhibited by antimycin* and Amytal, the pathway probably involves the cytochrome *c*–cytochrome *b* and flavoprotein–NAD^+ couples, and it is at these couples that energy must be invested to reverse the respiratory chain. The energy is provided by the aerobic oxidation of TMPD, which explains why azide inhibits the reduction of NAD^+ in this system. Thus high-energy intermediates of oxidative phosphorylation generated in the cytochrome oxidase region are probably able to react not only at the NAD^+–flavoprotein couple, as the studies on glutamate synthesis in the succinate–antimycin–TMPD system had already indicated, but at the cytochrome *b*–cytochrome *c* couple as well. The mechanism of the reversal of the respiratory chain will be further discussed in the following paper[4]. The uncouplers, dinitrophenol and arsenate, inhibit by reacting with the high-energy intermediate (see ref. 17). In agreement with previous work[13,18,19], oligomycin prevents uncoupling by arsenate.

Evidence has been presented that the reduction of α-oxoglutarate and ammonia by malate in rat-liver mitochondria requires the participation of high-energy intermediates of oxidative phosphorylation. As shown in Table IV of the present paper, these intermediates can be generated in the cytochrome oxidase region.

* It is not known why antimycin is relatively ineffective in inhibiting the synthesis of glutamate linked with the aerobic oxidation of TMPD by rat-liver mitochondria in contrast with the complete inhibition of the reduction of sarcosomal NAD^+. The reduction of ubiquinone in beef-heart sarcosomes coupled with the aerobic oxidation of TMPD was also almost completely inhibited by antimycin[11]. It is possible that the antimycin-insensitive pathway (*cf.* ref. 17) is involved in the liver-mitochondria experiments, but it would not be expected that this would be reversible, since it is not phosphorylative. Alternatively, it is possible that the antimycin-sensitive site was not completely inhibited, and the residual activity was sufficient not to be rate-limiting in the very slow reduction of NAD^+ by TMPD. There was always a small antimycin-resistant glutamate synthesis in the succinate system (see Table I and refs. 1 and 2).

ACKNOWLEDGEMENTS

We wish to thank Miss B. KELDER for her expert technical assistance.

This work was supported by a grant from the Life Insurance Medical Research Fund.

REFERENCES

1 J. M. TAGER AND E. C. SLATER, *Biochim. Biophys. Acta*, 77 (1963) 227.
2 J. M. TAGER AND E. C. SLATER, *Biochim. Biophys. Acta*, 77 (1963) 246.
3 J. M. TAGER, *Biochim. Biophys. Acta*, 77 (1963) 258.
4 E. C. SLATER AND J. M. TAGER, *Biochim. Biophys. Acta*, 77 (1963) 276.
5 E. E. JACOBS, *Biochem. Biophys. Res. Commun.*, 3 (1960) 536.
6 J. M. TAGER, J. L. HOWLAND AND E. C. SLATER, *Biochim. Biophys. Acta*, 58 (1962) 616.
7 L. PACKER AND M. D. DENTON, *Federation Proc.*, 21 (1962) 53.
8 L. PACKER, *Federation Proc.*, 20 (1961) 49.
9 L. PACKER, *J. Biol. Chem.*, 237 (1962) 1327.
10 H. LÖW AND I. VALLIN, *Biochem. Biophys. Res. Commun.*, 9 (1962) 307.
11 H. PENEFSKY, *Biochim. Biophys. Acta*, 58 (1962) 619.
12 B. CHANCE AND U. FUGMANN, *Biochem. Biophys. Res. Commun.*, 4 (1961) 317.
13 A. M. SNOSWELL, *Biochim. Biophys. Acta*, 60 (1962) 143.
14 B. CHANCE AND G. HOLLUNGER, *Federation Proc.*, 16 (1957) 163.
15 B. CHANCE, *J. Biol. Chem.*, 236 (1961) 1544.
16 M. KLINGENBERG AND P. SCHOLLMEYER, *Biochem. Z.*, 335 (1961) 243.
17 E. C. SLATER, *Symp. on Intracellular Respiration: Phosphorylating and Non-Phosphorylating Oxidation Reactions, Proc. 5th Intern. Congr. Biochem., Moscow, 1961*, Vol. 5, Pergamon Press, London, 1963, p. 325.
18 R. W. ESTABROOK, *Biochem. Biophys. Res. Commun.*, 4 (1961) 89.
19 F. HUIJING AND E. C. SLATER, *J. Biochem. Tokyo*, 49 (1961) 493.

Reprinted from
Biochimica et Biophysica Acta
sevier Publishing Company
Amsterdam
rinted in The Netherlands

SC 63003

Energy requirements for the reduction of mitochondrial NAD+ by succinate: Externally added ATP as an energy source in aerobic systems

A number of workers[1–4] has shown that under certain conditions externally added ATP can supply the energy necessary for the reduction of mitochondrial NAD^+ by succinate. CHANCE AND ITO[5] suggested that ATP reacted with the respiratory chain at the phosphorylation site between NAD^+ and flavoprotein by reversal of the normal phosphorylation reactions. Such a role of ATP in *aerobic* systems has been queried by other workers[3,4] who suggest that ATP merely restores "coupling" of the mitochondria in these systems.

In aerobic experiments with pigeon-heart sarcosomes CHANCE[6] found that the ATP-induced reduction of NAD^+ was completely inhibited by antimycin A (see

also ref. 7) and suggested that besides inhibiting the respiratory chain between cytochromes *b* and *c*, antimycin A also inhibited electron flow on the NAD^+ side of cytochrome *b*. However, this cannot be so in view of evidence presented by PACKER AND DENTON[8], LÖW AND VALLIN[9] and TAGER *et al.*[10]. Thus the inhibition of the ATP-induced reduction by antimycin A must be explained in other terms.

In the experiments described in this paper pigeon-heart sarcosomes were prepared by the method previously described for the preparation of rabbit-heart sarcosomes[11]. It was found unnecessary to age the pigeon-heart sarcosomes in order to produce preparations which did not reduce NAD^+ in the absence of added ATP. NAD^+ reduction was determined as described previously[11]. The reduction of ferricyanide was recorded on a Model 350 Perkin–Elmer spectrophotometer at 420 mμ and at 25° according to the method of ESTABROOK[12].

The results shown in Table I indicate that ATP was necessary to induce reduction of NAD^+ by succinate, as found by CHANCE[6]. Pre-incubation of the sarcosomes

TABLE I

THE EFFECT OF ATP AND SERUM ALBUMIN ON THE REDUCTION OF SARCOSOMAL NAD^+ BY SUCCINATE

The reaction mixture contained 0.22 M sucrose, 0.01 M EDTA (pH 7.4), 5–9 mg sarcosomal protein and additions in a total volume of 1.25 ml. The reaction time was 90 sec at 0°, followed by 2 min at room temperature. The figures quoted are the average of 4 experiments with the range shown in brackets.

Addition	$\frac{NADH}{NAD^+ + NADH}$
None	0.00 (0.00–0.008)
40 mM succinate	0.01 (0.00–0.03)
1 mM ATP*	0.03 (0.00–0.05)
40 mM succinate + 1 mM ATP	0.16 (0.10–0.21)
0.4% serum albumin**	0.21 (0.13–0.30)
40 mM succinate + 0.4% serum albumin	0.45 (0.36–0.53)

* ATP was added after the initial 90 sec and the incubation continued for a further 2 min at room temperature.

** The sarcosomes were pre-incubated with the serum albumin for 6 min at room temperature before the 90-sec incubation at 0°.

with serum albumin induced an even greater degree of reduction by succinate. The reduction induced by serum albumin in the absence of succinate was probably due to reducing equivalents supplied by endogenous substrate as this effect was eliminated by 1 mM arsenite (*cf.* SNOSWELL[11]). The effects of ATP and serum albumin were not additive.

The ATP-induced reduction of NAD^+ by succinate was completely inhibited by antimycin A at 1.25 μg/mg protein (*cf.* CHANCE[6]) as was the albumin-induced reduction. The ATP-induced reduction was not inhibited by oligomycin over the range of concentrations of 1.25–3.2 μg/mg protein. The albumin-induced reduction was not inhibited by oligomycin concentrations up to 1.25 μg/mg but at higher concentrations the reduction was markedly inhibited by oligomycin (Fig. 1).

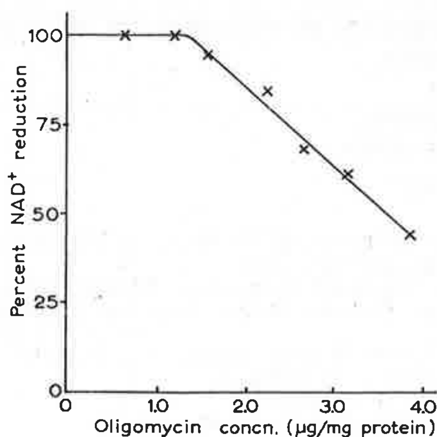


Fig. 1. Inhibition by oligomycin of the reduction of sarcosomal NAD^+ by succinate induced by serum albumin. Experimental conditions as in Table I. Oligomycin was added in ethanol, but the final concentration of ethanol did not exceed 2% and this was not inhibitory.

Pre-incubation of the pigeon-heart sarcosomes with serum albumin was found to restore coupling with α-oxoglutarate as substrate, as indicated by the three-fold increase in the rate of reduction of ferricyanide on the addition of ADP in contrast to only a 13% increase with the untreated sarcosomes (*cf.* ref. 1).

Thus it would appear from the results presented in this paper and those from other workers that ATP does not act as a direct energy source in the reduction of mitochondrial NAD^+ in *aerobic* systems for three reasons.

1. The fact that in the experiments reported here and in those described by KLINGENBERG AND SCHOLLMEYER[1,13] serum albumin as well as ATP restored the ability of succinate to reduce mitochondrial NAD^+ strongly suggests ATP does not act as a direct energy source in the reduction of NAD^+. ATP and serum albumin are known to prevent uncoupling caused by unsaturated fatty acids[14].

2. If ATP merely restores coupling in these pigeon-heart sarcosomes then the inhibition by antimycin A of the ATP and serum albumin-induced reduction can very simply be explained by the fact that antimycin A, by inhibition of the respiratory chain, prevents the formation of high-energy intermediates (necessary for NAD^+ reduction) during coupled succinate oxidation.

3. If ATP acts as a direct energy source in the reduction of NAD^+ its action should be blocked by oligomycin. In these experiments and those reported by ERNSTER[3] oligomycin had no effect on the ATP-induced reduction. CHANCE[6] reported that oligomycin (at 5 μg/mg protein) inhibited the ATP-induced reduction approx. 60%. Possibly, a considerable proportion of the inhibition found by CHANCE is due to a secondary effect of high concentration of oligomycin (*cf.* Fig. 1). These conclusions on the role of added ATP in the reduction of mitochondrial NAD^+ by succinate in *aerobic* systems certainly do not apply to the role of ATP in *anaerobic* systems.

The author is indebted to Professor SLATER and Dr. TAGER for copies of their manuscript prior to publication and to the Upjohn Co. for a gift of oligomycin. The skilful assistance of Mr. N. MUNK is gratefully acknowledged.

This work was supported by a Grant-in-Aid No. G157 from the Australian National Heart Foundation.

Department of Biochemistry,
University of New South Wales,
Sydney (Australia)

A. M. SNOSWELL

1 M. KLINGENBERG AND P. SCHOLLMEYER, *Biochem. Z.*, 333 (1960) 335.
2 B. CHANCE, *J. Biol. Chem.*, 236 (1961) 1544.
3 L. ERNSTER, *Symp. on Intracellular Respiration: Phosphorylating and Non-Phosphorylating Reactions, Proc. 5th Congr. Biochem., Moscow, 1961*, Vol. 5, Pergamon Press, London, 1963, p. 115.
4 E. C. SLATER AND J. M. TAGER, *Biochim. Biophys. Acta*, 77 (1963) 276.
5 B. CHANCE AND T. ITO, *Nature*, 195 (1962) 150.
6 B. CHANCE, *J. Biol. Chem.*, 236 (1961) 1569.
7 B. CHANCE AND T. ITO, *J. Biol. Chem.*, 238 (1963) 1509.
8 L. PACKER AND M. D. DENTON, *Federation Proc.*, 21 (1962) 53.
9 H. LÖW AND I. VALLIN, *Biochim. Biophys. Acta*, 69 (1963) 361.
10 J. M. TAGER, J. L. HOWLAND, E. C. SLATER AND A. M. SNOSWELL, *Biochim. Biophys. Acta*, 77 (1963) 266.
11 A. M. SNOSWELL, *Biochim. Biophys. Acta*, 60 (1962) 143.
12 R. W. ESTABROOK, *J. Biol. Chem.*, 236 (1961) 3051.
13 M. KLINGENBERG AND P. SCHOLLMEYER, *Biochem. Biophys. Res. Commun.*, 4 (1961) 38.
14 P. BORST, J. A. LOOS, E. J. CHRIST AND E. C. SLATER, *Biochim. Biophys. Acta*, 62 (1962) 509

Received September 16th, 1963

[Reprinted from Biochemistry, (1966) 5, 1660.]
Copyright 1966 by the American Chemical Society and reprinted by permission of the copyright owner.

Respiration-Dependent Proton Movements in Rat Liver Mitochondria*

Alan M. Snoswell

ABSTRACT: Rat liver mitochondria were found to extrude protons into the medium at a constant rate when incubated in various media. Proton extrusion was measured with a sensitive pH electrode system. The addition of adenosine diphosphate (ADP) to mitochondria respiring in a medium containing magnesium and phosphate caused an immediate halt in the liberation of protons and, as expected, H^+ concentration decreased during the phosphorylation of the ADP. In all media the extrusion of protons was dependent on respiration. It did not occur in the presence of antimycin or cyanide or in the absence of oxygen. It was not noticeably affected by the presence of other ions such as Mg^{2+}, $H_2PO_4^-$, Na^+, or K^+. The rate of proton extrusion varied between 10 and 100% of the rate of electron transport under varying conditions. Uncoupling agents increased the rates of both respiration and proton extrusion but substantially decreased the ratio of the rate of proton extrusion to the rate of electron transport. In the presence of cyanide and ferricyanide the rate of proton extrusion was approximately equal to the rate of electron transport. These results suggest that proton extrusion is a normal mitochondrial process and that the protons probably arise as direct products of respiration.

During the past few years a considerable number of reports have been published dealing with the translocation of various ions in mitochondrial preparations. While most of these reports have dealt with the respiration-dependent movement of Ca^{2+}, $H_2PO_4^-$, Mg^{2+}, Mn^{2+}, Na^+, and K^+, in a number of cases it was shown that there was an extrusion of protons from the mitochondria as other ions were taken up. It has been proposed that protons may be released from the mitochondria due to the deposition of insoluble salts within the mitochondria (*e.g.*, Brierley *et al.*, 1962) or by an exchange reaction with other cations (*e.g.*, Chappell and Greville, 1963; Chance, 1965). Such an exchange reaction for K^+ or Na^+ apparently requires the presence of an uncoupling agent (Moore and Pressman, 1964; Chappell and Crofts, 1965). These movements of protons are generally considered to be secondary reactions resulting from the movement of other cations. However, Chappell and Crofts (1965) suggested that a respiration-dependent H^+ pump mechanism might provide the underlying mechanism for mitochondrial ion movements. Mitchell (1963) has suggested that the separation of protons and OH^- ions on opposite sides of the mitochondrial membrane during respiration provides the driving potential for the synthesis of ATP.[1]

Thus in view of these suggestions and the fact that protons, either free or bound, are presumably produced during mitochondrial respiratory chain activity, it appeared important to study proton movements in respiring mitochondria *per se*.

Methods and Materials

Rat liver mitochondria were isolated by the method of Hogeboom (1955) as described by Myers and Slater

* From the Subdepartment of Veterinary Biochemistry, University of Melbourne, Victoria, Australia. *Received January 24, 1966.* This work was supported in part by a grant from the National Heart Foundation of Australia (G 157).

[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; TMPD, tetramethyl-*p*-phenylenediamine; DPN, 2-4-dinitrophenol.

(1957). The mitochondrial suspension, 35–45 mg of protein/ml, was stored at 0° and used within 4 hr. Mitochondrial protein was determined by the biuret method as described by Cleland and Slater (1953).

Oxygen utilization and H^+ concentration were measured simultaneously in a small water-jacketed reaction cell which was maintained at 30° and stirred with a magnetic stirrer. The volume of the reaction vessel was 2 ml and this volume completely filled the vessel. The electrodes passed through a tapered Teflon stopper which also contained a small hole (3.5-mm diameter) through which additions were made with 10-μl microsyringes.

Oxygen utilization was measured polarographically using a small Clark-type oxygen electrode (Titron Instrument Co., Sandringham, Australia) in conjunction with an Oxygraph, Model K (Gilson Medical Electronics, Wis.). The electrode was covered with 6 μ of Teflon membrane, a gift from Dr. I. A. Silver, which allowed a rapid response time (*ca.* 3 sec). Buffer solutions were calibrated for oxygen content according to the method of Chappell (1964).

H^+ concentration was measured with a small (4-mm o.d.) combination pH electrode (Titron Instrument Co., Sandringham, Australia) connected to a Radiometer pH meter, Model 27 (Radiometer, Copenhagen, Denmark). The electrolyte used in the electrode was saturated NaCl and the flow rate through the junction was only 1–2 μl/hr. The output voltage of the pH meter was first passed to a Rikadenki preamplifier, Model A-10 (Rikadenki Kogyo Co. Ltd., Tokyo, Japan), and then to a Heath servo-recorder, Model EUW-20 AE (Heath Co., Benton Harbour, Mich.). A suitable resistance network was used to match the output of the pH meter with the preamplifier over the desired range and a suitable backing-off circuit enabled a pH change of anywhere between 0.01 and 1.0 unit to give a full scale deflection on the recorder, starting at any initial pH value in the range 7.0–7.6. Changes in H^+ concentration were not determined by reference to the pH in view of the varying buffer capacities of the different media used and the suspension errors mentioned by Bartley and Davies (1954) which were found to be considerable in some of the weakly buffered media. Instead H^+ concentration was determined directly by reference to the recorder deflection caused by small additions of hydrochloric acid (3–10 μl of 50 mM HCl) at the end of each experiment, according to the method described by Nishimura *et al.* (1962). A weakly buffered medium composed of 0.17 M sucrose and 40 mM choline chloride described by Chappell and Crofts (1965) was found to be most satisfactory for recording small changes in H^+ concentration. In this medium some additions caused artefacts in the recording, as determined by control experiments with no mitochondria, and where these occurred a break has been left in the trace illustrated.

Ferricyanide reduction was followed in a Shimadzu recording spectrophotometer, Model SV 50 A (Seisakusho Ltd., Kyoto, Japan), at 420 mμ and 30°. The author is indebted to the C. S. I. R. O., Division of Animal Health, Parkville, Australia, for the use of this instrument. Incubation medium plus mitochondria were placed in both the reference and sample cuvets and the reaction was started by the addition of 15 μl of freshly prepared 0.2 M potassium ferricyanide to the sample cuvet.

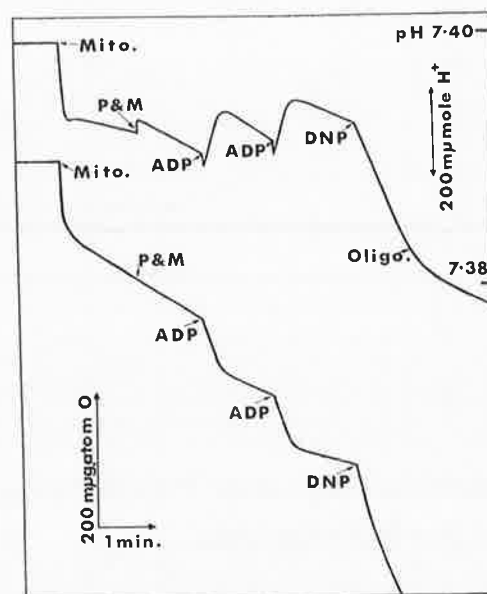


FIGURE 1: The effect of various additions on oxygen uptake and H^+ concentration in respiring preparations of rat liver mitochondria. The incubation mixture contained 50 mM sucrose, 130 mM KCl, 7.5 mM $MgCl_2$, and 30 mM sodium potassium phosphate buffer, pH 7.40, in a volume of 2.0 ml; temperature 30°. Additions in the order indicated were, mitochondria (equivalent to 4.2 mg of protein), 2 mM Tris–pyruvate plus 2 mM Tris–malate, 183 mμmoles of ADP (the same amount in both additions), 5×10^{-5} M 2,4-DNP, and oligomycin (equivalent to 2.2 μg/mg of mitochondrial protein). The upper trace represents the recording of H^+ concentration, a downward deflection indicating an increase in concentration. The lower trace represents the recording of oxygen concentration, a downward deflection indicating an uptake of oxygen.

Reagents. ADP was purchased from the Sigma Chemical Co. and the actual ADP content was determined by the method of Slater (1953). Antimycin A was purchased from the Kyowa Fermentation Co., Tokyo, Japan, and the oligomycin was a gift from the Upjohn Co., Mich. All substrates were AR grade acids which were neutralized with Tris.

Results

Response and Accuracy of the Apparatus for Recording Small Changes in H^+ Concentration. It seemed desirable to assess the response of the apparatus to various additions and changes in mitochondrial respiration and to determine the accuracy of the recording system at high amplification. Since during the formation of ATP from

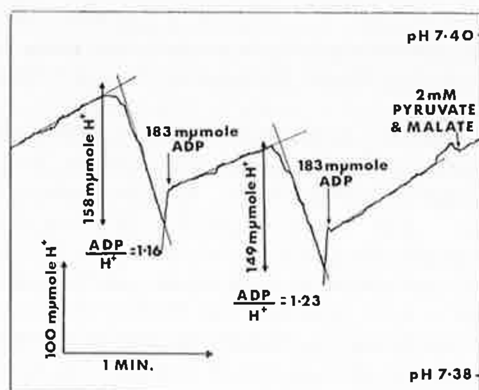


FIGURE 2: The effect of ADP on H+ concentration in a phosphorylating preparation of rat liver mitochondria. The figure shows an actual recorded trace with tangents drawn on the trace subsequently; the intersection of these tangents indicates the exact point at which the decrease in H+ concentration, caused by the ADP additions, ceases. Other experimental conditions as in Figure 1.

ADP there is an uptake of H+ ions according to the equation

$$ADP + P_i + nH^+ \rightleftharpoons ATP + H_2O \quad (1)$$

it was decided to examine the changes in H+ concentration of respiring rat liver mitochondrial preparations in response to small additions of ADP in a medium capable of supporting phosphorylation. Nishimura *et al.* (1962) have calculated theoretically the value of n, which equals $\Delta H^+/\Delta P_i$ in eq 1, as 0.851 at pH 7.4, assuming magnesium complexes of the adenine nucleotides, and 0.891 assuming no such complexes.

The results shown in Figure 1 indicate clearly that there is a decrease in H+ concentration which is initiated by the addition of ADP and continues while the ADP is phosphorylated (*cf.* Swanson, 1957). The decrease in H+ concentration ceases at exactly the same time as all the ADP is phosphorylated, this point being indicated by the cutoff point in the oxygen trace. This is followed by a reversion to the slower increase in H+ concentration which is dealt with in the next section. The exact point at which the decrease in H+ concentration ceased was determined by the intersection of tangents drawn on the trace as shown in Figure 2. This method is analogous to the one used by Chance and Williams (1955) to determine the exact amount of oxygen utilized during the phosphorylation of a small amount of added ADP.

Values for n, here $\Delta H^+/\Delta ADP$, at pH 7.40 were determined for three different substrates, *i.e.*, pyruvate plus malate, succinate, and glutamate (15 estimations in all). The average value of n was found to be 0.878 with a standard deviation of 0.066, which is in excellent agreement with the results of Nishimura *et al.* (1962) who found an experimental value of 0.882, with a

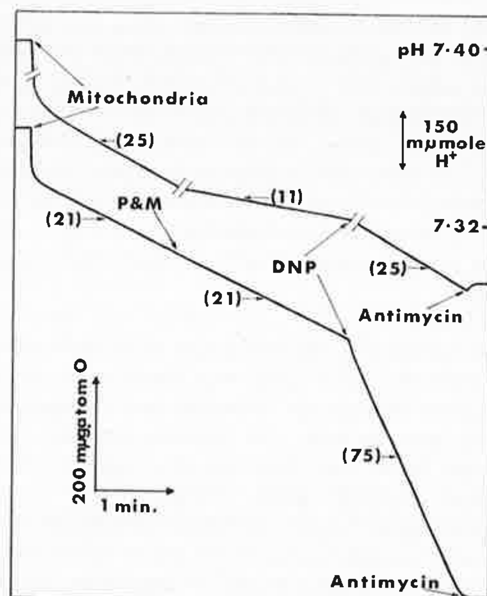


FIGURE 3: The effect on DNP and antimycin on oxygen uptake and H+ concentration in a respiring preparation of rat liver mitochondria. The incubation medium contained 0.17 M sucrose and 40 mM choline chloride adjusted to pH 7.4 with Tris; temperature 30°. Additions in the order indicated were, mitochondria (equivalent to 4.4 mg of protein), 2 mM Tris–pyruvate plus 2 mM Tris–malate, 5×10^{-5} M 2,4-DNP, and antimycin (2.1 μg/mg of mitochondrial protein). The traces are as described in Figure 1. The figures in brackets in relation to the upper trace represent the rate of increase in H+ concentration in millimicromoles of H+ per minute per milligram of mitochondrial protein and in relation to the bottom trace represent rates of oxygen uptake in millimicrogram-atoms of oxygen per minute per milligram of mitochondrial protein.

standard deviation of 0.071, for photophosphorylation in *Rhodospirillum rubrum* chromatophores.

Thus it appears from the results shown in Figures 1 and 2 and the above calculations that the apparatus is capable of responding to rapid changes in H+ concentration and that the recorded changes are quite accurate at high amplification.

Proton Extrusion from Mitochondria during Respiration. The results of a typical experiment are shown in Figure 3 and show that the H+ concentration of the medium increased at a constant rate when rat liver mitochondria were added to 0.17 M sucrose and 40 mM choline chloride at 30°. The rate was decreased by the addition of pyruvate plus malate and increased by the subsequent addition of 2,4-DNP. The addition of antimycin immediately stopped the increase in H+ concentration. As the increase in H+ concentration occurs only on the addition of the mitochondria it is considered that this represents an extrusion of protons from the mitochondria. In the experiment shown in Figure 3 the rate of proton extrusion was 11 mμmoles of H+/min

per mg of mitochondrial protein when the substrate oxidized was pyruvate plus malate while the rate of oxygen uptake was 21 mμg-atoms/min per mg of mitochondrial protein. If two electrons are transferred per oxygen atom utilized, the rate of the proton extrusion ie *ca.* 25% of the rate of electron transport. In different sxperiments in various media the rate of proton extrusion was found to vary between 25 and 70% of the rate of electron transport with pyruvate plus malate as substrate.

A. THE EFFECT OF VARIOUS INCUBATION MEDIA. The 0.17 M sucrose plus 40 mM choline chloride medium of Chappell and Crofts (1965) was found to be the most satisfactory medium for recording small changes in H^+ concentration as with this medium respiratory rates remained linear and there was no evidence of mitochondrial swelling. While changes similar to those shown in Figure 3 were recorded in unbuffered sucrose there was too much drift in the pH to allow accurate readings. The results obtained in a medium containing 50 mM sucrose, 130 mM KCl, 7.5 mM $MgCl_2$, and 30 mM sodium potassium phosphate, pH 7.4, as shown in Figure 1, were also similar. However, the effects of uncoupling agents on the rate of proton extrusion were complex and are considered in a later section.

B. THE EFFECT OF ADDED CATIONS. It is important to note that in the type of experiment shown in Figure 3 there are no added cations (with the exception of choline ions) in the incubation medium. The addition of 1.5 mM $MgCl_2$ plus 1.5 mM sodium phosphate, pH 7.4, or 6.5 mM KCl had virtually no effect on the rate of proton extrusion.

C. THE EFFECT OF VARIOUS SUBSTRATES. While the addition of pyruvate plus malate or glutamate had little effect on the rate of respiration, both reduced the rate of proton extrusion. The addition of succinate caused a greater reduction in the rate of proton extrusion, but at the same time slightly increased the rate of respiration (Table I).

It was of considerable interest to know whether oxidation in the terminal region of the respiratory chain also supported proton extrusion. A combination of ascorbate and TMPD (N,N,N′,N′-tetramethyl-*p*-phenylenediamine), first introduced by Jacobs (1960), provides an efficient electron donor system at the cytochrome *c* level. However this electron donor system could not be used successfully in these experiments as the oxidation of ascorbate is accompanied by a decrease in H^+ concentration due to the formation of dehydroascorbate and the consequent loss of the acidic enol group at position 3 of the molecule. This was demonstrated experimentally as the addition of antimycin, which blocked endogenous respiration and its associated proton extrusion, actually enhanced the rate of decrease in H^+ concentration with ascorbate and TMPD as substrate.

D. THE EFFECT OF RESPIRATORY INHIBITORS. Both antimycin A (at *ca.* 1 μg/mg of mitochondrial protein) and cyanide, 10^{-2} M, prevented the extrusion of protons immediately. Anaerobiosis had a similar effect. The phosphorylation inhibitor, oligomycin, had no noticeable effect on the rate of extrusion, except in special circumstances as mentioned below.

E. THE EFFECT OF UNCOUPLING AGENTS. A variety of uncoupling agents, *viz.*, 5×10^{-5} M 2,4-DNP, 10^{-5} M dicoumarol, and sodium oleate (20 μg/mg of mitochondrial protein), all considerably enhanced the rate of proton extrusion. Triton-X 100 at a concentration of 9.0 μg/mg of mitochondrial protein completely stopped the proton extrusion while respiration was increased slightly. The effect of 2,4-DNP is shown in Figure 3. The addition of DNP increased the rate of proton extrusion some 2.5-fold. However, although these uncoupling agents markedly increased the respiration rate (in the order of five–sixfold) the rate of proton extrusion compared with the rate of electron transport was significantly less in all cases than it was in the absence of the uncoupling agent. Considering the results shown in Figure 3, the rate of proton extrusion is equivalent to *ca.* 17% of the rate of electron transport in the presence of DNP compared with 30% before the addition of the DNP (see above for calculations).

In experiments carried out in the phosphorylating medium, such as in Figure 1, if DNP or the other uncoupling agents were added to the reaction mixture after the ADP additions there was a very marked increase in the rate of proton extrusion, up to 20-fold in some cases. Because this increase was markedly inhibited by oligomycin (Figure 1) and was not completely inhibited by antimycin it is probable that it was due mainly to the induced breakdown of preformed ATP and the consequent release of protons by a reversal of the reaction shown in eq 1. The increase in the rate of proton extrusion caused by the addition of the uncoupling agent was not nearly so marked if ADP had not been added previously. In this case oligomycin had

TABLE I: A Comparison between Rates of Oxygen Uptake and Proton Extrusion in Preparations of Rat Liver Mitochondria Oxidizing Different Substrates.[a]

Substrate	Oxygen Uptake (mμg-atoms/min mg of protein)	Proton Extrusion (mμmoles/min mg of protein)
Endogenous	14.0	16.7
2 mM glutamate	14.0	9.8
2 mM succinate	22.0	3.9
2 mM pyruvate plus 2 mM malate	13.9	9.7
2 mM pyruvate plus 2 mM malate plus 5×10^{-5} M DNP	73.0	25.5

[a] Experimental conditions as described in Figure 3. The figures are the average of three experiments.

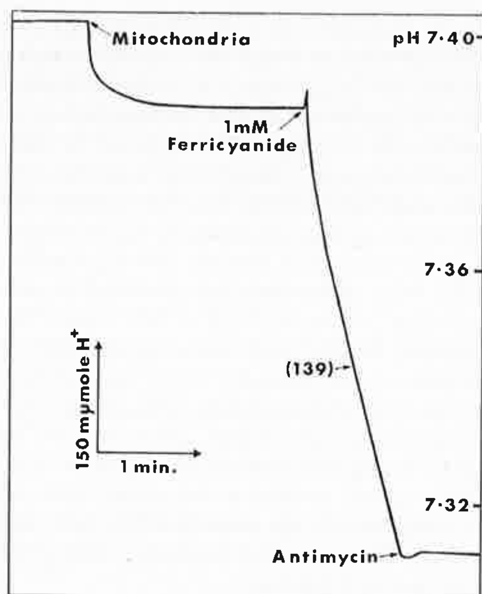


FIGURE 4: The effect of ferricyanide and antimycin on H^+ concentration in a respiring preparation of rat liver mitochondria. The incubation mixture contained 0.22 M sucrose, 25 mM Tris–chloride (pH 7.4), 10 mM KCN, and 2 mM Tris–succinate in a volume of 2 ml. Additions in the order indicated were, mitochondria (equivalent to 4.0 mg of protein), 1 mM potassium ferricyanide, and antimycin (2.5 μg/mg of mitochondrial protein). The figure in brackets represents the rate in decrease in H^+ concentration in millimicromoles per minute per milligram of mitochondrial protein.

little effect and antimycin completely stopped the proton extrusion.

F. THE EFFECT OF CYANIDE PLUS FERRICYANIDE. Ferricyanide may be used as an alternative electron acceptor to oxygen in the presence of cyanide, and electrons are accepted predominantly at the cytochrome *c* level (Estabrook, 1961). The results shown in Figure 4 indicate that the addition of mitochondria to a medium containing sucrose, Tris–chloride (pH 7.4), succinate, and 10^{-2} M cyanide caused a slight drop in pH but there was no extrusion of protons after the initial equilibration period. The addition of 1 mM ferricyanide caused a very rapid extrusion of protons which, after a short time, reached a constant rate of 139 mμmoles of H^+/min per mg of mitochondrial protein. The extrusion was completely stopped by the addition of antimycin. The experiment was repeated immediately, the rate of ferricyanide reduction being determined in a recording spectrophotometer. It was 147 mμmoles of ferricyanide reduced/min per mg of mitochondrial protein. Thus the rate of proton extrusion was 95% of the rate of electron transport, since ferricyanide is a one-electron acceptor. In a number of other experiments carried out under these conditions it was found that the rate of proton extrusion was approximately equal to the rate of electron transport.

Discussion

The results presented in this paper indicate that there is a constant increase in H^+ concentration of the medium when rat liver mitochondria are respiring. Similar observations have been made by other workers (see Figure 8 of Pressman, 1963, and Figure 2 of Brierley *et al.*, 1964). However, because of the preoccupation of these workers with other facets of mitochondrial ion movements these observations were not pursued. As the increase in H^+ concentration only occurs following the addition of mitochondria it is considered that this phenomenon represents an extrusion of protons from the mitochondria. A similar conclusion was very recently reached by Mitchell and Moyle (1965a) as a result of their ingenious experiments involving pH measurements following the addition of small amounts of oxygen to anaerobic suspensions of rat liver mitochondria. This concept of a respiration-dependent extrusion of protons then raises the question as to what is the source of protons within the mitochondria?

First, H^+ ions could be released by the breakdown of endogenous ATP according to eq 1. Koivusalo and Slater (1966) have recently determined the amount of reactive high-energy intermediates initially present in rat liver mitochondria to be equivalent to 0.20 μmole/mg of protein. If one assumed this is all in the form of ATP, or can be converted to ATP, then there are some 200 mμmoles of ATP/mg of protein which could give rise to an equivalent amount of H^+. This would be sufficient to maintain a rate of proton extrusion of 10 mμmoles of H^+/min per mg of protein for 20 min. It is, however, unlikely that ATP would break down during active respiration. Also, in experiments not recorded here the proton extrusion was found to continue for 1 hr or more providing respiration continued. Furthermore, if ATP breakdown were the source of protons one would expect oligomycin to inhibit this extrusion, which it does not. Also, it is difficult to see why the addition of a respiratory inhibitor, such as antimycin, should immediately stop proton extrusion. Indeed one would expect that if respiration were blocked, the rate of breakdown of endogenous ATP would increase, *i.e.*, native ATPase activity, and hence proton extrusion would be stimulated under these conditions (see Ter Welle and Slater, 1964).

Second, protons could be released from the mitochondria as other ions are taken up in an ion exchange reaction as indeed has been reported in a number of cases (Brierley *et al.* 1962; Chappell and Greville, 1963; Chance, 1965). However, in the experiments in sucrose–choline chloride medium described here there is no obvious source of cations in the medium for exchange reactions. Choline would be present in the form of a complex organic cation but the exchange reactions demonstrated have involved simple monovalent and divalent cations. Also the release of protons was observed when the mitochondria respired in 0.25 M sucrose although the actual measurement of the rates of proton extrusion were unreliable in this medium. Furthermore, the addition of ions such as Mg^{2+}, $H_2PO_4^-$, and K^+,

which have been shown to be involved in mitochondrial exchange reactions for protons (Brierley *et al.*, 1962; Moore and Pressman, 1964), had little or no effect on the rate of proton extrusion. Also in the experiments described here the addition of uncoupling agents increased the rate of proton extrusion, whereas the uptake of cations and the consequent release of protons is completely prevented by uncoupling agents (Lehninger, 1964). Finally, in reported experiments where protons were released from the mitochondria as a result of exchange with other ions, the subsequent addition of an uncoupling agent or antimycin caused an immediate increase in pH of the incubation medium as protons reentered the mitochondria and the previously absorbed cations were expelled (*e.g.*, see Moore and Pressman, 1964). In the present experiments, the addition of an uncoupling agent or antimycin, following a period in which there was a steady decrease in pH, was not accompanied by an increase in pH. The addition of antimycin, *e.g.*, merely stopped the decrease in pH. Thus the respiration-dependent extrusion of protons observed here would not appear to be due to the release of protons by an ion-exchange mechanism. However, there is the possibility that loosely bound cations such as magnesium and also phosphate may give rise to exchange reactions and a secondary movement of protons.

A third possible method by which protons may be produced in the mitochondria is directly as a product of electron transport. Mitchell (1963) has been a keen proponent of such a mechanism as providing the appropriate potential across the mitochondrial membrane to drive the formation of ATP. The results presented here certainly indicate that the proton extrusion is dependent on respiration as it is immediately stopped by the addition of antimycin or cyanide. In recent experiments Mitchell and Moyle (1965a) have demonstrated the respiration-dependent release of protons from mitochondria. Also in the present experiments, in the presence of cyanide and ferricyanide, the rate of proton extrusion was virtually the same as the rate of electron transport with succinate as substrate. The oxidation of substrates like glutamate and pyruvate plus malate, which should give rise to three protons per pair of electrons transferred during respiration, was associated with a greater rate of proton extrusion than the oxidation of succinate which gives rise to only two protons per pair of electrons. These latter findings are of particular interest in view of the recent experimental findings of Mitchell and Moyle (1965a). They found $\Delta H^+/O$ (*i.e.*, protons released per oxygen utilized) for succinate oxidation of four, and six for the oxidation of β-hydroxybutyrate.

Thus it appears that the most logical explanation of the respiration-dependent proton extrusion reported here is that protons are produced as a direct product of electron transport. A similar conclusion has recently been reached by Mitchell and Moyle (1955a) as a result of extensive studies on proton release associated with the uptake of small amounts of oxygen in rat liver mitochondria. Mitchell and Moyle (1965b) have provided additional support for this conclusion with their important findings with phosphorylating submitochondrial particles in which the respiration-dependent proton flow was in the reverse direction. Thus the concept of a H^+ pump mechanism, as suggested by Chappell and Crofts (1965), is now supported by some experimental evidence. Whether such a mechanism provides the underlying driving force for all mitochondrial ion transport, as also suggested by these authors, remains to be seen. Also whether this mechanism provides the basic electrochemical potential to generate ATP as suggested by Mitchell (1963) requires further investigation. The fact that in the present experiments the addition of ADP caused a halt in the proton extrusion which resumed immediately when all the ADP was phosphorylated is inconclusive. The very rapid uptake of protons during the phosphorylation of ADP (eq 1) may have merely masked a continuing slow rate of proton extrusion, or the extrusion may have stopped while the ADP was phosphorylated. This point requires further investigation.

Acknowledgments

The author is indebted to Dr. J. B. Chappell for advice and encouragement in the initial stages of this work and to Mr. L. Powe of Titron Instruments for invaluable help in the construction of the electrode system.

References

Bartley, W., and Davies, R. E. (1954), *Biochem. J. 57*, 37.

Brierley, G. P., Bachmann, E., and Green, D. E. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1928.

Brierley, G. P., Murer, E., and O'Brien, R. L. (1964), *Biochim. Biophys. Acta 88*, 645.

Chance, B. (1965), *J. Biol. Chem. 240*, 2729.

Chance, B., and Williams, G. R. (1955), *Nature 175*, 1120.

Chappell, J. B. (1964), *Biochem. J. 90*, 225.

Chappell, J. B., and Crofts, A. R. (1965), *Biochem. J. 95*, 393.

Chappell, J. B., and Greville, G. D. (1963), *Symp. Biochem. Soc. 23*, 39.

Cleland, K. W., and Slater, E. C. (1953), *Biochem. J. 53*, 547.

Estabrook, R. W. (1961), *J. Biol. Chem. 236*, 3051.

Hogeboom, G. H. (1955), *Methods Enzymol. 1*, 16.

Jacobs, E. E. (1960), *Biochem. Biophys. Res. Commun. 3*, 536.

Koivusalo, M., and Slater, E. C. (1966), *Biochem. Z.* (in press).

Lehninger, A. L. (1964), *in* The Mitochondrion, New York, N. Y., Benjamin, p 157.

Mitchell, P. (1963), *Symp. Biochem. Soc. 22*, 142.

Mitchell, P., and Moyle, J. (1965a), *Nature 208*, 147.

Mitchell, P., and Moyle, J. (1965b), *Nature 208*, 1205.

Moore, C., and Pressman, B. C. (1964), *Biochem. Biophys. Res. Commun. 15*, 562.

Myers, D. K., and Slater, E. C. (1957), *Biochem. J. 67*,

558.
Nishimura, M., Ito, T., and Chance, B. (1962), *Biochim. Biophys. Acta 59*, 177.
Pressman, B. C. (1963), *in* Energy-linked Functions of Mitochondria, Chance B., Ed., New York, N. Y., Academic, p 181.
Slater, E. C. (1953), *Biochem. J. 53*, 157.
Swanson, M. A. (1957), *Federation Proc. 16*, 258.
Ter Welle, H. F., and Slater, E. C. (1964), *Biochim. Biophys. Acta 89*, 385.

Reprinted from
Biochimica et Biophysica Acta
Elsevier Publishing Company
Amsterdam
Printed in The Netherlands

BBA 45609

THE USE OF A UBIQUINONE-DEFICIENT MUTANT IN THE STUDY OF MALATE OXIDATION IN *ESCHERICHIA COLI*

G. B. COX*, A. M. SNOSWELL AND F. GIBSON*

School of Microbiology and School of Veterinary Science, University of Melbourne, Melbourne (Australia)

(Received March 10th, 1967)
(Revised manuscript received June 12th, 1967)

SUMMARY

1. Malate oxidation catalyzed by sub-cellular fractions of a normal strain of *Escherichia coli* and a mutant strain unable to form ubiquinone has been compared.

2. The system catalyzing the aerobic oxidation of malate was localized in a membranous small particle fraction separated by $(NH_4)_2SO_4$ fractionation following disruption of the cells in a French pressure cell.

3. Comparison of malate oxidation catalyzed by particles from normal and mutant cells indicates that ubiquinone is concerned in malate oxidation. Malate oxidation proceeds at about half the normal rate in particles from cells lacking ubiquinone.

4. Malate oxidation catalyzed by small particles from cells lacking ubiquinone was insensitive to the low concentrations of dicoumarol which inhibited oxidation catalyzed by particles from normal cells. Malate oxidation catalyzed by particles from cells lacking vitamin K was even more sensitive to dicoumarol than that catalyzed by particles from normal cells. Therefore dicoumarol at low concentrations is not acting as a vitamin K antagonist.

INTRODUCTION

Ubiquinone is found in particulate structures such as mitochondria and bacterial chromatophores and this has led to the suggestion that the quinone is involved in electron transfer processes. The role played by ubiquinone in mitochondria has been subject to intensive research in recent years. (For summary and references see ref. 1.) The work of GREEN AND BRIERLEY[2] would suggest that ubiquinone plays a major part in electron transport processes while that of CHANCE and REDFEARN and their colleagues[3,4] suggests that ubiquinone may be concerned in reversed electron transport or a by-pass rather than on the main pathway of electron transport to oxygen. KRÖGER AND KLINGENBERG[5] suggest that ubiquinone is concerned in both forward and reversed pathways of electron transport.

Ubiquinone function in bacterial electron transport systems has not received

* Present address: Biochemistry Department, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia.

as much attention as in the mitochondrial systems. KNOWLES AND REDFEARN[6] have examined ubiquinone function in *Azotobacter vinelandii* using a technique which has been used with mitochondria, namely acetone extraction. Although activity was lost following extraction, it was not possible to restore oxidative activity by adding ubiquinone. KASHKET AND BRODIE[7,8] have studied ubiquinone function in *Escherichia coli*, an organism which contains both ubiquinone and vitamin K and concluded that ubiquinone is involved in succinate oxidation while vitamin K is involved in NAD-linked substrate oxidations.

It has been suggested previously that multiple aromatic auxotrophs could be grown in such a way as to provide cells deficient in the quinones and that such cells might be of use in the study of quinone function[9]. A more satisfactory method would be to obtain mutants which could not form ubiquinone and compare such strains with normal cells. Such a mutant of *E. coli* K12 (AB3285), which is unable to carry out the first specific step in ubiquinone biosynthesis, namely the conversion of chorismate into 4-hydroxybenzoate, has been isolated[10]. Part of the procedure used for the isolation of the mutant strain unable to form ubiquinone was to select strains unable to use malate as sole carbon source. It was observed, however, that the mutant still oxidized malate, suggesting that energy yielding processes might have been affected. The following experiments examine the relationship between ubiquinone and malate oxidation using sub-cellular fractions of Strain AB3285 and a revertant (AB3290) obtained from it.

METHODS

Organisms and media

The strains of *E. coli* K12 used were AB3285, AB3290 and AB3291. These strains are described in detail elsewhere[10]. Briefly, Strain AB3285 is a mutant unable to use malate as sole carbon source and which does not form detectable amounts of ubiquinone. This strain forms about 5 times the amount of vitamin K found in normal cells. The mutation in Strain AB3285 affects the first specific step in ubiquinone biosynthesis, namely the conversion of chorismate to 4-hydroxybenzoate.

Strain AB3290 is a revertant obtained from AB3285 which simultaneously regained the ability to grow on malate medium and to form ubiquinone and will be referred to in this paper as the 'normal strain'.

Strain AB3291 is a mutant unable to form detectable amounts of vitamin K but forming about 3 times the normal level of ubiquinone.

Cells were grown in a 0.5 % (w/v) glucose–mineral salts medium[11].

Chemicals

Chemicals used were obtained commercially and were not further purified. Ubiquinone (Q-2) was kindly provided by Merck, Sharp and Dohme, U.S.A. and piericidin A by Prof. S. TAMURA, Department of Agricultural Chemistry, University of Tokyo. L-Malate was used throughout except where otherwise indicated.

Preparation of sub-cellular fractions

Cells were grown as 1-l cultures in 2-l flasks, shaken on a New Brunswick gyro rotary shaker at 37°. Cultures were harvested in mid-exponential phase (about

0.6 mg dry wt./ml) and washed once in about 200 ml of cold potassium phosphate buffer (0.1 M, pH 7.0) per l of culture. The washed cells were resuspended in the above buffer (1 ml buffer/0.5 g wet wt. cells) and smashed in a French pressure cell at 20000 lb/inch^2. Examination by phase contrast microscopy indicated that these conditions caused disruption of almost all of the cells. The disrupted cells were then centrifuged at 25000 $\times$ *g* for 15 min to remove any whole cells and large pieces of membrane. The deposit will be referred to as the 'large particle' fraction. A 'small particle' fraction was separated from the supernatant by adding solid $(NH_4)_2SO_4$ slowly to give 20 % satn. and the solution was stirred for a further 20 min to ensure equilibration. The precipitate was collected following centrifugation at 25000 $\times$ *g* for 15 min and resuspended in 1 ml of phosphate buffer (0.1 M, pH 7) for each original gram wet weight of cells. Solid $(NH_4)_2SO_4$ was then added to the supernatant to give 80 % satn. and the precipitate collected as above. All operations on the washed cells and cell fractions were carried out at 0–4°. Proteins were estimated with Folin's phenol reagent[12].

Determination of quinone content of particles

The large particle fraction and the small particle fraction (as the $(NH_4)_2SO_4$ precipitate) were placed in Soxhlet thimbles, and the quinones extracted and chromatographed as described previously[9]. The yellow quinone bands were scraped off and eluted with diethyl ether. Absorption spectra of the solutions were measured in 1-cm cells in a Cary Model 11 spectrophotometer between 230 and 360 mμ to confirm the identity of the quinones. The absorbance at 248 mμ was used to estimate vitamin K and that at 275 mμ to estimate ubiquinone.

Test for ubiquinone reduction

The degree of reduction of ubiquinone was estimated after determining total ubiquinone and oxidized ubiquinone according to the method of HOFFMANN *et al.*[13]. Incubations were carried out in volumes of 1.5 ml at 30° and the reaction stopped by the addition of 5 ml of petroleum ether (b.p. 40–60°)–methanol (60:40, v/v) as described by KRÖGER AND KLINGENBERG[5].

Methods for measuring oxygen uptake

Oxygen uptakes were measured either in the conventional Warburg apparatus or by use of an Oxygraph Model K recording oxygen electrode (Gilson Medical Electronics, Wisc., U.S.A.). The electrode assembly of the latter apparatus was modified as described by SNOSWELL[14] and a medium composed of 30 mM sodium–potassium phosphate (pH 7.4) and 7.5 mM $MgCl_2$ was used. Buffer solutions were calibrated for oxygen content according to the method of CHAPPELL[15].

Reduction of pyridine nucleotides and cytochromes in small particles

The small particle preparation (0.2 ml) was added to 0.8 ml potassium phosphate buffer (0.1 M, pH 7). Fluorescence (activation 350 mμ; fluorescence 440 mμ, uncorrected) was measured in an Aminco Bowman spectrofluorimeter to which was attached a Moseley *xy* recorder with a 50 sec/inch time base. After measuring fluorescence for about 40 sec, 20 μmoles of DL-malate in 0.1 ml water were added, the contents of the cuvette mixed and fluorescence measurement continued. Increase

in fluorescence was taken as indicating pyridine nucleotide reduction. The reduced pyridine nucleotide was readily oxidized by aerating the contents of the cuvette by shaking.

The concentrations of the cytochromes in a number of preparations of small particles were compared after suspension in phosphate buffer as above and addition of malate or sodium hydrosulfite. The Soret peak in difference spectra was measured in a Cary Model 11 spectrophotometer.

RESULTS

Malate oxidation in sub-cellular fractions

A simple procedure for isolating a sub-cellular fraction which contained the enzyme system catalysing the oxidation of malate to oxaloacetate with the concomitant reduction of oxygen was developed. The fractionation procedure employed is outlined in METHODS. The data in Table I shows that malate oxidation is localised primarily in the 0–20 % $(NH_4)_2SO_4$ fraction. This fraction (see below) was shown to contain membranous particles and will be referred to hereafter as the 'small particle fraction'. The 20–80 % $(NH_4)_2SO_4$ fraction, while possessing little malate oxidation activity itself, was capable of stimulating the oxidation rate of the small particles. No membranous structures were detected in electron micrographs of the 20–80 % $(NH_4)_2SO_4$ fraction.

TABLE I

MALATE OXIDATION BY SUB-CELLULAR FRACTIONS

Oxygen uptake rates were measured in a conventional Warburg apparatus in a total volume of 3 ml. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), 15 mM $MgCl_2$ and 20 mM DL-malate. The small or large particles were added in a volume of 0.25 ml (2–3 mg protein). The reaction was followed for 30 min at 37°. (20–80) refers to the protein precipitated from cell extracts between 20 and 80 % satn. with $(NH_4)_2SO_4$ (see METHODS).

Expt. No.	*Organism used*	*Oxidation rate (mμgatoms O per min per mg protein)*				
		Large particles	*Small particles*	*(20–80)*	*Small particles + (20–80)*	*Large particles + (20–80)*
I	AB3290 (normal)	8	124	8	248	—
II	AB3290	20	116	0	272	52
III	AB3285 (mutant)	12	84	0	128	32
IV	AB3285	—	52	—	92	—

TABLE II

QUINONE CONTENT OF SMALL PARTICLES

Organism	*Quinone content (mμmoles/mg protein)*	
	Vitamin K_2	*Ubiquinone*
AB3285	20	Not detected (<0.2)
AB3290	4.5	2.2
AB3291	Not detected (<0.05)	6.0

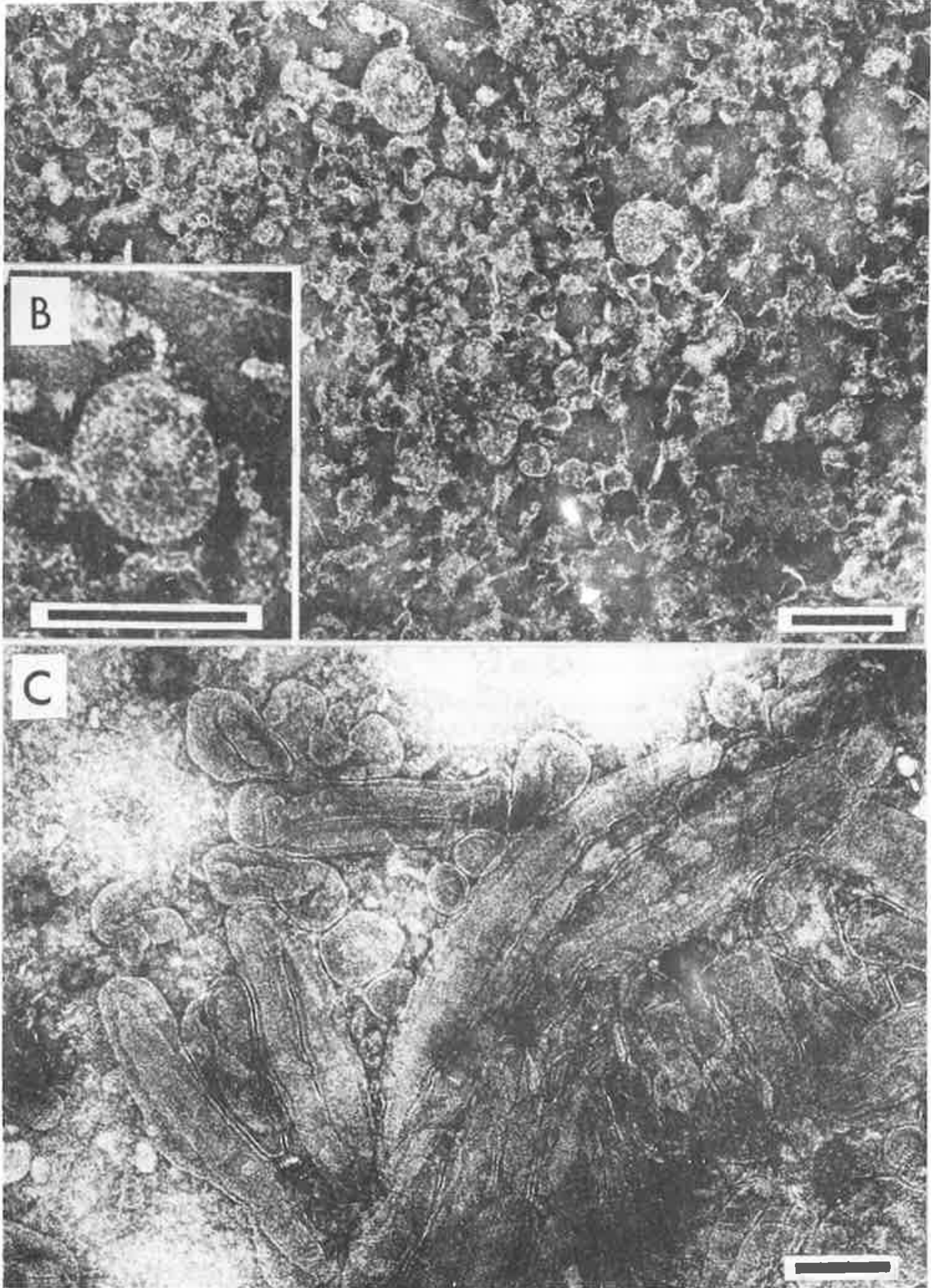


Fig. 1. Electron micrographs of small and large particle fractions. Fractions were negatively stained with 2 % potassium phosphotungstate (pH 7.0). A and B, small particle fraction; C, large particle fraction. Magnification marker in all cases = 0.2 μ.

Some properties of the small particle fraction

Electron microscopy

Examination of the small particle fraction from AB3290 by electron microscopy (Fig. 1) indicated the presence of membranous structures. The appearance of these structures was quite distinct from those found in the large particle fraction (Fig. 1) and resembled structures thought to be concerned in electron transport in mitochondria[16] and described in another bacterium[17].

Quinone content

An estimation of the quinone content of the particulate fractions from AB3290 indicated that the ratio of vitamin K to ubiquinone was constant at about 2 in both the small and large particle fractions. The small particles possessed 3 times the quinone content of the large particles. (Table II).

Substrates oxidized

The small particles from both the normal cells AB3290 and from the mutant AB3285 were capable of oxidizing a variety of substrates as shown in Table III. In general the oxidation rates of these substrates by the particles from the mutant were about a half of the rates with the normal particles with the exception of α-glycerophosphate. Pyruvate, succinate, β-hydroxybutyrate, oxaloacetate and glutamate were not oxidized to any significant extent by particles from either strain.

TABLE III

OXIDATION RATES OF VARIOUS SUBSTRATES WITH SMALL PARTICLES DERIVED FROM NORMAL *E. coli* AND A MUTANT UNABLE TO FORM UBIQUINONE

Rates of oxygen uptake were measured polarographically with an oxygen electrode in a volume of 2.0 ml at 30°. The reaction mixture contained 30 mM phosphate buffer (pH 7.4) and 7.5 mM $MgCl_2$ and small particles (0.7–1.4 mg protein). Substrates were added in a volume of 10 μl to give a final concentration of 2 mM.

Substrate added	*Number of experiments*	*Oxidation rate (mμgatoms O per min per mg protein)*	
		Normal strain	*Mutant strain*
None	6	Nil	Nil
α-Glycerophosphate	3	47	32
L-Malate	6	98	41
L-Lactate	3	131	76
Formate	2	158	81
$NADH_2$	1	435	180

Stoicheiometry of malate oxidation

As this study was concerned with the oxidation of malate by *E. coli* K12 it was necessary to establish that the oxygen uptakes observed when malate was used as the substrate were only due to the oxidation of malate and not subsequent reaction products. Table IV shows a stoicheiometric balance between oxaloacetate formed and oxygen utilized with normal particles of *E. coli* K12, indicating only a one-step oxidation was studied. Similar results were obtained for particles derived from the ubiquinone-deficient mutant.

TABLE IV

BALANCE BETWEEN OXALOACETATE FORMED AND OXYGEN UPTAKE DURING THE OXIDATION OF L-MALATE BY SMALL PARTICLES DERIVED FROM NORMAL *E. coli*

The reaction was started by the addition of 1.2 μmoles of malate and allowed to proceed for about 4 min at 30° in a volume of 2.4 ml. The reaction was then stopped by the addition of 0.2 ml of 40% trichloroacetic acid. Oxaloacetate was determined on the neutralized supernatant by the method of HORST AND REIM[18]. The reaction mixture as described in Table III.

Expt. No.	*Oxaloacetate formed (mμmoles/mg protein)*	*Oxygen used (mμgatoms/mg protein)*
I	242	237
II	215	212
III	223	219
IV	348	367

Malate-dependent reduction of bound pyridine nucleotides

A fluorimetric method (see METHODS) was used to test for substrate-dependent reduction of bound pyridine nucleotides in the small particles and in this way the small particles from the ubiquinone mutant and from the revertant could be compared. It was consistently found that higher levels of reduced pyridine nucleotides were detected in the particles from the mutant cells when they were incubated with malate than were detected in the particles from the normal cells. The addition of NAD to freshly prepared particles did not increase the amount of reduced pyridine nucleotide observed.

Role of ubiquinone in malate oxidation

The results shown in Table III indicate that, on the average, malate was oxidized by particles derived from the ubiquinone-deficient mutant at about half the rate observed with particles from normal cells. This is also illustrated in the experiment depicted in Fig. 2b. Addition of ubiquinone (Q-2) to particles from the mutant increased the rate of malate oxidation some 150 % while the oxidation rate of normal particles was only increased 30 % by a similar addition (Fig. 2b). In contrast, the addition of menadione, at five times the level of ubiquinone (Q-2) used, to particles from mutant cells increased the oxidation rate only 10 %. These observations would indicate that in the particles from mutant cells lack of ubiquinone is the rate-limiting factor in the malate-dependent oxygen uptake. Estimation of bound pyridine nucleotide (see earlier) and of cytochrome levels (see METHODS) in the particles from normal and mutant cells support this conclusion. Thus pyridine nucleotides reducible in the presence of malate are at much higher levels in particles from mutant cells than in those from normal cells and levels of cytochromes are similar in particles from both types of cells.

Furthermore, examination of the ubiquinone (Q-8) present in the normal particles showed that it was all present in the oxidized form in the absence of added substrate, but after incubation of the particles with malate at 30° for 2 min some 35 % of the total ubiquinone (Q-8) was reduced.

The effect of inhibitors on the oxidation of malate

The oxidation of malate by particles derived from the ubiquinone-deficient

mutant was not affected by dicoumarol at a concentration of 5 μM whereas the oxidation rate by particles from the normal cells was inhibited 56 % (Table V). This inhibition was only slightly reversed by ubiquinone (Q-2) (Table V).

The oxidation of malate by particles from the normal cells was also considerably more sensitive to inhibition by piericidin A than the oxidation by particles from the ubiquinone-deficient mutant. Subsequent addition of ubiquinone (Q-2) completely reversed the inhibition by piericidin A (Table V). The oxidation of malate by particles from both normal and ubiquinone-deficient cells was substantially inhibited by both 2-*n*-heptyl 4 hydroxyquinoline *N*-oxide and KCN and was not inhibited by antimycin A.

TABLE V

THE EFFECT OF VARIOUS INHIBITORS ON THE OXIDATION OF MALATE BY SMALL PARTICLES DERIVED FROM NORMAL *E. coli* AND A MUTANT UNABLE TO FORM UBIQUINONE

Rates of oxygen uptakes were measured as described in Table III. Piericidin A, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide or antimycin A were added in pure ethanol. The final ethanol concentration did not exceed 0.5 % and corrections for the effect of ethanol were made where required.

Inhibitor	*Final concn.*	*% Inhibition of oxidation rate*	
		Normal strain	*Mutant strain*
Dicoumarol	5 μM	56	Nil
Dicoumarol then Q-2 (0.11 mM)	5 μM	45	—
Piericidin A	10 μM	45	20
Piericidin A	20 μM	59	23
Piericidin A	40 μM	73	32
Piericidin A then Q-2 (0.11 mM)	20 μM	Nil	—
2-*n*-Heptyl-4-hydroxyquinoline *N*-oxide	0.5 μg/ml	63	66
2-*n*-Heptyl-4-hydroxyquinoline *N*-oxide	2.5 μg/ml	81	100
KCN	10 μM	100	75
Antimycin A	2.5 μg/ml	Nil	Nil

Further studies on the effects of dicoumarol on malate oxidation

As indicated in Table V 5 μM dicoumarol inhibited malate oxidation catalyzed by small particles from normal *E. coli* K12 cells by 56 %. The same concentration of dicoumarol did not inhibit the oxidation catalysed by small particles from a mutant strain unable to form ubiquinone (Fig. 2a). This mutant strain contains a derepressed level of vitamin K_2 (MK-8) (ref. 10).

The addition of ubiquinone (Q-2) to particles from the mutant deficient in ubiquinone restored the rate of malate oxidation to that catalyzed by particles from normal cells. Under these conditions 5 μM dicoumarol inhibited malate oxidation catalyzed by particles isolated from both the normal strain and ubiquinone-deficient mutant to the same extent (Fig. 2b). In contrast, added menadione had little effect on the oxidation rate catalyzed by particles from the mutant strain or on the dicoumarol inhibition.

The comparative insensitivity of particles from the ubiquinone-deficient mutant to inhibition by dicoumarol, in the absence of added ubiquinone, was seen over a wide range of concentrations of the inhibitor (Fig. 3). Furthermore malate

oxidation catalysed by particles from the mutant Strain AB3291 containing no vitamin K_2 (MK-8), but containing a derepressed level of ubiquinone (Q-8), was even more sensitive to inhibition by dicoumarol than the oxidation catalysed by particles from normal cells (Fig. 3).

Further evidence concerning the action of dicoumarol is provided by the experiments illustrated in Fig. 4. In these experiments the addition of ubiquinone (Q-2) to particles from the ubiquinone-deficient mutant not only restored oxidative

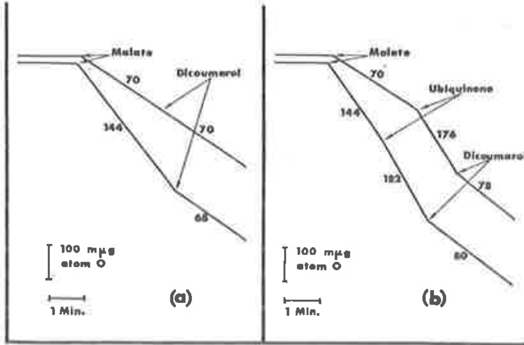


Fig. 2. The rate of malate oxidation catalysed by small particles derived from a normal and a ubiquinone-deficient strain of *E. coli* K12 and the effect of dicoumarol. The rate of oxygen uptake was measured with a recording oxygen electrode in a final volume of 2.0 ml at 30°. The reaction mixture contained 30 mM sodium phosphate buffer (pH 7.4), 7.5 mM $MgCl_2$ and small particles (0.9 mg protein); 4 μmoles DL-malate, 0.22 μmole Q-2 and 10 mμmoles of dicoumarol were added in 10-μl volumes where indicated. The upper line represents the rate of oxidation with particles from the ubiquinone-deficient mutant and the lower line the normal strain. The numbers represent rates of oxygen uptake in mμgatoms/min per mg protein.

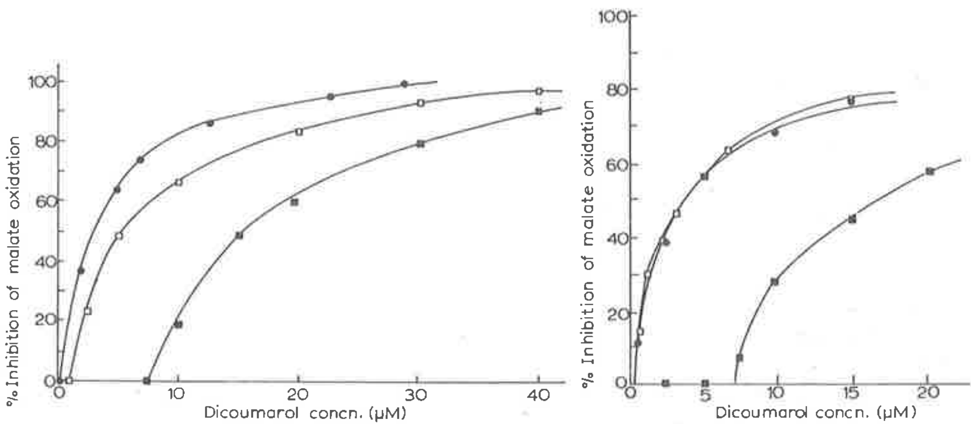


Fig. 3. Dicoumarol inhibition of malate oxidation catalysed by small particles from three strains of *E. coli*. Measurements and experimental conditions as in Fig. 2. ■—■, figures obtained with the mutant strain containing no ubiquinone; □—□, obtained for the normal strain; ●—●, obtained for the mutant containing no vitamin K_2.

Fig. 4. Dicoumarol titration curves for the inhibition of malate oxidation catalysed by small particles from a normal strain and ubiquinone-deficient mutant of *E. coli*. Measurements and experimental conditions as in Fig. 2. ■—■, figures obtained for the ubiquinone-deficient strain; □—□, obtained for the normal strain; ●—●, obtained for the ubiquinone-deficient strain where the malate oxidation rate had been restored by the prior addition of 0.33 μmole Q-2.

activity (see Fig. 2b) but also restored the sensitivity to dicoumarol to normal levels over a wide range of concentrations of inhibitor.

DISCUSSION

Studies on the role of ubiquinone in metabolism have, in general, been carried out either by experiments on the kinetics of oxidation–reduction changes in components of the respiratory chain or by the destruction or removal of ubiquinone from mitochondria or bacterial respiratory particles and restoration of activity by added ubiquinone. The latter techniques suffer from certain inherent difficulties. Extraction of mitochondria or other membranous structures with lipid solvents must necessarily lead to damage to the membranes. Irradiation to destroy quinones also leads to difficulties of interpretation, particularly in the case of the mixed quinone system in *E. coli* in which both ubiquinone and vitamin K are present.

In the present experiments a new approach to the study of ubiquinone function is presented. This approach involves a comparison of metabolic reactions which might involve ubiquinone in cells of a normal organism compared with a mutant of the same organism which has lost the ability to make ubiquinone. Such comparisons in the present paper are carried out between a mutant which lacks the ability to carry out the first specific reaction in ubiquinone biosynthesis and a revertant, which is taken as normal *E. coli*, obtained from that mutant.

It is clear that the system catalysing the aerobic oxidation of malate in *E. coli* K12 grown under the conditions described is localised in the small particle fraction. KASHKET AND BRODIE[8] working with *E. coli* W, found an approximately equal distribution of malate oxidation activity between the large and the small particles. This discrepancy may be due to the fact that in the present study glucose was used as carbon source for growth of the cells whereas KASHKET AND BRODIE[8] used either succinate or malate.

The nature of the factor in the 20–80 % $(NH_4)_2SO_4$ fraction stimulating malate oxidation has not been studied in detail. However, preliminary experiments show that freshly prepared small particles alone are incapable of reducing added (as distinct from endogenous) NAD in the presence of malate. However, if the 20–80 % $(NH_4)_2SO_4$ fraction is added the resultant system is then capable of reducing added NAD suggesting that there is a soluble malic dehydrogenase in the proteins precipitated at the higher concentrations of $(NH_4)_2SO_4$. A soluble malic dehydrogenase precipitated by high $(NH_4)_2SO_4$ concentrations has been reported in *A. vinelandii*[19].

The results of JONES AND REDFEARN[19] with particles from *A. vinelandii* suggested that pyridine nucleotides were not involved in malate oxidation as added NAD failed to stimulate the oxidation rate and a quantitative estimate of bound NAD indicated that little NAD was present in their particles. Although a quantitative estimate was not made in the present study, the fluorimetric technique used suggested that added NAD could not reach the site of malate dehydrogenation. However, reduction of endogenous pyridine nucleotide by malate could be detected readily. Aged particles, while having a lower level of bound pyridine nucleotides reducible by malate were able to reduce added NAD.

The distribution of ubiquinone and vitamin K between the large and small particle fractions found in the present experiments is at variance with that found by KASHKET AND BRODIE[8]. In the present study the K level was higher than the ubi-

quinone in both particulate fractions, but the quinone content of the small particles reported by KASHKET AND BRODIE[8] indicated that K was approximately one-twentieth the concentration of ubiquinone. This discrepancy also is probably due to the different carbon sources used. Thus the work of POLGLASE, PUN AND WITHAAR[20] indicates that under aerobic conditions the ubiquinone level is high and the vitamin K level is low. Under anaerobic conditions the relative levels are reversed. Oxygen would be an important electron acceptor for the facultative anaerobe *E. coli* growing with succinate or malate as sole carbon source. Glucose on the other hand would allow growth under semi-aerobic conditions tending to equalize the concentrations of the quinones.

A considerable amount of experimental evidence is presented in this paper which clearly indicates ubiquinone is involved in the oxidation of malate in *E. coli* K12.

(a) The endogenous ubiquinone (Q-8) present in the small particles is reduced when these particles are incubated with malate. This is in contrast to observations of KASHKET AND BRODIE[7]. However, in the experimental system used by these workers there was a considerable degree of reduction even in the absence of malate owing to the presence of KCN in the incubation mixture. In the present work the ubiquinone (Q-8) was all in the oxidized form prior to the addition of malate and the maximum degree of reduction in the presence of malate was 35 %, equivalent to the endogenous reduction reported by KASHKET AND BRODIE[7].

(b) The rate of malate oxidation in the ubiquinone-deficient mutant was about 40 % of the rate in normal cells and this rate could be restored to the oxidation rate observed with normal cells by the addition of ubiquinone (Q-2). Further, the addition of ubiquinone (Q-2) at the same concentration to particles from normal cells had little effect indicating that artificial pathways of electron transport are probably not operating. This is supported by the observation that the addition of a similar concentration of menadione had little effect on the rate of malate-dependent oxygen uptake with particles derived from either mutant or normal cells.

(c) Piericidin A, which appears to be a specific inhibitor of ubiquinone[21,22], inhibits the oxidation of malate in the normal cells and this inhibition can be completely reversed by the addition of ubiquinone (Q-2).

(d) The oxidation of malate by small particles from the ubiquinone-deficient mutant is not inhibited by 5 μM dicoumarol, whereas the oxidation rate in particles from normal cells is inhibited 56 % at the same concentration. However, if the oxidation rate of the small particles from the mutant is raised to that of the normal cells by the prior addition of ubiquinone (Q-2) then the oxidation in both particles is equally sensitive to dicoumarol.

Thus the evidence cited above clearly indicates that ubiquinone is involved in one pathway of malate oxidation. The results presented in this paper also indicate that some oxidation of malate in this organism proceeds without the participation of ubiquinone.

It is significant that of the three strains of *E. coli* K12 examined, the malate-oxidizing system of the mutant containing ubiquinone (Q-8), but no vitamin K_2 (MK-8), was the most sensitive to dicoumarol inhibition. In contrast, the malate-oxidizing system in the mutant containing no ubiquinone (Q-8), but containing vitamin K_2 (MK-8), was the least sensitive of the three strains examined. This indicates that, at low concentrations, dicoumarol preferentially inhibits the ubiquin-

one-dependent pathway at a site not involving vitamin K. It might be assumed that as the malate-oxidizing system in the mutant containing no ubiquinone, but containing vitamin K, is inhibited by dicoumarol at higher concentrations, vitamin K may be involved in malate-dependent oxygen uptake. While this may be true, no experimental evidence is provided in the present work to support this conclusion.

Dicoumarol has long been regarded as a vitamin K antagonist. In the present experiments, while the exact site of inhibition of oxygen uptake by low concentrations of dicoumarol has not been determined, it is not acting as a vitamin K antagonist. Another compound previously regarded as a typical vitamin K antagonist, namely SN5949, inhibits the succinoxidase system in animal mitochondria[23]. This system requires ubiquinone rather than vitamin K for activity. The present results would reinforce the suggestion[24] that a number of previous conclusions regarding functions attributed to vitamin K based on inhibitor studies may need to be revised. The availability of mutant strains of *E. coli*, lacking ubiquinone or vitamin K, provides useful experimental systems for the further investigation of the function of both ubiquinone and vitamin K.

ACKNOWLEDGEMENTS

We are grateful to Dr. I. HOLMES for the electron microscopy and valuable discussion and to Miss L. PURVIS for technical assistance. The Australian National Health and Medical Research Council, the Australian National Heart Foundation and the Institute af Arthritis and Metabolic Diseases of the National Institutes of Health, Washington, U.S.A. (Grant AM04632) are thanked for research grants.

REFERENCES

1 R. A. MORTON, *Biochemistry of Quinones*, Academic Press, London, 1965.
2 D. E. GREEN AND G. P. BRIERLEY, in R. A. MORTON, *Biochemistry of Quinones*, Academic Press, London, 1965, p. 405.
3 B. CHANCE, in R. A. MORTON, *Biochemistry of Quinones*, Academic Press, London, 1965, p. 460.
4 E. R. REDFEARN AND J. BURGOS, *Nature*, 209 (1966) 711.
5 A. KRÖGER AND M. KLINGENBERG, *Biochem. Z.*, 344 (1966) 317.
6 C. J. KNOWLES AND E. R. REDFEARN, *Biochem. J.*, 99 (1966) 33P.
7 E. R. KASHKET AND A. F. BRODIE, *J. Biol. Chem.*, 238 (1963) 2564.
8 E. R. KASHKET AND A. F. BRODIE, *Biochim. Biophys. Acta*, 78 (1963) 52.
9 G. B. COX AND F. GIBSON, *Biochem. J.*, 100 (1966) 1.
10 G. B. COX, F. GIBSON AND A. J. PITTARD, in preparation.
11 J. MONOD, G. COHEN-BAZIRE AND M. COHN, *Biochim. Biophys. Acta*, 7 (1951) 585.
12 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
13 P. C. HOFFMANN, H. W. KUNZ, W. SCHMID AND M. SIESS, *Biochem. Z.*, 339 (1964) 548.
14 A. M. SNOSWELL, *Biochemistry*, 5 (1966) 1660.
15 J. B. CHAPPELL, *Biochem. J.*, 95 (1964) 393.
16 H. FERNANDEZ-MORÁN, T. ODA, P. V. BLAIR AND D. E. GREEN, *J. Cell Biol.*, 22 (1964) 63.
17 D. ABRAM, *J. Bacteriol.*, 89 (1965) 855.
18 H. J. HORST AND M. REIM, in H. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1963, p. 335.
19 C. W. JONES AND E. R. REDFEARN, *Biochim. Biophys. Acta*, 113 (1966) 467.
20 W. J. POLGLASE, W. T. PUN AND J. WITHAAR, *Biochim. Biophys. Acta*, 118 (1966) 425.
21 C. HALL, M. WU, F. L. CRANE, N. TAKAHASHI, S. TAMURA AND K. FOLKERS, *Federation Proc.*, 25 (1966) 530.
22 A. M. SNOSWELL AND G. B. COX, in preparation.
23 F. L. CRANE, *Arch. Biochem. Biophys.*, 87 (1960) 198.
24 O. HOFFMANN-OSTENHOF, in R. M. HOCHSTER AND J. H. QUASTEL, *Metabolic Inhibitors*, Vol. II, Academic Press, New York, 1963, p. 145.

Reprinted from
Biochimica et Biophysica Acta
Elsevier Publishing Company
Amsterdam
Printed in The Netherlands

BBA 43206

Piericidin A and inhibition of respiratory chain activity in *Escherichia coli* K12

Piericidin A, an insecticide, was first isolated by TAKAHASHI, SUZUKI AND TAMURA[1]. In view of the apparent structural relationship of this compound to ubiquinone, HALL *et al.*[2] investigated the effects of piericidin A on electron transport systems in beef heart mitochondria. They suggested that piericidin A had two inhibitory effects. At low levels (0.04 nmole/mg protein) it specifically reacted at a site related to NADH dehydrogenase which they suggested might be identical to the site of rotenone inhibition. At high levels (0.3 µmole/mg protein for 50 % inhibition) it reacted in a reversible fashion in the region of ubiquinone in the succinoxidase system.

The isolation of a mutant strain of *Escherichia coli* K12 unable to form ubiquinone (COX, GIBSON AND PITTARD[3]) has provided an useful biological tool with which to investigate the function of ubiquinone (COX, SNOSWELL AND GIBSON[4]). Thus the relationship between piericidin A inhibition and ubiquinone function in respiratory systems of *E. coli* K12 using the ubiquinone deficient mutant and a revertant or normal strain was investigated.

The strains of *E. coli* K12 used were AB3285, a mutant strain unable to form ubiquinone and AB3290, a revertant strain of AB3285 (referred to here as the normal strain). These strains have been described in detail elsewhere[3]. The preparation of small respiratory particles, the measurement of oxygen uptakes and ubiquinone reduction have been described previously[4].

A comparison of the oxidation rates for various substrates catalysed by small particles from the normal strain of *E. coli* K12 and the mutant lacking ubiquinone is shown in Table I.

TABLE I

A COMPARISON OF THE OXIDATION RATES OF VARIOUS SUBSTRATES CATALYSED BY PARTICLES FROM NORMAL *E. coli* K12 AND A UBIQUINONE-DEFICIENT MUTANT AND THE INHIBITION BY PIERICIDIN A OF OXIDATIONS CATALYSED BY PARTICLES FROM NORMAL CELLS

Oxidation rates were determined polarographically. The percentage inhibition was determined by comparing the oxidation rate after the addition of the inhibitor to the rate obtained in the absence of inhibitor. The concentration of piericidin A used was 20 µM or 0.03–0.04 µmole/mg particle protein and that of ubiquinone-2 was 0.2 mM. Both piericidin A and ubiquinone-2 were added as solutions in absolute ethanol, 1–5 µl of solutions being added to a 2.0 ml reaction volume. Final substrate concentrations were 2 mM. The small-particle suspensions contained from 8.0 to 13.2 mg protein per ml and 0.1 ml fractions of these particle preparations were used in each experiment. The figures shown are an average of 5 experiments.

Substrate	*Oxidation rate (ngatoms O per min per mg protein)*		*Inhibition of oxidation rate of normal particles by piericidin A (%)*	
	Mutant	*Normal*	*−ubiquinone-2*	*+ubiquinone-2*
NADH	260	500	80	nil
L-Malate	51	141	54	nil
L-Lactate	156	225	65	6
α-Glycerophosphate	63	72	50	50
Dihydroorotate	45	42	100	100

It can be seen that the substrates NADH, malate and lactate are oxidised at a considerably slower rate by particles from the mutant cells compared with those from the normal cells. In contrast, the oxidation rates with α-glycerophosphate and dihydroorotate as substrates are virtually the same for particles from both the normal and mutant strains. Further, as shown in Table II endogenous ubiquinone-8 was not reduced in the presence of α-glycerophosphate and dihydroorotate, but was reduced in the presence of malate and lactate.

TABLE II

THE EFFECT OF PIERICIDIN A ON THE REDUCTION OF ENDOGENOUS UBIQUINONE-8 BY MALATE IN SMALL PARTICLES DERIVED FROM NORMAL *E. coli* K12 CELLS

0.5 ml of small-particle preparations containing 10.1–13.2 mg protein per ml were incubated at 25° for 2 min with 1.0 ml of buffer containing 30 mM phosphate buffer pH 7.4, 7.5 mM $MgCl_2$ *plus* 2 mM substrates and 20 μM piericidin A where indicated. The reaction was stopped by the addition of 5.0 ml of petroleum ether (b.p., 40–60°)–methanol (60:40, v/v) as described by KRÖGER AND KLINGENBERG[5] and oxidised and reduced ubiquinone estimated by the method of HOFFMANN *et al.*[6]. The figures shown are the average of 2 experiments.

Addition	*Reduction of endogenous ubiquinone-8* (%)
None	Nil
L-Malate	32
L-Malate + piericidin A	Nil
Piericidin A	Nil
L-Lactate	45
α-Glycerophosphate	Nil
Dihydroorotate	Nil

The above results suggest that ubiquinone is involved in the respiratory system associated with the oxidation of the substrates malate, lactate and NADH, and not in the system associated with the oxidation of α-glycerophosphate and dihydroorotate. This conclusion is supported by the observation that addition of ubiquinone-2 to particles from the mutant cells restores the oxidation rates with malate, lactate and NADH as substrates to the normal level. Addition of ubiquinone-2 at the same concentration to particles from normal cells had only a slight stimulatory effect on the oxidation rates of these substrates. Further, the cytochrome content of small particles from both mutant and normal cells is virtually the same[4], suggesting that the differences in respiratory rates described above are not due to an overall respiratory chain deficiency and indeed are due to a lack of ubiquinone.

Piericidin A (20 μM) substantially inhibited the oxidation of NADH, malate and lactate catalysed by small particles from the normal cells and this inhibition was completely reversed by the addition of ubiquinone-2 (Table I). The oxidation of α-glycerophosphate and dihydroorotate was also inhibited by piericidin A, but in contrast, this inhibition was not reversed by adding ubiquinone-2 (Table I).

The inhibition by piericidin A was examined more closely using malate as a substrate. The addition of ubiquinone-2 had little effect on the rate of malate oxidation

catalysed by particles from normal cells (Fig. 1a) but completely reversed the inhibition produced by the addition of piericidin A (Fig. 1b). Ubiquinone-1 was equally effective in reversing this inhibition although ubiquinone-6 only slightly reversed the inhibition. Menadione at a similar concentration to ubiquinone-2, *i.e.* 100 μM, only slightly reversed the piericidin A inhibition.

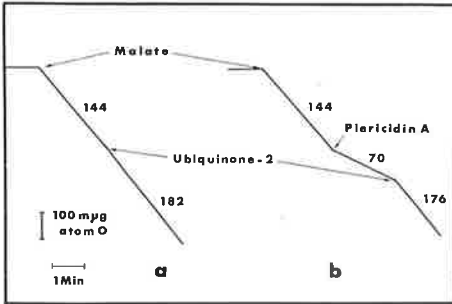


Fig. 1. The effect of piericidin A and ubiquinone-2 on the oxidation of malate catalysed by small particles from a normal strain of *E. coli* K12. Incubations were carried out at 25° in a volume of 2.0 ml containing small particles (equivalent to 1.0 mg protein), 30 mM phosphate pH 7.4 and 7.5 mM $MgCl_2$. 2 mM L-Malate, 100 μM ubiquinone-2 and 20 μM piericidin A were added at the points indicated. The figures shown indicate the rates of oxygen uptake in ngatoms O per min per mg protein.

The results shown in Table II indicate that no endogenous ubiquinone-8 is reduced in the absence of added substrate. However, when malate is added about 30 % of endogenous ubiquinone-8 is reduced (see also COX, SNOSWELL AND GIBSON[1]) and this reduction is completely prevented by the addition of piericidin A (Table II).

The results presented here indicate that piericidin A has two inhibitory effects on respiratory systems in *E. coli* K12. One effect is on respiratory systems not involving ubiquinone, such as that associated with the oxidation of α-glycerophosphate and dihydroorotate, and which is not reversed by added ubiquinone-2. The other inhibitory effect is seen with respiratory systems which do involve ubiquinone, such as those associated with the oxidation of NADH, malate and lactate, and this inhibition is completely reversed by the addition of ubiquinone-2. An important difference between these results and those obtained by HALL *et al.*[2] with beef heart mitochondrial systems is that in *E. coli* the two inhibitory effects are observed with the same level of piericidin A (*i.e.* 0.04 μmole/mg protein). JENG *et al.*[7] found that the NADH oxidase and succinoxidase systems in Azotobacter, unlike the mitochondrial systems are inhibited by the same level of piericidin A (1.5 μmoles/mg protein). Also it appears that high concentrations of piericidin A are generally required to inhibit bacterial respiratory systems[8].

The present results suggest that, in respiratory pathways in *E. coli* K12 which involve ubiquinone, piericidin A inhibits at or near the ubiquinone site. The site of the inhibitory action of piericidin A on respiratory pathways in *E. coli* K12 not involving ubiquinone is not yet known.

The authors are indebted to Professor S. TAMURA, Department of Agricultural Chemistry, University of Tokyo, for the sample of piericidin A and to Merck, Sharp and Dohme, U.S.A. for the ubiquinone-1 and -2. The authors are also grateful to

Professor F. GIBSON for advice and encouragement and to Mrs. J. MCDONALD for technical assistance.

Sub-department of Veterinary Biochemistry,
University of Melbourne,
Parkville, Victoria (Australia)

A. M. SNOSWELL*

Biochemistry Department,
John Curtin School of Medical Research,
Australian National University,
Canberra (Australia)

G. B. COX

1 N. TAKAHASHI, A. SUZUKI AND S. TAMURA, *J. Am. Chem. Soc.*, 87 (1965) 2066.
2 C. HALL, M. WU, F. L. CRANE, H. TAKAHASHI, S. TAMURA AND K. FOLKERS, *Biochem. Biophys. Res. Commun.*, 25 (1966) 373.
3 G. B. COX, F. GIBSON AND A. J. PITTARD, *J. Bacteriol.*, 95 (1968) 1591.
4 G. B. COX, A. M. SNOSWELL AND F. GIBSON, *Biochim. Biophys. Acta*, 153 (1968) 1.
5 A. KRÖGER AND M. KLINGENBERG, *Biochem. Z.*, 344 (1966) 317.
6 P. C. HOFFMANN, H. W. KUNZ, W. SCHMID AND M. SEISS, *Biochem. Z.*, 339 (1964) 548.
7 M. JENG, C. HALL, F. L. CRANE, N. TAKAHASHI, S. TAMURA AND K. FOLKERS, *Biochemistry*, 7 (1968) 1311.
8 C. J. KNOWLES, R. M. DANIEL, S. K. ERICKSON AND E. R. REDFEARN, *Biochem. J.*, 106 (1968) 49P.

Received June 25th, 1968

* Present address: Department of Agricultural Biochemistry, Waite Agricultural Research, University of Adelaide, Glen Osmond, S.A. (Australia).

Biochem. J. (1970) **117**, 551–562
Printed in Great Britain

The Function of Ubiquinone in *Escherichia coli*

BY G. B. COX, N. A. NEWTON, F. GIBSON, A. M. SNOSWELL*
AND J. A. HAMILTON

Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2601, *Australia, and *Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S. Austral.* 5064, *Australia*

(*Received* 17 *November* 1969)

1. The function of ubiquinone in *Escherichia coli* was studied by using whole cells and membrane preparations of normal *E. coli* and of a mutant lacking ubiquinone. 2. The mutant lacking ubiquinone, strain AN 59 (Ubi^-), when grown under aerobic conditions, gave an anaerobic type of growth yield and produced large quantities of lactic acid, indicating that ubiquinone plays a vital role in electron transport. 3. NADH and lactate oxidase activities in membranes from strain AN 59 (Ubi^-) were greatly impaired and activity was restored by the addition of ubiquinone (Q-1). 4. Comparison of the percentage reduction of flavin, cytochrome b_1 and cytochrome a_2 in the aerobic steady state in membranes from the normal strain (AN 62) and strain AN 59 (Ubi^-) and the effect of respiratory inhibitors on these percentages in membranes from strain AN 62 suggest that ubiquinone functions at more than one site in the electron-transport chain. 5. Membranes from strain AN 62, in the absence of substrate, showed an electron-spin-resonance signal attributed to ubisemiquinone. The amount of reduced ubiquinone (50%) found after rapid solvent extraction is consistent with the existence of ubiquinone in membranes as a stabilized ubisemiquinone. 6. The effects of piericidin A on membranes from strain AN 62 suggest that this inhibitor acts at the ubiquinone sites: thus inhibition of electron transport is reversed by ubiquinone (Q-1); the aerobic steady-state oxidation–reduction levels of flavins and cytochrome b_1 in the presence of the inhibitor are raised to values approximating those found in the membranes of strain AN 59 (Ubi^-); the inhibitor rapidly eliminates the electron-spin-resonance signal attributed to ubisemiquinone and allows slow oxidation of endogenous ubiquinol in the absence of substrate and prevents reduction of ubiquinone in the presence of substrate. It is concluded that piericidin A separates ubiquinone from the remainder of the electron-transport chain. 7. A scheme is proposed in which ubisemiquinone, complexed to an electron carrier, functions in at least two positions in the electron-transport sequence.

The function of ubiquinone has been the subject of intensive research since Crane, Hatefi, Lester & Widmer (1957) demonstrated that heptane extraction of ox heart mitochondria caused the loss of succinate oxidase activity. This activity could be restored by the addition of cytochrome *c* or a compound ('Q-275') present in the heptane extract and later named coenzyme Q (Lester, Crane & Hatefi, 1958) or ubiquinone (Morton, Wilson, Lowe & Leat, 1957). Szarkowska (1966) extracted freeze-dried ox heart mitochondria with pentane and demonstrated a loss of NADH oxidase activity that could be reversed by the addition of ubiquinone (Q-10) plus mitochondrial phospholipids. Ernster, Lee, Norling & Persson (1969) used the freeze-drying method of Szarkowska and examined the reduction of cytochromes *b*, c_1 and *a* by NADH and succinate in submitochondrial particles. They concluded that the site of inactivation caused by the extraction of ubiquinone (Q-10) occurred in the region between the NADH dehydrogenase or succinate dehydrogenase and cytochrome *b*.

Fragmentation of mitochondria or submitochondrial particles with appropriate reagents separates the respiratory chain into four segments or complexes (Green & Wharton, 1963). Studies of the interaction of the complexes with ubiquinone (Q-2) provided evidence that ubiquinone functions between the flavoproteins and the cytochromes (see Green & Brierley, 1965).

Redfearn & Pumphrey (1960) measured rates of reduction of endogenous ubiquinone by succinate

or NADH in mitochondrial fragments by a chemical extraction method. They found that the rates of reduction of ubiquinone were less than the overall oxidase rates, an observation confirmed by Chance & Redfearn (1961) using a direct spectrophotometric method. The conclusion from these observations was that ubiquinone was not involved directly in the pathway of electron transfer. Storey & Chance (1967), having found agreement between the spectrophotometric and extraction methods, also concluded that ubiquinone is not a component of the NADH oxidase system in the electron-transport particles prepared by alkaline treatment of ox heart mitochondria.

Klingenberg & Kröger (1966), using modified extraction and spectrophotometric techniques with sonicated mitochondrial particles, obtained kinetic data that were consistent with a function for ubiquinone on the main electron-transport pathway. Kröger & Klingenberg (1967) further concluded that ubiquinone functions between the flavoproteins and the cytochromes on the basis of changes in percentage reduction of the various electron carriers during steady-state transitions in whole mitochondria.

Bacterial membranes, unlike mitochondria, may contain ubiquinone or vitamin K_2, or both (Bishop, Pandya & King, 1962). Most of the Gram-positive organisms examined contain only vitamin K, whereas the Gram-negative organisms generally contain only ubiquinone. A few micro-organisms, including *Escherichia coli*, form both ubiquinone and vitamin K.

Azotobacter vinelandii is representative of the group containing only ubiquinone, and Swank & Burris (1969) have restored the NADH oxidase activity of freeze-dried pentane-extracted membranes from this organism by adding back ubiquinone (Q-8). The function of ubiquinone in membranes from *E. coli* has been studied by Kashket & Brodie (1963) using irradiation by light to deplete the membranes of quinones. Examination of the reactivation of NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase by the addition of either of the two quinones formed by *E. coli* led to the conclusion that vitamin K was involved in the NADH oxidase system and that ubiquinone was involved in the succinate oxidase system.

Bacterial mutants have been used only to a limited extent in the study of ubiquinone function (Jones, 1967; Cox, Snoswell & Gibson, 1968). Strains of *E. coli* containing mutations affecting the pathway of ubiquinone biosynthesis have recently been characterized both biochemically and genetically (Cox, Young, McCann & Gibson, 1969). One of these strains has now been used in the present more extensive study of ubiquinone function in *E. coli* K12.

MATERIALS AND METHODS

Chemicals. Piericidin A was kindly provided by Professor S. Tamura, Department of Agricultural Chemistry, University of Tokyo, Tokyo, Japan. Ubiquinone (Q-1) and vitamin K (MK-1) were kindly provided by Dr O. Isler of F. Hoffmann–La Roche and Co., Basle, Switzerland. Chemicals generally were of the highest purity available commercially and were not further purified.

Organisms. The strains of *E. coli* K12 used were AB2154, AN59 and AN62; these strains have been described previously (Cox *et al.* 1969). Strain AN59, the mutant lacking ubiquinone, is referred to throughout this paper as strain AN59 (Ubi^-).

Media and growth of organisms. The minimal medium used (medium 56) was that described by Monod, Cohen-Bazire & Cohn (1951). To the sterilized mineral salts base were added L-leucine, L-threonine and L-methionine each at a final concentration of 0.2mM and thiamin at a final concentration of 0.02μM. Glucose was added as a sterile solution either in excess at a final concentration of 30mM or at limiting concentrations as indicated.

For experiments on growth yields cells were grown at 37°C in 10ml volumes in 125ml conical flasks with a Klett–Summerson tube as a side arm. Limiting concentrations of glucose were used and growth was considered complete when two successive readings, taken at 30min intervals on the Klett–Summerson colorimeter, were similar. The flasks were aerated by shaking at 250rev./min in a New Brunswick Metabolyte shaker-bath. For anaerobic growth conditions the flasks were gassed with nitrogen for 1h while shaking. The medium was supplemented for the anaerobic experiments with $NaHCO_3$ at a final concentration of 25mM and, where indicated, KNO_3 at a final concentration of 0.1%.

For the preparation of cell extracts, organisms were grown at 37°C in 14-litre New Brunswick fermenters with aeration at 10l/min and stirring at 750rev./min. Strain AN59 (Ubi^-) had a tendency to revert, and to prepare inocula the stock culture was plated out on glucose-nutrient agar plates. If there were no revertant colonies (see Cox *et al.* 1969) after incubation overnight at 37°C the growth from three plates was emulsified and added to a 1-litre culture of the supplemented medium 56. After incubation with shaking at 37°C overnight this culture was used to inoculate 10 litres of medium in the 14-litre fermenter.

Preparation and fractionation of cell extracts. Cells were grown as described above and the cultures were harvested early in growth (0.2–0.4mg dry wt./ml). The cells were washed once in cold 0.1M-potassium phosphate buffer, pH7.0, and resuspended in fresh buffer (1ml of buffer/0.5g wet wt. of cells), and a cell extract was prepared by passing the suspension through a Sorvall Ribi Cell Fractionator at 20000lb/in^2. The membrane fraction was prepared from the cell extract by adding solid $(NH_4)_2SO_4$ slowly to give 30% saturation and stirring for a further 30min. The precipitate was collected after centrifugation at 25000g for 30min and resuspended in 1ml of 0.1M-phosphate buffer, pH7.0, for each original 1g wet wt. of cells. For the experiments described in Table 2 the precipitate was resuspended in 2ml of buffer per original 1g wet wt. of cells. For electron-spin-resonance measure-

ments the precipitate was resuspended in 0.2ml of buffer for each original 1g wet wt. of cells. All operations on the harvested cells and cell fractions were carried out at 0–4°C. Proteins were estimated with Folin's phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951) using bovine serum albumin (Fraction V, Sigma Chemical Co., St Louis, Mo., U.S.A.) as standard.

Determinations of glucose and lactic acid. Glucose was determined by the hexokinase–glucose 6-phosphate dehydrogenase method as described by Slein (1965). Lactic acid was determined colorimetrically after oxidation to acetaldehyde as described by Barker (1957).

Determination of fermentation products. The determinations of acetic acid, lactic acid and ethanol were kindly carried out by Dr H. Doelle of the University of Queensland using a g.l.c. technique (Doelle, 1969).

Measurement of oxygen uptake. Oxygen uptakes at 25°C were measured polarographically with a Titron oxygen electrode (Titron Instruments, Melbourne, Vic., Australia) modified as described by Snoswell (1966). The reaction mixture contained (final concentrations) 15mM-sodium–potassium phosphate buffer, pH7.4, 1.9mM-$MgCl_2$, 1–5mg of protein, 2mM-substrate (except for NADH, which was 1.2mM) and, where indicated, 0.6mM-NAD^+ and 0.6mM-$NADP^+$ in a final volume of 2.5ml. Buffer solutions were calibrated for oxygen content by the method of Chappell (1964).

Determinations of quinones. The ubiquinone and vitamin K contents of the membrane fraction were determined as described previously (Cox *et al.* 1968). The maximum value quoted for the ubiquinone content of strain AN59 (*Ubi*⁻) was determined after the extraction of large quantities of whole cells during the isolation of 2-octaprenylphenol (Cox *et al.* 1969).

Difference spectra. Difference spectra were recorded in a Cary 14R spectrophotometer with a scattered transmission accessory and a 0–0.1A slide-wire. Membrane preparations were diluted to a concentration of approx. 10mg of protein/ml and the differences between $Na_2S_2O_4$-reduced and oxygenated samples were recorded. The wavelength pairs and molar extinction coefficients were those employed by Jones & Redfearn (1966): cytochrome b_1, $\Delta E_{560}-\Delta E_{575}$ (ϵ_M 17500); cytochrome a_2, $\Delta E_{630}-\Delta E_{615}$ (ϵ_M 8500); total flavoprotein, $\Delta E_{465}-\Delta E_{510}$ (ϵ_M 11000). The determination of flavoprotein by this method may be subject to error due to absorption by non-haem iron. Cytochrome *o* concentrations were determined from the $Na_2S_2O_4$-reduced + CO minus $Na_2S_2O_4$-reduced difference spectra, by using the molar extinction coefficient calculated by Taber & Morrison (1964); $\Delta E_{415}-\Delta E_{430}$ (ϵ_M 80000). Cytochrome a_1 was not present in sufficient quantity for accurate determination.

Determinations of steady-state oxidation–reduction levels. The reduction kinetics of the individual cytochrome and flavin components in the membrane fractions were determined with an Aminco–Chance dual-wavelength spectrophotometer. A slit width of 0.25mm (dispersion, 5.5nm/mm) was used. The following wavelength pairs were employed: cytochrome b_1, 560 and 570nm; cytochrome a_2, 630 and 615nm; flavoprotein, 475 and 495nm. The measurements were carried out at 25°C in the 5% transmission range. The reaction mixture contained (final concentrations) approx. 5mg of membrane protein, 1.6mM-$MgCl_2$, 28mM-sodium–potassium phosphate buffer, pH7.4, in a final volume of 3ml. The mixture was well aerated by shaking and the reference and sample light-beams were balanced. The reaction was initiated by stirring in 30μl of substrate (5μmol of NADH or 15μmol of D-lactate). The reduction of a component in the aerobic steady state was expressed as a percentage of its final reduction after the exhaustion of all of the oxygen. Measurements could be repeated, after shaking the cuvette, until the substrate was exhausted. At the end of each experiment $Na_2S_2O_4$ was added to check total reduction and the results indicated that 80–90% of each component had been reduced by substrate. In the presence of cyanide (0.8mM) the cytochrome a_2 formed a complex of altered spectrum and therefore these steady-state measurements were only approximate. In cases where the percentage reduction of a component in the steady state was still rising when the system went anaerobic, the steady-state values determined after reshaking were taken as being more accurate. Inhibitors were added several minutes before the addition of substrate, with the exception of cyanide, which was incubated for 10min with undiluted membranes before the addition of buffer.

The degree of reduction of ubiquinone in the aerobic steady state was measured after determination of total ubiquinone and oxidized ubiquinone by the method of Hoffmann, Kunz, Schmid & Siess (1964). Incubations were carried out, with stirring, in volumes of 1.5ml at 25°C and the reaction was stopped by the addition of 5ml of light petroleum (b.p. 40–60°)–methanol (2:3, v/v) as described by Kröger & Klingenberg (1966).

Measurement of electron-spin-resonance spectra. The *X*-band spectra were recorded as first-derivative traces with a Varian V-4501 spectrometer. To an electron-spin-resonance tube was added 0.1ml of 15mM-sodium–potassium phosphate buffer, pH7.4, containing 1.9mM-$MgCl_2$ and, where indicated, 10μl of inhibitor. The membrane preparation (0.1ml containing 15mg of protein) was then added and mixed with a syringe. Spectra were recorded after freezing the samples in liquid nitrogen. Mixing and freezing the contents of the tube took 30s. The concentrations of unpaired spins were calculated by double integration by comparison with a standard Varian dilute pitch sample.

The electron-spin-resonance recording conditions were as follows: temperature, 77°K; microwave frequency, 9.05GHz; modulation frequency, 100kHz; modulation level, 3.78G peak-to-peak; power, 61mW; integrating time-constant, 1s; gain setting, ×1000; scanning rate, 500G/10min. A Fieldial was used for the direct calibration of field strength.

RESULTS

A ubiquinone-deficient strain of *E. coli* K12 (AB3285) has been used in a study of ubiquinone function in malate oxidation (Cox *et al.* 1968). This strain was derived by conjugation involving a male parent carrying a number of uncharacterized mutations, some of which affected growth on succinate (G. B. Cox, unpublished work), and was subject to the objection that an additional mutation modifying the effect of the ubiquinone deficiency may have been transferred. This objection has been

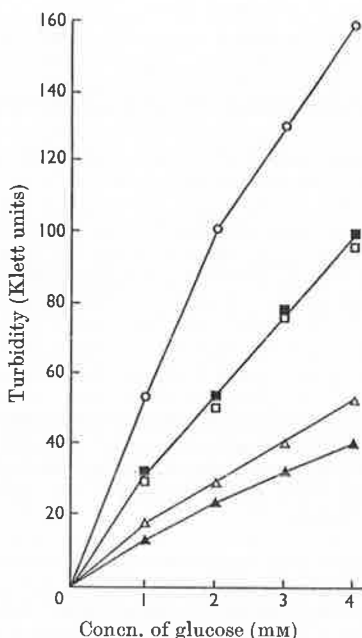


Fig. 1. Growth yields (turbidity) of strains AB 2154, AN 62 and AN 59 (Ubi^-) grown on limiting concentrations of glucose under various conditions. Cultures were aerated by shaking and anaerobic cultures were incubated under nitrogen as described in the Materials and Methods section. ○, Strain AB 2154 or strain AN 62, aerobic; △, strain AN 62, anaerobic; □, strain AN 62, anaerobic with nitrate; ▲, strain AN 59 (Ubi^-), aerobic or anaerobic; ■, strain AN 59 (Ubi^-), anaerobic with nitrate.

minimized by the use of a co-transduction system for the preparation of the ubiquinone-deficient strain AN 59 (Ubi^-) from strain AB 2154 (Cox *et al.* 1969). Strain AN 62 is a spontaneous revertant from strain AN 59 (Ubi^-) and is able to form ubiquinone (Cox *et al.* 1969). The mutation in strain AN 59 (Ubi^-) causes the accumulation in the cells of the intermediate 2-octaprenylphenol. Although this compound cannot be involved in functions requiring a quinone, it may assist in maintaining membrane structure in the absence of ubiquinone.

Growth yields. The growth yields obtained for strains AB 2154, AN 59 (Ubi^-) and AN 62 from limiting concentrations of glucose under various conditions of growth were estimated as turbidity in a Klett–Summerson colorimeter as described in the Materials and Methods section. Strains AB 2154 and AN 62, as expected, gave similar growth yields under aerobic conditions (Fig. 1). However, strain AN 59 (Ubi^-) under aerobic conditions gave a similar low growth yield to those obtained from both strain AN 59 (Ubi^-) and strain AN 62 under anaerobic conditions (Fig. 1). These results suggest that ubiquinone is essential for electron transport or for phosphorylation coupled to electron transport. The energy obtained from respiration with nitrate as terminal electron acceptor is apparently unaffected by the absence of ubiquinone, as the growth yields of both strains AN 59 (Ubi^-) and AN 62 are similar under anaerobic conditions in the presence of nitrate.

The metabolism of strains AN 59 (Ubi^-) and AN 62 under aerobic conditions was examined in more detail. Cultures were sampled at various stages of growth and the samples assayed for glucose and lactate. The results (Fig. 2) confirm the comparatively inefficient conversion of glucose into cell mass in strain AN 59 (Ubi^-). The lack of ubiquinone also causes the accumulation of D-lactate in the culture medium, further suggesting that ubiquinone-deficient cells derive their energy from glycolysis even when grown under aerobic conditions.

The normal products of glucose fermentation by *E. coli* in media of slightly acid pH are lactate, acetate, ethanol, carbon dioxide and hydrogen (Wood, 1961). Lactate, acetate and ethanol were determined in supernatants from cultures of strains AN 59 (Ubi^-) and AN 62, grown aerobically on excess of glucose. Strain AN 59 (Ubi^-) formed high concentrations of lactate compared with strain AN 62, but normal aerobic concentrations of the other fermentation products, acetate and ethanol (Table 1).

Oxidase systems in cell-free preparations from strain AN 62. The revertant strain, AN 62, was grown under conditions of good aeration with glucose as carbon source. Cell extracts were prepared by using a Sorvall–Ribi cell fractionator and examined for the presence of various oxidase systems by using an oxygen electrode. The NADH oxidase and D-lactate oxidase systems were quantitatively the most significant (Table 2). The pyruvate oxidase and α-oxoglutarate oxidase systems required the addition of NAD^+ and probably the oxidase activity was due to the presence of the NADH oxidase. The isocitrate oxidase activity required the addition of both NAD^+ and $NADP^+$ and presumably both transhydrogenase and NADH oxidase were involved. There was little or no activity obtained with any of the four substrates succinate, dihydro-orotate, α-glycerophosphate or formate.

The addition of ammonium sulphate (30% saturation) to the cell extract precipitated the NADH oxidase, malate oxidase and lactate oxidase systems without any significant loss of total activity (Table 2); this fraction is referred to below as the membrane fraction. The lactate oxidase and

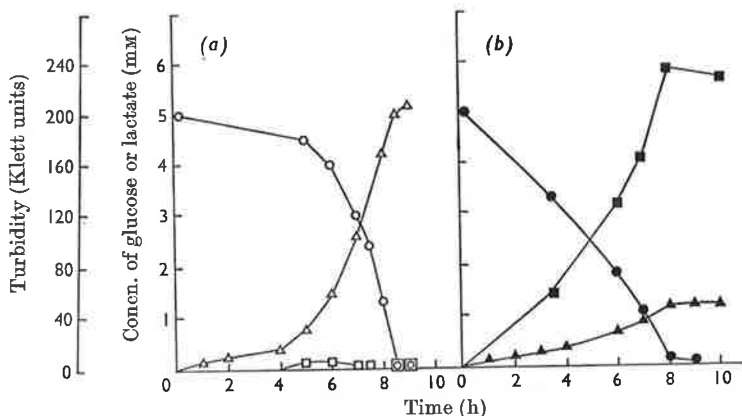


Fig. 2. Aerobic metabolism of glucose by (*a*) strain AN62 and (*b*) strain AN59 (*Ubi*⁻). △ and ▲, Growth; ○ and ●, glucose concentration; □ and ■, lactate concentration. The times were measured from the start of observable growth. Conditions for aerobic growth were as indicated in Fig. 1.

Table 1. *Products of glucose metabolism formed by strains AN62 and AN59* (Ubi⁻) *grown under aerobic conditions*

Determinations were made on supernatants from cultures of cells grown for the preparation of membranes as described in the Materials and Methods section. Products were determined by g.l.c. (Doelle, 1969).

Strain	Product (mg/l)		
	Ethanol	Acetate	Lactate
AN62	11	48	86
AN59 (*Ubi*⁻)	7	17	2500

malate oxidase activities were not stimulated by the addition of NAD^+. The loss of pyruvate oxidase and α-oxoglutarate oxidase activities is presumably due to the separation of the primary dehydrogenase and the NADH oxidase. The supernatant fraction after the ammonium sulphate precipitation did not have any detectable oxidase activity with the substrates tested, although a comparatively low NADH oxidase activity was detected on the addition of FAD.

The membrane fraction from strain AN62 was used to test the effect of some known electron-transport inhibitors on the NADH oxidase and lactate oxidase systems. Piericidin A and HQNO* were the most potent inhibitors of both oxidase systems (Table 3) and the inhibition by these compounds was reversed by the addition of ubiquinone (Q-1). Dicoumarol and sodium cyanide inhibited both systems, but sodium Amytal inhibited the lactate oxidase without having any effect on the NADH oxidase. It was not possible to demonstrate any inhibition of the NADH oxidase or lactate oxidase systems by rotenone or antimycin A.

* Abbreviation: HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide.

Table 2. *Oxidase systems in cell extract and the membrane fraction from strain AN62*

Rates of oxygen uptake were measured with an oxygen electrode in a volume of 2.5ml at 25°C. The reaction mixture contained 15mM-sodium–potassium phosphate buffer, pH 7.4, 1.9mM-$MgCl_2$ and about 4mg of protein for the cell extract or about 1.5mg of protein for the membrane preparation. The membrane fraction was prepared by $(NH_4)_2SO_4$ precipitation of the cell extract and resuspended to the original volume of cell extract as described in the Materials and Methods section. Substrates were added in a volume of 10μl to give a final concentration of 2mM except for NADH (1.2mM). NAD^+ and $NADP^+$ were added to give a final concentration of 0.6mM. Values given are the averages of several determinations on at least five different preparations of membranes and the endogenous oxygen uptakes (about 10ng-atoms of O/min per 100μl for cell extracts and about 2ng-atoms of O/min per 100μl for membrane fractions) have been subtracted.

Addition to buffer	O_2 uptake by preparation (ng-atoms of O/min per 100μl)	
	Cell extract	Membrane fraction
NADH	263	281
D-Lactate	80	76
DL-Malate	28	23
Pyruvate+NAD^+	90	0
α-Oxoglutarate+NAD^+	43	0
Isocitrate+NAD^++$NADP^+$	23	<5

Table 3. *Effects of inhibitors on NADH oxidase and lactate oxidase systems in membranes from strain AN 62*

Oxygen uptakes were measured as described in Table 2. The inhibitors were added to the reaction mixture 2 min before the addition of substrate, except for cyanide, which was incubated with membranes at 25°C for 5 min before addition to the electrode vessel. Ubiquinone was added after the addition of substrate.

Inhibitor and final concn. (μM)	Final concn. of ubiquinone (Q-1) (μM)	Inhibition (%) of the oxidation of	
		NADH	D-Lactate
Piericidin A (8)	0	69	32
Piericidin A (24)	0	86	64
Piericidin A (40)	0	92	—
Piericidin A (8)	32	46	—
Piericidin A (8)	64	33	12
HQNO (16)	0	57	—
HQNO (48)	0	82	—
HQNO (80)	0	88	71
HQNO (16)	32	40	—
HQNO (16)	64	28	—
NaCN (200)	0	50	63
NaCN (506)	0	84	—
Dicoumarol (200)	0	49	—
Dicoumarol (400)	0	75	67
Dicoumarol (400)	32	75	—
Sodium Amytal (2800)	0	0	40
Sodium Amytal (4000)	0	0	77

Ubiquinone requirement for oxidase activity. The NADH oxidase, lactate oxidase and malate oxidase systems present in cell extracts of strain AN 59 (*Ubi*⁻) were similarly precipitated by 30% ammonium sulphate saturation. The NADH oxidase, lactate oxidase and malate oxidase activities were all markedly decreased in the membrane fraction from strain AN 59 (*Ubi*⁻) as compared with those in membranes from strain AN 62 (Table 4). There was, however, some activity retained and this activity was stimulated to a value greater than that found in strain AN 62 by the addition of ubiquinone (Q-1). The addition of vitamin K (MK-1) had a relatively slight effect and the ubiquinone isoprenologues Q-6 and Q-8 were inactive.

Concentrations of membrane components in strains AN 59 (Ubi⁻) *and AN 62.* The concentrations of the flavins and cytochromes in the membrane fractions of strain AN 59 (*Ubi*⁻) and strain AN 62 were determined from the reduced minus oxidized difference spectra. The cytochrome b_1 concentrations were the same in each strain (Table 5) and, although the flavin and cytochrome a_2 concentrations were higher and the cytochrome *o* concentration was lower in strain AN 59 (*Ubi*⁻), the differences are small considering the different metabolism of the two strains. The ubiquinone and vitamin K contents were determined after extraction and purification as described in the Materials and Methods section. Ubiquinone was present in strain AN 62 at a concentration 25 times that of cytochrome b_1. The vitamin K concentration was derepressed fourfold in the absence of ubiquinone.

Table 4. *Comparison of the oxidase systems in strain AN 62 and AN 59* (Ubi⁻) *and the effects of ubiquinone (Q-1) and vitamin K (MK-1)*

Oxygen uptakes were measured as described in Table 2. Ubiquinone (Q-1) or vitamin K (MK-1) were added after the addition of substrate. Values represent the averages of experiments with at least ten different preparations of membranes.

Substrate	Quinone added and final concn. (μM)	O_2 uptake (ng-atoms/min per mg of protein) by membranes from	
		Strain AN 62	Strain AN 59 (*Ubi*⁻)
NADH	—	230	25
NADH	Q-1 (32)	430	560
NADH	Q-1 (48)	430	560
NADH	MK-1 (32)	230	82
NADH	MK-1 (128)	210	72
D-Lactate	—	68	19
D-Lactate	Q-1 (32)	150	210
D-Lactate	MK-1 (32)	77	30
D-Lactate	MK-1 (128)	—	26
DL-Malate	—	31	5
DL-Malate	Q-1 (32)	50	42

Table 5. *Concentrations of some membrane components in strains AN 62 and AN 59* (Ubi⁻)

Flavins and cytochromes were determined by direct spectrophotometric examination of suspensions of the membranes. Quinones were first extracted and partially purified before spectrophotometric determination. Details of methods are given in the Materials and Methods section.

Component	Concn. of component (nmol/mg of protein) in	
	Strain AN 62	Strain AN 59 (*Ubi*⁻)
Total flavin	0.25	0.39
Cytochrome b_1	0.19	0.19
Cytochrome a_2	0.027	0.047
Cytochrome *o*	0.073	0.04
Cytochrome a_1*	+	+
Ubiquinone	4.7	<0.05
Vitamin K_2	0.67	2.7

* Cytochrome a_1 was present but the quantities were too low for determination.

Percentage reduction of membrane components in the aerobic steady state. During the passage of electrons from substrate to oxygen via the respiratory chain, the oxidation–reduction levels of various components in this steady state may be determined by direct spectrophotometric methods. The values obtained depend on the balance of oxidase and dehydrogenase activities in the preparation. If, however, an inhibitor is added, then the components of the chain on the substrate side of the point of inhibition become more reduced and those on the oxygen side become more oxidized (Chance & Williams, 1956). Ubiquinone deficiency causes 'inhibition' of the various oxidase systems and, in an attempt to localize the point of inhibition, a comparison of the steady state levels of the components in the membrane fractions from strains AN 59 (*Ubi*⁻) and AN 62 was made.

The percentage reduction in the aerobic steady state of total flavin, cytochrome b_1 and cytochrome a_2 for strains AN 59 (*Ubi*⁻) and AN 62 with NADH or lactate as substrate may be seen in Table 6 and Fig. 3. The percentage of flavin and cytochrome b_1 reduced in the aerobic steady state was increased in strain AN 59 (*Ubi*⁻) with either NADH or lactate as substrate when compared with strain AN 62. These results indicate that ubiquinone has a function after cytochrome b_1. The oxidation–reduction levels in strain AN 59 (*Ubi*⁻) were returned to those in strain AN 62 by the addition of ubiquinone (Q-1). The percentage reduction of cytochrome a_2 in the aerobic steady state was low in membranes prepared from either strain.

The effects of the inhibitors piericidin A, HQNO, sodium cyanide and sodium Amytal on the percentage reduction, in the steady state, of membrane components from strain AN 62 with NADH or lactate as substrate were examined (Table 6 and Fig. 3). Sodium cyanide, at a concentration inhibiting electron transport less than ubiquinone deficiency, caused a greater increase in the percentage of cytochrome b_1 reduced than did ubiquinone

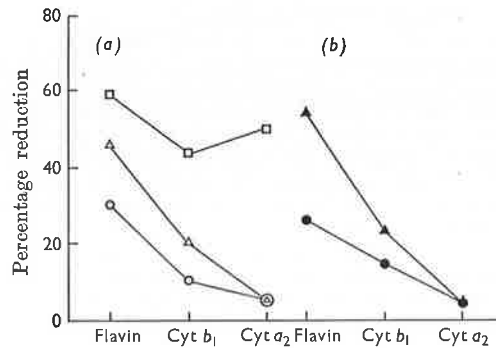


Fig. 3. Percentage reduction of respiratory components in the steady state, with NADH as substrate, in membranes from (*a*) strain AN 62 and (*b*) strain AN 59 (*Ubi*⁻): the effects of piericidin A or cyanide on strain AN 62 and the effect of added ubiquinone (Q-1) on strain AN 59 (*Ubi*⁻). Conditions of experiments were as described in Table 6. ○, Strain AN 62; △, strain AN 62 plus piericidin A; □, strain AN 62 plus cyanide; ▲, strain AN 59 (*Ubi*⁻); ●, strain AN 59 (*Ubi*⁻) plus ubiquinone (Q-1). Abbreviation: Cyt, cytochrome.

Table 6. *Percentages of flavin, cytochrome* b_1 *and cytochrome* a_2 *reduced in the aerobic steady state in membranes from strains AN 62 and AN 59* (Ubi⁻)

The steady state oxidation–reduction levels were determined by using a dual-wavelength spectrophotometer as described in the Materials and Methods section. The values for the steady state levels represent the averages of several determinations on at least six different membrane preparations. The times taken for membranes to remove the oxygen are given as an indication of the oxidase rates.

Membrane from	Addition and final concn. (μM)	Approximate time (s) taken to remove dissolved oxygen in the presence of: NADH	Lactate	Steady-state percentage reduction of components in the presence of: NADH: Flavin	NADH: Cytochrome b_1	NADH: Cytochrome a_2	Lactate: Flavin	Lactate: Cytochrome b_1	Lactate: Cytochrome a_2
AN 62	—	7	20	30	10	<5	19	8	<5
AN 62	NaCN (800)	20	80	59	43	>50	27	29	>50
AN 62	Piericidin A (130)	70	70	46	20	<5	30	13	<5
AN 62	HQNO (130)	80	120	42	18	<5	18	12	<5
AN 62	Sodium Amytal (3000)	7	50	30	10	<5	17	2	<5
AN 59	—	60	140	54	23	<5	31	24	<5
AN 59	Ubiquinone (Q-1) (40)	<5	10	26	14	<5	15	7	<5

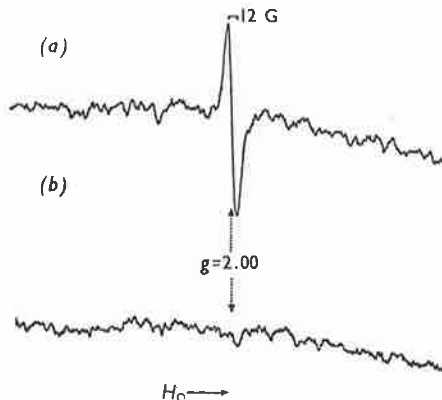


Fig. 4. Effect of inhibitors on the electron-spin-resonance spectrum of membranes from strain AN62 in the absence of added substrate. (*a*) Spectrum from membranes in buffer. A similar spectrum was obtained in the presence of sodium cyanide (12.5mM). (*b*) Spectrum of the same membrane preparation in the presence of either piericidin A (2mM) or HQNO (2mM). The preparation of the membrane samples and the technique used for the preparation of the electron-spin-resonance samples are described in the Materials and Methods section.

Table 7. *Percentage reduction of ubiquinone in the aerobic steady state and the effect of piericidin A*

Membrane preparations (0.2ml) containing about 6mg of protein were incubated with constant shaking at 25°C with 1.3ml of 30mM-sodium–potassium phosphate buffer, pH7.4, containing 7.5mM-$MgCl_2$. The percentage reduction of ubiquinone was measured as described in the Materials and Methods section. Piericidin A, where included, was added (final concn. 107μM) to the buffer before the addition of the membranes.

Time of incubation without substrate (min)	Final concn. of NADH* (mM)	Percentage reduction of ubiquinone in: Membranes	Membranes plus piericidin A
2	0	52	38
10	0	51	2
20	0	32	3
20	2.0	55	0

*Incubation was continued for 30s after addition of NADH.

deficiency. The effect of piericidin A and HQNO on the steady state oxidation–reduction levels of both flavin and cytochrome b_1 was, however, similar to the effect of ubiquinone deficiency (Fig. 3). Therefore, although ubiquinone deficiency, piericidin A, HQNO and sodium cyanide all inhibit after cytochrome b_1, the effect of sodium cyanide differs from those of the other three in the degree of increase in the percentage reduction of cytochrome b_1 in the steady state (see the Discussion section). Sodium Amytal, which inhibits only the lactate oxidase system, appears to inhibit before cytochrome b_1.

Formation of ubisemiquinone. Electron-spin-resonance studies on membrane fractions from strains AN59 (*Ubi*$^-$) and AN62 indicated the presence of a signal in the strain AN62 preparations attributed to the semiquinone of ubiquinone (Hamilton, Cox, Looney & Gibson, 1970). The signal was present in the absence of substrate, reaching a maximum value of approx. 2% of the total ubiquinone present, and rapidly disappeared after the addition of NADH. The effect of the inhibitors, sodium cyanide, piericidin A and HQNO on the appearance of the signal in membranes from strain AN62 in the absence of substrate are shown in Fig. 4. Although sodium cyanide had little effect on ubisemiquinone formation, the signal was not observed in the presence of piericidin A or HQNO.

Extraction of the ubisemiquinone from membranes by organic solvents would presumably yield a quinol–quinone mixture by a disproportionation reaction. It was therefore decided to examine the percentage of ubiquinone in the reduced form in membrane preparations from strain AN62 by the rapid solvent-extraction method of Kröger & Klingenberg (1967). The percentage of ubiquinone reduced after 2min incubation under aerobic conditions was 50% (Table 7) and this percentage remained constant for at least a further 8min of incubation. However, the addition of piericidin A caused the percentage reduction of ubiquinone to decrease to 38% after 2min of incubation and after a further 8min the ubiquinone was essentially fully oxidized. Addition of the substrate NADH to membranes, whether preincubated for 2, 10 or 20min, caused the percentage of ubiquinone reduced in the aerobic steady state to rise to 55%. If, however, the membranes were preincubated in the presence of piericidin A the addition of the substrate NADH did not change the ubiquinone from its fully oxidized state. This is in marked contrast with the effect of piericidin A on the percentage reduction of flavin and cytochrome b_1 in the aerobic steady state (see above).

The effect of HQNO on the percentage reduction of ubiquinone was similar to, but not as pronounced as, that of piericidin A. Thus the effect of incubating the membranes with HQNO (107μM) for 10min was to lower the percentage reduction of ubiquinone in the absence of substrate from 51 to 38%.

DISCUSSION

The relationship between growth yield and the amount of substrate utilized in cultures growing

under aerobic or anaerobic conditions is well established (see, for examples, Bauchop & Elsden, 1960; Kormančíková, Kováč & Vidová, 1969). Comparison of the growth yields given by strains AN 62 and AN 59 (Ubi^-) indicate that phosphorylation coupled to electron transport with oxygen as terminal acceptor does not occur in the absence of ubiquinone. The accumulation of lactic acid by strain AN 59 (Ubi^-) also suggests that, even under aerobic growth conditions, glycolysis is the main source of energy. However, the metabolism of strain AN 59 (Ubi^-) has not simply been changed to an anaerobic type of mixed acid fermentation (Wood, 1961), since only relatively small amounts of acetic acid and ethanol are formed. *Escherichia coli*, when grown aerobically on glucose as carbon source, forms an NAD-linked lactate dehydrogenase that functions unidirectionally in producing the D-isomer of lactic acid (Tarmy & Kaplan, 1968). In addition, a flavoprotein-linked membrane-bound D-lactate oxidase is formed that functions in the opposite direction, i.e. formation of pyruvate from D-lactate (Kline & Mahler, 1965). The accumulation of D-lactate in aerobic cultures of strain AN 59 (Ubi^-) is due not only to the decrease in D-lactate oxidase activity but also to a fourfold increase in the NAD-linked lactate dehydrogenase (P. Stroobant, unpublished work).

A number of *E. coli* K 12 strains grown on glucose as carbon source have been examined for the presence of various oxidase systems; the NADH oxidase and D-lactate oxidase systems were always quantitatively the most important, whereas the presence of other systems, such as the malate oxidase and the succinate oxidase, varied from strain to strain (G. B. Cox, unpublished work). It is clear that ubiquinone is involved in the NADH oxidase, lactate oxidase and malate oxidase systems, since the oxidase activities were much lower in strain AN 59 (Ubi^-) than in the revertant strain AN 62. Further, the addition of ubiquinone (Q-1) to membranes from AN 59 (Ubi^-) caused an immediate increase in oxidase activities to values greater than those found in membranes from strain AN 62.

The inhibitions of the NADH oxidase system by dicoumarol and HQNO found in the present work are similar to those found by Bragg & Hou (1967) and Jones (1967), whereas our preparations were somewhat more sensitive to cyanide. The inhibitors piericidin A and HQNO are alike in that they inhibited at low concentrations and both inhibitions were reversed by added ubiquinone (Q-1).

The results of the steady state experiments indicate that, whereas ubiquinone deficiency, piericidin A, HQNO and sodium cyanide all inhibit electron transport after cytochrome b_1, the effect of cyanide differs from those of the other three in the degree of increase in the percentage reduction of cytochrome b_1 in the steady state. Thus, with NADH as substrate, cyanide used at a concentration causing less inhibition of electron transport than ubiquinone deficiency increased the percentage reduction of cytochrome b_1 some fourfold while increasing that of flavin twofold. However, ubiquinone deficiency, and the inhibitors piericidin A and HQNO added at a concentration giving a greater inhibition of electron transport than by cyanide, caused only a twofold increase in the percentage reduction of cytochrome b_1. These observations are consistent with ubiquinone functioning, and piericidin A and HQNO inhibiting, both before and after cytochrome b_1. Jones (1967) has proposed that ubiquinone functions between flavin and cytochrome b_1 in the NADH oxidase system of *E. coli*, although Kashket & Brodie (1963) have suggested that vitamin K rather than ubiquinone functions at this site. Evidence for a quinone functioning after cytochrome b_1 is provided by Krogstad & Howland (1966) working with the succinate oxidase system of *Corynebacterium diphtheriae*, in which the only quinone is vitamin K.

In the lactate oxidase system of strain AN 62 ubiquinone appears to function only after cytochrome b_1, since the percentage reduction of this cytochrome in membranes from strain AN 59 (Ubi^-) was not markedly different from that in membranes from strain AN 62 in the presence of cyanide. The lactate oxidase system, as distinct from the NADH oxidase system, was inhibited by Amytal, and the percentage reduction of cytochrome b_1 in the aerobic steady state was lower in the presence of Amytal, indicating that it inhibits before cytochrome b_1. However, the percentage reduction of flavin was about the same in the presence or absence of Amytal, and therefore Amytal may inhibit, not immediately before cytochrome b_1, but between two flavoproteins.

The high percentage of ubiquinone in the reduced form in membranes from strain AN 62 in the absence of substrate is in sharp contrast with the other electron carriers examined, all of which are essentially fully oxidized before the addition of substrate. Further, ubiquinone is present in the membranes at a concentration some 25 times that of cytochrome b_1. The values found for the percentage reduction of ubiquinone could possibly arise from disproportionation of ubisemiquinone on extraction into organic solvent. However, quantitative determination, from electron-spin-resonance spectra of the ubisemiquinone present in membranes indicates that about 2% of the ubiquinone was present in the radical form. If the values found for reduced ubiquinone (up to 50%), as measured by extraction, were due to disproportionation then essentially all of the ubiquinone should be in the radical form.

The explanation for this discrepancy is not known. However, electron-spin-resonance measurements were recorded under quite different conditions (e.g. of aeration, temperature and concentration of membranes) from those used in the measurements of the amounts of reduced ubiquinone by the rapid solvent-extraction technique. Also, there are difficulties associated with the quantitative determination of free radicals (Beinert & Palmer, 1965), and there is also the possibility that an appreciable amount of the ubisemiquinone radical is involved in a metal chelate complex (see below) devoid of an electron-spin-resonance signal (cf. Beinert & Hemmerich, 1965).

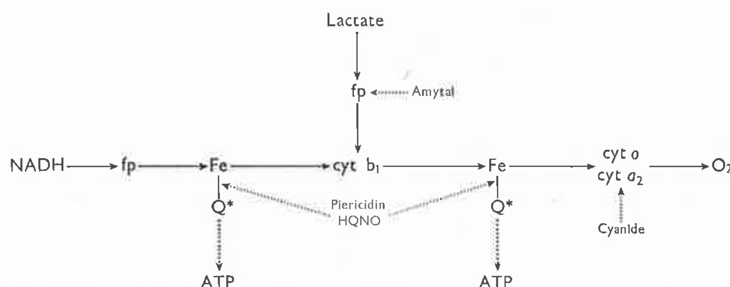
Piericidin A, in the absence of substrate, eliminated the electron-spin-resonance signal and caused the oxidation of reduced ubiquinone (as measured by solvent extraction), although at a lower rate than the elimination of the electron-spin-resonance signal. This inhibitor also prevented a reduction of ubiquinone after the addition of substrate, an observation in marked contrast with its effects in raising the values for the percentage reduction of flavin and cytochrome b_1 in the steady state.

The inhibition by piericidin A of ubiquinone reduction on the addition of substrate has also been found previously (Snoswell & Cox, 1968) with a different strain of *E. coli*. However, in the earlier work ubiquinone was found to be fully oxidized in the absence of substrate, an effect probably due to the use of a different membrane ('small-particle') preparation rather than a difference in strains since similar results were obtained with small-particle preparations from strain AN 62. The percentage reduction of ubiquinone in small-particle preparations from this strain in the absence of substrate rose to about 50% on storage of the membranes at 0°C (A. M. Snoswell, unpublished work). The inhibition of reduction of ubiquinone by piericidin A has also been shown in ox heart mitochondria (Jeng *et al.* 1968) under conditions where, from the data presented, it is likely that reduction of the cytochromes occurred.

Scheme 1 is advanced as a basis for further work, taking into account the various observations reported above.

From the evidence discussed above, ubiquinone has been placed both before and after, cytochrome b_1 in the electron-transport chain. However, it is difficult to envisage ubiquinone functioning as a direct electron carrier at two sites, since its redox potential has not been demonstrated to be markedly changed by environment (see Boyer, 1968). The effects of the inhibitor piericidin A in fact suggest that ubiquinone is not acting as a direct electron carrier. Thus, although this inhibitor, with NADH as substrate, causes an increase in the percentage reduction of flavin and cytochrome b_1 in the steady state, it completely inhibits reduction of ubiquinone. However, lack of ubiquinone or the presence of piericidin A caused marked inhibition of forward electron transport. Therefore we propose that ubiquinone is complexed with an electron carrier and that the carrier alone does not function as efficiently in electron transport as the electron carrier–ubiquinone complex. Ubiquinone would be in the semiquinone form in the complex and would disproportionate on extraction into organic solvent giving about 50% of ubiquinol. The elimination of the electron-spin-resonance signal by piericidin A would reflect disruption of the complex with subsequent inhibition of electron transport. Estimation of the percentage reduction of ubiquinone in the membranes, after the addition of piericidin A, indicated that complete oxidation of the ubiquinol (formed in the membranes by disproportionation of ubisemiquinone after addition of inhibitor) took several minutes, even though the radical was eliminated in less than 30s. Thus piericidin A, as well as preventing reduction of ubiquinone on the addition of substrate, also prevents rapid oxidation of endogenous ubiquinol via the electron-transport chain, implying that piericidin A effectively separates ubiquinone from the remainder of the electron-transport chain.

The most likely electron carrier to form a complex



Scheme 1. Ubiquinone function in *E. coli*. Abbreviations: fp, flavoprotein; Q*, ubisemiquinone; cyt, cytochrome.

with ubiquinone would be non-haem iron, since non-haem iron can function at different redox potentials (see Lardy & Ferguson, 1969). An electron-spin-resonance signal attributed to non-haem iron has been found in membranes from strain AN 62 (Hamilton *et al.* 1970), and, further, iron analysis on membrane preparations (D. J. David, unpublished work) indicated that there was more than sufficient iron to allow formation of the proposed complex. A non-haem iron–ubiquinone–protein complex in mitochondria has been suggested (Vallin & Löw, 1968) as the primary high-energy intermediate in oxidative phosphorylation (see also Blumberg & Peisach, 1965; Moore & Folkers, 1964). In this regard it may be noted that an anaerobic-type growth yield is given by strain AN 59 (*Ubi*$^-$) even though low oxidase activities can be demonstrated. It is possible that evidence for an interaction between the ubisemiquinone and non-haem iron could be obtained from electron-spin relaxation studies similar to those carried out by Beinert and co-workers (see Beinert & Hemmerich, 1965) on flavin–metal systems.

The terminal region of the electron-transport chain has not been studied extensively in the present work because of the difficulty of measuring the steady-state oxidation–reduction levels of cytochrome *o* in the presence of cytochrome b_1. Cytochrome *o* is likely to be the important terminal oxidase in the membranes of *E. coli* cells harvested in the early stages of growth (Castor & Chance, 1959). Vitamin K has not been included in Scheme 1, although it has been suggested that this vitamin is the quinone involved in NADH oxidation in *E. coli* (Kashket & Brodie, 1963). Preliminary experiments with a vitamin K-deficient strain of *E. coli* indicate that this quinone is not concerned in aerobic respiration.

We thank Miss B. Craker, Mrs J. McDonald and Miss B. Humphrey for technical assistance. Dr H. Doelle, Department of Microbiology, University of Queensland, is thanked for examining culture supernatants for fermentation products, and Mr P. Stroobant for the determinations of glucose and the colorimetric determination of lactate. We are indebted to Dr M. E. Winfield and Mr F. D. Looney of the Division of Applied Chemistry, Commonwealth Scientific and Industrial Research Organisation, Melbourne, Vic., Australia, for the generous assistance in the measurement of electron spin resonance. Dr D. J. David, Division of Plant Industry, Commonwealth Scientific and Industrial Research Organisation, Canberra, A.C.T., Australia, is thanked for determinations of iron.

REFERENCES

Barker, S. B. (1957). In *Methods in Enzymology*, vol. 3, p. 241. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Bauchop, T. & Elsden, S. R. (1960). *J. gen. Microbiol.* **23**, 457.

Beinert, H. & Hemmerich, P. (1965). *Biochem. biophys. Res. Commun.* **18**, 212.

Beinert, H. & Palmer, G. (1965). *Adv. Enzymol.* **27**, 105.

Bishop, D. H. L., Pandya, K. P. & King, H. K. (1962). *Biochem. J.* **83**, 606.

Blumberg, W. E. & Peisach, J. (1965). In *Non-Heme Iron Proteins*, p. 101. Ed. by San Pietro, A. Yellow Springs: Antioch Press.

Boyer, P. D. (1968). In *Biological Oxidations*, p. 193. Ed. by Singer, T. P. New York: Interscience Publishers Inc.

Bragg, P. D. & Hou, C. (1967). *Archs Biochem. Biophys.* **119**, 194.

Castor, L. N. & Chance, B. (1959). *J. biol. Chem.* **234**, 1587.

Chance, B. & Redfearn, E. R. (1961). *Biochem. J.* **80**, 632.

Chance, B. & Williams, G. R. (1956). *Adv. Enzymol.* **17**, 65.

Chappell, J. B. (1964). *Biochem. J.* **90**, 225.

Cox, G. B., Snoswell, A. M. & Gibson, F. (1968). *Biochim. biophys. Acta*, **153**, 1.

Cox, G. B., Young, I. G., McCann, L. M. & Gibson, F. (1969). *J. Bact.* **99**, 450.

Crane, F. L., Hatefi, Y., Lester, R. L. & Widmer, C. (1957). *Biochim. biophys. Acta*, **25**, 220.

Doelle, H. W. (1969). *J. Chromat.* **39**, 398.

Ernster, L., Lee, I.-Y., Norling, B. & Persson, B. (1969). *Eur. J. Biochem.* **9**, 299.

Green, D. E. & Brierley, G. P. (1965). In *Biochemistry of Quinones*, p. 405. Ed. by Morton, R. A. London and New York: Academic Press Inc.

Green, D. E. & Wharton, D. C. (1963). *Biochem. Z.* **338**, 335.

Hamilton, J. A., Cox, G. B., Looney, F. D. & Gibson, F. (1970). *Biochem. J.* **116**, 319.

Hoffmann, P. C., Kunz, H. W., Schmid, W. & Siess, M. (1964). *Biochem. Z.* **339**, 548.

Jeng, M., Hall, C., Crane, F. L., Takahashi, N., Tamura, S. & Folkers, K. (1968). *Biochemistry, Easton*, **7**, 1311.

Jones, C. W. & Redfearn, E. R. (1966). *Biochim. biophys. Acta*, **113**, 467.

Jones, R. G. W. (1967). *Biochem. J.* **103**, 714.

Kashket, E. R. & Brodie, A. F. (1963). *J. biol. Chem.* **238**, 2564.

Kline, E. S. & Mahler, H. R. (1965). *Ann. N.Y. Acad. Sci.* **119**, 905.

Klingenberg, M. & Kröger, A. (1966). *Abstr. FEBS 3rd Meet., Warsaw*, no. M3.

Kormančíková, V., Kováč, L. & Vidová, M. (1969). *Biochim. biophys. Acta*, **180**, 9.

Kröger, A. & Klingenberg, M. (1966). *Biochem. Z.* **344**, 317.

Kröger, A. & Klingenberg, M. (1967). In *Current Topics in Bioenergetics*, vol. 2, p. 151. Ed. by Sanadi, D. R. New York: Academic Press Inc.

Krogstad, D. J. & Howland, J. L. (1966). *Biochim. biophys. Acta*, **118**, 189.

Lardy, H. A. & Ferguson, S. M. (1969). *A. Rev. Biochem.* **38**, 991.

Lester, R. L., Crane, F. L. & Hatefi, Y. (1958). *J. Am. chem. Soc.* **80**, 4751.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Monod, J., Cohen-Bazire, G. & Cohn, M. (1951). *Biochim. biophys. Acta*, **7**, 585.

Moore, H. W. & Folkers, K. (1964). *J. Am. chem. Soc.* **86**, 3393.

Morton, R. A., Wilson, G. M., Lowe, J. S. & Leat, W. M. F. (1957). *Chemy Ind.* p. 1649.

Redfearn, E. R. & Pumphrey, A. M. (1960). *Biochem. J.* **76**, 64.

Slein, M. W. (1965). In *Methods of Enzymatic Analysis*, p. 117. Ed. by Bergmeyer, H. New York and London: Academic Press Inc.

Snoswell, A. M. (1966). *Biochemistry, Easton*, **5**, 1660.

Snoswell, A. M. & Cox, G. B. (1968). *Biochim. biophys. Acta*, **162**, 455.

Storey, B. T. & Chance, B. (1967). *Archs Biochem. Biophys.* **121**, 279.

Swank, R. T. & Burris, R. H. (1969). *J. Bact.* **98**, 311.

Szarkowska, L. (1966). *Archs Biochem. Biophys.* **113**, 519.

Taber, H. W. & Morrison, M. (1964). *Archs Biochem. Biophys.* **105**, 367.

Tarmy, E. M. & Kaplan, N. O. (1968). *J. biol. Chem.* **243**, 2587.

Vallin, I. & Löw, H. (1968). *Eur. J. Biochem.* **5**, 402.

Wood, W. A. (1961). In *The Bacteria*, vol. 2, p. 59. Ed. by Gunsalus, I. C. & Stanier, R. Y. New York: Academic Press Inc.

Aust. J. Biol. Sci., 1976, **29**, 33–42

Production of Endogenous Acetate by the Liver in Lactating Ewes

N. D. Costa[A], *G. H. McIntosh*[B] *and A. M. Snoswell*[A]

[A] Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S.A. 5064.
[B] Division of Human Nutrition, CSIRO, Kintore Avenue, Adelaide, S.A. 5000.

Abstract

The production of endogenous acetate by the liver has been investigated in lactating ewes using animals with indwelling arterial, and portal and hepatic venous cannulae. The capacity of the liver to produce acetate from acetyl-CoA *in vitro* has also been examined using homogenates prepared from liver biopsy samples.

Mean arterial, portal and hepatic venous blood acetate concentrations in four ewes at 4 weeks lactation were 0·40, 1·00 and 1·46 mM respectively. The mean exogenous and endogenous acetate production rates were 56 and 54 mmol/h respectively, giving a total of 110 mmol/h. The mean portal–hepatic venous difference in free fatty acid concentration was 81 μM. Converting this uptake of free fatty acids by the liver (based on palmitate as a standard) to 2-carbon equivalents, the acetate produced accounted for 70% of the fatty acids taken up. The correlation coefficient (r^2) between uptake of free fatty acids and production of acetate by the liver was 0·83 ($P < 0·01$).

Calculation of the net acetate production *in vivo* gave a mean value for the production of acetate of 0·75 mmol/min. Calculation of the *in vitro* enzymic capacity of the liver to produce acetate from acetyl-CoA gave a mean of 0·94 mmol/min. These results indicate that enzymic production of acetate from acetyl-CoA, via carnitine acetyltransferase and acetylcarnitine hydrolase (see Costa and Snoswell 1975*a*), can adequately account for the substantial production of acetate by the liver in lactating ewes.

Introduction

Acetate is the major product of fermentation in the rumen and caecum of sheep and other ruminants, and this volatile fatty acid is an important source of energy in these animals (Annison and Armstrong 1970). In addition to this acetate produced in the alimentary tract, endogenous acetate contributes some 25% of total acetate turnover in normal sheep (Annison and White 1962; Bergman and Wolf 1971) and in fed steers and mature cows (Lee and Williams 1962). In fasted sheep this value may rise to 50–75% (Annison and White 1962; Bergman and Wolf 1971).

Although the importance of endogenous acetate in ruminant metabolism has been recognized for more than a decade, we still know little of the control of its release from tissues (see Annison 1973). The carbon source for this endogenous acetate is unclear. All metabolic processes proceeding via acetyl-CoA are potential sources of endogenous acetate; this acetyl-CoA would be formed in the mitochondrial matrix, and the inner mitochondrial membrane is impermeable to acetyl-CoA (Tubbs and Garland 1968). Thus a mechanism for effective release of acetyl-CoA from the mitochondria must exist, or acetyl-CoA must be first hydrolysed to acetate.

A number of authors have suggested that endogenous acetate is probably derived from acetyl-CoA via the acetyl-CoA hydrolase (EC 3.1.2.1) reaction (Annison *et al.*

1967; Ballard 1972; Knowles *et al.* 1974). However, our recent work (Costa and Snoswell 1975*b*) has shown that acetyl-CoA hydrolase is an artifact and that the activity is due to the combined action of carnitine acetyltransferase (EC 2.3.1.7), located in the inner mitochondrial membrane, and acetylcarnitine hydrolase (Costa and Snoswell 1975*a*), located in the outer mitochondrial membrane. The combined action of these two enzymes could account for the production of endogenous acetate.

Baird *et al.* (1974) have recently reported a very substantial production of acetate by the liver of a lactating cow *in vivo* as measured by hepatic–portal differences in blood acetate concentration. Thus, we decided to examine endogenous acetate production in the lactating ewe drawing on our previous experience with the surgical preparation of sheep with indwelling portal and hepatic venous and arterial cannulae (Snoswell and McIntosh 1974). We attempted to associate any production of acetate by the liver, as measured by hepatic–portal differences, with the enzymic capacity of the liver to produce acetate from acetyl-CoA.

The present work has established that there is a substantial net production of endogenous acetate by the liver of lactating ewes. Free fatty acids simultaneously taken up by the liver could account for the acetate produced by the combined carnitine acetyltransferase–acetylcarnitine hydrolase system, which has adequate capacity to account for this endogenous acetate production.

Materials and Methods

Animals

The sheep used were 5-year-old Merino ewes weighing approximately 50 kg. One ewe was a Dorset × Merino. The animals were chosen so that, at the time of intensive sampling, they were at the height of lactation (i.e. 4 weeks into the lactation cycle). The sampling was repeated on one ewe after 8 weeks of lactation. The lambs were left with the ewes to maintain active lactation. Under these conditions milk yields after 8 weeks fall to 1·0 l/day compared with 2·9 l/day after 4 weeks lactation (Snoswell and Linzell 1975). The animals were maintained in pens and fed chaffed lucerne hay *ad libitum*.

Surgical Cannulation of Blood Vessels

Indwelling cannulae were inserted into the portal and hepatic veins under general anaesthesia as described previously (Snoswell and McIntosh 1974). A cannula was also inserted into the femoral artery under local anaesthesia on the morning of sampling by the technique described previously (Snoswell and McIntosh 1974). A further cannula (polyvinyl tube 1·00 mm i.d., 1·5 mm o.d.) was also inserted through a 13-gauge needle into the superficial mammary vein, which was clearly visible on the abdomen anterior to the udder. The cannula was fixed in place with metal clips. All cannulae were kept patent by flushing with heparin–saline mixture (100 i.u. heparin/ml).

Measurement of Hepatic Blood Flow

Total hepatic venous blood flow through the liver was assessed using the bromosulphophthalein (BSP) clearance technique as described by Shoemaker (1964) and Katz and Bergman (1969). A priming dose of 100 mg BSP was administered intravenously followed by a constant infusion of 10 mg/min into the jugular vein over a period of 2·5 h. Thirty min were allowed for equilibration of the dye with the blood, after which simultaneous portal and hepatic venous samples (5 ml) were withdrawn into heparinized syringes every 20 min for the following 2 h. BSP concentrations were determined on plasmas which were prepared on the same day. Packed cell volumes were determined on the blood samples and a sample of hepatic venous blood was drawn prior to commencing the infusion, as an analytical blank. Plasma BSP concentrations were determined by the method of Varley (1963).

Blood flow determinations were made midway through the experimental blood sampling periods, on either one or two occasions, depending on the length of the sampling period. In all blood flow

determinations (with one exception), blood levels of BSP were constant over the period of analysis. There was no evidence of changing packed cell volumes, as might be expected if excessive removal of blood was occurring. Arterial samples drawn together with portal venous samples were shown to contain equivalent BSP concentrations.

Tissue Preparations

The sheep were killed by severing the necks, and samples of liver were immediately freeze-clamped with aluminium-faced tongs previously cooled in liquid N_2 (Wollenberger *et al.* 1960). The $HClO_4$ extracts of frozen tissue were prepared as described by Snoswell and Henderson (1970). Fresh liver samples were collected into 0·25M sucrose–23 mM potassium phosphate (pH 7·4).

Blood Samples

Blood samples for metabolite assays were drawn from all four cannulae (mammary, arterial, and portal and hepatic venous) simultaneously at hourly intervals for 12 h. Blood fractions of 2·0 ml were immediately added to 2·0 ml of 15% perchloric acid for acetate assays and 1·0-ml fractions added to a mixture of 20 ml chloroform plus 6·625 ml of 0·1M sodium phosphate buffer (pH 6·2) for the determination of free fatty acids.

Metabolite Assays

The perchloric acid extracts of the blood samples were adjusted to pH 6·5 with 3M KOH and centrifuged at 5°C to remove potassium perchlorate.

Acetate was measured in duplicate on 30–60-µl aliquots of the neutralized extract using a specific enzyme assay described by Knowles *et al.* (1974). Acetate kinase used in the assay may utilize propionate at 2% of the rate of acetate (Bergmeyer and Möllering 1974) but the propionate concentration even in portal blood is only one-seventh of the acetate concentration (Bergman and Wolf 1971). Thus any interference in the assay of acetate by propionate would be negligible.

Free fatty acids were estimated in the chloroform extracts of the blood samples in the Auto Analyzer (Technicon Instruments Corporation, Ardsley, N.Y.) according to the method described by Dalton and Kowalski (1967).

Alanine was measured using alanine dehydrogenase. The assay system contained 20 mM sodium carbonate (pH 10·0), 5 mM NAD, 25 µg enzyme protein and neutralized extract in a final volume of 1 ml. Reduction of NAD was monitored at 340 nm.

Acetylcarnitine was measured in the neutralized extracts of blood and freeze-clamped livers by the method of Pearson and Tubbs (1964).

Free carnitine was measured in the neutralized extracts of freeze-clamped livers by the method of Marquis and Fritz (1964).

Carnitine Acetyltransferase

This enzyme was assayed in the supernatant fractions obtained by centrifuging (8000 *g* for 3 min) frozen and thawed homogenates (20 % w/v) of liver prepared in hypotonic 0·025M sucrose containing 2·3 mM potassium phosphate (pH 7·4) and 0·1% Triton X-100. The reaction mixture consisted of 400 mM tris–HCl (pH 8·0), 100 µM acetyl-CoA, 330 µM L-carnitine, 100 µM 5,5′-dithio-bis(2-nitrobenzoic acid) and tissue enzyme (2 mg wet weight of liver) in a total volume of 1·0 ml. The reaction was monitored spectrophotometrically by the increase in absorbance at 412 nm due to the yellow 5-thio-2-nitrobenzoic anion at 37°C. The mixture was preincubated at 37°C for 10 min and the reaction started by the addition of L-carnitine.

Carnitine Palmitoyltransferase (EC 2.3.1.21)

The activity of this enzyme was measured in homogenates of liver prepared as described for the carnitine acetyltransferase assay. The reaction mixture consisted of 200 mM tris–HCl (pH 8·2), 100 µM palmitoyl-CoA, 330 µM L-carnitine, 100 µM 5,5′-dithio-bis(2-nitrobenzoic acid) and tissue homogenate (2 mg wet weight liver) in a total volume of 1·0 ml. The reaction was monitored spectrophotometrically by the increase in absorbance at 412 nm and 37°C. The reaction was started by addition of L-carnitine. The increase in absorbance in the absence of L-carnitine was due to palmitoyl-CoA hydrolase (EC 3.1.2.2) activity. The difference in rates of absorbance at 412 nm and 37°C was the true carnitine palmitoyltransferase activity.

Instrumentation for these assays was described previously in Costa and Snoswell (1975*a*).

Results

The results in Table 1 show that, although there is a considerable variation in the concentration of acetate in the portal and hepatic venous blood and arterial blood of the four ewes, there is a substantial and significant ($P < 0 \cdot 01$) production of endogenous acetate by the liver of each animal as judged by hepatic–portal differences in blood acetate. The mean hepatic–portal difference for the four ewes at 4 weeks lactation is 0·46 μmol/ml and the average hepatic venous blood flow is 1·74 l/min (Table 4), giving a mean net endogenous acetate production of 48 mmol/h. This value is increased to 54 mmol/h if a correction is made for the input into liver via the hepatic artery in addition to the main input via the portal vein (see Bergman and Wolf 1971). This production of endogenous acetate by the liver is almost equal to the production of exogenous acetate in the portal-drained viscera, viz. 56 mmol/h calculated from the mean portal–arterial difference in blood acetate concentration of 0·60 μmol/ml (Table 1) and a calculated portal blood flow rate of 1·56 l/min.

Table 1. Acetate concentrations in arterial, and in portal and hepatic venous blood of lactating ewes
Acetate was estimated in duplicate as described in the text. The values shown are means ± S.E.M. with the number of blood samples assayed given in parentheses. The significance of the differences between hepatic–portal and portal–arterial samples, as determined by paired *t* test, is also indicated

Ewe	Blood acetate concentration (μmol/ml)				
	Hepatic (H)	Portal (P)	Arterial (A)	H−P	P−A
1	2·70±0·18 (6)	1·82±0·19 (6)	0·95±0·17 (6)	0·88±0·24 (6) $P < 0 \cdot 01$	0·88±0·17 (6) $P < 0 \cdot 01$
2	1·08±0·08 (6)	0·66±0·11 (6)	0·20±0·04 (6)	0·42±0·06 (6) $P < 0 \cdot 01$	0·46±0·05 (6) $P < 0 \cdot 05$
3	1·31±0·13 (11)	1·05±0·13 (11)	0·21±0·05 (11)	0·26±0·06 (11) $P < 0 \cdot 01$	0·82±0·16 (11) $P < 0 \cdot 001$
4a[A]	0·76±0·10 (12)	0·46±0·05 (12)	0·22±0·02 (12)	0·29±0·06 (12) $P < 0 \cdot 01$	0·23±0·05 (12) $P < 0 \cdot 001$
Mean	1·46	1·00	0·40	0·46	0·60
4b	0·46±0·02 (12)	0·40±0·03 (12)	0·24±0·02 (12)	0·07±0·02 (12) $P < 0 \cdot 001$	0·16±0·03 (12) $P < 0 \cdot 001$

[A] Ewe 4a sampled after 4 weeks lactation, 4b is the same ewe sampled after 8 weeks lactation.

Thus the mean total (endogenous plus exogenous) acetate production rate was 110 mmol/h for these lactating ewes. Simultaneously with the production of acetate by the liver, there was a significant ($P < 0 \cdot 01$) uptake of acetate across the udder, as determined by the arterial–mammary venous difference in blood acetate concentration, with an approximate 50% extraction after 4 weeks of lactation.

The portal blood concentration of free fatty acids exceeded that in the hepatic blood in each ewe (Table 2). In three of the five cases, the portal–hepatic difference was significant. The mean portal–hepatic difference for the four ewes was 81 nmol/ml. The portal and hepatic venous blood concentrations of free fatty acids were significantly ($P < 0 \cdot 01$ and $P < 0 \cdot 05$ respectively) less for ewe No. 4 at 8 weeks lactation than those determined at 4 weeks lactation (Table 2). The free fatty acid concentrations are based on the use of palmitic acid as a standard, so that the uptake of fatty acids from the blood across the liver is equivalent to 8×81 or 648 nmol/ml potential 2-carbon

units. Thus acetate produced by the liver, viz. 0·46 μmol/ml (Table 1), represents 70% of the 2-carbon potential of the free fatty acids extracted by the liver.

The portal–hepatic difference in the concentration of free fatty acids in the blood varied considerably over the total sampling periods in each case. Indeed it was found essential to take a considerable number of blood samples over an extended period to

Table 2. Free fatty acid concentrations in hepatic and portal venous blood of lactating ewes
Free fatty acids were determined as described in the text. The values shown are means ± S.E.M. with the number of blood samples given in parentheses. The significance of the differences between portal and hepatic concentrations were determined by paired *t* test. n.s., No significant difference

Ewe	Free fatty acid concentration in blood (nmol/ml)			
	Hepatic (H)	Portal (P)	P−H	Significance
1	251 ± 51 (4)	314 ± 67 (4)	63 ± 33 (4)	n.s.
2	251 ± 29 (9)	365 ± 66 (9)	114 ± 41 (9)	$P < 0\cdot05$
3	346 ± 53 (10)	403 ± 73 (10)	57 ± 24 (10)	n.s.
4a[A]	448 ± 16 (10)	535 ± 25 (10)	87 ± 26 (10)	$P < 0\cdot01$
Mean	324	404	81	
4b	365 ± 24 (11)	394 ± 20 (11)	29 ± 8 (11)	$P < 0\cdot01$

[A] Ewe 4a sampled after 4 weeks lactation, 4b is the same ewe sampled after 8 weeks lactation.

obtain statistically significant results. The results shown in Fig. 1 indicate a significant correlation ($P < 0\cdot01$) between the portal–hepatic difference for free fatty acids and the hepatic–portal difference in blood acetate concentrations, with a correlation coefficient (r^2) of 0·83. After 4 weeks of lactation the portal–hepatic difference in free fatty acid concentration for ewe No. 4 was 87 nmol/ml while the hepatic–portal

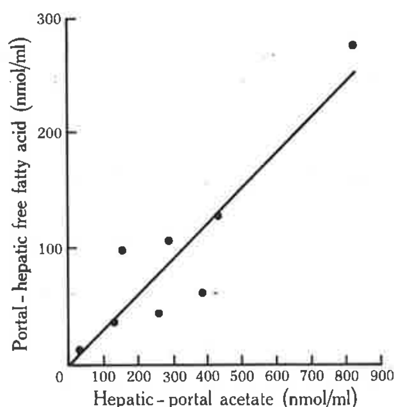


Fig. 1. Portal–hepatic differences in blood free fatty acid *versus* hepatic–portal differences in blood acetate for hourly blood samples. Simultaneous hepatic and portal blood samples were taken from a ewe (No. 4) after 4 weeks lactation. Blood acetate and free fatty acid concentrations were determined as in Tables 1 and 2.

difference in acetate concentration was 0·29 μmol/ml, but after 8 weeks of lactation these values had fallen significantly ($P < 0\cdot01$) to 29 nmol/ml and 0·07 μmol/ml respectively. At this later stage of lactation there was no correlation between free fatty acid uptake and acetate production. The portal–hepatic difference in concentration of L-alanine in ewe No. 1 was 0·016 mM. There was no detectable acetylcarnitine in portal or hepatic venous blood.

The activity of carnitine acetyltransferase in the liver of these lactating ewes was 2·2 μmol min^{-1} (g tissue)$^{-1}$ (see Table 3), which is similar to that in normal wethers, as reported previously (Snoswell and Koundakjian 1972). The activity of carnitine

Table 3. The activity of carnitine acetyltransferase, carnitine palmitoyltransferase and enzymic conversion of acetyl-CoA to acetate in liver biopsies from lactating ewes

Liver biopsy samples were removed at the time of surgical insertion of the cannulae and again at autopsy. The activities shown are the means of assays on these two samples. Activities were measured as described in the text. The mean values ±S.E.M. are those for all assays on four ewes. The number of assays is shown in parentheses

Ewe	Liver enzyme activities [μmol min^{-1} (g wet tissue)$^{-1}$]		
	Carnitine acetyltransferase	Carnitine palmitoyltransferase	Acetate production from acetyl-CoA
1	2·1	4·3	0·7
2	3·0	4·2	1·2
3	2·4	5·3	1·5
4a[A]	1·5	2·2	1·3
Mean	2·2±0·23 (10)	4·1±0·33 (8)	1·1±0·14 (10)
4b	2·4	4·2	1·1

[A] Ewe 4a sampled after 4 weeks lactation, 4b is the same ewe sampled after 8 weeks lactation.

palmitoyltransferase, relative to carnitine acetyltransferase activity, of 4·1 μmol min^{-1} (g tissue)$^{-1}$ (see Table 3) is very much greater than that reported previously for wethers (Snoswell and Henderson 1970). The average enzymic activity for converting acetyl-CoA to acetate, via the carnitine acetyltransferase and acetylcarnitine hydrolase reactions (see Costa and Snoswell 1975*b*) in homogenates of liver, was 1·1 μmol min^{-1} (g wet weight)$^{-1}$ (see Table 3).

Table 4. Comparison of the actual *in vivo* production rate of acetate by the liver with the *in vitro* enzymic capacity of the liver to convert acetyl-CoA to acetate as determined on biopsy samples from lactating ewes

Blood and liver samples were collected and assayed as in Tables 1 and 3. Hepatic venous blood flows are the means of two determinations measured by BSP extraction (see Methods)

Ewe	Blood acetate hepatic–portal concentration (μmol/ml)	Hepatic venous blood flow (l/min)	Hepatic acetate production (mmol/min)	Enzymic acetate production rate (μmol $min^{-1}g^{-1}$)	Total liver wet weight (g)	Total enzymic acetate production capacity (mmol/min)
1	0·88	1·35	1·19	0·7	914	0·64
2	0·42	1·74	0·73	1·2	840	1·01
3	0·26	2·06	0·54	1·5	818	1·28
4a[A]	0·29	1·81	0·52	1·3	620[B]	0·81
Mean	0·46	1·74	0·75	1·2	798	0·94
4b	0·07	1·73	0·12	1·1	620	0·68

[A] Ewe 4a sampled after 4 weeks lactation, 4b is the same ewe sampled after 8 weeks lactation.
[B] Liver weight after 8 weeks lactation used, at 4 weeks the weight was probably greater.

The concentrations of free carnitine and acetylcarnitine in the livers of lactating ewes were 117±25 and 90±31 nmol/g respectively (mean±S.E.M. for six samples). This concentration of acetylcarnitine was much greater than that reported by Snoswell

and Koundakjian (1972) for normal wethers fed on a similar diet. The mean total enzymic capacity of the liver to produce acetate from acetyl-CoA for the four ewes after 4 weeks lactation, calculated by multiplying the enzymic capacity determined *in vitro* by the total liver weight, was 0·94 mmol/min (Table 4). The actual mean *in vivo* acetate production by the livers, determined by multiplying the mean hepatic–portal difference in blood acetate concentration by the mean hepatic venous blood flow rate (1·74 l/min), was 0·75 mmol/min (Table 4). The ewe sampled at 8 weeks after lactation showed an *in vitro* enzymic capacity to produce acetate of 0·68 mmol/min but an actual acetate production of only 0·12 mmol/min (Table 4).

Discussion

The results presented here clearly indicate a substantial net production of endogenous acetate by the liver in lactating ewes. The net endogenous acetate production was almost equivalent to the rate of exogenous acetate production. These results differ from those obtained by Bergman and Wolf (1971) working with fed and fasted sheep. These workers observed only a small net production of endogenous acetate by the liver, both in fed and fasted sheep, but in the latter case the total acetate turnover was considerably reduced. However, the total acetate production (i.e. exogenous and endogenous) in lactating ewes was 110 mmol/h, which is considerably higher than the 75 mmol/h production reported by Bergman and Wolf in non-lactating sheep. The net production of endogenous acetate by the liver does appear to be related to the lactation state. The ewe sampled after 8 weeks of lactation showed an *in vivo* acetate production rate of only 0·12 mmol/min compared with 0·52 mmol/min after 4 weeks lactation. Snoswell and Linzell (1975) report that milk production decreases substantially after 8 weeks of lactation and acetate thiokinase activity in the mammary glands is only about 15% of that after 4 weeks lactation (Snoswell and Linzell, unpublished data). Finally, Baird *et al.* (1974) have shown very substantial hepatic–portal differences in blood acetate concentration in the lactating cow, indicating net endogenous acetate production by the liver during lactation in the cow.

In the experiments described here with lactating ewes there was a significant uptake by the liver of free fatty acids from the blood as well as a significant production of endogenous acetate across the liver. On the basis of 2-carbon equivalents, acetate produced accounted for 70% of the free fatty acids taken up. This of course does not prove that free fatty acids are the source of the acetate (this could only be determined by infusing labelled fatty acids), but it does establish that there is sufficient potential carbon in the fatty acids taken up to account for the acetate produced. Further, when the uptake of free fatty acids and the output of acetate by the liver for each successive blood sample were plotted (see Fig. 1), there was a significant correlation ($r^2 = 0\cdot83$) between the free fatty acids taken up and the acetate produced. With the reservation that correlation does not imply causation, it would appear likely that free fatty acids could supply the major portion of the acetate produced. Both Palmquist (1972) and West and Annison (1964) have shown that significant incorporation of label from [^{14}C]palmitate into acetate occurs in the sheep.

The production of endogenous acetate from other sources is possible, however. In ewe No. 1 the portal–hepatic difference in alanine concentration was 0·016 μmol/ml compared with a hepatic–portal difference in acetate of 0·88 μmol/ml. Thus alanine

could possibly have contributed up to 18% of the acetate formed. Other amino acids might also have been utilized to a minor extent. If ethanol was utilized, it could be converted to acetate without being metabolized via acetyl-CoA. However, this would appear most unlikely from the work of Baird *et al.* (1974) with the lactating cow, where the small uptake of ethanol from the liver contributed only to a very minor extent to the large amount of acetate produced.

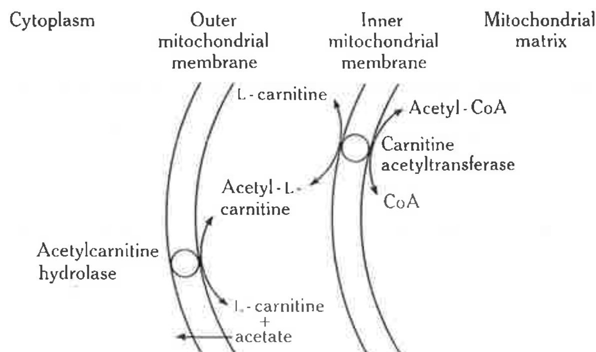


Fig. 2. Proposed mode of action of carnitine acetyltransferase and acetylcarnitine hydrolase in releasing acetate from mitochondrial acetyl-CoA.

The mean enzymic capacity of the liver to produce acetate from acetyl-CoA, as determined *in vitro* using homogenates of biopsy samples, was 0·94 mmol/min, compared with an actual mean net acetate production rate of 0·75 mmol/min. The net production rate may be an underestimate of the actual production rate since some acetate could be used by the liver at the same time as acetate is being produced. This could be demonstrated by infusion experiments with labelled acetate. In spite of this reservation, it would appear that the enzymic capacity of the liver to produce acetate, as measured *in vitro*, could account for the actual endogenous production of acetate observed in the whole animal. The enzymic measurement of the conversion of acetyl-CoA to acetate used here involves a combination of two enzymes, carnitine acetyltransferase associated with the inner mitochondrial membrane (see also Edwards *et al.* 1974) and acetylcarnitine hydrolase associated with the outer mitochondrial membrane (see Costa and Snoswell 1975*a*, 1975*b*).

This enzyme system then establishes a flow, as shown in Fig. 2, which adequately accounts for the breakdown of mitochondrial acetyl-CoA leading to the release of acetate.

The reason why sheep liver should produce considerable amounts of acetate during lactation is of interest. We suggest here that acetate is produced to relieve an intramitochondrial build up of acetyl-CoA derived from fatty acid oxidation. This would appear to be an energetically wasteful process, yet it is no more wasteful than the production of ketone bodies and should be regarded as an alternative process to ketone body production in producing an important metabolite for use in the mammary glands. Sheep liver mitochondria oxidize fatty acids at a relatively slow rate (Koundakjian and Snoswell 1970) and this appears to be due to a lesser ability to oxidize Krebs cycle intermediates. The high activity of carnitine palmitoyltransferase in the livers of lactating ewes reported here (Table 3), together with the significant uptake of fatty acids and acetate production by the livers *in vivo*, suggests that the

limitation on total fatty acid oxidation is not due to a limitation on the conversion of fatty acids to acetyl-CoA but rather on the subsequent oxidation of acetyl-CoA. Baird and Heitzman (1970) found significantly reduced levels of a number of Krebs cycle intermediates in the liver of lactating cows compared with non-lactating cows, suggesting a decreased turnover of the Krebs cycle in bovine liver during lactation. Thus, if Krebs cycle activity in sheep liver is depressed during lactation, the fate of excess acetyl-CoA would largely be determined by the relative activities of carnitine acetyltransferase and of the ketone body-producing enzymes and their affinities for acetyl-CoA. In this respect the activity of carnitine acetyltransferase in liver of lactating sheep is considerable [$2 \cdot 22\ \mu$mol min^{-1} (g wet tissue)$^{-1}$; see Table 3]. The question of mechanisms controlling acetate production obviously requires further investigation.

Acknowledgments

The authors are indebted to Mr D. Pethick and Mr G. D. Henderson for assistance in the collection of the blood samples and to Mr R. Fishlock for skilled technical assistance. The authors are also grateful to Mr O. H. Filsell for help in the analyses of the free fatty acids in blood. Grants from the Australian Meat Research Committee and the Australian Research Grants Committee to A.M.S. are gratefully acknowledged. N.D.C. is supported by a grant from the Australian Wool Corporation.

References

Annison, E. F. (1973). In 'Production Disease in Farm Animals'. (Eds J. M. Payne, K. G. Hibbitt and B. F. Sansom.) p. 140. (Bailliere Tindall: London.)

Annison, E. F., and Armstrong, D. G. (1970). In 'Physiology of Digestion and Metabolism in the Ruminant'. (Ed A. T. Phillipson.) pp. 422–37. (Oriel Press: Newcastle.)

Annison, E. F., Brown, R. E., Leng, R. A., Lindsay, D. B., and West, C. E. (1967). *Biochem. J.* **104**, 135–47.

Annison, E. F., and White, R. R. (1962). *Biochem. J.* **84**, 546–52.

Baird, G. D., and Heitzman, R. J. (1970). *Biochem. J.* **116**, 865–74.

Baird, G. D., Symonds, H. W., and Ash, R. (1974). *Proc. Nutr. Soc.* **33**, 70a.

Ballard, F. J. (1972). *Am. J. Clin. Nutr.* **25**, 773–9.

Bergman, E. N., and Wolf, J. E. (1971). *Am. J. Physiol.* **221**, 586–92.

Bergmeyer, H. U., and Möllering, H. (1974). In 'Methods of Enzymatic Analysis'. Vol. 3, 2nd Edn. (Ed. H. U. Bergmeyer.) pp. 1520–8. (Academic Press: New York.)

Costa, N. D., and Snoswell, A. M. (1975*a*). *Biochem. J.* **152**, 161–6.

Costa, N. D., and Snoswell, A. M. (1975*b*). *Biochem. J.* **152**, 167–72.

Dalton, C., and Kowalski, C. (1967). *Clin. Chem.* **13**, 744–51.

Edwards, Y. H., Chase, J. F. A., Edwards, M. R., and Tubbs, P. K. (1974). *Eur. J. Biochem.* **46**, 209–15.

Katz, M. L., and Bergman, E. N. (1969). *Am. J. Physiol.* **216**, 946–52.

Knowles, S. E., Jarrett, I. G., Filsell, O. H., and Ballard, F. J. (1974). *Biochem. J.* **142**, 401–11.

Koundakjian, P. P., and Snoswell, A. M. (1970). *Biochem. J.* **119**, 49–57.

Lee, S. D., and Williams, W. F. (1962). *J. Dairy Sci.* **45**, 893–6.

Marquis, N. R., and Fritz, I. B. (1964). *J. Lipid Res.* **5**, 184–7.

Palmquist, D. L. (1972). *J. Nutr.* **102**, 1401–6.

Pearson, D. J., and Tubbs, P. K. (1964). *Nature (London)* **202**, 91.

Shoemaker, W. C. (1964). In 'The Liver'. (Ed C. Roullier.) pp. 243–66. (Academic Press: New York.)

Snoswell, A. M., and Henderson, G. D. (1970). *Biochem. J.* **119**, 59–65.

Snoswell, A. M., and Koundakjian, P. P. (1972). *Biochem. J.* **127**, 133–41.
Snoswell, A. M., and Linzell, J. L. (1975). *J. Dairy Res.* **42**, 371–80.
Snoswell, A. M., and McIntosh, G. H. (1974). *Aust. J. Biol. Sci.* **27**, 645–50.
Tubbs, P. K., and Garland, P. B. (1968). *Brit. Med. Bull.* **24**, 158–64.
Varley, H. (1963). In 'Practical Clinical Biochemistry'. 3rd Edn. pp. 313–14. (Heinemann: London.)
West, C. E., and Annison, E. F. (1964). *Biochem. J.* **92**, 573–8.
Wollenberger, A., Ristan, O., and Schoffa, G. (1960). *Pflügers Arch. Gesamte Physiol. Menschen Tiere* **270**, 399.

Manuscript received 24 September 1975

Biochimica et Biophysica Acta, 716 (1982) 290–297
Elsevier Biomedical Press

BBA 21121

METABOLIC EFFECTS OF ACETATE IN PERFUSED RAT LIVER

STUDIES ON KETOGENESIS, GLUCOSE OUTPUT, LACTATE UPTAKE AND LIPOGENESIS

ALAN M. SNOSWELL [a], RODNEY P. TRIMBLE [b], RICHARD C. FISHLOCK [a],
GERALD B. STORER [b] and DAVID L. TOPPING [b,*]

[a] *Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, SA 5064 and* [b] *CSIRO, Division of Human Nutrition, Glenthorne Laboratory, O'Halloran Hill, SA 5158 (Australia)*

(Received December 29th, 1981)

Key words: Acetate effect; Ketogenesis; Glucose output; Lactate uptake; Lipogenesis; (Rat liver)

1. Livers from fed male rats were perfused in situ in a non-recirculating system with whole rat blood containing acetate at six concentrations, from 0.04 to 1.5 μmol/ml, to cover the physiological range encountered in the hepatic portal venous blood in vivo. 2. Below a concentration of 0.25 μmol/ml there was net production of acetate by the liver, while above it there was net uptake with a fractional extraction of 40%. 3. No relationship was observed between blood [acetate] and hepatic ketogenesis, the ratio [3-hydroxybutyrate]/[acetoacetate] or glucose output, either at low fatty acid concentrations or during oleate infusion. 4. Following the increase in serum fatty acid concentration, induced by oleate infusion, there were sequential increases in ketogenesis and the ratio of [3-hydroxybutyrate]/[acetoacetate] while hepatic glucose output rose and lactate uptake fell significantly after the shift in redox state. 5. There was a highly significant negative correlation between blood [acetate] and hepatic lactate uptake during oleate infusion. At the highest acetate concentration of 1.5 μmol/ml there was a small net hepatic lactate output. After oleate infusion ceased, lactate uptake increased, but the negative correlation between blood [acetate] and hepatic lactate uptake persisted. 6. Livers were also perfused with either [1-^{14}C]acetate or [U-^{14}C]lactate at a concentration of acetate of either 0.3 or 1.3 μmol/ml of blood. With [1-^{14}C]acetate, most of the radioactivity was recovered as fatty acids at the lower concentration of blood acetate. At the higher blood [acetate] a considerably smaller proportion of the radioactivity was recovered in lipids. With [U-^{14}C]lactate the reverse pattern obtained i.e., recovery was greater at the high concentration of acetate and fell at the low concentration. Fatty acid biosynthesis, measured with $^{3}H_2O$, was stimulated from 2.4 to 6.6 μmol of fatty acid/g of liver per h by high blood [acetate] although the contribution of (acetate+lactate) to synthesis remained constant at 33–38% of the total. 7. These results emphasize the important role of the liver in regulating blood acetate concentrations and indicate that it can be a major hepatic substrate. Acetate taken up by the liver appeared to compete directly with lactate for lipogenesis and metabolism and acetate uptake was inhibited by raised blood [lactate].

Acetate plays a central role in the metabolism of ruminants and its role as a major energy source in these animals is well known [1]. On the other hand, acetate can also be a significant fuel for mammals in general [2] and for skeletal muscle in humans in particular [3].

It has been known for some time that non-ruminant liver can both release and take up acetate [4,5]. However, only recently it has been realized

* To whom correspondence should be addressed.

0304-4165/82/0000–0000/$02.75 © 1982 Elsevier Biomedical Press

that acetate is a potentially significant hepatic substrate for monogastric omnivores. Buckley and Williamson [6] have clearly demonstrated that, under most nutritional conditions, there is substantial production of acetate by the gastrointestinal tract in the rat. They showed that this substrate was delivered to the liver by the portal blood supply and above a critical concentration of 0.20 μmol/ml acetate was removed by the liver, while below this concentration there was a net hepatic production.

Metabolic studies in vitro, both in recirculating liver perfusions and with isolated hepatocytes, are complicated by the fact that the liver both utilizes and produces acetate. Soling and co-workers [7,8] have overcome this problem by using a non-recirculating liver perfusion system. These authors found that infusion of hexanoate in this system stimulated the production of both ketone bodies and acetate. However, no exogenous acetate was added except for the trace initially present in the albumin. Further, hexanoate is not a physiological fatty acid substrate for the rat liver.

To date there have been no studies on the effects of blood acetate concentration on ketogenesis and related metabolic functions of the liver in vitro. Thus, we decided to examine these relationships in a non-recirculating rat liver perfusion system under normal conditions and where hepatic ketogenesis was stimulated by oleate.

Materials and Methods

Liver perfusions

Livers from fed male Hooded Wistar rats (195–205 g of body weight) were perfused in situ with defibrinated undiluted rat blood, dialyzed against a modified Krebs and Henseleit buffer [9] to remove vasoconstrictive factors [10]. In preliminary experiments it was found that the dialyzed perfusate contained 0.3–0.4 μmol of acetate/ml. The source of the acetate was found to be the dialysis tubing (Visking Co., Chicago, IL, USA) which contained 40 μmol free acetate/g. This contamination was inimical to the objectives of the experiment and was prevented by heating the tubing to 90°C for 15 min with 500 ml distilled water, the procedure being repeated three times with fresh water.

Because of the large volume of blood and, hence, blood donor rats (in excess of 35 per experiment) for non-recirculating perfusion, we chose to examine effects of acetate at six points over the physiological concentration range [11]. Thus, perfusion was initiated with 70 ml of blood with a recirculating perfusate before transferring to the non-recirculating system. During the 15 min equilibration period the rate of flow was 8 ml/min (approx. 0.75 ml/g of liver per min) and the pO_2 of the blood was maintained at 12–13 kPa. Non-recirculating perfusion was commenced (zero time) by transferring the inflow supply and collecting the hepatic venous outflow. The perfusate consisted of 2 equal portions of 110 ml of blood contained in separate reservoirs. Reservoirs A and B contained equal concentrations of acetate, while to B was added a 6 ml bovine albumin:oleate complex to give a final concentration of 1.0 μmol fatty acid/ml of serum. The complex was prepared by adding 2.448 g bovine serum albumin (Sigma, Fraction V, essentially fatty acid-free) to 57.6 mg oleic acid (Calbiochem-A grade) brought into solution at 40°C with 1 M NaOH. Final volume was 20 ml and the concentration of NaCl adjusted to 0.15 M. To minimize changes in perfusate haematocrit, which might per se modify changes in ketogenesis and carbohydrate metabolism [12,13], blood from reservoir B was centrifuged and 6 ml serum removed before addition of fatty acid solution. To maintain a physiological PO_2 both reservoirs were equilibrated with humidified air:CO_2:N_2 (70:5:25). Throughout the experiment the temperature of the liver was kept at 37°C.

Each experiment acted as its own control with the order of perfusion: reservoir A, 4 min; reservoir B, 12 min; reservoir A, 8 min. Effluent samples were collected at 1 min intervals and immediately chilled in ice. As it had been found that contamination occurred due to release of free acetate from plastic centrifuge tubes [14], blood was collected and processed in glass ones. 3 ml of blood was also taken at 2, 20 and 22 min after zero time from reservoir A and at 4 and 12 min from reservoir B.

At the end of the experiment the liver was rapidly excised, blotted dry and weighed.

To determine the relative contributions of

acetate and lactate to fatty acid biosynthesis at high and low concentrations of acetate (see Results and Discussion) livers from fed rats were perfused initially as described above. Non-recirculating perfusion was then started with 190 ml blood containing 2 μCi of either [1-^{14}C]acetate or [U-^{14}C]lactate (both obtained from Amersham International, Amersham, U.K.) and maintained for 20 min. These additions were made in 1 ml 0.15 M NaCl which also contained sufficient sodium acetate to raise the inflow concentration to either 0.3 or 1.3 μmol/ml blood. Total lipogenesis was measured with 10 mCi ^3H_2O also added in 1 ml 0.15 M NaCl. In these experiments the rate of perfusion was again 8 ml/min but because mean liver weights were rather lighter than the previous perfusions (9.2 g vs. 10.7 g), blood flow per unit weight of liver was higher. Blood samples were collected in ice as described above and at the end of the experiment the liver was also quickly blotted dry and weighed and then frozen for lipid analysis. Because of the non-recirculating perfusion, it was not possible to measure oxidation of [^{14}C]acetate or lactate. This was because of the low incorporation of isotope into $^{14}CO_2$ and the presence of high radioactivity in the substrates in the effluent blood.

Analytical techniques

Blood acetate was determined enzymatically by the method of Snoswell et al. [15]. Lactate and ketone bodies in whole perfusate were measured by the respective methods of Hohorst [16] and Williamson et al. [17]. Blood glucose was measured with a commercial glucose oxidase preparation (Boehringer Mannheim Pty. Ltd., Victoria, Australia). Fatty acids in serum were measured by GLC as the methyl esters [18] using $C_{17:0}$ as internal standard.

For determination of lipogenesis, serum (2 ml) was separated from samples taken at 2, 5, 10, 15 and 20 min and extracted with $CHCl_3$:CH_3OH (2:1, v/v). The solvent was partitioned with 0.03 M HCl and the lipids hydrolyzed and fatty acids extracted [13]. Liver was homogenized and similarly extracted and fatty acids and cholesterol and water-soluble radioactivity measured [13]. 0.2 ml portions of inflow perfusate were taken at 0, 10 and 20 min and deproteinized as for blood glucose determinations. The specific radioactivity of ^3H_2O was determined [11] by counting 0.2 ml of the supernatant. Further portions (1 ml) of the deproteinized samples were made alkaline with 0.1 ml 4% NaOH and evaporated to dryness at 70°C to remove ^3H_2O. The residual material was redissolved in 0.1 ml H_2O and counted for calculation of the specific radioactivities of [^{14}C]acetate or [^{14}C]lactate. In obtaining rates of incorporation of the latter substrates into perfusate lipids, radioactivities (dpm) were averaged for each time interval and multiplied by the rate of flow [18]. A correction was applied for incorporation of [U-^{14}C] lactate into fatty acids and cholesterol because of decarboxylation at carbon 1. Rates of total lipogenesis were determined from the incorporation of ^3H_2O using the factors and assumptions of Windmueller and Spaeth [19].

Statistical methods

The results before and after oleate infusion were compared on the basis of paired *t*-tests within each experiment. Linear regression analysis was used to examine correlations.

Results and Discussion

Hepatic acetate uptake

We examined the effects of acetate at six concentrations calculated to cover the range likely to be encountered in the hepatic portal vein of the rat in vivo, i.e., 0.04, 0.07, 0.21, 0.68, 1.07 and 1.48 μmol/ml. These concentrations were attained by addition of acetate to both blood reservoirs and remained constant throughout the experiment.

At the two lowest concentrations examined (i.e., 0.04 and 0.07 μmol/ml of blood) there was net production of acetate by the liver, while at the three higher concentrations, net hepatic removal occurred (Fig. I). A highly significant ($r = 0.985$, $P < 0.001$) correlation was obtained between acetate uptake (or output) and the inflow acetate concentration when the former was averaged for the experimental period 4–24 min for the five experiments. A balance between acetate uptake and output was obtained at an inflow concentration of 0.25 μmol/ml of blood, a figure remarkably close to that obtained in vivo by Buckley and Williamson [6].

Infusion of oleate: albumin had no significant

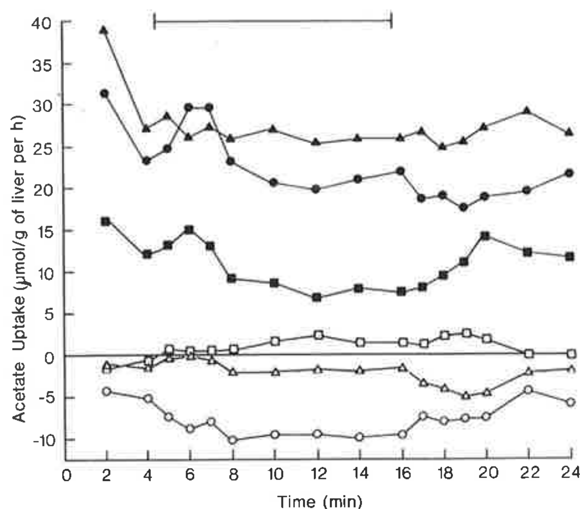


Fig. 1. Concentration dependence of the effects of blood acetate and serum fatty acids in perfused rat liver. Livers from fed rats were perfused in situ with defibrinated, dialyzed whole rat blood, containing sodium acetate at the indicated concentrations, as described in the text. During the period 4–16 min perfusion was with blood containing additionally oleate-albumin to a concentration of total fatty acids of 1.11 μmol/ml of serum. Uptake or output of acetate was calculated by direct difference from concentrations in inflow and outflow blood at the times indicated. Acetate concentrations (μmol/ml) of blood: ○———○, 0.04; △———△, 0.07; □———□, 0.21; ■———■, 0.68; ●———●, 1.07; ▲———▲, 1.48. The bar indicates the period oleate: albumin infusion.

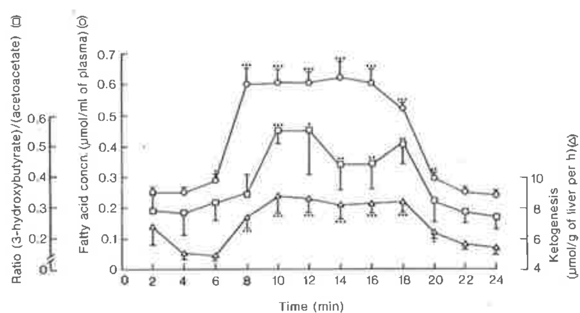


Fig. 2. Concentrations of serum fatty acids in the hepatic outflow, the rate of net ketogenesis and the ratio [3-hydroxybutyrate]/[acetoacetate] in perfused rat liver. Livers from fed rats were perfused in situ with defibrinated, dialyzed, whole rat blood containing sodium acetate, at concentrations of 0.04, 0.07, 0.21, 0.68, 1.07 and 1.48 μmol/ml of blood, as described in the text. During the period 4–16 min perfusion was with blood plus oleate-albumin to a concentration of total fatty acids of 1.11 μmol/ml of serum. Serum fatty acids and blood ketone bodies were determined and net ketogenesis calculated by direct difference from concentrations in inflow and outflow blood at the times indicated. No relationship was detected between blood acetate concentration and serum fatty acid concentration, ketogenesis or redox state and the data are shown as the means ± S.E. for the six perfusions. ○———○, serum fatty acid concentration (μmol/ml); △———△, ketogenesis (μmol/g of liver per h); □———□, ratio [3-hydroxybutyrate]/[acetoacetate]. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ by paired t-test against 4 min value.

effect on uptake or output of acetate at all inflow concentrations (Fig. I). In those experiments at higher acetate concentrations, where uptake occurred, the fractional extraction of acetate was approx. 40%.

Serum fatty acids, ketogenesis and redox state

Mean concentrations of serum long-chain fatty acids in the liver outflow are shown in Fig. 2 together with the rate of ketone body production and the ratio [3-hydroxybutyrate]/[acetoacetate].

Concentrations of fatty acids in the two reservoirs were constant throughout the perfusion, with mean values of 0.47 ± 0.04 [6] μmol/ml and 1.11 ± 0.06 [6] μmol/ml of serum, respectively. During the initial period of perfusion, the concentration of fatty acids in the liver effluent was steady at approx. 0.25 μmol/ml of serum. Following transfer to the reservoir containing oleate: albumin, concentrations rose rapidly and remained constant at 0.60 μmol/ml of serum for the period 8–16 min of perfusion (Fig. 2). The return to pre-infusion concentrations was slightly delayed compared to the initial transfer but serum concentrations returned to 0.25 μmol/ml for the period 22–24 min.

Rates of hepatic fatty acid uptake were calculated for the three steady-state periods of perfusion (i.e., 2–4 min, 8–16 min and 22–24 min) with mean values of 5.85 ± 0.57 [6], 14.45 ± 0.84 [6] and 6.12 ± 0.62 [6] μmol/g of liver/h.

Fractional and absolute uptake of plasma fatty acids by the liver also were independent of blood acetate concentration. Mean values for fractional extraction during the periods of high and low serum fatty acid uptake were the same, i.e., 44.3 and 46.2%, respectively. This quantity is very similar to that for acetate in experiments where there was net uptake and also to the fractional extraction of fructose by the perfused liver and similar rates of blood flow [20].

Over the entire range of blood [acetate] examined we found no relationship at any time interval between acetate concentration and either ketogenesis or the ratio [3-hydroxybutyrate]/[acetoacetate]. For example, during the period of oleate infusion, mean production was 7.9 μmol/g of liver/h at 0.04 μmol of acetate/m of blood and 8.4 μmol/g of liver/h at 1.45 μmol of acetate/m of blood. Analysis of the relationship between blood [acetate] and ketogenesis by linear regression analysis gave very low values of r (<0.05) at each time point over the whole experiment. Similar low values were obtained for the redox state. vs. blood acetate concentration. Therefore for both these parameters mean values are shown. Ketogenesis rapidly declined from a high value at 2 min and remained constant up to 6 min after zero time. The ratio [3-hydroxybutyrate]/[acetoacetate] also remained constant prior to fatty acid infusion. It can be seen that although fatty acid concentrations in the hepatic effluent and the rate of ketogenesis had risen significantly at 8 min, there was a delay of 2 min before the ratio [3-hydroxybutyrate]/[acetoacetate] became reduced. The latter reached a maximum at 10 min of perfusion. This period which elapsed between the increase in ketogenesis and the appearance of a more reduced mitochondrial redox state presumably reflects the time required to saturate NADH-consuming reactions following increased supply from β-oxidation. The reverse change which occurred at the end of fatty acid infusion also indicated that the consumption of NADH was less easily saturated than that of acetyl-CoA.

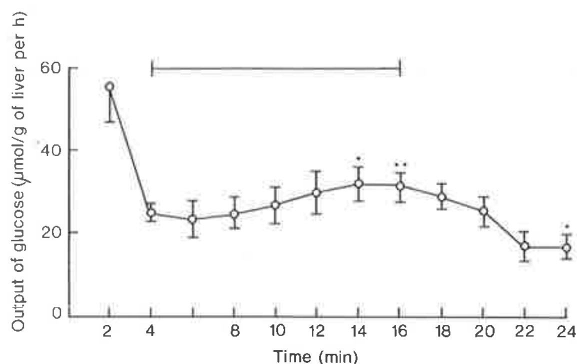


Fig. 3. Output of glucose by perfused rat liver. Livers from fed rat were perfused in situ, with defibrinated, dialyzed whole rat blood containing sodium acetate, at concentrations of 0.04, 0.07, 0.21, 0.68, 1.07 and 1.48 μmol/ml of blood, as described in the text. During the period 4–16 min perfusion was with blood plus oleate-albumin to a concentration of total fatty acids of 1.11 μmol/ml of serum (indicated by the bar). Blood glucose was determined and net output calculated by direct difference from concentrations in inflow and outflow blood. No relationship was detected between blood acetate concentration and hepatic glucose output and therefore data are shown as the mean±S.E. for the six perfusions. *$P<0.05$; **$P<0.01$ by paired t-test against 4 min value.

Hepatic carbohydrate metabolism

Mean concentrations of glucose and lactate in the inflow perfusate were 6.1 and 3.1 μmol/ml of blood, respectively. These rates of hepatic output and uptake were determined by direct difference and are shown in Fig. 3 and Table 1.

As with ketone body metabolism, no relationship was observed between hepatic glucose output and blood acetate concentration ($r=0.03$) and so, mean values are shown (Fig. 3). Glucose output fell rapidly from an initial high rate and at 4 min averaged 25 μmol/g of liver per h. Following fatty acid infusion output rose and by 14–16 min the rate was significantly higher than the pre-infusion value. After the concentration of fatty acid in the effluent perfusate fell, hepatic glucose output also declined rapidly and at the end of the experiment was significantly lower than the preinfusion rate.

Following infusion of oleate, uptake of lactate by the liver fell. Uptake was lowest at 14–16 min and rose rapidly on cessation of fatty acid infusion, i.e., a time course that was the mirror image of that seen with glucose output. However, graphic representation of these data is complex as, unlike the other metabolic processes examined, hepatic uptake of lactate was found to be sensitive to blood acetate concentrations. Accordingly, we have shown individual values for the periods of maximum effect, i.e., 14–16 and 22–24 min of perfusion (Table 1). It can be seen that uptake of lactate showed an extremely strong negative correlation with blood [acetate] at both time intervals. Removal was considerably less during the period of oleate infusion and at the highest concentration of blood acetate, there was a small net hepatic output of lactate.

TABLE I

THE UPTAKE OR OUTPUT OF LACTATE IN PERFUSED RAT LIVER

Livers from fed rats were perfused in situ with defibrinated, dialyzed whole rat blood containing sodium acetate, at the indicated concentrations, as described in the text. During the period 4–16 min, perfusion was with blood plus oleate-albumin to a concentration of total fatty acids of 1.11 μmol of serum. Blood lactate was determined and net uptake or output calculated by direct difference from concentrations in inflow and outflow blood for the period of 14–16 min and 22–24 min of perfusion. Negative values indicate net output. 14–16 min $r = -0.966$, $P < 0.001$; 22–24 min $r = -0.785$, $P < 0.02$.

Inflow acetate Conc. (μmol/ml)	Mean lactate uptake Perfusion period (μmol/g/h)	
	14–16 min	22–24 min
0.04	20.2	54.7
0.07	16.5	22.5
0.21	14.6	17.1
0.68	13.4	16.5
1.07	2.8	4.0
1.45	−3.5	0.3

The fall in lactate uptake and stimulation of glucose output with fatty acid infusion accord with similar recent observations (Topping D.L. and Mayes, P.A., unpublished data) in a recirculating perfusion system where a strong positive relationship was observed between equilibrium blood [glucose] and [lactate] and fatty acid concentrations. Inhibition of lactate uptake by oleate, with a simultaneous stimulation of glucose output, accord with the view that exogenous fatty acids do not stimulate gluconeogenesis in perfused liver [21]. Rather it would appear that there is a more efficient conversion of the lactate taken up to glucose, possibly by inhibition of pyruvate dehydrogenase, an enzyme extremely sensitive to fatty acid concentration [22]. The data also appear to complete the 'glucose-fatty acid cycle' proposed by Randle et al. [23,24] based on studies in perfused heart. These authors showed an inverse relationship between tissue glucose and fatty acid metabolism. Our results, obtained in livers perfused with whole blood, show that the higher concentrations of blood glucose attending oleate infusion were due to enhance glucose output while lactate uptake was impaired. Thus, the extrahepatic changes in carbohydrate metabolism due to long-chain fatty acids also occur in liver.

Metabolic fate of acetate in perfused liver

The data obtained above clearly indicate that at the higher concentrations in the inflow blood, acetate becomes a major metabolic substrate for the liver. If one were to assume complete oxidation via the critic acid cycle, acetate could account for some 20% of hepatic O_2 consumption [12,13]. The lack of any relationship between acetate uptake and ketogenesis was surprising and would suggest that C_2 units were not channeled towards oxidation. Both Damgaard et al. [4] and Buckley and Williamson [6] have suggested that acetate was preferentially directed towards de novo fatty acid

TABLE II

METABOLISM OF [1-^{14}C]ACETATE OR [U-^{14}C]LACTATE IN PERFUSED RAT LIVER

Livers from fed rats were perfused in situ with defibrinated dialyzed whole rat blood containing sodium acetate, at the indicated concentrations, and 2 μCi of either [1-^{14}C]acetate or [U-^{14}C]lactate as described in the text. Blood acetate and lactate and incorporation of radioactivity into liver and perfusate lipids per h were determined as described in the text. Values for acetate and lactate metabolism represent μmol/g liver per h.

Inflow acetate conc. (μmol/ml of blood)	Acetate metabolism				Lactate metabolism			
	Uptake	Incorporation of [1-^{14}C]acetate into:			Uptake	Incorporation of [U-^{14}C]lactate into		
		Glycerol	Fatty acids	Cholesterol		Glycerol	Fatty acids	Cholesterol
0.3	6.1	0.02	4.21	0.15	43.9	1.78	2.90	0.30
1.3	21.6	0.06	5.65	0.26	24.4	2.80	17.19	1.04

synthesis. This view is supported by the observation that lactate uptake fell with increasing blood [acetate] as a number of studies have indicated that the former is a major oxidative substrate in liver and contributes significantly to lipogenesis [25–27]. If acetyl-CoA derived from acetate were to compete with that from lactate oxidation for lipogenesis, this would readily explain the diminished uptake.

Accordingly, livers were perfused at either low (0.3 μmol/ml) or high (1.3 μmol/ml) blood [acetate] and at an inflow lactate concentration of 2.8 μmol/ml. Although absolute rates of acetate and lactate uptake were rather higher than in the first series of perfusions (due to the higher rate of flow/g of liver), the same relationship was obtained. Thus, with increased blood acetate concentration, lactate uptake fell (Table II) At the low levels of blood acetate, most of the [^{14}C] acetate taken up was recovered in liver and perfusate lipids, the major proportion of which was in fatty acids + cholesterol and only a trace in acylglyceride glycerol. In this experiment incorporation of radioactivity into these fractions accounted for over 70% of total uptake. At the higher blood [acetate], incorporation of ^{14}C into fatty acids + cholesterol had fallen to 27% of a considerably increased total uptake of acetate. At this level more radioactivity was found in acylglyceride glycerol but it still remained a very small fraction of the total and presumably reflects randomization of label via the citric acid cycle.

When [^{14}C]lactate was included in the perfusate, the pattern of incorporation was reversed so that, at the high concentration of acetate, nearly 75% of the total lactate taken up by the liver was recovered in fatty acids + cholesterol (Table II). At 0.3 μmol of acetate/ml of blood, incorporation of lactate into these lipids had fallen to 7% of total lactate uptake which had risen nearly 2-fold. Recovery of [^{14}C] lactate in acylglyceride glycerol was similar at both levels of blood acetate.

The respective poor recoveries of the two substrates at the extremes of acetate concentration strongly suggest oxidation and, indeed, lactate is recognized as a good oxidative substrate in liver [25–27].

In these experiments with ^{14}C labelled substrates, where the rate of total lipogenesis was measured with $^{3}H_2O$, we found that at low blood [acetate] mean lipogenesis was 2.4 μmol of fatty acid/g of liver per h (individual values of 2.7 and 2.1). The rate of synthesis was increased to 6.6 μmol of fatty acid/g of liver per h (individual values of 5.3 and 7.9) in livers perfused at the higher concentration of blood acetate. Assuming an average chain length of C_{18} for newly-synthesised fatty acids [19] it can be seen that the contribution of C_2 units from acetate + lactate recovered in fatty acids was low relative to total synthesis. At 0.3 μmol of acetate/ml of blood, the ^{14}C labelled substrates contributed approx. 33% to lipogenesis while the corresponding figure was 38% at the higher blood [acetate]. Thus, it would appear that the simulatory effect of acetate on synthesis is not due to an increase in the relative contributions of the C_2 units taken up by the liver from the perfusate. The source of acetyl CoA for lipogenesis not accounted for by [^{14}C]acetate and [^{14}C]lactate is almost certainly glycogen [25–28] and exogenous acetate appears to enhance its contribution also.

Conclusions

Our studies clearly show in vitro the buffering role of the liver with respect to blood acetate which has been observed in vivo [6]. Good agreement was found between the blood acetate concentration at which the perfused liver switched over from output of acetate to uptake and that at which the organ in the whole rat did the same, i.e., 0.25 μmol/ml. It is probable that this reflects the use of whole blood, providing a supply of O_2 similar to that enjoyed by the liver in vivo [12,13] and also the use of substrates, particularly lactate, at concentrations close to the physiological.

The data show that there is considerable metabolic interaction between acetate and lactate, particularly as lipogenic substrates. It appears that both compete for oxidation or fatty acid biosynthesis but that these processes are mutually exclusive. Further evidence of this competition was obtained in a separate experiment when blood lactate was raised to 3.9 μmol/ml from the more physiological concentrations otherwise employed. In this perfusion at an acetate concentration of 0.7 μmol/ml of blood, uptake was abolished and there

was a net output of 2.1 μmol of acetate/g of liver per h. However, our results do not equate with the stimulation, by acetate, of gluconeogenesis from lactate in isolated hepatocytes from fasted rats as reported by Whitton et al. [29]. In this case, the difference probably reflects one of nutritional status i.e. fed vs. fasting.

Acknowledgements

We wish to thank Mr. R.A. Weller for useful discussion and Misses V.A. Hill and G. Nangle for expert technical assistance. A.M.S. was supported by a grant from the Australian Research Grants Committee (No. D27715558).

References

1 Annison, E.F. and Lewis, D. (1959) Metabolism in the Rumen, p. 65, Methuen, London
2 Ballard, F.J. (1972) Am. J. Clin. Nutr. 25, 773–779
3 Lundquist, F., Sestoft, L., Damgaard, S., Clausen, J.P. and Trap-Jensen, J. (1973) J. Clin. Invest. 52, 3231–3235
4 Damgaard, S.E., Lundquist, F., Tonnesen, K., Hansen, F.V. and Sestoft, L. (1973) Eur. J. Biochem. 33, 87–97
5 Knowles, S.E., Jarrett, I.G., Filsell, O.H. and Ballard, F.J. (1974) Biochem. J. 142, 401–411
6 Buckley, B.M. and Williamson, D.H. (1977) Biochem. J. 166, 539–545
7 Seufert, C.D., Graf, M., Janson, G., Kuhn, A. and Soling, H.D. (1974) Biochem. Biophys. Res. Commun. 57, 901–909
8 Soling, H.D., Graf, M. and Seufert, D. (1974) in Regulation of Hepatic Metabolism. A. Benzon Sympsosium VI (Lundquist, F. and Tygstrup, N., eds.), pp. 695–704, Scand. University Books, Munksgaard, Copenhagen
9 Krebs, H.A. and Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33–66
10 Mayes, P.A. and Felts, J.M. (1966) Proc. Eur. Soc. Study Drug Tox. 7, 16–29
11 Mayes, P.A. and Topping, D.L. (1974) Biochem. J. 140, 111–114
12 Storer, G.B., Trimble, R.P. and Topping, D.L. (1980) Biochem. J. 192, 219–222
13 Topping, D.L., Trimble, R.P. and Storer, G.B. (1981) Biochem. Int. 3, 101–106
14 Topping, D.L., Fishlock, R.C., Trimble, R.P., Storer, G.B. and Snoswell, A.M. (1981) Biochem. Int. 3, 157–163.
15 Snoswell, A.M., Costa, N.D., McLean, J.G., Baird, G.D., Lomax, M.A. and Symonds, H.W. (1978) J. Dairy Res. 45, 331–338
16 Hohorst, H.J. (1963) in Methods of Enzymatic Analysis (H.U. Bergmeyer, ed.), edn. 1., pp. 266–272, Academic Press, New York
17 Williamson, D.H., Mellanby, J. and Krebs, M.A. (1962) Biochem. J. 108, 353–361
18 Topping, D.L., Clark, D.G., Storer, G.B., Trimble, R.P. and Illman, R.J. (1979) Biochem. J. 184, 97–106
19 Windmueller, H.G. and Spaeth, A.E. (1967) Arch. Biochem. Biophys. 122, 362–369
20 Topping, D.L. and Mayes, P.A. (1976) Br. J. Nutr. 36, 113–126
21 Exton, J.H. and Park, C.R., (1967) J. Biol. Chem. 242, 2622–2636
22 Wieland, O.H., Patzelt, C. and Loffler, G. (1972) Eur. J. Biochem. 26, 426–433
23 Randle, P.J., Gardland, P.B., Hales, C.N. and Newsholme, E.A. (1963) Lancet i, 785–789
24 Randle, P.J., Garland, P.B., Hales, C.N., Newsholme, E.A., Denton, R.M. and Pogson, C.J. (1966) Recent Prog. Horm Res. 22, 1–49
25 Clark, D.G., Rognstad, R. and Katz, J. (1974) J. Biol. chem. 249, 2028–2036
26 Salmon, D.M.W., Bowen, N.L. and Hems, D.A. (1974) Biochem. J. 142, 611–618
27 Bloxham, D.P., Fitzsimons, J.T.R. and York, D.A. (1977) Horm. Metab. Res. 9, 304–309
28 Clark, D.G., Topping, D.L., Illman, R.J., Trimble, R.P. and Malthus, R.S. (1980) Metabolism 29, 415–420
29 Whitton, P.D., Rodrigues, L.M. and Hems, D.A. (1979) FEBS Lett. 98, 85–87

Vol. 3, No. 2, August, 1981

BIOCHEMISTRY INTERNATIONAL

Pages 157-163

12

CARBOXYHAEMOGLOBIN INHIBITS THE METABOLISM OF ETHANOL BY PERFUSED RAT LIVER

David L. Topping,[1] Richard C. Fishlock,[2] Rodney P. Trimble,[1] Gerald B. Storer,[1] and Alan M. Snoswell[2]

[1]Division of Human Nutrition, CSIRO, Glenthorne Laboratory, O'Halloran Hill, South Australia 5158, Australia.

[2]Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064, Australia.

Received July 8, 1981

SUMMARY

Livers from fed male rats were perfused *in situ* in a non-recirculating manner with whole rat blood containing 20 mM ethanol. Carboxyhaemoglobin (COHb), at a level of 20% of total haemoglobin depressed O_2 consumption and lowered ethanol utilisation from 4.4 to 2.5 µmol/g/min. In the absence of COHb, acetate release represented 32% of total ethanol uptake. This fraction rose to 60% of total ethanol utilisation with COHb although there was no change in the absolute rate of net acetate production. When livers were perfused with ethanol and COHb there was a change in the net equilibrium resulting in a release of lactate coincident with the fall in ethanol utilisation. Ketogenesis was not altered significantly by COHb. We conclude that, in this system, ethanol taken up by the liver is directed towards lipogenesis and COHb lowers uptake by inhibiting this process.

INTRODUCTION

Carbon monoxide (CO) combines with haemoglobin to give a stable complex, carboxyhaemoglobin (COHb), which lowers the O_2-carrying capacity of the blood by physical combination and also by modifying the O_2 dissociation curve of the remaining haemoglobin (1). Levels of COHb in cigarette smokers are not insignificant and may reach 20% of total haemoglobin (2). At these concentrations COHb can considerably affect hepatic metabolic activity *in vitro* (3-5).

0158-5231/81/020157-07$01.00/0

Copyright © 1981 by Academic Press Australia. All rights of reproduction in any form reserved.

As the liver is the major site of ethanol metabolism in the body, we set out to investigate effects of COHb on its utilisation by the perfused rat liver. Also, as free acetate appears to be a major end product of this hepatic metabolism (6) we chose to examine the relationship between ethanol uptake and acetate output, and the effects of COHb on these parameters, in a non-recirculating perfusion system (7).

MATERIALS AND METHODS

Liver perfusion. Livers from fed adult, male Hooded Wistar rats (200-220 g body weight) were perfused *in situ*. The perfusate was defibrinated whole rat blood, dialysed to remove vasoconstrictive factors (8). Perfusion was initially with 60 ml of recirculating medium and then, after 15 min, changed to a non-recirculating one. This changeover was designated zero-time. For the non-recirculating perfusion there were two reservoirs, A and B. Blood in both reservoirs contained 20 mM ethanol while that in reservoir B also contained blood equilibrated against CO to give a concentration of COHb of 20% of total haemoglobin (3). Blood in both vessels was equilibrated against humidified air:CO_2:N_2 (70:5:25) to give a $\underline{P}O_2$ of approximately 100 mm Hg.

Perfusion followed the order A, 5 min; B, 9 min, and the flow rate was kept at 1.1 ml/g liver/min. Outflow blood samples were taken at 1 min intervals and rapidly chilled in ice. Blood was taken from the inflow reservoirs at 1, 5, 7 and 13 min of perfusion. At the end of the experiment the liver was removed, blotted dry and weighed for calculation of metabolic rates per gm wet weight.

Analytical techniques. Concentrations of ethanol, lactate, glucose and ketone bodies (3-hydroxybutyrate + acetoacetate) in blood were measured as described previously (9). O_2 consumption was measured polarographically (3) at 4 and 12 min after zero time in 2 experiments.

Blood acetate was determined enzymically (10). Previously we noted that the dialysis tubing used for preparation of the blood perfusate contained a significant quantity of acetate. This contamination was prevented by pre-washing the tubing with water at 90-100°C. In the present studies we found that a similar contamination also occurred if the deproteinisation (1 ml of blood + 1 ml of 15% $HClO_4$) was carried out in polythene tubes. Therefore for all samples this step was carried out in glass tubes. It should be noted that in a study of blood acetate levels in human subjects (11), an apparent increase in concentration occurred during storage and may represent similar contamina[tion].

Statistical methods. Metabolic activities before and after COHb infusion were compared on the basis of paired *t* tests within each experiment. Values are shown as the mean±SEM of five perfusions.

RESULTS AND DISCUSSION

Ethanol uptake and acetate output. The mean concentration of ethanol in the hepatic inflow was 19.7±0.8 µmol/ml of blood. Ethanol levels in the liver effluent rose rapidly reaching equilibrium at 4 min.

During the period prior to perfusion with COHb, utilisation was constant at 4.4 µmol/g liver/min (Fig. 1). Following the switch to blood containing 20% COHb there was a dramatic fall in ethanol utilisation. This depression was discernible at 9 min and between 10 and 14 min the mean rate of uptake was constant at 2.5 µmol/g liver/min. To establish conclusively that this decline in ethanol uptake was due solely to perfusion with COHb, a liver was similarly perfused but the blood in reservoir B did not contain COHb. The rate of ethanol removal, as well as the other metabolic activities which were modified by COHb, remained constant throughout this experiment (data not shown).

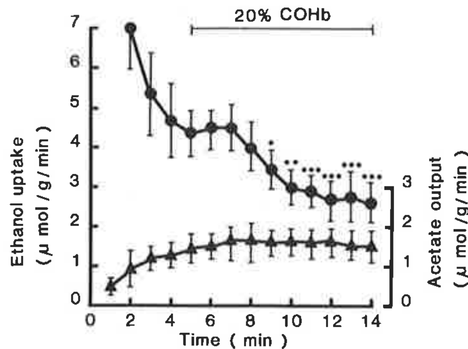


Fig. 1. Effects of 20% carboxyhaemoglobin on the uptake of ethanol (●) and output of acetate(▲) by rat livers perfused with whole blood containing 20 mM ethanol. *,P<0.05; **,P<0.01; ***,P<0.001 by paired t test on 4 min values.

When livers were perfused with ethanol alone, acetate output averaged 1.4 μmol/g liver/min or approximately 32% of ethanol uptake (Fig. 1). Production was essentially unchanged during perfusion with COHb and ethanol although the fractional conversion of ethanol to acetate rose to approximately 60%.

Ketogenesis and carbohydrate metabolism.

In comparison to the rates of ethanol uptake or acetate release, production of ketone bodies was very low. During perfusion with ethanol alone, the mean rate of ketogenesis at 4-6 min was only 0.09±0.1 μmol/g liver/min rising with COHb to 0.11±0.02 μmol/g liver/min with COHb. These rates did not differ significantly from each other or from experiments where livers were similarlyperfused in the absence of ethanol where the mean production was 0.08 μmol/g liver/min (7).

Prior to perfusion with 20% COHb, the liver was in equilibrium with perfusate lactate with essentially zero uptake (Fig.2) at a concentration of 2.10±0.16 μmol/ml in the inflow blood. Following the change to the second reservoir there was a highly significant switch to lactate output. Prior to perfusion with COHb, hepatic glucose output averaged 0.97±0.17 μmol/g liver/min. There was a transient inhibition in production ($P<0.01$) during the period 8-11 when the rate fell to 0.75±0/20 μmol/g liver/min. However, for the remainder of the experiment glucose release was essentially the same as during the period before perfusion with COHb. From these data it is clear that the change in hepatic lactate uptake due to COHb + ethanol did not reflect a sustained inhibition of gluconeogenesis. However they do offer a possible metabolic fate for the 60% of ethanol C_2 units not accountable by acetate production in the control period

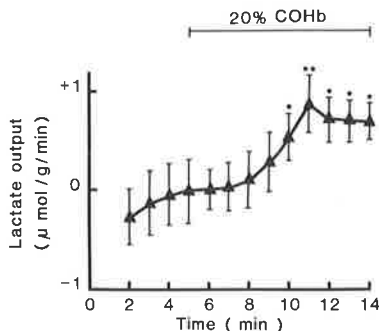


Fig. 2. Effects of 20% carboxyhaemoglobin on the output of lactate by rat livers perfused with whole blood containing 20 mM ethanol (negative values indicate net uptake). *,$P<0.05$; **,$P<0.01$ by paired *t* test on 4 min value.

and also for the metabolic effects of COHb. We have suggested (7) that, in the fed state, acetate taken up by the perfused liver was directed towards lipogenesis. This conclusion was based on the observations that, over a wide range of concentrations, acetate did not affect ketogenesis but depressed lactate uptake as the concentrations of acetate increased. As lactate is a preferred lipogenic substrate in liver (12,13) it appeared that both lactate and acetate were competitive precursors for fatty acid biosynthesis. In the present experiments, as in those of Soling *et al*. (6) with non-recirculating perfusion, ethanol did not alter hepatic ketogenesis but increased the output of acetate. Therefore it would appear that the acetate formed from ethanol was also directed towards lipognesis and not oxidation. This hypothesis would also explain the effects of COHb. In perfused liver COHb lowers O_2 consumption (3-5), an effect which is proportional to its concentration (5), and in 2 of the perfusions in the present series COHb lowered O_2 consumption from 5.4 to 4.2 μmol/g liver/min. COHb also inhibits lipogenesis

(5) and so, ethanol uptake would fall as the main product, acetate, could not be used so efficiently for lipogenesis

It ought to be noted that the effects of ethanol in the present system differ somewhat from those in livers perfused with recirculating blood (9). Thus, in the latter the rate of ethanol uptake is lower and ethanol stimulates both ketogenesis and gluconeogenesis - consistent with enhanced oxidation. We must assume that this reflects accumulation of acetate to a point where it is able to penetrate the mitochondrion. Indeed the situation in the intact animal is much closer to that in non-recirculating perfusion as acetate released by the liver would be rapidly removed by extrahepatic tissues such as heart (14) and skeletal muscle (15).

CONCLUSIONS

To our knowledge, this is the first direct study of effects of COHb on hepatic ethanol metabolism *in vitro*. At a level of 20% of total hemoglobin, COHb virtually halved the rate of ethanol utilisation. It should be considered that, under similar conditions *in vivo*, elimination of ethanol from the body would be considerably impaired.

ACKNOWLEDGEMENTS

We thank Dr. J.D. Potter for useful discussion and Miss L. Maland for technical assistance. A.M.S. was supported by a grant from the Australian Research Grants Committee (No. D277 15558).

REFERENCES

1. Roughton, F.J.W., and Darling, R.C. (1944) Am. J. Physiol. 141, 17-31.
2. Astrup, P., Kjeldsen, K., and Wanstrup, J. (1970) Ann. N.Y. Acad. Sci. 174, 294-300.
3. Topping, D.L. (1975) Biochem. J. 152, 425-527.
4. Gardner, R.S., Topping, D.L., and Mayes, P.A. (1978) Biochem. Biophys. Res. Comm. 82, 526-531.
5. Topping, D.L. (1980) in *Biochemistry of Cellular Regulation* (Clemens, M.J., ed.) vol. 2, pp 165-183, CRC Press, Florida.
6. Soling, H.D., Graf, M., and Seufert, D. (1974) in Regulation of Hepatic Metabolism (Lundquist, F. and Tygstrup, N., eds.) pp 695-704, Scand. University Books, Munksgaard, Copenhagen.
7. Snoswell, A.M., Trimble, R.P., Fishlock, R.C., Storer, G.B., and Topping, D.L. (1981) Proc. Aust. Biochem. Soc. 14, Q5.

8. Mayes, P.A., and Felts, J.M. (1966) Proc. Eur. Soc. Study Drug Toxicity 7, 16-29.
9. Topping, D.L., Clark, D.G., Storer, G.B., Trimble, R.P., and Illman, R.J. (1979) Biochem. J. 184, 97-106.
10. Snoswell, A.M., Costa, N.D., McLean, J.G., Baird, G.D., Lomax, M.A., and Symonds, H.W. (1978) J. Dairy Res. 45, 331-338.
11. Tollinger, C.D., Vreman, H.J., and Werner, M.W. (1979) Clin. Chem. 25, 1787-1790.
12. Clark, D.G., Rognstad, R., and Katz, J. (1974) J. Biol. Chem. 249, 2028-2036.
13. Salmon, D.M.W., Bowen, N.L., and Hems, D.A. (1974) Biochem. J. 142, 611-618.
14. Ballard, F.J. (1972) Am. J. Clin. Nutr. 25, 773-779.
15. Lundquist, F., Sestoft, L., Damgaard, S., Clausen, J.P. and Trap-Jensen, J. (1973) J. Clin. Invest. 52, 3231-3235.

NUTRITION REPORTS INTERNATIONAL

DAILY VARIATIONS IN THE CONCENTRATIONS OF VOLATILE FATTY ACIDS IN THE SPLANCHNIC BLOOD VESSELS OF RATS FED DIETS HIGH IN PECTIN AND BRAN

R.J. Illman,[1] R.P. Trimble,[1] A.M. Snoswell,[2] and D.L. Topping[1]
[1]CSIRO Division of Human Nutrition,
Glenthorne Laboratory, O'Halloran Hill, South Australia 5158, Australia
and
[2]Department of Agricultural Biochemistry,
Waite Agricultural Research Institute, University of Adelaide,
Glen Osmond, South Australia 5064, Australia

SUMMARY

Adult male rats were maintained on one of two diets containing either 10% citrus pectin (CP) or wheat bran (WB) for two weeks. Concentrations of plasma volatile fatty acids (VFA) were significantly higher in both hepatic portal venous and arterial blood at 10.00, 15.00 and 20.00 h in rats fed CP compared with WB. In CP rats total plasma VFA in the hepatic portal vein were constant at approximately 1.0 µmol/ml at 10.00 and 15.00 h falling to 0.7 µmol/ml at 20.00 h. In WB rats comparable values were 0.6, 0.4 and 0.5 µmol/ml. Acetate was the only significant VFA present in arterial samples at 20-30% of hepatic portal venous concentrations. The rise in glucose release from the intestines was considerably delayed on CP compared with WB and occurred when VFA had fallen. Plasma triacylglycerols were relatively constant on diet CP at all sampling times. Levels at 10.00 h were lower on WB but showed a sharp increase in concentration at 15.00 h. It is clear that VFA are major metabolic fuels for the rat liver and are transported in proportion to apparent digestibility of fibre type. However, any chronic effects of dietary fibre in modifying blood glucose or plasma lipids are not directly related to daily variations in VFA.

INTRODUCTION

The importance of volatile fatty acids (VFA), formed by the ruminal degradation of plant fibre, to the metabolism of ruminant herbivores is widely recognised (1). That a similar process occurs in monogastric omnivores may be inferred from the observations that some dietary fibre components, particularly pectins and hemicelluloses, are nearly completely degraded during passage through the human gut (2,3) and high levels of VFA have been found in the caecal contents of the rat and pig (4). Evidence of their absorption has been obtained in rat with the observation of significant concentrations of acetate (5,6) and other VFA (6) in blood from the hepatic portal vein.

Relatively little is known about daily variations in VFA concentrations in hepatic portal venous blood in the rat, particularly in relation to diet. Here we report the effects of adaptation to diets high in pectin and bran on such variations in VFA concentrations in blood from this vessel as well as the aorta. Bran was chosen as it is relatively rich in lignin which is largely resistant to bacterial action, while pectin is highly degradable. Further, as high fibre diets (particularly those containing viscous gums)

have been shown to lower both blood glucose and plasma lipids in diabetic humans (7), blood glucose and plasma triacylglycerols were also measured.

MATERIALS AND METHODS

Animals and diets

Adult male rats of the Hooded Wistar strain (190-200 g of body weight) were used. They were housed in groups of six in cages with wire mesh bottoms (to prevent coprophagy) under conditions of controlled lighting (10.00-22.00 h light) and temperature (21-22°C). Each rat received water *ad libitum* and 21 g/day of a diet containing cornflour, skim milk powder, maize oil, vitamins and salts and either 10% by weight wheat bran (diet WB) (8) or citrus pectin (diet CP). The pectin (degree of methoxylation 72%) was obtained from A.C. Hatrick Pty. Ltd., Botany, NSW 2019, Australia. Samples were also taken from rats consuming a standard chow diet (Diet C).All food presented was consumed.

Sample collection

Food was withdrawn at 09.00 h and all animals were weighed. Rats were taken from the colony at 10.00, 15.00 and 20.00 h. Blood samples were drawn (10) from the hepatic portal vein (2 ml) and systemic aorta (5 ml) under light ether anaesthesia (9) and collected into ice-cold tubes containing lithium heparin as anticoagulant.

Analytical techniques

Blood glucose was assayed directly on 0.2 ml of whole blood using a commercial glucose oxidase method (Boehringer Mannheim Pty. Ltd., Mt. Waverley, Vic. 3149, Australia). TAG in plasma were analysed as described previously (8). VFA were measured by a modification of the method of Tollinger *et al*. (10). Briefly, plasma (250-500 µl) was deproteinized with an equal volume of 10% sulphosalicylic acid, centrifuged and an aliquot of the supernatant, containing 50 µl of 0.1M caproate as internal standard, was vacuum distilled (10). The distillate was adjusted to pH10 with 0.1M NaOH and redistilled under vacuum. The residual VFA sodium salts were dissolved in 20-50 µl of 0.2 M phosphoric acid and 1-2 µl injected on to a 2 mm x 2 mm glass column of Tenax GC (Altech Associates, Sydney, NSW 2130, Australia) coated with 3% phosphoric acid. The latter was chosen in preference to the formic acid normally used (10,11) because of the presence of unacceptable levels of acetate in all samples tested. Temperature programming was from 130 to 160°C at 4°C/min with injector and flame ionization detector temperatures of 150°C and 200°C, respectively. To avoid contamination by exogenous acetate (11) all operations were carried out in glass containers.

VFA concentrations were calculated automatically (by a Hewlett-Packard 3388 computing integrator) relative to caproate. This acid was chosen as internal standard as it was found to be absent in rat plasma.

Tenax GC does not appear to have been used previously for VFA analysis but is an excellent phase for the purpose. Baseline resolution of each acid up to caproate was achieved in 12 min with no tailing and with an extremely long column life.

Statistical methods

The statistical significance of differences between groups was established by the analysis of variance. Data are shown as the means±SEM of six animals per group.

RESULTS AND DISCUSSION

Body weight

The mean starting body weight (all groups combined) was 210 g. In chow-fed controls body weight rose to 274±4 g (n=18) but, as in other recent studies (13,14), mean weight gain was significantly less in rats fed bran and pectin, mean values being 240±4 (n=18) g ($P<0.001$) and 222±3 (n=18) g ($P<0.001$), respectively.

Plasma volatile fatty acids

In samples from the hepatic portal vein of rats fed all three diets, acetate propionate and butyrate were the only significant VFA (Table 1). *Iso*-butyrate, *iso*-valerate and valerate accounted for less than 10% of the total and are not shown individually.

The highest concentrations of total VFA were found at 10.00 h in chow-fed rats with concentrations of acetate similar to those reported by Buckley and Williamson (5) using an enzymic determination. Total VFA at this

Table 1. Concentrations of plasma volatile fatty acids (VFA) in hepatic portal venous blood of rats fed chow (C) or diets containing citrus pectin (CP) or wheat bran (WB).

Time (h)	Group	Acetate	Propionate	Butyrate	Total
		(μmol/ml)			
10.00	C	1.08±0.05[a]	0.21±0.03[a]	0.34±0.05[a,b]	1.67±0.03[a]
	CP	0.67±0.05[a]	0.14±0.03[a]	0.12±0.02[a]	0.95±0.07[a]
	WB	0.42±0.03[a]	0.07±0.01[a]	0.11±0.02[b]	0.61±0.05[a]
15.00	C	0.50±0.04[a]	0.12±0.01[a]	0.20±0.03[a]	0.90±0.08[a]
	CP	0.66±0.05[a]	0.15±0.04[b]	0.16±0.05[b]	1.01±0.05[b]
	WB	0.26±0.02[a]	0.05±0.01[a,b]	0.08±0.01[a,b]	0.42±0.03[a,b]
20.00	C	0.50±0.05[a]	0.15±0.03[a,b]	0.21±0.03[a,b]	0.90±0.10[a]
	CP	0.46±0.05[b]	0.09±0.01[a]	0.08±0.02[a]	0.66±0.07[b]
	WB	0.30±0.03[a,b]	0.07±0.01[b]	0.09±0.01[b]	0.47±0.02[a,b]

Individual or total VFA with the same superscripts at any one sampling time are significantly different ($P<0.05$).

sampling interval were generally similar in composition (but rather higher in concentration) to the study of Remesy and Demigne (6). VFA concentrations in rats fed the stock diet declined to 0.90 μmol/ml at 15.00 h and 20.00 h due to equivalent falls in all constituent acids.

Total plasma VFA in rats fed diet CP were generally lower than chow-fed rats at 10.00 h and 20.00 h (Table 1). VFA levels in these rats were constant at 10.00 and 15.00 h but fell at 20.00 h. In the chow-fed animals the proportions of the major acids were approximately acetate:propionate: butyrate 1:0.2:0.3. However, in plasma samples from the hepatic blood portal vein of rats fed CP the proportions were 1:0.2:0.2.

Concentrations of total and individual VFA in hepatic portal venous samples of rats fed diet WB were significantly lower than in both other groups at all times. Concentrations were highest at 10.00 h and remained constant at approximately 0.4 μmol/ml at 15.00 and 20.00 h. In all samples the proportions of the major acids were similar to those in animals fed the commercial diet.

In contrast to the above, acetate was the only VFA present in significant quantities in arterial samples from all three groups (Table 2). In general, the same relativity of concentration and diurnal variation observed in the hepatic portal vein was also obtained in arterial samples, with the lowest concentrations being observed in bran-fed rats at the later time intervals. Concentrations of acetate were only some 30% of those found in blood draining the gut. It is likely that this is due to both dilution

Table 2. Concentrations of plasma acetate and total volatile fatty acids (VFA) in arterial blood of rats fed chow (C) or diets containing citrus pectin (CP) or wheat bran (WB).

Time (h)	Group	Acetate (μmol/ml)	Total acids (μmol/ml)
10.00	C	0.32±0.03[a]	0.33±0.02[a]
	CP	0.24±0.04[a]	0.25±0.03[a]
	WB	0.16±0.02[a]	0.17±0.02[a]
15.00	C	0.15±0.01[a,b]	0.17±0.02[a,b]
	CP	0.11±0.01[a,b]	0.13±0.02[a]
	WB	0.10±0.01[b]	0.12±0.01[b]
20.00	C	0.12±0.01[a,b]	0.15±0.01[a,b]
	CP	0.09±0.02[a]	0.11±0.02[a]
	WB	0.08±0.01[b]	0.10±0.01[b]

Concentrations of acetate or total VFA with the same superscripts at any one sampling time are significantly different ($P<0.05$).

of splanchnic blood in the vena cava and also to the fact that there is a substantial extraction of acetate by liver (5,15) and heart (16) *in vivo*. For other VFA the fraction remaining in arterial plasma was even lower and Remesy *et al*. (17) have reported hepatic fractional extractions approaching 90% for these acids.

Concentrations of blood glucose and plasma triacylglycerols

We have observed a powerful interaction between net acetate uptake and both carbohydrate metabolism (11,15) and lipogenesis (15) in perfused rat liver. Therefore, it was of interest to document any changes in blood glucose and plasma triacylglycerols in relation to VFA transport.

In control rats, as for VFA, blood glucose concentrations were highest at 10.00 h (Table 3). At this time only arterial levels were measured and in arterial blood glucose concentrations fell to approximately 8 µmol/ml at 15.00 and 20.00 h. At the two latter sampling times, blood from the hepatic portal vein was also analyzed. From the data in Table 3 it can be seen that there was net glucose uptake by the intestines in these animals. Arterial blood glucose levels in rats fed diet CP were generally lower than in controls but the difference was only significant at 20.00 h. In animals on diet CP the arterio-hepatic portal venous concentration difference was

Table 3. Concentrations of blood glucose in the hepatic portal vein (HP) and systemic aorta (SA) and their concentration difference (HP-SA) in rats fed chow (C) or diets containing citrus pectin (CP) or wheat bran (WB).

Time (h)	Group	HP	SA (µmol/ml)	(HP-SA)
10.00	C	-	9.7±0.04	-
	CP	-	9.1±0.03	-
	WB	-	9.6±0.03	-
15.00	C	7.8±0.4[a]	8.1±0.3[a]	-0.2±0.2
	CP	7.6±0.6[b]	7.5±0.6[b]	+0.6±0.4
	WB	9.2±0.5[a,b]	8.9±0.3[a,b]	+0.4±0.4
20.00	C	7.8±0.2[a]	8.4±0.2[a]	-0.6±0.1[a]
	CP	8.9±0.4[a,b]	7.7±0.5	+1.2±0.3[a,b]
	WB	7.4±0.2[b]	7.3±0.2[a]	0.0±0.3[b]

For (HP-SA) negative values indicate net intestinal uptake.

Values with the same superscripts at one any sampling time are significantly different ($P<0.05$).

significantly positive only at 20.00 h. This suggests a considerable delay in absorption and is consistent with the lowering effect of pectin on postprandial glycaemia (18). Arterial blood glucose in rats fed WB did not differ from controls at 10.00 and 15.00 h and was significantly lower at 20.00 h. The pattern of extraction of glucose by the gut in WB rats was similar to those on diet C.

In rats fed the commercial diet, plasma TAG concentrations were constant over the three sampling times with mean values of 1.09±0.16, 1.15±0.13 and 1.09±0.10 μmol/ml, respectively. In rats fed diet CP, TAG concentrations were significantly ($P<0.001$) lower than controls at 10.00 h, mean value 0.50±0.11 μmol/ml. In animals fed this diet TAG rose to 0.98±0.18 μmol/ml at 15.00 h before falling to 0.78±0.06 μmol/ml at the last sampling time. Plasma TAG were much less stable in animals fed diet WB. Thus, at 10.00 h the mean concentration was similar to group CP, 0.36±0.04 μmol/ml. However, at 15.00 h plasma concentrations rose to 1.42±0.15 μmol/ml before falling to 0.43±0.06 μmol/ml at 20.00 h. Although we cannot differentiate between hepatic and intestinal lipoprotein secretion, some of the rise must have been due to the latter as chyle was seen in the thoracic lymph duct at 15.00 h. Whether this phenomenon contributes to the small rise in plasma cholesterol in many dietary trials with bran in human volunteers (18) remains to be determined.

CONCLUSIONS

To our knowledge, this is the first study to relate dietary fibre to splanchnic levels of VFA and circulating glucose and TAG in the rat. It is clear that, for CP and WB, hepatic portal venous VFA were roughly in proportion to the apparent susceptibility of fibre type to microbial degradation with low levels (approaching those following antibiotic treatment (5)) in later samples from rats on WB. In rats fed C and CP, concentrations of VFA in the hepatic portal vein are not insignificant and are sustained for a large part of the day, approaching those of dietary fructose in this blood vessel (9). The net rates of transport of VFA from the intestines were calculated as described by Buckley and Williamson assuming a blood flow of 9 ml/min through the hepatic portal vein (5). Average rates of transport over the period 10.00-20.00 h were 5.6, 4.7 and 2.2 μmol/min for diets C, CP and WB, respectively. At these rates of presentation acetate becomes a significant metabolic substrate for the rat liver perfused *in vitro* with whole blood (15). However, while there is an interaction with the secretion of TAG and the metabolism of carbohydrate (particularly lactate), it is clear that long term effects of dietary pectin on blood glucose and plasma lipids are not directly related to daily alterations in VFA transport.

ACKNOWLEDGEMENTS

We wish to thank Miss G. Nangle for technical assistance. AMS was supported by a grant from the Australian Research Grants Committee (D2771 5558).

REFERENCES

1. Blaxter, K.L. The Energy Metabolism of Ruminants. Hutchinson, London (1962).

2. Werch, S.C., Young, R.W., Day, A.A., Friedemann, T.E. and Ivy, A.C. The decomposition of pectin and galacturonic acid by intestinal bacteria. J. Infect. Dis. 70, 231 (1942).

3. Holloway, W.D., Tasman-Jones, C. and Lee, S.P. Digestion of certain fractions of dietary fiber in humans. Am. J. Clin. Nutr. 31, 927 (1978).

4. Elsden, S.R., Hitchock, M.W.S., Marshall, R.A. and Phillipson, A.T. Volatile acid in the digesta of ruminants and other animals. J. Exp. Biol. 22, 191 (1946).

5. Buckley, B.M. and Williamson, D.H. Origins of blood acetate in the rat. Biochem. J. 166, 539 (1977).

6. Remesy, C. and Demigne, C. Determination of volatile fatty acids after ethanolic extraction. Biochem. J. 141, 85 (1974).

7. Mann, J.I. Diet and Diabetes. Diabetologia 18, 89 (1980).

8. Oakenfull, D.G., Fenwick, D.E., Hood, R.L., Topping, D.L., Illman, R.J. and Storer, G.B. Effects of saponins on bile acids and plasma lipids in the rat. Brit. J. Nutr. 42, 209 (1979).

9. Topping, D.L. and Mayes, P.A. The concentrations of fructose, glucose and lactate in the splanchnic blood vessels of rats absorbing fructose. Nutr. Metabol. 13, 331 (1971).

10. Tollinger, C.D., Vreman, H.J. and Weiner, M.W. Measurement of acetate in human blood by gas chromatography: effects of sample preparation, feeding and various diseases. Clin. Chem. 25, 1787 (1979).

11. Dankaert, J., Zilstra, J.B. and Wolthers, B.G. Volatile fatty acids in human peripheral and portal blood. Quantitative determination by vacuum distillation and gas chromatography. Clin. Chim. Acta 110, 301 (1981).

12. Topping, D.L., Fishlock, R.C., Trimble, R.P., Storer G.B. and Snoswell, A.M. Carboxyhaemoglobin inhibits the metabolism of ethanol by perfused rat liver. Biochem. Int. 3, 157 (1981).

13. Chen, W.J.H., Anderson, J.W. and Gould, M.R. Effects of oat bran, oat gum and pectin on lipid metabolism of cholesterol-fed rats. Nut. Rep. Int. 24, 1093 (1981).

14. Story, J.A., Baldino, A., Czarnecki, S.K. and Kritchevsky, D. Modification of liver cholesterol accumulation by dietary fiber in rats. Nut. Rep. Int. 24, 1213 (1981).

15. Snoswell, A.M., Trimble, R.P., Fishlock, R.G., Storer, G.B. and Topping, D.L. Metabolic effects of acetate in perfused rat liver: studies on ketogenesis, glucose output, lactate uptake and lipogenesis. Biochim. Biophys. Acta In the Press (1982).

16. Ballard, F.J. The supply and utilization of acetate in mammals. Am. J. Clin. Nutr. 25, 773 (1972).

17. Remesy, C., Demigne, C. and Chartier, F. Origin and utilization of volatile fatty acids in the rat. Reprod. Nutr. Develop. 20, 1339 (1980).

18. Jenkins, D.J.A., Leeds, A.R., Gassull, M.A., Cochet, B. and Alberti, K.G.M.M. Decrease in postprandial insulin and glucose concentrations by guar and pectin. Ann. Int. Med. 86, 20 (1977).

19. Stasse-Wolthuis, M. Influence of dietary fibre on cholesterol metabolism and colonic function in healthy subjects. World Rev. Nutr. Diet. 36, 100 (1981).

Accepted for publication: June 8, 1982.

Biochem. J. (1970) **119**, 49–57
Printed in Great Britain

14

Ketone Body and Fatty Acid Metabolism in Sheep Tissues

3-HYDROXYBUTYRATE DEHYDROGENASE, A CYTOPLASMIC ENZYME IN SHEEP LIVER AND KIDNEY

BY PATRICIA P. KOUNDAKJIAN AND A. M. SNOSWELL
Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S. Austral. 5064, Australia

(*Received* 11 *March* 1970)

1. 3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) activities in sheep kidney cortex, rumen epithelium, skeletal muscle, brain, heart and liver were 177, 41, 38, 33, 27 and 17 μmol/h per g of tissue respectively, and in rat liver and kidney cortex the values were 1150 and 170 respectively. 2. In sheep liver and kidney cortex the 3-hydroxybutyrate dehydrogenase was located predominantly in the cytosol fractions. In contrast, the enzyme was found in the mitochondria in rat liver and kidney cortex. 3. Laurate, myristate, palmitate and stearate were not oxidized by sheep liver mitochondria, whereas the L-carnitine esters were oxidized at appreciable rates. The free acids were readily oxidized by rat liver mitochondria. 4. During oxidation of palmitoyl-L-carnitine by sheep liver mitochondria, acetoacetate production accounted for 63% of the oxygen uptake. No 3-hydroxybutyrate was formed, even after 10 min anaerobic incubation, except when sheep liver cytosol was added. With rat liver mitochondria, half of the preformed acetoacetate was converted into 3-hydroxybutyrate after anaerobic incubation. 5. Measurement of ketone bodies by using specific enzymic methods (Williamson, Mellanby & Krebs, 1962) showed that blood of normal sheep and cattle has a high [3-hydroxybutyrate]/[acetoacetate] ratio, in contrast with that of non-ruminants (rats and pigeons). This ratio in the blood of lambs was similar to that of non-ruminants. The ratio in sheep blood decreased on starvation and rose again on re-feeding. 6. The physiological implications of the low activity of 3-hydroxybutyrate dehydrogenase in sheep liver and the fact that it is found in the cytoplasm in sheep liver and kidney cortex are discussed.

The production of ketone bodies in sheep has been investigated extensively since these animals are more prone to ketosis than non-ruminants. However, the majority of investigations have been made *in vivo*, with whole animals. Further, it has been assumed that the various enzyme reactions involved are similar to those occurring in the rat, the animal most frequently used in detailed studies on ketone-body formation. D(−)-3-Hydroxybutyrate–NAD oxidoreductase (3-hydroxybutyrate dehydrogenase, EC 1.1.1.30), the enzyme catalysing the interconversion of the two major ketone bodies, 3-hydroxybutyrate and acetoacetate, is regarded as a classical 'marker enzyme' for the inner mitochondrial membranes (e.g. see Tubbs & Garland, 1968). In attempting to use the enzyme to identify mitochondrial membrane fractions prepared from sheep liver, we failed to detect activity in any membrane fractions. Thus we have examined the intracellular distribution of this enzyme in liver and other tissues of the sheep.

Ketone bodies are produced in the mitochondria of liver cells from acetyl-CoA derived from oxidation of fatty acids (Lehninger, 1964), particularly under conditions where high concentrations of free fatty acids prevail (e.g. in starvation). However, these relationships have not previously been examined at the mitochondrial level in tissues of sheep. Thus we have investigated the oxidation of long-chain fatty acids and associated ketone-body formation in isolated sheep liver mitochondria.

In sheep, as in other ruminants, the investigation of ketone-body formation is complicated by the fact that ketone bodies are produced in two main tissues, i.e. the rumen epithelium and liver (Annison & Lewis, 1959). Work by Katz & Bergman (1969) on whole animals suggests that in normal sheep the tissues supplying the portal vascular system are the main areas for ketone-body production but in the starved animal the liver is the major site of ketone-body formation. We have determined the amounts of acetoacetate and 3-hydroxybutyrate in the blood

of sheep under different conditions in order to attempt to make some correlation between these blood concentrations, the known sites of ketone body production and the tissue distribution of 3-hydroxybutyrate dehydrogenase. Some of these results have been published in a preliminary form (Snoswell, 1968; Snoswell, Broadhead & Henderson, 1969).

MATERIALS AND METHODS

Animals. The sheep used were 4-year-old Merino or Merino × Dorset Horn wethers weighing between 35 and 45kg. The animals were pen-fed on 1:1 (w/w) mixture of lucerne-hay chaff and wheaten-hay chaff, lucerne-hay chaff or pelleted sheep cubes (Barastoc and Co., Melbourne, Vic., Australia) as indicated. The lambs were day-old Merinos. The cattle were Aberdeen Angus steers, which were stall-fed on hay chaff. The horses were thoroughbred race-horses, which were maintained on a relatively high plane of nutrition. The rats were males of the Wistar strain (250–300g); the pigeons were of mixed strain. Both the rats and pigeons were maintained on a pelleted diet suitable for small animals (Barastoc and Co., Melbourne, Vic., Australia).

Tissue samples. Blood samples for ketone-body assays were taken from the jugular vein of the sheep, cattle and horses, from the tail of the rats and from the pigeons after decapitation. Samples (2.0ml) of the whole blood were immediately added to 1.0ml of ice-cold 30% (w/v) $HClO_4$ with the sheep and lambs and 0.2–0.3ml to 2.0ml of 3% (w/v) $HClO_4$ for the blood from the other species.

Samples of liver, kidney, heart, rumen wall, skeletal muscle, spleen, brain and small intestine of sheep were collected into ice-cold 0.25M-sucrose immediately after slaughter. Liver and kidney samples from rats and cattle were treated similarly. Adipose tissue from sheep was collected into 0.25M-sucrose at 37°C.

Homogenates and subcellular fractions. Homogenates (5%, w/v) of all tissues except adipose tissue and rumen epithelium were prepared in 0.25M-sucrose at 0°C with the aid of a Potter–Elvehjem homogenizer. Liver and kidney-cortex homogenates were centrifuged at 700*g* for 5min to remove cell debris and nuclei. The supernatant fractions were then centrifuged at 7000*g* for 10min to sediment the mitochondria. The mitochondrial pellets were washed in 0.25M-sucrose and recentrifuged at 13000*g* for 10min and finally suspended in 0.25M-sucrose. Microsome and supernatant (cytosol) fractions were prepared by centrifuging at 100000*g* for 30min the supernatants obtained after sedimentation of the mitochondria. The microsomal pellet was resuspended in 0.25M-sucrose and the supernatant represented the cytosol fraction. Other tissue homogenates were centrifuged directly at 100000*g* to give cytosol and particulate fractions.

The mitochondria prepared from sheep liver and kidney by the method outlined above appeared intact as seen under the electron microscope and had low adenosine triphosphatase activity. With palmitoyl-L-carnitine as substrate the respiratory control index was 3.8 ± 2 (4 samples) and the P/O ratio was 2.4 ± 1 (4). These results are approximately the same as those for rat liver mitochondria assayed under similar conditions, and suggest that the mitochondria isolated were not extensively damaged.

Assays of succinate–cytochrome *c* dehydrogenase, glutamate dehydrogenase and monoamine oxidase activities of sheep liver and kidney mitochondria and cytosol fractions showed that contamination of cytosol with mitochondria was less than 4%.

Rumen epithelium was stripped from the rumen wall and 5% (w/v) homogenates in 0.25M-sucrose were prepared with a high-speed homogenizer (Edmund Buhler and Co., Tubingen, Germany). Subcellular fractions were prepared as outlined above. Some samples of rumen epithelium (and liver, for comparison) were frozen in liquid nitrogen before homogenization.

Adipose tissue was homogenized in 0.25M-sucrose at 37°C.

Assay procedures. (*a*) Ketone bodies. Acetoacetate and 3-hydroxybutyrate were determined by the specific enzymic methods developed by Williamson, Mellanby & Krebs (1962), with 3-hydroxybutyrate dehydrogenase isolated from *Rhodopseudomonas spheroides* (Williamson *et al.* 1962) with further purification on a DEAE-Sephadex column as suggested by Williamson, Lund & Krebs (1967). 3-Hydroxybutyrate dehydrogenase activities were determined on all particulate fractions before and after a total of 2min sonication (in 30s periods) in an MSE sonic disintegrator (Measuring and Scientific Equipment Ltd., London S.W.1, U.K.) at 20kHz and 1.5A.

Sheep liver homogenates and mitochondria were also subjected to the following treatments before assay of the enzyme: addition of glycerol to 50% (w/v) concentration; freezing and thawing three times; shaking for 3min with glass beads in the presence of 1% digitonin; addition of 5% (w/v) Tween 80; exposure to 0.125M-, 0.05M- and 0.025M-sucrose for 15min at 0°C.

The 3-hydroxybutyrate dehydrogenase activity was measured in the sonicated fractions (see above) by a modification of the method of Lehninger, Sudduth & Wise (1960). The assay mixture consisted of 33mM-tris–HCl buffer, pH 8.5, 1.8mM-NAD, 50mM-nicotinamide, 20mM-dithiothreitol, 1mM-$CaCl_2$, 1mM-KCN, 0.75mg of asolectin (Associated Concentrates Inc., New York, N.Y., U.S.A.) and 22mM-sodium DL-3-hydroxybutyrate in a final volume of 1.0ml. KCN was added to suppress NADH oxidase activity and asolectin (purified soya phosphatides) to meet the known requirement of mitochondrial 3-hydroxybutyrate dehydrogenase for phospholipid. The tissue fraction was added to start the reaction, which was followed at 20°C and at 366nm in semi-micro cuvettes with an Eppendorf spectrophotometer fitted with an automatic sample-changer and recording attachment (Eppendorf, Netheler und Hinz, Hamburg, Germany). All activities presented were corrected for blank values.

(*b*) Oxidation experiments. Oxidation rates were measured polarographically with a small Clark-type oxygen electrode (Titron Instrument Co., Sandringham, Vic., Australia) as described previously (Snoswell, 1966). Oxidation rates with free fatty acids and fatty acyl-carnitine derivatives were measured in a medium described by Bode & Klingenberg (1964).

Chemicals. Bovine serum albumin (fraction V; Calbiochem, Los Angeles, Calif., U.S.A.) was freed from fatty acids by the method of Chen (1967). The free fatty acids were combined with this bovine serum albumin in a molar ratio of 5:1 by the method of Björntorp (1968). Lauroyl, myristoyl, palmitoyl and stearoyl esters of L-carnitine

were kindly synthesized by Mr K. C. Reed from the corresponding acid chlorides and L-carnitine hydrochloride by the method of Bremer (1968). D- and L-Carnitine hydrochloride and O-acetyl-L-carnitine hydrochloride were generously supplied by the Otsuka Pharmaceutical Factory (Osaka, Japan) and were recrystallized from ethanol. Other compounds used were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

RESULTS

Distribution of 3-hydroxybutyrate dehydrogenase in various tissues. Kidney cortex was found to have the highest 3-hydroxybutyrate dehydrogenase activity of the various sheep tissues examined (Table 1). The activity in sheep kidney cortex was approximately the same as in rat kidney cortex (Table 2). However, the activity of this enzyme in sheep liver was less than 2% of that in rat liver (Table 2). The figures for rat liver and kidney are comparable with those reported by Lehninger *et al* (1960). Rumen epithelium also had appreciable 3-hydroxybutyrate dehydrogenase activity but considerable difficulty was encountered in preparing homogenates from this tissue. The most active homogenates were made from tissue that had been first frozen in liquid nitrogen and then powdered before homogenization. This treatment was not necessary for the softer tissues, such as liver, and indeed the activity of homogenates of liver prepared in this way was the same as that obtained by the direct homogenization of fresh tissue.

Table 1. *Distribution of 3-hydroxybutyrate dehydrogenase in various tissues of the sheep*

Homogenates (5% w/v) of the tissues were sonicated and assayed as described in the Materials and Methods section. Two values are given for rumen epithelium, one for tissue treated as described above, the other for tissue prefrozen in liquid N_2. The values are given as means ±S.E.M. when more than two assays were done.

Tissue	No. of determinations	3-Hydroxybutyrate dehydrogenase activity (μmol/h per g of tissue)
Kidney cortex	6	177 ±26
Rumen epithelium	2 (N_2)	41
	6	11 ± 4
Skeletal muscle	2	38
Brain	2	32.5
Heart	7	27.4±16.1
Liver	6	16.5± 2.2
Kidney medulla	2	12
Spleen	2	3.4
Small intestine	2	3.3
Adipose tissue	2	0

The most striking characteristic of the 3-hydroxybutyrate dehydrogenase in sheep liver and kidney cortex is that in these tissues the enzyme is found predominantly in the cytosol fraction, whereas the activity in rat liver and kidney cortex is mainly in the mitochondria (Table 2). The lack of activity in the sheep liver mitochondria is not due to the presence of an inhibitor, as addition of the liver mitochondria to cytosol fractions of both liver and kidney cortex did not diminish the activity of the enzyme in these fractions. Moreover, disruption of the sheep liver mitochondria by techniques other than the standard sonication procedure, e.g. repeated freezing and thawing, exposure to 50% glycerol and various sucrose solutions, treatment with 5% Tween 80 etc., did not result in any increase in the amount of enzyme activity detected.

Although asolectin was included as a routine in the assay mixture for all tissue fractions, the

Table 2. *Subcellular distribution of 3-hydroxybutyrate dehydrogenase in the liver and kidney cortex of the rat and the sheep*

Sonicated subcellular fractions were prepared and assayed as described in the Materials and Methods section. The values shown are the means ±S.E.M. of two determinations (rats) and six determinations (sheep). Activities are expressed in μmol/h per g of tissue.

Tissue fraction	Rat		Sheep	
	Activity	%	Activity	%
Liver				
Homogenate	1150±11	100	16.5± 2.2	100
Mitochondria	1110±13	97	0.3± 0.2	1.6
Microsomes	12± 0.8	1.4	2.2± 1.0	13.2
Cytosol	35± 3	3.1	14.3± 2.8	87.2
Kidney cortex				
Homogenate	170±15	100	177 ±26	100
Mitochondria	167± 5	98.3	9.9± 3.8	5.6
Microsomes	0	0	8.3± 2.1	4.7
Cytosol	2± 2	1.2	163 ±21.3	92

cytosol 3-hydroxybutyrate dehydrogenase did not require added phospholipid to elicit maximal activity.

In contrast with the results with sheep liver and kidney cortex the enzyme activity of rumen epithelium was mainly associated with the particulate fractions and appeared to be associated with mitochondria. However, it is difficult to prepare uncontaminated subcellular fractions from this tissue, owing to the problems mentioned above. In other sheep tissues where appreciable 3-hydroxybutyrate dehydrogenase activity was found, e.g. skeletal muscle, brain and heart (Table 1), the enzyme was also predominantly in the particulate fraction and again was probably located in the mitochondria.

The activity of 3-hydroxybutyrate dehydrogenase in bovine liver and kidney cortex was 3 and 21 μmol/h per g of tissue respectively (values are the means of two assays). In the bovine liver all the activity was present in the cytosol fraction and in the kidney cortex 80% was in this fraction.

Oxidation of fatty acids by sheep liver mitochondria and associated ketone-body production. The results presented in Table 3 indicate that long-chain fatty acids were not oxidized by sheep liver mitochondria. Subsequent additions of 4 mM-L-carnitine, but not D-carnitine, resulted in quite significant rates of oxidation of long-chain fatty acids by these mitochondria (e.g. for palmitate 18 ng-atoms of O/min per mg of mitochondrial protein). Butyrate and crotonate were oxidized slowly. Acetate was not oxidized but acetyl-L-carnitine was oxidized slowly (Table 3). The L-carnitine esters of the long-chain fatty acids were oxidized at appreciable rates although the overall oxidative activity of sheep liver mitochondria appeared to be only about one-third of that for rat liver mitochondria oxidizing the same substrate (Table 3).

In contrast, the results presented in Table 3 also show that free long-chain fatty acids, especially laurate and myristate, were oxidized at quite high rates by rat liver mitochondria. The results for oxidation of the L-carnitine esters of these fatty acids are also shown in Table 3.

In view of the fact that free long-chain fatty acids were not oxidized by sheep liver mitochondria in the absence of added L-carnitine, palmitoyl-L-carnitine was used as substrate in subsequent experiments. During the oxidation of this substrate by sheep liver mitochondria, 84 nmol of acetoacetate was formed with a concomitant uptake of 235 nmol of oxygen (Table 4). Thus acetoacetate formation accounts for 63% of the oxygen uptake observed, based on the equation:

$$\text{Palmitate} + 7O_2 \rightarrow 4\ \text{acetoacetate} + 4H_2O$$

Similarly, acetoacetate production accounted for approx. 60% of the oxygen uptake by rat liver mitochondria.

When the incubation mixtures were allowed to remain under anaerobic conditions for 10 min after all the oxygen had been utilized, about half of the acetoacetate formed was reduced to 3-hydroxybutyrate in the experiments with rat liver mitochondria (Table 4). Similar results with rat liver mitochondria have been reported by Portenhauser, Schäfer & Lamprecht (1969). In contrast no 3-hydroxybutyrate was produced in the experiments with sheep liver mitochondria. This difference

Table 3. *Oxidation of free fatty acids and their corresponding L-carnitine esters by isolated sheep and rat liver mitochondria*

Oxygen uptake was measured polarographically by using an oxygen electrode in a volume of 2.5 ml at 25°C. The incubation medium used was that described by Bode & Klingenberg (1964) including 6 μmol of ADP. Free fatty acid (FFA) (0.4 μmol) or L-carnitine esters (0.12 μmol), both combined with bovine serum albumin (free from bound fatty acids), were used per assay. Sheep or rat liver mitochondria equivalent to 2–4 mg of mitochondrial protein were used per assay. The values are corrected for the rates obtained in the absence of added substrate and are the means ±S.E.M. for three experiments.

	Oxygen uptake (ng-atoms/min per mg of mitochondrial protein)			
	Sheep liver mitochondria		Rat liver mitochondria	
Chain length of saturated fatty acid	FFA	Carnitine ester	FFA	Carnitine ester
C_2	0	9±2	—	—
C_4	4±1	—	—	—
C_6	9±1	—	—	—
C_{12}	0	29±3	76±6	84±4
C_{14}	0	26±2	65±4	80±4
C_{16}	0	23±3	51±3	77±3
C_{18}	0	21±2	8±1	74±2
Glutamate	21±2		51±4	

Table 4. *Oxidation of palmitoylcarnitine by sheep and rat liver mitochondria and the associated production of ketone bodies*

The standard mixture contained 80mM-KCl, 20mM-tris–chloride buffer, pH 7.4, 2mM-$MgCl_2$, 1mM-EDTA, 1.25mM-AMP, 1.25mM-sodium–potassium phosphate, pH 7.4, and 25mg of bovine serum albumin (free from fatty acids), in a total volume of 2.5ml. The substrate was 45μM-palmitoyl-L-carnitine and the reaction was started by the addition of 5–7mg of protein of sheep or rat liver mitochondria. Oxygen uptakes were measured as described in Table 3. The incubations were allowed to proceed until almost all of the oxygen in the solution was utilized and then the reaction was stopped by the addition of 0.2ml of 30% $HClO_4$. Acetoacetate and 3-hydroxybutyrate in the neutralized supernatants were measured by the enzymic method of Williamson *et al.* (1962). The results are means ±S.E.M. and in each case are corrected for the values obtained with no substrate. The number of experiments is shown in parentheses. N.D., not detectable. The limit of detection was considered to be 1nmol under the conditions of assay.

	Sheep			Rat		
Incubation conditions	Oxygen uptake (nmol)	Acetoacetate formed (nmol)	3-Hydroxy-butyrate formed (nmol)	Oxygen uptake (nmol)	Acetoacetate formed (nmol)	3-Hydroxy-butyrate formed (nmol)
Standard	235±3 (3)	84±1 (3)	N.D. (3)	180±4 (3)	62±2 (3)	5±1 (3)
Plus 10min subsequent anaerobic incubation	239±3 (3)	87±1 (3)	N.D. (3)	183±4 (3)	32±2 (3)	33±2 (3)
*Plus 3nmol of rotenone after half the oxygen utilized	220±5 (2)	96±2 (2)	N.D. (2)	239±5 (2)	38±3 (2)	141±6 (2)
Plus 1ml of liver cytosol and 10min anaerobic incubation	276±4 (2)	51±2 (2)	20±2 (2)	—	—	—

* 10mM-Malonate and 10μM-fluorocitrate were added to the standard incubation medium.

between sheep and rat liver mitochondria was further demonstrated when ketone-body production was increased to a maximum rate by the addition of fluorocitrate and malonate during the oxidation of palmitoyl-L-carnitine. Under these conditions, the addition of rotenone, after about half of the total oxygen in the reaction mixture had been utilized, resulted in the production of 3-hydroxybutyrate as the main end product with rat liver mitochondria (Table 4). Again, there was no 3-hydroxybutyrate produced with sheep liver mitochondria (Table 4).

The results shown in Table 4 indicate that, if cytosol fraction of sheep liver was added to the reaction mixture containing sheep liver mitochondria and the incubation continued for 10min under anaerobic conditions, a significant amount of 3-hydroxybutyrate was produced.

Blood ketone bodies. In Table 5 acetoacetate and 3-hydroxybutyrate concentrations in sheep blood are contrasted with those in cattle blood and in the blood of non-ruminant species. The results in this table show that cattle and sheep have a significantly higher ($P<0.01$) [3-hydroxybutyrate]/[acetoacetate] ratio in the blood than have the non-ruminants, pigeons and rats. The results for horses are intermediate between these two groups. The horse, although a non-ruminant, produces large quantities of volatile fatty acids in the caecum and colon (Dukes, 1955). The variation between the results for the two groups of sheep may be in part dietary or may be due to the difference in breed. For example, Merino wethers showed significantly lower [3-hydroxybutyrate]/[acetoacetate] ratios in the blood than did Merino×Dorset Horn wethers (see Table 6). Also, differences in diet and strain of the rats used here may well account for the variation between the results reported here and those reported for rat blood by Berry, Williamson & Wilson (1965). Eggleston & Krebs (1969) have reported strain differences in the activities of various enzymes in rat liver. Such differences could affect metabolite concentrations.

The values shown in Table 5 for the blood ketone-body concentration in lambs indicate [3-hydroxybutyrate]/[acetoacetate] ratios similar to those of non-ruminant species. The lambs were 1 day old and thus there was no active rumen fermentation. Knodt, Shaw & White (1942) found in young calves a gradual increase in ketone-body production, particularly of 3-hydroxybutyrate, which roughly paralleled the development of the rumen.

Table 5. *Acetoacetate and 3-hydroxybutyrate in the blood of various species*

The smaller group of sheep were Merino × Dorset Horn wethers, fed on pelleted sheep cubes (see the text), in contrast with the larger group, which were Merino wethers fed on an equal mixture of lucerne and oaten chaff. Acetoacetate and 3-hydroxybutyrate were determined as described in Table 4. The mean value for the acetoacetate of the cattle is shown as <0.002 as the actual values were less than 0.002 μmol/ml in a number of cases. This value was the lowest that could be detected. The results shown are means ±S.E.M.

Species	No. of animals	Acetoacetate (μmol/ml)	3-Hydroxybutyrate (μmol/ml)	[3-Hydroxybutyrate]/[acetoacetate] ratio
Cow	12	< 0.002	0.102±0.011	> 38
Sheep	12	0.058±0.004	0.623±0.034	12 ±1
Sheep	4	0.010±0.002	0.250±0.064	24 ±3
Horse	10	0.008±0.001	0.080±0.006	11 ±2
Pigeon	12	0.035±0.006	0.168±0.025	5.8±1.0
Rat	10	0.029±0.006	0.096±0.016	4.3±0.8
Lamb	6	0.044±0.006	0.207±0.032	5.0±0.2

Table 6. *Acetoacetate and 3-hydroxybutyrate in the blood of normal, starved and re-fed sheep*

Each group of four animals was stabilized on a diet of lucerne-hay chaff before the experiment and the normal values shown are the means for 14 successive daily samples for the Merino wethers, and three for the Merino × Dorset Horn wethers. The values for the starved condition are the means for six successive daily samples taken after a period of 4 days starvation and the values for the re-fed period are the means for four successive daily samples after re-feeding. Acetoacetate and 3-hydroxybutyrate were determined as described in Table 4. The values are the means ±S.E.M. for four animals in each group.

Sheep	Dietary status	Acetoacetate (μmol/ml)	3-Hydroxybutyrate (μmol/ml)	[3-Hydroxybutyrate]/[acetoacetate] ratio
Merino wethers	Normal	0.026±0.003	0.252±0.014	11.2±0.9
	Starved	0.111±0.005	0.723±0.018	6.6±0.3
	Re-fed	0.030±0.006	0.254±0.023	9.9±2.4
Merino × Dorset Horn wethers	Normal	0.010±0.001	0.241±0.009	26.8±2.4
	Starved	0.106±0.011	0.895±0.062	10.8±1.9
	Re-fed	0.024±0.003	0.423±0.040	22.7±2.8

The results presented in Table 6 show that the [3-hydroxybutyrate]/[acetoacetate] ratio in sheep blood falls on starvation and increases again on re-feeding. Total 3-hydroxybutyrate plus aceto-acetate increases on starvation, as expected, but the amount of acetoacetate increases relatively more than the amount of 3-hydroxybutyrate, thus exerting a major influence on the ratio of these two ketone bodies. The effects of starvation on this ratio are more pronounced in the crossbred sheep than with the pure Merinos, the former sheep showing a higher [3-hydroxybutyrate]/[aceto-acetate] ratio in the blood under normal feeding conditions.

DISCUSSION

The most striking feature of the results presented here is the fact that 3-hydroxybutyrate dehydro-genase is present in the cytosol fraction of the sheep liver and kidney cortex. This is in direct contrast with the rat, where the enzyme is found in the mitochondrial fraction of the same tissues. Of the other tissues of the sheep examined, appre-ciable 3-hydroxybutyrate dehydrogenase activity was found in rumen epithelium, skeletal muscle, heart and brain, and the enzyme was located pre-dominantly in the particulate fractions, probably in the mitochondria. The absence of this enzyme in liver mitochondria of sheep is particularly unusual as this organ under certain conditions pro-duces ketone bodies in large quantities (see Katz & Bergman, 1969) and ketone bodies are known to be produced in mitochondria (Lehninger, 1964). The experiments reported above with isolated sheep liver mitochondria show that during the oxidation of palmitoyl-L-carnitine, acetoacetate was produced but no 3-hydroxybutyrate. Even when ketone-body formation was raised to a maxi-mal rate by the addition of malonate and fluoro-citrate to block oxidation in the tricarboxylic acid cycle and an increase in reducing potential was provided (by the addition of rotenone or by anaero-

bic conditions), no 3-hydroxybutyrate was produced by the sheep liver mitochondria. Under similar conditions the major end-product of palmitoyl-L-carnitine oxidation by the rat liver mitochondria was 3-hydroxybutyrate. Thus in sheep liver mitochondria internally generated acetoacetate is not reduced to 3-hydroxybutyrate even under reducing conditions. These results thus confirm the absence of 3-hydroxybutyrate dehydrogenase in sheep liver mitochondria.

The work of Katz & Bergman (1969) shows that some 3-hydroxybutyrate is produced by sheep liver, particularly during starvation. The results presented above show that if a cytosol fraction of sheep liver is added to isolated sheep liver mitochondria oxidizing palmitoyl-L-carnitine, then some 3-hydroxybutyrate is produced under reducing conditions. This result illustrates that 3-hydroxybutyrate can be produced in sheep liver under appropriate conditions even though the enzyme is located in the cytosol fraction. However, the $[NAD^+]/[NADH]$ ratio in the cytoplasm of the liver is not particularly favourable for the reduction of acetoacetate to 3-hydroxybutyrate. Veech, Eggleston & Krebs (1969) reported an $[NAD^+]/[NADH]$ ratio of 1164 for the rat liver cytoplasm in normal animals which fell to 564 in starved rats. The ratio in normal sheep liver is slightly lower but does not decrease on starvation (A. M. Snoswell, unpublished work). Thus sheep liver would appear to have a very limited capacity for producing 3-hydroxybutyrate.

3-Hydroxybutyrate dehydrogenase is associated only with the inner mitochondrial membrane, at least in rat liver (Schnaitman, Erwin & Greenawalt, 1967). This fact has been used by Williamson *et al.* (1967) to assess the $[NAD^+]/[NADH]$ ratio in the mitochondrial cristae from the [3-hydroxybutyrate]/[acetoacetate] ratio determined in freeze-clamped rat liver. This technique for determining redox states in intracellular compartments from a knowledge of the amounts of metabolites of a redox pair is very useful (see Williamson *et al.* 1967) but the intracellular distribution of the appropriate enzyme in the tissue being examined must be known. Quite obviously knowledge of the $[NAD^+]/[NADH]$ ratio in the mitochondrial cristae could not be obtained from the [3-hydroxybutyrate]/[acetoacetate] ratio in sheep liver since the 3-hydroxybutyrate dehydrogenase in this tissue is mainly found in the cytosol. Ballard, Hanson & Kronfeld (1968) have used the [3-hydroxybutyrate]/[acetoacetate] ratio to determine the $[NAD^+]/[NADH]$ ratio for the mitochondrial cristae in bovine liver. However, this approach would appear invalid because in bovine liver, as in sheep liver, the 3-hydroxybutyrate dehydrogenase is predominantly in the cytosol fraction.

A further feature of the results presented here is the profound difference in the activity of 3-hydroxybutyrate dehydrogenase in different tissues of sheep when compared with those of the rat. Lehninger *et al.* (1960) have reported that the activity of this enzyme in rat liver is some eight times greater than the activity in any other rat tissues. The activity in sheep liver, however, is less than 2% of that in rat liver and is about 10% of that in sheep kidney cortex, the tissue with the highest activity in the sheep. Lehninger *et al.* (1960) found considerable differences in the activity of 3-hydroxybutyrate dehydrogenase in the liver of various species although no ruminant species were examined. Baird *et al.* (1968) report that the activity in bovine liver is less than one-thirtieth of that in rat liver. It is probable that the relatively high activity of 3-hydroxybutyrate dehydrogenase in the kidney is indicative of the kidney being a major tissue for the utilization of 3-hydroxybutyrate in the sheep. Leng & Annison (1964) found that kidney had the highest rate of uptake of 3-hydroxybutyrate when slices from a number of sheep tissues were examined. Weidemann & Krebs (1969) have shown that ketone bodies are a preferred metabolic fuel for rat kidney. The reason for the occurrence of 3-hydroxybutyrate dehydrogenase in the cytosol fraction of sheep kidney cortex is obscure but may be related to efficient utilization of 3-hydroxybutyrate by this tissue.

The results presented above show that sheep, like cattle, have a high [3-hydroxybutyrate]/[acetoacetate] ratio in the blood under normal conditions. This has been generally accepted to be the case for cattle since Thin & Robertson (1952) found that 3-hydroxybutyrate was the only ketone body that could be detected in the blood of normal dairy cattle. However, the results published for individual ketone-body concentrations in sheep blood have been variable. Values reported for the [3-hydroxybutyrate]/[acetoacetate] ratio in normal sheep blood have ranged from 0.25:1 (Procos, 1962) to 2:1 (Reid, 1960), and Leng & Annison (1963) implied that in normal sheep 3-hydroxybutyrate represents about 85% of the blood ketone bodies. This wide range of values is undoubtedly due to the difficulty in determining accurately the concentration of individual ketone bodies in sheep blood by the older chemical methods (see Roe, Bergman & Kon, 1966, for a discussion of this point). The values reported above were determined by the specific enzyme procedures developed by Williamson *et al.* (1962) and appear to be the first values published for sheep blood obtained by these methods.

The high [3-hydroxybutyrate]/[acetoacetate] ratio in the blood is probably a general characteristic of ruminant animals, as it seems to be associated with active rumen fermentation because it

is not observed in young lambs (the present study) or in young calves (Knodt *et al.* 1942). The results presented above show that the rumen epithelium has significant 3-hydroxybutyrate dehydrogenase activity and it is known that butyrate absorbed from the rumen is substantially converted into ketone bodies (Hungate, 1966). Oxidation of fatty acids leads to a low [NAD$^+$]/[NADH] ratio within rat heart mitochondria (Klingenberg & Kröger, 1966), and it is reasonable to assume that fatty acid oxidation in rumen epithelium mitochondria has the same effect. Thus a favourable environment is provided within the mitochondria of the rumen epithelium for the conversion of acetoacetate (derived from oxidation of butyrate) into 3-hydroxybutyrate by 3-hydroxybutyrate dehydrogenase. In this tissue the enzyme is probably associated with mitochondria.

In starved sheep the [3-hydroxybutyrate]/[acetoacetate] ratio in the blood falls markedly, particularly in crossbred animals. This is in direct contrast with the results for the rat where the blood ratio rises on starvation (Berry *et al.* 1965). In the rat this increase in the ratio is attributed to increased fatty acid oxidation in the liver and the subsequent effect on the concentration of NADH in the mitochondria of hepatic cells (Williamson & Wilson, 1965). Katz & Bergman (1969) have shown that in the sheep there is a close correlation between the concentration of free fatty acids in the blood and ketone production in the liver, particularly in starvation, and the present results show that isolated sheep liver mitochondria readily form acetoacetate, but not 3-hydroxybutyrate, during the oxidation of palmitoyl-L-carnitine. Thus the reason for the decrease in the [3-hydroxybutyrate]/[acetoacetate] ratio in the blood of sheep on starvation appears to be twofold. First, we have suggested that the normal high ratio is mainly due to the production of 3-hydroxybutyrate in the mitochondria of the rumen epithelium; this production will diminish greatly on prolonged starvation. Secondly, on starvation ketone-body production occurs in sheep liver (Katz & Bergman, 1969) and since sheep liver has relatively low 3-hydroxybutyrate dehydrogenase activity, and the enzyme is in the cytosol fraction, it is probable that the conversion of the primary ketone body, acetoacetate, into 3-hydroxybutyrate is severely limited.

The presence of 3-hydroxybutyrate dehydrogenase in the cytosol of sheep liver cells rather than in the mitochondria, and its comparatively low activity in sheep liver, may well have some important physiological implications. It appears that it is the accumulation of acetoacetate that depresses the functioning of the central nervous system in ketosis, as the acidosis of 3-hydroxybutyrate accumulation is readily compensated (Behnke, 1964). The conversion of acetoacetate into 3-hydroxybutyrate in the liver therefore may be regarded as a detoxification process. Thus, as cattle and sheep liver contain only relatively small amounts of 3-hydroxybutyrate dehydrogenase, and this small amount is present in the cytosol, this may be one reason why these animals are more prone to severe ketosis than non-ruminants.

The experimental conditions chosen in the present work for the study of the oxidation of long-chain fatty acids by mitochondria are known (for the rat) to result in appreciable oxidation of these fatty acids independent of L-carnitine (see Greville & Tubbs, 1968, for discussion of this point). Yet under the same conditions the oxidation of long-chain fatty acids by sheep liver mitochondria was completely dependent on the presence of L-carnitine. This could indicate that a different system operates for the activation of fatty acids in sheep liver mitochondria from that in rat liver mitochondria (see Van Den Bergh, 1967; Garland & Yates, 1967). An alternative explanation may be that the high activity of carnitine acetyltransferase in sheep liver mitochondria, in contrast with the low activity of carnitine palmitoyltransferase (Snoswell & Henderson, 1970) results in the former enzyme competing more effectively for endogenous L-carnitine, thus severely limiting the oxidation of long-chain fatty acids in the absence of added carnitine. The fact that sheep liver contains only small amounts of long-chain carnitine esters relative to acetylcarnitine, and this long-chain ester fraction increases only slightly on starvation (Snoswell & Henderson, 1970), would support this view.

In the final stages of the preparation of this manuscript a report by Nielsen & Fleischer (1969) appeared indicating that ruminant liver mitochondria lack 3-hydroxybutyrate dehydrogenase. Thus the present work confirms this point and also establishes that homogenates of sheep liver (and bovine liver) do have demonstrable enzyme activity, the enzyme being present in the cytosol. This is also the case for sheep kidney cortex, which has much higher 3-hydroxybutyrate dehydrogenase activity.

The authors are indebted to Mr J. G. McLean and Mr I. W. Caple of the Sub-Department of Veterinary Biochemistry, University of Melbourne, for assistance in the collection of blood samples and the assay of 3-hydroxybutyrate and acetoacetate in cattle, horse and pigeon blood. The assistance of Mr J. M. Gooden and Dr J. R. Sabine of the Department of Animal Physiology, University of Adelaide, in the provision and accommodation of sheep and collection of blood samples is gratefully acknowledged. Dr I. G. Jarrett and Dr F. J. Ballard are thanked for valuable advice and discussions, and Mr R. Fishlock for skilful technical assistance. A grant for capital equipment was provided by the Australian Meat

Research Committee and P. P. K. holds a Commonwealth Scholarship and Fellowship Plan Australian award.

REFERENCES

Annison, E. F. & Lewis, D. (1959). *Metabolism in The Rumen*, pp. 70, 159. London: Methuen and Co. Ltd.

Baird, G. D., Hibbitt, K. G., Hunter, G. D., Lund, P., Stubbs, M. & Krebs, H. A. (1968). *Biochem. J.* **107**, 683.

Ballard, F. J., Hanson, R. W. & Kronfeld, D. S. (1968). *Biochem. biophys. Res. Commun.* **30**, 100.

Behnke, A. R. (1964). In *Fat as a Tissue*, p. 379. Ed. by Rodahl, K. & Issekutz, B. New York: McGraw-Hill Book Co.

Berry, M. N., Williamson, D. H. & Wilson, M. B. (1965). *Biochem. J.* **94**, 17c.

Björntorp, P. (1968). *J. biol. Chem.* **243**, 2130.

Bode, C. & Klingenberg, M. (1964). *Biochim. biophys. Acta*, **84**, 93.

Bremer, J. (1968). *Biochem. Prep.* **12**, 69.

Chen, R. F. (1967). *J. biol. Chem.* **242**, 173.

Dukes, H. H. (1955). *The Physiology of Domestic Animals*, 7th ed., p. 380. Ithaca: Comstock Publishing Associates.

Eggleston, L. V. & Krebs, H. A. (1969). *Biochem. J.* **114**, 877.

Garland, P. B. & Yates, D. W. (1967). In *Mitochondrial Structure and Compartmentation*, p. 385. Ed. by Quagliariello, E., Papa, S., Slater, E. C. & Tager, J. M. Bari: Adriatica Editrice.

Greville, G. D. & Tubbs, P. K. (1968). In *Essays in Biochemistry*, vol. 4, p. 155. Ed. by Campbell, P. N. & Greville, G. D. London: Academic Press (Inc.) Ltd.

Hungate, R. E. (1966). *The Rumen and its Microbes*, p. 245. New York: Academic Press Inc.

Katz, M. L. & Bergman, E. N. (1969). *Am. J. Physiol.* **216**, 953.

Klingenberg, M. & Kröger, A. (1966). *FEBS Colloq. Biochemistry of Mitochondria, Warsaw*, p. 11. Ed. by Slater, E. C., Kaniuga, Z. & Wojtczak, L. London and New York: Academic Press.

Knodt, C. B., Shaw, J. C. & White, G. C. (1942). *J. Dairy Sci.* **25**, 851.

Lehninger, A. L. (1964). *The Mitochondrion*, p. 53. New York: W. A. Benjamin Inc.

Lehninger, A. L., Sudduth, H. C. & Wise, J. B. (1960). *J. biol. Chem.* **235**, 2450.

Leng, R. A. & Annison, E. F. (1963). *Biochem. J.* **86**, 319.

Leng, R. A. & Annison, E. F. (1964). *Biochem. J.* **90**, 464.

Nielsen, N. C. & Fleischer, S. (1969). *Science, N.Y.*, **166**, 1017.

Portenhauser, R., Schäfer, G. & Lamprecht, W. (1969). *Hoppe-Seyler's Z. physiol. Chem.* **350**, 641.

Procos, J. (1962). *Onderstepoort J. Vet. Res.* **29**, 259.

Reid, R. L. (1960). *Analyst, Lond.*, **85**, 265.

Roe, W. E., Bergman, E. N. & Kon, K. (1966). *Am. J. vet. Res.* **27**, 729.

Schnaitman, C., Erwin, V. G. & Greenawalt, J. W. (1967). *J. Cell Biol.* **32**, 719.

Snoswell, A. M. (1966). *Biochemistry, Easton*, **5**, 1660.

Snoswell, A. M. (1968). *Proc. Aust. biochem. Soc.* p. 71.

Snoswell, A. M., Broadhead, P. P. & Henderson, G. D. (1969). *Proc. Aust. biochem. Soc.* p. 34.

Snoswell, A. M. & Henderson, G. D. (1970). *Biochem. J.* **119**, 59.

Thin, C. & Robertson, A. (1952). *Biochem. J.* **51**, 218.

Tubbs, P. K. & Garland, P. B. (1968). *Br. med. Bull.* **24**, 158.

Van Den Bergh, S. G. (1967). In *Mitochondrial Structure and Compartmentation*, p. 400. Ed. by Quagliariello, E., Papa, S., Slater, E. C. & Tager, J. M. Bari: Adriatica Editrice.

Veech, R. L., Eggleston, L. V. & Krebs, H. A. (1969). *Biochem. J.* **115**, 609.

Weidemann, M. J. & Krebs, H. A. (1969). *Biochem. J.* **112**, 149.

Williamson, D. H., Lund, P. & Krebs, H. A. (1967). *Biochem. J.* **103**, 514.

Williamson, D. H., Mellanby, J. & Krebs, H. A. (1962). *Biochem. J.* **82**, 90.

Williamson, D. H. & Wilson, M. B. (1965). *Biochem. J.* **94**, 19c.

Biochem. J. (1970) **119**, 59–65
Printed in Great Britain

15

Aspects of Carnitine Ester Metabolism in Sheep Liver

By A. M. SNOSWELL and G. D. HENDERSON
Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S. Austral. 5064, Australia

(*Received* 11 *March* 1970)

1. Carnitine acetyltransferase (EC 2.3.1.7) activity in sheep liver mitochondria was 76nmol/min per mg of protein, in contrast with 1.7 for rat liver mitochondria. The activity in bovine liver mitochondria was comparable with that of sheep liver mitochondria. Carnitine palmitoyltransferase activity was the same in both sheep and rat liver mitochondria. 2. The [free carnitine]/[acetylcarnitine] ratio in sheep liver ranged from 6:1 for animals fed *ad libitum* on lucerne to approx. 1:1 for animals grazed on open pastures. This change in ratio appeared to reflect the ratio of propionic acid to acetic acid produced in the rumen of the sheep under the two dietary conditions. 3. In sheep starved for 7 days the [free carnitine]/[acetylcarnitine] ratio in the liver was 0.46:1. The increase in acetylcarnitine on starvation was not at the expense of free carnitine, as the amounts of free carnitine and total acid-soluble carnitine rose approximately fivefold on starvation. An even more dramatic increase in total acid-soluble carnitine of the liver was seen in an alloxan-diabetic sheep. 4. The [free CoA]/[acetyl-CoA] ratio in the liver ranged from 1:1 in the sheep fed on lucerne to 0.34:1 for animals starved for 7 days. 5. The importance of carnitine acetyltransferase in sheep liver and its role in relieving 'acetyl pressure' on the CoA system is discussed.

The oxidation of long-chain fatty acids by isolated rat liver mitochondria may be studied under conditions where the oxidation is completely dependent on the presence of L-carnitine or under conditions where the oxidation is largely independent of carnitine (see Greville & Tubbs, 1968). In the accompanying paper (Koundakjian & Snoswell, 1970) it is shown that the oxidation of long-chain fatty acids (C_{12} to C_{18}, saturated) by sheep liver mitochondria is completely dependent on the presence of L-carnitine under conditions where the oxidation by rat liver mitochondria is largely carnitine-independent. Also, Shepherd, Yates & Garland (1966) have shown that the reaction catalysed by carnitine palmitoyltransferase is the rate-limiting step in the oxidation of palmitate in rat liver mitochondria. Therefore we have examined carnitine acyltransferase activity in sheep liver. Friedman & Fraenkel (1955) have reported that sheep liver showed carnitine acetyltransferase activity, although no quantitative results were given.

The concentrations of free carnitine and of various carnitine esters in rat tissues have been reported by a number of workers (e.g. Marquis & Fritz, 1965; Bøhmer, 1967; Pearson & Tubbs, 1967). However, there are no reports on the amounts of carnitine and carnitine esters in ruminant tissues. In the present paper values for free carnitine and various carnitine esters in sheep liver are presented.

Pearson & Tubbs (1967) showed in experiments with perfused rat heart that, when acetate and more particularly propionate were added to the perfusate, the relative proportions of free carnitine and the various carnitine esters were altered markedly. As acetate and propionate are important metabolites in sheep and other ruminants and the ratio of propionate to acetate produced in the rumen varies considerably with different diets (Hungate, 1966), the effect of different diets on the amounts of various carnitine esters in sheep liver was examined. In particular, the effects of a high lucerne diet, which gives rise to high concentrations of propionate in the rumen, were examined. Some of these results have been reported in a preliminary form (Snoswell, Broadhead & Henderson, 1969). The interrelationships between carnitine and acetylcarnitine, and CoA and acetyl-CoA, and the importance of carnitine acetyltransferase in sheep liver are also discussed.

MATERIALS

Animals. The sheep used were Merino wethers, approx. 4 years old and weighing between 38 and 48kg. In some experiments the animals were used after grazing for at

least 2 weeks on open pastures that consisted mainly of wheaten and oaten stubble. In other experiments the sheep were grazed for 1 week on similar pasture clover but were supplemented *ad libitum* with fresh lucerne and lucerne hay.

The rats used were Wistar males weighing 250–300g and were fed on a pelleted rat diet (Barastoc and Co., Melbourne, Vic., Australia).

Tissue preparations. The sheep were killed by severing the necks and the livers were immediately freeze-clamped *in situ* with aluminium-faced tongs previously cooled in liquid N_2 (Wollenberger, Ristau & Schoffa, 1960). The time between the severing of the necks of the animals and the actual freeze-clamping of the liver varied between 10 and 20s. However, analysis of freeze-clamped samples obtained from control sheep with a 'spinal block' (achieved by administering 8ml of Xylocaine into the sacral–lumbar region of the spinal cord) gave results similar to those obtained by the method described above. Other samples of liver were immediately placed in ice-cold 0.25M-sucrose.

The rats were killed by a blow on the head and the livers immediately freeze-clamped *in situ* as described above. Liver samples were also collected into ice-cold 0.25M-sucrose.

The frozen liver samples were powdered in a stainless steel mortar with a heavy stainless steel pestle. Frozen liver powder (5g) was added to 5ml of 30% (w/v) $HClO_4$, previously frozen in liquid N_2, in the mortar of a glass Potter–Elvehjem homogenizer. The homogenizer was then placed in an alcohol bath at −15°C and the powdered liver was homogenized in the $HClO_4$ without the tissue powder thawing (see Lowry, Passonneau, Hasselberger & Schulz, 1964). Ice-cold water (7ml) was subsequently added to facilitate the final homogenization. The homogenate was centrifuged at 10000*g* for 20min and the supernatant used in the assay of the various metabolites after neutralization with 3M-KOH and removal of the $KClO_4$ precipitate by centrifuging.

Mitochondria. These were isolated from both rat and sheep liver by the method of Hogeboom (1955) as described by Meyers & Slater (1957).

METHODS

All assays involved the use of an Eppendorf spectrophotometer fitted with an automatic cell-changer and recorder (Eppendorf Gerätebau, Netheler und Hinz G.m.b.H., Hamburg, Germany) or a Zeiss PMQ II spectrophotometer fitted with an automatic sample-changer and TE converter (Carl Zeiss, Oberkocken, Germany) connected to a Rikadenki model B140 recorder (Rikadenki Kogyo Co. Ltd., Tokyo, Japan). Both instruments were fitted with temperature-controlled cell holders and the temperature was maintained at 20°C for all assays.

Carnitine acetyltransferase. This was measured by the method of Beenakkers & Klingenberg (1964). Rotenone (3μM) was included in the assay mixture to inhibit NADH oxidase.

Carnitine palmitoyltransferase. This was measured spectrophotometrically by the method of Shepherd *et al.* (1966) based on the measurement of CoA released with α-oxoglutarate oxidase (Garland, Shepherd & Yates, 1965). α-Oxoglutarate oxidase was isolated from bovine heart by the method of Sanadi, Littlefield & Bock (1952) as modified by Hirashima, Hayakawa & Koike (1967).

Both carnitine acetyl- and palmitoyl-transferase were measured in mitochondrial preparations after sonication. The mitochondrial pellets were suspended in cold 0.1M-sodium–potassium phosphate buffer, pH7.4, to give a concentration of approx. 40mg of mitochondrial protein/ml. The suspensions were disrupted in an MSE sonic disintegrator (Measuring and Scientific Equipment Ltd., London S.W.1, U.K.) at 20kHz for 20s. Enzyme activities were determined in the supernatants obtained after centrifuging the sonicated mitochondrial suspensions at 14000*g* for 10min.

Carnitine compounds. Acetylcarnitine was measured by the method of Pearson & Tubbs (1964) and free carnitine by the method of Marquis & Fritz (1964). High blank values in the latter assay, due to endogenous free thiol groups, were decreased to quite acceptable values by adjusting the extracts to pH8.5 and heating at 90°C for 5min before assay, as suggested by Marquis & Fritz (1964). Pearson, Chase & Tubbs (1969) suggest that significant hydrolysis of short-chain carnitine esters may occur under these conditions but we could detect no breakdown of a 0.2mM solution of *O*-acetyl-L-carnitine at pH8.5 and 90°C even after 15min heating. Total acid-soluble carnitine and acid-insoluble carnitine were measured by the method of Pearson & Tubbs (1967).

Acetyl-CoA and CoA. Acetyl-CoA and acetyl-CoA plus CoA were measured by the kinetic method of Allred & Guy (1969). Free CoA was determined by taking the difference between acetyl-CoA plus CoA and acetyl-CoA. In these kinetic determinations a standard curve was prepared on each occasion by using a CoA standard solution, the concentration of which was determined with phosphotransacetylase (EC 2.3.1.8) by the method of Michal & Bergmeyer (1963). However, as this method only assays reduced CoA the standards were preincubated for 10min at 25°C with 2μl of 0.2M-dithiothreitol to ensure that all the CoA was in the reduced form.

Protein. This was measured by the biuret method (Gornall, Bardawill & David, 1949).

Chemicals. D- and L-Carnitine hydrochlorides and *O*-acetyl-L-carnitine chloride were generously supplied by Dr Otsuka of Otsuka Pharmaceuticals, Osaka, Japan. All products were recrystallized from ethanol. Other biochemicals were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., and enzymes from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Palmitoyl-CoA was prepared by the method of Seubert (1960) and propionyl- and butyryl-CoA from free CoASH and the appropriate anhydrides (redistilled) by the method of Stadtman (1957).

RESULTS

Carnitine acyltransferase activities in sheep liver mitochondria. The results in Table 1 indicate that the activity of carnitine palmitoyltransferase of sheep liver mitochondria is the same as that of rat liver mitochondria. However, the activity of carnitine acetyltransferase is some 40–50 times that of rat liver mitochondria. The high activity of carnitine acetyltransferase appears to be an

Table 1. *Carnitine acyltransferase activities in sheep liver mitochondria*

The mitochondria were suspended in 0.1M-sodium–potassium phosphate buffer to give a concentration of 40mg of protein/ml and were sonicated at 20kHz for 20s. The sonicated suspensions were centrifuged at 14000*g* for 10min and the supernatants used for assay. The enzyme activities were measured as described in the text. The results, expressed as nmol/min per mg of protein, are means ± S.E.M. for five individual preparations in each case.

Species	Carnitine acetyltransferase	Carnitine palmitoyltransferase
Rat	1.7 ± 0.4	1.1 ± 0.1
Sheep	76.0 ± 6.9	1.1 ± 0.1

Table 2. *Free carnitine and various carnitine fractions in the liver of sheep under different dietary conditions*

One group of Merino wethers was grazed on open pasture consisting mainly of wheaten and oaten stubble and dried rye grass, and the diet of the second group was supplemented with lucerne hay *ad libitum*. The third group was starved for 7 days. The animals were killed by severing the necks and samples of liver were immediately frozen *in situ* with aluminium-faced tongs previously cooled in liquid N_2. The frozen liver powders were extracted with $HClO_4$ and assayed as described in the text. Values are means ± S.E.M. for four animals in each case. N.D. indicates not detectable, i.e. <0.5nmol/g wet wt. under the conditions of assay.

Dietary condition	Acetylcarnitine (nmol/g wet wt.)	Free carnitine (nmol/g wet wt.)	Total acid-soluble carnitine (nmol/g wet wt.)	Acid-insoluble carnitine (nmol/g wet wt.)
Grazed plus lucerne *ad libitum*	15 ± 2	82 ± 7	143 ± 15	N.D.
Grazed on open pasture	80 ± 23	55 ± 18	148 ± 41	15 ± 8
Starved for 7 days	442 ± 46	192 ± 15	695 ± 43	30 ± 5

intrinsic feature of the species and is not associated with active rumen fermentation as young lambs (2 weeks old) had enzyme activities of 79 ± 6 (3) nmol/min per mg of protein in liver mitochondria, which are similar to those of adult sheep (Table 1). Mitochondria isolated from bovine liver and assayed in the same manner showed carnitine acetyltransferase activity of 69 ± 5 (2) nmol/min per mg, which again is similar to the activity in sheep liver mitochondria.

The specificity of the carnitine acetyltransferase reaction of sheep liver was examined by using various CoA esters as substrates and by using the carnitine palmitoyltransferase assay system. If the activity with the acetyl ester was expressed as 100, then the activities found with the propionyl and butyryl esters were 121 and 95 respectively. This pattern of specificity of carnitine acetyltransferase from sheep liver is very similar to that reported by Bøhmer & Bremer (1968) for the commercially available enzyme, which is isolated from pigeon breast muscle.

Free carnitine and carnitine esters in sheep liver and the effect of diet. The results presented in the previous section revealed a relatively high activity of carnitine acetyltransferase in sheep liver. It was therefore considered important to determine the amounts of the various carnitine esters in sheep liver.

The results in Table 2 for freeze-clamped liver samples show that when sheep were grazed on pastures consisting of wheaten and oaten stubble and dried grass the amount of acetylcarnitine was approximately the same as the amount of free carnitine. In contrast, in the animals that grazed on pastures and whose diet was supplemented *ad libitum* with fresh lucerne and lucerne hay the [free carnitine]/[acetylcarnitine] ratio was markedly ($P<0.001$) different, being approx. 6:1, although the amount of total acid-soluble carnitine was similar in both cases (Table 2). In the former group of sheep the sum of the free carnitine plus acetylcarnitine is not significantly different from the total acid-soluble carnitine, whereas in the latter group free carnitine plus acetylcarnitine represents only 74% of the total acid-soluble carnitine. Samples of neutralized perchloric acid extracts of powdered liver from the sheep whose diet was supplemented with lucerne were incubated with 10 μmol of CoA and 20 units of carnitine acetyltransferase at pH 8.0 for 30min. Under these conditions, due to the specificity of this enzyme (see Bøhmer & Bremer, 1968), both acetylcarnitine and propionylcarnitine would be converted into the

Table 3. *Free carnitine, acetylcarnitine, free CoA and acetyl-CoA in the liver of sheep under different dietary conditions*

The various groups of sheep, the dietary conditions and the removal of liver samples and their subsequent treatment were as described in Table 2. The liver samples were extracted and assayed as described in the text. Results are means ±S.E.M. for four animals in each case.

Dietary condition	Acetyl carnitine (nmol/g wet wt.)	Free carnitine (nmol/g wet wt.)	Acetyl-CoA (nmol/g wet wt.)	Free CoA (nmol/g wet wt.)	[Free carnitine]/[acetyl-carnitine] ratio	[Free CoA]/[acetyl-CoA] ratio
Grazed plus lucerne *ad libitum*	15±2	82±7	51±6	52±5	6.1 ±0.8	1.04±0.10
Grazed on open pasture	80±23	55±15	77±7	29±1	0.77±0.22	0.38±0.04
Starved for 7 days	442±46	192±15	70±7	23±1	0.46±0.09	0.34±0.08

corresponding CoA derivatives and an equivalent amount of free L-carnitine would be released. Any butyrylcarnitine would also be utilized in a similar manner. However, most of the butyrate produced in the rumen of sheep is converted into ketone bodies in the rumen epithelium (Pennington, 1952). After the incubation described above, total carnitine was measured in these mixtures. The values for acetylcarnitine and free carnitine were deducted from these total carnitine values. The remaining portion was tentatively considered to have been derived from propionylcarnitine and the amount of extra carnitine liberated by the procedure outlined above was found to be equal to approx. 80% of the total acid-soluble carnitine minus free carnitine plus acetylcarnitine. This experiment, however, only gives a qualitative indication that the unaccounted fraction of total acid-soluble carnitine is propionylcarnitine, as the carnitine transferase reaction may not have gone to completion and the presence of unchanged CoA in the carnitine assay gave very high blank values.

The results in Table 3 also indicate that acid-insoluble carnitine was not detectable in the livers of the sheep on diets supplemented with lucerne, whereas in the non-supplemented animals there was 15nmol of this acid-insoluble fraction/g wet wt. This fraction is considered by Pearson & Tubbs (1967) to represent long-chain fatty-acylcarnitine esters.

Effects of starvation on the amounts of carnitine and carnitine esters in sheep liver. The results presented in Table 2 indicate that the amount of acetylcarnitine in the liver of the starved sheep increased fivefold over the amount found in the livers of sheep grazed on open pastures. This was a highly significant increase ($P<0.001$) but it did not occur at the expense of free carnitine, for the total acid-soluble carnitine also increased some four- to five-fold ($P<0.001$) on starvation. This is in marked contrast with the situation in rat liver, where the amount of acetylcarnitine does increase on starvation (about twofold) but at the expense of carnitine (Pearson & Tubbs, 1967). If the amounts of acetylcarnitine in liver of the starved sheep are contrasted with the amounts present in the livers of the grazing animals that were supplemented with lucerne (Table 2) then the increase of acetylcarnitine on starvation is even more marked, being some thirty-fold.

The acid-insoluble carnitine fraction in the liver only increased twofold on starvation (when compared with the sheep on open grazing) and in the starved sheep only constitutes about 5% of the total carnitine (Table 3). Again this is in marked contrast with the situation in rat liver, where this fraction increases sevenfold on starvation and constitutes some 20% of the total carnitine in the starved state (Pearson & Tubbs, 1967).

Striking increases in free carnitine, acetylcarnitine and total acid-soluble carnitine concentrations have also been observed under other conditions. In an alloxan-diabetic sheep (60mg of alloxan/kg body wt. given intravenously 60h before slaughter) the total acid-soluble carnitine had reached 824nmol/g wet wt. compared with a normal average of 148nmol/g (Table 2). Also, in one sheep that was in rather poor condition initially (weight 36kg) the total acid-soluble carnitine in the liver was found to be 1550nmol/g wet wt. after 7 days starvation.

CoA, acetyl-CoA, carnitine and acetylcarnitine in the livers of normal and starved sheep. In the previous section the amounts of various carnitine fractions in sheep liver were reported. The proportions of these various fractions were found to be markedly different from those found in rat liver. In view of this fact and because carnitine acetyltransferase allows equilibration between acetyl-CoA and acetylcarnitine, at least in rat tissues (Pearson &

Tubbs, 1967), the relationship between acetyl-CoA and acetylcarnitine was examined in sheep liver.

The results presented in Table 3 show that in the sheep that received lucerne the [free carnitine]/[acetylcarnitine] ratio in the liver was 6:1 and the [free CoA]/[acetyl-CoA] ratio was 1:1. In contrast, in the sheep that were grazed with no lucerne, the [free carnitine]/[acetylcarnitine] ratio was approx. 1:1 and the [free CoA]/[acetyl-CoA] ratio was 0.38:1 (Table 3). On starvation the [free carnitine]/[acetylcarnitine] ratio in a sheep liver was further lowered to 0.46:1, although the total amounts of both fractions increased markedly (Table 3), and the [free CoA]/[acetyl-CoA] ratio was 0.34:1. The total content of CoA plus acetyl-CoA was not significantly different in any of the three groups of sheep and the overall mean was 101nmol/g wet wt. compared with a mean value of 173nmol/g for rat liver as reported by Allred & Guy (1969). Jarrett & Filsell (1964) have reported that sheep liver contains less CoA than rat liver.

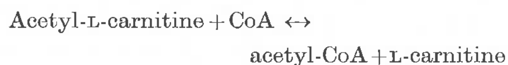
DISCUSSION

The most noteworthy results presented in this paper are the remarkable changes in the [free carnitine]/[acetylcarnitine] ratio in the liver of the sheep when the animals were fed on a diet of mainly lucerne, in contrast with animals grazed on open pastures, and the striking increase in total acid-soluble carnitine in the sheep liver on starvation. These two findings are discussed in turn.

In the sheep that were fed *ad libitum* on lucerne, the [free carnitine]/[acetylcarnitine] ratio in the liver was 6:1. In the rat, the [free carnitine]/[acetylcarnitine] ratio in the liver of animals fed on a normal diet is reported to be 3:1 by Bøhmer (1967) and 4:1 by Pearson & Tubbs (1967). Thus the ratio in the liver of these sheep indicated an even greater proportion of free carnitine than is observed in the rat. In these particular sheep the free carnitine plus acetylcarnitine was found to constitute 74% of the total acid-soluble carnitine in the liver. This is comparable with the situation in the rat (Pearson & Tubbs, 1967). Bøhmer & Bremer (1968) have shown a considerable proportion of the remaining acid-soluble carnitine (i.e. total minus free plus acetyl) in rat liver is propionylcarnitine and the results presented here tentatively suggest that most of the unspecified acid-soluble carnitine is also propionylcarnitine. It should be noted that the crude carnitine acetyltransferase of sheep liver, as reported here, shows a greater activity with the propionyl ester than the acetyl ester, as does the purified enzyme from pigeon breast muscle (Bøhmer & Bremer, 1968).

In the sheep grazed on open pastures the free carnitine plus acetylcarnitine in the liver is approximately equal to the total acid-soluble carnitine, whereas in the animals fed on lucerne *ad libitum* free carnitine plus acetylcarnitine represents only 74% of the total acid-soluble carnitine. Also, in the former group of sheep the [free carnitine]/[acetylcarnitine] ratio is approx. 1:1, in contrast with the value of 6:1 for the latter group of sheep. These changes are undoubtedly due to the different diets affecting the proportions of the various volatile fatty acids produced in the rumen of the sheep. A high rate of lucerne intake by sheep under the conditions used here results in large amounts of propionate being produced in the rumen (A. R. Egan, unpublished work). Thus the dramatic changes in the [free carnitine]/[acetylcarnitine] ratio reported here appear due to changes in the relative proportions of propionate and acetate reaching the liver from the rumen. Pearson & Tubbs (1967) have shown that perfusion of rat hearts with propionate induces an almost complete disappearance of acetylcarnitine from the heart-muscle tissue. A similar situation is observed here in the sheep liver but is undoubtedly of considerable physiological significance as far as the overall metabolism of the sheep is concerned.

The results presented here indicate that the metabolism of sheep liver is under much more 'acetyl pressure' than is the system in the rat liver, as even in the starved rat the [free carnitine]/[acetylcarnitine] ratio is never less than 1:1 (Pearson & Tubbs, 1967; Bøhmer, 1967). The effects of this 'acetyl pressure' in the sheep liver on the CoA system are probably of considerable physiological significance. Even in the sheep fed on lucerne the [free CoA]/[acetyl-CoA] ratio in the liver was only slightly greater than 1:1 and in the starved sheep the [free CoA]/[acetyl-CoA] ratio is 0.34, whereas in normal rat liver this ratio is approx. 4:1 in favour of free CoA (Allred & Guy, 1969). As the [free CoA]/[acetyl-CoA] ratio has an important controlling effect on metabolism generally (Tubbs & Garland, 1964; Wieland & Weiss, 1963; Garland *et al.* 1965), and the CoA system is under considerable 'acetyl pressure' in sheep liver, the buffering role of the carnitine system would appear to be of particular importance in the starved sheep. Under these conditions the amount of acetyl-CoA in the liver is the same as in the normal sheep, but the content of acetylcarnitine is five times greater after starvation. Also, in the starved sheep an apparent equilibrium constant for the reaction:



$$\text{Acetyl-L-carnitine} + \text{CoA} \leftrightarrow \text{acetyl-CoA} + \text{L-carnitine}$$

may be calculated from the data in Table 3 to be 1.3. Fritz, Schultz & Srere (1963) determined the apparent equilibrium constant for carnitine acetyltransferase, that has been partially purified from

pig heart, to be 0.6 at pH 7.0. If one assumes a similar equilibrium constant for the sheep liver enzyme, then the system in the starved sheep liver would appear to be close to equilibrium. The situation in the normal sheep liver is less clear and is complicated by the fact that the carnitine acetyltransferase also reacts quite strongly with the propionyl ester.

The results discussed above thus seem to point to an important role for the relatively high activity of the carnitine acetyltransferase in sheep liver. The physiological function of this enzyme is uncertain (see Fritz, 1967). However, the results presented here for the starved sheep strongly suggest that the presence of carnitine acetyltransferase in large amounts in sheep liver allows the 'acetyl pressure' in the starved condition to be shifted from the vital CoA system to the carnitine system. This is probably true in other ruminant species. A similar 'buffering' role for carnitine acetyltransferase has been suggested by Pearson and Tubbs (1967) on the basis of studies in perfused rat heart.

It is difficult to envisage any other function than that of an 'acetyl buffer' system as suggested here for the high activity of carnitine acetyltransferase. Very little synthesis of fatty acids takes place in ruminant liver compared with rat liver (Hanson & Ballard, 1967), and fatty acid synthesis occurs in the cytoplasm, mainly from acetate, whereas carnitine acetyltransferase is associated with the inner mitochondrial membrane in sheep liver mitochondria (G. D. Henderson, unpublished work). Homogenates of sheep liver are capable of oxidizing acetate (Mayfield, Smith & Johnson, 1965) and the results presented above are certainly indicative of acetate utilization, although the main oxidation of acetate in the whole animal would appear to be by extrahepatic tissues (Mayfield, Bensadoun & Johnson, 1966). The oxidation of short-chain fatty acids is considered to be carnitine-independent, at least in the rat (Bremer, 1967).

However, carnitine does increase acetate oxidation in sheep liver homogenates (Mayfield *et al.* 1966) and we find isolated sheep liver mitochondria do not oxidize acetate but oxidize acetylcarnitine very slowly (Koundakjian & Snoswell, 1970). Thus carnitine acetyltransferase may have a minor role in acetate oxidation.

The other particularly striking feature of the results presented here is the remarkable increase in total acid-soluble carnitine that is seen in the sheep liver on starvation (nearly fivefold). This is in marked contrast with the rat, where only a 10–20% increase is seen on starvation (Pearson & Tubbs, 1967). As suggested above, this increase in total acid-soluble carnitine on starvation is probably related to the role of carnitine acetyltransferase in relieving 'acetyl pressure' on the CoA system in the sheep liver. Free fatty acid turnover, giving rise to acetyl-CoA, increases markedly on starvation (Katz & Bergman, 1969) and acetylcarnitine is the largest fraction of the total acid-soluble carnitine. However, free carnitine also increases in starvation in the sheep liver. Also the amount of total acid-soluble carnitine was found to increase similarly or even to greater extent in an alloxan-diabetic sheep.

The striking increases in total acid-soluble carnitine in the sheep liver on starvation imply that there is synthesis of carnitine under these conditions. The values presented here are given in nmol/g wet wt. of tissue; and, although the weight of sheep liver increases by approx. 13% after 5 days starvation (O. H. Filsell & I. G. Jarrett, unpublished work), the increase in total acid-soluble carnitine on starvation is still over fourfold, even allowing for the slight increase in liver weight.

Little is known of the biosynthesis of carnitine, apart from the source of the methyl groups (Bremer, 1962), and it would appear that these remarkable changes in the amounts of carnitine in sheep liver indicate an important system for the study of the biosynthesis of carnitine and its control.

The authors are indebted to Dr I. G. Jarrett and Dr F. J. Ballard for helpful advice and discussions, to Mr R. Fishlock for skilful technical assistance and to the Australian Meat Research Committee for a grant for capital equipment.

REFERENCES

Allred, J. B. & Guy, D. G. (1969). *Analyt. Biochem.* **29**, 293.

Beenakkers, A. M. T. & Klingenberg, M. (1964). *Biochim. biophys. Acta*, **84**, 205.

Bøhmer, T. (1967). *Biochim. biophys. Acta*, **144**, 259.

Bøhmer, T. & Bremer, J. (1968). *Biochim. biophys. Acta*, **152**, 559.

Bremer, J. (1962). *Biochim. biophys. Acta*, **57**, 327.

Bremer, J. (1967). *FEBS Symp. Cellular Compartmentalization and Control of Fatty Acid Metabolism, Oslo*, p. 65. Ed. by Gran, F. C. Oslo: Universitetsforlaget.

Friedman, S. & Fraenkel, G. (1955). *Archs Biochem. Biophys.* **59**, 491.

Fritz, I. B. (1967). *FEBS Symp. Cellular Compartmentalization and Control of Fatty Acid Metabolism, Oslo*, p. 39. Ed. by Gran, F.C. Oslo: Universitetsforlaget.

Fritz, I. B. Schultz, S. K. & Srere, P. A. (1963). *J. biol. Chem.* **238**, 2509.

Garland, P. B., Shepherd, D. & Yates, D. W. (1965). *Biochem. J.* **97**, 587.

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.

Greville, G. D. & Tubbs, P. K. (1968). In *Essays in Biochemistry*, vol. 4, p. 155. Ed. by Campbell, P. M. & Greville, G. D. London: Academic Press (Inc.) Ltd.

Hanson, R. W. & Ballard, F. J. (1967). *Biochem. J.* **105**, 529.

Hirashima, H., Hayakawa, T. & Koike, M. (1967). *J. biol. Chem.* **242**, 902.
Hogeboom, G. H. (1955). In *Methods in Enzymology*, vol. 1, p. 16. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Hungate, R. E. (1966). *The Rumen and its Microbes*, p. 376. New York: Academic Press Inc.
Jarrett, I. G. & Filsell, O. H. (1964). *Biochem. J.* **91**, 217.
Katz, M. L. & Bergman, E. N. (1969). *Am. J. Physiol.* **216**, 953.
Koundakjian, P. P. & Snoswell, A. M. (1970). *Biochem. J.* **119**, 49.
Lowry, O. H., Passonneau, J. V. Hasselberger, F. X. & Schulz, D. W. (1964). *J. biol. Chem.* **239**, 18.
Marquis, N. R. & Fritz, I. B. (1964). *J. Lipid Res.* **5**, 184.
Marquis, N. R. & Fritz, I. B. (1965). In *Recent Research on Carnitine*, p. 27. Ed. by Wolf, G. Cambridge, Mass.: Massachusetts Institute of Technology Press.
Mayfield, E. D., Bensadoun, A. & Johnson, B. C. (1966). *J. Nutr.* **89**, 189.
Mayfield, E. D., Smith, J. L. & Johnson, B. C. (1965). *J. Dairy Sci.* **48**, 93.
Meyers, D. K. & Slater, E. C. (1957). *Biochem. J.* **67**, 558.
Michal, G. & Bergmeyer, H.-U. (1963). In *Methods of Enzymatic Analysis*, p. 512. Ed. by Bergmeyer, H.-U. New York and London: Academic Press.
Pearson, D. J., Chase, J. F. A. & Tubbs, P. K. (1969). In *Methods in Enzymology*, vol. 14, p. 612. Ed. by Lowenstein, J. M. New York: Academic Press Inc.
Pearson, D. J. & Tubbs, P. K. (1964). *Nature, Lond.*, **202**, 91.
Pearson, D. J. & Tubbs, P. K. (1967). *Biochem. J.* **105**, 953.
Pennington, R. J. (1952). *Biochem. J.* **52**, 251.
Sanadi, D. R., Littlefield, J. W. & Bock, R. M. (1952). *J. biol. Chem.* **197**, 851.
Seubert, W. (1960). In *Biochemical Preparations*, vol. 7, p. 80. Ed. by Lardy, H. A. New York and London: John Wiley and Sons (Inc.) Ltd.
Shepherd, D., Yates, D. W. & Garland, P. B. (1966). *Biochem. J.* **98**, 3c.
Snoswell, A. M., Broadhead, P. P. & Henderson, G. D. (1969). *Proc. Aust. biochem. Soc.* p. 34.
Stadtman, E. R. (1957). In *Methods in Enzymology*, vol. 3, p. 931. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Tubbs, P. K. & Garland, P. B. (1964). *Biochem. J.* **93**, 550.
Wieland, O. & Weiss, L. (1963). *Biochem. biophys. Res. Commun.* **10**, 333.
Wollenberger, A., Ristau, O. & Schoffa, G. (1960). *Pflügers Arch. ges. Physiol.* **270**, 399.

Biochem. J. (1972) **127**, 133–141
Printed in Great Britain

Relationships between Carnitine and Coenzyme A Esters in Tissues of Normal and Alloxan-Diabetic Sheep

By A. M. SNOSWELL* and PATRICIA P. KOUNDAKJIAN
Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K., and Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S. Austral. 5064, *Australia*

(*Received* 18 *October* 1971)

1. The total acid-soluble carnitine concentrations of four tissues from Merino sheep showed a wide variation not reported for other species. The concentrations were 134, 538, 3510 and 12900nmol/g wet wt. for liver, kidney cortex, heart and skeletal muscle (M. biceps femoris) respectively. 2. The concentration of acetyl-CoA was approximately equal to the concentration of free CoA in all four tissues and the concentration of acid-soluble CoA (free CoA plus acetyl-CoA) decreased in the order liver > kidney cortex > heart > skeletal muscle. 3. The total amount of acid-soluble carnitine in skeletal muscle of lambs was 40% of that in the adult sheep, whereas the concentration of acid-soluble CoA was 2.5 times as much. A similar inverse relationship between carnitine and CoA concentrations was observed when different muscles in the adult sheep were compared. 4. Carnitine was confined to the cytosol in all four tissues examined, whereas CoA was equally distributed between the mitochondria and cytosol in liver, approx. 25% was present in the cytosol in kidney cortex and virtually none in this fraction in heart and skeletal muscle. 5. Carnitine acetyltransferase (EC 2.3.1.7) was confined to the mitochondria in all four tissues and at least 90% of the activity was latent. 6. Acetate thiokinase (EC 6.2.1.1) was predominantly (90%) present in the cytosol in liver, but less than 10% was present in this fraction in heart and skeletal muscle. 7. In alloxan-diabetes, the concentration of acetylcarnitine was increased in all four tissues examined, but the toatl acid-soluble carnitine concentration was increased sevenfold in the liver and twofold in kidney cortex. 8. The concentration of acetyl-CoA was approximately equal to that of free CoA in the four tissues of the alloxan diabetic sheep, but the concentration of acid-soluble CoA in liver increased approximately twofold in alloxan-diabetes. 9. The relationship between CoA and carnitine and the role of carnitine acetyltransferase in the various tissues is discussed. The quantitative importance of carnitine in ruminant metabolism is also emphasized.

It has been shown (Snoswell & Henderson, 1970) that the [free carnitine]/[acetylcarnitine] ratio in sheep liver varies widely with different dietary conditions, whereas much smaller variations in the ratio [free CoA]/[acetyl-CoA] were observed. In view of these results, plus the fact that acetate, a major fuel in ruminants, is metabolized mainly in extrahepatic tissues (Mayfield *et al.*, 1966), we have investigated the relationships between various carnitine and CoA fractions in several tissues of normal sheep. Also, in order to understand more fully the role played by carnitine acetyltransferase (EC 2.3.1.7) in the conversions between acetylcarnitine, CoA, acetyl-CoA and carnitine, we have examined the intracellular distribution of carnitine acetyltransferase, carnitine and CoA and also the intracellular distribution of acetate thiokinase in these tissues.

A marked increase in acetylcarnitine concentration in sheep liver occurs on starvation (Snoswell & Henderson, 1970). However, this increase does not occur at the expense of free carnitine (as observed in the rat by Pearson & Tubbs, 1967), for the concentrations of free and total acid-soluble carnitine also increased markedly on starvation (Snoswell & Henderson, 1970). Alloxan-diabetes causes an even more striking increase in the total acid-soluble carnitine content of sheep liver. Mehlman *et al.* (1969) reported that the carnitine content of rat skeletal muscle is approximately halved in alloxan-diabetes. Thus, we have extended our observations on the effects of alloxan-diabetes in sheep and have examined carnitine and CoA fractions in various

* Present address: Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S. Austral. 5064, Australia.

sheep tissues in order to investigate intra-tissue relationships between carnitine and CoA under conditions of severe metabolic stress.

Experimental

Methods

Animals. The adult sheep used were Merino wethers, approx. 4 years old, and weighing between 35 and 45kg; they were given a diet of lucerne–hay chaff. The lambs were between 5 and 16 days old. They were bottle-fed on reconstituted skim-milk powder with a vitamin supplement. Tissues from Suffolk sheep were obtained from the abattoirs at Bristol directly after slaughter.

Alloxan-diabetic animals. Alloxan-diabetes was produced in adult Merino wethers by injecting a sterile solution of alloxan (60mg/kg body wt.) into the jugular vein. The animals were killed 3 days later. Results are presented only for those animals in which the blood glucose concentration had risen to 200mg/100ml.

Tissue preparations. The sheep were killed by severing the necks, and samples of liver, kidney cortex, heart and skeletal muscle (M. biceps femoris and M. sternothyreoidus) from adult animals, and liver and skeletal muscle (M. biceps femoris) from lambs, were immediately freeze-clamped with aluminium-faced tongs previously cooled in liquid N_2 (Wollenberger *et al.*, 1960). The $HClO_4$ extracts of the frozen tissues were prepared as described for liver (Snoswell & Henderson, 1970).

Homogenates and subcellular fractions. Fresh samples of sheep liver and kidney cortex were collected directly into 0.25M-sucrose containing 23mM-potassium phosphate (pH7.2). Homogenates (10%, w/v) were then prepared in the same sucrose–phosphate solution with a Potter–Elvehjem homogenizer. These homogenates were then centrifuged at 700*g* to remove cell debris and nuclei. The supernatant fractions were centrifuged at 10000*g* for 10min to sediment mitochondria. The mitochondrial pellets were washed twice in the sucrose–phosphate medium and re-centrifuged at 13000*g* for 10min. Microsomal and supernatant (cytosol) fractions were prepared by centrifugation (for 1h at 100000*g*) of the supernatants obtained after sedimentation of the mitochondria. The mitochondrial and microsomal fractions were finally suspended in the sucrose–phosphate medium described above.

Homogenates (10%, w/v) of sheep heart and skeletal muscle were prepared in the electrolyte buffer described by Chappell & Perry (1954) by using a ground-glass homogenizer.

The preparation of subcellular fractions from sheep heart and skeletal muscle was much more difficult than the preparation of similar fractions from sheep liver and kidney cortex. Most methods for preparing subcellular fractions from heart and skeletal muscle involve the use of a bacterial proteinase. However, there have been a number of reports of the destruction of mitochondrial enzymes, particularly carnitine acyltransferases, by these methods (see, e.g., De Jong & Hülsmann, 1970). Thus we have avoided using fractionation methods involving the use of a proteinase. Unfortunately, all other methods for preparing subcellular fractions from heart and skeletal muscle require more extensive homogenization in a ground-glass homogenizer. These methods led to extensive loss of the mitochondrial-matrix marker enzyme, citrate synthase, into the cytosol fractions. Thus a direct preparation of subcellular fraction from sheep heart and skeletal muscle was not practicable. Instead, an indirect method was employed to prepare cytosol fractions of these tissues relatively free of mitochondrial contamination. 'Press' fractions of heart and skeletal muscle (M. sternothyreoidus) were prepared by direct centrifugation of whole muscle (cut into small pieces with scissors) at 100000*g* for 4h (skeletal muscle) or 33000*g* for 1½h (heart) in a procedure similar to that described by Amberson *et al.* (1964). The small amounts of supernatants thus obtained were designated 'press' fractions.

Homogenates, mitochondrial and microsomal fractions were disrupted by sonic disintegration for 3min at 0°C (in 15s intervals with 15s cooling in between) by using a Soniprobe (Dawe Instruments Ltd., London W.3, U.K.) at 20kHz and 2A. The preparations were subsequently centrifuged at 20000*g* for 2min and the supernatants were used for the assay of enzymes.

Immediately after isolation, samples (1.5ml) of the various homogenates and subcellular fractions were treated with 0.25ml of 30% (w/v) $HClO_4$ and the supernatants were neutralized with saturated $KHCO_3$ before assay of carnitine and CoA.

Enzyme assays. All assays were done at 30°C with a Hilger–Gilford recording spectrophotometer or a Unicam spectrophotometer (model SP. 500) fitted with a Gilford automatic cell-positioner and recorder.

Acetate thiokinase (*EC* 6.2.1.1). This was assayed in sonic extracts of the various subcellular fractions by coupling the reaction with that catalysed by arylamine acetyltransferase (EC 2.3.1.5) and measuring the change in extinction at 460nm caused by acetylation of (*p*-aminophenylazo)benzene-*p*-sulphonic acid in an assay system similar to that used for pyruvate dehydrogenase (EC 1.2.4.1) by Denton *et al.* (1971). The arylamine acetyltransferase was prepared from acetone-dried powder of pigeon liver by the method of Tabor *et al.* (1953) and was kindly supplied by Dr. R. M. Denton. The assay system contained 100mM-tris–HCl (pH7.8), 0.5mM-EDTA, 1mM-$MgCl_2$, 5mM-mercaptoethanol, 100mM-potassium acetate, 10mM-ATP and 0.3 unit of

arylamine acetyltransferase plus the sample in total volume of 1.0ml. The reaction was started by the addition of 0.3 μmol of CoA and a linear rate of reaction was observed after 5 to 10min. A molar extinction coefficient (substrate–acetylated substrate) at 460nm of 7.11×10^6 litre·mol^{-1}·cm^{-1} (Jacobson, 1961) was used to calculate enzyme activity, which was expressed as nmol of acetyl-CoA produced/min at 30°C. All subcellular fractions were assayed for acetate thiokinase immediately after preparation, as it was found that the activity of this enzyme decreased rapidly after isolation of these fractions, particularly in the cytosol fractions.

Carnitine acetyltransferase. This was assayed in the various subcellular fractions that had been exposed to hypo-osmotic sucrose (0.025M) plus 0.1% Triton X-100 for 30min. Activity was also assayed in whole mitochondrial suspensions. The assay system was similar to that described by Barker *et al.* (1968) but included 0.18M-sucrose, and the acetyl-CoA was added 5min before the addition of 5,5′-dithiobis-(2-nitrobenzoic acid) to ensure maximum activity.

Citrate synthase (*EC* 4.1.3.7). This was assayed spectrophotometrically by using dithiobis(nitrobenzoic acid) as described by Shepherd & Garland (1969). Enzyme activity was assayed in sonic extracts of the various subcellular fractions of heart and skeletal muscle.

Glutamate dehydrogenase (*EC* 1.4.1.3). This was assayed in sonic extracts of various subcellular fractions from kidney cortex and liver by the method of Barker *et al.* (1968), but with 1.5mM-ADP to ensure maximum activity and 10μM-rotenone in place of 3mM-KCN and P_i in place of tris–HCl buffer. Recovery of the glutamate dehydrogenase present in homogenates in the various subcellular fractions could only be achieved if P_i was included in the homogenizing medium and in the assay medium (Walter & Anabitarte, 1971).

Lactate dehydrogenase (*EC* 1.1.1.27). This was assayed in sonic extracts of the various subcellular fractions by spectrophotometric assay at 340nm. The system contained 200mM-tris–HCl (pH7.4), 0.2mM-NADH, 10μM-rotenone and enzyme fraction in a final volume of 1.0ml. The reaction was started by addition of 1mM-pyruvate.

Metabolite assays. Acetylcarnitine was measured by the method of Pearson & Tubbs (1964), and free carnitine by the method of Marquis & Fritz (1964). High blank values in the latter assay, due to endogenous free thiol groups, were decreased to acceptable values by adjusting the extracts to pH8.5 with 1M-tris base and heating at 90°C for 5min before assay, as suggested by Marquis & Fritz (1964). Pearson *et al.* (1969) suggest that significant hydrolysis of short-chain carnitine esters may occur under these conditions. We could detect no breakdown of a 0.2mM solution of *O*-acetyl-L-carnitine in 20mM-tris–HCl, pH8.5, at 90°C after 15min heating. However, owing to the temperature coefficient of the tris the actual pH at 90°C would have been nearer 6.5. The total amounts of acid-soluble carnitine and acid-insoluble carnitine were measured by the method of Pearson & Tubbs (1967).

Acetyl-CoA and acetyl-CoA plus free CoA were measured by the kinetic method of Allred & Guy (1969); free CoA was determined by difference. In these kinetic determinations a standard curve was prepared on each occasion by using a CoA standard solution, the concentration of which was determined with phosphotransacetylase (EC 2.3.1.8) by the method of Michal & Bergmeyer (1963). However, as this method only assays reduced CoA, the standards were preincubated for 10min at 25°C with 2μl of 0.2M-dithiothreitol to ensure that all the CoA was in the reduced form.

Instrumentation was as described by Snoswell & Henderson (1970).

Table 1. *Free carnitine, carnitine esters, free CoA and acetyl-CoA in sheep tissues*

Merino wethers that had been given a diet of lucerne chaff were killed and tissue samples were immediately frozen with aluminium-faced tongs previously cooled in liquid N_2. The frozen tissue powders were extracted with $HClO_4$ and assayed as described in the text. Results are means ± S.E.M. for four animals.

	Concentration (nmol/g wet wt.)					
Tissue	Acetylcarnitine	Free carnitine	Total acid-soluble carnitine	Acid-insoluble carnitine	Acetyl-CoA	Free CoA
Liver	3.5±2.6	74±7	134±9	<0.1	46±3	50±6
Kidney cortex	67±18	415±45	538±64	4±1	31±1	34±3
Heart	812±83	2060±323	3510±143	12±8	12±3	16±1
Skeletal muscle (M. biceps femoris)	1820±478	9860±1380	12900±880	17±5	1.0±0.1	1.3±0.2

Chemicals

L-Carnitine hydrochloride and *O*-acetyl-L-carnitine chloride were generously supplied by Dr. Otsuka of Otsuka Pharmaceuticals, Osaka, Japan, and were recrystallized from ethanol. CoA was obtained from Calbiochem, Los Angeles, Calif., U.S.A., enzymes from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and alloxan from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Acetyl-CoA was prepared from free CoA and redistilled acetic anhydride by the method of Stadtman (1957).

Results

Free carnitine, carnitine esters, free CoA and acetyl-CoA in normal sheep tissues

The results in Table 1 show that acetylcarnitine constitutes a relatively small portion of the total acid-soluble carnitine in all tissues shown except the heart, where it represents approx. 25%. Acid-insoluble carnitine (long-chain fatty-acyl carnitine esters) constitutes a very minor fraction of the total carnitine in all the sheep tissues examined. The total carnitine content of the four tissues examined had a wide range (Table 1). The concentrations of the various carnitine fractions in sheep liver recorded in Table 1 are comparable with those in the liver of sheep fed on lucerne *ad libitum*, as reported by Snoswell & Henderson (1970).

The concentrations of acetyl-CoA are approximately equal to the concentrations of free CoA in all four sheep tissues (Table 1) and the acid-soluble CoA content of these tissues decreases in the order liver > kidney > heart > skeletal muscle.

The carnitine and CoA concentrations in different muscles were found to vary and also in the same muscle from different species, e.g. M. sternothyreoidus from Merino sheep was found to contain 3.3 and 8390 nmol of acid-soluble CoA and total acid-soluble carnitine/g wet wt. respectively, whereas the same muscle from Suffolk sheep contained 9.5 and 2260 nmol/g wet wt. respectively. M. biceps femoris from Merino sheep contained 2.3 and 12900 nmol/g wet wt. of acid-soluble CoA and total acid-soluble carnitine respectively (Table 1). These results, together with those for M. biceps femoris for Merino lambs (Table 2), suggest an inverse relationship between CoA and carnitine concentrations in sheep muscles.

Free carnitine, carnitine esters, free CoA and acetyl-CoA in lamb tissues

The concentration of total acid-soluble carnitine in the liver of lambs (Table 2) is comparable with that of adult sheep (Table 1) although the proportion of acetylcarnitine is considerably greater in the lamb. Similarly the lamb liver contains a greater proportion of acid-insoluble carnitine (compare Table 2 with Table 1), which undoubtedly reflects the milk diet of the lambs. The total acid-soluble carnitine content of skeletal muscle in the lamb (Table 2) is significantly ($P<0.01$) less than that of the adult sheep (Table 1), being only about 40% of the adult value. The concentrations of acetyl-CoA and free CoA in the lamb liver (Table 2) are very similar to those of the adult sheep (Table 1), but the total concentration of acid-soluble CoA in lamb skeletal muscle is about 2.5 times ($P<0.05$) that of the adult sheep.

Subcellular distribution of carnitine, CoA, carnitine acetyltransferase and acetate thiokinase in sheep tissues

The subcellular distribution of these metabolites and enzymes, together with the marker enzymes, glutamate dehydrogenase and lactate dehydrogenase, in sheep liver is shown in Table 3. As it has been reported that sheep liver is rather difficult to fractionate (Taylor *et al.*, 1971), the fact that 96% of the mitochondrial marker glutamate dehydrogenase, was found in the mitochondrial fraction indicates a very satisfactory fractionation.

Table 2. *Free carnitine, carnitine esters, free CoA and acetyl CoA in liver and skeletal muscle of lambs*

Merino lambs 5 to 16 days old were bottle-fed on reconstituted skim-milk powder with vitamin supplement. Tissue samples were prepared and assayed as described in Table 1. Results are means ± S.E.M. for four animals.

	Concentrations (nmol/g wet wt.)					
Tissue	Acetylcarnitine	Free carnitine	Total acid-soluble carnitine	Acid-insoluble carnitine	Acetyl-CoA	Free CoA
Liver	35 ± 24	86 ± 18	153 ± 11	10 ± 1	46 ± 3	51 ± 4
Skeletal muscle (M. biceps femoris)	175 ± 43	3590 ± 585	4780 ± 456	33 ± 1	2.8 ± 0.2	3.3 ± 0.6

Table 3. *Subcellular distribution of carnitine, CoA, acetate thiokinase and carnitine acetyltransferase in sheep liver*

Homogenates and subcellular fractions of sheep liver were prepared and assayed as described in the text. The values are means ± S.E.M. of four experiments. N.D., non-detectable.

Metabolite or enzyme	Enzyme activity in homogenate (nmol/min per ml)	Metabolite concentration in homogenate (nmol/ml)	% of activity or concentration in homogenate		
			Cytosol	Mitochondria	Microsomal fraction
Free CoA + acetyl-CoA		8.6 ± 1.1	48 ± 3	49 ± 4	2 ± 1
Total acid-soluble carnitine		9.2 ± 2.4	96 ± 2	N.D.	N.D.
Acetate thiokinase	13 ± 3.1		90 ± 1	8 ± 1	N.D.
Carnitine acetyltransferase	122 ± 12		6 ± 5	96* ± 9	N.D.
Glutamate dehydrogenase	3790 ± 87		2 ± 1	96 ± 3	3 ± 1
Lactate dehydrogenase	5610 ± 892		98 ± 2	2 ± 1	2 ± 1

* Whole mitochondrial suspensions (i.e. not previously exposed to 0.1% Triton X-100 in hypo-osmotic sucrose) contained <10% of this activity.

Table 4. *Subcellular distribution of carnitine, CoA, acetate thiokinase and carnitine acetyltransferase in sheep kidney cortex*

Homogenates and subcellular fractions of sheep kidney cortex were prepared and assayed as described in the text. The values are means ± S.E.M. of four experiments. N.D., non-detectable.

Metabolite or enzyme	Enzyme activity in homogenate (nmol/min per ml)	Metabolite concentration in homogenate (nmol/ml)	% of activity or concentration in homogenate		
			Cytosol	Mitochondria	Microsomal fraction
Free CoA + acetyl-CoA		4.5 ± 0.4	23 ± 4	56 ± 4	N.D.
Total acid-soluble carnitine		50 ± 12	101 ± 1	2 ± 1	N.D.
Acetate thiokinase	70 ± 5		32 ± 1	68 ± 1	3 ± 1
Carnitine acetyltransferase	241 ± 51		N.D.	97* ± 1	N.D.
Glutamate dehydrogenase	4220 ± 753		8 ± 1	87 ± 1	6 ± 1
Lactate dehydrogenase	24500 ± 3990		94 ± 1	5 ± 1	2 ± 1

* Whole mitochondrial suspensions (i.e. not previously exposed to 0.1% Triton X-100 in hypo-osmotic sucrose) contained <10% of this activity.

Acid-soluble CoA (free CoA plus acetyl-CoA) was distributed equally between the mitochondrial and cytosol fractions (Table 3) whereas the carnitine was present solely in the cytosol, as it was in all tissues examined (see also Tables 4 and 5). In kidney cortex only approx. 25% of the acid-soluble CoA was present in the cytosol (Table 4).

Determination of the degree of acetylation of the CoA in the various subcellular fractions of liver and kidney cortex is meaningless, as this may change during isolation of the fractions. In the present work this change did occur, especially in the kidney cortex; thus only values for free CoA plus acetyl-CoA were used. It might even be argued that CoA may move from one subcellular compartment to another during the fractionation procedure. Skrede & Bremer (1970)

have shown there is considerable loss of CoA from rat liver mitochondria that were incubated at 30°C for 20min. However, at 0°C in homogenizing medium the mitochondrial CoA is apparently stable for several hours (J. Bremer, personal communication); thus any movement of CoA during fractionation would seem unlikely.

Carnitine acetyltransferase was mainly confined to the mitochondria in sheep liver (Table 3) and in kidney cortex (Table 4). Also, over 90% of the activity of this enzyme in the mitochondria was latent, as the activity measured in whole mitochondria was less than 10% of that in preparations disrupted in 0.025M-sucrose containing 0.1% Triton X-100.

About 90% of the acetate thiokinase was present in the cytosol in sheep liver (Table 3) but only 30% was present in this fraction in kidney cortex (Table 4).

Owing to the difficulties in preparing subcellular fractions from sheep heart and skeletal muscle, as outlined in the Experimental section, the amounts of metabolites and enzymes in 'press' fractions of these were related to those in whole homogenates. These 'press' fractions were considered to be relatively clean cytosol fractions as they contained very little citrate synthase, the mitochondrial matrix marker (Table 5). To express the results obtained with the 'press' fractions quantitatively and to compare them with those for homogenates all concentrations and activities were related to the amount of lactate dehydrogenase, the cytoplasmic marker enzyme. The results presented in Table 5 show that the amount of lactate dehydrogenase in the 'press' fractions was 10 times that in the corresponding 1-in-10 homogenates. On this basis the cytosol contained very little acid-soluble CoA, acetate thiokinase or carnitine acetyltransferase in either sheep heart or skeletal muscle (Table 5). It is assumed that these compounds and enzymes are found in the mitochondria of these tissues. In support of this assumption, mitochondrial fractions prepared from these tissues, although having lost some of their contents during homogenization, showed very much higher specific activities than those found in microsomal fractions from the corresponding tissues. Again approx. 90% of the carnitine acetyltransferase activity in such mitochondria prepared from heart and skeletal muscle was latent.

The loss of acetate thiokinase into the cytosol fraction during homogenization paralleled the release of citrate synthase. The latter enzyme is present in the matrix of mitochondria (Tubbs & Garland, 1968) and it is thus assumed that the acetate thiokinase is present in the matrix fraction of the sheep muscle tissues.

Free carnitine, carnitine esters, free CoA and acetyl-CoA in the tissues of alloxan-diabetic sheep

The amounts of acetylcarnitine in the liver, kidney cortex, heart and skeletal muscle of alloxan-diabetic sheep (Table 6) are considerably greater than in the same tissues of normal sheep (Table 1). Also, although the total acid-soluble carnitine concentrations of heart and skeletal muscle are the same in alloxan-diabetic sheep and normal sheep, the concentration of this carnitine fraction is approx. 7 times as great in the liver of alloxan-diabetic sheep ($P<0.001$) and nearly twice as much in the kidney ($P<0.05$).

An even more dramatic increase in the total acid-soluble carnitine fraction of liver was observed when insulin was withdrawn from an alloxan-diabetic

Table 5. *Carnitine, CoA, acetate thiokinase and carnitine acetyltransferase in homogenates and 'press' fractions of sheep heart and skeletal muscle, relative to lactate dehydrogenase activities*

Tissue homogenates and 'press' fractions were prepared and assayed as described in the text. The values shown are for 3 animals, and are in nmol/ml or nmol/min per ml divided by the appropriate lactate dehydrogenase activity. N.D., non-detectable. The values in parentheses refer to amounts in 'press' fractions expressed as percentages of those for the homogenates.

Fraction	Lactate dehydrogenase (μmol/min per ml)	Total acid-soluble carnitine	Acetyl-CoA plus free CoA	Acetate thiokinase	Carnitine acetyltransferase	Citrate synthase
Heart						
Homogenate	18.8 ± 1.96	7.28	0.095	3.78	64.5	568
'Press'	214 ± 20	7.32	0.00635	0.169	0.503	15.8
			(7)	(5)	(1)	(3)
Skeletal muscle						
Homogenate	39.7 ± 5.05	7.15	0.0031	0.52	3.27	32.7
'Press'	403 ± 36.1	7.13	N.D.	0.04	N.D.	0.084
				(7)		(<1)

Table 6. *Free carnitine, carnitine esters, free CoA and acetyl-CoA in tissues of alloxan-diabetic sheep*

Merino wethers were given an intravenous injection of alloxan (60mg/kg body wt.) 3 days before slaughter. Tissue samples and assays were as described in Table 1. Results are means ± S.E.M. for three animals.

	Concentrations (nmol/g wet wt.)					
Tissue	Acetylcarnitine	Free carnitine	Total acid-soluble carnitine	Acid-insoluble carnitine	Acetyl-CoA	CoA
Liver	461 ± 24	203 ± 15	886 ± 52	2 ± 2	81 ± 9	88 ± 18
Kidney cortex	509 ± 121	274 ± 73	993 ± 98	2 ± 1	49 ± 8	37 ± 6
Heart	1450 ± 168	1520 ± 361	3270 ± 440	96 ± 26	11 ± 2	9 ± 2
Skeletal muscle	4530 ± 471	7200 ± 574	12800 ± 820	34 ± 11	2.3 ± 0.6	1.7 ± 0.6

sheep that was previously stabilized by continuous intravenous infusion of insulin. Samples of liver were removed surgically. The first one contained 66nmol/g wet wt. of total acid-soluble carnitine, but 24h after the withdrawal of insulin this value, measured in a second sample of liver, was 2210nmol/g wet wt. Similar surgical removal of two liver samples from a normal control sheep showed that no variation in total acid-soluble carnitine was caused by the surgery alone.

As in the normal sheep, acid-insoluble carnitine is only a very minor fraction of the total carnitine in all four tissues of the alloxan-diabetic sheep (Table 6), although the increase in this fraction in the heart of the alloxan-diabetic sheep may be significant ($P=0.05$).

The concentrations of acetyl-CoA are approximately the same as the concentrations of free CoA in all four tissues of the alloxan-diabetic sheep (Table 6). The concentration of acid-soluble CoA in the liver of alloxan-diabetic sheep (Table 6) is approximately twice ($P<0.01$) that of the same tissue in the normal sheep (Table 1).

Discussion

The wide range in the concentration of total acid-soluble carnitine in the four tissues examined is very prominent. This range is much greater than that reported for other species, e.g. rat skeletal muscle has only about twice the total acid-soluble carnitine concentration of rat liver (Pearson & Tubbs, 1967). A similar range to that of the rat tissues is observed in the rabbit and a fivefold concentration difference between the liver and skeletal muscle was found in the dog (Fraenkel & Friedman, 1957). The total acid-soluble carnitine concentration of sheep skeletal muscle is about 20 times greater than that of rat skeletal muscle (Pearson & Tubbs, 1967). This difference is not due to the use of different assay procedures. We find the total acid-soluble carnitine content of rat liver and skeletal muscle is 232 and 634nmol/g wet wt. respectively (mean values), which is comparable with the figures of 296 and 627nmol/g wet wt. respectively (mean values) reported by Pearson & Tubbs (1967).

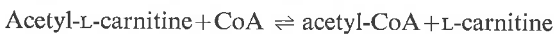
The acid-soluble CoA concentration is greatest in sheep liver and least in sheep skeletal muscle (of the tissues examined); this is in contrast with the variation observed for total acid-soluble carnitine concentrations. Thus in these sheep tissues there appears to be a reciprocal relationship between the concentration of total acid-soluble carnitine and of acid-soluble CoA. A similar gradation in CoA content of these four tissues has been observed in other species (Glock, 1961), but no obvious relationship between carnitine and CoA concentrations can yet be inferred for any other species. This reciprocal relationship between the concentration of total acid-soluble carnitine and acid-soluble CoA may be observed in a single sheep tissue: the skeletal muscle of lamb has only 40% of the total acid-soluble carnitine concentration of that of the adult sheep but 2.5 times the concentration of acid-soluble CoA (Table 2). This inverse relationship between the concentration of acid-soluble CoA and total acid-soluble carnitine was also seen when various muscles were compared and would suggest that the synthesis and/or degradation of carnitine and CoA in these tissues must be carefully integrated. This inverse relationship also emphasizes the role of carnitine acetyltransferase, particularly in muscle tissues of the sheep. The enzyme is localized in the mitochondria of all the four tissues examined and most of the activity is latent, i.e. only released after exposure of the mitochondria to hypo-osmotic solutions plus detergent. A similar situation occurs with the carnitine acetyltransferases of rat, guinea-pig, goat and sheep liver mitochondria (Barker *et al.*, 1968) and it was concluded that the enzyme is mainly membrane-bound and is not available to acetyl-CoA outside the mitochondria.

The high concentrations of carnitine in the sheep

muscle tissues examined suggest that the carnitine in these tissues, in conjunction with carnitine acetyltransferase, might be involved in acetate oxidation, since acetate is metabolized mainly in the extrahepatic tissues of the sheep (Mayfield *et al.*, 1966). However, the acetate thiokinase of sheep heart and skeletal muscle is predominantly present in the mitochondrial matrix, and the activity of this enzyme in sheep skeletal-muscle mitochondria is low (Cook *et al.*, 1969). Thus, it seems unlikely that carnitine acetyltransferase plays a significant role in acetate oxidation by the heart and skeletal muscle of sheep.

It would seem much more likely that the role of carnitine acetyltransferase and carnitine is in a buffer system, as suggested by Pearson & Tubbs (1967) after their studies with perfused rat hearts. In sheep muscle tissues the main reactions generating acetyl-CoA are localized in the mitochondria, i.e. the fatty acid oxidation system and the acetate thiokinase reaction. Also, the present studies show that CoA is virtually confined to the mitochondria and carnitine to the cytoplasm. Yates & Garland (1966) have shown, by using rat liver mitochondria, that the inner mitochondrial membrane is impermeable to carnitine. Thus the carnitine acetyltransferase located in the inner mitochondrial membrane would appear to act as a vectorial transferase that reacts with CoA and acetyl-CoA on the inside and carnitine and acetylcarnitine on the outside. A similar role in a vectorial transfer system has been suggested by Yates & Garland (1970) for the membrane-bound carnitine palmitoyltranferase. The net effect would be to remove acetyl groups from the mitochondria in times of increased 'acetyl pressure', i.e. during increased fatty acid oxidation, and transfer these out of the mitochondria to be 'stored' as acetylcarnitine, thereby relieving pressure on the CoA system. The amount of acetyl groups 'stored' in such a way is considerable, being about 6g in a 50kg sheep, if the total muscle mass is taken into account. In support of this concept the results presented here for alloxan-diabetic sheep indicate a marked increase in the acetylcarnitine concentration of skeletal muscle, but relatively little change in the ratio [acetyl-CoA]/[free CoA] compared with that of the normal animal.

For enzyme to function in a buffer system *in vivo* the reactants and products should be near or at equilibrium. Fritz *et al.* (1963) have calculated the apparent equilibrium constant for the reaction:



$$\text{Acetyl-L-carnitine} + \text{CoA} \rightleftharpoons \text{acetyl-CoA} + \text{L-carnitine}$$

to be 0.6 at pH7.0 by using a partially purified enzyme from pig heart. From the results in Table 1, $K_{app.}$ = [acetyl-CoA][L-carnitine]/[acetyl-L-carnitine]-[CoA]=1.9 for sheep heart and 4.2 for skeletal muscle. In alloxan-diabetes (Table 6) the values are 1.3 and 2.1 respectively. Thus the reaction in these sheep muscles *in vivo* appears to be near equilibrium, assuming that the carnitine acetyltransferase in these tissues is accessible to intramitochondrial CoA and acetyl-CoA and to cytoplasmic carnitine and acetylcarnitine. However, the situation in sheep liver and kidney cortex is considerably more complex, as in these tissues CoA is present both inside and outside the mitochondria.

The approximately sevenfold rise in total acid-soluble carnitine content of sheep liver in alloxan-diabetes is striking. Marked increases in the individual fractions of the total acid-soluble carnitine, i.e. free carnitine and acetylcarnitine also occur (compare Table 6 with Table 1). A significant but less pronounced (twofold) rise in acid-soluble CoA also occurs in sheep liver in alloxan-diabetes, in contrast with the rat, where there is little change (Tubbs & Garland, 1964). The [free CoA]/[acetyl-CoA] ratio in normal sheep liver is approx. 1:1 (Table 1), which is considerably lower than the ratio of 3–4:1 in rat liver (Allred & Guy, 1969; Bode *et al.*, 1970). This ratio remains at approx. 1:1 in the liver of alloxan-diabetic sheep (Table 3); thus, it seems that the CoA system in sheep liver has only a limited capacity to accommodate the increased 'acetyl pressure' presumably arising in alloxan-diabetes because of the increased fatty acid oxidation. This limitation in the CoA system of the liver appears to be compensated by the marked rise in carnitine concentration that occurs during alloxan-diabetes in sheep.

The other feature of the results obtained with alloxan-diabetic sheep is that the acid-insoluble carnitine fraction constituted only a very minor proportion of the total carnitine in any tissue examined. There was a significant increase in this fraction in the heart in alloxan-diabetic sheep, but even in this tissue it was only 3% of the total carnitine. This contrasts with the situation in the rat, where the amount of acid-insoluble carnitine fraction increases markedly in alloxan-diabetes and is a very significant proportion of the total carnitine (Bøhmer *et al.*, 1966; Pearson & Tubbs, 1967).

The very large amounts of carnitine in the skeletal muscle of sheep (over 2mg/g wet wt.) indicate an important quantitative role for carnitine in the overall metabolism of the sheep, as the muscles are the largest tissue mass of an animal. Fraenkel & Friedman (1957) reported very high concentrations of carnitine in commercial beef extracts. A high carnitine output in the milk of dairy cows, and particularly of acetylcarnitine in ketotic cows, has been observed (Erfle *et al.*, 1970). Thus it would appear that carnitine may have a particularly important role in the metabolism of ruminant animals, especially under conditions of metabolic stress.

We are indebted to Dr. I. G. Jarrett for providing the alloxan-diabetic sheep stabilized on insulin and for the surgical removal of liver samples. A. M. S. is indebted to

Professor P. J. Randle for providing laboratory facilities and encouragement during part of this work. Mr. R. Fishlock is thanked for skilled technical assistance. Grants from the Australian Meat Research Committee and the Australian Research Grants Committee are gratefully acknowledged. P. P. K. holds a Commonwealth Scholarship and Fellowship Plan Australian award.

References

Allred, J. B. & Guy, G. D. (1969) *Anal. Biochem.* **29**, 293
Amberson, W. R., Bauer, A. C., Philpott, D. E. & Rosen, F. (1964) *J. Cell. Comp. Physiol.* **63**, 7
Barker, P. J., Fincham, N. J. & Hardwick, D. C. (1968) *Biochem. J.* **110**, 739
Bode, Ch., Stähler, E., Kono, H. & Goebell, H. (1970) *Biochim. Biophys. Acta* **210**, 448
Bøhmer, T., Norum, K. R. & Bremer, J. (1966) *Biochim. Biophys. Acta* **125**, 244
Chappell, J. B. & Perry, S. V. (1954) *Nature (London)* **173**, 1094
Cook, R. M., Liu, S. C. & Quraishi, S. (1969) *Biochemistry* **8**, 2966
De Jong, J. W. & Hülsmann, W. C. (1970) *Biochim. Biophys. Acta* **210**, 499
Denton, R. M., Coore, H. G., Martin, B. R. & Randle, P. J. (1971) *Nature (London)* **231**, 115
Erfle, J. D., Fisher, L. J. & Sauer, F. (1970) *J. Dairy Sci.* **53**, 486
Fraenkel, G. & Friedman, S. (1957) *Vitam. Horm. (New York)* **15**, 73
Fritz, I. B., Schultz, S. K. & Srere, P. A. (1963) *J. Biol. Chem.* **238**, 2509
Glock, G. E. (1961) in *Biochemists' Handbook* (Long, C., ed.), p. 790, Spon, London
Jacobson, K. B. (1961) *J. Biol. Chem.* **236**, 343
Marquis, N. R. & Fritz, I. B. (1964) *J. Lipid Res.* **5**, 184
Mayfield, E. D., Bensadoun, A. & Johnson, B. C. (1966) *J. Nutr.* **89**, 189
Mehlman, M. A., Kader, M. M. A. & Therriault, D. G. (1969) *Life Sci.* **8**, 465
Michal, G. & Bergmeyer, H.-U. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), p. 512, Academic Press, New York and London
Pearson, D. J. & Tubbs, P. K. (1964) *Nature (London)* **202**, 91
Pearson, D. J. & Tubbs, P. K. (1967) *Biochem. J.* **105**, 953
Pearson, D. J., Chase, J. F. A. & Tubbs, P. K. (1969) *Methods Enzymol.* **14**, 612
Shepherd, D. & Garland, P. B. (1969) *Biochem. J.* **114**, 597
Skrede, S. & Bremer, J. (1970) *Eur. J. Biochem.* **14**, 465
Snoswell, A. M. & Henderson, G. D. (1970) *Biochem. J.* **119**, 59
Stadtman, E. R. (1957) *Methods Enzymol.* **3**, 931
Tabor, H., Mehler, A. H. & Stadtman, E. R. (1953) *J. Biol. Chem.* **204**, 127
Taylor, P. H., Wallace, J. C. & Keech, D. B. (1971) *Biochim. Biophys. Acta* **237**, 179
Tubbs, P. K. & Garland, P. B. (1964) *Biochem. J.* **93**, 550
Tubbs, P. K. & Garland, P. B. (1968) *Brit. Med. Bull.* **24**, 158
Walter, P. & Anabitarte, M. (1971) *FEBS Lett.* **12**, 289
Wollenberger, A., Ristau, O. & Schoffa, G. (1960) *Pflügers Arch. Gesamte Physiol.* **270**, 399
Yates, D. W. & Garland, P. B. (1966) *Biochem. Biophys. Res. Commun.* **23**, 460
Yates, D. W. & Garland, P. B. (1970) *Biochem. J.* **119**, 547

Biochem. J. (1972) **127**, 449–452
Printed in Great Britain

3-Hydroxy Acid Dehydrogenases in Sheep Tissues

By Patricia P. Koundakjian and A. M. Snoswell
Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S. Austral. 5064, Australia

(*Received* 2 *February* 1972)

Williamson & Kuenzel (1971) have shown that the 'soluble' 3-hydroxybutyrate dehydrogenase activity of sheep kidney cortex described by Koundakjian & Snoswell (1970) is, in fact, that of an L-3-hydroxy acid dehydrogenase (probably L-gulonate–NAD^+ oxidoreductase, EC 1.1.1.45). Smiley & Ashwell (1961) have shown that this enzyme reacts with a large number of 3-hydroxy acids provided that the 3-hydroxyl group is in the L-configuration. It does not react with D-3-hydroxy acids (Smiley & Ashwell, 1961). Koundakjian & Snoswell (1970) had used DL-3-hydroxybutyrate as substrate in the original assay of the dehydrogenase and implied that the activity found was that of D(−)-3-hydroxybutyrate–NAD^+ oxidoreductase (EC 1.1.1.30). In order to ascertain the contribution of L-3-hydroxy acid dehydrogenase to the overall 3-hydroxybutyrate dehydrogenase activity of sheep tissues we have examined the activity of dehydrogenases of L- and D-3-hydroxybutyrate in various tissues of the sheep. The results obtained are discussed in relation to ketone-body metabolism in sheep.

Experimental

The sheep used were 3-year-old Merino wethers, weighing about 40kg and pen-fed on lucerne hay.

Samples of liver, kidney cortex, heart, skeletal muscle and rumen epithelium were collected, and homogenates, subcellular fractions and sonicated fractions were prepared as described previously (Koundakjian & Snoswell, 1970), except that the medium used was 0.25M-sucrose in 23mM-potassium phosphate buffer, pH7.2. Phosphate-buffered sucrose was used because recovery of glutamate dehydrogenase (EC 1.4.1.3) activity is impaired in the absence of phosphate (Walter & Anabitarte, 1971).

3-Hydroxy acid dehydrogenase activities were measured as described for 3-hydroxybutyrate dehydrogenase (Koundakjian & Snoswell, 1970) with either 22mM-sodium DL-3-hydroxybutyrate or 11mM-sodium L-3-hydroxybutyrate as substrate. Enzyme activity was also measured in the reverse direction, the reaction mixture consisting of (final concentrations) 33mM-tris–HCl buffer, pH7.0, 0.2mM-NADH, 50mM-nicotinamide, 1mM-$CaCl_2$ and 10mM-sodium acetoacetate in a final volume of 1.0ml. All activities recorded were corrected for blank values.

Glutamate dehydrogenase was assayed by the method of Barker *et al.* (1968), but 1.5mM-ADP was used to ensure maximal activity, 10μM-rotenone replaced 3mM-KCN and phosphate was used instead of tris–HCl buffer.

Further assessment of the activities of 3-hydroxy acid dehydrogenases was made by incubating tissue fractions with substrates and measuring the consequent changes in the concentrations of D-3-hydroxybutyrate and acetoacetate. The assay systems used were (final concentrations): for acetoacetate reduction (final vol. 3ml), 33mM-tris–HCl buffer, pH7.0, 0.2mM-NADH, 67mM-nicotinamide, 1mM-$CaCl_2$, 10μM-rotenone and 3.28mM-sodium acetoacetate; for 3-hydroxybutyrate oxidation (final vol. 3ml), 33mM-tris–HCl buffer, pH8.5, 1.8mM-NAD^+, 67mM-nicotinamide, 1mM-$CaCl_2$, 20mM-dithiothreitol, 1mM-KCN, 2.25mg of asolectin (see the Materials and Methods section of Koundakjian & Snoswell, 1970) and either 22mM-sodium DL-3-hydroxybutyrate or 11mM-sodium L-3-hydroxybutyrate. A 0.2ml portion of tissue fraction was used in each assay and the reaction was started by the addition of substrate. After 1h at 20°C, 0.3ml of 30% (w/v) $HClO_4$ was added to stop the reaction. The protein-free solution was neutralized, and acetoacetate and D-3-hydroxybutyrate were measured by the enzymic method of Williamson *et al.* (1962). All values presented were corrected for zero-time ketone-body concentrations.

The L-isomer of 3-hydroxybutyric acid was prepared from the DL-acid by the method of McCann & Greville (1962). This preparation of L-isomer contained only 1.2% of D-isomer. We were unable to obtain a sufficiently pure sample of D-isomer either with this method (McCann & Greville, 1962) or with several others tried.

Results and discussion

The results presented in Table 1 show that for all tissues recorded here the glutamate dehydrogenase activity was associated almost exclusively with the particulate fractions. Since glutamate dehydrogenase is used as a marker for mitochondria, the cytosol fractions prepared were considered to be free of significant contamination with mitochondrial enzymes. Values for the glutamate dehydrogenase

Table 1. *Distribution of glutamate dehydrogenase and 3-hydroxy acid dehydrogenase activities in various tissues of the sheep*

Tissue fractions were prepared and assayed as described in the text. Results are expressed in μmol/h per g of tissue, and are given as mean values±S.E.M. for three (glutamate dehydrogenase), five (3-hydroxy acid dehydrogenase with acetoacetate) and seven (3-hydroxy acid dehydrogenase with 3-hydroxybutyrate) observations.

	Glutamate dehydrogenase activity	3-Hydroxy acid dehydrogenase activity		
Substrate ...		Acetoacetate	L-3-Hydroxybutyrate	DL-3-Hydroxybutyrate
Liver				
Homogenate	2450 ±181	26.9± 7.3	9.6 ± 7.7	17.3 ± 4.7
Mitochondria	1990 ± 48	4.5± 1.6	0.16± 0.11	0.66± 0.47
Cytosol	272 ± 78	19.1± 7.4	7.3 ± 5.3	9.8 ± 3.7
Kidney cortex				
Homogenate	634 ± 9	116 ± 9	126 ±23	160 ±19
Mitochondria	555 ± 42	14 ± 2.1	2.24± 1.14	6.9 ± 2.9
Cytosol	69 ± 13	118 ± 5.9	110 ±14	122 ±14
Heart				
Homogenate	128 ± 6	25.6± 6.1	9.3 ± 5.7	13.9 ± 7
Cytosol	22 ± 7	11.1± 4.4	5.5 ± 3.6	4.5 ± 2.9
Rumen epithelium				
Homogenate	38 ± 8	65.1±12	9.5 ± 5.3	15.5 ± 5.8
Cytosol	8.7± 2.2	10.3± 4.8	4 ± 2.1	7.8 ± 3.7

activity of skeletal-muscle fractions indicated extensive mitochondrial breakdown (see Snoswell & Koundakjian, 1972), so results obtained for this tissue have been omitted.

The dehydrogenase activities detected with DL-3-hydroxybutyrate as substrate (Table 1) were similar to those reported by Koundakjian & Snoswell (1970) except that those for rumen epithelium were somewhat lower. However, this tissue is extremely difficult to homogenize and fractionate, and any enzyme-activity results obtained in this manner for rumen epithelium are likely to be greatly underestimated. A major proportion of the dehydrogenase activity with DL-3-hydroxybutyrate was cytoplasmic in liver and kidney cortex, and some cytoplasmic activity was detected in heart and rumen epithelium (Table 1). When the L-isomer was used as substrate the cytoplasmic activities observed were similar to those with the DL-salt, whereas the activities of the particulate fractions were lower. Thus it would appear that the cytoplasmic activity of all the tissues examined is attributable to L-3-hydroxy acid dehydrogenase, and that the D(−)-3-hydroxybutyrate–NAD^+ oxidoreductase activity is mitochondrial. The results shown for assays with acetoacetate as substrate indicate that measuring the activity in this direction generally gives higher apparent activities for both the D- and the L-3-hydroxybutyrate dehydrogenase, particularly for rumen epithelium.

The results of assay of reactant formation and removal when tissue fractions were incubated with the different substrates are shown in Table 2. With all the tissues examined D-3-hydroxybutyrate was formed by the particulate fractions from acetoacetate, but, with the exceptions of kidney-cortex mitochondria and liver homogenate and mitochondria, the amount formed was considerably less than the amount of acetoacetate removed. With kidney-cortex mitochondria the amount of substrate removed was roughly equal to that of D-3-hydroxybutyrate produced; and with liver homogenate and mitochondria there was an increase in the concentration of acetoacetate during the incubation period owing to concomitant oxidation of endogenous fatty acids.

When L-3-hydroxybutyrate was used as substrate (Table 2) there was very little change in D-3-hydroxybutyrate concentrations compared with the endogenous values. There was significant acetoacetate production by all tissue fractions, particularly kidney-cortex homogenate and cytosol. However, the production of acetoacetate by the liver homogenate and mitochondrial fractions in the presence of L-3-hydroxybutyrate (and also the DL-salt) was surprisingly low. It is probable that L-3-hydroxybutyrate inhibits endogenous acetoacetate formation.

The use of DL-3-hydroxybutyrate as substrate resulted in the formation of more acetoacetate by the particulate fractions than when the L-isomer was

Table 2. *Oxidation of* D- *and* L-*3-hydroxybutyrate and reduction of acetoacetate by fractions prepared from various sheep tissues*

Tissue fractions were prepared, incubated and assayed as described in the text. The results are in μmol and were corrected for zero-time ketone-body concentrations. They are the mean values for five to seven animals; S.E.M. values ranged from 1.5 to 40% of the mean values.

Substrate	Reactant determined	Liver			Kidney cortex			Heart		Rumen epithelium	
		Homogenate	Mitochondria	Cytosol	Homogenate	Mitochondria	Cytosol	Homogenate	Cytosol	Homogenate	Cytosol
Acetoacetate	D-3-Hydroxybutyrate	+30	+24	0	+117	+400	0	+95	+15	+316	+7
L-3-Hydroxybutyrate	D-3-Hydroxybutyrate	+2	+4	+1	+1	+4	+1	+1	+2	+2	+1
DL-3-Hydroxybutyrate	D-3-Hydroxybutyrate	0	−28	0	−1504	−962	0	−177	0	−783	0
Acetoacetate	Acetoacetate	+480	+460	−575	−450	−465	−860	−520	−450	−855	−250
L-3-Hydroxybutyrate	Acetoacetate	+40	+23	+58	+155	+62	+146	+46	+33	+17	+8
DL-3-Hydroxybutyrate	Acetoacetate	+44	+42	+87	+191	+127	+142	+90	+43	+117	+9

used, but the amounts produced by the cytosol fractions were virtually the same (Table 2). The amount by which the D-3-hydroxybutyrate concentration was decreased during incubation with the particulate fractions was much greater than could be accounted for by acetoacetate production, especially when the fact that acetoacetate can also be formed by the homogenate fractions from the L-isomer is taken into account. There was no evidence of breakdown of 3-hydroxybutyrate during the incubation period. It seems likely that some of the formed acetoacetate was further metabolized, and that under the conditions used the L-3-hydroxy acid dehydrogenase in particular could be using the acetoacetate, thus leading to low estimates of the amount formed. The tissues showing the greatest rate of removal of D-3-hydroxybutyrate were kidney cortex and rumen epithelium (Table 2). This is in accord with the facts that the kidney cortex has been shown by Koundakjian & Snoswell (1970) to have the highest D-3-hydroxybutyrate–NAD^+ oxidoreductase activity of sheep tissues and that since rumen epithelium produces D(−)-3-hydroxybutyrate from butyrate in the fed animal (Annison & Lewis, 1959) it is likely to have much higher D(−)-3-hydroxybutyrate–NAD^+ oxidoreductase activity than is readily demonstrable.

The results of these tissue-incubation experiments therefore further substantiate the conclusions drawn from the results of direct measurement of the apparent tissue enzyme activities: the D(−)-3-hydroxybutyrate–NAD^+ oxidoreductase activity of all the sheep tissues examined is associated with the mitochondria, and is very low in liver and kidney cortex, and there is cytoplasmic activity of L-3-hydroxy acid dehydrogenase, which is in some tissues (liver and kidney cortex) much greater than the activity of the D-3-hydroxybutyrate dehydrogenase. It must be emphasized that values given for both the D- and the L-hydroxybutyrate dehydrogenase activities may not be absolute values, but are comparative values between the various tissues for the two enzymes since only one aliquot size of the fractions was used in each case, and the assay conditions for the L-acid dehydrogenase may not have been optimal for this enzyme.

Although there is comparatively high activity of L-3-hydroxy acid dehydrogenase in the liver and kidney cortex of the sheep, both organs being particularly involved in ketone-body metabolism, it is not likely that this enzyme has any important role in the metabolism of ketone bodies by sheep. The high NAD^+/NADH concentration ratio of the cytosol of sheep liver and kidney cortex (A. M. Snoswell, unpublished work) is unfavourable for acetoacetate reduction; and also Williamson & Kuenzel (1971) have shown that the K_m value of the kidney-cortex enzyme for acetoacetate is high.

Mr. R. Fishlock is thanked for skilful technical assistance. A grant was provided by the Australian Research Grants Committee, and P. P. K. holds a Commonwealth Scholarship and Fellowship Plan Australian award.

Annison, E. F. & Lewis, D. (1959) *Metabolism in the Rumen*, p. 70, Methuen and Co. Ltd., London

Barker, P. J., Fincham, N. J. & Hardwick, D. C. (1968) *Biochem. J.* **110**, 739

Koundakjian, P. P. & Snoswell, A. M. (1970) *Biochem. J.* **119**, 49

McCann, W. P. & Greville, G. D. (1962) *Biochem. Prep.* **9**, 63

Smiley, J. D. & Ashwell, G. (1961) *J. Biol. Chem.* **236**, 357

Snoswell, A. M. & Koundakjian, P. P. (1972) *Biochem. J.* **127**, 133

Walter, P. & Anabitarte, M. (1971) *FEBS Lett.* **12**, 289

Williamson, D. H. & Kuenzel, P. (1971) *Biochem. J.* **121**, 569

Williamson, D. H., Mellanby, J. & Krebs, H. A. (1962) *Biochem. J.* **82**, 90

Eur. J. Biochem. *29*, 104–109 (1972)

Effects of a Glucocorticoid on the Concentrations of CoA and Carnitine Esters and on Redox State in Bovine Liver

G. David BAIRD, Raymond J. HEITZMAN, and Alan M. SNOSWELL

Department of Biochemistry, Agricultural Research Council, Institute for Research on Animal Diseases, Compton,
and Department of Biochemistry, University of Bristol

(Received March 23/May 23, 1972)

1. In this study, the hepatic concentrations of free and acetylated CoA and carnitine, and also hepatic redox state, have been determined in normal lactating cows and in lactating cows to which voren (dexamethasone 21-pyridine-4-carboxylate) was administered 48 h previously.

2. The values for the hepatic [CoA]/[acetyl-CoA] and [carnitine]/[acetylcarnitine] ratios in the normal cow were found to be lower than the corresponding values previously reported for other species. Voren administration brought about an increase in both these ratios. Only the increase for the [CoA]/[acetyl-CoA] ratio was statistically significant, however.

3. Using the lactate dehydrogenase and glutamate dehydrogenase systems, respectively, values were obtained for the cytosolic and mitochondrial $[NAD^+]_{free}/[NADH]_{free}$ ratios in normal bovine liver that were similar to the corresponding values previously obtained for rat liver. Following voren administration there was a fall in the cytosolic value for this ratio and a rise in the mitochondrial value, so that the equilibrium between the cytosolic and mitochondrial redox states appeared to be disrupted.

4. Following voren administration there was an increase in the degree of reduction of total NAD.

5. The results are discussed in relation to the antiketogenic action of voren in the cow.

Following administration of the glucocorticoid voren (dexamethasone 21-pyridine-4-carboxylate) to ketotic cows, there ensues a decline in blood ketone-body concentrations that is not initially associated with any decline in blood free-fatty-acid concentrations [1]. This observation suggests that voren might have a direct antiketogenic action within the liver. One possible mechanism by which voren could exert this action would be by increasing the supply of oxaloacetate and its precursors [2,3]. The fact that voren increases the steady-state concentrations of various intermediates of the citric acid cycle in both healthy and ketotic cows suggests that this mechanism may indeed be in operation [1,4].

Besides regulation of the supply of oxaloacetate and its precursors, several other mechanisms have been postulated for the control of hepatic ketogenesis. Included among these are regulation of the ratio of the concentrations of free CoA and acetyl-CoA and regulation of the redox ratio of the whole cell and of the subcellular compartments [3,5].

The work described in this communication consists of a study of the effect of voren on both these parameters in healthy bovine liver. This has involved measuring relevant metabolite concentrations in liver tissue obtained from healthy untreated lactating cows and from healthy voren-treated lactating cows. In conjunction with the determination of the [CoA]/[acetyl-CoA] ratio it seemed also of interest to determine the concentration of free carnitine and acetylcarnitine in view of the possible role of carnitine in relieving "acetyl pressure" on the CoA system in sheep liver under stress conditions, *e.g.* starvation [6]. With regard to redox control, attempts have been made to determine the redox state of free NAD in the mitochondrial and cytoplasmic compartments, and of total NAD.

Enzymes. Carnitine acetyltransferase (EC 2.3.1.7); glutamate dehydrogenase (EC 1.4.1.3); lactate dehydrogenase (EC 1.1.1.27).

MATERIALS AND METHODS

Materials

Substrates and enzymes for metabolite determinations were obtained either from Boehringer Corp. (London) Ltd or from Sigma (London) Chemical Co. Ltd. Other chemicals were of analytical grade. Double distilled water, both distillations being from glass, was used throughout. Voren (dexamethasone 21-pyridine-4-carboxylate), manufactured by Cela

GmbH (Ingelheim/Rhein, W. Germany) was obtained from Abbott Laboratories Ltd (Agro-Vet Division, Queenborough, Kent, U.K.). The compound is supplied as an 0.1% w/v suspension in isotonic aqueous solution.

Animals

Ayrshire cross Friesian lactating dairy cows were used. The animals had all been calved for less than 12 weeks and had all been through at least one previous lactation. They received a standard dairy concentrate ration, containing 15.3% protein, 58.5% nitrogen-free extract and 3.5% fibre, together with hay of medium quality and silage containing a high proportion of dry matter.

Experimental Procedure

In this communication hepatic steady-state metabolite concentrations in untreated lactating cows are compared with those in voren-treated lactating cows. All the values for metabolic concentrations in the voren-treated group, except those for total NAD, were obtained from the same four animals. In the case of the untreated group, the values for lactate, pyruvate, oxoglutarate and glutamate were those obtained previously [4]. The values for the other metabolites were obtained from further untreated animals during the course of this study. Voren-treated cows each received 10 mg voren, corresponding to an approximate dosage rate of 1 mg/50 kg, intramuscularly 48 h before liver biopsy (*cf.* [4]). In accordance with observations made in the previous study [4], voren administration had no noticeable effect on appetite.

Liver Biopsy

Liver tissue was obtained by biopsy, freeze-clamped and extracted for metabolite assays as described previously [4]. The extracts used for the determination of free and acetylated CoA and carnitine were not neutralised until immediately before the assays took place.

Steady-State Metabolite Concentrations

CoA and acetyl-CoA were assayed by the method of Allred and Guy [7], acetylcarnitine by the method of Pearson and Tubbs [8] and free carnitine by the method of Marquis and Fritz [9]. These assays were modified in minor ways as described by Snoswell and Henderson [6]. Lactate, pyruvate and 2-oxoglutarate were assayed as described previously [10]. Glutamate was assayed by the method of Bernt and Bergmeyer [11]. Ammonium ion concentration was determined by the method of Kirsten, Gerez and Kirsten [12] after preliminary adsorption on Amberlite IR-120 resin, followed by elution with 4 M NaCl, as described by Williamson, Lund and Krebs [13]. Total NAD^+ and NADH were assayed as described previously [1].

Lactate Dehydrogenase

Lactate dehydrogenase was assayed by the method of Bergmeyer, Bernt and Hess [14] in subcellular fractions prepared from the livers of untreated and voren-treated cows by the procedure used by Heitzman, Herriman and Mallinson [15].

Statistics

The probability values (P) were obtained by Student's t test.

RESULTS

Hepatic Content of Free and Acetylated CoA and Carnitine

The concentrations of free CoA, acetyl-CoA, free carnitine and acetylcarnitine in the livers of untreated and voren-treated cows are listed in Table 1. It can be seen that voren administration brought about a statistically significant increase in the $[CoA]_{free}/[acetyl\text{-}CoA]$ ratio as compared with the untreated group. This increase was achieved largely by a statistically significant decrease in the concentration of acetyl-CoA. Voren administration also caused a slight but nonsignificant increase in the concentration of free CoA. In parallel with the increase in the $[CoA]_{free}/[acetyl\text{-}CoA]$ ratio, voren administration also appeared to bring about an

Table 1. *Hepatic content of free CoA, acetyl-CoA, free carnitine and acetylcarnitine in untreated and voren-treated lactating cows*
The values are means ± S.D. with the numbers of observations in parentheses. The liver was obtained by biopsy. In the case of the voren-treated group this was at 48 h after voren administration

Cows	Free CoA	Acetyl-CoA	$[CoA]_{free}$ / [Acetyl-CoA]	Free carnitine	Acetylcarnitine	$[Carnitine]_{free}$ / [Acetylcarnitine]
	nmol/g wet weight			nmol/g wet weight		
Untreated	11.9 ± 1.7 (3)	60.3 ± 12.8 (3)	0.20 ± 0.04 (3)	6.7 ± 2.4 (3)	17.5 ± 4.3 (3)	0.43 ± 0.24 (3)
Voren-treated	14.5 ± 1.8 (4)	42.2 ± 5.7 (4)[a]	0.34 ± 0.08 (4)[a]	16.3 ± 6.7 (4)	20.9 ± 10.6 (4)	0.87 ± 0.43 (4)

[a] $P < 0.05$ compared with the untreated group.

Table 2. *Hepatic-metabolite concentrations and cytoplasmic and mitochondrial redox states in untreated and voren-treated lactating cows*
The values are means ± S.D. with the numbers of observations in parentheses. The liver was obtained by biopsy. In the case of the voren-treated group this was at 48 h after voren administration. c = cytoplasmic, m = mitochondrial

Cows	Lactate	Pyruvate	Glutamate	Oxoglutarate	NH_4^+	$\frac{[NAD^+]_{free}}{[NADH]_{free}}$ Cytoplasmic	$\frac{[NAD^+]_{free}}{[NADH]_{free}}$ Mitochondrial	$\frac{[NAD^+]_c/[NADH]_c}{[NAD^+]_m/[NADH]_m}$
	µmol/g wet weight							
Untreated	0.51 ± 0.14 (6) [a]	0.038 ± 0.006 (6) [a]	3.34 ± 1.19 (5) [a]	0.099 ± 0.044 (5) [a]	0.57 ± 0.02 (4)	671	4.4	153
Voren-treated	0.60 ± 0.22 (4)	0.036 ± 0.004 (4)	4.80 ± 0.77 (4)	0.446 ± 0.174 (4)	0.56 ± 0.08 (4)	541	13.5	40

[a] Values from [4].

increase in the $[carnitine]_{free}/[acetylcarnitine]$ ratio, although in this case the change was not significant. The increase in the $[carnitine]_{free}/[acetylcarnitine]$ ratio was in turn due to an apparent increase in the concentration of free carnitine. Statistical significance was not achieved for this increase either, due to the large variance in the results.

Hepatic Redox States

In this study, the [oxoglutarate] $[NH_4^+]$/[glutamate] ratio was used to calculate the $[NAD^+]_{free}/[NADH]_{free}$ ratio for the mitochondrial matrix [13,16]. The justification for this procedure in rat liver is that free NAD in the mitochondrial matrix is considered to be in equilibrium with the glutamate dehydrogenase system according to the mass action equation. For application to other tissues it must be shown that glutamate dehydrogenase is confined to the mitochondrial matrix in the tissue in question and is present at a sufficiently high activity to establish equilibrium between reagents and products. That these criteria are satisfied in liver from both untreated and voren-treated lactating cows has recently been shown [15]. The concentrations of the components of the glutamate dehydrogenase equilibrium, other than NAD, *i.e.* oxoglutarate, glutamate and NH_4^+, in livers from untreated and voren-treated cows are listed in Table 2. It can be seen from the table that, using these values, the mitochondrial $[NAD^+]/[NADH]$ ratio was calculated to be 4.4 for the untreated group and 13.5 for the voren-treated group. For these calculations the equilibrium constant for glutamate dehydrogenase was taken to be 3.87 µM [13].

For comparison, the redox state of the cytoplasmic compartment was also calculated in the two groups of cows. In this case the [pyruvate]/[lactate] ratio was used to calculate the cytosolic $[NAD^+]_{free}/[NADH]_{free}$ ratio and the assumption is made that the cytoplasmic NAD is in equilibrium with the lactate dehydrogenase system according to the mass action equation [13]. Again lactate dehydrogenase must be present in the cytosolic compartment and must be of sufficient activity to achieve equilibrium. That this is the case in bovine liver is indicated by the data in Table 3. As Table 3 shows, there is substantial lactate dehydrogenase activity in liver from both untreated and voren-treated cows, and at least 95% of the activity is located within the cytoplasmic fraction. The hepatic concentrations of lactate and pyruvate in the two groups of cows are also shown in Table 2, where it can be seen that, using these values and assuming the equilibrium constant of the lactate dehydrogenase reaction to be 1.11×10^{-4} [13], the cytosolic $[NAD^+]/[NADH]$ ratio was calculated to be 671 for the untreated animals and 541 for the voren-treated animals.

Table 3. *Activity of lactate dehydrogenase in cytoplasmic and particulate fractions from the livers of untreated and voren-treated lactating cows*

The values are means ± S.D. with the numbers of observations in parentheses. The liver was obtained by biopsy. In the case of the voren-treated group this was at 48 h after voren administration. Activity is expressed as NAD formed at 25 °C in μmol/min per g wet wt.

Cows	Activity		Distribution	
	Cytoplasmic fraction	Particulate fraction	Cytoplasmic fraction	Particulate fraction
	$\mu mol \times min^{-1} \times g^{-1}$		%	
Untreated	109 ± 25 (5)	5.1 ± 1.3 (5)	95.5 ± 0.9 (5)	4.5 ± 0.9 (5)
Voren-treated	105 ± 44 (3)	5.3 ± 1.8 (3)	95.0 ± 0.5 (3)	5.0 ± 0.5 (3)

Table 4. *Hepatic concentrations of total NAD^+ and total NADH in untreated and voren-treated lactating cows*

The values are means ± S.D. with the numbers of observations in parentheses. The liver was obtained by biopsy. In the case of the voren-treated group this was at 48 h after voren administration

Cows	NAD^+	NADH	$[NAD^+]/[NADH]$
	μmol/g wet weight		
Untreated	0.776 ± 0.077 (7)	0.129 ± 0.020 (4)	6.0
Voren-treated	0.609 ± 0.077 (6)[a]	0.205 ± 0.029 (5)[a]	3.0

[a] $P < 0.01$ compared with the untreated group.

Finally, Table 2 also records the values for the hepatic ratio of cytosolic $[NAD^+]/[NADH]$ to mitochondrial $[NAD^+]/[NADH]$ in the two groups. The value of this ratio is found to be about 150 to 200 in most physiological states when the glutamate dehydrogenase equilibrium is used to calculate the redox state of mitochondrial NAD in rat liver [16]. A value within this range, *i.e.* 153, was found for this ratio in the liver of untreated cows. However, following voren treatment the value of the ratio decreased to only 40 (Table 2).

The concentrations of total NAD^+ and total NADH were also determined in the livers of untreated and voren-treated cows. As can be seen from Table 4, the effect of voren was to cause a decrease in the $[NAD^+]_{total}/[NADH]_{total}$ ratio from 6.0 to 3.0.

DISCUSSION

Hepatic Concentrations of Free and Acetylated CoA and Carnitine and the Effect of Voren

The value of 0.2 for the $[CoA]_{free}/[acetyl\text{-}CoA]$ ratio in normal bovine liver is lower than the corresponding values found in the livers of other species so far examined. For example, in the sheep this value varies between about 0.4 for animals grazed on open pasture to 1.0 for animals supplemented with lucerne *ad libitum* [6], while in the fed rat it is about 3.5 [7]. Similarly, the value of the $[carnitine]_{free}/[acetylcarnitine]$ ratio in bovine liver is lower than that in sheep or rat liver [6, 17, 18]. The low values for these two ratios in the cow as compared with the sheep appear to be due to much lower concentrations of free CoA and free carnitine respectively rather than to any major differences in the concentrations of the acetylated derivatives. An indication of the relative size of the [CoA]/[acetyl-CoA] ratio in normal bovine liver is given by the fact that the value for this ratio is even lower than that in the liver of the starved sheep [6] and considerably lower than that in the liver of the starved rat [19]. In both these latter species the decrease in the value of the [CoA]/[acetyl-CoA] ratio with starvation is accompanied by an elevated rate of hepatic ketogenesis [19, 20] in line with the observed inverse proportionality between the magnitude of the ratio and the rate of ketogenesis [5]. The fact that in the healthy lactating cow the value of the ratio is already below the level that would be associated with a high rate of ketogenesis in other species may be of aetiological importance with regard to the susceptibility of the dairy cow to ketosis in early lactation. In the current study, voren treatment was found to increase the magnitude of the ratio in healthy liver. A similar response to voren in the ketotic cow could be an important facet of the antiketogenic activity of the glucocorticoid, since a rise in the [CoA]/[acetyl-CoA] ratio would presumably be accompanied by a fall in the rate of hepatic ketogenesis.

It is of interest that the sum of free CoA plus acetyl-CoA decreased following voren treatment while the sum of free carnitine plus acetylcarnitine increased. An inverse relationship between acid-soluble CoA and acid-soluble carnitine has been observed in a number of sheep tissues and even in a single tissue under various conditions, suggesting that the synthesis and/or degradation of CoA and carnitine are carefully integrated [21]. In sheep tissues the high carnitine content and the presence of an active carnitine acetyltransferase are considered to be important in relieving the CoA system of "acetyl

pressure" [6,21]. However, the concentration of free carnitine plus acetylcarnitine in the liver of lactating cows, as reported here, is much less than in normal or starved sheep (*cf.* [6]). Thus it would appear unlikely that carnitine plays an important role in an acetyl buffer system in normal cow liver.

Redox

If use of the glutamate dehydrogenase system does indeed provide a reliable measure of mitochondrial redox state under these circumstances, then it would appear that voren could exert an antiketogenic effect by increasing the degree of oxidation of mitochondrial NAD. This increase would in turn lead to a rise in the intramitochondrial concentration of oxaloacetate, in the presence of a constant intramitochondrial malate concentration. Any increase in malate concentration of the type observed following voren administration [1,4] would further augment the concentration of oxaloacetate. An increase in mitochondrial oxaloacetate concentration would in turn be expected to decrease ketogenesis by directing acetyl-CoA from ketone body formation to citrate formation (see [3]).

The possibility that there is an uneven distribution of oxoglutarate between the subcellular compartments has led to recent criticism of the use of the glutamate dehydrogenase system for caclulating mitochondrial redox state, and it has been suggested that use of the D-3-hydroxybutyrate dehydrogenase system is to be preferred [22,23]. In the present instance, use of this latter system is precluded, however, since the activity of the enzyme is virtually negligible in bovine liver [10,24]. The fact also remains that the hydroxybutyrate and glutamate dehydrogenase systems yield parallel values for the mitochondrial $[NAD^+]/[NADH]$ ratio in a variety of circumstances in rat liver [16,23].

Nevertheless, there is still the possibility that, following voren treatment, a disparity could arise between the cytoplasmic and mitochondrial concentrations of oxoglutarate, that would in turn lead to an erroneous value for the mitochondrial $[NAD^+]/[NADH]$ ratio. Such compartmentation could develop, for example, if voren interfered with the oxoglutarate translocase (*cf.* [16]). There are a number of considerations which suggest that the ratio could be in error. Thus, the calculated changes in redox in the two subcellular compartments do not appear to account adequately for the marked fall in the $[NAD^+]_{total}/[NADH]$ ratio. There is also the striking similarity between several of the metabolic changes elicited by voren in bovine liver and by quinolinic acid in starved rat liver [25]. Following quinolinic acid treatment, there was a fall in the $[NAD^+]_{total}/[NADH]_{total}$ ratio that was accompanied by a disequilibrium between the hydroxybutyrate and glutamate dehydrogenase systems, so that the mitochondrial $[NAD^+]/[NADH]$ ratio appeared to fall when calculated using the former system and to rise when calculated using the latter system. It was concluded that the value for the ratio that was obtained using the hydroxybutyrate dehydrogenase system was the correct one and that the value obtained using the glutamate dehydrogenase system was in error owing to compartmentation of oxoglutarate [25].

It is clear, therefore, that for the present the calculated value for the mitochondrial $[NAD^+]/[NADH]$ ratio following voren administration must be treated with reservation.

The authors thank Mrs J. L. Young for skilled technical assistance. A. M. S. in indebted to Professor P. J. Randle for providing laboratory facilities and encouragement during this work, and to the Australian Research Grants Committee for financial assistance.

REFERENCES

1. Baird, G. D. & Heitzman, R. J. (1971) *Biochim Biophys. Acta, 252,* 184–198.
2. Krebs, H. A. (1966) *Vet. Rec. 78,* 187–192.
3. Wieland, O. (1968) in *Advances in Metabolic Disorders* (Levine, R. & Luft, R., eds) vol. 3, pp. 1–47, Academic Press, London and New York.
4. Baird, G. D. & Heitzman, R. J. (1970) *Biochem. J. 116,* 865–874.
5. Greville, G. D. & Tubbs, P. K. (1968) in *Essays in Biochemistry* (Campbell, P. N. & Greville, G. D., eds) vol. 4, pp. 155–212, Academic Press, London and New York.
6. Snoswell, A. M. & Henderson, G. M. (1970) *Biochem. J. 119,* 59–65.
7. Allred, J. B. & Guy, D. G. (1969) *Anal. Biochem. 29,* 293–299.
8. Pearson, D. J. & Tubbs, P. K. (1964) *Nature (London) 202,* 91.
9. Marquis, N. R. & Fritz, I. B. (1964) *J. Lipid. Res. 5,* 184–187.
10. Baird, G. D., Hibbitt, K. G., Hunter, G. D., Lund, P., Stubbs, M. & Krebs, H. A. (1968) *Biochem. J. 107,* 683–689.
11. Bernt, E. & Bergmeyer, H. U. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) pp. 384 to 388, Academic Press, London and New York.
12. Kirsten, E., Gerez, C. & Kirsten, R. (1963) *Biochem. Z. 337,* 312–319.
13. Williamson, D. H., Lund, P. & Krebs, H. A. (1967) *Biochem. J. 103,* 514–527.
14. Bergmeyer, H. U., Bernt, E. & Hess, B. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) pp. 736–741, Academic Press, London and New York.
15. Heitzman, R. J., Herriman, I. D. & Mallinson, C. M. (1972) *FEBS Lett. 20,* 19–21.
16. McLean, P., Gumaa, K. A. & Greenbaum, A. L. (1971) *FEBS Lett. 17,* 345–350.
17. Bøhmer, T. (1967) *Biochim. Biophys. Acta, 144,* 259 to 270.
18. Pearson, D. J. & Tubbs, P. K. (1967) *Biochem. J. 105,* 953–963.

19. Williamson, D. H., Veloso, D., Ellington, E. V. & Krebs, H. A. (1969) *Biochem. J. 114*, 575—584.
20. Katz, M. L. & Bergman, E. N. (1969) *Amer. J. Physiol. 216*, 953—960.
21. Snoswell, A. M. & Koundakjian, P. P. (1972) *Biochem. J. 127*, 133—141.
22. Williamson, J. R. (1969) in *Energy Level and Metabolic Control in Mitochondria* (Papa, S., Tager, J. M., Quagliariello, E. & Slater, E. C., eds) pp. 385—400, Adriatica Editrice, Bari.
23. Greenbaum, A. L., Gumaa, K. A. & McLean, P. (1971) *Arch. Biochem. Biophys. 143*, 617—663.
24. Koundakjian, P. P. & Snoswell, A. M. (1970) *Biochem. J. 119*, 49—57.
25. Williamson, D. H., Mayor, F. & Veloso, D. (1971) in *Regulation of Gluconeogenesis* (Söling, H.-D. & Willms, B., eds) pp. 92—102, Academic Press, London and New York.

G. D. Baird and R. J. Heitzman
A.R.C. Institute for Research on Animal Diseases
Compton, Newbury, Berkshire, Great Britain

A. M. Snoswell's present address:
Department of Agricultural Biochemistry
Waite Agricultural Research Institute
University of Adelaide
Glen Osmond, South Australia 5064, Australia

Aust. J. Biol. Sci., 1974, **27**, 645–50

19

The Liver as the Site of Carnitine Biosynthesis in Sheep with Alloxan-induced Diabetes

A. M. Snoswell[A] *and G. H. McIntosh*[B]

[A] Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S.A. 5064.
[B] Division of Nutritional Biochemistry, CSIRO, Kintore Avenue, Adelaide, S.A. 5000.

Abstract

The total acid-soluble carnitine concentration in the livers of three sheep increased 20-fold, to 4530 nmol/g wet weight, 10–14 days after induction of the diabetic state by alloxan. There was a threefold increase in the total carnitine concentration of the kidney cortex and no significant change in that of heart or skeletal muscle (M. biceps femoris).

In normal animals no significant difference was observed between the carnitine concentrations of blood taken from indwelling hepatic and portal cannulae. However, 10–14 days after administration of alloxan there was a substantial increase in the carnitine concentration of both portal and hepatic venous blood in most animals. An hepatic–portal difference in carnitine concentration became apparent only when the daily food intakes of the animals dropped to a third or less of the normal value, at which stage there was a net production of carnitine by the liver. No significant difference was observed between the carnitine concentrations of femoral arterial and femoral venous blood samples in either the normal or the alloxan-induced diabetic state.

The results indicate that the increased amounts of carnitine observed in the circulating blood and in the livers of sheep in the terminal diabetic state are not due to mobilization of carnitine from the muscle tissues but result from increased synthesis of carnitine within the liver itself.

Introduction

Previous work has shown that the total carnitine concentration in sheep liver increases in starvation (Snoswell and Henderson 1970) and in alloxan-induced diabetes (Snoswell and Koundakjian 1972). This is in direct contrast to the situation in the rat, where the total concentration of carnitine in liver remains essentially the same in the normal and diabetic states (Pearson and Tubbs 1967), and also to that in the guinea pig, where the sum of acetyl- and free carnitine falls during diabetes (Erfle and Sauer 1967). Also, alloxan-induced diabetes in rats leads to a decrease of about 50% in the carnitine concentration in the muscle tissues and in the total body pool (Mehlman *et al.* 1969).

It has been suggested that the increases in liver carnitine concentration in starved and diabetic sheep may indicate increased biosynthesis of this compound in the liver (Snoswell and Henderson 1970). However, in view of the very high concentrations of carnitine in muscle tissues of sheep (Snoswell and Koundakjian 1972), the possibility arises that the accumulation of carnitine in the liver could be due to mobilization of carnitine from the muscle tissues in times of metabolic stress. The aim of this investigation was to determine which tissue was the source of the carnitine that accumulates in the liver during alloxan-induced diabetes.

Sheep were surgically prepared with indwelling portal and hepatic venous cannulae and femoral venous and arterial cannulae in order to examine differences in blood

carnitine concentrations across the liver and hind limb during development of diabetes following administration of alloxan.

Materials and Methods

Animals

Merino wethers weighing 30–35 kg were used. They were kept in metabolism cages and were provided with water *ad libitum* and a daily ration of 750 g wheaten hay chaff–250 g lucerne chaff or 1000 g lucerne chaff. Prior to surgery the animals were fasted for 48 h.

Surgical Cannulation of Hepatic and Portal Veins

Two methods have been reported for placing cannulae into portal and hepatic veins of sheep for repeated sampling in the conscious animal (Harrison 1969; Katz and Bergman 1969). In the present study difficulties were experienced in obtaining workable preparations with either method. Therefore an improvement of the method described by Katz and Bergman (1969) was developed.

Anaesthesia was induced with thiopental sodium (25 mg/kg body weight) and maintained with Fluothane and oxygen. The sheep was laid on its left side, clipped and sterilized with nitromersol (5 mg/ml) over the right flank and thorax. Access was gained to the liver via a paracostal incision 3–4 cm behind the last rib and extending from the sternum to the midflank region. The ventral lobe of the liver was retracted from the diaphragm to expose the posterior vena cava where it passes through the diaphragm. The entrance of the hepatic vein to the vena cava could be palpated through the wall of the vena cava and the hepatic vein was followed as it passed centrally down the ventral lobe of the liver. The hepatic vein was cannulated from the visceral surface about 5 cm from the ventral border by making a stab incision with a large-bore hypodermic needle attached to a suitable handle. The bevel of the needle faced towards the liver surface. A polyvinyl tube (1·5 mm i.d., 2·5 mm o.d. and 60 cm in length) was passed through the lumen of the needle into the vein and the needle withdrawn while keeping the tube in place. Correct positioning of the tube at this stage was indicated by a free flow of blood from the tube. The tube was then passed further into the hepatic vein until it could be palpated within the lumen of the vena cava. The tip was subsequently withdrawn into the hepatic vein until it was about 2 cm from the vena cava (as determined by palpation). The tube was then fixed to the surface of the liver with tissue adhesive (methyl α-cyanoacrylate) and exteriorized.

The portal vein was cannulated near its entry to the liver by means of a stab incision, after a purse-string suture had first been placed in position to stop subsequent haemorrhaging. A similar cannula to that used for the hepatic vein was then passed 4–5 cm towards the liver and fixed in position with tissue adhesive. This cannula was exteriorized, attached to the skin with sutures (size 0 braided silk) and taken up onto the back of the sheep for ease of sampling.

The operation was completed in 1·5 h and the sheep was returned to its cage. The animals normally took 4–5 days to return to full food intake and then daily blood sampling was commenced. The cannulae were kept patent by flushing daily with heparin–saline mixture (100 units heparin/ml).

Following the intravenous (femoral venous) infusion of sulphobromophthalein sodium, blood samples taken from the hepatic venous cannulae showed very low concentrations of the dye compared with those taken from the portal cannulae. This indicated that an effective removal of the dye by the liver had occurred, and that the hepatic and portal cannulae had been correctly placed. The positions of the cannulae were also checked by post-mortem examination and in all cases were found to be correct.

Cannulation of the Femoral Arteries and Veins

Polyvinyl cannulae were inserted into the femoral vein and artery via the saphenous branches of the vessels. Access to the vessels was gained via a skin incision on the inside of the hind limb near the pelvic symphysis. The artery and vein were dissected free from the surrounding tissues and ligated. Cannulae (1·0 mm i.d., 1·5 mm o.d.) were introduced through incisions and passed for several centimetres up into the deep femoral vessels. The cannulae were tied in place with ligatures, the skin incision closed with metal clips and the cannulae taken up behind the leg and tied to the wool on the back of the sheep. The cannulae were kept patent by flushing with heparin–saline mixture (100 units heparin/ml) daily.

Induction of Diabetes with Alloxan

The sheep were allowed to stabilize on full food intake for 5–7 days after surgery. Blood samples were then taken on several consecutive days to establish normal values, after which alloxan (Koch-Light Ltd., 65 mg/kg body weight) was dissolved in sterile saline (0·9%) and immediately injected into a jugular vein.

Blood glucose, as monitored with Dextrostix (Ames Pharmaceuticals Ltd), rose to approximately 180 mg/100 ml within 2 days of alloxan administration and total blood ketones (acetoacetate plus 3-hydroxybutyrate) rose to 5–7 mM within 3 days. After approximately 7 days most of the diabetic sheep spontaneously decreased their food intakes and in the terminal stages (10–14 days after alloxan administration) these animals were eating a third of their normal intakes or less. The animals were slaughtered on the day the food intakes fell to zero.

Some animals did not reduce their food intakes in this manner and these animals have not been included in the main body of observations reported here. However, they were used to check whether the deprivation of food had any influence on carnitine metabolism. This variable response of sheep to alloxan-induced diabetes may be related to the degree of acidosis associated with the ketosis. In the present study the animals that reduced their food intakes spontaneously all showed acidosis as judged by the low pH of urine samples.

Measurement of Free Carnitine and Carnitine Esters

Total acid-soluble carnitine in the neutralized perchloric acid extracts of the blood samples following alkaline hydrolysis was measured using the method described by Marquis and Fritz (1964) for free carnitine.

Tissue samples and perchloric acid extracts of these were prepared as described previously (Snoswell and Henderson 1970). Acetylcarnitine was estimated by the method of Pearson and Tubbs (1964) and total acid-soluble and acid-insoluble carnitine were measured by the method of Pearson and Tubbs (1967).

Chemicals

L-Carnitine hydrochloride and *O*-acetyl-L-carnitine hydrochloride were generously supplied by Otsuka Pharmaceuticals, Japan.

Results

Tissue Carnitine Concentrations

The results presented in Table 1 show a 20-fold increase in total acid-soluble carnitine and a 30-fold increase in acetylcarnitine in livers of sheep with severe alloxan-induced diabetes. These increases are much more pronounced than those previously reported for sheep 3 days after alloxan administration (Snoswell and Koundakjian 1972) and appear in the present work to be correlated with the drastically reduced food intakes in the later stages of the diabetic state. Despite the fact that the livers of the diabetic sheep were extremely fatty, the acid-insoluble carnitine fraction (not shown in the table), which contains the long-chain fatty acylcarnitine esters, constituted less than 2% of the total tissue carnitine, while acetylcarnitine contributed two-thirds of the total carnitine.

The total carnitine concentration in the kidney cortex increased threefold in the diabetic animals (Table 1). This result is probably related to the raised blood concentration of carnitine and increased clearance of carnitine into the urine. In sheep Al-567 the total output of carnitine in the urine on the day prior to slaughter was 1600 μmol and on the previous day 1500 μmol. The daily output of carnitine in the urine of normal sheep maintained under comparable conditions was 73 ± 13 μmol/day (mean ± S.E.M. for nine observations on three animals).

No significant changes in the total carnitine concentration of muscle tissue (heart or skeletal) were observed in the diabetic animals (Table 1).

Blood Carnitine Concentrations

The values shown in Table 2 indicate marked hepatic–portal differences in the concentrations of carnitine in all sheep in the latter stages of the diabetic state and this is particularly pronounced in sheep no. Al-761. This hepatic–portal difference did not become apparent until 9–10 days after the administration of alloxan, i.e. until the food intake dropped to a third or less of the normal daily intake. Data for the daily blood samples up to 9 days after alloxan administration are not included in Table 2, as no hepatic–portal difference was apparent. No significant difference was observed

Table 1. Free carnitine and carnitine ester concentrations in tissues of normal sheep and sheep with alloxan-induced diabetes

Tissues from three Merino wethers were immediately frozen with aluminium-faced tongs previously cooled in liquid nitrogen and the frozen powders were extracted with $HClO_4$ and assayed as described in the text. The normal liver samples were removed within 2 min of the administration of thiopental sodium at the time of surgery. The sheep were subsequently made diabetic by the intravenous injection of alloxan (65 mg/kg body wt). The animals were slaughtered 10–14 days later and the tissues immediately freeze-clamped as above. The normal kidney, heart and skeletal muscle samples were obtained from another group of four Merino wethers maintained under similar conditions, as reported previously (Snoswell and Koundakjian 1972). Concentrations, given as means ± S.E.M., are expressed as nmol/g wet wt

Tissue	State	Acetyl-carnitine	Free carnitine	Total acid-soluble carnitine
Liver	Normal	98±46	79±11	227±59
	Diabetic	3090±340	797±184	4530±610
Kidney cortex	Normal	67±18	415±45	538±64
	Diabetic	999±32	438±153	1740±432
Heart	Normal	812±83	2060±323	3510±143
	Diabetic	1150±115	1740+275	3830±217
Skeletal muscle	Normal	1820±478	9860±1380	12900±880
(M. biceps femoris)	Diabetic	12000±2460	3240±1190	17100±2600

between the carnitine concentrations of hepatic and portal blood in normal animals: the hepatic concentration was 36·1 ± 1·8 nmol/ml and the portal concentration was 35·3 ± 1·7 nmol/ml (means ± S.E.M. of 23 observations on nine animals).

There was no significant difference in the carnitine concentration of femoral venous compared with femoral arterial blood in either the normal or diabetic state. In the normal state the femoral arterial concentration was 36·4±2·5 nmol/ml and the femoral venous concentration was 36·3 ± 2·5 nmol/ml (means ± S.E.M. of 16 observations on five animals); in the terminal stages of the diabetic state the femoral arterial concentration was 104 ± 7·6 nmol/ml, and the femoral venous concentration was 102 ± 5·6 nmol/ml (means ± S.E.M. of 10 observations on three animals). At this stage the portal carnitine concentration was equal to that of the femoral artery.

The pronounced increases in the concentration of carnitine in the blood (particularly hepatic blood) after administration of alloxan (Table 2) only occurred after appreciable spontaneous reductions in food intake in the latter stages of diabetes. This effect was not due to starvation *per se*, as normal sheep completely deprived of food for 7 days showed no significant increase in blood carnitine concentration, nor was any hepatic–portal difference in blood carnitine concentration observed. Similarly,

sheep that had been made diabetic with alloxan and that did not decrease their food intake within 14 days of alloxan administration were subsequently deprived of food for a 7-day period; these sheep also failed to show any significant rise in blood carnitine concentration.

Table 2. Carnitine concentrations in portal and hepatic venous blood samples from normal sheep and sheep with alloxan-induced diabetes

Blood samples from normal sheep were collected daily prior to alloxan administration and those from diabetic sheep were collected 10–14 days after the intravenous administration of alloxan (65 mg/kg body wt). The samples were collected simultaneously from indwelling hepatic and portal cannulae. Duplicate 5-ml aliquots were immediately mixed with an equal volume of 15% $HClO_4$ and total acid-soluble carnitine was estimated as described in the Methods. The values for each duplicate assay are recorded; n.d., not determined

Sheep	Sample	Blood carnitine concentrations (nmol/ml)						
		Normal sheep			Diabetic sheep			
		Successive daily samples:			Days prior to slaughter:			
		1	2	3	3	2	1	0
A1-761	Hepatic	21,22	34,32	34,33	98,97	70,68	59,59	279,273
	Portal	24,22	38,34	34,32	38,34	23,21	37,35	201,193
A1-567	Hepatic	45,46	51,46	40,39	n.d.	78,76	60,56	108,102
	Portal	44,44	48,43	42,40	n.d.	70,69	45,43	95,94
A1-742	Hepatic	n.d.	n.d.	46,42	n.d.	n.d.	123,128	138,135
	Portal	n.d.	n.d.	47,45	n.d.	n.d.	115,111	116,116

Discussion

The results presented here indicate that even in the terminal stages of alloxan-induced diabetes in sheep there is no reduction in the concentration of carnitine in the muscle tissues as is seen in the alloxan-diabetic rat (Mehlman *et al.* 1969). Thus, it is unlikely that the accumulation of carnitine in the livers of diabetic sheep is due to mobilization from muscle tissues. Further, in the terminal stages of the diabetic state, the concentration of carnitine in the circulating blood increased, as did the excretion of carnitine in the urine. At the same time, an appreciable hepatic–portal difference in the concentration of carnitine in the blood was observed. As the concentration of carnitine in the arterial blood at that stage was equal to that in the portal blood, the apparent production by the liver could not have been due to carnitine production in the gut or a contribution to the hepatic venous output from the hepatic artery. Thus, the observed result must have been due to production of carnitine in the liver.

The marked hepatic–portal differences in blood carnitine concentrations were only seen in the terminal stages of the diabetic state and were probably due to reduced blood flow rates through the liver at that stage. The spontaneous reduction in food intake seemed to be an essential feature of this phenomenon, as the deliberate withholding of food from diabetic sheep that did not spontaneously reduce their food intakes failed to produce any significant change in blood carnitine concentrations. Although the physiological basis of the spontaneous reduction in food intake in the terminal stages of the diabetic state is unclear, this phenomenon appears to provide the metabolic inducement for the increased synthesis of carnitine by sheep liver. The synthesis of carnitine at this stage is probably related to the acetyl-buffering action of

carnitine and carnitine acetyltransferase (see Snoswell and Koundakjian 1972 for discussion), as the bulk of carnitine in the liver in the terminal stages of the diabetic state is in the form of acetylcarnitine.

Acknowledgments

The authors are grateful to Mr G. R. Lewis for assistance with the surgery, to Mr R. Fishlock for skilled analytical assistance and to Mrs P. P. Koundakjian for helpful discussions. Grants from the Australian Meat Research Committee and the Australian Research Grants Committee to A.M.S. are gratefully acknowledged.

References

Erfle, J. D., and Sauer, F. (1967). Acetyl coenzyme A and acetylcarnitine concentration and turnover rates in muscle and liver of the ketotic rat and guinea pig. *J. Biol. Chem.* **242**, 1988–96.

Harrison, F. A. (1969). The introduction and maintenance of permanently indwelling catheters in portal and hepatic veins of the sheep. *J. Physiol.* **200**, 28–30P.

Katz, M. L., and Bergman, E. N. (1969). A method for the simultaneous cannulation of the major splanchnic blood vessels of the sheep. *Am. J. Vet. Res.* **30**, 655–61.

Marquis, N. R., and Fritz, I. B. (1964). Enzymological determination of free carnitine concentrations in rat tissues. *J. Lipid Res.* **5**, 184–7.

Mehlman, M. A., Abdel-Kader, M., and Therriault, D. G. (1969). Metabolism turnover time, half life and body pool of carnitine-^{14}C in normal, alloxan diabetic and insulin treated rats. *Life Sci.* **8**, 465–72.

Pearson, D. J., and Tubbs, P. K. (1964). Acetylcarnitine in heart and liver. *Nature* (*Lond.*) **202**, 91.

Pearson, D. J., and Tubbs, P. K. (1967). Carnitine derivatives in rat tissues. *Biochem. J.* **105**, 953–63.

Snoswell, A. M., and Henderson, G. D. (1970). Aspects of carnitine ester metabolism in sheep liver. *Biochem. J.* **119**, 59–65.

Snoswell, A. M., and Koundakjian, P. P. (1972). Relationships between carnitine and coenzyme A esters in tissues of normal and alloxan-diabetic sheep. *Biochem. J.* **127**, 133–41.

Manuscript received 28 December 1973

Journal of Dairy Research (1975), **42**, 371–380

Carnitine secretion into milk of ruminants

By A. M. SNOSWELL

Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064, *Australia*

and J. L. LINZELL

*Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge, CB*2 4*AT*

(*Received* 13 *March* 1975)

Summary. Total acid-soluble carnitine concentration in cow's, goat's and ewe's milk was 117, 101 and 872 nmol/ml respectively, of which acetylcarnitine made up 30 % in goats, 10 % in cows and 11 % in ewes. The concentration of carnitine in the arterial blood of goats decreased significantly ($P < 0{\cdot}01$) with the onset of lactation from 18·1 to 8·4 nmol/ml and during lactation in goats and cows there was a significant arterio-venous difference of carnitine across the udder, with mean extractions of 14 and 5 % respectively. Calculation of the udder uptake of carnitine, from these figures and from udder blood-flows, showed that in goats the amount lost in the milk was much less than that taken from the blood, but in cows about the same. Two groups of lactating ewes on low and high nutritional planes were sampled at 2-weekly intervals from 2 to 8 weeks of lactation. The concentrations of total acid-soluble carnitine and acetylcarnitine in the milk were similar in the 2 groups and remained relatively constant over this period, but the total acid-soluble carnitine concentration in jugular blood from the ewes on the low nutritional plane was significantly ($P < 0{\cdot}01$) higher than from the ewes on the higher nutritional plane from the fourth week of lactation. The total acid-soluble carnitine concentration in liver of goats was 290 nmol/g wet wt; mammary gland, 324; kidney-cortex, 692; heart, 2030 and skeletal muscle, 14300. Carnitine acetyltransferase (E.C. 2.3.1.7) activity of mammary tissue from lactating ewes was 0·6 μmol per min per g wet wt of which approximately half appeared to be 'latent' or membrane bound. Acetate thiokinase (E.C. 6.2.1.1) activity in this tissue was found to be 1·5 μmol per min per g wet wt and was predominantly localized in the cytoplasm. Carnitine palmitoyltransferase (E.C. 2.3.1.21) activity in the same tissue was 0·8 μmol per min per g wet wt while no acetylcarnitine hydrolase activity could be detected. The results suggest that carnitine in mammary tissue is extracted from the blood for the oxidation of both acetate and long-chain fatty acids and that some is lost in the milk.

Carnitine is an essential cofactor in the oxidation of fatty acids and plays a vital role in the transport of long-chain fatty acyl groups across the inner mitochondrial membrane to the site of β-oxidation (Fritz, 1963). Carnitine is particularly high in sheep muscle and the quantitative importance of carnitine in ruminant metabolism

generally has been emphasized previously (Snoswell & Henderson, 1970; Snoswell & Koundakjian, 1972). Free carnitine was first reported in cow's milk by Broekhuysen & Deltour, (1961). Erfle, Fisher & Sauer (1970) subsequently showed that in dairy cows there was a loss of 0·5 g/d of carnitine in the milk which rose to 0·9 g/d in ketotic cows. In the latter animals, a large proportion of the total carnitine secreted in the milk was acetylcarnitine. This considerable loss in the milk of a vital coenzyme involved in lipid metabolism of cows poses a number of important questions. Do similar losses of carnitine in the milk occur in other ruminant species? Is the carnitine secreted in the milk synthesized in the mammary glands or is it taken up from the blood?

MATERIALS AND METHODS

Animals

Goats

Saanen goats, both dry and at various stages of lactation at Babraham were fed a diet of hay *ad lib.*, and a standard cereal ration twice daily according to milk yield. The lactating animals were in their second–fifth lactation, each lasting about 42 weeks, total milk yields 350–1080 l, peak yields 3·2–5·3 l/d. The yields at the times of sampling are shown in the Tables.

The goats had been surgically prepared for sampling arterial and mammary venous blood by exteriorizing a carotid artery and a milk vein in skin covered loops and removing minor veins and those crossing between the left and right side of the udder. Mammary venous blood-flow was measured by a thermodilution method (Linzell, 1966) immediately after blood sampling.

Sheep

Eight Dorset × Merino ewes, in their third lactation and yielding 1·3–2·9 l milk/d were studied at Martlock, South Australia. Half grazed on a pasture with abundant legumes and grasses, a high plane of nutrition, while the rest grazed on a somewhat sparse pasture with few legumes and a lower plane.

Cows

Jersey cows at Babraham, in their second and third lactations yielding 10–20 l milk/d, and Holstein cows at Camden, New South Wales, in their third lactation, yielding 11–12 l milk/d, were surgically prepared like the goats for blood sampling and udder blood-flow measurements. The Jersey cows were fed the same hay and concentrates diet as the goats and the Camden animals grazed grass–legume pasture, supplemented with 4 kg/d lucerne chaff and crushed oats (3:1 w/w).

At Werribee, Victoria, Jersey × Friesian cows in their second lactation, yielding 10–14 l milk/d, and dry Jersey cows, grazed a grass–clover pasture; dry Friesians were stall-fed on hay.

Tissue samples

Blood

Samples were collected from the jugular vein, carotid artery and mammary vein by needle puncture or, in a later series of experiments, arterial and mammary

venous blood were sampled at a continuous rate for 30–60 min (using peristaltic pumps) via indwelling catheters (Fleet & Linzell, 1974). Five ml blood were added to 5 ml 15% (w/v) perchloric acid. The precipitated protein was removed by centrifugation and a sample of the supernatant adjusted to pH 6·5 with 3 N-KOH prior to assay.

Milk

The cows were milked by machine and the goats by hand twice daily. The ewes were milked by hand, after being given oxytocin (5 i.u.) intravenously and again after oxytocin 4 h later. Five ml milk were immediately added to 5 ml 15% (w/v) perchloric acid and then frozen if not assayed immediately. In these conditions no significant change in metabolites assayed could be detected after several months of storage.

Organs

From goats, liver samples were freeze-clamped within 20s of stunning and then small pieces of kidney, heart, skeletal muscle and mammary tissue were placed in N_2. The frozen tissues were powdered, extracted into perchloric acid and neutralized as described previously (Snoswell & Henderson, 1970) for metabolite assays.

Mammary tissue for enzyme assays was collected from lactating sheep in 0·25 M-sucrose and subsequently homogenized (1 in 5) in 0·25 M-sucrose, or added directly to centrifuge tubes and centrifuged at 100000 ***g*** for 2 h to yield a supernatant 'press' or a cytosol fraction, as described previously (Snoswell & Koundakjian, 1972).

Metabolic assays

Acetylcarnitine was measured by the method of Pearson & Tubbs (1964) and free carnitine by the method of Marquis & Fritz (1964). Total acid-soluble carnitine and acid-insoluble carnitine were measured by the method of Pearson & Tubbs (1967). Some very low blood carnitine concentrations were checked using the isotopic method of Cederblad & Lindstedt (1972). Good agreement was found between the 2 methods.

Enzyme assays

All assays were carried out at 30 °C in a Zeiss spectrophotometer (Model PMQ II) fitted with an automatic cell changer and transmittance extinction converter (TE) (Carl Zeiss, Oberkochen, Germany) connected to a Rikadenki Model B16 (Rikadenki Kogyo Co. Ltd, Tokyo, Japan).

Acetate thiokinase. (E.C. 6.2.1.1) was measured spectrophotometrically at 340 nm. The assay mixture contained 45 mM-tris-chloride buffer (pH 8·2), 100 mM-K acetate, 1 mM-$MgCl_2$, 20 mM-DL-malate, 0·3 mM-CoA, 0·5 mM-NAD^+, 9·1 mM-GSH, 5 μl malate dehydrogenase (5500 U/ml), 5 μl citrate synthase (220 U/ml) in a final volume of 1 ml. The reaction was started by the addition of 10 mM-ATP.

Citrate synthase. (E.C. 4.1.3.7) was assayed spectrophotometrically using 5,5′-dithiobis-(2-nitrobenzoic acid) as described by Shepherd & Garland (1969). Enzyme activity was measured in fractions which had been sonicated for 20 s in an MSE

sonic disintegrator (Measuring & Scientific Equipment Ltd, London W.1, England) at 20 kH_z and 1·5 A.

Carnitine acetyltransferase. (E.C. 2.3.1.7). This enzyme was assayed in fractions which had been exposed to hypo-osmotic sucrose (0·025 M) plus 0·1 % Triton X-100. The assay system depended on CoASH liberated from acetyl-CoA in the presence of carnitine reacting with 5,5′-(2-nitrobenzoic acid) as described by Barker, Fincham & Hardwick (1968).

Carnitine palmitoyltransferase was assayed in the same system as carnitine acetyltransferase (CAT), except palmitoyl-CoA replaced acetyl-CoA as the substrate.

Acetylcarnitine hydrolase. Fresh tissues were homogenized in 0·25 M-sucrose containing 23 mM-K phosphate buffer (pH 7·4) to give a 40 % (w/w) homogenate; 0·6 ml of this homogenate was then incubated in a system containing 0·1 M-K phosphate buffer (pH 7·4), 5 mM-acetylcarnitine and 1·5 mM-bromo-acetylcarnitine in a volume of 1·0 ml at 37 °C for 30 min. The reaction was stopped by the addition of 0·2 ml of 15% perchloric acid and the mixture centrifuged. Acetylcarnitine, acetate and carnitine were then measured in the neutralized supernatant. Acetylcarnitine hydrolase activity was expressed as μmol of substrate utilized, or product formed per min under the above assay conditions.

Chemicals

L-Carnitine chloride and O-acetyl-L-carnitine chloride were generously supplied by Otsuka Pharmaceuticals, Osaka, Japan, and were recrystallized from ethanol. CoA and palmitoyl-CoA were obtained from P.L. Biochemicals Inc., Milwaukee, Wisconsin, U.S.A., and enzymes from C. F. Boehringer & Soehne G.m.b.H., Mannheim, Germany. Acetyl-CoA was prepared from free CoA and acetic anhydride by the method of Stadtman (1957).

RESULTS

The concentration of carnitine in cow's milk was comparable to that previously reported by Erfle, Fisher & Sauer (1970), but the concentration in ewe's milk was considerably greater than that in milk of cows and goats (Table 1). Acetylcarnitine represents a significant proportion of the total acid-soluble carnitine in milk of goats (30 %), cows (10 %) and ewes (11 %).

The concentrations of carnitine in liver and mammary tissue of lactating goats were relatively low compared with other tissues, particularly skeletal muscle (Table 2), which had a very high concentration as in sheep (Snoswell & Koundakjian, 1972; Snoswell & McIntosh, 1974).

Using the mean mammary tissue concentration and the milk yields and empty udder weights (1·5–2·6 kg) of the goats in Table 4, it may be calculated that total mammary carnitine in these animals was half to one mmol and yet they were losing nearly a half mmol each day in the milk. Since there is no evidence that mammary tissue can synthesize carnitine, the amount passing into milk must be replenished from the blood and the present results provide evidence for this. In lactating, but not in dry, goats there were significant arteriovenous differences with a mean extraction

Table 1. *Acetylcarnitine and carnitine concentrations in milk*

(Values are means ±S.E.M. with the number of samples in brackets.)

	Milk yield, l/d	Acetylcarnitine, nmol/ml	Free carnitine, nmol/ml	Total acid-soluble carnitine, nmol/ml
Cows	10–20	11 ± 3 (11)	107 ± 25 (11)	117 ± 24 (11)
Goats	2·5–4·0	31 ± 3 (9)	65 ± 13 (9)	101 ± 10 (13)
Ewes	1·3–2·9	101 ± 22 (32)	—	872 ± 67 (32)

Table 2. *Carnitine compounds in tissues of lactating goats*

(Values are means with the individual figures in brackets.)

	Carnitine concentration, nmol/g wet wt			
	Acetyl	Free	Total acid-soluble	Acid-insoluble
Liver	7 (3, 10)	278 (157, 400)	290 (171, 409)	25 (22, 27)
Kidney-cortex	13 (9, 17)	612 (459, 765)	692 (524, 859)	20 (19, 20)
Heart	262 (195, 329)	1530 (1330, 1730)	2030 (1870, 2180)	28 (27, 29)
Skeletal muscle	1050 (815, 1280)	12900 (11190, 13800)	14300 (13900, 14600)	35 (32, 37)
Mammary gland	47 (62, 62, 16)	248 (216, 419, 110)	324 (322, 523, 127)	41 (41, –, –)

of 19% and arterial and mammary venous concentrations were significantly lower in lactating than in dry animals (Table 3).

In cows, the evidence for mammary uptake of carnitine was at first less convincing because blood concentrations were higher in lactating than in dry animals and arteriovenous differences were not significant in all lactating animals (Table 3). However, Table 3 also shows that blood concentrations were higher in Holsteins and Friesians than in Jerseys, but raises the possibility that diet might be a factor, which was investigated later. Nevertheless, for the Jersey cows at Babraham, milk yield and udder blood-flow were known and using the mean carnitine concentration in cow's milk (Table 1) it may be calculated that the udder uptake indicated by the small arteriovenous difference was more than sufficient to account for the carnitine lost in the milk (uptake/output ratio 1·85). Similar calculations for the lactating goats at Babraham indicated an even higher ratio of 7·5.

In order to check whether there is a true difference between the species more detailed measurements of udder uptake and milk output of carnitine were made in individual animals. The results are shown in Table 4. The arterial concentration and arteriovenous difference varied very little over 30 min, but the simultaneously measured blood-flow, milk yield and milk carnitine concentration again revealed high uptake/output ratios of 8·4–14·0 in goats, but about 1 in a cow. In the next lactation the improved method of sampling where arterial and mammary venous

Table 3. *Carnitine concentrations in blood of lactating and dry goats and cows*

(Values are means ± S.E.M. with the numbers of samples in brackets. Statistical differences were determined by paired '*t*' tests. NS, not significant.)

Species	Lactation state	Breed	Location	Carnitine concentration in blood, nmol/ml: Arterial (A)	Venous (V)	A–V	P	Extraction (A–V/A) × 100)
Goats*	Dry*	Saanen	Babraham	18·1 ± 2·2 (5)	18·4 ± 2·8 (5)	−0·3 ± 0·9 (5)	NS	−1·4 ± 5·6 (5)
Goats*	Lactating*	Saanen	Babraham	8·4 ± 1·2 (12)	7·0 ± 1·1 (12)	1·5 ± 0·2 (12)	< 0·001	19 ± 1·8 (12)
Cows	Lactating	Jersey	Babraham	6·9 ± 1·8 (8)	6·4 ± 1·7 (8)	0·5 ± 1·1 (8)	NS	9 ± 14 (8)
Cows	Lactating	Holstein	Camden	19·2 ± 2·3 (6)	16·6 ± 1·8 (6)	2·6 ± 0·8 (6)	< 0·05	13 ± 2·6 (6)
				Jugular				
Cows	Dry	Friesian	Werribee	10·0 ± 2·8 (7)				
Cows	Dry	Jersey	Werribee	2·5 ± 0·4 (4)				
Cows	Lactating	Jersey × Friesian	Werribee	9·1 ± 1·1 (4)				

* Values for the same group of goats with more samples analysed in lactation.

Table 4. *Uptake and output of carnitine by the udder*

	Blood carnitine, nmol/ml: Arterial (A)	Venous (V)	A–V	Extraction ((A–V/A) × 100), %	Udder blood flow, l/min	Udder carnitine uptake, mmol/d	Milk carnitine, nmol/ml	Milk yield, l/d	Milk carnitine, mmol/d	Uptake output ratio
				Intermittent sampling						
Goat: Winnie	22·8 ± 0·1	20·6 ± 0·1	2·1 ± 0·1	9·7 ± 0·3	1·25	3·78	126	3·55	0·45	8·4
Sabrina	19·8 ± 0·6	17·1 ± 0·4	2·7 ± 0·3	13·7 ± 1·2	1·1	4·28	111	4·0	0·44	9·7
Zsa-Zsa	27·5 ± 0·5	22·3 ± 1·3	5·3 ± 0·8	19·3 ± 2·9	0·7	5·34	99	3·81	0·38	14·0
Cow: R23	5·6 ± 0·3	5·3 ± 0·3	0·3 ± 0·2	5·3 ± 2·0	3·7	1·62	179	10·95	1·96	0·83
				Continuous sampling						
Goat: Sabrina	8·6 ± 0·1	7·4 ± 0·1	1·2 ± 0·1	14 ± 1·1	0·74	1·28	131	2·8	0·38	3·36
Cow: R16	5·2 ± 0·1	4·8 ± 0·1	0·4 ± 0·1	7 ± 2·5	4·72	2·54	237	10·3	2·44	1·04
R23	8·7 ± 0·6	8·3 ± 0·5	0·4 ± 0·1	5 ± 0·6	6·24	3·59	211	18·4	3·88	0·92

In lactating goats and cows blood samples were collected by 2 methods. For intermittent sampling 3 pairs were taken at 10-min intervals. The values shown are the means ± S.E.M. for duplicate assays on these 3 samples. The goat Sabrina (in the next lactation) and the 2 cows were sampled continuously over 60 min from exteriorized carotid arteries and mammary veins as described in the text. Two samples from each vessel were assayed in duplicate and the figures recorded are the means of these assays. Udder blood-flows and milk yields were measured as described in the text. Carnitine concentration was determined on samples of bulk milk collected daily as described in Table 1.

Table 5. *Milk and blood carnitine concentrations in ewes during the course of lactation*

Metabolite	Plane of nutrition	Metabolite concentration (nmol/ml) at lactation weeks						
		2	4	*P*	6	*P*	8	*P*
Acetylcarnitine (milk)	Low	86 ± 18	77 ± 2		92 ± 23		98 ± 7	
	High	121 ± 17	56 ± 11		114 ± 41		166 ± 63	
Total acid soluble carnitine (milk)	Low	968 ± 39	883 ± 49		983 ± 77		871 ± 73	
	High	811 ± 96	569 ± 36	< 0·01	936 ± 64		961 ± 104	
Carnitine (blood)	Low	27 ± 5	41 ± 2		56 ± 4		44 ± 3	
	High	30 ± 8	23 ± 4	< 0·01	32 ± 2	< 0·001	29 ± 1	< 0·01
Milk yield, ml/d	Low	1590 ± 204	2030 ± 281		—		634 ± 140	
	High	2260 ± 419	2910 ± 360		—		1035 ± 35	

Eight Dorset × Merino ewes in their third lactation were divided into 2 equal groups. Those on a 'high' nutritional plane were grazed on a pasture with abundant grasses and legumes, while those on the 'low' nutritional plane were grazed on sparse pasture with few legumes. The figures are means ± S.E.M. Statistical differences were determined by '*t*' test.

Table 6. *Enzyme activities in mammary tissue from lactating ewes*

Enzyme	Activity (μmol per min per g wet tissue)
Carnitine acetyltransferase	$0{\cdot}61 \pm 0{\cdot}12$
Carnitine palmitoyltransferase	$0{\cdot}75 \pm 0.22$
Acetate thiokinase	$1{\cdot}47 \pm 0{\cdot}54$
Acetylcarnitine hydrolase	ND

Tissue was collected into 0·25 M-sucrose. Homogenates were prepared and assayed as described in the text. The values are means ± S.E.M. for 5 samples from 3 animals. ND, non-detectable.

blood are pumped out continuously at identical and constant rates for 30–60 min (Fleet & Linzell, 1974) were applied (Table 4). The animals were eating the same food and blood carnitine concentrations were similar. Nevertheless, in the cows the uptake/output ratios were close to one but over 3 in the goat.

The effects of diet and stage of lactation were studied in ewes where the lactation is shorter than in cows and goats.

The concentrations of total acid-soluble carnitine and acetylcarnitine in the milk of lactating ewes on high and low planes of nutrition were examined every 2 weeks for 8 weeks, and although there were large differences between individuals the mean concentrations remained relatively constant (Table 5). At the height of lactation (4 weeks) the carnitine concentration in the milk was significantly higher ($P < 0{\cdot}01$) in ewes on the low plane of nutrition (Table 5). However, due to the lower milk yields in this group (Table 5) the quantities of carnitine lost in the milk were not significantly different between the 2 groups of ewes. The concentration of total acid-soluble carnitine in the jugular blood of the ewes on the low nutritional plane was also significantly ($P < 0{\cdot}01$) higher than in ewes maintained on the high nutritional plane from 4 to 8 weeks (Table 5).

The activity of CAT in sheep mammary tissue homogenates (Table 6) was comparable to that previously reported for sheep liver and kidney (Snoswell & Koundakjian, 1972). Some 50 % of the CAT activity detected in homogenates treated with 0·1 % Triton X-100 was found in homogenates prepared with 0·25 M-sucrose, suggesting that only half the CAT activity was 'latent', or membrane bound.

The activity of carnitine palmitoyltransferase in sheep mammary homogenates was 0·57 μmol per min per g tissue and the activity of acetate thiokinase was 1·5 μmol per min per g tissue (Table 6). The activity of the latter enzyme is higher than that previously reported for sheep liver and kidney, i.e. 0·13 and 0·7 μmol per min per g tissue repectively (Snoswell & Koundakjian, 1972). The acetate thiokinase in sheep mammary tissue would appear to be predominantly located in the cytosol, since a 'press' or cytosolic fraction (see Methods) contained virtually all the acetate thiokinase found in a whole tissue homogenate. The same press fraction contained only 2 % of the mitochondrial matrix marker enzyme, citrate synthase, when compared with a whole tissue homogenate, indicating that the press fraction was relatively free of mitochondrial contamination.

Acetylcarnitine hydrolase activity was not detected in sheep mammary tissue (Table 6) indicating that acetylcarnitine would not be cleaved to acetate and carnitine as in sheep liver (Costa & Snoswell, 1974).

DISCUSSION

The present results confirm that there is a considerable daily loss of carnitine in the milk in cows, goats and particularly ewes and that the carnitine secreted in the milk appears to have been taken up from the blood passing through the udder.

Whilst the concentration of carnitine in the milk of goats is comparable to that in the milk of cows, in ewe's milk it is 8–14 times greater. Although the goats and ewes were of a comparable size and the goats produced more milk/d, the amount of carnitine secreted daily in the milk by the ewes was still much greater than that secreted by the goat, and was as high as 0·4 g. This is a very large loss of a vital cofactor of mammary lipid metabolism and its significance is obscure. It may be that, since the ewe also has a higher blood carnitine concentration she has a greater capacity to synthesize carnitine. Recent work (Snoswell & McIntosh, 1974) indicates that the liver of the sheep does indeed have a significant capacity to synthesize carnitine in times of metabolic stress. It may also be that the cow and the goat, which are bred for milk production, have evolved a more efficient mechanism for retaining this vital cofactor.

The activity and distribution of CAT, carnitine palmitoyltransferase and acetate thiokinase in mammary tissue from lactating ewes suggest that carnitine is an essential cofactor in the oxidation of both acetate and long-chain fatty acids in mammary tissue and that acetate thiokinase could provide acetyl-CoA for fatty-acid synthesis in the cytoplasm. These suggestions would need to be confirmed by further in vitro experiments with mammary tissue. However, Annison & Linzell (1964) have shown that in goats approximately half the acetate taken up by the udder is used to form milk fatty acids up to C_{14} in chain length, while the other half is oxidized.

The present results suggest that the amount of carnitine taken up by the udder from the blood in both goats and cows is adequate to account for the carnitine secreted in the milk; in fact, in goats the uptake is in excess of output. Further evidence for the blood origin of milk carnitine is that the amount lost in the milk would completely deplete the mammary tissue in 1–2 d. Thus, either the total daily carnitine output must be synthesized in the mammary glands or must be taken up from the blood. There is no evidence in the literature to suggest that carnitine is synthesized in any tissue other than the liver, either in the ruminant (Snoswell & McIntosh, 1974) or in rats (Bøhmer, 1974; Haigler & Broquist, 1974).

The mechanism by which carnitine is secreted is a matter of speculation. In rat epididymal fluid, the carnitine concentration is as high as 63000 nmol/ml (Brooks, Hamilton & Mallek, 1974) and the use of the labelled compound shows that it is derived from plasma (Brooks, Hamilton & Mallek, 1973), so that there must be an active concentrating mechanism. However, this may not apply to mammary tissue. It may be calculated from the concentration of carnitine in goat mammary tissue reported here and the proportion of intracellular fluid in the tissue (Linzell & Peaker, 1971*a*) that the concentration of carnitine in the cytosol must be approximately 400 nmol/ml, which is some 4 times greater than the mean concentration of carnitine in goat's milk and some 20-fold greater than the blood carnitine concentration. Because of its trimethylammonium group, carnitine acts as a cation at neutral pH, and since the insides of mammary secretory cells are 35–40 mV negative with

respect to blood and milk (Linzell & Peaker, 1971*b*), it will tend to be held inside the cell by electrical forces. To maintain a concentration gradient of 20 would require a potential difference (P.D.) of 90 mV so there is probably some active means of moving carnitine from blood into the cells as well. The concentration gradient of 4 across the apical cell membrane would be accounted for by the P.D. which would suggest that carnitine passes passively from the cell into milk according to the electrical and concentration gradients, as has been suggested for Na^+ and K^+ (Linzell & Peaker, 1971*b*). Although some cytoplasm is lost as milk fat globules are extruded, surrounded by apical cell membrane (Linzell & Peaker, 1971*b*), this volume could not account for all the carnitine in milk.

In the final stages of the preparation of this paper a report by Erfle *et al.* (1974) appeared, indicating that the carnitine concentration in cow's milk decreased to about half its initial value after 8 weeks of lactation. In contrast, no significant variation in the concentration of carnitine in ewe's milk over the same lactation period was observed in the present study. This may be due to the fact that ewe's milk contains much higher concentrations of carnitine and sheep appear to have a considerable capacity to synthesize carnitine in the liver (Snoswell & McIntosh, 1974).

The authors are indebted to Mr I. R. Fleet for assistance in the collection of samples and the measurement of udder blood-flow in cows and goats, and to Mr N. D. Costa for the determination of the enzyme activities in mammary glands. Drs J. G. McLean and J. M. Gooden are thanked for their assistance in collecting blood samples from cows at Werribee and Camden respectively, and Mr R. Fishlock for skilled analytical assistance. The co-operation of Mr P. Geytenbeek in the experiments with lactating ewes is gratefully acknowledged. Support to A.M.S. from the Wellcome Trust, the Australian Research Grants Committee and the Australian Meat Research Committee is also gratefully acknowledged.

REFERENCES

ANNISON, E. F. & LINZELL, J. L. (1964). *Journal of Physiology* **175**, 372.
BARKER, P. J., FINCHAM, N. J. & HARDWICK, D. C. (1968). *Biochemical Journal* **110**, 739.
BØHMER, T. (1974). *Biochimica et Biophysica Acta* **343**, 551.
BROEKHUYSEN, J. & DELTOUR, G. (1961). *Annales de Biologie Clinique* **9**, 549.
BROOKS, D. E., HAMILTON, D. W. & MALLEK, A. H. (1973). *Biochemical and Biophysical Research Communications* **52**, 1354.
BROOKS, D. E., HAMILTON, D. W. & MALLEK, A. H. (1974). *Journal of Reproduction and Fertility* **36**, 141.
CEDERBLAD, G. & LINDSTEDT, S. (1972). *Clinica Chimica Acta* **37**, 235.
COSTA, N. D. & SNOSWELL, A. M. (1974). *Proceedings of the Australian Biochemical Society* **7**, 36.
ERFLE, J. D., FISHER, L. J. & SAUER, F. (1970). *Journal of Dairy Science* **53**, 486.
ERFLE, J. D., SAUER, F. D. & FISHER, L. J. (1974). *Journal of Dairy Science* **57**, 671.
FLEET, I. R. & LINZELL, J. L. (1974). *Journal of Physiology* **242**, 1*P*.
FRITZ, I. B. (1963). *Advances in Lipid Research* **1**, 285.
HAIGLER, H. T. & BROQUIST, H. P. (1974). *Biochemical and Biophysical Research Communications* **56**, 676.
LINZELL, J. L. (1966). *Circulation Research* **18**, 745.
LINZELL, J. L. & PEAKER, M. (1971*a*). *Journal of Physiology* **216**, 683.
LINZELL, J. L. & PEAKER, M. (1971*b*). *Physiological Reviews* **51**, 564.
MARQUIS, N. R. & FRITZ, I. B. (1964). *Journal of Lipid Research* **5**, 184.
PEARSON, D. J. & TUBBS, P. K. (1964). *Nature* **202**, 91.
PEARSON, D. J. & TUBBS, P. K. (1967). *Biochemical Journal* **105**, 953.
SHEPHERD, D. & GARLAND, P. B. (1969). *Biochemical Journal* **114**, 597.
SNOSWELL, A. M. & HENDERSON, G. D. (1970). *Biochemical Journal* **119**, 59.
SNOSWELL, A. M. & KOUNDAKJIAN, P. P. (1972). *Biochemical Journal* **127**, 133.
SNOSWELL, A. M. & MCINTOSH, G. H. (1974). *Australian Journal of Biological Sciences* **27**, 645–650.
STADTMAN, E. R. (1957). *Methods in Enzymology* **3**, 931.

Printed in Great Britain

Biochem. J. (1975) **152**, 161–166
Printed in Great Britain

21

Enzymic Hydrolysis of Acetylcarnitine in Liver from Rats, Sheep and Cows

By N. D. COSTA and A. M. SNOSWELL
Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S. Austral. 5064, Australia

(*Received* 4 *March* 1975)

1. The enzymic utilization of *O*-acetyl-L-carnitine other than via carnitine acetyltransferase (EC 2.3.1.7) was investigated in liver homogenates from rats, sheep and dry cows. 2. An enzymic utilization of *O*-acetyl-L-carnitine via hydrolysis of the ester bond to yield stoicheiometric quantities of acetate and L-carnitine was demonstrated; 0.55, 0.53 and 0.30 μmol of acetyl-L-carnitine were utilized/min per g fresh wt. of liver homogenates from rats, sheep and dry cows respectively. 3. The acetylcarnitine hydrolysis activity was not due to a non-specific esterase or non-specific cholinesterase. *O*-Acetyl-D-carnitine was not utilized. 4. The activity was associated with the enriched outer mitochondrial membrane fraction from rat liver. Isolation of this fraction resulted in an eightfold purification of acetylcarnitine hydrolase activity. 4. The K_m for this acetylcarnitine utilization was 2 mM and 1.5 mM for rat and sheep liver homogenates respectively. 6. There was a significant increase in acetylcarnitine hydrolase in rats on starvation and cows on lactation and a significant decrease in sheep that were severely alloxan-diabetic. 7. The physiological role of an acetylcarnitine hydrolase is discussed in relation to coupling with carnitine acetyltransferase for the relief of 'acetyl pressure'.

The only known pathway to date for the production and utilization of *O*-acetyl-L-carnitine is via the enzyme, carnitine acetyltransferase (Friedman & Fraenkel, 1955; Bremer, 1962; Fritz *et al.*, 1963). Lowenstein (1964) observed a low incorporation of the acetyl group of acetylcarnitine into fatty acids. He postulated a possible hydrolysis to yield acetate as the simplest interpretation of this observation. In his investigations into the reversibility of the carnitine acetyltransferase reaction in rat liver, Bremer (1962) observed some alternative utilization of acetylcarnitine. Mahadevan & Sauer (1969), in experiments with rat liver microsomal preparations, established a hydrolytic utilization of C_6–C_{18} acylcarnitines but did not detect any hydrolysis of C_2–C_4 acylcarnitines. Thus a more specific investigation into the enzymic utilization of acetylcarnitine seemed warranted. In none of the above reports was the utilization of acetylcarnitine by carnitine acetyltransferase prevented. In our current investigations, carnitine acetyltransferase was completely inhibited by the addition of the specific inhibitor bromoacetyl-L-carnitine (Chase & Tubbs, 1969).

We report an alternative utilization of acetylcarnitine via hydrolysis of the ester bond to yield acetate and L-carnitine. Some of these results have been presented in a preliminary form (Costa & Snoswell, 1974).

Experimental

Methods

Animals. The rats used were hooded Wistar males weighing 250–300 g and were fed on a pelleted rat diet (Charlicks, Adelaide, S. Austral., Australia). Food was withheld from the starved rats for 48 h.

The sheep used were 4-year-old Merino wethers, weighing between 38 and 48 kg. These animals were fed *ad libitum* on lucerne-hay chaff. Tissue from Merino sheep was also obtained from the abattoirs directly after slaughter.

Non-lactating cows were predominantly Jersey and were all approx. 3 years of age. The lactating cows were all Jersey × Friesian animals in the fifth month of their second lactation. The cattle were grazed on irrigated perennial pasture consisting mainly of white clover, cocksfoot, paspallum and rye grass. No supplementary feed was given.

Alloxan-diabetic animals. Alloxan-diabetes was produced in adult Merino wethers by injecting a sterile solution of alloxan (60 mg/kg body weight) into the jugular vein. The animals were killed 5–6 days later. These animals spontaneously reduced their food intakes. Blood total acid-soluble carnitine had doubled in the 2 days before slaughter.

Tissue preparations and homogenates. Rats were killed by cervical dislocation and exsanguination. The

sheep were killed by severing the necks. Liver tissue from the non-lactating and lactating cows was obtained by biopsy as described by Baird & Heitzman (1970). Fresh samples of liver were collected directly into 0.25M-sucrose containing 23mM-potassium phosphate, pH7.4. The liver tissue was homogenized with a Thomas homogenizer in either 0.25M-sucrose containing 23mM-potassium phosphate, pH7.4, as the homogenizing buffer (40%, w/v) or 0.025M-sucrose containing 2.3mM-potassium phosphate, pH7.4, and 0.1% Triton X-100 as the homogenizing buffer (20%, w/v).

Tissue fractionation. Rat liver was collected directly into 0.25M-sucrose containing 23mM-potassium phosphate, pH7.2. Homogenates (10%, w/v) were then prepared in the same sucrose–phosphate solution with a Potter–Elvehjem homogenizer with a tight-fitting Teflon pestle. These homogenates were then centrifuged at 700 *g* to remove all debris and nuclei. The supernatant fractions were centrifuged at 10000*g* for 10min to sediment mitochondria. The supernatant fractions were recentrifuged at 100000 *g* for 1h. The supernatants from this centrifugation were designated cytosol fractions and the pellets were resuspended in sucrose–phosphate medium to give microsomal fractions. The mitochondrial pellets were washed twice in the sucrose–phosphate medium and recentrifuged at 13000*g* for 10min.

Mitochondrial fractionation. Rat liver mitochondria were isolated in 0.44M-sucrose–10mM-triethanolamine (pH7.6)–2mM-EDTA by the method of Brdiczka *et al.* (1969). A slight modification of the method of Hoppel & Tomec (1972) was used to separate the submitochondrial fractions in that all pellet fractions were resuspended in 0.25M-sucrose–23mM-potassium phosphate, pH7.4.

Enzyme activity assays. All assays were performed on a Zeiss PMQ II spectrophotometer fitted with an automatic sample changer and TE converter (Carl Zeiss, Oberkochen, Germany) connected to a Rikadenki model B140 recorder (Rikadenki Kogyo Co., Tokyo, Japan). The instrument was fitted with a temperature-controlled cell holder and the temperature was maintained at 37°C for all assays.

Carnitine acetyltransferase. Liver homogenates (20%, w/v) homogenized in 0.025M-sucrose–2.3mM-potassium phosphate–0.1% Triton X-100 were frozen in liquid N_2 and thawed. The homogenates were then centrifuged at 8000 *g* for 3min in an Eppendorf centrifuge model 3200 (Eppendorf Geratebau Netheter and Hinz, G.m.b.H., Hamburg, Germany) and the supernatants used for assay. The assay system was similar to that described by Solberg *et al.* (1972) and contained 400mM-Tris–HCl, pH8.0, 100μM-5,5′-dithiobis-(2-nitrobenzoic acid), 100μM-acetyl-CoA and enzyme fraction in a total volume of 1ml. The reaction was started by the addition of 3.3mM-L-carnitine. The inhibition of carnitine acetyltransferase by bromoacetylcarnitine was investigated in the same assay system. The carnitine acetyltransferase was pre-incubated with bromoacetylcarnitine (1–20μM).

Enzymic utilization of O-acetyl-L-carnitine. Liver homogenates (40%, w/v) homogenized in 0.25M-sucrose–23mM-potassium phosphate, pH7.4, were used. Activities were measured by determining the rate of acetylcarnitine utilization and L-carnitine and acetate formation. The incubation series for each tissue consisted of a boiled-homogenate reaction, zero-time reaction, and two tubes to determine acetyl-L-carnitine utilization in the presence and absence of bromoacetyl-L-carnitine. Eppendorf tubes contained, in a total volume of 1.0ml, 0.3M-Tris–HCl, pH8.0, 70μM-bromoacetylcarnitine, 5mM-acetyl-L-carnitine and tissue homogenate. The tubes were incubated for 0, 5, 10, 15, 20 or 30min at 37°C and the reaction was stopped with 200μl of 15% (w/v) $HClO_4$; after centrifugation at 8000 *g* for 2–5min, the supernatant was neutralized with 3M-KOH. The enzymic activity was linear with respect to time for 20min and therefore a 20min incubation period was used as a routine.

Specificity of acetylcarnitine hydrolase. Utilization of *O*-acetyl-D-carnitine, *O*-propionyl-L-carnitine and *O*-butyryl-L-carnitine was determined in the same manner as utilization of *O*-acetyl-L-carnitine.

Rotenone-insensitive NADH–cytochrome c reductase. This was assayed in the digitonin-treated submitochondrial fractions of rat liver in a system similar to that described by Sottocasa *et al.* (1967). The spectrophotometric assay was performed at 550nm and the assay system contained 240mM-potassium phosphate, pH7.6, 5mM-EDTA, 20μM-rotenone, 0.08mM-cytochrome *c* and submitochondrial fraction in a final volume of 1.0ml. The reaction was started with 0.1mM-NADH.

Adenylate kinase. This was determined in the submitochondrial fractions from rat liver by spectrophotometric assay at 340nm. The system contained, in a final volume of 1.0ml, 50mM-triethanolamine buffer, pH7.6, 5mM-EDTA, 8mM-$MgSO_4$, 75mM-KCl, 0.22mM-NADH, 3mM-ATP, 0.8mM-phosphoenolpyruvate, 10μg of pyruvate kinase, 50μg of lactate dehydrogenase and enzyme fraction. The reaction was started with 3mM-AMP.

Succinate dehydrogenase. This was assayed in the submitochondrial fractions from rat liver by spectrophotometric assay at 550nm. The assay system contained, in a total volume of 1.0ml, 100mM-potassium phosphate, pH7.6, 5mM-EDTA, 20μM-rotenone, 0.1mM-phenazine methosulphate, 0.08mM-cytochrome *c* and enzyme fraction. The reaction was started with 5mM-succinate.

Metabolite assays. Acetylcarnitine was measured by the method of Pearson & Tubbs (1964) and free carnitine by the method of Marquis & Fritz (1964).

Free acetate was determined by the kinetic method of Knowles *et al.* (1974).

Preparation of bromoacetyl-L-carnitine. The bromoacetyl derivative of L-carnitine was prepared by modification of several methods (Chase & Tubbs, 1969; Fraenkel & Friedman, 1957; Zeigler *et al.*, 1967). First 2g (15.3mmol) of bromoacetic acid (recrystallized) was allowed to react with 24ml (26.4mmol) of bromoacetyl bromide for 3h at 75°C. Then 1g (5.9mmol) of L-carnitine was added and the mixture allowed to react for a further hour. The mixture was then cooled and extracted eight times with ice-cold diethyl ether. The solid mass remaining was dried with a stream of N_2 gas. The solid mass was dissolved in 4ml of ethanol, and 3ml of 70% (w/v) $HClO_4$ was added. Bromoacetyl-L-carnitine perchlorate was recrystallized from isopropyl alcohol. The resultant crystals were stored frozen *in vacuo*. A sample of bromoacetyl-L-carnitine was also kindly supplied by Dr. P. K. Tubbs, Department of Biochemistry, University of Cambridge, Cambridge, U.K.

Chemicals

L-Carnitine hydrochloride and *O*-acetyl-L-carnitine chloride were generously supplied by Otsuka Pharmaceuticals, Osaka, Japan, and were recrystallized from ethanol. Propionyl-L-carnitine and butyryl-L-carnitine chloride were prepared by the method of Bøhmer & Bremer (1968). CoA was obtained from Calbiochem, Los Angeles, Calif., U.S.A., enzymes were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and alloxan was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Acetyl-CoA was prepared by the method of Stadtman (1957).

Results

Enzymic utilization of O-acetyl-L-carnitine by normal sheep liver homogenates

The results in Table 1 show a small amount of chemical hydrolysis of acetylcarnitine at the pH of the incubation. In addition to the chemical hydrolysis of acetylcarnitine there is a significantly greater ($P<0.001$) enzymic utilization of acetylcarnitine. This acetylcarnitine utilization and carnitine production may proceed *via* the carnitine acetyltransferase reaction. In the presence of bromoacetylcarnitine, a potent specific inhibitor of carnitine acetyltransferase (Chase & Tubbs, 1969), there is a small decrease in acetylcarnitine utilization ($P<0.05$) proceeding via carnitine acetylcarnitine, but there is still a significant ($P<0.001$) and substantial utilization of acetylcarnitine of 0.55 μmol/min per g of fresh liver tissue. The carnitine acetyltransferase activity present in the sheep liver homogenates is completely inhibited by the concentration of bromoacetylcarnitine used in the incubations (i.e. 70 μM). This was also established by independent spectrophotometric assay of carnitine acetyltransferase inhibition as described under 'Methods'. In subsequent assays of acetylcarnitine utilization, this concentration of bromoacetylcarnitine was included in all incubations. Thus the enzymic utilization of acetylcarnitine and production of L-carnitine proceeds via an alternative enzymic mechanism to the carnitine acetyltransferase reaction.

Enzymic hydrolysis of acetylcarnitine by homogenates of normal rat, sheep and cow liver

Results in Table 2 show an overall balance between acetylcarnitine utilized and acetate and L-carnitine produced in incubations of liver homogenates from

Table 1. *Enzymic utilization of O-acetyl-L-carnitine by normal sheep liver homogenates*

Liver samples were obtained from sheep killed at the abattoirs. Incubations of acetyl-L-carnitine with sheep liver homogenates were performed in the presence and absence of bromoacetylcarnitine as described in the text. Results are means±S.E.M. for six animals.

	Activity (μmol/min per g of liver tissue)	
	Acetylcarnitine utilized	Carnitine produced
Normal−inhibitor	0.715±0.05	0.680±0.10
Normal+inhibitor	0.550±0.05	0.515±0.05
Boiled homogenate	0.155±0.025	0.100±0.020

Table 2. *Enzymic hydrolysis of acetylcarnitine by homogenates of normal rat, sheep and cow liver*

Homogenates were prepared and assayed as described in the text. N.A., Not assayed. The values shown are means±S.E.M. with the number of determinations in parentheses.

	Activity (μmol/min per g of fresh liver tissue) as per method of assay		
	Acetylcarnitine utilized	Carnitine produced	Acetate produced
Rat	0.50 (4)±0.10	0.50 (4)±0.05	0.45 (4)±0.10
Sheep	0.55 (6)±0.05	0.52 (6)±0.05	N.A.
Dry cow	0.30 (3)±0.05	0.30 (3)±0.10	0.30 (3)±0.05

these species. Although the amount of acetylcarnitine utilized varies between the different species, this balanced relationship holds throughout. Within each species there is no significant difference with respect to the use of any of the three compounds for determining acetylcarnitine hydrolysis activity. These results then establish a stoicheiometric relationship between acetylcarnitine utilized and acetate and carnitine produced in the absence of carnitine acetyltransferase activity. Thus the enzymic utilization of acetylcarnitine in the presence of bromoacetylcarnitine in liver homogenates of these species would then appear to proceed via the hydrolytic cleavage of the ester bond of acetylcarnitine.

Enzymic specificity of acetylcarnitine hydrolysis

The hydrolysis of the ester bond of acetylcarnitine could be catalysed by a non-specific esterase. The main non-specific esterase present in liver in various species is carboxylic ester hydrolase (Shibko & Tappet, 1964; Chow & Ecobichon, 1973; Ecobichon, 1973). This enzyme is inhibited by iodoacetamide (Shibko & Tappet, 1964), but the addition of iodoacetamide had no effect on the rate of utilization of acetylcarnitine by liver homogenates. Purified preparations of acylcholine acylhydrolase (EC 3.1.1.8) did not hydrolyse acetylcarnitine to any significant extent. These results imply that acetylcarnitine hydrolysis is catalysed by a specific enzymic mechanism. Liver homogenates from rats, sheep and cows did not hydrolyse the unnatural acetyl-D-carnitine as measured by production of acetate. Thus acetylcarnitine hydrolysis appears to proceed *via* a stereospecific enzyme subsequently referred to as acetylcarnitine hydrolase.

Substrate specificity of acetylcarnitine hydrolase

Acetylcarnitine hydrolase from rat liver also hydrolysed propionyl and butyryl esters of L-carnitine. The rate of utilization of propionyl-L-carnitine by acetylcarnitine hydrolase was 9.0nmol of carnitine liberated/min per mg of tissue protein, which was greater than the rate of utilization of acetyl-L-carnitine of 5.8nmol/min per mg of tissue protein. In contrast, butyryl-L-carnitine was hydrolysed at a lower rate than acetyl-L-carnitine (2.8 and 5.8nmol/min per mg of tissue protein respectively).

Michaelis–Menten constants for acetylcarnitine hydrolase from rat and sheep liver

The kinetic constants for acetylcarnitine hydrolase from the livers of the two species were calculated by using crude enzymic preparations from these two sources. The K_m for acetylcarnitine of rat liver acetylcarnitine hydrolase was 2mM, and that for sheep liver acetylcarnitine hydrolase was 1.5mM. These values were determined from Lineweaver–Burk plots by a statistical weighting method for $1/v$ described by Wilkinson (1961).

pH optimum of acetylcarnitine hydrolase

The pH optimum for acetylcarnitine hydrolysis was 8.0. There was a sharp increase in activity in the pH range from 7.4 to 8.0 with a fall in activity after pH 8.0.

Intracellular localization of acetylcarnitine hydrolase in rat liver

Preliminary fractionation of rat liver homogenates into mitochondrial, microsomal and cytosol fractions and subsequent analysis of these fractions for acetylcarnitine hydrolase activity showed the activity to be confined to the mitochondrial fraction. Submitochondrial fractions were then prepared by using the modifications described in the text. Rotenone-insensitive NADH–cytochrome *c* reductase was chosen as the marker enzyme for the outer-membrane fraction. Ernster & Kuylenstierna (1968) showed that contamination by microsomal rotenone-insensitive NADH–cytochrome *c* reductase could be significantly decreased after a few washings of the mitochondrial preparation. The advantages of using this marker enzyme instead of the more commonly used monoamine oxidase were greater ease and sensitivity of measurement. There was difficulty in preparing a rigidly pure outer-membrane fraction, as the results in Table 3 indicate. Thus the preparation was rather

Table 3. *Submitochondrial localization of acetylcarnitine hydrolase activity within rat liver mitochondria*

Submitochondrial fractions were prepared and assayed as described in the text. The values are means±S.E.M. of three experiments. N.D., Not detectable.

	% of total activity in submitochondrial fractions		
	Outer membrane	Intermembrane space	Inner-membrane matrix
Rotenone-insensitive cytochrome *c*–succinate dehydrogenase	13± 6	4± 1	85±7
Adenylate kinase	12± 6	88± 6	N.D.
Rotenone-insensitive cytochrome *c*–NADH reductase	64± 8	35± 8	N.D.
Acetylcarnitine hydrolase activity	72±10	23±10	N.D.

Table 4. *Enzymic hydrolysis of acetyl-L-carnitine by homogenates of rat, sheep and cow liver under various conditions*

Liver homogenates were prepared and assayed as described in the text. The results are means ± S.E.M. with the number of assays in parentheses. Significance of the difference from normal or between dry and lactating cow was determined by the Student's t test.

Animal	Physiological state	Activity of acetylcarnitine hydrolase (μmol/min per g of fresh liver tissue)	
Rat	Normal	0.55 (4) ± 0.10	
	Starved	0.95 (2) ± 0.25	$P<0.05$
Sheep	Normal	0.55 (6) ± 0.05	
	Alloxan-diabetic	0.35 (2) ± 0.10	$P<0.01$
Cow	Dry	0.30 (3) ± 0.05	
	Lactating	0.95 (4) ± 0.05	$P<0.01$

an enrichment of the outer-membrane fraction of the mitochondria. The results in Table 3 show that the acetylcarnitine hydrolase is associated with this fraction and its activity represented an eightfold purification over the activity in the crude homogenates from rat liver.

Effect of various physiological states on enzymic hydrolysis of acetyl-L-carnitine by homogenates of rat, sheep and cow liver

The results in Table 4 show a significant ($P<0.05$) almost twofold increase in acetylcarnitine hydrolase in liver from rats starved for 48 h over liver from normal rats. Also the activity of acetylcarnitine hydrolase increased threefold ($P<0.01$) in liver of cows on lactation. In direct contrast with these two results, in liver from alloxan-diabetic sheep there was a significant ($P<0.01$) decrease in acetylcarnitine hydrolase activity when compared with the activity in normal sheep liver. All values for acetylcarnitine hydrolase activity shown in Table 4 are an overall average of measurement of acetylcarnitine utilized and L-carnitine and acetate produced.

Discussion

The results presented for sheep liver homogenates show that utilization of acetylcarnitine within the assay incubation is mainly enzymic rather than chemical. This enzymic utilization was demonstrated in the liver homogenates from at least three different species and the stoicheiometry of acetylcarnitine utilization is consistent with a concept of hydrolysis of the ester bond to yield equimolar proportions of acetate and L-carnitine. These findings are not entirely inconsistent with the observations of Bremer (1962) who, using rat liver mitochondria, found some alternative utilization of acetylcarnitine other than via the carnitine acetyltransferase reaction. Also Bremer & Davis (1974) reported acetylcarnitine concentrations lower than expected in their experiments with rat liver mitochondria, which is again consistent with an alternative utilization of acetylcarnitine.

The utilization of acetylcarnitine in the presence of iodoacetamide and non-utilization of acetyl-L-carnitine by non-specific cholinesterase indicates that hydrolysis of acetyl-L-carnitine is due to a specific enzyme entity rather than a non-specific esterase. The stereospecificity of acetylcarnitine utilization is also indicative of an enzymic rather than chemical utilization and further indicates a specific enzyme is involved. This specific hydrolase activity in rat liver cells is associated with the outer mitochondrial membrane. Thus attempts by Mahadevan & Sauer (1969) to establish a short-chain carnitine ester hydrolase in enriched microsomal fractions from rat liver were unlikely to have been successful. The isolation of an enriched outer mitochondrial membrane fraction also resulted in an eightfold purification of acetylcarnitine hydrolase activity over that present in the crude rat liver homogenate. Further attempts to purify this enzyme met with difficulty owing to its highly unstable nature.

The precise physiological role of an acetylcarnitine hydrolase within rat liver in the normal and starved state is difficult to delineate, compounded in part by the lack of a precise role for carnitine acetyltransferase. However, the increase in acetylcarnitine hydrolase activity in the starved rat is consistent with the hypothesis of the short-chain carnitine ester system buffering 'acetyl pressure' (Pearson & Tubbs, 1967) generated in starvation. The increase in acetylcarnitine hydrolase activity would allow the carnitine released to be recycled through the system or complexed with long-chain fatty acids via carnitine palmitoyltransferase. Thus the acetylcarnitine hydrolase in rat liver may maintain a high concentration of free carnitine during the starved state. The energy lost in this carnitine-maintenance system during 'acetyl pressure' would be equivalent to that lost by diverting acetyl groups into ketone-body production.

The physiological role of an acetylcarnitine hydrolase in sheep liver is also not easy to rationalize. The high activity of an acetylcarnitine hydrolase in the presence of a high carnitine acetyltransferase activity (in the normal sheep liver) could help to ease a high ratio of acetyl-CoA/CoA (Snoswell & Henderson, 1970) with the resultant bonus of producing acetate, a major metabolic fuel used in peripheral tissues in ruminants. In the severely alloxan-diabetic animal there is a significant decrease in acetylcarnitine hydrolase activity. This would firstly decrease the amount of L-carnitine recycled through the coupled system, which may be reflected in the need to synthesize more carnitine as a compensating measure, and secondly result in an accumulation of acetyl-L-carnitine (see

Snoswell & Koundakjian, 1972). In the severely diabetic state there is a 20-fold increase in total acid-soluble carnitine and a 30-fold increase in acetylcarnitine (Snoswell & McIntosh, 1974).

The increase in acetylcarnitine hydrolase activity in livers from lactating cows relative to dry cows also suggests that the carnitine acetyltransferase–acetylcarnitine hydrolase-coupled system could operate to relieve 'acetyl pressure' in the lactating state. The carnitine acetyltransferase activity in liver from lactating cows was 2.2μmol of CoA produced/min per g of fresh tissue (N. D. Costa, unpublished work). Thus coupling this activity with the acetylcarnitine hydrolase activity would result in a facile carnitine-recycling system in the lactating cow. The observations of Baird *et al.* (1972) where they failed to observe a significant rise in total acid-soluble carnitine could be rationalized on the basis of active carnitine recycling. This coupled-enzyme system releases a non-toxic end product, acetate, which would then be available to the mammary gland for the synthesis *de novo* of milk fatty acids, and oxidation (Annison & Linzell, 1964).

We are indebted to Dr. J. McLean for providing dry and lactating cows and for the surgical removal of liver biopsy samples. Mr. R. Fishlock is thanked for his skilled technical assistance. Grants from the Australian Research Grants Committee are gratefully acknowledged. N. D. C. holds an Australian Wool Corp. Postgraduate Scholarship.

References

Annison, E. F. & Linzell, J. L. (1964) *J. Physiol.* (*London*) **175**, 372–385
Baird, G. D. & Heitzman, R. J. (1970) *Biochem. J.* **116**, 865–874
Baird, G. D., Heitzman, R. J. & Snoswell, A. M. (1972) *Eur. J. Biochem.* **29**, 104–109
Bøhmer, T. & Bremer, J. (1968) *Biochim. Biophys. Acta* **152**, 559–567
Brdiczka, D., Gerbitz, K. & Pette, D. (1969) *Eur. J. Biochem.* **11**, 234–240
Bremer, J. (1962) *J. Biol. Chem.* **237**, 2228–2231
Bremer, J. & Davis, E. J. (1974) *Biochim. Biophys. Acta* **370**, 564–572
Chase, J. F. A. & Tubbs, P. K. (1969) *Biochem. J.* **111**, 225–235
Chow, A. Y. K. & Ecobichon, D. J. (1973) *Biochem. Pharmacol.* **22**, 689–701
Costa, N. D. & Snoswell, A. M. (1974) *Proc. Aust. Biochem. Soc.* **7**, 36
Ecobichon, D. J. (1973) *Can. J. Biochem.* **51**, 506–513
Ernster, L. & Kuylenstierna, B. (1968) in *FEBS. Symp. Mitochondria Structure and Function, Prague*, (Ernster, L. & Drahota, Z., eds.), pp. 5–31, Academic Press, London and New York
Fraenkel, G. & Friedman, S. (1957) *Vitam. Horm.* (*N.Y.*) **15**, 74–115
Friedman, S. & Fraenkel, G. (1955) *Arch. Biochem. Biophys.* **59**, 409–501
Fritz, I. B., Schultz, S. K. & Srere, P. A. (1963) *J. Biol. Chem.* **238**, 2509–2517
Hoppel, C. L. & Tomec, R. J. (1972) *J. Biol. Chem.* **247**, 832–841
Knowles, S. E., Jarrett, I. G., Filsell, O. H. & Ballard, F. J. (1974) *Biochem. J.* **142**, 401–412
Lowenstein, J. M. (1964) in *Recent Research in Carnitine* (Wolf, G., ed.), pp. 97–112, Massachusetts Institute of Technology Press, Cambridge, Mass.
Mahadevan, S. & Sauer, F. (1969) *J. Biol. Chem.* **244**, 4448–4453
Marquis, N. R. & Fritz, I. B. (1964) *J. Lipid Res.* **5**, 184–187
Pearson, D. J. & Tubbs, P. K. (1964) *Nature* (*London*) **202**, 91
Pearson, D. J. & Tubbs, P. K. (1967) *Biochem. J.* **105**, 953–963
Shibko, S. & Tappet, A. L. (1964) *Arch. Biochem. Biophys.* **106**, 259–266
Snoswell, A. M. & Henderson, G. D. (1970) *Biochem. J.* **119**, 59–65
Snoswell, A. M. & Koundakjian, P. P. (1972) *Biochem. J.* **127**, 133–141
Snoswell, A. M. & McIntosh, G. H. (1974) *Aust. J. Biol. Sci.* **27**, 645–650
Solberg, H. E., Aas, M. & Daae, L. N. W. (1972) *Biochim. Biophys. Acta* **280** 434–439
Sottocasa, G. L., Ernster, L., Kuylenstierna, B. & Bergstrand, A. (1967) in *FEBS. Symp. Mitochondria Structure and Compartmentation, Polignano a Mare* (Quagliariello, E., Papa, S., Slater, E. C. & Tager, J. M. eds.), pp. 74–89, Adriatica Editrice, Bari
Stadtman, E. R. (1957) *Methods Enzymol.* **3**, 931–941
Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332
Zeigler, H. J., Bruckner, P. & Binon, F. (1967) *J. Org. Chem.* **32**, 3989–3991

Biochem. J. (1975) **152**, 167–172
Printed in Great Britain

Acetyl-Coenzyme A Hydrolase, an Artifact?

THE CONVERSION OF ACETYL-COENZYME A INTO ACETATE BY THE COMBINED ACTION OF CARNITINE ACETYLTRANSFERASE AND ACETYLCARNITINE HYDROLASE

By N. D. COSTA and A. M. SNOSWELL
Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S. Austral. 5064, Australia

(*Received* 4 *March* 1975)

1. The nature of the acetyl-CoA hydrolase (EC 3.1.2.1) reaction in rat and sheep liver homogenates was investigated. 2. The activity determined in an incubated system was 5.10 and 3.28nmol/min per mg of protein for rat and sheep liver homogenate respectively. This activity was not affected by the addition of L-carnitine, but was decreased by the addition of D-carnitine. 3. No acetyl-CoA hydrolase activity could be detected in rat or sheep liver homogenates first treated with Sephadex G-25. This treatment decreased the carnitine concentrations of the homogenates to about one-twentieth. Subsequent addition of L-carnitine, but not D-carnitine, restored the apparent acetyl-CoA hydrolase activity. 4. Sephadex treatment did not affect acetylcarnitine hydrolase activity of the homogenates, which was 5.8 and 8.1 nmol/min per mg of protein respectively for rat and sheep liver. 5. Direct spectrophotometric assay of acetyl-CoA hydrolase, based on the reaction of CoA released with 5,5′-dithiobis-(2-nitrobenzoic acid), clearly demonstrated that after Sephadex treatment no activity could be measured. 6. Carnitine acetyltransferase (EC 2.3.1.7) activity measured in the same assay system in response to added L-carnitine was very low in normal rat liver homogenates, owing to the apparent high acetyl-CoA hydrolase activity, but was increased markedly after Sephadex treatment. The $V_{max.}$ for this enzyme in rat liver homogenates was increased from 3.4 to 14.8nmol/min per mg of protein whereas the K_m for L-carnitine was decreased from 936 to 32μM after Sephadex treatment. 7. Acetyl-CoA hydrolase activity could be demonstrated in disrupted rat liver mitochondria but not in separated outer or inner mitochondrial membrane fractions. Activity could be demonstrated after recombination of outer and inner mitochondrial membrane fractions. The outer mitochondrial membrane fraction showed acetylcarnitine hydrolase activity and the inner mitochondrial membrane fraction showed carnitine acetyltransferase activity. 8. The results presented here demonstrate that acetyl-CoA hydrolase activity in rat and sheep liver is an artifact and the activity is due to the combined activity of carnitine acetyltransferase and acetylcarnitine hydrolase.

The initial report describing the activity of an acetyl-CoA hydrolase in a pig heart preparation only implied the presence of such an enzyme (Gergely *et al.*, 1952), and on the basis of that report the enzyme was classified and numbered. The enzyme has been reported to be widely distributed in rat and sheep tissues (Knowles *et al.*, 1974) and in bovine tissues (Quraishi & Cook, 1972). In each case a high activity was reported in the liver with the activity predominantly localized in the mitochondria. Murthy & Steiner (1973) have claimed that, in rat-liver, this enzyme is associated with the inner mitochondrial membrane. It is difficult to conceive a physiological function of such an enzyme in the inner mitochondrial membrane since the well-characterized enzyme, carnitine acetyltransferase, is localized in this membrane (Edwards *et al.*, 1974). This latter enzyme has a K_m for acetyl-Co A of approx. 0.04mM (Fritz *et al.*, 1963; Chase, 1967) whereas the K_m for acetyl-CoA of the acetyl-CoA hydrolase is approx. 0.7mM (Knowles *et al.*, 1974), and the concentration of acetyl-CoA in rat liver or sheep liver is in the order of 0.04–0.08mM (Allred & Guy, 1969; Snoswell & Henderson, 1970).

In our investigations on the acetylcarnitine hydrolase of rat and sheep liver (see the preceding paper, Costa & Snoswell, 1975) we observed that acetyl-CoA hydrolase was completely inhibited by low concentrations of bromoacetyl-L-carnitine (Costa & Snoswell, 1974), which is a potent inhibitor of carnitine acetyltransferase (Chase & Tubbs, 1969). This suggested that acetyl-CoA hydrolase activity might be due to the combined

action of carnitine acetyltransferase and acetylcarnitine hydrolase. In the present work we have examined the nature of the acetyl-CoA hydrolase reaction and have found that in both sheep and rat liver the activity is due to a combination of carnitine acetyltransferase present in the inner mitochondrial membrane and acetylcarnitine hydrolase present in the outer mitochondrial membrane.

Experimental

Methods

Animals. The rats used were hooded Wistar females (150–200g) which had been fed on a pelleted rat diet (Charlicks, Adelaide, S. Austral., Australia).

The sheep used were 9-month-old Merino wethers which had been fed on a diet of lucerne-chaff.

The rats and the sheep were killed by severing the necks and samples of liver were collected into 0.25M-sucrose–23mM-potassium phosphate, pH 7.4.

Tissue preparation and homogenates. Homogenates (20%, w/v) were prepared in 0.025M-sucrose–2.3mM-potassium phosphate (pH 7.4)–0.1% Triton X-100 solution with a Potter–Elvehjem homogenizer. These homogenates were then centrifuged at 8000 **g** for 3min and the supernatants used for assay. In addition, 2ml fractions of supernatant were passed down a column (22.5cm × 1.5cm) of Sephadex G-25 (coarse) in 25mM-potassium phosphate, pH 7.4. The protein peak and total acid-soluble carnitine peak were monitored. Fractions at the beginning of the protein peak were taken for enzyme assay.

Subcellular fractions. Mitochondria were prepared from rat liver by the method described by Snoswell & Koundakjian (1972). The mitochondria were suspended in 0.44M-sucrose–10mM-triethanolamine (pH 7.6)–2mM-EDTA as described by Brdiczka *et al.* (1969). The method of Hoppel & Tomec (1972) was used to separate the submitochondrial fractions which were resuspended in 0.25M-sucrose–23mM-potassium phosphate, pH 7.4. The integrity of each fraction was established by using the marker enzymes as described in the preceding paper (Costa & Snoswell, 1975).

Enzyme assays. All assays were determined at 37°C by using the instrumentation described in the preceding paper (Costa & Snoswell, 1975).

Carnitine acetyltransferase. This was assayed in homogenates from sheep and rat liver both before and after treatment with Sephadex G-25, and in submitochondrial fractions from rat liver. The reaction was monitored spectrophotometrically at 412nm as described in the preceding paper (Costa & Snoswell, 1975). The kinetics of carnitine acetyltransferase before and after G-25 Sephadex treatment were investigated over the range of 2μM–4.5mM-L-carnitine. The data from these experiments were fitted to Lineweaver–Burk plots by statistically weighting $1/v$ by the method described by Wilkinson (1961).

Acetylcarnitine hydrolase. This was assayed in homogenates from sheep and rat liver both before and after treatment with Sephadex G-25 and also in submitochondrial fractions derived from rat liver. The activity was determined by the method described in the preceding paper (Costa & Snoswell, 1975).

Acetyl-CoA hydrolase. This was assayed in homogenates from sheep and rat liver both before and after treatment with Sephadex G-25 and also in submitochondrial fractions derived from rat liver. The activity was assayed spectrophotometrically at 412nm. The assay contained 400mM-Tris–HCl (pH 8.2), 100μM-5,5′-dithiobis-(2-nitrobenzoic acid) and 100μM-acetyl-CoA and enzyme preparation in a total volume of 1.0ml. The 5,5′-dithiobis-(2-nitrobenzoic acid) was added first and the rate allowed to become linear before the addition of acetyl-CoA.

Also the activity was assayed in an incubated system similar to that described by Knowles *et al.* (1974). L- or D-Carnitine (3.3mM) was added to this system. The reaction was started by adding 4.0mM-acetyl-CoA.

Metabolite assays. Total acid-soluble carnitine was measured by the method of Pearson & Tubbs (1964) and free carnitine by the method of Marquis & Fritz (1964).

Acetyl-CoA and acetyl-CoA plus free CoA were measured by the kinetic method of Allred & Guy (1969); free CoA was determined by difference. In these kinetic determinations a standard curve was prepared on each occasion by using a CoA standard solution, the concentration of which was determined with phosphotransacetylase (EC 2.3.1.8) by the method of Michal & Bergmeyer (1963). However, as this method only assays reduced CoA, the standards were preincubated for 10min at 25°C with 2μl of 0.2M-dithiothreitol to ensure that all the CoA was in a reduced form.

Acetate was determined by the kinetic method of Knowles *et al.* (1974).

Protein was determined by the biuret method.

Chemicals

L-Carnitine and D-carnitine hydrochloride were generously supplied by Otsuka Pharmaceuticals, Osaka, Japan. CoA and acetyl-CoA were obtained from Calbiochem, Los Angeles, Calif., U.S.A., and enzymes from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Acetyl-CoA was also prepared from free CoA and redistilled acetic anhydride by the method of Stadtman (1957).

Results

The results in Table 1 indicate that, after the passage of homogenates of both rat and sheep

Table 1. *Effect of stereoisomers of carnitine on acetyl-CoA hydrolase activity in normal and Sephadex G-25-treated homogenates of rat and sheep liver*

Homogenates from rat and sheep liver were prepared and assayed as described in the text. The values are means±S.E.M. of three experiments. N.D., Not detectable.

	Acetyl-CoA hydrolase activity in rat liver (nmol/min per mg of protein)		Acetyl-CoA hydrolase activity in sheep liver (nmol/min per mg of protein)	
Additions	Normal	Sephadex G-25-treated	Normal	Sephadex G-25-treated
None	5.10±0.98	N.D.	3.28±0.28	N.D.
+L-Carnitine	4.87±0.87	5.98±1.26	3.27±0.14	11.56±1.26
+D-Carnitine	3.23±1.00	N.D.	2.39±0.15	N.D.

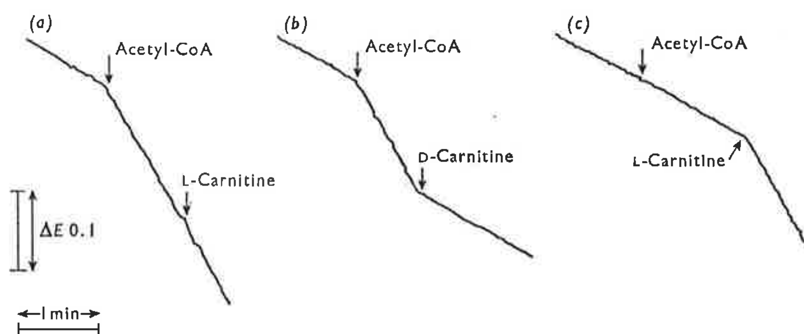


Fig. 1. *Spectrophotometric assay of acetyl-CoA hydrolase and carnitine acetyltransferase in normal and Sephadex-treated homogenates from rat liver*

Recording of E_{412} is shown; a downward deflexion represents an increase in absorbance. The reaction was started by adding 5,5′-dithiobis-(2-nitrobenzoic acid) and the recording shown was obtained after this initial reaction became linear. Homogenates were prepared and assayed as described in the text. Acetyl-CoA (0.1mM) and L- or D-carnitine (3.3mM) were added as indicated. (*a*) and (*b*) Normal homogenate. (*c*) Homogenate that had first been passed down a Sephadex G-25 column (1.5cm×22.5cm) equilibrated with potassium phosphate (pH7.4). The initial tubes of peak protein concentration were used.

liver down columns of Sephadex G-25, no acetyl-CoA hydrolase activity could be detected, whereas appreciable activity could be measured in untreated homogenates from both species. The total acid-soluble carnitine concentration in the homogenates initially was 40 and 24nmol/ml for rat and sheep liver respectively, and this was decreased to approximately one-twentieth of these values by the Sephadex treatment (not shown in Table 1).

Addition of L-carnitine to untreated homogenates of either rat or sheep liver had no effect on the acetyl-CoA hydrolase activity but resulted in appreciable apparent acetyl-CoA hydrolase activity in the Sephadex-treated homogenates (Table 1). In fact the activity in the Sephadex-treated sheep liver homogenate supplemented with L-carnitine was considerably higher than activity in the untreated homogenate. The reason for this is not clear.

Addition of D-carnitine significantly ($P<0.01$) decreased the activity of acetyl-CoA hydrolase in untreated homogenates of sheep liver and decreased the activity in rat liver homogenate but failed to result in any activity in the Sephadex-treated extracts, in marked contrast with the addition of L-carnitine (Table 1).

It should be emphasized that the acetylcarnitine hydrolase activity determined at the same pH, 8.2, as the acetyl-CoA hydrolase activity, was 5.8 and 8.1nmol/min per mg of protein respectively for the rat and sheep liver homogenate. This activity remained after the extracts were treated with Sephadex. Also carnitine acetyltransferase activity was present after Sephadex treatment and was 14.8 and 22.2nmol/min per mg respectively for the rat and sheep liver homogenate.

All the acetyl-CoA hydrolase activities shown in Table 1 were determined on the basis of acetate

and free CoA released in an incubated system; in each case there was a stoicheiometric balance between acetyl-CoA utilized and acetate and free CoA formed. An alternative direct spectrophotometric assay of this enzyme activity, based on the development of a yellow complex absorbing at 412nm formed by reaction of the free CoA released with 5,5′-dithiobis-(2-nitrobenzoic acid), is often used in the assay of this enzyme and demonstrates even more clearly some of the features shown in Table 1.

The results shown in Fig. 1 clearly show that there is no acetyl-CoA hydrolase activity in rat liver homogenates treated with Sephadex G-25 (compare Fig. 1*c* with 1*a*). Fig. 1(*b*) shows a much more dramatic inhibition of added D-carnitine on acetyl-CoA hydrolase activity with normal homogenates than is shown in Table 1, where the incubated assay system was used. In the spectrophotometric assay system the ratio of added D-carnitine to endogenous L-carnitine is considerably greater than in the incubated system.

The results shown in Fig. 1(*a*) indicate that in rat liver homogenates the activity of carnitine acetyltransferase appears to be very low owing to the apparent high rate of the acetyl-CoA hydrolase reaction which is deducted from the rate in the presence of L-carnitine. However, prior treatment of the rat liver homogenate with Sephadex abolished the apparent acetyl-CoA hydrolase activity and a substantial carnitine acetyltransferase activity was then observed (Fig. 1*c*). This effect of Sephadex treatment on carnitine acetyltransferase activity is illustrated in Fig. 2 which shows Lineweaver–Burk plots of computer-fitted experimental data. $V_{max.}$ was increased from 3.4 to 14.8nmol/min per mg of protein after Sephadex treatment and the K_m for L-carnitine was substantially decreased from 936 to 32μM.

The results presented in Fig. 3 show the measurement of acetyl-CoA hydrolase activity in rat liver mitochondria. No acetyl-CoA hydrolase activity was detected in other subcellular fractions. Appreciable activity was measured in disrupted whole mitochondria (Fig. 3*a*); however, no activity could be detected in either the inner mitochondrial membrane

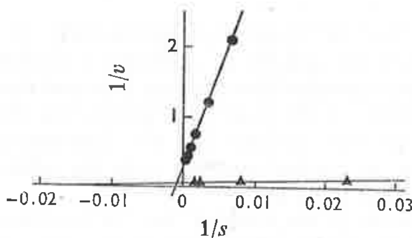


Fig. 2. *Lineweaver–Burk plot for the carnitine acetyltransferase reaction in rat liver homogenate and for homogenate treated with Sephadex G-25*

The Sephadex-treated homogenate prepared as in Fig. 1 was used. Carnitine acetyltransferase activity was determined as in Fig. 1. Acetyl-CoA concentration was 0.1mM and L-carnitine concentration varied between 2μM and 4.5mM. Experimental data were weighted for accuracy by the statistical method of Wilkinson (1961) by using an appropriate computer program. ●, Normal; ▲, Sephadex G-25-treated.

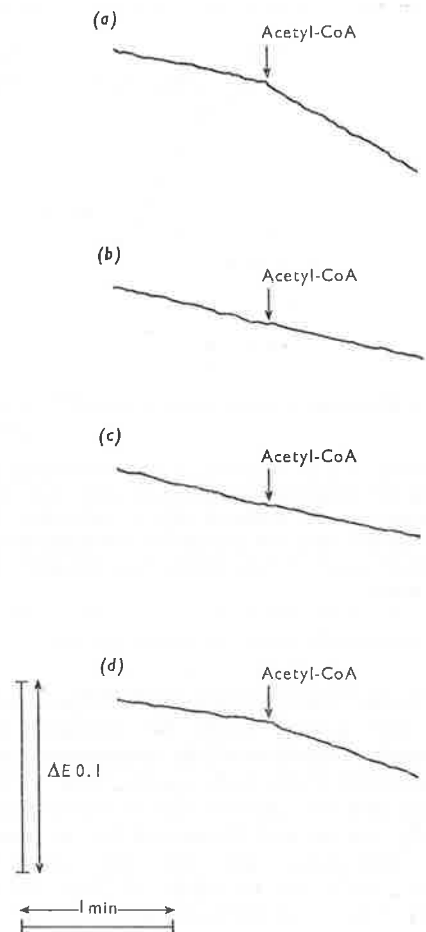


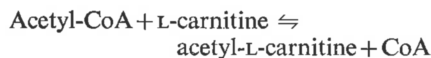
Fig. 3. *Acetyl-CoA hydrolase activity in rat liver mitochondria and in inner and outer mitochondrial membrane fractions*

Acetyl-CoA hydrolase was assayed as described in Fig. 1. Acetyl-CoA (0.1mM) was added as indicated. (*a*) Disrupted rat liver mitochondria; (*b*) inner mitochondrial membrane fraction; (*c*) outer mitochondrial membrane fraction; (*d*) recombined inner and outer membrane fractions (1:1 ratio).

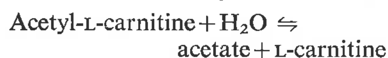
fraction (Fig. 3*b*) or the outer mitochondrial membrane fraction (Fig. 3*c*), but on recombination of these two membrane fractions acetyl-CoA hydrolase activity could again be detected (Fig. 3*d*). The inner mitochondrial membrane fraction showed appreciable carnitine acetyltransferase activity, whereas the outer membrane fraction showed acetylcarnitine hydrolase activity.

Discussion

The results presented here clearly demonstrate that in both rat and sheep liver the measurement of acetyl-CoA hydrolase activity depends on the presence of endogenous carnitine. When this was substantially removed by passage through a Sephadex G-25 column, no acetyl-CoA hydrolase activity was observed. Also addition of D-carnitine, a competitive inhibitor of carnitine acetyltransferase (Fritz & Schultz, 1965), decreased acetyl-CoA hydrolase activity in both rat and sheep liver homogenates, particularly in the spectrophotometric assay with normal rat liver homogenate where the ratio of D-carnitine to endogenous L-carnitine was greater than in the incubated enzyme assay system. On the other hand, when L-carnitine was added to homogenates from which endogenous L-carnitine was previously removed, then the apparent hydrolase activity reappeared. Finally, it was demonstrated that, although acetyl-CoA hydrolase activity could be measured in whole rat liver mitochondria, neither the outer membrane, which contained acetylcarnitine hydrolase (see preceding paper, Costa & Snoswell, 1975), nor the inner membrane, which contained carnitine acetyltransferase, showed any acetyl-CoA hydrolase. However, on combination of these two membrane fractions acetyl-CoA hydrolase activity could again be measured. These results then clearly show that, in sheep and rat liver, acetyl-CoA hydrolase is an artifact and the activity measured is due to the combined action of two enzymes, carnitine acetyltransferase present in the inner mitochondrial membrane and acetylcarnitine hydrolase present in the outer mitochondrial membrane. The sum of these two reactions does lead to an overall acetyl-CoA hydrolase reaction as indicated below:



$$\text{Acetyl-CoA} + \text{L-carnitine} \leftrightharpoons \text{acetyl-L-carnitine} + \text{CoA}$$



$$\text{Acetyl-L-carnitine} + H_2O \leftrightharpoons \text{acetate} + \text{L-carnitine}$$

$$\text{Sum: Acetyl-CoA} + H_2O \leftrightharpoons \text{acetate} + \text{CoA}$$

This finding has a number of important implications. First, it explains the anomalous situation referred to in the introduction of having two enzymes, namely carnitine acetyltransferase and acetyl-CoA hydrolase, present in the inner mitochondrial membrane both using acetyl-CoA. Second, it provides a system whereby acetyl groups may be removed from the mitochondria as acetate in times of increased 'acetyl pressure'.

The other important finding arising from these studies is that use of the spectrophotometric method for the assay of carnitine acetyltransferase based on the reaction of CoA liberated with 5,5′-dithiobis-(2-nitrobenzoic acid) at 412nm, after adding L-carnitine, underestimates the activity of the enzyme. The initial rate of acetyl-CoA breakdown is attributed to acetyl-CoA hydrolase, but in fact is due to carnitine acetyltransferase reacting with endogenous carnitine. This is particularly so with rat liver. It has been assumed in the past that in this type of assay the small amount of endogenous carnitine present would be insufficient to allow any appreciable measurement of carnitine acetyltransferase. However, the results presented here indicate that the K_m of the rat liver enzyme for L-carnitine, namely 32μM, is considerably less than 120μM reported for pigeon breast-muscle enzyme (Chase, 1967) or 0.31mM for pig heart enzyme (Fritz *et al.*, 1963). Thus previous reports of low carnitine acetyltransferase in rat liver from this (Snoswell & Henderson, 1970) and other laboratories (Barker *et al.*, 1968; Solberg *et al.*, 1972) appear to be underestimates. This also raises some doubts about various activities of carnitine acetyltransferase and short-chain transferases obtained with this assay procedure after various treatments, e.g. clofibrate treatment of rats (Solberg *et al.*, 1972). Slight variations in the concentrations of endogenous carnitine in the liver under various conditions could lead to considerable differences in carnitine acetyltransferase activity.

We are indebted to Dr. F. J. Ballard for helpful discussions and to Mr. R. Fishlock for skilled technical assistance. The work was supported by a grant from the Australian Research Grants Committee. N.D.C. holds an Australian Wool Corp. Postgraduate Scholarship.

References

Allred, J. B. & Guy, G. D. (1969) *Anal. Biochem.* **29**, 293–299

Barker, P. J., Fincham, N. J. & Hardwick, D. C. (1968) *Biochem. J.* **110**, 739–746

Brdiczka, D., Gerbitz, K. & Pette, D. (1969) *Eur. J. Biochem.* **11**, 234–240

Chase, J. F. A. (1967) *Biochem. J.* **104**, 510–518

Chase, J. F. A. & Tubbs, P. K. (1969) *Biochem. J.* **111**, 225–235

Costa, N. D. & Snoswell, A. M. (1974) *Proc. Aust. Biochem. Soc.* **7**, 36

Costa, N. D. & Snoswell, A. M. (1975) *Biochem. J.* **152**, 161–166

Edwards, Y. H., Chase, J. F. A., Edwards, M. R. & Tubbs, P. K. (1974) *Eur. J. Biochem.* **46**, 209–215

Fritz, I. B. & Schultz, S. K. (1965) *J. Biol. Chem.* **240**, 2188–2192

Fritz, I. B., Schultz, S. K. & Srere, P. A. (1963) *J. Biol. Chem.* **238**, 2509–2517

Gergely, J., Hele, P. & Ramakrishnan, C. V. (1952) *J. Biol. Chem.* **198**, 323–334

Hoppel, C. L. & Tomec, R. J. (1972) *J. Biol. Chem.* **247**, 832–841

Knowles, S. E., Jarrett, I. G., Filsell, O. H. & Ballard, F. J. (1974) *Biochem. J.* **142**, 401–411

Marquis, N. R. & Fritz, I. B. (1964) *J. Lipid Res.* **5**, 184–187

Michal, G. & Bergmeyer, H. U. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 517–520, Academic Press, New York and London

Murthy, V. K. & Steiner, G. (1973) *Metab. Clin. Exp.* **22**, 81–84

Pearson, D. J. & Tubbs, P. K. (1964) *Nature (London)* **202**, 91

Quraishi, S. & Cook, R. D. (1972) *J. Agric. Food Chem.* **20**, 91–95

Snoswell, A. M. & Henderson, G. D. (1970) *Biochem. J.* **119**, 59–65

Snoswell, A. M. & Koundakjian, P. P. (1972) *Biochem. J.* **127**, 133–141

Solberg, H. E., Aas, M. & Daae, L. N. W. (1972) *Biochim. Biophys. Acta* **280**, 434–439

Stadtman, E. R. (1957) *Methods Enzymol.* **3**, 931–941

Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332

Biochem. J. (1978) **171**, 299–303
Printed in Great Britain

Deacylation of Acetyl-Coenzyme A and Acetylcarnitine by Liver Preparations

By ALAN M. SNOSWELL* and PHILIP K. TUBBS
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

(*Received* 12 *September* 1977)

The breakdown of acetylcarnitine catalysed by extracts of rat and sheep liver was completely abolished by Sephadex G-25 gel filtration, whereas the hydrolysis of acetyl-CoA was unaffected. Acetyl-CoA and CoA acted catalytically in restoring the ability of Sephadex-treated extracts to break down acetylcarnitine, which was therefore not due to an acetylcarnitine hydrolase but to the sequential action of carnitine acetyltransferase and acetyl-CoA hydrolase. Some 75% of the acetyl-CoA hydrolase activity of sheep liver was localized in the mitochondrial fraction. Two distinct acetyl-CoA hydrolases were partially purified from extracts of sheep liver mitochondria. Both enzymes hydrolysed other short-chain acyl-CoA compounds and succinyl-CoA (3-carboxypropionyl-CoA), but with one acetyl-CoA was the preferred substrate.

The deacylation of both acetyl-CoA and acetylcarnitine could lead to the formation of acetate *in vivo*, and significant amounts of acetate have been shown to be released from the liver in rats (Seufert, *et al.*, 1974), lactating cows (Baird *et al.*, 1975) and sheep (Costa *et al.*, 1976). In the last paper the acetyl-CoA-cleavage activity of liver extracts *in vitro*, prepared from biopsy samples, was sufficient to account for the rates of liver acetate production *in vivo*.

It has been suggested that acetylcarnitine is deacylated in liver by a specific acetylcarnitine hydrolase (Costa & Snoswell, 1975*a*) and that the breakdown of acetyl-CoA in cell-free preparation is due to the combined action of carnitine acetyltransferase (EC 2.3.1.7) and the acetylcarnitine hydrolase (Costa & Snoswell, 1975*b*). By contrast a number of workers had previously suggested that an acetyl-CoA hydrolase (EC 3.1.2.1), originally classified on the basis of a very brief report in a paper dealing with the deacylation of succinyl-CoA (3-carboxypropionyl-CoA) in pig liver (Gergely *et al.*, 1952), was the main enzyme responsible for the deacylation of acetyl-CoA. This enzyme was reported to be predominantly found in the mitochondria and widely distributed in bovine (Quraishi & Cook, 1972) and in rat and sheep tissues (Knowles *et al.*, 1974), with highest activity in the liver. A particularly rich source is the brown fat of the hamster (Bernson, 1976).

In view of the significant rates of production of acetate by the liver under some physiological conditions, it seemed important to investigate the enzymic deacylation of both acetyl-CoA and acetylcarnitine in more detail in order to understand the basis of this acetate production.

* Permanent address: Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064.

Materials and Methods

Tissue preparations

Rat liver homogenates (20%, w/v) were prepared in 25mM-sucrose containing 2.3mM-potassium phosphate buffer (pH 7.4) and 0.1% Triton X-100 by the method of Costa & Snoswell (1975*a*). These homogenates were frozen in liquid N_2, thawed and centrifuged at 10000*g* for 5min, and the supernatant was used to examine the hydrolysis of acetylcarnitine and acetyl-CoA. Where indicated, 2ml samples of supernatant were passed down columns (1.9cm × 15cm) of Sephadex G-25 (medium grade) equilibrated in 25mM-phosphate buffer (pH 7.4). Only the first few ml of eluate containing protein were used in subsequent assays.

Sheep liver, kindly provided by Mr. D. W. Pethick of the A.R.C. Institute of Animal Physiology, Babraham, was collected immediately after slaughter. Homogenates (40%, w/v) were prepared in 0.3M-sucrose/10mM-4-morpholinepropanesulphonic acid/1mM-EGTA adjusted to pH 7.2, by using an electric blender. The homogenate was centrifuged at 500*g* for 10min, and the supernatant filtered through muslin and re-centrifuged at 15000*g* for 10min. The pellet was resuspended in 3vol. of homogenizing buffer and re-centrifuged at 15000*g* for a further 10min. The washed mitochondria were suspended in

a minimum volume of homogenizing buffer and stored at −20°C.

Protein concentrations were measured by a modification of the method of Gornall *et al.* (1949).

Assay of acetylcarnitine hydrolysis

Extracts of rat liver described above were incubated in a mixture containing 100mM-Tris/HCl buffer (pH 8.0) and 10mM-acetyl-L-carnitine in a volume of 1.0ml at 37°C for 20min. The reaction was stopped by the addition of 0.2ml of 15% (w/v) $HClO_4$. L-Carnitine was measured in the neutralized supernatant by using 4,4′-dithiobispyridine in the assay system described by Ramsay & Tubbs (1975).

Assay of acetyl-CoA hydrolysis

At first sight the assay of acetyl-CoA hydrolase presents no difficulty, since the liberated CoA can react with chromophoric reagents such as 5,5′-dithiobis-(2-nitrobenzoic acid) or 4,4-dithiobispyridine. However, tissues contain very large amounts of 3-oxoacyl-CoA thiolases (EC 2.3.1.9 and EC 2.1.3.16) (Middleton, 1975), which can form CoA by the reaction:

$$2\ \text{Acetyl-CoA} \rightleftharpoons \text{acetoacetyl-CoA} + \text{CoA}$$

Although the equilibrium is very unfavourable, a CoA-trapping system can cause the continuous consumption of acetyl-CoA, as mentioned by Kohlhaw & Tan-Wilson (1977). It was also pointed out by Middleton (1974) that under appropriate conditions thiolases can rapidly liberate CoA from acetyl-CoA. In liver mitochondrial extracts this interference by thiolase might be exacerbated by the presence of 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) and 3-hydroxy-3-methylglutaryl-CoA lyase (EC 4.1.3.4), which together in effect deacylate acetoacetyl-CoA. The thiolase interference is avoided by allowing the acetyl-CoA hydrolase reaction to proceed in the absence of a CoA trap, since the formation of acetoacetyl-CoA will be very small (and transient if a hydrolase is also liberating CoA). For this reason we have used an incubated system and subsequent CoA measurement as outlined below for the experiments described in this paper. A direct spectrophotometric assay involving continuous reaction of CoA with 4,4′-dithiobispyridine was only used for the preliminary screening of fractions during enzyme purification. The discontinuous assay was also used for CoA derivatives other than acetyl-CoA, although in these cases thiolase would probably not have caused any interference.

In the routine assay 4mM-acetyl-CoA, 100mM-Tris/HCl buffer (pH 8.0), 10mM-KCl and enzyme were incubated in a volume of 0.1ml for 5min at 30°C. After 2.5 and 5min 30μl samples were removed and placed in cuvettes containing 2.0ml of 100mM-potassium phosphate buffer (pH 7.2), 1mM-EGTA and 125μM-4,4′-dithiobispyridine. The increase in A_{324} was followed for 2min and then extrapolated back to the time of adding the sample to the cuvette, so permitting calculation of the CoA released during the incubation. The rate of CoA release was linear with time for 5min. One unit of enzyme activity is the amount that caused the release of 1μmol of CoA/min under the conditions described. Various concentrations of acetyl-CoA and other acyl-CoA derivatives were used in kinetic experiments.

Preparation and assay of acyl-CoA compounds

Acetyl-CoA, propionyl-CoA, butyryl-CoA and succinyl-CoA were prepared from free CoA and the acid anhydrides, and decanoyl-CoA, lauroyl-CoA and palmitoyl-CoA were prepared from free CoA and the corresponding acyl thioglycollates (Chase & Tubbs, 1972).

The concentrations of acetyl-, propionyl, butyryl- and decanoyl-CoA were determined by reaction with 4,4′-dithiobispyridine at 324nm, after the addition of carnitine acetyltransferase in the presence of excess of carnitine. The concentrations of lauroyl-CoA and palmitoyl-CoA were determined in a similar system but containing ox liver carnitine palmitoyltransferase (prepared by Dr. M. R. Edwards in this Department) in place of carnitine acetyltransferase. Succinyl-CoA was measured by determining the increase in free CoA after alkaline hydrolysis.

Enzymes and chemicals

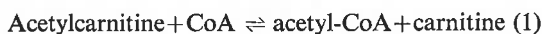
Carnitine acetyltransferase and CoA were obtained from the Boehringer Corp. (London), London W.5, U.K. 4-4′-Dithiobispyridine (4-Aldrithiol) was obtained from the Aldrich Chemical Co., Wembley, Middx., U.K., and L-carnitine was generously supplied by Otsuka Pharmaceutical Co., Tokushima, Japan.

Results

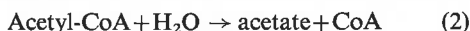
Deacylation of acetylcarnitine by rat liver homogenates

Costa & Snoswell (1975*a*) suggested that the breakdown of acetylcarnitine was catalysed by a specific acetylcarnitine hydrolase. However, in the present work it was found that passage of a high-speed supernatant of either rat (Table 1) or sheep liver homogenate through a column of Sephadex G-25 completely abolished the breakdown of acetylcarnitine, but had no effect on the hydrolysis of acetyl-CoA (cf. Seufert *et al.*, 1976). These results were in direct contrast with those reported by Costa & Snoswell (1975*a*). Further, the addition of small amounts of either CoA or acetyl-CoA to the Sephadex-treated extracts of rat liver acted catalyti-

cally and led to the extensive release of carnitine (Table 1). CoA, acetyl-CoA and carnitine were not detected in the Sephadex-treated extracts. These findings suggested that the breakdown of acetylcarnitine is due to the coupled reactions:



$$\text{Acetylcarnitine} + \text{CoA} \rightleftharpoons \text{acetyl-CoA} + \text{carnitine} \quad (1)$$



$$\text{Acetyl-CoA} + H_2O \rightarrow \text{acetate} + \text{CoA} \quad (2)$$

catalysed respectively by carnitine acetyltransferase and an acetyl-CoA hydrolase.

Purification of mitochondrial acetyl-CoA hydrolases from sheep liver mitochondria

Initial experiments indicated that some 75% of the acetyl-CoA hydrolase activity in sheep liver was found in the mitochondrial fraction, in agreement with Knowles *et al.* (1974). The results in Table 2 show that the specific activity of the mitochondrial fraction was 8-fold greater than that of the initial liver homogenate, and a preparation of sheep liver mitochondria was used as the starting material for enzyme purification.

Frozen mitochondrial suspension (51ml, about 40mg of protein/ml) was thawed and mixed with an equal volume of 50mM-potassium phosphate buffer (pH7.5). The mixture was then sonicated with an MSE Soniprobe at an amplitude of 12μm peak-to-peak for three 20s periods with cooling in an ice bath in between. The preparation was then centrifuged at 106000*g* for 30min.

The supernatant was adjusted to 40% saturation by the addition of 242g of solid $(NH_4)_2SO_4$/litre and after 30min was centrifuged at 30000*g* for 10min. The supernatant was then adjusted to 70% saturation by the further addition of solid $(NH_4)_2SO_4$ (205g/litre) and centrifuged as above. The pellet was dissolved in 5mM-phosphate (pH7.5): this, and all buffers used in later steps, contained 10% (v/v) glycerol and also 0.5mM-dithiothreitol, the omission of which led to rapid inactivation.

The solution was then desalted by passage down a column of Sephadex G-25 (medium grade) previously equilibrated in 5mM-potassium phosphate buffer (pH7.5), and applied to a column (11.5cm × 6.5cm) of DEAE-cellulose previously equilibrated in the same buffer at 5°C. The column was washed with 1 bed vol. of the same buffer and then a linear gradient of phosphate to 50mM applied (total volume 1 litre).

One peak of acetyl-CoA hydrolase activity was eluted with 5mM-phosphate from this DEAE-cellulose column, immediately after the main peak of protein (Fig. 1). A second peak, completely separated from the first, was eluted when the phosphate concentration reached about 25mM. Most of

Table 1. *Deacylation of acetylcarnitine by preparations of rat liver*

Homogenates of rat liver were prepared in hypo-osmotic sucrose containing Triton X-100 as described in the text and, where appropriate, were treated with Sephadex G-25. Release of carnitine from 10mM-acetylcarnitine was measured in a 1ml incubation system as described in the text.

Treatment/addition	Carnitine released (nmol)
Untreated extract (10.7mg protein)	540
Sephadex-treated extract (5.1mg of protein)	None
Sephadex-treated extract+20nmol of acetyl-CoA	316
Sephadex-treated extract+19nmol of CoA	354

Table 2. *Purification of acetyl-CoA hydrolases from sheep liver mitochondria*

Homogenates of sheep liver, the isolation of mitochondria and the purification and assay of acetyl-CoA hydrolase are described in the text.

Fraction	Volume (ml)	Acetyl-CoA hydrolase (units/ml)	Protein (mg/ml)	Specific activity (units/mg of protein)	Purification (fold)
Crude homogenate	410	0.340	52	0.0065	—
Mitochondrial extract	69	1.56	30	0.052	8
40–70%-satd.-$(NH_4)_2SO_4$ fraction	10	8.5	62	0.136	21
Peak 1 from DEAE-cellulose column (pooled)	2.5	8.2	16	0.51	78
Peak 2 from DEAE-cellulose column (pooled)	2.6	5.8	12	0.48	74

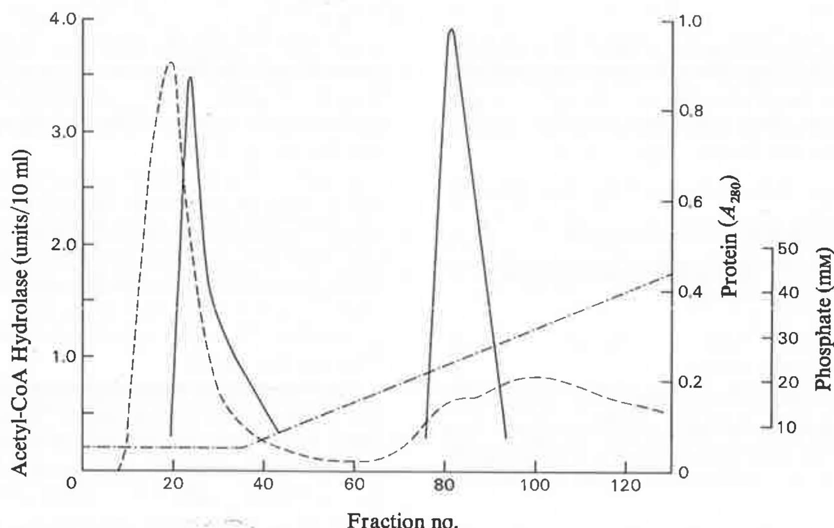


Fig. 1. *Elution profile of acetyl-CoA hydrolase activity and protein from DEAE-cellulose*
A DEAE-cellulose column (11 cm × 6.5 cm diam) previously equilibrated in 5mM-phosphate buffer (pH 7.5) containing 10% glycerol and 0.5mM-dithiothreitol was loaded with about 600mg of protein; for details see the text. The column was washed with 1 bed volume of this buffer and then a linear gradient to 50mM-phosphate was applied: 10ml fractions were collected. Fractions 21–40 and 77–90 were pooled separately. ——, Acetyl-CoA hydrolase activity; ----, protein (A_{280}); –·–·–·, phosphate concentration.

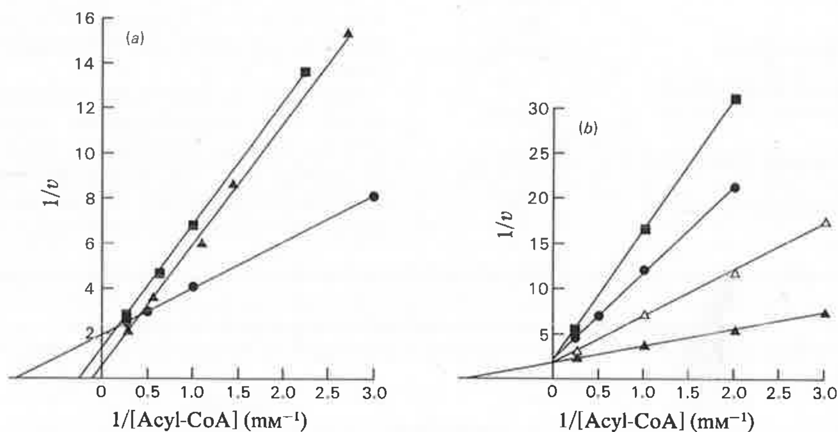


Fig. 2. *Utilization of various acyl-CoA compounds by the first* (*a*) *and second* (*b*) *peaks of acetyl-CoA hydrolase activity isolated from the DEAE-cellulose column* (*see Fig.* 1)
Hydrolase activity was measured as described for acetyl-CoA in the text. For succinyl-CoA, correction was made for the non-enzymic rate of hydrolysis. v is expressed as μmol of CoA released/min per mg of protein. ●, Propionyl-CoA; ▲, acetyl-CoA; ■, succinyl-CoA, △, acetyl-CoA in the presence of 0.8mM-CoA (*b* only).

the protein adsorbed on the column was not eluted with 50mM-phosphate and required higher concentrations for elution, but no further acetyl-CoA hydrolase activity was obtained by this treatment.

The fractions containing the two peaks of acetyl-CoA hydrolase activity were pooled separately and precipitated by adding solid $(NH_4)_2SO_4$ (529 g/litre). After centrifugation at 30000g for 10 min the pellets were dissolved in 5mM-phosphate buffer (pH 7.5) containing 40% (v/v) glycerol and 0.5mM-dithio-

threitol. This concentration step caused some loss of activity, especially for the second peak. Both peaks still contained thiolase activity, with acetoacetyl-CoA as substrate, but in the first peak the specific activity was decreased some 6-fold compared with the original mitochondrial extract and in the second peak by 20-fold.

Properties of the two peaks from the DEAE-cellulose column showing acetyl-CoA hydrolase activity

The material in the first peak eluted from the DEAE-cellulose column hydrolysed propionyl-CoA more rapidly than acetyl-CoA at low substrate concentrations (Fig. 2*a*). Activity with succinyl-CoA was similar to that with acetyl-CoA. Butyryl-CoA was hydrolysed at a similar rate to propionyl-CoA, and decanoyl-CoA, lauroyl-CoA and palmitoyl-CoA were scarcely hydrolysed when tested at concentrations of 0.5–4mM.

By contrast the material in the second peak from the DEAE-cellulose column showed its highest activity with acetyl-CoA (Fig. 2*b*): the K_m value, about 1mM, was considerably lower than that for the first enzyme. Propionyl-CoA, butyryl-CoA and succinyl-CoA showed higher K_m values than acetyl-CoA. Free CoA was a competitive inhibitor (Fig. 2*b*), as it also was with the first enzyme (results not shown). Decanoyl-CoA was a very poor substrate, and lauroyl-CoA and palmitoyl-CoA were not appreciably utilized.

Discussion

In the present work we have not detected acetylcarnitine hydrolase activity in either rat or sheep liver, but have found that extracts of mitochondria from sheep liver contain at least two distinct enzymes that hydrolyse acetyl-CoA and other acyl-CoA compounds. Prass *et al.* (1977) have extensively purified an acetyl-CoA hydrolase from rat liver cytosol which is cold-labile and activated by ATP. An acyl-CoA hydrolase has been partially purified from pig heart mitochondria (Lee, 1977): this enzyme was activated by Ca^{2+} and was more active with decanoyl-CoA than with acetyl-CoA. Neither of the sheep liver hydrolases described here was activated by ATP or Ca^{2+}, and both were relatively inactive with decanoyl-CoA, were stable to storage at −20°C, and had much higher K_m values than the very active enzyme from hamster brown-fat mitochondria (Bernson, 1976). It appears that there are several enzymes capable of hydrolysing acetyl-CoA.

The K_m values for the sheep liver mitochondrial hydrolases are relatively high with respect to acetyl-CoA and the other acyl-CoA compounds utilized, but the mitochondrial concentration of acetyl-CoA can reach more than 1mM (e.g. Garland *et al.*, 1965): the same is true for succinyl-CoA (Smith *et al.*, 1974). The physiological function of the acetyl-CoA hydrolases is obscure, but presumably one or more of them is responsible for the acetate formation by the liver referred to in the introduction. Their competition with other enzymes utilizing acetyl-CoA remains to be elucidated.

The award of a Royal Society and Nuffield Foundation Commonwealth Bursary to A. M. S. is gratefully acknowledged.

References

Baird, G. D., Symonds, H. W. & Ash, R. (1975) *J. Agric. Sci.* **85**, 281–296
Bernson, V. S. M. (1976) *Eur. J. Biochem.* **67**, 403–410
Chase, J. F. A. & Tubbs, P. K. (1972) *Biochem. J.* **129**, 55–62
Costa, N. D. & Snoswell, A. M. (1975*a*) *Biochem. J.* **152**, 161–166
Costa, N. D. & Snoswell, A. M. (1975*b*) *Biochem. J.* **152**, 167–172
Costa, N. D., McIntosh, G. H. & Snoswell, A. M. (1976) *Aust. J. Biol. Sci.* **29**, 33–42
Garland, P. B., Shepherd, D. & Yates, D. W. (1965) *Biochem. J.* **97**, 587–594
Gergely, J., Hele, P. & Ramakrishnan, C. V. (1952) *J. Biol. Chem.* **198**, 323–334
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
Kohlhaw, G. B. & Tan-Wilson, A. (1977) *J. Bacteriol.* **129**, 1159–1161
Knowles, S. E., Jarrett, I. G., Filsell, O. H. & Ballard, F. J. (1974) *Biochem. J.* **142**, 401–412
Lee, K. Y. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 788
Middleton, B. (1974) *Biochem. J.* **139**, 109–121
Middleton, B. (1975) *Methods Enzymol.* **35**, 128–136
Prass, R. L., Isohashi, F. & Utter, M. F. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 761
Quraishi, S. & Cook, R. M. (1972) *J. Agric. Food Chem.* **20**, 91–95
Ramsay, R. R. & Tubbs, P. K. (1975) *FEBS Lett.* **54**, 21–25
Seufert, C. D., Graf, M., Janson, G., Kuhn, A. & Soling, H. D. (1974) *Biochem. Biophys. Res. Commun.* **57**, 901–909
Seufert, C. D., Grigat, K. P., Koppe, K. & Soling, H. D. (1976) *Abstr. Int. Congr. Biochem. 10th* p. 244
Smith, C. M., Bryla, J. & Williamson, J. R. (1974) *J. Biol. Chem.* **249**, 1497–1505

Journal of Dairy Research (1978), **45**, 331–338

Interrelationships between acetylation and the disposal of acetyl groups in the livers of dairy cows

BY ALAN M. SNOSWELL,* NICHOLAS D. COSTA*[1] AND JOCK G. McLEAN†

** Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia and † Department of Veterinary Pre-Clinical Science, University of Melbourne, Parkville, Victoria, Australia*

AND G. DAVID BAIRD, MICHAEL A. LOMAX[2] AND HERBERT W. SYMONDS

Agricultural Research Council, Institute for Research on Animal Diseases, Compton, Newbury, Berkshire, RG16 0NN

(*Received 2 February 1978*)

SUMMARY. Possible mechanisms by which the degree of acetylation of hepatic CoA might be regulated were examined in lactating and in non-lactating dairy cows. This involved the measurement of the hepatic steady-state concentrations of free CoA, acetyl-CoA, free carnitine, acetylcarnitine and total acid-soluble carnitine in freeze-clamped biopsy samples, the measurement of the hepatic release of acetate from acetylcarnitine in vitro, and the measurement of the rate of hepatic output of acetate and ketone bodies in vivo.

The hepatic ratio of [free CoA]/[acetyl-CoA] was 0·11 in lactating cows and 0·59 in non-lactating cows. There was a significant rise in the hepatic concentration of acetyl-CoA and a significant fall in that of total acid-soluble carnitine in the lactating cows as compared with the non-lactating cows.

There was a net in vivo output of acetate and of ketone bodies from the livers of both lactating and non-lactating cows. The rates of output amounted to 7·77 and 2·00 mmole/min for acetate and ketone bodies respectively in the lactating cows, and 4·95 and 2·14 mmole/min in the non-lactating cows.

The rate of enzymic release of acetate from acetylcarnitine in vitro amounted to 0·93 and 0·34 μmole min^{-1} (g wet wt)$^{-1}$ at 37 °C for liver homogenates derived from lactating and non-lactating cows respectively. The activity observed in the case of the lactating cows was sufficient to account for the rate of hepatic acetate output observed in vivo.

Baird, Heitzman & Snoswell (1972) obtained a value of only 0·2 for the ratio of [free CoA]/[acetyl-CoA] in the liver of lactating cows. This value is even lower than that found in sheep liver (0·4–1·0; Snoswell & Henderson, 1970), which in turn is substantially lower than that found in rat liver (3·5; Allred & Guy, 1969). Snoswell & Henderson (1970) suggested that the low [free CoA]/[acetyl-CoA] ratio in sheep liver

[1] Present address: School of Veterinary Studies, Murdoch University, Western Australia.
[2] Present address: Biological Laboratory, The University, Canterbury, Kent, CT2 7NJ, U.K.

implied that the metabolically important coenzyme A system was under substantial 'acetyl-pressure' – tendency to acetylation – in this species and that mechanisms must therefore exist for the relief of this pressure, if sufficient free CoA were to be kept available to allow other CoA-requiring reactions to proceed adequately. From this viewpoint, the necessity for the relief of acetyl-pressure must be even greater in the lactating cow since the value of the ratio is lower, as is the concentration of free CoA (Snoswell & Henderson, 1970).

Snoswell & Koundakjian (1972) considered that the presence of an active carnitine acetyltransferase and relatively high concentrations of carnitine were important in buffering acetyl-pressure in the sheep. However, buffering by this means is unlikely to be of importance in lactating cows since the hepatic concentration of carnitine is low in these animals (Baird *et al.* 1972). The removal of the acetyl moiety from acetyl-CoA and the subsequent release of free acetate would provide an alternative means of disposing of acetyl groups. This could be achieved either by direct enzymic hydrolysis of acetyl-CoA (Quraishi & Cook, 1972; Knowles *et al.* 1974; Snoswell & Tubbs, 1978) or, possibly, by the initial conversion of acetyl-CoA to acetylcarnitine which was then hydrolysed (Costa & Snoswell, 1975*b*). In fact, the net production of acetate by the liver in vivo has now been demonstrated in sheep and has been equated with the enzymic capacity of the ovine liver to produce free acetate (Costa, McIntosh & Snoswell, 1976), while preliminary evidence has been obtained that the bovine liver also produces acetate in vivo (Baird, Symonds & Ash, 1975). Yet another mechanism for disposing of acetyl groups is the conversion of the acetyl moiety of acetyl-CoA into ketone bodies, which are then released. There is good evidence for a net output of ketone bodies from the liver of both sheep and cows (Katz & Bergman, 1969*b*; Baird *et al.* 1975). However, little attempt has been made to compare systematically the relative magnitudes of the hepatic production of acetate and ketone bodies. In fact, the only comparison of this type that has been made in any species appears to be that of Seufert *et al.* (1974), who examined acetate and ketone-body production by the isolated, perfused rat liver, using hexanoate as substrate.

In order to obtain more information on how the cow might deal with the problem of acetyl-pressure it seemed appropriate, therefore, to compare the hepatic concentrations of components of the CoA and carnitine systems with the enzymic ability of the liver to release acetate, and with the actual hepatic output of both acetate and ketone bodies in vivo. The greater susceptibility of lactating cows than of non-lactating cows to ketosis (*cf.* Baird, 1977), and presumably, therefore, to increased hepatic ketogenesis, suggested that acetyl-pressure might be higher in the lactating state than in the non-lactating state since, in the case of the rat at least, low values for the [free CoA]/[acetyl-CoA] ratio tend to be associated with elevated rates of hepatic ketogenesis (e.g. Williamson *et al.* 1969). Consequently, in the present work, the relationship between acetyl-pressure and possible mechanisms for its relief has been examined in non-lactating as well as in lactating cows.

MATERIALS AND METHODS

Animals

For determination of hepatic metabolite concentrations and the enzymic release of acetate in vitro. Four non-lactating and 4 lactating cows were used. The non-lactating cows were predominantly Jersey and were all 3 years of age. The lactating cows were all Jersey ×Friesian animals in the fifth month of their second lactation. The cows were grazed on irrigated perennial pasture consisting mainly of white clover, cocksfoot, paspallum and ryegrass. No supplementary feed was given. The cows were generously supplied by the Victorian Department of Agriculture.

For determination of hepatic production rates in vivo. Four non-pregnant Friesian × Ayrshire cows were used, 2 of which were non-lactating and 2 lactating. The cows had all been catheterized as described by Symonds & Baird (1973) at least 3 weeks prior to the experiment. At the time of the experiment health and appetite were normal. One of the lactating cows had calved 1 month previously and the other 2 months previously, while the milk yield of each of the lactating animals was about 13–14 kg/d. All 4 cows were fed a daily maintenance ration of 5 kg hay and 1 kg dairy concentrate. In addition, the lactating cows received 0·4 kg concentrate/kg milk produced daily. Half the daily feed was given at 07.30 h and half at 13.30 h.

Materials

Substrates and enzymes for the determination of metabolite concentrations in liver and blood, and of the enzymic release of acetate in liver, were obtained from Boehringer Corporation (London) Ltd, Lewes, Sussex, England. Para-aminohippuric acid, used for the determination of blood flow-rate, was obtained from Sigma Chemical Co. Ltd, Kingston-upon-Thames, Surrey, England. Other chemicals were of analytical grade. Double distilled water, the second distillation being from glass, was used throughout.

Tissue preparations and homogenates

Liver tissue for the determination of hepatic metabolite concentrations and the enzymic release of acetate was obtained from the non-lactating and lactating cows by biopsy according to the method of Baird & Heitzman (1970). Separate portions of biopsy samples were freeze-clamped and collected fresh into cold 0·25 M-sucrose–23 mM-potassium phosphate buffer (pH 7·4). Perchloric acid extracts of all freeze-clamped tissue were prepared as described by Snoswell & Henderson (1970) and used for the determination of metabolite concentrations. The fresh liver tissue was homogenized with a Potter–Elvehjem homogenizer (Jencons (Scientific) Ltd, Hemel Hempstead, Herts., England) in 0·25 M-sucrose–23 mM-potassium phosphate (pH 7·4) to yield homogenates with a liver tissue content of 40 % (w/v). These were then used to measure acetate production in vitro.

Production rates in vivo

Hepatic production rates of acetate, D-3-hydroxybutyrate and acetoacetate were determined in each animal on each of 3 separate d. On each of these days, beginning at 09.30 h, 2 % (w/v) Na *p*-aminohippurate was first infused via a mesenteric-vein catheter at a constant rate of about 5 ml/min for at least 90 min. Subsequently,

while the infusion continued, sets of blood samples were taken simultaneously from a carotid artery, the portal vein and an hepatic vein and collected either in heparin (for measurement of the concentration of *p*-aminohippurate and of packed cell volume) or in 6 % (w/v) perchloric acid (for determination of metabolite concentrations). Following measurement of the concentration of *p*-aminohippurate and of the relevant metabolites in the blood samples taken from the 3 different locations, portal and hepatic flow-rates and hepatic metabolite production rates were calculated as described for sheep by Katz & Bergman (1969*a*). In order to calculate the production rates, whole-blood flow-rates were used. These were computed from the plasma flow-rates, determined from the concentrations of *p*-aminohippurate in plasma, by multiplying by 1/(1-haematocrit).

Metabolite assays

Hepatic metabolites. The perchloric-acid extracts of liver samples were adjusted to pH 6·5 with 3N-KOH and centrifuged at 5 °C to remove potassium perchlorate. The supernatants were then used for subsequent assays. Acetylcarnitine was determined by the method of Pearson & Tubbs (1964) and total acid-soluble carnitine by the method of Pearson & Tubbs (1967). Free carnitine was determined by the method of Marquis & Fritz (1964). Acetyl-CoA and total CoA (that is acetyl-CoA + free CoA) were determined by the kinetic method of Allred & Guy (1969) with some modifications as described by Snoswell & Koundakjian (1972).

Blood metabolites. Para-aminohippurate was determined in plasma, obtained by centrifuging whole blood, by the method of Harvey & Brothers (1962). The concentrations of D-3-hydroxybutyrate, acetoacetate and acetate were determined in the neutralized perchloric-acid extracts of whole blood (see Baird & Heitzman, 1970). D-3-Hydroxybutyrate and acetoacetate were determined by the methods of Williamson & Mellanby (1974) and Mellanby & Williamson (1974) respectively. Acetate was measured spectrophotometrically by reaction with CoA in the presence of acetyl-CoA synthetase (E.C. 6.2.1.1) to form acetyl-CoA. In the presence of both malate dehydrogenase (E. C. 1.1.1.37) and citrate synthase (E.C. 4.1.3.7) the acetyl-CoA then condensed with oxaloacetate, derived from malate, to form citrate. The reduction of NAD^+ in the malate dehydrogenase reaction then gave a measure of acetate concentration. The final volume in the cuvette of 1-cm light path was 3·03 ml and this contained 100 mM-potassium phosphate buffer (pH 7·6); 20 mM-potassium malate; 5 mM-neutralized ATP; 10 mM-neutralized glutathione; 0·2 mM-CoA; 1 mM-NAD; 0·1 mM-NADH; 3·3 mM-$MgCl_2$; 20 μg citrate synthase; 50 μg malate dehydrogenase and 200 μg acetyl-CoA synthase. Increase in absorbance was measured at 340 nm.

Enzymic production of acetate in vitro

The enzymic production of acetate by the liver homogenates was measured by hydrolysis of acetylcarnitine at 37 °C according to the method of Costa & Snoswell (1975*a*). It should be noted that subsequent work (Snoswell & Tubbs, 1978) has shown that the enzymic hydrolysis of acetylcarnitine in fact proceeds via acetyl-CoA, and thus this activity also gives an indication of acetyl-CoA hydrolysis (see also Results and Discussion).

Table. 1. *Liver concentrations of free CoA, acetyl-CoA, total CoA, free carnitine, acetylcarnitine and total acid-soluble carnitine in non-lactating and lactating cows*

	Concentrations			[free CoA]/[acetyl-CoA]
Cows	Free CoA	Acetyl-CoA	Total CoA	ratio
Non-lactating	34·2 ± 13·1	60·1 ± 3·7	94·4 ± 17·2	0·59 ± 0·31
Lactating	9·1 ± 1·7	86·1 ± 2·1**	92·8 ± 3·8	0·11 ± 0·02*
	Concentrations			[free carnitine]/[acetylcarnitine]
Cows	Free carnitine	Acetyl-carnitine	Total acid-soluble carnitine	ratio
Non-lactating	26·5 ± 4·3	23·9 ± 4·9	67·3 ± 7·4	1·34 ± 0·42
Lactating	14·8 ± 4·0	11·2 ± 3·6	24·4 ± 5·6**	2·11 ± 0·98

Concentrations are expressed as nmole/g wet wt of tissue and the values are means for 4 animals in each group ± S.E.M. * $P < 0·05$ and ** $P < 0·01$ compared with the non-lactating cows.

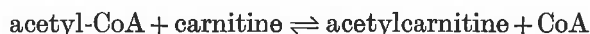
Statistics

Probability values (P) were obtained by Student's t test.

RESULTS AND DISCUSSION

As Table 1 shows, the hepatic concentration of acetyl-CoA was significantly higher in the lactating cows than in the non-lactating cows, although the hepatic concentration of total CoA was very similar in the 2 groups. The proportion of total CoA that was in the acetylated form was thus higher in the lactating cows and this fact is reflected in the hepatic [free CoA]/[acetyl-CoA] ratio, which was significantly lower in the lactating cows than in the non-lactating cows. The value for the [free CoA]/[acetyl-CoA] ratio in the lactating cows, i.e. 0·11, is similar to the value of 0·2 previously found in lactating cows by Baird *et al.* (1972). By contrast, there was little difference in the hepatic [free carnitine]/[acetylcarnitine] ratios found in the lactating and non-lactating groups of cows. In spite of this lack of difference, the hepatic concentration of total acid-soluble carnitine was significantly lower in the lactating than in the non-lactating cows.

The decrease in the hepatic [free CoA]/[acetyl-CoA] ratio from 0·59 in the non-lactating cows to 0·11 in the lactating cows suggests that the bovine hepatic CoA system is indeed subject to increased acetyl-pressure during lactation. However, there was clearly a substantial degree of acetylation of CoA in both groups of cows. In fact, the data in Table 1 confirm the tentative conclusion of Baird *et al.* (1972) that the hepatic ratio of [free CoA]/[acetyl-CoA] is low, and the acetyl-pressure high, in cows as compared with other species so far examined. Thus, even the value of 0·59 found in the non-lactating cows is still only of a similar order to the lowest values found in fed sheep (Snoswell & Henderson, 1970). In the non-lactating state the acetyl-pressure may be buffered by the transfer of acetyl groups to carnitine via carnitine acetyltransferase (E.C. 2.3.1.7), since substitution of the figures given in Table 1 yields a value of 0·5 for the mass action ratio of the reaction:



$$\text{acetyl-CoA} + \text{carnitine} \rightleftharpoons \text{acetylcarnitine} + \text{CoA}$$

Table 2. *Rates of production of acetate,* D*-3-hydroxybutyrate and acetoacetate by the liver in non-lactating and in lactating cows in vivo*

Metabolite	Production rate		
	Non-lactating	Lactating	Pooled
Acetate	+4·95±0·84	+7·77±2·89	+6·36±1·49
D-3-hydroxybutyrate	+2·93±0·31	+2·83±0·35	+2·88±0·22
Acetoacetate	−0·79±0·05	−0·83±0·08	−0·81±0·04
D-3-hydroxybutyrate +acetoacetate	+2·14±0·26	+2·00±0·28	+2·07±0·18

Rates of production are expressed as mmole/min ± S.E.M. + indicates net output and − net uptake. Each value for the non-lactating cows and the lactating cows represents the mean of values obtained on 3 separate d from each of 2 cows, i.e. 6 observations in all. Each pooled value then represents the mean of all 12 observations.

in the non-lactating group. This value is very close to the apparent equilibrium constant of 0·6 that was determined at pH 7·0 using partly purified carnitine acetyltransferase from pig liver (Fritz, Schultz & Srere, 1963). In the lactating cows, however, the computed value for the mass action ratio is 0·08. Besides this, there was only a very low hepatic concentration of total acid-soluble carnitine in this group. The value of 0·08 deviates substantially from the value of 0·6 obtained by Fritz *et al.* (1963), and consequently the carnitine buffer system is not likely to be of importance for relieving acetyl-pressure during lactation (*cf.* Baird *et al.* 1972).

Three possible means by which acetate could be removed from combination with coenzyme A within the liver are by hydrolysis of acetyl-CoA to yield free acetate, by condensation of acetyl-CoA molecules to yield acetoacetyl-CoA and hence ketone bodies, and by condensation of acetyl-CoA with oxaloacetate and consequent oxidation in the tricarboxylic acid cycle. The extent to which the first 2 of these processes are occurring in vivo can conveniently be determined in cows which have been appropriately catheterized so that net output of metabolites from the liver can be measured under normal husbandry conditions (see Methods).

The hepatic production rates of acetate and ketone bodies that were observed in 2 catheterized non-lactating cows and in 2 catheterized lactating cows are given in Table 2. The livers of both the lactating and the non-lactating cows were found to produce acetate, and the rates of production were similar in the 2 instances. The livers of both groups of cows also produced hydroxybutyrate, but took up acetoacetate. Since acetoacetate uptake was less than hydroxybutyrate output there was a net production of ketone bodies. Once again, however, there were no differences in the rates of production in the 2 groups of cows. The pooled value for hepatic acetate production in all the cows was 6·36 mmole/min (Table 2), while that for net ketone-body production was 2·07 mmole/min. It is clear, therefore, that even on a carbon basis the hepatic production of acetate was as great or greater than the net hepatic production of ketone bodies. Table 3 gives the mean concentrations of acetate, hydroxybutyrate and acetoacetate found in carotid, portal and hepatic-venous blood in the catheterized cows and used for calculating the production rates given in Table 2. It will be seen that hydroxybutyrate concentrations decrease in the order hepatic-venous > portal > carotid. The situation with acetate, however, is that

Table 3. *Concentrations of acetate,* D-*3-hydroxybutyrate and acetoacetate in blood at the 3 sampling sites in non-lactating and in lactating cows*

Metabolite	C	P	H
		Non-lactating	
Acetate	1·59 ± 0·08	2·71 ± 0·07	2·71 ± 0·08**
D-3-hydroxybutyrate	0·61 ± 0·04	0·76 ± 0·05	0·88 ± 0·07*
Acetoacetate	0·025 ± 0·002	0·068 ± 0·007	0·018 ± 0·002***
		Lactating	
Acetate	1·51 ± 0·04	2·44 ± 0·15	2·45 ± 0·04**
D-3-hydroxybutyrate	0·63 ± 0·05	0·77 ± 0·04	0·84 ± 0·05*
Acetoacetate	0·035 ± 0·002	0·072 ± 0·004	0·029 ± 0·001***

Concentrations are expressed as μmole/ml of whole blood and are each the means of 6 values (2 cows sampled on 3 d, see Table 2) ± S.E.M. C, carotid site; P, portal site; H, hepatic-venous site. *$P < 0·05$ with respect to carotid value; **$P < 0·01$ with respect to carotid value; ***$P < 0·001$ with respect to portal value.

hepatic-venous = portal > carotid. With acetoacetate it is hepatic-venous < carotid < portal. It thus appears that free CoA can be liberated from acetyl-CoA by the net formation of ketone bodies and acetate in both lactating and non-lactating cows. The results therefore extend the original observation of Baird *et al.* (1975) that there is a net production of acetate by the liver of the lactating cow in vivo, since they demonstrate that hepatic production of acetate can occur in the non-lactating cow as well. Furthermore, the rate of acetate production was found to be very similar in the 2 groups of cows. The rates of hepatic acetate output that were determined in the present work are lower than those previously observed by Baird *et al.* (1975) in lactating cows. The rate of hepatic output of acetate measured in the present study appears, however, to correspond more closely than did the earlier values to the enzymic capacity for acetate release from the liver (see below).

In order to consolidate the claim that there is a net output of acetate from bovine liver in vivo, it is necessary to demonstrate that sufficient activity of an appropriate enzyme is present to account for the observed in vivo rate of acetate release. Measurement of the hydrolysis of acetylcarnitine (which proceeds via acetyl-CoA) in the same cows in which the hepatic metabolite concentrations were determined gave values for acetate release in vitro of $0·34 \pm 0·04\ \mu$mole min^{-1} (g wet wt)$^{-1}$ and $0·93 \pm 0·04\ \mu$mole min^{-1} (g wet wt)$^{-1}$ in the non-lactating and lactating cows respectively (mean of 4 cows in each group ± S.E.M.). If liver weight is taken to be 6000 g, then these values correspond to total hepatic outputs of about 2·0 and 5·6 mmole/min respectively. The capacity of this reaction therefore corresponds reasonably closely to observed in vivo acetate output in the lactating cows, but not in the non-lactating cows. At the time when these experiments were being carried out it was considered that the hydrolysis of acetylcarnitine was the primary reaction leading to the production of acetate (see Costa & Snoswell, 1975*b*). Very recently, however, Snoswell & Tubbs (1978) have shown that in sheep liver, at least, the primary reaction is in fact the direct hydrolysis of acetyl-CoA, catalysed by an acetyl-CoA hydrolase (E.C. 3.1.2.1), since sheep liver homogenates hydrolysed acetyl-CoA at 3 times the rate of acetylcarnitine. Use of acetylcarnitine as substrate

would, therefore, give an underestimate of the capacity of sheep liver for acetate production. If the same situation applies in bovine liver, then the values for acetate production in vitro, obtained with acetylcarnitine as substrate, would suggest that sufficient enzymic capacity would be present to account for the observed rate of acetate output in vivo in the non-lactating cows as well.

If the hepatic productions of acetate and ketone bodies are assumed to represent mechanisms for relieving acetyl-pressure, it then becomes somewhat difficult to reconcile the observed differences in the hepatic [free CoA]/[acetyl-CoA] ratio with the fact that the rates of production of acetate and ketone bodies were similar in the lactating and non-lactating cows in the present study. Clearly, other factors must also be involved in determining the prevailing value of the [free CoA]/[acetyl-CoA] ratio.

The Australian authors are indebted to the State Research Farms of the Victorian Department of Agriculture for access to their animals, and to Mr R. Fishlock for skilled technical assistance. N.D.C. was supported by a Scholarship from the Australian Wool Corporation. A.M.S. is grateful for the support of a Royal Society and Nuffield Foundation Commonwealth Bursary during the preparation of the manuscript. The work was supported by grants from the Australian Research Grants Committee and the University of Melbourne Veterinary Research Fund.

The British authors thank Mrs Denise Mather and Mrs Allison Brown for skilled technical assistance. M.A.L. was in receipt of an Agricultural Research Council Research Studentship.

REFERENCES

ALLRED, J. B. & GUY, D. G. (1969). *Analytical Biochemistry* **29**, 293.
BAIRD, G. D. (1977). *Biochemical Society Transactions* **5**, 819.
BAIRD, G. D. & HEITZMAN, R. J. (1970). *Biochemical Journal* **116**, 865.
BAIRD, G. D., HEITZMAN, R. J. & SNOSWELL, A. M. (1972). *European Journal of Biochemistry* **29**, 104.
BAIRD, G. D., SYMONDS, H. W. & ASH, R. (1975). *Journal of Agricultural Science* **85**, 281.
COSTA, N. D. & SNOSWELL, A. M. (1975*a*). *Biochemical Journal* **152**, 161.
COSTA, N. D. & SNOSWELL, A. M. (1975*b*). *Biochemical Journal* **152**, 167.
COSTA, N. D., MCINTOSH, G. H. & SNOSWELL, A. M. (1976). *Australian Journal of Biological Sciences* **29**, 33.
FRITZ, I. B., SCHULTZ, S. K. & SRERE, P. A. (1963). *Journal of Biological Chemistry* **238**, 2509.
HARVEY, R. B. & BROTHERS, A. J. (1962). *Annals of the New York Academy of Sciences* **102**, 46.
KATZ, M. L. & BERGMAN, E. N. (1969*a*). *American Journal of Physiology* **216**, 946.
KATZ, M. L. & BERGMAN, E. N. (1969*b*). *American Journal of Physiology* **216**, 953.
KNOWLES, S. E., JARRETT, I. G., FILSELL, O. H. & BALLARD, F. J. (1974). *Biochemical Journal* **142**, 401.
MARQUIS, N. R. & FRITZ, I. B. (1964). *Journal of Lipid Research* **5**, 184.
MELLANBY, J. & WILLIAMSON, D. H. (1974). In *Methods of Enzymatic Analysis*, 2nd edn, vol. 4, p. 1840. (Ed. H. U. Bergmeyer). New York: Academic Press.
PEARSON, D. J. & TUBBS, P. K. (1964). *Nature, London* **202**, 91.
PEARSON, D. J. & TUBBS, P. K. (1967). *Biochemical Journal* **105**, 953.
QURAISHI, S. & COOK, R. M. (1972). *Journal of Agricultural and Food Chemistry* **20**, 91.
SEUFERT, C. D., GRAF, M., JANSON, G., KUHN, A. & SÖLING, H. D. (1974). *Biochemical and Biophysical Research Communications* **57**, 901.
SNOSWELL, A. M. & HENDERSON, G. D. (1970). *Biochemical Journal* **119**, 59.
SNOSWELL, A. M. & KOUNDAKJIAN, P. P. (1972). *Biochemical Journal* **127**, 133.
SNOSWELL, A. M. & TUBBS, P. K. (1978). *Biochemical Journal* **171**, 299.
SYMONDS, H. W. & BAIRD, G. D. (1973). *Research in Veterinary Science* **14**, 267.
WILLIAMSON, D. H. & MELLANBY, J. (1974). In *Methods of Enzymatic Analysis*, 2nd edn, vol. 4, p. 1836. (Ed. H. U. Bergmeyer). New York: Academic Press.
WILLIAMSON, D. H., VELOSO, D., ELLINGTON, E. V. & KREBS, H. A. (1969). *Biochemical Journal* **114**, 575.

Printed in Great Britain

Quarterly Journal of Experimental Physiology (1979) **64**, 7–15

Reprinted from the QUARTERLY JOURNAL OF EXPERIMENTAL PHYSIOLOGY
VOL. LXIV, 1979

Effect of Cold Exposure on Mammary Gland Uptake of Fat Precursors and Secretion of Milk Fat and Carnitine in the Goat

E. M. THOMSON,* A. M. SNOSWELL,** P. L. CLARKE*** and G. E. THOMPSON****

From the Departments of Physiology, Hannah Research Institute, Ayr KA6 5HL, Scotland, and Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, England

(RECEIVED FOR PUBLICATION 1 JUNE 1978)

Measurements of mammary gland uptake of milk fat precursors and milk lipid secretion and composition were made in lactating goats exposed for 2 days to each of three different environments. These were designated thermoneutral (21°C), mildly cold (0°C, still air) and moderately cold (0°C with a wind speed of $3{\cdot}6\,m\,.\,s^{-1}$). Milk and blood carnitine concentrations were measured in lactating goats exposed for 1 day to thermoneutral and moderately cold environments.

Exposure to cold significantly decreased milk secretion rate. During moderate, but not mild, cold exposure mammary blood flow also was decreased significantly. Cold exposure caused a decrease in the arterial plasma concentrations of acetate and triglyceride but an increase in plasma free fatty acid concentration. Mammary gland uptake of acetate and triglyceride was decreased while net free fatty acid uptake was increased in the cold. Total milk triglyceride secretion rate was maintained in the cold, despite the fall in milk volume, but its composition was altered with the % of triglyceride fatty acids 16 carbons and greater in length being increased while the % of fatty acids less than 16 carbons in length was decreased. Blood carnitine concentration and carnitine secretion in milk was decreased during cold exposure.

These results suggest that the effect of cold exposure on milk fat composition is due to changes in the relative rates of supply and uptake of short and long-chain fatty acids by the mammary gland.

The decreased availability of carnitine in the milk could have important consequences in the metabolism and survival of the offspring.

In cows and goats milk yield is reduced during exposure to a cold environment [Cobble and Herman, 1951; Thompson and Thomson, 1977]. There are also changes in milk composition and, in particular, milk fat composition in the cold. Fatty acids of chain length C_8–C_{14}, which are normally synthesised *de novo* in the mammary gland from circulating acetate and β hydroxybutyrate, constitute a smaller proportion of milk fat, whereas C_{18} acids, which are

*Present address: Max-Planck-Institut für Physiologische und Klinische Forschung, W. G. Kerckhoff-Institut, D-6350 Bad Nauheim. West Germany.

**Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064, Australia.

***Department of Physiology, Hannah Research Institute, Ayr KA6 5HL, Scotland.

****Department of Physiology, Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, England.

Printed in Great Britain

synthesised from triglyceride and free fatty acids taken up by the gland, are proportionately increased [Clarke, Thomson and Thompson, 1976]. These changes in milk fat composition could be the result of changes in the rate of supply of fat precursors to the mammary gland. It was the purpose of the present study to examine the effect of cold stress on the supply of lipid precursors to the mammary gland in goats, their uptake by the gland and their subsequent output in the form of milk fat. In addition carnitine, which is an essential co-factor in the oxidation of fatty acids [Fritz, 1964], was measured in blood and milk during cold exposure, as cold stress in rats produced a marked increase in body pool of carnitine [Therriault and Mehlman, 1965].

Methods

Animals. Ten female British Saanen goats aged from 4 to 10 years, in their third to ninth lactations, and weighing 49–57 kg were used. They were housed in an insulated byre and fed hay *ad libitum* and 1200 g of concentrates per day, given in two equal meals at the times of milking (07.30 and 16.00 h). The animals ate all their concentrate ration in each environment. Although hay intake was not measured in the present experiments it has been shown previously that these levels and duration of cold stress do not significantly affect the free intake of hay by goats [Thompson and Thomson, 1977]. Animals were surgically prepared for measurement of mammary blood flow [Thompson and Thomson, 1977] before lactation. Experiments were carried out between weeks 4 and 35 in lactation.

Experimental procedure. Five animals were exposed for 2 days to each of three different environments designated thermoneutral, mild cold and moderate cold. The thermoneutral room temperature was $21{\cdot}0 \pm 1{\cdot}0$°C while the mildly cold room temperature was $0 \pm 0{\cdot}5$°C. A moderately cold environment was provided by blowing air at $3{\cdot}6\,m\,.\,s^{-1}$ from a large fan placed behind the animal in a room temperature of $0 \pm 0{\cdot}5$°C. Mild and moderate cold environments have been shown previously to increase metabolism by 18% and 46% respectively above that in a thermoneutral environment [Thompson and Thomson, 1977]. In each experiment two sets of blood samples were taken daily, at 10.00 h and 15.00 h to measure mammary blood flow twice. The 15.00 h arterial and venous samples were also used for measurement of plasma triglyceride, free fatty acid and acetate concentrations. Milk collected from the 16.00 h milking on each day was used for measurement of milk triglyceride concentration and fatty acid composition.

In a further series of experiments five animals were exposed to thermoneutral and moderately-cold environments for 1 day only. In these experiments carnitine concentration was measured in arterial blood sampled at 10.00 h and 15.00 h and also in milk from the 16.00 h milking.

Measurements. Milk secretion rate, mammary blood flow and haematocrit were measured as previously described [Thompson and Thomson, 1977].

The concentration of acetate in plasma was estimated by the method of Gardner and Thompson [1974] but using *n*-valeric acid as an internal standard. The concentration of free fatty acids (C_{16}–C_{18}) in plasma was estimated by the method described by Darling, Findlay and Thompson [1974] but the initial lipid extraction was by the method of Folch, Lees and Sloane-Stanley [1957] adapted by Nelson and Freeman [1959]. Plasma glycerol was measured after hydrolysing plasma triglyceride with potassium hydroxide and then estimating total glycerol concentration using glycerokinase (EC 2.7.1.30) (Boehringer Corporation, London, U.K.) and conversion of $NADH_2$ to NAD. On a duplicate (non-hydrolysed) sample of blood, free glycerol was measured as above. Triglyceride glycerol was calculated by subtraction of the free glycerol value from the total glycerol value.

The uptake of substrates by the mammary gland was calculated from measurements of mammary blood flow, haematocrit, and carotid arterial and subcutaneous venous plasma concentrations of various substrates according to the equation

$$U_M = Q_M \times \frac{1}{1000}\left(\frac{100 - Ht}{100}\right)(C_A - C_V)$$

where U_M = mammary uptake (μmol . min^{-1})
Q_M = mammary blood flow (ml . min^{-1})
Ht = haematocrit (%)
C_A = concentration of substrate in carotid arterial plasma (μmol . l^{-1})
C_V = concentration of substrate in subcutaneous venous plasma (μmol . l^{-1})

The extraction of a substrate was calculated from the equation

$$E = \frac{A - V}{A} \times 100$$

where
E = extraction percentage
A = arterial concentration of substrate (μmol . l^{-1})
V = venous concentration of substrate (μmol . l^{-1})

Blood and milk carnitine was measured using a modification of the radiochemical enzymic method of Cederblad and Lindsted [1972]. Two ml samples of blood or milk were collected directly into 2 ml of 15% perchloric acid, centrifuged to remove protein and 1 ml of the supernatant was neutralized with 3·5 N potassium hydroxide and 2 N potassium bicarbonate. Fifty μl of sample was incubated with 33 mM sodium phosphate, 0·8 mM 4,4′dihyridyl disulphide (Aldrich Chemical Co. Ltd., 264 Walter Road, Wembley, Middlesex, U.K.), 80 μM ^{3}H acetyl-CoA (25 μC . μmole^{-1} provided by Dr. Stephen Mann, ARC Institute of Animal Physiology, Babraham, Cambridge) and 60 μg bovine plasma albumin (Armour Pharmaceutical Co., Eastbourne, U.K.) in a final volume of 120 μl. The mixture was incubated at 39°C for 20 min and the

reaction started by the addition of 1 μl (400 u . ml^{-1}) of carnitine acetyltransferase (EC 2.3.1.7) (Boehringer Corporation). The reaction was stopped by the addition of 80 μl of 0·15 N perchloric acid and the residual ^{3}H acetyl CoA separated from the ^{3}H acetylcarnitine formed on small columns (0·65 × 10 cm) of Deacidite FF-IP SRA 63 (Permutit Co., 632/652 London Road, Isleworth, Middlesex, U.K.) and radioactivity measured in a procedure described by Hebb, Mann and Mead [1975]. In this method a linear response between ^{3}H acetylcarnitine formed and carnitine added, up to 1000 p moles, was obtained.

Milk triglyceride fatty acid composition was measured as follows. After addition of a known amount of internal standard (triheptadecanoin), the triglycerides were extracted with 2:1 chloroform:methanol and the organic layer separated. This was extracted with 0·88% aqueous potassium chloride and separated again. The extract was then evaporated to dryness under reduced pressure and the triglyceride fatty acids transesterified with sodium methoxide. When the reaction was complete, hexane and anhydrous calcium chloride were added and the mixture left to stand at room temperature for 1 h. The mixture was finally centrifuged at 3000 r.p.m. for 3 min and 6 μl of the supernatant was injected onto a gas–liquid chromatographic column. A Pye Unicam Model 104 dual-column gas chromatograph fitted with flame ionization detectors was used and analyses were carried out using glass columns (214 mm × 6 mm o.d.) packed with 15% EGGS-X on Gas-Chrom P (100–120 mesh) (Field Instruments). Separation of the fatty acids was effected at a temperature of 90°C for an initial period of 4 min, followed by a rise of 5°C . min^{-1} to a final temperature of 190°C. The seven largest fatty acid peaks were chosen for measurement and these represented approximately 90% of the total fatty acids in milk. The fatty acid nomenclature used in the results is that of Farquhar, Insull, Rosen, Stoffel and Ahrens [1959].

Statistics. There was no significant difference between the mean values obtained in day 1 and day 2 in each treatment for all variables measured and so the average value over the 2-day period has been used. Results obtained in the thermoneutral environment were compared with those from the mildly- and moderately-cold environments using Student's paired t-test.

Results

Effect of cold exposure on milk secretion and mammary blood flow

As found previously [Thompson and Thomson, 1977], milk secretion rate decreased as the severity of cold stress increased (Table I). Milk secretion in the mildly cold environment just failed to be significantly different from that in the thermoneutral environment but was significantly decreased ($P < 0·05$) to 65% of its thermoneutral value in the moderately cold environment.

Mammary blood flow and plasma flow were unaffected by the mild cold stress but decreased to approximately 75% of the thermoneutral value during moderate cold stress ($P < 0·05$). Haematocrit increased in the cold but this change was not significant.

Effect of cold on plasma levels and uptake of fat precursors by the mammary gland

The arterial concentration of acetate fell below thermoneutral values ($P < 0{\cdot}05$) (Table II) during cold exposure and this was associated with a decrease in acetate uptake by the mammary gland, which was significant in the moderately cold environment ($P<0{\cdot}05$). The extraction of circulating acetate, which averaged 75 %, was not significantly affected by environment.

There was a small net uptake of free fatty acids by the mammary gland in a thermoneutral environment but this changed to a large net uptake in the moderately-cold environment ($P<0{\cdot}01$). Net extraction of free fatty acids by the gland was significantly increased ($P<0{\cdot}05$) during moderate cold exposure. Fatty acids are released into the blood leaving the mammary gland as a result of hydrolysis of plasma triglyceride [West, Annison and Linzell, 1967] and thus the net free fatty acid difference across the gland does not give a true indication of fatty acid uptake. Consequently the total fatty acid (free fatty acid + triglyceride fatty acid) concentration was calculated for arterial and venous

Table I. *The effect of cold exposure on milk secretion and mammary blood flow. Mean ± s.e. of mean for five animals in each treatment.*

	Thermoneutral	Mild cold	Moderate cold
Milk secretion rate ($ml.min^{-1}$)	0·668 ± 0·052	0·583 ± 0·079	0·437 ± 0·066*
Mammary blood flow ($ml.min^{-1}$)	349 ± 41	384 ± 51	268 ± 31*
Haematocrit (%)	22 ± 1	24 ± 1	24 ± 1
Mammary plasma flow ($ml.min^{-1}$)	274 ± 24	290 ± 27	203 ± 24*

Significance of difference from thermoneutral value *$P<0{\cdot}05$.

Table II. *The effect of cold exposure on arterial (A), mammary venous (V) concentration, and mammary uptake of substrates. Mean ± s.e. of mean for five animals in each treatment.*

		Thermoneutral	Mild cold	Moderate cold
Acetate	*A*	1553 ± 204	934 ± 114*	801 ± 48*
	V	333 ± 59	247 ± 74	225 ± 18
	$(A-V)100/A$	79 ± 3	75 ± 5	72 ± 2
	Uptake	363 ± 88	197 ± 37	100 ± 12*
Free fatty acids	*A*	372 ± 49	613 ± 201	868 ± 41†
	V	357 ± 31	530 ± 95	584 ± 36
	$(A-V)100/A$	−2 ± 13	−4 ± 13	32 ± 3*
	Uptake	3·2 ± 10·0	21·1 ± 30·4	58·1 ± 8·7†
Total (free + triglyceride fatty acids)	*A*	1192 ± 40	1318 ± 200	1492 ± 103
	V	772 ± 56	912 ± 140	815 ± 95
	$(A-V)100/A$	35 ± 6	29 ± 7	46 ± 4
	Uptake	108·2 ± 21·1	115·0 ± 33·0	148·5 ± 16·8
Triglyceride glycerol	*A*	124 ± 17	94 ± 12	82 ± 9
	V	75 ± 10	60 ± 13	54 ± 9
	$(A-V)100/A$	38 ± 6	37 ± 9	35 ± 8
	Uptake	10·9 ± 2·6	8·7 ± 3·1	5·0 ± 1·6*
Free glycerol	*A*	23 ± 1	32 ± 8	39 ± 5
	V	19 ± 3	25 ± 4	22 ± 3
	$(A-V)100/A$	23 ± 10	13 ± 12	41 ± 9*
	Uptake	1·0 ± 0·8	1·4 ± 1·6	3·5 ± 1·0*

Significance of difference from thermoneutral value:

*$P<0{\cdot}05$ plasma substrate concentration $\mu moles.l^{-1}$

†$P<0{\cdot}01$ substrate uptakes $\mu moles.min^{-1}$.

blood. Total fatty acid concentration was increased slightly in both arterial and venous blood during cold exposure although this change was not significant. Cold exposure also caused a small increase in total fatty acid uptake by the gland but this just failed to be statistically significant. Triglyceride glycerol uptake fell in the cold ($P<0.05$ in moderate cold exposure) as did the circulating level although this change was not significant.

The arterial concentration of free glycerol increased during cold exposure, though not significantly, and extraction by the udder increased ($P<0.05$ in moderate cold exposure).

Effect of cold on milk triglyceride output

Despite the fact that milk secretion rate fell significantly during cold exposure (Table I), total triglyceride output was not affected by environment (Table III) but there were changes in milk triglyceride fatty acid composition. Cold stress caused an increase in the % of triglyceride fatty acids 16 carbons in length or longer, especially oleic acid, and a decrease in the % of acids less than 16 carbons in length. This effect was significant ($P<0.05$) during moderate cold exposure.

Table III. *Effect of cold on milk triglyceride output (μmoles.min^{-1}). Values are means for five animals.*

	Thermoneutral		Mild cold		Moderate cold	
	Output	% of milk triglyceride	Output	% of milk triglyceride	Output	% of milk triglyceride
$C_{6:0}$	1·08	3·2	1·05	3·0	0·94	2·9
$C_{8:0}$	1·25	3·7	1·20	3·4	1·11	3·5
$C_{10:0}$	3·72	11·0	3·72	10·7	3·18	10·0
$C_{12:0}$	1·63	4·8	1·56	4·5	1·27	4·0
$C_{14:0}$	3·43	10·1	3·50	10·1	2·59	8·2
$C_{16:0}$	8·26	24·4	8·72	25·1	7·14	22·5
$C_{16:1}$	0·14	0·4	0·23	0·7	0·22	0·7
$C_{18:0}$	5·25	15·5	5·20	14·9	5·27	16·6
$C_{18:1}$	8·63	25·5	9·03	25·9	9·54	30·1
$C_{18:2}$	0·40	1·2	0·58	1·7	0·45	1·4
Total	33·8		34·8		31·7	

Effect of cold on arterial carnitine concentration and carnitine concentration in milk

Regardless of environment, arterial carnitine concentration was lower in the sample taken at 10.00 h than in the sample taken at 15.00 h ($P<0.05$) (Table IV). Cold exposure,

Table IV. *Effect of cold on carnitine concentration in arterial blood and milk, and carnitine secretion in milk. Mean ±s.e. of mean for five animals.*

		Thermoneutral	Moderate cold
Arterial carnitine (nmoles.ml^{-1})	10.00 h	4·2±1·0	2·8±0·5 †
	15.00 h‡	4·8±1·0	3·2±0·8 †
Milk carnitine (nmoles.ml^{-1})		54±3	47±3
Milk yield (g)§		511±64	223±68*
Carnitine secretion in milk (μmoles)		28±5	12±7*

*$P<0.05$ significance of difference from thermoneutral values.
†$P<0.02$ significance of difference from thermoneutral values.
‡$P<0.05$ significance of difference from 10.00 h values.
§Afternoon milk yield only.

at the two times studied, caused a decrease in circulating carnitine level which was statistically significant ($P<0·02$). Milk carnitine concentration fell slightly in the cold but the total carnitine secreted in milk fell significantly ($P<0·05$) due to the decrease in milk yield ($P<0·05$).

Discussion

When mammals are exposed to a cold environment their heat production increases, and this increase is associated with changes in the blood concentrations of various energy substrates. Cold exposure of non-lactating sheep results in increases in circulating free fatty acid [Bost and Dorleac, 1965] and glycerol [Aulie, Astrup, Nedkvitne and Velle, 1971] concentrations, probably from the mobilization of adipose tissue triglycerides. No change in plasma triglyceride concentration was detected during cold exposure of non-lactating cattle which have a low blood triglyceride concentration [Thompson and Clough, 1972] but decreases have been reported in cold-exposed rats [Radomski, 1966]. Plasma acetate concentration increases during acute cold exposure in male cattle [Bell, Gardner and Thompson, 1974]. In the present experiments, on lactating goats, there was an increase in circulating free fatty acid concentration, a decrease in circulating triglyceride and a decrease in plasma acetate concentration during cold exposure. The change in plasma acetate concentration was in the opposite direction to that previously reported for cattle [Bell, Gardner and Thompson, 1974] in comparable severities of cold stress; however the duration of cold exposure in the previous experiments was approximately 4 h compared with 48 h in the present experiment.

The supply of milk fat precursors to the mammary gland is determined by their concentration in blood and by blood flow to the gland. Mammary blood flow decreased during moderate, but not mild, cold exposure in the present experiment, which confirms similar previous observations [Thompson and Thomson, 1977]. Free fatty acid supply to the gland increased with increasing severity of cold exposure, due to the large increase in arterial level, and the net uptake of free fatty acids by the gland also increased. The net arteriovenous difference of free fatty acids across the mammary gland is affected by both the direct uptake of arterial free fatty acids and also the release of free fatty acids into venous plasma due to triglyceride hydrolysis during the uptake of plasma triglyceride [West *et al.*, 1967]. It is not possible to ascertain from the present data the exact quantitative importance of these two processes to the net increase in free fatty acid uptake in the cold. However, the supply and uptake of triglyceride was decreased during cold exposure in the present experiments and this implies that the 'true' uptake from the free fatty acid fraction of the plasma must be increased during cold exposure. Normally plasma free fatty acids and triglycerides are used for the synthesis of milk triglyceride fatty acids greater than or equal to 16 carbons in length [Annison, Linzell, Fazakerley and Nichols, 1967]. The net secretion rate of these milk fatty acids was maintained or slightly increased in the cold despite the fact that milk volume was much reduced. In contrast, the net secretion rate of milk fatty acids less than 16 carbons in length was slightly reduced during cold exposure. These fatty acids are synthesized from acetate and β-hydroxybutyrate in the normal animal [Linzell, Annison, Fazakerley and Leng, 1967]. Acetate supply and uptake was decreased markedly during cold exposure in the present experiments and we have recently found that β-hydroxybutyrate uptake is also decreased by these levels of cold stress [Faulkner, Thomson and Thompson, unpublished]. Thus it appears that the changes in milk fat composition which occur during cold exposure are probably a result of changes in the rate of supply and uptake of short and long chain fatty acids in blood. Similar changes in milk fat composition occur in the lactating goat

when it is fasted for 24 h [Annison, Linzell and West, 1968]. In the present experiments the animals were fed hay *ad libitum*. In previous experiments it has been shown that these levels and duration of cold stress do not affect free intake of hay by goats [Thompson and Thomson, 1977]. It can be calculated from the measurements of oxygen consumption, food intake and milk composition that were made in these experiments [Thompson and Thomson, 1977] that animals were in positive energy balance in the neutral environment and during mild but not moderate cold exposure when they lapsed into a negative energy balance. Thus in the present experiments the metabolic effects of moderate cold may be complexed with the effect of a negative energy balance.

The decrease in the concentration of circulating carnitine observed was in contrast to the substantial increase reported for rats exposed to cold over a period of several weeks [Delisle and Radomski, 1968]. The explanation of the two different responses probably lies in the time of cold exposure used with the two species. In the experiments with rats, where several weeks of cold exposure occurred, there was ample time for increased carnitine synthesis to result in an increased body pool of carnitine [see Therriault and Mehlman, 1965]. The turnover time of carnitine in female rats is 6–10 days [Khairallah and Mehlman, 1965] which is reduced to about half after 6 weeks exposure at 5°C [Therriault and Mehlman, 1965], but is still a substantial period. The rate of carnitine biosynthesis in sheep liver, the main site of biosynthesis, is similar to that in rat liver [Costa, 1977] and probably similar rates occur in goats. Thus, in the 1 day cold exposure used in the present experiments, there is unlikely to have been time for any significant increase in carnitine concentrations to occur.

In the short period of cold exposure that the goats were subjected to in the present experiment the decreased blood carnitine concentrations, coupled with the decreased loss in the milk, saving some 52 μmoles of carnitine daily, probably represent a conservation of carnitine for increased fatty acid oxidation in body tissues of the mother. This in turn would result in a decreased intake of carnitine, *via* the milk, by the offspring. This may have important metabolic consequences for the offspring as Robles-Valdes, McGarry and Foster [1976] have shown that mother's milk is a vital source of carnitine to suckling rats. Further, Henderson [1978] has suggested that the very marked increase in muscle carnitine content in new born lambs in the first few days after birth is probably due to a transfer of carnitine from the ewe's milk, which has a high carnitine concentration [Snoswell and Linzell, 1975]. During cold exposure there is an increase in fatty acid oxidation by the new born [Alexander, 1961] and this may require an increased supply of carnitine from the milk. Thus the decrease in carnitine output in the milk, following short-term cold exposure, could ultimately affect the metabolism and survival of young animals, particularly lambs, and may be an important factor in their survival which warrants further investigation.

Acknowledgments

The authors are most grateful for the technical assistance of Misses J. G. Veitch, E. R. Waugh and E. Y. Brechany. E.M.T. was supported by an Agricultural Research Council Studentship. A.M.S. was supported by a Royal Society and Nuffield Foundation Commonwealth Bursary and by laboratory facilities kindly provided by Dr. R. M. C. Dawson, Dr. D. B. Lindsay and Dr. S. P. Mann.

References

ALEXANDER, G. (1961). Temperature regulation in the newborn lamb. III. Effect of environmental temperature on metabolic rate, body temperature and respiratory quotient. *Australian Journal of Agricultural Research*, **12**, 1152–1174.

ANNISON, E. F., LINZELL, J. L., FAZAKERLEY, S. and NICHOLS, B. W. (1967). The oxidation and utilization of palmitate, stearate, oleate and acetate by the mammary gland of the fed goat in relation to their overall metabolism and the role of plasma phospholipids and neutral lipids in milk fat synthesis. *Biochemical Journal*, **102**, 637–647.

ANNISON, E. F., LINZELL, J. L. and WEST, C. E. (1968). Mammary and whole animal metabolism of glucose and fatty acids in fasting lactating goats. *Journal of Physiology*, **197**, 445–459.

AULIE, A., ASTRUP, H. N., NEDKVITNE, J. J. and VELLE, W. (1971). Serum non-esterified fatty acids and plasma glycerol as indicators of fat mobilization in pregnant sheep subjected to cold stress. *Acta Veterinaria Scandinavica*, **12**, 496–503.

BELL, A. W., GARDNER, J. W. and THOMPSON, G. E. (1974). The effects of acute cold exposure and feeding on volatile fatty acid metabolism in the hind leg of the young ox. *British Journal of Nutrition*, **32**, 471–477.

BOST, J. and DORLEAC, E. (1965). Action due froid sur le taux plasmatique des acides gras non estérifiés (FFA) chez le mouton. *Comptes rendus des séances de la Société de Biologie*, **159**, 2209–2212.

CEDERBLAD, G. and LINDSTEDT, S. (1972). A method for the determination of carnitine in the picomole range. *Clinica Chimica Acta*, **37**, 235–243.

CLARKE, P. L., THOMSON, E. M. and THOMPSON, G. E. (1976). Acute cold exposure and milk secretion in the goat. *Journal of Physiology*, **263**, 176–177P.

COBBLE, J. W. and HERMAN, H. A. (1951). The influence of environmental temperatures on the composition of milk of the dairy cow. *Missouri Agricultural Experimental Station Research Bulletin*, No. 485.

COSTA, N. D. (1977). Aspects of the metabolic role and biosynthesis of carnitine. Ph.D. Thesis, University of Adelaide.

DARLING, K. F., FINDLAY, J. D. and THOMPSON, G. E. (1974). Effect of intraventricular acetylcholine and eserine on the metabolism of the sheep. *Pflugers Archiv*, **349**, 235–245.

DELISLE, G. and RADOMSKI, M. W. (1968). The role of carnitine in the animal exposed to cold. *Canadian Journal of Physiology and Pharmacology*, **46**, 71–75.

FARQUHAR, J. W., INSULL, W., ROSEN, P., STOFFEL, W. and AHRENS, E. H. (1959). The analysis of fatty acid mixtures by gas–liquid chromatography: Construction and operation of an ionization chamber instrument. *Nutrition Reviews*, **17**, suppl. 18, part 2.

FOLCH, J., LEES, M. and SLOANE-STANLEY, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*, **226**, 497–509.

FRITZ, I. B. (1964). Carnitine and its role in fatty acid metabolism. In *Advances in Lipid Research*, Vol. 1, pp. 285–334. (R. Paoletti and D. Krtichevsky, editors), New York: Academic Press.

GARDNER, J. W. and THOMPSON, G. E. (1974). A method for the determination of volatile fatty acids in the blood plasma of ruminant animals. *Analyst*, **99**, 326–329.

HEBB, C., MANN, S. P. and MEAD, J. (1975). Measurement and activation of choline acetyltransferase. *Biochemical Pharmacology*, **24**, 1007–1012.

HENDERSON, G. D. (1978). Interrelationships between carnitine, choline and creatine metabolism in sheep. Ph.D Thesis, University of Adelaide.

KHAIRALLAH, E. A. and MEHLMAN, M. A. (1965). The turnover, body pool, and daily excretion of carnitine as determined by isotope-dilution techniques. In *Recent Research on Carnitine*, pp 57–62 (G. Wolf, editor). Massachusetts: MIT Press.

LINZELL, J. L., ANNISON, E. F., FAZAKERLEY, S. and LENG, R. A. (1967). The incorporation of acetate, stearate and D(-) β-hydroxybutyrate into milk fat by the isolated perfused mammary gland of the goat. *Biochemical Journal*, **104**, 34–42.

NELSON, G. T. and FREEMAN, N. K. (1959). Serum phospholipid analysis by chromatography and infra-red spectrophotometry. *Journal of Biological Chemistry*, **234**, 1375–1380.

RADOMSKI, M. W. (1966). Effect of cold exposure on serum lipids and lipoproteins in the rat. *Canadian Journal of Physiology and Pharmacology*, **44**, 711–719.

ROBLES-VALDES, C., MCGARRY, J. D. and FOSTER, D. W. (1976). Maternal-fetal carnitine relationships and neonatal ketosis in the rat. *Journal of Biological Chemistry*, **251**, 6007–6012.

SNOSWELL, A. M. and LINZELL, J. L. (1975). Carnitine secretion into the milk of ruminants. *Journal of Dairy Research*, **42**, 371–380.

THERRIAULT, D. G. and MEHLMAN, M. A. (1965). Carnitine ^{14}C metabolism in cold acclimated rats. In *Recent Research on Carnitine*, pp 141–145 (G. Wolf, editor). Massachusetts: MIT Press.

THOMPSON, G. E. and CLOUGH, D. P. (1972). The effect of cold exposure on plasma lipids of the new born and adult ox. *Quarterly Journal of Experimental Physiology*, **57**, 192–198.

THOMPSON, G. E. and THOMSON, E. M. (1977). Effect of cold exposure on mammary circulation, oxygen consumption and milk secretion in the goat. *Journal of Physiology*, **272**, 187–196.

WEST, C. E., ANNISON, E. F. and LINZELL, J. L. (1967). Plasma free fatty acid uptake and release by the goat mammary gland. *Journal of Biochemistry*, **102**, 23P.

J. Reprod. Fert. (1979) **56**, 105–111 0022–4251/79/030105–08$02.00/0

The concentration of carnitine in the luminal fluid of the testis and epididymis of the rat and some other mammals

B. T. Hinton, A. M. Snoswell* and B. P. Setchell

Department of Biochemistry, A.R.C. Institute of Animal Physiology, Babraham, Cambridge, CB2 4AT, U.K.

Summary. Luminal fluid was collected by micropuncture techniques from the testis and epididymis of the rat, hamster, rabbit, boar and ram and the concentration of free L-carnitine in the fluid was estimated using enzymic methods. Carnitine was present in the testicular fluid of the rat in concentrations <1 mM but increased down the epididymis to reach 53 mM in luminal fluid from the cauda epididymidis, approximately 2000 times higher than in blood plasma. A high concentration was first found in the luminal fluid from the distal caput epididymidis, at about the point where the spermatozoa become motile. Carnitine was also present in the epididymal luminal fluid of the other species studied; the amounts were not as high as those in the rat but were still higher than those in blood plasma.

Introduction

It was first shown by Marquis & Fritz (1965) and later by Pearson & Tubbs (1967) that L-carnitine is present in higher concentrations in the rat epididymis than in other body tissues. These estimations were made on whole homogenates of the epididymis or on fluid extruded from different regions along the epididymis using techniques which would have led to considerable cell damage. In a later study by Brooks, Hamilton & Mallek (1974), fluid was expressed from the lumen of the rat cauda epididymidis. This fluid contained about 60 mM-carnitine compared with about 25 mM for whole tissue and 0·02 mM in blood. It has been suggested that the high concentration of carnitine in the luminal fluid of the epididymis may be involved in the maturation of spermatozoa. Therefore, we have estimated the concentration of carnitine in uncontaminated luminal fluid collected by micropuncture from different regions of the rat epididymis. It seemed particularly important to determine the precise area where carnitine is first secreted into the lumen as our previous studies (Hinton, Dott & Setchell, 1979) have shown that there is a definite region along the epididymis where spermatozoa first show motility. Our investigations have concentrated on the rat but we have also estimated carnitine in the epididymal luminal fluid from the hamster, rabbit, boar and ram, because there appears to be a lack of information regarding the concentration of carnitine in the epididymal luminal fluid of these species.

Materials and Methods

Chemicals

4,4′-Dithiobispyridine (Aldrithiol-4, 4PDS; Aldrich Chemical Company, Middlesex, U.K.), carnitine acetyltransferase (80 U/ml, EC 2.3.1.7; Boehringer Corporation, London, U.K.), De Acidite FF-IP SRA 63 resin (Permutit Company, Middlesex, U.K.), bovine plasma albumin (Armour Pharmaceutical Company, Eastbourne, U.K.), carnitine (L-chloride) and EGTA

* Present address: Waite Agricultural Institute, University of Adelaide, Glen Osmond, South Australia, 5064, Australia.

(ethylene glycol bis (2-amino ethyl) tetra acetic acid; Koch-Light Laboratories, Bucks, U.K.) were used as purchased from the manufacturers. [^{3}H]Acetyl CoA (sp. act. 25 μCi/μmol) was prepared and purified by the method described by Hebb, Mann & Mead (1975) and was kindly supplied by Dr S. P. Mann of this Institute.

Animals

Adult male animals were used throughout this study. Porton–Wistar rats weighing 350–450 g were obtained from the Institute colony. Golden hamsters, 120–160 g, were purchased from Wright's of Essex, U.K., and New Zealand White rabbits from Morton Commercial Rabbits, U.K. Clun Forest rams and Large White boars, weighing 60–80 kg and 200–500 kg respectively, were from the Institute's experimental farm stock.

Micropuncture

Rats and hamsters were anaesthetized by an intraperitoneal injection of urethane (ethyl carbamate, 1·2 mg/kg; Fisons, Loughborough, U.K.) and Inactin (sodium 5-ethyl-5-(1-methyl propyl)-2-thiobarbiturate, 200 mg/kg; Promonta, Hamburg, West Germany) respectively. Rabbits were anaesthetized by an intravenous injection of a solution of 25% (w/v) urethane and 1·5% (w/v) α-chloralose (4 ml/kg; Hopkin and Williams, Romford, Essex, U.K.). The rams and boars were anaesthetized by an intravenous injection of pentobarbitone sodium (Sagatal; May and Baker, Dagenham, Essex, U.K.) and anaesthesia was maintained by a halothane (Fluothane: ICI, Macclesfield, U.K.)–oxygen gas mixture.

In the smaller animals, the testis and epididymis on one side were exposed through a scrotal incision and prepared for micropuncture as previously described (Hinton *et al.*, 1979). Samples were obtained from the epididymides of the rams and boars either by micropipettes or by inserting a polythene cannula (0·8 mm i.d., 1·0 mm o.d. or 0·5 mm i.d., 0·8 o.d.; Dural Plastics, N.S.W., Australia) into the tubules of the cauda epididymidis. In one experiment, samples were removed from the proximal regions of the boar epididymis immediately after death of the boar; samples were also removed from the cauda epididymidis in the same boar before and after death to see whether there were changes in the carnitine concentration after death. The epididymal luminal contents collected were centrifuged as previously described (Hinton *et al.*, 1976). Rete testis fluid (RTF) was collected from boars by the method of Evans & Setchell (1979) and from rams by the method of Voglmayr, Scott, Setchell & Waites (1967) as modified by Suominen & Setchell (1972).

The term 'luminal fluid' will be used to refer to the fluid in the duct in which the spermatozoa are suspended, whereas 'luminal contents' will be used to mean both fluid and spermatozoa.

Carnitine was measured in seminiferous tubule fluid (STF) and in fluid from as many sites as possible along the epididymis in each species (see Plate 1 and Hinton *et al.*, 1979). In the rat epididymis, two extra sites were chosen equidistant between Sites 2 and 3 (caput 2a and 2b, see Plate 1 and Hinton *et al.*, 1979) in order to find the precise area where carnitine secretion begins.

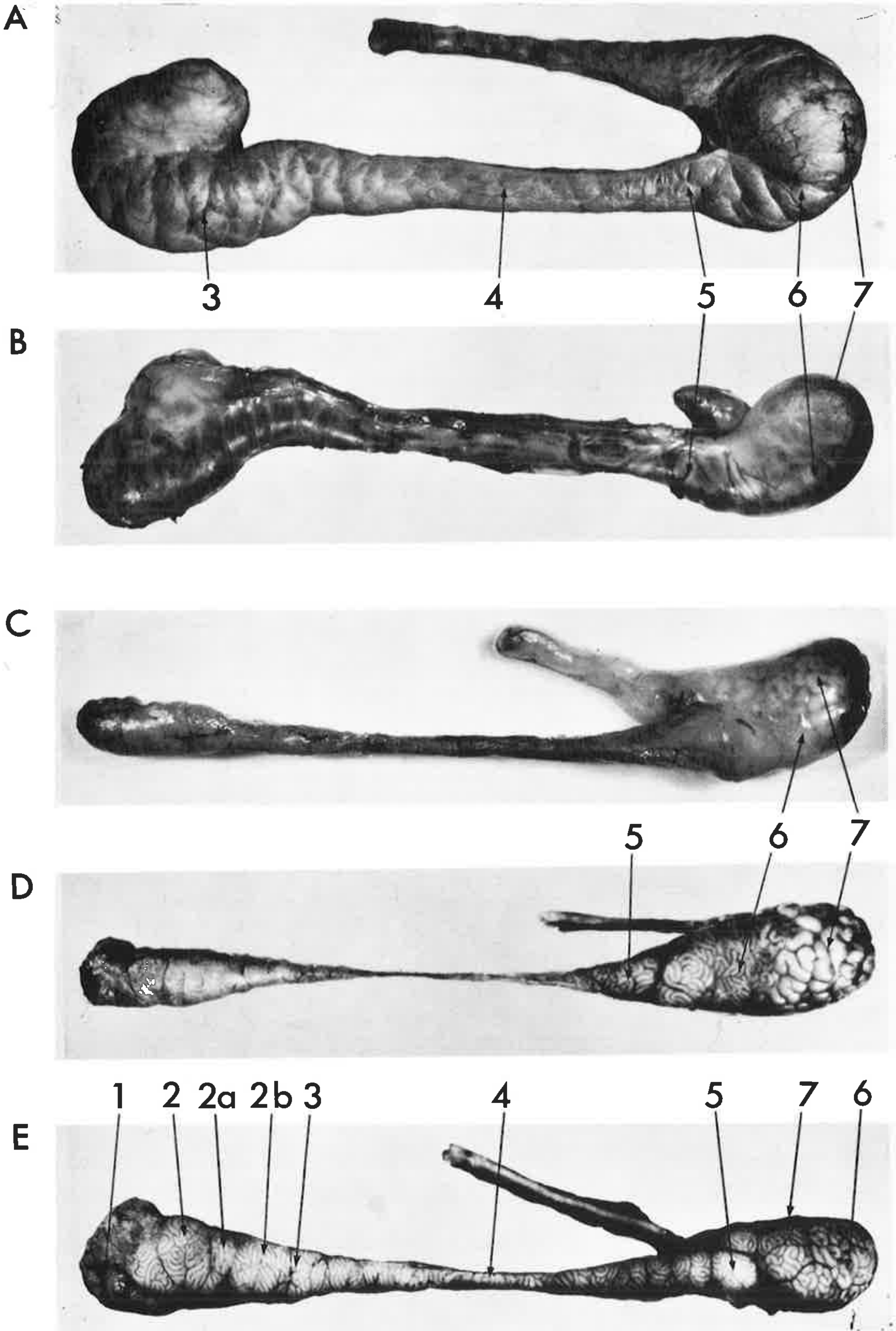
Analysis of micropuncture samples

The spectrophotometric enzymic method of Ramsey & Tubbs (1975) for estimation of carnitine was modified for micropuncture samples (samples containing 0·05–0·2 nmol carnitine); 50–70 nl sample and 4 μl 15–20 mM-acetyl CoA (synthesized by the method of Stadtman, 1957) were added to 295 μl 100 mM-phosphate buffer, pH 7·2, containing 1 mM-EGTA and 125 μM-

EXPLANATION OF PLATE 1

Photographs of the epididymides of the species of animals used in this investigation: A, boar; B, ram; C, rabbit; D, hamster; E, rat. The numbers refer to the sites where micropuncture samples were collected. 2, Proximal caput; 3, distal caput; 4, mid-corpus; 5, distal corpus; 6, proximal cauda; 7, distal cauda. Samples were not removed from the initial segment (Site 1).

PLATE 1



(*Facing p.* 106)

4PDS (25 μl of a stock solution of 50 mM-4PDS in ethanol was added to 10 ml buffer solution immediately before each series of assays) in a microcuvette. Then 1 μl carnitine acetyltransferase was added and the change in optical density was measured at 324 nm using a Gilford spectrophotometer (model 250).

4-Thiopyridone formed by the reaction between 4PDS and the free CoA has an extinction coefficient of $19 \cdot 8 \times 10^3$ M^{-1} cm^{-1} at 324 nm (Grassetti & Murray, 1967) and the amount of carnitine in the sample was calculated from this figure.

Analysis of RTF and plasma samples

A modified radiochemical enzymic method was used to measure carnitine in RTF and plasma because of the lower concentrations of carnitine present in these fluids. After removal of a blood sample from the animal, the plasma was separated by centrifuging at 1500 ***g*** for 15 min. The proteins were removed by centrifugation through an Amicon CG 50 A Centriflo membrane cone at 4°C (800 ***g*** for 10 min) and the ultrafiltrates were stored at −70°C until analysed. A similar procedure was used with sperm-free RTF samples. The ultrafiltrates were then analysed by a modification of the radiochemical–enzymic method of Cederblad & Lindstedt (1972). The original method did not give a linear response to carnitine and Bøhmer, Rydning & Solberg (1974) suggested the use of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to trap the free CoA released during the reaction to give a linear response. However, DTNB inhibits carnitine acetyltransferase and we have used 4PDS which traps the free CoA but does not affect the enzyme at the concentration used. The incubation mixture contained 33 mM-sodium phosphate buffer, pH 7·4, 0·8 mM-4PDS, 80 μM [^{3}H]acetyl CoA, 60 μg bovine plasma albumin, and 50 μl sample in a final volume of 120 μl. The reaction was begun by the addition of 1 μl carnitine acetyltransferase and the mixture was incubated for 20 min at 39°C. The reaction was stopped by the addition of 80 μl 0·15 N-perchloric acid and the residual [^{3}H]acetyl CoA was separated from the [^{3}H]acetyl-L-carnitine formed by using small glass columns (0·65 mm i.d.; 10 cm long) containing De Acidite FF-1P SRA 63 resin as described by Hebb *et al.* (1975). Standards in the range of 10–2000 pmol were run with each series of samples and a linear response was obtained over this range. All samples and standards were assayed in duplicate. The recovery of carnitine by this procedure was 97%, the accuracy to within ±2·5% and the limit of detection was 0·1 nmol/ml serum. This method estimates free carnitine and short chain acylcarnitine compounds (equivalent to total acid-soluble carnitine).

Results

The greatest concentration of carnitine was found in the rat, the lowest in the hamster while the rabbit, ram and boar were intermediate. There were no differences in the concentration of carnitine in the fluid removed from the boar cauda epididymidis before or immediately after death of the animal. In each of the species studied there was a progressive increase along the epididymis in the concentration of carnitine in the luminal fluid (Table 1); in the rat there was a large difference between the concentration of carnitine in the luminal fluid of the proximal and distal caput epididymidis. Consequently, attention was focused on this region by sampling from two intermediate sites (Sites 2a and 2b); fluid from Site 2a contained 9·3 ± 0·55 mM- and that from Site 2b contained 14·1 ± 1·27 mM-carnitine (5 observations).

Discussion

The results presented in this paper are the first estimates of the concentration of carnitine in the luminal fluid from distinct sites along the epididymis. Our collected samples were free from blood and other contamination and probably represent the true physiological fluid in which spermatozoa are surrounded during their transit along the epididymis. Our values for fluid from

Table 1. Mean ± s.e.m. concentrations of carnitine (mM) in the plasma and the tubular fluid of the testis and epididymis of the rat, hamster, boar, ram and rabbit

	No. of animals	Seminiferous tubule	Rete testis	Epididymis						Ductus deferens	Plasma
				Proximal caput	Distal caput	Mid corpus	Distal corpus	Proximal cauda	Distal cauda		
Rat	14	<1	<1	<1–3	19·1 ±0·55	22·2 ±1·65	30·8 ±4·02	51·7 ±5·47	53·7 ±1·26	53·3 ±1·99	*0·0343 ±0·0023
Hamster	5	<1	<1	—	—	—	2·36 ±0·32	8·74 ±1·29	10·7 ±0·66	12·1 ±1·26	*0·0061 ±0·0005
Boar	3	—	*0·0122 ±0·0012	—	†5·84 ±0·65	†4·06 ±0·27	15·6 ±1·15	17·9 ±0·82	16·1 ±1·15	22·0 ±1·56	*0·0078 ±0·0006
Ram	3	<1	*0·0278 ±0·0048	—	—	—	11·2 ±0·05	10·6 ±0·55	14·5 ±0·88	14·7 ±1·07	*0·0436 ±0·0050
Rabbit	2	—	—	—	—	—	—	14·5 ±1·08	19·0 ±0·33	22·9 ±0·28	*0·0202 ±0·0005

* Estimation by radiochemical method, see 'Materials and Methods'.
† Samples removed from epididymis, *post mortem*.

the rat ductus deferens are very similar to those reported by Brooks *et al.* (1974) but our values for luminal fluid from elsewhere in the epididymis of this species are much higher than those reported for equivalent epididymal tissue by Marquis & Fritz (1965), Pearson & Tubbs (1967), and Brooks *et al.* (1974). However, this pattern of concentration of carnitine in the luminal fluid is similar for the combined samples of fluid and tissue.

We have shown that a high concentration of carnitine first appears in a confined region of the rat caput epididymidis (caput 2a; see Plate 1), the area where accumulation of carnitine in the whole epididymal tissue appears to be particularly active (Bøhmer & Johansen, 1978) and where there is a sudden increase in the potential for motility of the spermatozoa (Hinton *et al.*, 1979). It therefore seems possible that carnitine may play a role in the initiation of the motility of rat spermatozoa. This suggestion is supported by the fact that Casillas (1973) found that bovine spermatozoa from the caput epididymidis readily accumulate carnitine whereas those from cauda epididymidis do not.

Rat spermatozoa contain high concentrations of carnitine acetyltransferase and acetylcarnitine (see Marquis & Fritz, 1965) and several important functions have been suggested for these two components. Acetylcarnitine may serve as a readily accessible energy pool for use in both activation of respiration and motility in mammalian spermatozoa (Milkowski, Babcock & Lardy, 1976; Harrison, 1977), and the maturing spermatozoa of *Drosphilia melanogaster* have been shown to contain high levels of carnitine acetyltransferase and acetylcarnitine (Geer & Newburgh, 1970). When acetylcarnitine or carnitine were added to ejaculated human spermatozoa, an increase in the motility was demonstrated (Tanphaichitr, 1977), although it was suggested that carnitine is first converted to acetylcarnitine.

Casillas & Erickson (1975b) have suggested that acetylcarnitine and carnitine acetyltransferase may act to buffer the sperm acetyl CoA:CoA ratio as originally suggested by Pearson & Tubbs (1967) for rat heart muscle and by Snoswell & Koundakjian (1972) for sheep muscle tissue. Furthermore, high concentrations of carnitine have been shown to inhibit oxygen uptake by bull spermatozoa (Hamilton & Olsen, 1976). Bøhmer & Johansen (1978) confirmed these findings but found that no inhibition could be demonstrated with ejaculated human spermatozoa or epididymal bull or rat spermatozoa. For several tissues, carnitine has been shown to have an important cellular function in the transfer of long chain fatty acids across the inner mitochondrial membrane for β-oxidation (see Bremer, 1977 for recent review); carnitine may therefore have a similar function in the developing spermatozoa as discussed by Casillas (1972). Sperm mitochondria, like those of heart, have an active acetylcarnitine–carnitine exchange carrier (Calvin & Tubbs, 1976). The high concentration of carnitine in the rat epididymis appears to be achieved by transport from blood plasma and not local synthesis, as there is evidence that the rat epididymis does not possess the hydroxylase enzyme necessary for the conversion of γ-butyrobetaine to carnitine, the final step in the biosynthetic pathway of carnitine (Casillas & Erickson, 1975a). Only the liver (with a slight capacity in the testis) appears to be capable of the final conversion step, and carnitine is then rapidly transported to other tissues (Lindstedt, 1967; Haigler & Broquist, 1974; Bøhmer, 1974). Other studies have shown that the accumulation mechanism is under androgenic control (Marquis & Fritz, 1965; Brooks *et al.*, 1973; Bøhmer & Hansson, 1975; Bøhmer & Johansen, 1978) and that it is influenced by circulating androgens rather than by androgens bound to the intraluminal androgen-binding protein (Bøhmer *et al.*, 1977).

The concentrations of carnitine in the luminal fluid of the epididymis of the hamster, boar, rabbit and ram were never as high as those in the rat. No studies on the accumulation of radioactive carnitine have been made in these species, but as the concentrations found in the luminal fluid are still much higher than those in blood plasma, it seems likely that a similar concentration mechanism is operating in these species as in the rat. The estimations of carnitine in the luminal fluid of the rat cauda epididymidis and in blood plasma indicate a concentration gradient of carnitine across the epididymal epithelium of some 2000 which is much higher than for most

physiological compounds. However, the sarcoplasmic reticulum of muscle, which has a high affinity for calcium ions, is capable of creating a concentration gradient of calcium higher than 10 000 during active calcium transport, probably due to the presence of the large internal surface of the sarcoplasmic reticulum membrane together with a very high surface density of transporting units (see Hasselbach, 1978). A similar mechanism may operate for carnitine in the rat epididymis.

In the rat, carnitine contributes significantly to the osmotic pressure of the luminal fluid but in other species studied some other organic compounds must also be present in the epididymal fluid to account for the osmotic pressure not contributed by carnitine and ions. Glycerylphosphorylcholine (GPC) is present in almost equimolar amounts with carnitine in the rat epididymis (Brooks *et al.*, 1974), but is found in higher concentrations in the luminal fluid of the cauda epididymidis of the rabbit (60 mM; Jones & Glover, 1973; B. T. Hinton, unpublished observations) and boar (80 mM, B. T. Hinton, unpublished observations). In the hamster, inositol may contribute to the osmotic pressure rather than GPC since the concentration of inositol in the luminal fluid from the cauda epididymidis exceeds 90 mM (B. T. Hinton, R. W. White & B. P. Setchell, unpublished observations).

There appear to be large species differences in the composition of epididymal luminal fluid and choice of a suitable animal model for studying possible contraceptive agents acting on the epididymis will be difficult, especially as so little information is available about the composition of the epididymal luminal fluid in man.

This investigation received the financial support from the World Health Organisation. A.M.S. was supported by a Royal Society and Nuffield Commonwealth Bursary.

References

Bøhmer, T. (1974) Conversion of butyrobetaine to carnitine in the rat *in vivo*. *Biochim. biophys. Acta* **343**, 551–557.

Bøhmer, T. & Hansson, V. (1975) Androgen-dependent accumulation of carnitine by rat epididymis after injection of [^{3}H]butyrobetaine *in vivo*. *Mol. cell. Endocr.* **3**, 103–115.

Bøhmer, T. & Johansen, L. (1978) Inhibition of sperm maturation through intervention of the carnitine system. *Int. J. Androl., Suppl.* **2**, 565–572.

Bøhmer, T., Rydning, A. & Solberg, H.-E. (1974) Carnitine levels in human serum in health and disease. *Clin. chim. Acta* **57**, 55–61.

Bøhmer, T., Weddington, S.C. & Hansson, V. (1977) Effect of testosterone propionate on levels of carnitine and testicular androgen binding protein (ABP) in rat epididymis. *Endocrinology* **100**, 835–838,

Bremer, J. (1977) Carnitine and its role in fatty acid metabolism. *Trends Biochem. Sci.* **2**, 207–209.

Brooks, D.E., Hamilton, D.W. & Mallek, A.H. (1973) The uptake of L-[methyl-^{3}H] carnitine by the rat epididymis. *Biochem. Biophys. Res. Commun.* **52**, 1354–1360.

Brooks, D.E., Hamilton, D.W. & Mallek, A.H. (1974) Carnitine and glycerylphosphorylcholine in the reproductive tract of the male rat. *J. Reprod. Fert.* **36**, 141–160.

Calvin, J. & Tubbs, P.K. (1976) A carnitine: acetylcarnitine exchange system in spermatozoa. *J. Reprod. Fert.* **48**, 417–420.

Casillas, E.R. (1972) The distribution of carnitine in male reproductive tissues, and its effect on palmitate oxidation by spermatozoal particles. *Biochim. biophys. Acta* **280**, 545–551.

Casillas, E.R. (1973) Accumulation of carnitine by bovine spermatozoa during maturation in the epididymis. *J. biol. Chem.* **248**, 8227–8232.

Casillas, E.R. & Erickson, B.J. (1975a) Studies on carnitine synthesis in the rat epididymis. *J. Reprod. Fert.* **44**, 287–291.

Casillas, E.R. & Erickson, B.J. (1975b) The role of carnitine in spermatozoan metabolism: substrate-induced elevations in the acetylation state of carnitine and coenzyme A in bovine and monkey spermatozoa. *Biol. Reprod.* **12**, 275–283.

Cederblad, G. & Lindstedt, S. (1972) A method for the determination of carnitine in the picomole range. *Clin. Chim. Acta* **37**, 235–243.

Evans, R.W. & Setchell, B.P. (1979) Lipid changes in boar spermatozoa during epididymal maturation with some observations on the flow and composition of boar rete testis fluid. *J. Reprod. Fert.* (in press).

Geer, B.W. & Newburgh, R.W. (1970) Carnitine acetyltransferase and spermatozoan development in *Drosophilia melanogaster*. *J. biol. Chem.* **245**, 71–79.

Grassetti, D.R. & Murray, J.F., Jr (1967) Determination of sulphydryl groups with 2,2′ or 4,4′-dithiodipyridine. *Archs Biochem. Biophys.* **119**, 41–49.

Haigler, H.T. & Broquist, H.P. (1974) Carnitine synthesis in rat tissue slices. *Biochem. Biophys. Res. Commun.* **56**, 676–681.

Hamilton, D.W. & Olsen, G.E. (1976) Effects of carnitine on oxygen uptake and utilisation of [U-^{14}C]palmitate by ejaculated bull spermatozoa. *J. Reprod. Fert.* **46**, 195–202.

Harrison, R.A.P. (1977) The metabolism of mammalian spermatozoa. In *Frontiers in Reproduction and Fertility Control*, pp. 379–401. Eds R. O. Greep & M. A. Koblinsky. M.I.T. Press, Cambridge, Massachusetts.

Hasselbach, W. (1978) The reversibility of the sarcoplasmic calcium pump. *Biochim. biophys. Acta* **515**, 23–53.

Hebb, C., Mann, S.P. & Mead, J. (1975) Measurement and activation of choline acetyltransferase. *Biochem. Pharmacol.* **24**, 1007–1011.

Hinton, B.T., Setchell, B.P. & White, R.W. (1976) The determination of myo-inositol in micropuncture samples from the testis and epididymis of the rat. *J. Physiol., Lond.* **265**, 14–15P.

Hinton, B.T., Dott, H.M. & Setchell, B.P. (1979) Measurement of the motility of rat spermatozoa collected by micropuncture from the testis and from different regions along the epididymis. *J. Reprod. Fert.* **55**, 167–172.

Jones, R. & Glover, T.D. (1973) The collection and composition of epididymal plasma from the cauda epididymidis of the rabbit. *J. Reprod. Fert.* **34**, 395–403.

Lindstedt, G. (1967) Hydroxylation of γ-butyrobetaine to carnitine in rat liver. *Biochemistry, N.Y.* **6**, 1271–1282.

Marquis, N.R. & Fritz, I.B. (1965) Effects of testosterone on the distribution of carnitine, acetylcarnitine, and carnitine acetyltransferase in tissues of the male reproductive system of the male rat. *J. biol. Chem.* **240**, 2197–2200.

Milkowski, A.L., Babcock, D.F. & Lardy, H.A. (1976) Activation of bovine epididymal sperm respiration by caffeine; its transient nature and relationship to utilisation of acetylcarnitine. *Archs Biochem. Biophys.* **176**, 250–256.

Pearson, D.J. & Tubbs, P.K. (1967) Carnitine and derivatives in rat tissues. *Biochem. J.* **105**, 953–963.

Ramsay, R.R. & Tubbs, P.K. (1975) The mechanism of fatty acid uptake by heart mitochondria: an acylcarnitine–carnitine exchange. *FEBS Letters* **54**, 21–25.

Snoswell, A.M. & Koundakjian, P.P. (1972) Relationships between carnitine and coenzyme A esters in tissues of normal and alloxan-diabetic sheep. *Biochem. J.* **127**, 133–141.

Stadtman, E.R. (1957) Preparation and assay of acylcoenzyme A and other thiol esters: use of hydroxylamine. *Methods Enzymol.* **3**, 931–941.

Suominen, J. & Setchell B.P. (1972) Enzymes and trypsin inhibitor in the rete testis fluid of rams and boars. *J. Reprod. Fert.* **30**, 235–245.

Tanphaichitr, N. (1977) *In vitro* stimulation of human sperm motility by acetylcarnitine. *Int. J. Fert.* **22**, 85–91.

Voglmayr, J.K., Scott, T.W., Setchell, B.P. & Waites, G.M.H. (1967) Metabolism of testicular spermatozoa and characteristics of testicular fluid collected from conscious rams. *J. Reprod. Fert.* **14**, 87–89.

Received 26 July 1978

Volume 126, number 1 FEBS LETTERS April 1981

KINETICS OF CARNITINE UPTAKE BY RAT EPIDIDYMAL CELLS

Androgen-dependence and lack of stereospecificity

M. J. JAMES*, D. E. BROOKS+ and A. M. SNOSWELL

Department of Animal Physiology, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, SA 5064, Australia

Received 23 January 1981

1. Introduction

Carnitine, a vital cofactor in lipid metabolism, is derived from the diet and is also synthesized in the liver. Other tissues of the body acquire carnitine by uptake from the bloodstream [1]. The process of carnitine uptake into tissues is of considerable importance since one of the factors contributing to human carnitine deficiency is impaired active-uptake into tissues such as muscle [2,3]. There is also good evidence that carnitine plays an important role in the maturation and maintenance of spermatozoa within the epididymal duct [4–6]. It is in this region of the male reproductive tract that immature spermatozoa from the testis develop the potential to fertilize eggs.

It was shown in [7] that the epididymis contains the greatest concentration of carnitine of any tissue in the body. In [8,9] this carnitine was shown to be transported from the blood into the epididymal lumen against a gradient of 2000:1 [6] to establish an intraluminal concentration as high as 60 mM [4,10]. However, little is known of the actual mechanism of carnitine uptake in the epididymis. In vivo the process is androgen-dependent [7,10] and is likely to involve two vectorial pumping sites [6].

Here, we report that dispersed epididymal cells take up carnitine by a saturable process which is apparently not stereospecific since D- and L-isomers display similar kinetics. After castration the rate of carnitine uptake decreased by ~90% and was no longer a saturable process, indicating that androgens are necessary to maintain the normal carnitine-transport system in the epididymis.

* Present address: Department of Surgery, Flinders Medical Centre, Bedford Park, SA 5042, Australia

+ Address correspondence to D. E. B.

2. Materials and methods

2.1. *Resolution of D-[^{3}H]carnitine and L-[^{3}H]carnitine*

D,L-[^{3}H]Carnitine (1 mCi/μmol from Amersham) was resolved into its isomers by a procedure based on that in [11]. Briefly, 9.6 mM D,L-[^{3}H]carnitine was incubated with 7.7 mM acetyl-CoA, 1.2 mM 4,4′-dithio-bis-pyridine and 10 μg carnitine acetyltransferase in 52 μl total vol. of 0.1 M potassium phosphate (pH 7.0) at 30°C for 1 h. The reaction mixture was then loaded onto pre-coated silica gel aluminium sheets (E. Merck) and developed with methanol/chloroform/water conc. ammonia/formic acid (55:50:10:7.5:2.5 by vol.) [12]. Acetyl-L-[^{3}H]carnitine and D-[^{3}H]carnitine were located using a radiochromatogram scanner; the radioactivity was eluted from these areas (4 ×) with water. Acetyl-L-[^{3}H]carnitine was hydrolysed with 4.4 M ammonia (30°C for 1 h). The samples containing L-[^{3}H]carnitine and D-[^{3}H]carnitine were dried with a stream of nitrogen and the residues were dissolved in 0.1 M potassium phosphate buffer (pH 7.4). The D-[^{3}H]carnitine was taken through the complete procedure again to check that it was free of L-[^{3}H]carnitine. The specific radioactivity of the labelled D-carnitine was assumed to be equal to that of the labelled L-carnitine.

2.2. *Dispersion of epididymal cells*

Isolated cells were obtained after digestion of rat epididymis with collagenase and protease based on the

method in [13]. Rats were decapitated and the epididymides were perfused with saline administered into the aorta [14] until they were visibly clear of blood. That region of the epididymis known to be active in carnitine uptake [8] was dissected free of fat, chopped finely with a razor blade and depleted of sperm by washing twice (10 min at 33°C) with culture medium (1 ml contained 11.1 mg powdered tissue culture medium (TC 199), 4.8 mg Hepes, 1 mg BSA) (pH 7.4). Weighed tissue mince from 4 epididymides was incubated (2 h at 33°C) with 15 ml TC 199 containing 15 mg protease (Sigma, type VI), 15 mg collagenase (Sigma, type I), and 2 mg DNase (Sigma, DN-100). The mixture was agitated by suction with a Pasteur pipette at 20 min intervals. After straining the mixture through nylon mesh, the dispersed cells were gently pelleted by centrifugation and resuspended in TC 199 containing DNase (150 μg/ml). Viability determined by trypan blue exclusion was usually ~90%.

2.3. *Measurement of carnitine uptake*

Radiolabelled carnitine was prepared at various concentrations by dilution with non-radioactive carnitine in TC 199 and added to microfuge tubes (final vol. 50 μl). In each case the radioactive isomer was diluted with the appropriate steric form of the non-radioactive isomer. For L-carnitine, the concentration of the non-radioactive stock solution was checked by enzymatic assay [15]; the D-carnitine stock solution contained 0.7% L-carnitine. Incubations (25 min at 33 °C) began with the addition of 50 μl cell suspension. Carnitine uptake was linear during this period and the radioactivity taken up was decreased with increasing amounts of non-radioactive carnitine in the medium. Incubations were terminated by loading the cell suspension onto 150 μl silicone oil (Dow Corning grades 500 and 200 mixed in proportion 8:2 to give a final specific gravity of 1.025) and rapidly pelleting the cells through the oil by centrifugation at 10 000 × *g* for 10 s in a Beckman microfuge. The silicone oil and incubation medium were decanted and the bottom of the tube containing the cells was cut off with a razor blade and transferred to a scintillation vial for the determination of radioactivity. The amount of extracellular radioactivity adhering to the cells was <0.5% of the volume of the cells. This was accounted for by separating cells and radioactive supernatant at 'zero' time and subtracting these counts from those at the end of the incubation. Typically, the cells accumulated 2–3-times the radioactivity measured at 'zero' time.

Radioactivity was determined by adding the cut-off tube to 1 ml toluene:Triton X-100 (2:1, v/v) containing 5 g PPO/l. Samples were left overnight to allow full extraction of radioactivity into the scintillation fluid. Final counting efficiency was 27%.

3. Results

Dispersed epididymal cells accumulated both L-carnitine and its biologically inactive isomer, D-carnitine, by an apparently saturable mechanism (fig.1). The apparent K_m and V_{max} values for each isomer were calculated from several experiments (table 1). There was no significant difference between the two isomers in either K_m or V_{max} when the results were analysed by Student's *t*-test. Because the results indicated that the carnitine-transport system was not stereospecific, we used a racemic mixture of carnitine for the remainder of this study.

Since epididymal carnitine uptake in vivo and epididymal lipid oxidation is decreased in androgen-deprived animals [7,10,16], we measured the effect of castration on the apparent K_m and V_{max} of D,L-carnitine uptake into dispersed epididymal cells. Prior castration not only decreased the velocity of carnitine uptake (e.g., 10% of the rate in cells from intact ani-

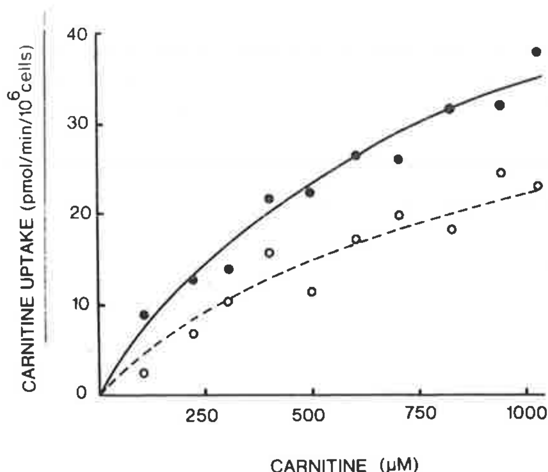


Fig.1. The effect of carnitine concentration on its uptake by dispersed epididymal cells. Each incubation tube contained 0.15 μCi L-[^{3}H]carnitine or 0.24 μCi D-[^{3}H]carnitine at the indicated concentrations and 7.9 × 10^5 cells. Incubation conditions are described in the text and the data represent the means of duplicate determinations. (•) L-carnitine; (○) D-carnitine.

Table 1
K_m and V_{max} values for carnitine uptake

Isomer	App. K_m (μM)	V_{max} (pmol . 10^6 cells . min^{-1})
L-Carnitine	927 ± 174	62 ± 14
D-Carnitine	995 ± 164	49 ± 7

Kinetic parameters were obtained by a direct fit to the hyperbolic plot by the procedure in [27]. The values represent the means and their standard errors from 4 determinations for L-carnitine, and 3 determinations for D-carnitine. In these experiments, the cell no./incubation tube was $5.5 - 7.9 \times 10^5$

mals at 50 μM), but the data further indicate that carnitine uptake is not a saturable process in cells from androgen-deprived rats (fig.2). This was confirmed in other experiments in which the concentration range was extended up to 10 mM.

4. Discussion

The kinetics of L-carnitine uptake have been examined in several rat tissues such as liver, muscle and kidney cortex [12,17–19] and in cultured human heart cells [20]. In these studies estimates of K_m have ranged from 5 μM for human heart cells to 5.6 mM for

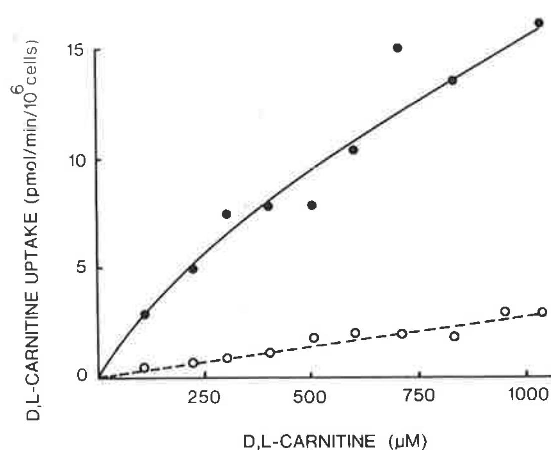


Fig.2. The effect of prior castration on D,L-[^{3}H]carnitine uptake into dispersed epididymal cells. Each incubation tube contained 0.25 μCi D,L-[^{3}H]carnitine and 9.6×10^5 cells from intact rats, or 0.49 μCi D,L-[^{3}H]carnitine and 1.3×10^6 cells from rats castrated for at least 8 weeks. Incubation conditions are described in the text and the data represent the means of duplicate determinations. (•) Cells from intact rats; (○) cells from castrated rats.

rat liver cells. The apparent K_m obtained for epididymal cells in this study was ~1 mM, being lower than liver [12] but greater than muscle and kidney [17–19]. Since the apparent K_m is significantly above the normal serum concentration of carnitine (50–90 μM) [1,21] it is expected that the transport system would not be saturated with substrate in vivo.

D-Carnitine was transported with the same kinetics as L-carnitine and hence the transporting system shows no stereospecificity between the two isomers. In [22] the overall carnitine transport process in the perfused caudal region of the epididymis was stereospecific. However, overall transport is likely to involve both an inwardly-directed basal pump and an outwardly-directed apical pump located in the epithelial cells [6]. It is probable that only the basal pump was measured in our cell preparations and hence the combined results may suggest that the basal pump is not stereospecific whereas the apical pump is.

In a direct study of D-carnitine transport with liver cells a lack of stereospecificity was also found [12]. On the other hand, competition studies suggest that the transport system may be stereospecific in muscle and cultured heart cells [17,23]. Since butyrobetaine is apparently transported by the same carrier as carnitine in liver, heart and muscle [12,18,20] it would seem that the carrier primarily recognizes the quaternary ammonium portion of the molecule. This would explain the ability of other compounds containing this grouping to reduce carnitine transport [17,23]. However, a negatively charged moeity within the molecule may also be important since choline, which contains the quaternary ammonium but lacks a carboxyl group, is a poor inhibitor of carnitine transport [17,22].

By using the protein content of epididymal cells (0.28 mg/10^6 cells) and the total protein content of the epididymis (122 mg/g wet wt tissue [24]), it is possible to convert our results for V_{max} into units equivalent to those used for liver [12] and for muscle [17]. When this is done the maximum velocity of uptake in the epididymis is ~10-times slower than liver but 100-times faster than muscle.

In contrast to the saturable process of carnitine uptake by epididymal cells from normal animals, uptake was slower and non-saturable in cells from castrated animals. The non-saturable uptake by castrate cells may reflect a carrier-independent mode of uptake, in which case it could be postulated that castration results in a loss from the cell membrane of specific carrier molecules. Whatever the mechanism for carni-

tine uptake, the results (fig.2) clearly show that at the concentration of carnitine normally present in plasma (50–90 μM), uptake by cells from normal rats is 10-times greater than that of cells from castrated rats. The difference in uptake kinetics for epididymal cells from the two endocrine states is fully consistent with the observed effect of androgens on epididymal carnitine accumulation in vivo [7,10].

The control of carnitine transport by sex hormones may be a feature of most body tissues since carnitine in blood plasma is raised by androgens and reduced by oestrogens [25] and marked sex differences are noted in carnitine content of liver and muscle and in the rate of tubular excretion in the kidney [21]. Moreover, there is some evidence that the concentration of plasma carnitine may, in itself, regulate the number of membrane carriers [26].

Acknowledgements

This work was supported by the Australian Research Grants Committee. D- and L-carnitine were donated by Otsuka Pharmaceutical Co., Japan and Sigma Tau, Italy.

References

[1] Brooks, D. E. and McIntosh, J. E. A. (1975) Biochem. J. 148, 439–445.
[2] Van Dyke, D. H., Griggs, R. C., Markesbury, W. and Di Mauro, S. (1975) Neurology 25, 149–154.
[3] Engel, A. G. (1980) in: Carnitine Biosynthesis, Metabolism and Functions (Frenkel, R. A. and McGarry, J. D. eds) pp. 271–284, Academic Press, New York.
[4] Hinton, B. T., Snoswell, A. M. and Setchell, B. P. (1979) J. Reprod. Fert. 56, 105–111.
[5] Hinton, B. T., Brooks, D. E., Dott, H. M. and Setchell, B. P. (1981) J. Reprod. Fert. 61, in press.
[6] Brooks, D. E. (1980) in: Carnitine Biosynthesis, Metabolism and Functions (Frenkel, R. A. and McGarry, J. D. eds) pp. 219–235, Academic Press, New York.
[7] Marquis, N. R. and Fritz, I. B. (1965) J. Biol. Chem. 240, 2197–2200.
[8] Brooks, D. E., Hamilton, D. W. and Mallek, A. H. (1973) Biochem. Biophys. Res. Commun. 52, 1354–1360.
[9] Hinton, B. T. and Setchell, B. P. (1980) in: Carnitine Biosynthesis, Metabolism and Functions (Frenkel, R. A. and McGarry, J. D. eds) pp. 237–250, Academic Press, New York.
[10] Brooks, D. E., Hamilton, D. W. and Mallek, A. H. (1974) J. Reprod. Fert. 36, 141–160.
[11] Ramsay, R. R. and Tubbs, P. K. (1975) FEBS Lett. 54, 21–25.
[12] Christiansen, R. Z. and Bremer, J. (1976) Biochim. Biophys. Acta 448, 562–577.
[13] Killian, G. J., Amann, R. P. and Snyder, J. (1976) Biol. Reprod. 15, 266–279.
[14] Brooks, D. E. and Higgins, S. J. (1980) J. Reprod. Fert. 59, 363–375.
[15] Marquis, N. R. and Fritz, I. B. (1964) J. Lipid Res. 5, 184–187.
[16] Brooks, D. E. (1978) Biol. Reprod. 18, 629–638.
[17] Rebouche, C. J. (1977) Biochim. Biophys. Acta 471, 145–155.
[18] Willner, J. H., Ginsburg, S. and Di Mauro, S. (1978) Neurology 28, 721–724.
[19] Huth, P. J., Thomsen, J. H. and Shug, A. L. (1978) Life Sci. 23, 715–722.
[20] Böhmer, T., Eiklid, K. and Jonsen, J. (1977) Biochim. Biophys. Acta 465, 627–633.
[21] Borum, P. R. (1978) Biochem. J. 176, 677–681.
[22] Yeung, C. H., Cooper, T. G. and Waites, G. M. H. (1980) Biol. Reprod. 23, 294–304.
[23] Mølstad, P., Bøhmer, T. and Eiklid, K. (1977) Biochim. Biophys. Acta 471, 296–304.
[24] Brooks, D. E. (1977) J. Reprod. Fert. 49, 383–385.
[25] Borum, P. R. (1980) in: Carnitine Biosynthesis, Metabolism and Functions (Frenkel, R. A. and McGarry, J. D. eds) pp. 115–126, Academic Press, New York.
[26] Mølstad, P., Bøhmer, T. and Hovig, T. (1978) Biochim. Biophys. Acta 512, 557–565.
[27] Wilkinson, G. N. (1961) Biochem. J. 80, 324–332.

Vol. 4, No. 4, April 1982 **BIOCHEMISTRY INTERNATIONAL**
Pages 451-456

CARNITINE ESTERS AND CARNITINE ACYLTRANSFERASE ACTIVITY IN NORMAL AND ALLOXAN-DIABETIC SHEEP

R. C. Fishlock and A. M. Snoswell
Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, 5064, Australia
and
K. Valkner and L. L. Bieber
Department of Biochemistry, Michigan State University, East Lansing, Michigan, U.S.A. 48824.

Received February 2, 1982

SUMMARY

Short-chain acyl-carnitines were separated from perchloric acid extracts of freeze-clamped sheep tissues and the acyl residues analyzed by gas chromatography. Acetylcarnitine was the major carnitine ester present in both sheep liver and skeletal muscle. Acetyl, propionyl, isobutyryl, butyryl and isovaleryl esters all increased markedly in the diabetic liver. Acetyl-, isobutyryl- and isovaleryl-carnitines also increased significantly in skeletal muscle in the diabetic animals. Carnitine acetyltransferase and carnitine isobutyryltransferase increased significantly in the liver in the diabetic state. The results suggest that in addition to an important role of carnitine in 'acetyl' metabolism, carnitine plays a small but significant role in propionate metabolism and branched chain acid metabolism in sheep.

INTRODUCTION

Marked increases in the concentration of acetylcarnitine occur in sheep liver on starvation (1) and even more pronounced increases have been observed in diabetic sheep (2,3). These changes are much greater than in other species and are also accompanied by large increases in free carnitine and total acid-soluble carnitine (4). The nature of the acid-soluble carnitine fraction, other than free and acetyl-carnitine, is not known, but knowledge of this fraction could give important insights into the metabolic roles of carnitine in sheep.

Choi and Bieber (5) developed a method for the isolation, identification and quantitation of water-soluble aliphatic acylcarnitines. Using gas chromatography combined with mass spectrometry they identified a variety of carnitine esters in beef heart (6) and in rat tissues (7). In the latter studies acetylcarnitine was the most abundant acylcarnitine, but appreciable quantities of propionyl-, isobutyryl-, isovaleryl- and tiglyl-carnitines were found. Because branched chain acylcarnitines were found, Bieber and Choi

0158-5231/82/040451-06$01.00/0

Copyright © 1982 by Academic Press Australia.
All rights of reproduction in any form reserved.

(6) suggested that carnitine played a role in branched-chain amino acid metabolism. This view was reinforced by the finding that significant increases in the amount of isobutyryl- and isovaleryl-carnitine occurred in the muscle of fasted rats (8).

Since very large changes in the amounts of water-soluble acylcarnitines occur in diabetic sheep and since this species depends on the metabolism of volatile fatty acids for energy production and depends on propionate for gluconeogenesis we have examined the nature of the water-soluble acyl-carnitine fraction and carnitine acyltransferase activity in normal and diabetic sheep.

METHODS AND MATERIALS

Sheep. Merino ewes approximately 2 years old and weighing between 40-45 kg were used. The animals were maintained on 1000 g of lucerne-chaff per day. Diabetic animals were produced by intravenous injection of 50 mg/kg body weight of alloxan in isotonic saline (prepared immediately prior to injection). The animals were slaughtered one week after alloxan administration. At this time blood glucose concentrations were greater than 10 mM and total blood ketones (acetoacetate plus 3-hydroxybutyrate) were 5-7 mM.

Tissue preparations. Liver and skeletal muscle (biceps femoris) were freeze-clamped immediately upon slaughter. An additional sample of liver was collected into ice-cold 0.25 M sucrose, 2.5 mM HEPES, 0.25 mM EDTA medium (pH 7.5) and a 10% (w/v) homogenate was prepared in the same medium. This homogenate was centrifuged at 500 x g for 10 min, and the supernatant fluid used for the assay of carnitine acyltransferase.

Carnitine acyltransferase was measured spectrophotometrically using acetylCoA, isobutyrylCoA, octanoylCoA and palmitoylCoA as substrates, see Choi *et al.* (7).

Acetyl-, free and total acid-soluble carnitine. Perchloric acid extracts of the tissues were prepared as described previously (1). Free carnitine was measured in neutralized perchloric acid extracts by the method of Marquis and Fritz (9), acetylcarnitine by the method of Pearson and Tubbs (10) and total acid-soluble carnitine by a later method of Pearson and Tubbs (11).

Isolation and quantification of water-soluble acyl-carnitines. The water-soluble acylcarnitine fractions were isolated from frozen tissue preparations and the volatile fatty acids identified by gas chromatography according to the method of Choi and Bieber (5). The tissue extracts were lyophilized at the end of step 7 of this method and sealed in ampoules under nitrogen. The samples to this stage were prepared in Adelaide and then airfreighted to East Lansing for the final stages of the separation and the gas chromatography. Known amounts of crotonyl- and valeryl-carnitines were added as internal standards to the perchloric acid extraction stage prior to homogenization to determine recoveries in the isolation and quantitation of

of the acylcarnitines. Valerylcarnitine is the preferable internal standard as the crotonylcarnitine is unstable over extended periods. The acylcarnitines separated by gas chromatography were also measured by reference to acetylcarnitine determined spectrophotometrically in the samples at the time of collection.

RESULTS

Acetylcarnitine is the major short-chain acylcarnitine in both sheep liver and skeletal muscle (Table 1). A 90-fold increase in liver acetylcarnitine occurred with diabetic animals, which was greater than previously reported (3). This large increase was due however to the relatively low value for the normal sheep fed on lucerne chaff (cf. Snoswell and Henderson (1)).

In addition to the increase in acetylcarnitine in the liver of the diabetic animals, even larger increases were found for the other acylcarnitines. Propionyl-, isobutyryl-, butyryl and isovaleryl-carnitines increased 104, 1620, 220 and 37-fold respectively (Table 1). In skeletal

Table 1. Short chain acylcarnitines in liver and skeletal muscle of normal and alloxan-diabetic sheep

Tissue	Condition	Actylcarnitine (nmoles/gm wet wt.)				
		Acetyl	Propionyl	Isobutyryl	Butyryl	Isovaleryl
Liver	normal	13.9 ± 2.8	3.6 ± 2.3	0.1 ± 0.1	1.3 ± 0.6	2.1 ± 1.1
"	diabetic	1260 ± 191	377 ± 12	162 ± 12	288 ± 19	78 ± 19
		$p<0.01$	$p<0.001$	$p<0.05$	$p<0.001$	$p<0.02$
Skeletal muscle (b.femoris)	normal	1360 ± 157	238 ± 41	54 ± 8	156 ± 16	23 ± 13
"	diabetic	6280±1740	411 ± 180	219 ± 24	267 ± 130	156 ± 46
		$p<0.05$	n.s.	$p<0.01$	n.s.	$p<0.05$

The figures shown are the means ± SEM for 3 animals in each group. Significance was determined by *t* test comparing the values for the diabetic sheep with those for the normal sheep for each tissue. The diabetic animals were assayed 1 week after the intravenous administration of 50 mg/kg body weight of alloxan.

muscle, in addition to the 5-fold increase in acetylcarnitine, significant increases in isobutyrylcarnitine (4-fold) and isovaleryl-carnitine (6-fold) were obtained for the diabetic state (Table 1).

In sheep liver carnitine acetyltransferase activity was considerably higher than the other carnitine acyltransferase activities measured (Table 2). This is in contrast with the situation in rat liver, where carnitine octanoyltransferase activity was greatest (7). Both carnitine acetyltransferase and carnitine isobutyryltransferase activities increased significantly (approx. 2-fold) in sheep liver in the diabetic state (Table 2). Again, this is in contrast to the rat where, with streptozotocin-induced diabetic animals, all carnitine acyltransferase activities (determined using acetyl-, octanoyl-, isobutyryl-, isovaleryl- and palmitoyl-CoA as substrates) increased significantly in the diabetic state (12).

Table 2. Carnitine acyltransferase activity in normal and alloxan-diabetic sheep liver

Condition	Carnitine acyltransferase activity (nmoles/min/mg protein)			
	Acetyl	Isobutyryl	Octanoyl	Palmitoyl
Normal	3.2 ± 0.6	1.6 ± 0.2	1.4 ± 0.3	2.0 ± 0.5
Diabetic	6.2 ± 0.5	3.4 ± 0.2	2.0 ± 0.3	1.0 ± 0.4
	p<0.02	p<0.01	n.s.	n.s.

The figures shown are the means ± SEM for 3 animals in each group.

DISCUSSION

The results presented here confirm earlier observations (2-4) that acetylcarnitine is the major water-soluble carnitine ester in both sheep liver and skeletal muscle. However, in addition, the relatively greater amounts of propionylcarnitine present in sheep liver compared to rat liver (7) are in accord with the role of propionate as an important glucoeogenic precursor in ruminant animals. Most of the propionate produced in the rumen

of sheep is utilized by the liver (13) via formation of propionyl-CoA. Presumably the propionyl-CoA is readily utilized by carnitine acetyltransferase (14), producing propionylcarnitine. Recent evidence (15,16) strongly indicates this role for carnitine is indirect; it apparently modulates the CoASH/short-chain acylCoA ratio in the matrix of mitochondria. Since propionyl-CoA is a potent inhibitor of the pyruvate dehydrogenase in pig kidney mitochondria (17), it seems likely that carnitine could affect the propionylCoA inhibition via propionylcarnitine formation.

The increased amounts of isobutyryl- and isovaleryl-carnitine in the diabetic sheep muscle point to a role of carnitine in branched chain amino acid metabolism in this species, as first suggested by Bieber and Choi (6) following their studies in beef heart. Snoswell and Henderson (4) previously found that carnitine stimulated the oxidation of α-ketoisocaproic acid (the keto acid derived from leucine) by isolated skeletal muscle mitochondria from diabetic sheep and here we report increased amounts of isobutyryl- and isovaleryl-carnitines in the sheep liver in the diabetic state.

Together, these findings point to a new role of carnitine in inter-organ metabolism, which may operate as follows: carnitine compounds are derived from the oxidation of branched-chain amino acids in skeletal muscle mitochondria, then leave the muscle tissues and travel to the liver for subsequent oxidation. Such a process was first suggested by van Hinsberg, Veerkamp and Cordewener (18) but requires verification with labelled compounds.

ACKNOWLEDGEMENTS

A.M.S. was supported by a grant from the Australian Research Grants Committee (No.D277.15558). Support in part by grant AM 18427 from N.I.H.

REFERENCES

1. Snoswell, A.M. and Henderson, G.D. (1970) Biochem. J. 119, 59-65.
2. Snoswell, A.M. and Koundakjian, P.P. (1972) Biochem. J. 127, 133-141.
3. Snoswell, A.M. and McIntosh, G.H. (1974) Aust. J. Biol. Sci. 27, 645-650.
4. Snoswell, A.M. and Henderson, G.D. (1980) In Carnitine biosynthesis metabolism and functions (Frenkel, R.A. and McGarry, J.D. eds.) pp. 191-204, Academic Press.
5. Choi, Y.R. and Bieber, L.L. (1977) Anal. Biochem. 79, 413-418.
6. Bieber, L.L. and Choi, Y.R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2795-2798.

7. Choi, Y.R., Fogle, P.J., Clarke, P.R.H. and Bieber, L.L. (1977) J. Biol. Chem. 252, 7930-7931.
8. Choi, Y.R., Fogle, P.J. and Bieber, L.L. (1979) J. Nutr. 109, 155-161.
9. Marquis, N.R. and Fritz, I.B. (1964) J. Lipid Res. 5, 184-187.
10. Pearson, D.J. and Tubbs, P.K. (1964) Nature (Lond.) 202, 91.
11. Pearson, D.J. and Tubbs, P.K. (1967) Biochem. J. 105, 953-963.
12. Fogle, P.J. and Bieber, L.L. (1979) Biochem. Med. 22, 119-126.
13. Bergman, E.N. (1975) In Digestion and metabolism in the ruminant (McDonald, I.W. and Warner, A.C.I. eds.) pp. 292-305, The University of New South Wales Publishing Unit, Sydney.
14. Chase, J.F.A. (1967) Biochem. J. 104, 510-518.
15. May, M.E., Aftring, R.P. and Buse, M.G. (1980) J. Biol. Chem. 255, 8394-8397.
16. Bieber, L.L., Emaus, R., Valkner, K. and Farrell, S. (1981) Mini Symposium "Carnitine Biosynthesis and Function", Amer. Soc. Biol. Chem. in press.
17. Gregersen, N. (1981) Biochem. Med. 26, 20-27.
18. Van Hinsbergh, V.W.M., Veerkamp, J.H. and Cordewener, J.H.G. (1980) Int. J. Biochem. 12, 559-565.

CARNITINE AND METABOLISM IN RUMINANT ANIMALS[1]

A. M. Snoswell
G. D. Henderson

Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia.

INTRODUCTION

Carnitine generally plays the same important metabolic roles in the tissues of ruminant animals as in other species, viz. as an essential factor in fatty acid oxidation and in the transfer of acyl groups across intracellular membranes (1). However, there are a number of quantitative aspects of the role of carnitine, particularly in relation to metabolic stress, which are highlighted in this group of animals. References to this work are scattered throughout the literature and these have been consolidated here, together with a number of new features not previously reported. In retrospect it is perhaps not surprising that carnitine should be quantitatively more important in ruminant animals where tissues are relatively more dependent on the oxidation of fatty acids than carbohydrate. Particular attention will be devoted to the role of carnitine in skeletal muscle and liver in this paper.

CARNITINE AND METABOLISM IN SKELETAL MUSCLE

It is appropriate that this paper should begin with this topic as it is over 70 years ago since carnitine was first isolated from meat extracts and identified (2). The data shown in Table 1 indicate that muscles of sheep, cattle and goats contain quite large amounts of carnitine, most of which is present in the free form, under normal conditions.

[1]Supported by grants from the Australian Research Grants Committee.

Copyright © 1980 by Academic Press, Inc.
All rights of reproduction in any form reserved.
ISBN: 0-12-267060-4

TABLE 1. Carnitine and Carnitine Esters in Muscle Tissues of Ruminants

Species	Age	No., Sex & Conditions	Muscle	μmoles/gm wet wt.				Ref.
				Free	Acetyl	Total acid-soluble	Acid-Insoluble	
Sheep	5-16 days	4 various	*M. biceps femoris*	3.59 ± 0.59	0.175 ± 0.043	4.78 ± 0.46	0.033 ± 0.001	3
"	4 years	5 wethers, winter	"	6.88 ± 0.64	1.20 ± 0.15	9.10 ± 0.43		*
"	4 "	4 wethers, summer	"	9.86 ± 1.38	1.82 ± 0.48	12.9 ± 0.88	0.017 ± 0.005	3
"	2 "	4 ewes	"	9.78 ± 1.40		12.6 ± 0.94		*
"	2 "	4 wethers	*M. masseter*	3.49 ± 0.60	0.69 ± 0.070	6.4 ± 0.56		*
"	4-5 "	2 rams	various			9.09 - 11.0		4
Cattle	3 weeks	4 various	*M. diaphragma*	3.59 ± 0.72	0.98 ± 0.08	5.54 ± 0.58		*
"	Adult	4 steers	"	9.39 ± 1.40	1.52 ± 0.19	12.5 ± 0.97		*
"	Adult	4 cows	various			3.69 - 4.25		4
Goats	Adult	3 male	*M. biceps femoris*	4.70 ± 1.19	1.25 ± 0.14	7.86 ± 0.49		*
"	Adult	2 females lactating	"	12.9	1.05	14.3		5

The figures quoted are means ± S.E.M. where indicated.
*Unpublished work relating to Merino sheep, Ayrshire cattle and Saanen goats.

The total carnitine content of a particular muscle (e.g. *M.biceps femoris*) can vary with the season (Table 1). This variation is probably related to the different pasture conditions. Pastures are of poorer quality in Australian summer conditions and muscle carnitine content is significantly greater than in winter. In this respect the carnitine content of blood of lactating ewes on a low nutritional plane was found to be significantly greater than those on a high nutritional plane (5). The data in Table 1 also show that the total carnitine content of the same muscle varies with age nd is considerably less in younger animals. This is highlighted in Fig.1, which shows a dramatic rise in the total carnitine content of *M. biceps femoris* of lambs immediately after birth. Only a very small rise in liver carnitine is seen at the same time (Fig.1). A 2-3-fold increase in muscle carnitine was observed in rats during the first week after birth (6).

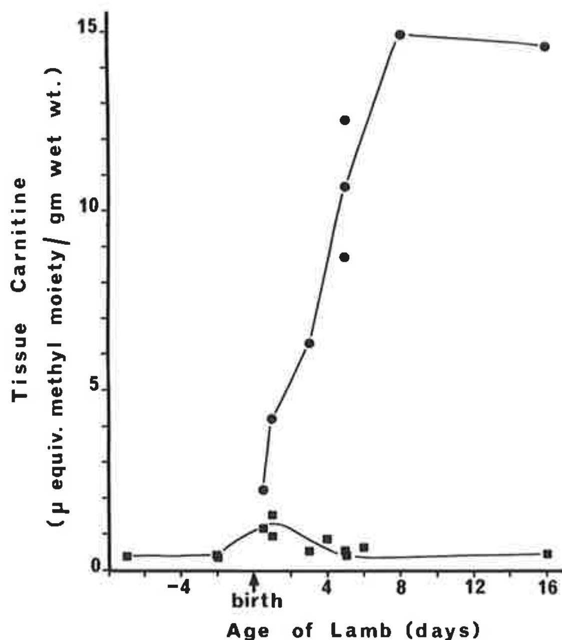


FIGURE 1. Carnitine content of skeletal muscle and liver of young lambs in relation to age. Total acid-soluble carnitine was assayed in freeze-clamped samples. For details see ref. 3.

● Skeletal muscle (*M.biceps femoris*), ■ liver.

Thus it would appear that the rapid rise in muscle carnitine is not due to biosynthesis in the liver. However, biosynthesis of carnitine in the muscle tissue of the lamb is a possibility. Hydroxylation of γ-butyrobetaine to carnitine by skeletal muscle from adult sheep has been demonstrated by three groups (7,8,9). Moreover, a significant increase in the carnitine content of muscle tissue occurs in adult diabetic sheep (see Table 2), suggesting possible biosynthesis in this tissue as no significant uptake of carnitine from the blood by skeletal muscle was observed in the severe diabetic state (12). The most likely source of this muscle carnitine in the lamb is carnitine derived from the mother's milk. Ewes' milk is relatively rich in carnitine (5) and a young lamb could receive 30-40 gm of carnitine via the milk during a 10-week lactation. It has been shown that in suckling rats uptake of carnitine from the mother's milk gives rise to an increased carnitine content of liver and heart tissue (10). The rapid rise of muscle carnitine in the young lamb following birth and its possible derivation from the mother's milk could mean that the newborn lamb is at risk in cold stress situations, for short term cold stress in goats has been shown to significantly decrease carnitine secretion in the milk (11).

TABLE 2. Carnitine and Carnitine Esters in Muscle (*M.biceps femoris*) of Alloxan-Diabetic Sheep

Condition	μ mole/gm wet wt.			
	Free	Acetyl	Total Acid-Sol.	Acid-insol.
3 days after alloxan	7.20	4.53	12.8	0.034
7-10 days after alloxan	5.76	4.78	12.4*	
10-12 days after alloxan	3.24	12.0	17.1	

Values are the means for 4 animals taken from ref.3, 5 animals (unpublished data) and 3 animals taken from ref.12, respectively. All sheep were Merino wethers.

*The total carnitine figure here [12.4 ± 1.1 (5)] was significantly ($P<.05$) higher than the value for a corresponding group of normal animals (see line 2, Table 1).

The carnitine content of a particular muscle may vary between breeds in one species (e.g. sheep), as seen in Fig.2 for *M.sternothyroideus*, although this could be related to differing nutritional conditions, as discussed above. However, more importantly, the carnitine content of muscle appears to be inversely related to the CoA content (Fig.2), those muscles with a very low CoA content containing relatively high levels of carnitine. These observations may suggest that carnitine transport into muscle tissues, or biosynthesis is regulated by the CoA content or vice versa.

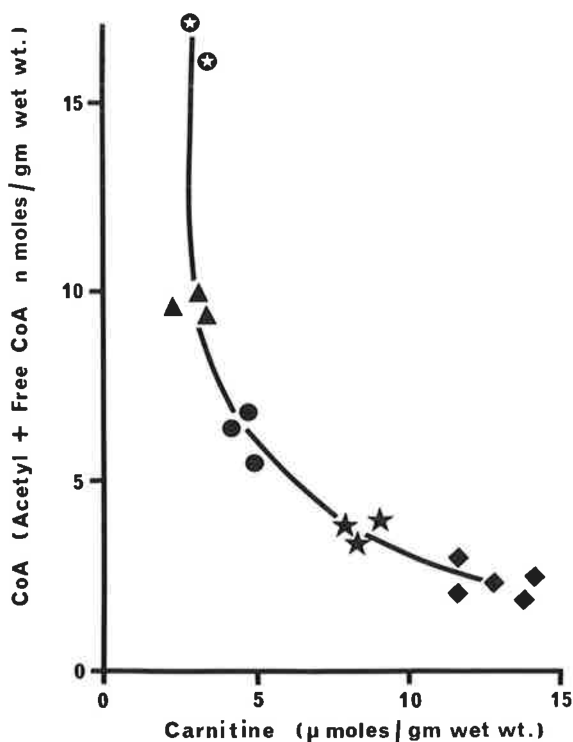


FIGURE 2. Carnitine and coenzyme A contents of muscle tissue of sheep. Total acid-soluble carnitine and acetyl plus free CoA were measured as described in ref.3.

M.biceps femoris ✪ from 5-day-old Merinos, ● from 8-day-old Merinos, ◆ from 2-year-old Merinos; *M.sternothyroideus* ▲ from adult Merinos, ✱ from adult Suffolks.

These observations also raise the possibility that muscle myopathies due to a deficiency of carnitine, or defective carnitine transport into muscle, as seen in humans (13,14) may well be discovered in domestic ruminant animals in the future.

The varying carnitine content of muscles with age and in particular the inverse relationship to CoA content raise questions as to the main function of carnitine in muscle tissues. Obviously carnitine is essential for the oxidation of long chain fatty acids as these form a significant proportion of the metabolizable fuel in ruminant muscle (15). However, it is surprising that long chain acylcarnitine (acid-insoluble fraction) constitutes only a very small fraction of the total carnitine in the muscles of sheep (Table 1), even in the diabetic state (Table 2). The high concentration of carnitine present in ruminant muscles could be necessary for the oxidation of acetate. Acetate is a major calorific fuel in ruminants which is mainly oxidized in extra hepatic tissues. However, as acetate readily penetrates the inner mitochondrial membrane, and in muscle tissues of the sheep the acetate thiokinase is located mainly in the mitochondria, in the matrix (3), the high content of carnitine in ruminant muscles is not necessary for acetate oxidation.

It would seem that a major role of carnitine in muscle tissues, particularly in stress conditions, is in an acetyl buffer system, removing acetyl groups from inside the muscle mitochondria and thereby relieving 'acetyl pressure' on the very small amounts of CoA present in muscle, 95% of which is localized in the mitochondria (3). Calculation of the Mass Action Ratios according to the equation for the carnitine acetyltransferase reaction

Acetyl-L-carnitine + CoA $\rightleftharpoons$ acetyl-CoA + L-carnitine

from metabolite concentrations determined in freeze-clamped muscle tissue gives values of 1.9-4.2 (3). These values are close to the value of the apparent equilibrium constant of 0.6 for the isolated enzyme reaction (16) and suggest that the muscle carnitine acetyltransferase is sufficiently active to allow acetyl buffering, even under extreme 'acetyl pressure' as seen in the severe diabetic state (Table 2), where more than 60% of the muscle carnitine may be present as acetylcarnitine. This represents an extremely large reserve, up to 30 gm in a whole sheep, of a substance which is a 'high energy' storage compound.

A further significant role of carnitine in muscle tissues, particularly in times of metabolic stress, may be in the metabolism of branched-chain amino acids. Skeletal muscle is the main site for the oxidation of branched-chain amino acids in the rat and the capacity to degrade these acids is

increased 3-5-fold in fasting and diabetes (17). Significant quantities of isobutyryl-carnitine have been found in skeletal muscle of sheep (L.L. Bieber and Y.R. Choi, unpublished results). Isobutyryl-carnitine is derived from the oxidation of valine. There was a slight net uptake of valine by muscles of the hind limb of the sheep, as judged by tarsal venous *versus* femoral arterial concentrations, in the diabetic state (P.J. Buttery, D.B. Lindsay and D.W. Pethick, unpublished results). Simultaneously there was a significant net output from the muscles of the hind limb of virtually all other amino acids, with the output of the other branched chain amino acids, leucine and isoleucine being relatively small. Further, as shown in Figure 3, carnitine stimulated the oxidation of α-keto isocaproate, the keto acid derived from leucine, by isolated skeletal muscle mitochondria from a

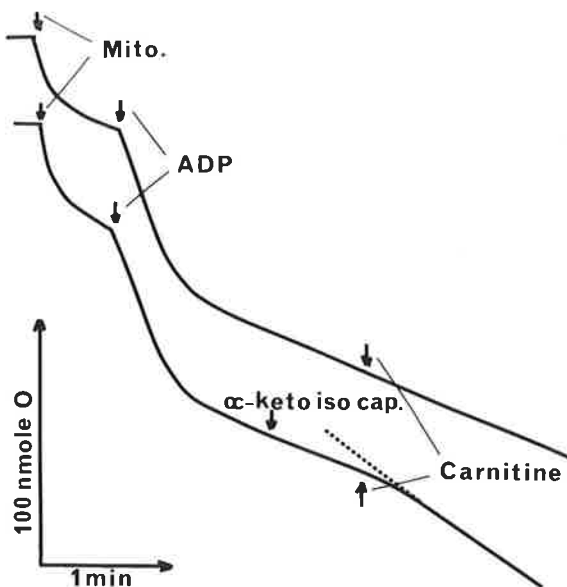


FIGURE 3. Effect of carnitine on the oxidation of α-keto isocaproate by skeletal muscle mitochondria from a diabetic sheep. Mitochondria were isolated by the method described in ref.18 and incubated in a medium containing 225 mM sucrose, 20 mM KCl, 10 mM pot. phosphate (pH 7.4), 20 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 25 μM cytochrome c, 0.1 mM CoA, and 0.1% bovine serum albumen in a volume of 1.35 ml at 30°C. Oxygen uptake was monitored with an oxygen electrode. Mitochondria (1.8 mg protein), 5 μmole ADP and 10 μmole L-carnitine were added where indicated.

diabetic sheep. There is a lag in the stimulation of the rate of oxidation by added carnitine (Fig.3), and this may imply that carnitine plays a role in the transport of the isovaleryl group out of the mitochondria, as suggested for rat skeletal muscle (19).

CARNITINE AND METABOLISM IN LIVER

Data for the liver carnitine content of sheep, cattle and goats are presented in Table 3. The carnitine content of liver varies significantly with the season, as in muscle. Also, the type of feed affects the proportion of acetylcarnitine and is probably related to the ratio of major volatile fatty acids produced in the rumen under varying feed conditions (20). Lactation in cows causes a significant reduction in liver carnitine (Table 3).

Carnitine is obviously necessary for the oxidation of long chain fatty acids in ruminant liver. However, as in muscle, relatively small amounts of long chain acylcarnitine are found even in the severe diabetic state where, in sheep, over 60% of the carnitine is in the form of acetyl-carnitine (12). This is probably a reflection of the relatively high proportion of carnitine acetyltransferase to carnitine palmitoyltransferase in sheep liver (20,21).

Undoubtedly the most outstanding feature of carnitine metabolism, at least in the sheep, is the dramatic increase in liver carnitine seen in various metabolic stress conditions. The data in Table 4 show that in fasting there is a 4-5-fold increase in liver carnitine, a 28-fold increase in severe diabetes, a 40-fold increase in moderate cases of pregnancy toxaemia and an 85-fold increase in one severe case of pregnancy toxaemia. The data are expressed as μmole/gm dry wt. of fat-free tissue to allow for variations in water content and particularly the fat content of the liver in these conditions (22). We have also found a 12-fold increase in liver carnitine in the diabetic goat. In addition, there is a significant increase in the amount of total acid-soluble carnitine secreted into the milk of spontaneously ketotic cows compared with normal lactating cows (23) which may reflect an increased amount of carnitine in the liver of this species in this metabolic disease.

The carnitine content of sheep liver appears to increase in response to triacylglycerol up to about 1 gm of total lipid per gm dry wt. of fat-free tissue, but beyond that point the carnitine content rises sharply (Fig.4). The large increase in liver carnitine seen in the sheep in the diabetic state (Table 4) is due to production in the liver and not mobiliza-

TABLE 3. Carnitine and Carnitine Esters in Liver of Ruminants

Species	No.	Sex and Conditions	nmoles/gm. wet wt.				Ref.
			Free	Acetyl	Total acid-soluble	Acid-Insoluble	
Sheep	4	lambs 5-16 days old	86 ± 18	35 ± 24	153 ± 11	10 ± 1	3
"	4	4-year-old wethers fed lucerne chaff	74 ± 7	3.5 ± 2.6	139 ± 9	<0.1	3
"	4	4-year-old wethers on open pasture	55 ± 18	80 ± 7	148 ± 41	15 ± 8	20
"	4	wethers, summer pasture	79 ± 22	21 ± 3	168 ± 22		*
"	5	wethers, winter pasture	47 ± 11	13 ± 5	110 ± 10		*
"	2	4-5-year-old rams			160		4
Cattle	4	Non lactating cows	26.5 ± 4.3	23.9 ± 4.9	67.3 ± 7.4		24
"	4	Lactating cows	14.8 ± 4.0	11.2 ± 3.6	24.4 ± 5.6		24
"	4	Dry and late lactating cows			160 ± 10		4
"	4	3-week-old calves	80 ± 6	9 ± 2	87 ± 5		*
"	4	Adult steers	91 ± 25	25 ± 6	198 ± 25		*
Goats	2	Lactating	278	7	290	25	5
"	2	6-year-old males	18	24	67		*

*Unpublished results from Merino sheep and Ayrshire cattle and Saanen goats.

TABLE 4. Carnitine Content of Sheep Liver under Various Conditions

Condition	Total acid-soluble carnitine (μmole/gm dry wt. fat-free tissue)
Normal	0.678 ± 0.124 (15)
Fasted	3.23 ± 0.20 (4)
Diabetic:	
- mild	4.32 ± 0.25 (4)
- severe	25.2 ± 3.4 (8)
Pregnancy toxaemia:	
- mild	7.43 ± 1.1 (5)
- moderate	32.1 ± 5.5 (3)
- severe	76.7

Values are means ± S.E.M. with the number of sheep in parentheses. The fasted animals were sampled after 7 days; the mild diabetic animals 3 days after and the severe group 7-10 days after alloxan administration. The severity of field cases of pregnancy toxaemia was judged on the basis of clinical observations.

tion from the muscle, for muscle carnitine content is maintained in the diabetic state (Table 2). Also, a significant hepatic-portal difference in blood carnitine concentration was found in surgically cannulated sheep in the terminal diabetic state (12). The blood carnitine concentration rose up to 10-fold and the daily loss of carnitine in the urine increased markedly.

It appears from the foregoing considerations that hepatic carnitine biosynthesis is regulated by the demand placed on the liver by incoming fatty acids, but beyond a certain point hepatic carnitine biosynthesis runs out of control and carnitine passes into the blood and is lost in the urine.

The unique dramatic response of liver carnitine levels to liver lipid levels in sheep may be related to the very low rates of fatty acid biosynthesis in ruminant liver (25,26). Thus, the proposed regulatory role of malonyl-CoA in hepatic fatty acid metabolism (27) may not be of significance in sheep. Further, the unusual structure of the hepatic sinusoids (28) and the apparent low rate of VLDL biosynthesis in sheep liver (29) may prevent the ready removal of accumulated

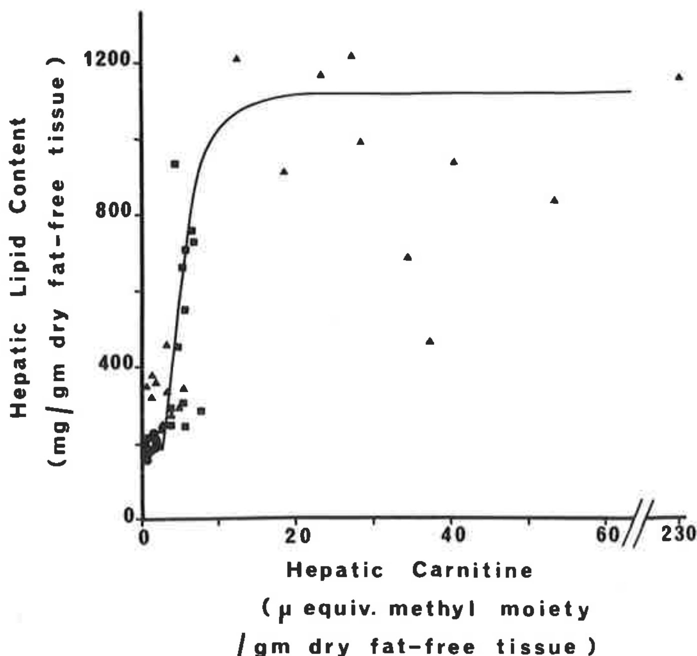


FIGURE 4. The relationship between total hepatic lipid and carnitine content of adult sheep. Total acid-soluble carnitine and total lipid (predominantly triacylglycerol) were measured as described in ref.22. The data shown are for 48 sheep in a variety of conditions ranging from normal to diabetic animals and severe cases of pregnancy toxaemia.

triacylglycerols from the liver. Consequently, hepatic carnitine levels may assume a more significant quantitative role in regulating the flux of fatty acids via the oxidative pathway.

These findings raise important questions regarding the turnover of methionine and methyl groups. Carnitine has three methyl groups which are derived from methionine (via S-adenosylmethionine) and when large amounts of carnitine are synthesized in the liver, as in the severe diabetic state, and then pass into the blood and urine, this represents a major drain on the methyl pool and hence methionine. This is particularly critical in the sheep where methionine is the first limiting amino acid (30). The question arises as to how so much of the methyl pool can be diverted into carnitine biosynthesis under these conditions?

TABLE 5. Creatine Content of Liver and Muscle and Urinary Excretion in Normal and Diabetic Sheep

Condition	Creatine* Content µmole/gm dry wt. fat-free tissue		Urinary Creatine* Excretion µmole/kg body wt. /24 h
	Liver	Muscle	
Normal	10.4 ± 1.3	106 ± 5	219 ± 11
Diabetic	4.2 ± 0.4	112 ± 11	[1]129 ± 20
	P < 0.01	N.S.	P < 0.05

Values are means ± S.E.M. for 5 sheep.
*Measured enzymically as creatine plus creatinine.
[1]Measured 7 days after alloxan administration (i.e. severe diabetic state).

The major methyl output in animals is creatine, which is largely excreted in the urine as creatinine. Creatine metabolism accounts for over 80% of methyl output in humans (31) and appears of a similar order in other animals. Thus it seemed important to examine creatine metabolism in the sheep. The results in Table 5 indicate that the creatine content of the liver of diabetic sheep is significantly (P < 0.01) reduced to 40% of that in normal animals, in contrast to the situation for carnitine (Table 4). The excretion of creatine (as creatine and creatinine) into the urine is also substantially reduced in the diabetic state, while muscle creatine content remains unchanged (Table 5). Thus it is clear that in the diabetic state in sheep, creatine retention by muscle is maintained and biosynthesis in the liver is reduced. The activity of guanidinoacetate methyltransferase (EC 2.1.1.2) in the liver may be reduced in the diabetic state (results not shown). Thus it appears that in the diabetic state increased hepatic carnitine biosynthesis in sheep can proceed to a large extent because the major route of methyl group output via creatine biosynthesis, has been substantially reduced. These and other findings, such as the inverse interrelationship between carnitine and choline biosynthesis (22), raise further important questions on the regulation of carnitine biosynthesis in response to various metabolic demands, such as occur in pregnancy, lactation, growth, nutritional and physiological stress, which will need to be examined in the future.

REFERENCES

1. Bremer, J., Trends in Biochem. Sci., 2:207 (1977).
2. Fraenkel, G. and Friedman, S., Vitams. Horm., 15:73 (1957)
3. Snoswell, A.M. and Koundakjian, P.P., Biochem.J., 127:133 (1972).
4. Erfle, J.D., Sauer, F.D. and Fisher,L.J., J. Dairy Sci., 57:671 (1974).
5. Snoswell, A.M. and Linzell, J.L., J. Dairy Res., 42:371 (1975).
6. Borum, P.R., Biochem. J., 176:677 (1978).
7. Erfle, J.D., Biochem. Biophys. Res. Commun., 64:553 (1975).
8. Costa, N.D., Ph.D. Thesis, University of Adelaide (1977)
9. Cederblad, G., Holm, J., Lindstedt, G., Lindstedt, S., Nordin, I. and Schérsten, T., FEBS Lett., 98:57 (1979).
10. Robles-Valdes, C., McGarry, J.D. and Foster, D.W., J. Biol. Chem., 251:6007 (1976).
11. Thomson, E.M., Snoswell, A.M., Clarke, P.L. and Thompson, G.E., Quart. J. Exptl. Physiol., in press (1979).
12. Snoswell, A.M. and McIntosh, G.H., Aust. J. Biol. Sci., 27:645 (1974).
13. Karpati, G., Carpenter, S., Engel, A.G., Watters, G., Allen, J., Rothman, S., Klassen, G. and Mamer, O.A. Neurology, Minneap., 25:16 (1975).
14. Angelini, C., Lücke, S. and Cantarutti, F., Neurology, Minneap., 26:633 (1976).
15. Jarrett, I.G., Filsell, O.H. and Ballard, F.J., Metabolism, 25:523 (1976).
16. Fritz, I.B., Schultz, S.K. and Srere, P.A., J. Biol. Chem., 238:2509 (1963).
17. Goldberg, A.L. and Chang, T.W., Fed. Proc., 37:2301 (1978).
18. Godwin, K.O., Kuchel, R.E. and Fuss, C.N., Aust.J. Biol. Sci., 27:633 (1974).
19. Van Hinsbergh, V.W.M., Veerkamp, J.H. and Zuurveld, J.G.E.M., FEBS Lett., 92:100 (1978).
20. Snoswell, A.M. and Henderson, G.D., Biochem. J., 119:59 (1970).
21. Barker, P.J., Fincham, N.J. and Hardwick, D.C., Biochem. J., 110:739 (1968).
22. Henderson, G.D., Ph.D. Thesis, University of Adelaide, (1978).

23. Erfle, J.D., Fisher, L.J. and Sauer, F., J. Dairy Sci. 53:486 (1970).
24. Snoswell, A.M., Costa, N.D., McLean, J.G., Baird, G.D., Lomax, M.A. and Symonds, H.W., J. Dairy Res., 45:331, (1978).
25. Ingle, D.L., Bauman, D.E. and Garrigus, U.S., J. Nutr., 102:609 (1972).
26. Liepa, G.U., Beitz, D.C. and Linder, J.R., J. Nutr., 108:1733 (1978).
27. McGarry, J.D., Mannaerts, G.P. and Foster, D.W., J. Clin. Invest., 60:265 (1977).
28. Gemmell, R.T. and Heath, T., Anat. Record, 172:57 (1972)
29. Leat, W.M.F., Kubasek, F.O.T. and Buttress, N., Quart. J. Exptl. Physiol., 61:193 (1976).
30. Chalupa, W., Fed. Proc., 31:1152 (1972).
31. Mudd, S.H. and Poole, J.R., Metabolism, 24:721 (1975).

DISCUSSION

D. SACHAN: Since acetate and carnitine seem to coexist and large amounts of acetate are produced in the rumen, is there any indication of carnitine biosynthesis in the rumen wall? Also, is any of the acetate in the rumen carried to the liver as acetylcarnitine?

A. SNOSWELL: There is no evidence that I know of indicating carnitine biosynthesis in the rumen wall. Acetate travels from the alimentary tract to the liver as such and not as acetylcarnitine. This is clear from studies on portal versus arterial blood.

N. SILIPRANDI: As far as the methylation process is concerned, we have tried to deplete carnitine by injecting rats with azo-adenosine to inhibit transmethylation processes. However, to our surprise we found that creatine decreased while the liver carnitine content increased. We have been unable to explain this strange situation.

A. SNOSWELL: That is a very interesting observation that fits in with the results I presented. It suggests that in the rat, as well as in the sheep, hepatic carnitine synthesis takes preference over the major methyl output system via creatine.

R. REITZ: In the pregnancy toxemia model, you showed an increase in liver lipid and an increase in carnitine levels. Is this related to a change in long-chain acyl-CoA:carnitine acyltransferase activity?

A. SNOSWELL: We have not examined this question in animals with pregnancy toxemia because these were field cases. However, in the closely related severely alloxan diabetic animal there is little change in the enzyme. Moreover, because of the dramatic increase in ketogenesis there would appear to be no limitation at the carnitine acyltransferase level.

J. McLEAN: In view of the diversion of methyl groups for the synthesis of carnitine in the metabolic stress states, what are the effects on choline synthesis and subsequent phospholipid synthesis?

A. SNOSWELL: We have done some extensive studies on choline-carnitine interrelationships in sheep liver in some metabolic stress states. In the diabetic state choline synthesis via the methylation pathway in the liver is markedly depressed, leading to lowered levels of phophatidyl choline in this tissue. We think this is related to the degeneration of intracellular membranes which we see and the general breakdown in cellular function in the diabetic state.

Comp. Biochem. Physiol. Vol. 76B, No. 2, pp. 295–298, 1983
Printed in Great Britain

30

0305-0491/83 $03.00 + 0.00
© 1983 Pergamon Press Ltd

CARNITINE AND CREATINE CONTENT OF TISSUES OF NORMAL AND ALLOXAN-DIABETIC SHEEP AND RATS

GRAHAM D. HENDERSON, GANG-PING XUE* and ALAN M. SNOSWELL
Department of Animal Sciences, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064 (Tel: 79-7901)

(*Received* 3 *March* 1983)

Abstract—1. The concentration of carnitine in liver increased 28-fold and urinary carnitine excretion 5-fold in alloxan-diabetic sheep. In contrast there were no similar increases in alloxan-diabetic rats.

2. The creatine content of liver decreased 3-fold and creatine excretion decreased 2-fold in diabetic sheep. In contrast the creatine content of liver increased nearly 4-fold in diabetic rats with no change in creatine excretion.

3. The marked increased in production of carnitine by the liver of the diabetic sheep appears possible because of decreased production and excretion of creatine.

INTRODUCTION

The concentration of carnitine (total acid-soluble carnitine) in the liver of sheep increases significantly in fasting (Snoswell and Henderson, 1970), in alloxan-diabetes (Snoswell and Koundakjian, 1972; Snoswell and MacIntosh, 1974) and in pregnancy toxaemia (Snoswell and Henderson, 1980). These increases are very much more pronounced than changes seen in non-ruminant animals in similar metabolic stress states, although they may be characteristic of ruminant animals (Snoswell and Henderson, 1980). The increases in carnitine in sheep liver appear to be due to a marked increase in carnitine production in the liver (Snoswell and MacIntosh, 1974).

These marked increases in carnitine production in the liver of sheep in metabolic stress states seem rather surprising as carnitine is a vital co-factor involved in fatty acid oxidation (see Bremer, 1977). Carnitine also contains three methyl groups, initially derived from methionine (via *S*-adenosylmethionine) (Rebouche, 1980) and thus a large increase in carnitine production would result in a significant drain on labile methyl groups and hence methionine. Again, a large increase in carnitine production in stress states seems surprising in the sheep as methionine is the first limiting amino acid in this species (Chalupa, 1972). Thus it would appear that these marked increases in carnitine production in the sheep could only take place if there was some modification in the normal major output of methyl groups. Mudd and Poole (1975) pointed out that creatine (or creatinine) is the major methyl containing compound excreted in humans and represents 80% of the labile methyl output. Similar figures probably pertain to most mammals.

Thus we have examined the carnitine and creatine content of liver, the major biosynthetic site, and skeletal muscle, the main storage site, together with the urinary excretion of these compounds in diabetic versus normal sheep. We have also compared the situation in the sheep with that in the rat since most of the detailed work on carnitine metabolism has been carried out in the rat and the carnitine responses to diabetes in the rat are relatively small (Fogle and Bieber, 1979).

MATERIALS AND METHODS

Animals

Female Wistar rats (*Rattus norvegicus*) weighing approximately 200–250 g were used. The animals were maintained in metabolism cages and urine was collected daily. Diabetes was induced by intraperitoneal injection of alloxan in sterile saline at a dose of 230 mg/kg body weight. The rats were slaughtered approximately 60 hr after alloxan administration by which time the blood glucose was greater than 14 mM and urinary ketones were moderate, as determined by Ketostix test strips (Ames Company, Melbourne, Australia).

The sheep used were 2-yr-old Merino (*Ovis aries*) wethers maintained in metabolism crates. Diabetic animals were produced by injecting alloxan (60 mg/kg body wt) into the jugular vein. The animals were slaughtered approximately 1 week after alloxan treatment by which time blood glucose concentration was greater than 10 mM and total blood ketones were 5–7 mM. At this stage the diabetic state in the sheep was comparable to that in the rats as judged by hyperglycaemia and ketonaemia.

Tissue preparations

Freeze-clamped samples of liver and skeletal muscle (M. biceps femoris) were extracted into perchloric acid as described by Snoswell and Henderson (1970). Blood was obtained from the jugular vein on the day before slaughter. Urine was collected daily and perchloric acid was added as preservative. All samples were stored at −14°C until analysis.

Metabolite assays

Total acid-soluble carnitine was measured by method of

*Permanent address: Department of Animal Sciences, Zhejiang Agricultural University, Hangzhou, Zhejiang, The People's Republic of China.

Table 1. Carnitine content of tissues and carnitine excretion in sheep and rats in the normal and alloxan-diabetic state

Animal	Condition	Carnitine content: Liver (μmol/g wet wt)	Carnitine content: Muscle (μmol/g wet wt)	Carnitine excretion (μmol/24 hr/kg body wt)
Sheep	Normal (5)	0.11 ± 0.01	9.32 ± 0.54	5.07 ± 1.47
	Diabetic (5)	3.04 ± 0.41	12.5 ± 1.08	27.9 ± 4.1
		$P < 0.01$	$P < 0.05$	$P < 0.01$
Rats	Normal (4)	0.274 ± 0.038	0.632 ± 0.057	7.33 ± 1.08
	Diabetic (6)	0.302 ± 0.035	0.607 ± 0.108	7.02 ± 1.19
		n.s.	n.s.	n.s.

Carnitine was determined as total acid-soluble carnitine. The values shown are means ± SEM with the number of animals in parentheses. Significance was determined by t-test comparing the values for the diabetic animals with those for the normal animals for each tissue.

Pearson and Tubbs (1967). Creatine and creatinine were measured by a specific enzymatic method as described by Wahlefeld *et al.* (1974), but for measurement of creatine, the creatininase was omitted from the assay.

Guanidoacetate methyltransferase (E.C. 2.1.1.2.) *assay*

Homogenates (20%, w/v) of fresh livers were prepared in ice-cold 0.1 M Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose with a Potter–Elvehjem homogenizer and centrifuged for 30 min at 4°C and 12,000 **g**. The supernatants were used for the enzyme assay. The enzyme was assayed by a modification of the method of Cantoni and Vignos (1954). The assay mixture in 0.5 ml contained 0.1 M Tris–HCl buffer (pH 7.4), 0.2 mM dithiothreitol, 5 mM guanidoacetic acid, 2 mM S-adenosylmethionine and 5 mg of crude enzyme protein. The mixture was incubated at 37°C and the reaction was stopped by adding 30 μl of (50% w/v) perchloric acid after 1-hr incubation. The amount of creatine formed was measured as above.

RESULTS

Carnitine content of tissues and carnitine excretion in normal and alloxan-diabetic sheep and rats

Table 1 shows that the carnitine content in the liver and skeletal muscle of the alloxan-diabetic sheep was markedly greater than in normal sheep. In particular, there was a 28-fold rise in the carnitine content in the liver of severely alloxan-diabetic sheep as we have previously noted (Snoswell and Henderson, 1980). In contrast, there was no significant change in the carnitine content of the liver and skeletal muscle of the alloxan-diabetic rats. Urinary carnitine excretion in the alloxan-diabetic sheep was five times higher than in the normal sheep. Also there was no difference in the urinary carnitine excretion between the alloxan-diabetic and normal rats. The values of carnitine in Table 1 were measured as total acid-soluble carnitine, since it accounts for more than 99% total carnitine in the liver and skeletal muscle of both the normal and alloxan-diabetic sheep (Snoswell and Koundakjian, 1972).

Creatine content of tissues and creatine excretion in normal and alloxan-diabetic sheep and rats

Results in Table 2 show that the creatine (as creatine plus creatinine) content in the liver of the alloxan-diabetic sheep compared to normal sheep was markedly reduced and that there was no significant change, in the creatine content of skeletal muscle or of the blood in the diabetic state. Daily urinary creatine excretion was also reduced in the alloxan-diabetic sheep. In contrast the creatine content in the liver of the alloxan-diabetic rats showed a significant rise, instead of the fall as observed in the alloxan-diabetic sheep, but no changes in the creatine content of the muscle or in daily urinary creatine excretion were observed.

Guanidoacetate methyltransferase activity in normal and alloxan-diabetic sheep and rats

As the final step in creatine biosynthesis occurs in the liver and involves the transfer of the methyl group from S-adenosyl-methionine to guanidoacetate and is catalyzed by the guanidoacetate methyltransferase (E.C.2.1.2.2) it was considered important to examine the activity of this enzyme in normal and diabetic rats

Table 2. Creatine content of tissues and creatine excretion in sheep and rats in the normal and alloxan-diabetic state

Animal	Condition	Creatine content: Liver (μmol/g wet wt)	Creatine content: Muscle (μmol/g wet wt)	Blood (μmol/ml)	Creatine excretion (μmol/24 hr/kg body wt)
Sheep	Normal (4)	2.43 ± 0.31	20.6 ± 1.0	0.43 ± 0.01	219 ± 11
	Diabetic (4)	0.83 ± 0.07	21.8 ± 2.2	0.47 ± 0.05	129 ± 20
		$P < 0.001$	n.s.	n.s.	$P < 0.05$
Rats	Normal (4)	0.40 ± 0.01	28.2 ± 1.4		184 ± 15
	Diabetic (6)	1.50 ± 0.18	27.7 ± 1.7		192 ± 37
		$P < 0.01$	n.s.		n.s.

Creatine is the sum of creatine and creatinine. The values shown are means ± SEM with the number of animals in parentheses. Significance was determined by t-test comparing the values for the diabetic animals with those for the normal animals for each tissue.

Table 3. Guanidoacetate methyltransferase activity in normal and alloxan-diabetic sheep and rat liver

Animal	Condition	Enzyme activity (nmol/hr/mg protein)	
Sheep	Normal (4)	2.65 ± 0.18	n.s.
	Diabetic (5)	2.33 ± 0.35	
Rat	Normal (4)	6.21 ± 0.51	$P < 0.01$
	Diabetic (6)	2.98 ± 0.55	

The values shown are means ± SEM with the number of animals in parentheses. Significance was determined by *t*-test comparing the values for the diabetic animals with those for the normal animals.

and sheep. Guanidoacetate methyltransferase activity showed no significant difference between the normal and alloxan-diabetic sheep, but it was significantly higher in the normal rats than in the alloxan-diabetic rats (Table 3).

Daily urinary creatine excretion pattern in alloxan-diabetic sheep

It is known that daily urinary creatine excretion may show large individual variations, but that it is reasonably constant in the same individuals (Berg and Kolenbrander, 1970). In order to clarify whether the decreased creatine excretion in these alloxan-diabetic sheep represented a true difference from the normal state or was due to individual variation, we measured daily creatine excretion in two sheep before and after injection of alloxan. The mean values (mmol/24 hr) of creatine (as creatine plus creatinine) excretion in the two sheep were 12.1 prior to alloxan injection and 11.6, 7.3 and 4.4 on day 2, 4 and 7 after injection of alloxan, respectively. Thus a pronounced decrease of daily creatine excretion occurred.

DISCUSSION

In the present study, the finding of the effect of alloxan-diabetes on carnitine (total acid-soluble carnitine) concentration in sheep tissues confirms earlier observations that carnitine concentration in the liver of severely alloxan-diabetic sheep was markedly increased (Snoswell and Henderson, 1980) and was also significantly increased in the skeletal muscle. In contrast we did not find any changes of carnitine concentration in the alloxan-diabetic rat liver and skeletal muscle. These latter observations are in agreement with those of Bøhmer *et al.* (1966) but in contrast to that of McGarry *et al.* (1975) who found a 4-fold increase in total carnitine content in the liver of alloxan-diabetic rats. The discrepancy is probably due to the unusually high-carbohydrate and low-fat diet employed by the latter workers since dietary fat level significantly influences the carnitine concentration in animals. This may explain the very low hepatic carnitine concentrations seen in the control animals in the experiments of McGarry *et al.* (1975).

The markedly increased tissue carnitine concentrations together with the significant increase of urinary carnitine excretion in the severely alloxan-diabetic sheep reflects an enhanced carnitine synthesis in the animals, but these changes did not occur in the alloxan-diabetic rats. Our observations suggest that there exists a real difference in carnitine metabolism between sheep and rats in response to alloxan-diabetes. This difference may well be related to the availability of labile methyl groups in the two species.

The synthesis of both carnitine and creatine require labile methyl groups derived from methionine via *S*-adenosylmethionine. The increased synthesis of carnitine in the diabetic sheep must then require an increased proportion of the labile methyl pool. Mudd and Poole (1975) have pointed out that creatine (or creatinine) is the major methyl compound excreted by humans and probably also by other animals. Thus, the decreased concentration of creatine in the liver of the diabetic sheep and the decreased urinary excretion is of particular significance. These findings indicate a decreased synthesis of creatine, the major form of methyl output in animals, which would thus allow for a diversion of additional methyl group to carnitine synthesis in the diabetic sheep.

It could be argued that the decreased creatine excretion in the diabetic sheep might be due to renal lesion which may have been caused by injection of a large dose of alloxan, since renal insufficiency will reduce creatine excretion. However, this does not seem to be the case because if renal damage occurs it is most conspicuous in the first four days after injection of alloxan and thereafter it returns to normal (Lukens, 1948) and blood creatine concentration in renal insufficiency is much higher than in normal state (Syllm-Rapoport *et al.*, 1981). The results of our study showed that the blood creatine concentration in the severely alloxan-diabetic sheep was the same as that in the normal sheep (Table 2) and that the creatine excretion did not decrease in the first two days, which suggested that the animals in our experiments did not have renal insufficiency. In addition, a pronounced decrease of creatine excretion observed in the same sheep with aloxan-diabetes indicates that the decreased excretion of creatine is part of the diabetic syndrome rather than an effect on renal function. Hence, it is clear that the reduced hepatic creatine concentration, with its decreased excretion, reflects the reduced creatine synthesis in the severely alloxan-diabetic sheep.

The reason why carnitine biosynthesis appears to take preference over creatine biosynthesis in the diabetic sheep is not clear. Snoswell and Henderson (1980) have shown a direct relationship between hepatic carnitine concentration and hepatic lipid in the sheep and it may be that in some way hepatic carnitine biosynthesis is regulated by incoming fatty acids which increase dramatically in the diabetic state. Also, even in rats, when transmethylation is blocked by injecting azo-adenosine, liver carnitine increases while liver creatine decreases (Siliprandi, 1980) suggesting that under these circumstances in the rat carnitine biosynthesis takes preference for available methyl groups.

There are a number of metabolic features of the sheep (and other ruminants) which should also be considered when comparisons are made with the rat. (1) The normal diet of sheep (mainly plant material) contains virtually no creatine and low carnitine (Mitchell, 1978) but the amount of total creatine and carnitine excreted daily is comparable to that of humans, whose diet contains considerable amounts

of creatine and carnitine. (2) Methionine is known to be the first limiting amino acid in sheep (Chalupa, 1972). (3) There is low availability of dietary choline, also a methyl donor, because dietary choline is mostly broken down to trimethylamine by rumen micro-organisms (Neill *et al.* 1979).

All of these factors would thus contribute to a relatively smaller input and larger output of methyl compounds in sheep than in humans or other carnivorous and omnivorous animals. Thus, in sheep the methyl group of urinary creatine must be essentially derived from "methionine", either as such, or synthesized from the C1 pool, and creatine becomes a major output of methionine methyl group. This concept is supported by our recent studies which showed that the amount of ^{14}C in creatine (as creatine plus creatinine), excreted by a normal sheep infused with [^{14}C-methyl]methionine, accounted for approximately 70% of the total ^{14}C-excreted in the urine in the first 36 hr (unpublished data).

The question of the regulation of creatine biosynthesis also remains obscure. Guanidoacetate methyltransferase activity did not appear to be rate limiting in either species examined as the enzyme activity was not correlated to liver creatine concentration in the normal or diabetic states. This is in agreement with the observations of Walker (1960). The possibility that creatine synthesis is regulated by the activity of arginine–glycine transamidinase (Walker, 1960, 1961) in the diabetic sheep remains. However, it is clear that the body appears to maintain its creatine pool in the diabetic sheep by reducing excretion as the creatine concentration in muscle and blood was maintained.

In conclusion we suggest that carnitine and creatine metabolism are regulated somewhat differently in the sheep, compared to the rat, because of a relatively reduced methyl intake in the sheep and thus in the diabetic state carnitine synthesis takes preference over creatine synthesis for methyl groups.

REFERENCES

Berg C. P. and Kolendrander H. M. (1970) Nitrogen metabolism in the mammal. In *Comparative Biochemistry of Nitrogen Metabolism*, (Edited by Campbell J. W.), Vol. 2, pp. 796–898. Academic Press, London.

Bøhmer T., Norum K. R. and Bremer J. (1966) The relative amounts of long-chain acylcarnitine, acetylcarnitine and free carnitine in organs of rats in different nutritional states and with alloxan diabetes. *Biochim. biophys. Acta* **125,** 244–251.

Bremer J. (1977) Carnitine and its role in fatty acid metabolism. *Trends Biochem. Sci.* **2,** 207–209.

Cantoni G. L. and Vignos P. J. (1954) Enzymatic mechanism of creatine synthesis. *J. biol. Chem.* **209,** 647–659.

Chalupa W. (1972) Metabolic aspects of nonprotein nitrogen utilization in ruminant animals. *Fedn. Proc.* **31,** 1152–1164.

Fogle P. J. and Bieber L. L. (1979) Effect of streptozotocin on carnitine acyltransferases in rat heart, liver and kidney. *Biochem. Med.* **22,** 119–126.

Luckens F. D. W. (1948) Alloxan diabetes. *Physiol. Rev.* **28,** 304–330.

McGarry J. D., Robles-Valdes C. and Foster D. W. (1975) Role of carnitine in hepatid ketogenesis. *Proc. natn. Acad. Sci. U.S.A.* **72,** 4385–4388.

Mitchell M. E. (1978) Carnitine metabolism in human subjects. I. Normal metabolism. *Am. J. clin. Nutr.* **31,** 293–306.

Mudd S. H. and Poole J. R. (1975) Labile methyl balances for normal human on various dietary regimens. *Metabolism* **24,** 721–735.

Neill A. R., Grime D. W., Snoswell A. M., Northrop A. J., Lindsay D. B. and Dawson R. M. C. (1979) The low availability of dietary choline for the nutrition of the sheep. *Biochem. J.* **180,** 559–565.

Pearson D. J. and Tubbs P. K. (1967) Carnitine and derivatives in rat tissues. *Biochem. J.* **105,** 953–963.

Rebouche C. J. (1980) Comparative aspects of carnitine biosynthesis in microorganisms and mammals with attention to carnitine biosynthesis in man. In *Carnitine Biosynthesis, Metabolism and Functions* (Edited by Frenkel R. A. and McGarry J. D.), pp. 57–72. Academic Press, New York.

Siliprandi N. (1980) In discussion of "Carnitine and Metabolism in Ruminant Animals" by Snoswell, A. M. and Henderson, G. D. In *Carnitine Biosynthesis, Metabolism and Function* (Edited by Frenkel R. R. and McGarry J. D.), pp. 191–205. Academic Press, New York.

Snoswell A. M. and Henderson G. D. (1970) Aspects of carnitine ester metabolism in sheep liver. *Biochem. J.* **119,** 59–65.

Snoswell A. M. and Henderson G. D. (1980) Carnitine and metabolism in ruminant animals. In *Carnitine Biosynthesis, Metabolism and Function* (Edited by Frenkel R. A. and McGarry J. D.), pp. 191–205. Academic Press, New York.

Snoswell A. M., and Koundakjian P. P. (1972) Relationships between carnitine and coenzyme-A esters in tissues of normal and alloxan-diabetic sheep. *Biochem. J.* **127,** 133–141.

Snoswell A. M. and MacIntosh G. H. (1974) the liver as the site of carnitine biosynthesis in sheep with alloxan induced diabetes. *Aust. J. biol. Sci.* **27,** 645–650.

Syllm-Rapoport, Daniel A., Starck H., Götze W., Hartwig A., Gross J. and Rapoport S. (1981) Creatine in red cell. *Acta biol. med. germ.* **40,** 653–659.

Wahlefeld A. W., Holz G. and Bergmeyer H. U. (1974) Creatinine. In *Methods of Enzymatic Analysis* (Edited by Bergmeyer H. U.), Vol. 4, pp. 1786–1790. Academic Press, New York.

Walker J. B. (1960) Metabolic control of creatine biosynthesis. I. Effect of dietary creatine. *J. biol. Chem.* **235,** 2357–2361.

Walker J. B. (1961) Metabolic control of creatine biosynthesis. II. Restoration of transamidinase activity following creatine repression. *J. biol. Chem.* **236,** 493–498.

Vol. 3, No. 5, November 1981 BIOCHEMISTRY INTERNATIONAL
Pages 441-447

31

ACUTE EFFECTS OF GLUCAGON ON FATTY ACID METABOLISM AND ENZYMES OF GLYCEROLIPID SYNTHESIS IN PERFUSED RAT LIVER

E. David Saggerson,[1,3] Alan M. Snoswell,[2] Rodney P. Trimble,[1] Richard J. Illman[1] and David L. Topping[1]

[1]Division of Human Nutrition, CSIRO, Glenthorne Laboratory, O'Halloran Hill, South Australia 5158, Australia.

[2]Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064, Australia.

Received August 31, 1981

SUMMARY

In homogenates prepared from livers of fed male rats which had been perfused in situ for 30 min. with whole rat blood containing glucagon both peroxisomal and mitochondrial fatty oxidation were stimulated over controls. The increase parallelled that in the rate of ketogenesis of whole liver but was unaccompanied by any change in the malonyl CoA sensitivity of carnitine palmitoyl transferase of isolated mitochondria. Glucagon inhibited hepatic triacylglycerol secretion but did not affect the activities of either mitochondrial or microsomal glycerol phosphate acyl transferase or of dihydroxyacetone phosphate acyltransferase. As bile flow was inhibited by glucagon, it is suggested that acute effects of the hormone on fatty acid metabolism may have resulted from changes in ionic balance.

INTRODUCTION

The role of pancreatic polypeptide hormones in the regulation of hepatic fatty acid metabolism is well-recognized (1). Thus, net esterification of exogenous fatty acids is promoted by insulin (2) while glucagon stimulates their oxidation (3). In the case of insulin, enhancement of esterification may reflect either diminished lipolysis of intracellular acylglycerols (2) or stimulation of mitochondrial glycerol phosphate acyl transferase (GPAT) activity (4). The mechanism of action of glucagon is unclear but may reflect stimulation of endogenous lipolysis (5).

---

[3]Permanent address: Department of Biochemistry, University College, London WC1E 6BT, UK.

0158-5231/81/050441-07$01.00/0

Copyright © 1981 by Academic Press Australia.
All rights of reproduction in any form reserved.

In hepatocytes long-chain fatty acid oxidation occurs in two organelles, i.e. the mitochondria and peroxisomes. The relative contribution of peroxisomal (cyanide-insensitive) oxidation to total oxidation has been variously estimated at less than 10% (6) up to 30% (7). Although fatty acid oxidation in peroxisomes has been shown to be stimulated by nutritional means (8) little is known about its hormonal regulation. We therefore decided to examine effects of glucagon on mitochondrial and peroxisomal oxidation in homogenates from rat livers perfused with and without glucagon. As we considered that any observed changes might have reflected alterations in the enzymes of glycerolipid synthesis, dihydroxyacetone phosphate acyl transferase (DHAPAT) and GPAT activities were also studied.

MATERIALS AND METHODS

Livers from fed adult male rats of the Hooded Wistar strain (295-305 g of body weight) were perfused *in situ* for 30 min with 80 ml of defibrinated rat blood, dialyzed to remove vasoconstrictive factors (9). Crystalline glucagon (a generous gift from Eli Lilly and Co.) was added at the start of the experimental period (zero time) to give a plasma concentration of 5×10^{-9}M. Blood samples were taken at zero time and at 30 min and rapidly chilled in ice. Whole perfusate was analyzed for ketones (3-hydroxybutyrate + acetoacetate) while triacylglycerols and fatty acids were determined in plasma (10).

At the end of each perfusion three portions of liver (1-2 g each) were rapidly taken. One portion was frozen *in situ* with tongs cooled in liquid N_2 and weighed. This sample was used for measurement of GPAT and DHAPAT activities (11). A second sample of liver was weighed fresh, homogenised and mitochondria isolated for determination of the sensitivity of carnitine palmitoyl transferase (CPT) to malonyl-CoA inhibition (12). The third portion was weighed fresh and homogenised in 0.25M sucrose containing 0.1% ethanol and this homogenate (5% w/v) was used to obtain rates of peroxisomal (CN-insensitive) and mitochrondrial (CN-sensitive) oxidation. These were measured by the incorporation of [U-^{14}C]palmitate into acid-soluble products as described by Mannaerts *et al.* (6) using a molar ratio of palmitate/albumin of 1.67:1. The combined weights of these liver samples and the unused portion were used for calculation of rates of metabolic activity/g of liver.

Data are shown as the mean±SEM for the numbers of observations in parentheses and statistical evaluation was by the analysis of variance.

RESULTS AND DISCUSSION

The weight of the liver at the end of perfusion was unaffected by glucagon treatment with a mean value of 11.2 ± 0.3 (8) g.

Ketogenesis and subcellular fatty acid oxidation

The mean rate of ketogenesis was raised from 1.66±0.39 (4) μmol/g liver/hr to 2.82±0.33 (4) μmol/g liver/hr ($P<0.05$) by treatment with glucagon. A similar stimulation, by the hormone, was observed in the oxidation of [U-^{14}C]palmitate by liver homogenates. Thus, the incorporation of ^{14}C fatty acid into acid-soluble products was increased from 290 nmol/g liver/min in controls to 510 nmol/g liver/min in glucagon-treated livers (Table 1). Both peroxisomal (CN-insensitive) and mitochondrial (CN-sensitive) oxidation were increased by the hormone and at first sight it would appear that the increase was greatest in the former which rose by 143% as compared with 62% in mitochondrial oxidation. However, as a percentage of total oxidation, CN-insensitive activity was 17% in untreated livers and approximately 22% following glucagon treatment. Thus the stimulation of fatty acid oxidation would seem to be general and not confined to one particular compartment. To our knowledge

TABLE 1. Effects of glucagon on mitochondrial (cyanide-sensitive) and peroxisomal (cyanide-insensitive) fatty acid oxidation in perfused rat liver

Group	Mitochondrial oxidation	Peroxisomal oxidation	Total
	(nmol [U-^{14}C]palmitate/g liver/min)		
Control (3)	241±8	49±5	290±12
	**	*	***
Glucagon (3)	391±42	119±23	510±29

* $P<0.05$; ** $P<0.025$; *** $P<0.001$

this is the first demonstration of an acute effect of glucagon, or any other hormone, on peroxisomal oxidation.

We considered it possible that the glucagon-induced rise in CN-sensitive oxidation could have reflected a modulation in the activity of CPT as this enzyme is potentially rate-limiting for the entry of fatty acids into the mitochondrion (13). However, after only 4 perfusions (two in each group) it became apparent that there was no change in the malonyl CoA sensitivity of this enzyme. The mean concentration for 50% inhibition was 1.2 μM malonyl CoA. Therefore the loss of sensitivity of this enzyme to malonyl CoA inhibition which is observed in the fed-fasting transition (14) is not necessarily due to an acute effect of glucagon.

Triacylglycerol secretion and enzymes of glycerolipid synthesis

In control livers the mean rate of triacylglycerol release was 3.9± 0.5 (4) μmol/g liver/min. Secretion was significantly ($P<0.05$) inhibited by glucagon to 2.7±0.2 (4) μmol/g liver/min. Of particular interest is the fact that, in percentage terms, the inhibition was similar to the stimulation which insulin exerts on the secretory process in this preparation (15).

An inverse relationship exists between hepatic fatty acid oxidation and esterification in perfused liver (16) and when oxidation is inhibited, hepatic triacylglycerol secretion is enhanced. It has also been noted *in vivo* (11) that when mitochondrial GPAT is altered by experimental treatments such as anti-insulin serum or starvation, the changes parallel those in triacylglycerol and export in perfused rat liver. We have shown previously that, in perfused liver, insulin enhances the activity of mitochondrial GPAT (4). To determine whether glucagon might also have persistent effects on this enzyme and also DHAPAT, their activities were measured. Mitochondrial and microsomal GPAT activity may be readily distinguished by the fact that the latter is inhibited by N-ethylmaleimide

(NEM) whereas the mitochondrial enzyme is not (11). Rather surprisingly glucagon had no effect on the activity of either NEM-sensitive or insensitive activity measured with 45 μM palmitoyl-CoA and 0.5 mM glycerol 3-phosphate (Table 2) GPAT activity was also unaffected by glucagon treatment when measured with oleyl-CoA, a substrate giving essentially microsomal activity (12,17). A similar lack of hormonal effect was observed when these activities were measured with the appropriate substrate at 20, 75 and 120 μM (data not shown).

NEM inhibits microsomal DHAPAT activity but accelerates that in peroxisomes and other subcellular fractions (4, 18-20). However, activity with and without this reagent was completely unaffected by glucagon (Table 2). It is apparent, therefore, that the alterations in mitochondrial and peroxisomal fatty acid oxidation and in triacylglycerol

TABLE 2. Effects of glucagon on the activities of glycerol phosphate acyl transferase (GPAT) and dihydroxyacetone phosphate acyltransferase (DHAPAT) activites in perfused rat liver

Activity	Substrate	NEM (10 mM)	Control (nmol/g liver/min)	Glucagon (nmol/g liver/min)
GPAT	Palmitoyl CoA (45 μM)	-	319±18 (3)	317±9 (3)
	Palmitoyl CoA (45 μM)	+	165±10 (3)	160±7 (3)
NEM-sensitive GPAT (by difference)	Palmitoyl CoA (45 μM)		154±9 (3)	157±5 (3)
GPAT	Oleoyl CoA (45 μM)	-	195±6 (3)	197±15 (3)
DHAPAT	Palmitoyl CoA (60 μM)	-	41±3 (4)	44±1 (4)
DHAPAT	Palmitoyl CoA (60 μM)	+	45±2 (4)	47±3 (4)

secretion were not due to persistent modifications in measured enzyme activities. This view is supported by the fact that 30 min plasma fatty acid concentrations were also unaffected by the hormone with mean values of 0.21 ± 0.02 (4) and 0.22 ± 0.03 (4) μmol/ml, control and glucagon, respectively. In contrast, when GPAT is altered by insulin, plasma fatty acids fall in parallel with the enhancement of esterification (4).

The question remains: how might the effects of glucagon be mediated ? It has been considered that the hormone may alter fatty acid oxidation *via* cyclic AMP-dependent mechanisms (3) and dibutyryl cyclic AMP does cause inhibition of microsomal GPAT in perfused rat liver (21). However, in the present study no such changes in enzymic activity were observed. It is possible that changes in ionic balance were involved. A number of hormones appear to act on hepatic metabolism by altering the hepatocellular distribution of cations, particularly Ca^{2+} (22, 23) and we have sought to explain some of the effects of insulin in these terms (24). The latter suggestion was based on stimulation, by the hormone, of bile flow in the absence of bile acids. In the present study, glucagon also affected bile secretion but in the opposite direction to insulin and lowered flow from 780 ± 20 (4) μ/liver/h in controls to 640 ± 40 (4) μ/liver/h ($P<0.001$). As microsomes contain a Ca^{2+} pump, activity of which has been implicated in the regulation of enzymes of glycerolipid synthesis (25), it may be that the observed effects of glucagon on endogenous fatty acid oxidation and triacylglycerol secretion reflect a change in intracellular ionic balance.

ACKNOWLEDGEMENTS

We wish to thank Miss V.A. Hill and Miss L. Maland for technical assistance and Dr. B.S. Hetzel for arranging the visit of EDS. AMS was supported by a grant from the Australian Research Grants Committee (No. D 277 15558).

REFERENCES

1. Saggerson, E.D. (1979) Trends Biochem. Sci. 4, 33-35.
2. Topping, D.L. and Mayes, P.A. (1972) Biochem. J. 126, 295-311.
3. Heimberg, M., Weinstein, I. and Kohout, M. (1969) J. Biol. Chem. 244, 5131-5139.
4. Bates, E.J., Topping, D.L., Sooranna, S.R., Saggerson, E.D. and Mayes, P.A. (1977) FEBS Lett. 84, 225-228.
5. Poledne, R. and Mayes, P.A. (1970) Biochem. J. 119, 47P.
6. Mannaerts, G.P., Debeer, L.J., Thomas, J. and Schepper, P.J. (1979) J. Biol. Chem. 254, 4585-4595.
7. Krahling, J.B., Gee, R., Gauger, J.A. and Tolbert, N.E. (1979) J. Cell. Physiol. 101, 374-390.
8. Neat, C.E., Thomassen, M.S. and Osmundsen, H. (1981) Biochem. J. 196, 149-159.
9. Mayes, P.A. and Felts, J.M. (1966) Proc. Eur. Soc. Study Drug Toxicity 7, 16-29.
10. Topping, D.L., Clark, D.G., Storer, G.B., Trimble, R.P. and Illman, R.J. (1979) Biochem. J. 184, 97-106.
11. Bates, E.J. and Saggerson, E.D. (1979) Biochem. J. 184, 751-762.
12. Saggerson, E.D. and Topping, D.L. (1981) FEBS Lett. In the press.
13. McGarry, J.D. and Foster, D.W. (1979) J. Biol. Chem. 254, 8163-8168.
14. Saggerson, E.D. and Carpenter, C.A. (1981) FEBS Lett. In the press.
15. Topping, D.L., Trimble, R.P. and Storer, G.B. (1981) Biochem. Internat. 3, 101-106.
16. Mayes, P.A. and Felts, J.M. (1967) Nature 215, 716-718.
17. Monroy, G., Pola, F.H. and Pullman, M.E. (1972) J. Biol. Chem. 247, 6884-6894.
18. Bates, E.J. and Saggerson, E.D. (1981) FEBS Lett. 128, 230-232.
19. Jones, C.L. and Hajra, A.K. (1980) J. Biol. Chem. 255, 8289-8295.
20. Schlossman, D.W. and Bell, R.M. (1972) Arch. Biochem. Biophys. 182, 732-742.
21. Soler-Argilaga, C., Russell, R.L. and Heimberg, M. (1978) Arch. Biochem. Biophys. 190, 367-372.
22. Hems, D.A. (1979) Clin. Sci. Mol. Med. 56, 197-202.
23. Exton, J.H. (1979) J. Cyclic Nucleotide Res. 5, 277-287.
24. Storer, G.B., Trimble, R.P. and Topping, D.L. (1980) Biochem. J. 192, 219-222.
25. Soler-Argilaga, C., Russell, R.L. and Heimberg, M. (1977) Biochem. Biophys. Res. Comm. 78, 1053-1059.

0148-6071/83/0702-0176$02.00/0
JOURNAL OF PARENTERAL AND ENTERAL NUTRITION
Vol. 7, No. 2
Printed in U.S.A.
Copyright © 1983 by the American Society for Parenteral and Enteral Nutrition

Carnitine Deficiency with Hyperbilirubinemia, Generalized Skeletal Muscle Weakness and Reactive Hypoglycemia in a Patient on Long-term Total Parenteral Nutrition: Treatment with Intravenous L-Carnitine

L. I. G. WORTHLEY,* R. C. FISHLOCK,† AND A. M. SNOSWELL†

*From the *Intensive Care Unit, Royal Adelaide Hospital, Adelaide, South Australia, 5000, and the †Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia, 5064.*

ABSTRACT. Low levels of plasma carnitine and reduced urinary carnitine excretion with persistently elevated plasma bilirubin levels, reactive hypoglycemia and generalized skeletal muscle weakness are described in a patient requiring long-term total parenteral nutrition (TPN). Intravenous administration of L-carnitine at 400 mg/day for 7 days and subsequently a maintenance dose of 60 mg/day corrected the plasma carnitine deficiency and reactive hypoglycemia and was associated with a return to normal plasma bilirubin levels and a restoration of skeletal muscle strength.

L-Carnitine is required for the transport of activated long chain fatty acids into the matrix compartment of mitochondria of tissues for subsequent oxidation.[1] In carnitine deficiency, fatty acid oxidation is reduced and fatty acids may be diverted into triglyceride synthesis, particularly in the liver. Also, there is a readjustment of the balance between carbohydrate and fat metabolism and glycolysis is increased.

Carnitine deficiency may occur as a genetically determined metabolic defect or as an acquired disorder associated with severe nutritional protein deficiency and cirrhosis due to a diminished endogenous production and exogenous intake of carnitine.[2] It may also occur due to an excess dialysate loss of carnitine in patients requiring hemodialysis for chronic renal failure.[3] However, in normal patients, carnitine deficiency is rare, since the daily requirement may be met by endogenous hepatic synthesis from methionine and lysine,[4] in addition to the dietary intake.

Patients receiving parenteral nutrition may develop hepatic fatty infiltration with liver function tests indicative of cholestasis.[5] Reactive hypoglycemia may also occur with the abrupt cessation of the parenteral nutrition infusion.[6] These clinical manifestations may also occur with carnitine deficiency.[2] Neonatal and premature infants when receiving TPN show a marked reduction in plasma and tissue carnitine when compared to infants receiving human milk or carnitine-containing milk formulae.[7, 8] To date, however, carnitine deficiency in adults receiving TPN has not been described.[9]

We have investigated the possibility of carnitine deficiency in an adult male patient, who had been maintained on TPN for 1 year and who had a persistently elevated plasma bilirubin concentration generalized skeletal muscle weakness, and reactive hypoglycemia.

Received for publication, February 18, 1982.
Accepted for publication, September 23, 1982.
Reprint requests to: L.I.G. Worthley, Intensive Care Unit, Royal Adelaide Hospital, North Terrace, Adelaide, South Australia 5000.

CASE REPORT

A 41-year-old, previously fit man was admitted to the intensive care unit with gas gangrene of the anterior abdominal wall and *Clostridium welchii* septicemia developing 24 hr after a laparotomy for small bowel obstruction.

The patient was anuric and in shock. Intravenous penicillin, (4×10^6 U four times hourly) and Isoprenaline (6 μg/min) were administered and the patient underwent an operation to excise the necrotic anterior abdominal wall, leaving the peritoneal cavity and underlying bowel exposed. Postoperatively he required hemodialysis for 6 weeks and intermittent positive pressure ventilation for 4 weeks. Although split skin grafts were placed directly onto the exposed bowel wall to cover the anterior abdominal surface, 6 small bowel fistulae developed, necessitating prolonged intravenous alimentation to maintain normal nutrition. During this period he also developed a cholestatic jaundice with peak plasma values of total bilirubin 555 μmol/liter (normal; 6–24 μmol/liter), conjugated bilirubin 510 μmol/liter (normal; 1–4 μmol/liter), aspartate amino transferase 270 U/liter (normal; 5–40 U/liter), and lactate dehydrogenase 407 U/liter (normal; 110–230 U/liter), occurring 6 weeks postoperatively. The only medications the patient received during this time were parenteral vitamins, penicillin, gentamycin, and lincomycin.

Abdominal ultrasound scans revealed an enlarged liver without evidence of cholelithiasis or enlarged bile ducts. Serum tests to detect hepatitis B surface antigen and antibody, infectious mononucleosis, toxoplasmosis, and cytomegalic inclusion virus were also negative. The hepatic failure was thought to be due to the severe abdominal sepsis and TPN, producing hepatic steatosis and a predominantly cholestatic jaundice.

Total parenteral nutrition was required throughout his hospital stay and was administered during a 12-hr period at night in 4.5 liters of fluid. 2,600 non-nitrogen calories were given as dextrose; 18 gm of nitrogen were given as

TABLE I
Plasma metabolite concentrations for the patient before and during L-carnitine administration

Metabolite	Normal values[a]	Before L-carnitine administration	L-carnitine administration	
			400 mg/day	60/mg/day
			mmol/liter	
Glucose	4–6	3.8 ± 0.3 (7)	4.2 ± 0.2 (3)	5.6 ± 0.6 (5)[b]
3-Hydroxybutyrate	0.1–0.18	0.042 ± 0.003 (7)	0.021 ± 0.004 (8)	0.037 ± 0.006 (8)
Triglycerides	0.5–1.9	1.6 ± 0.6 (5)	1.5 ± 0.1 (7)	1.6 ± 0.04 (6)
Total cholesterol	4.0 − 7.0	4.4 ± 0.1 (5)	4.2 ± 0.3 (7)	4.5 ± 0.1 (6)
			μmol/liter	
Free carnitine	38.9 ± 6.7	19.5 ± 0.8 (7)	62.1 ± 3.1 (9)[c]	27.0 ± 2.2 (8)[b]
Short chain acyl-carnitine	3.9 ± 1.8	1.8 ± 0.5 (7)	11.6 ± 2.3 (9)[c]	2.5 ± 1.2 (8)[b]
Long chain acyl-carnitine	10.4 ± 2.6	7.4 ± 0.4 (7)	24.2 ± 1.2 (9)[c]	14.4 ± 1.0 (8)[c]
Total carnitine	52.0 ± 6.7	28.6 ± 1.4 (7)	97.8 ± 3.9 (9)[c]	44.0 ± 1.3 (8)[c]
Conjugated bilirubin	1–4	47 ± 3.7 (7)	14 ± 0.3 (3)[c]	7 ± 0.4 (5)[c]
Total bilirubin	6–24	65 ± 5.4 (7)	25 ± 1.5 (3)[c]	15 ± 0.7 (5)[c]

[a] Values are means ± SEM with the number of samples in brackets.
[b] Significantly different from the figures before L-carnitine administration at $p < 0.01$ level.
[c] Significantly different from the figures before L-carnitine administration at $p < 0.001$ level.

800 ml of Synthamin-17®‡ and 50 mmole lysine, 60 mmole leucine, 40 mmol isoleucine, 40 mmole valine, and 30 mmole histidine. The latter five amino acids were added to produce a normal fasting serum amino acid profile. Sodium, potassium, phosphate, calcium, magnesium, acetate, zinc, copper, selenium and vitamins A, D, E, K, folic acid, B_{12}, C, and B group were given in amounts necessary to maintain normal physiological functions and serum levels. Intralipid-10% (500 to 1000 ml) were infused each week to maintain the triene/tetrene ratio < 0.2. Intravenous lipid solutions were not given daily, since the patient developed an elevated temperature and rigors each time intralipid-10% was infused.

The patient underwent a further operation to close the six abdominal fistulae, although two subsequently reopened. Twelve months after the initial operation the patient was discharged from the hospital to manage his parenteral nutrition and convalescence at home. However, he remained icteric with persistently elevated plasma bilirubin levels (Table I), complaining of symptomatic reactive hypoglycemia with cessation of the TPN (the plasma glucose on one such occasion was 0.5 mmol/liter) and generalized muscular weakness. The latter confined him to bed throughout most of the day.

Plasma carnitine measurements revealed markedly low levels of free-, long and short chain acyl carnitine and urinary carnitine excretion was less than normal. 400 mg of L-carnitine was administered intravenously daily for 7 days followed by 40 mg/day for 3 weeks and thereafter 60 mg/day.

Plasma-free, short and long chain acylcarnitine, urinary free, short chain acyl- and total acid-soluble carnitine, plasma lipids, and three hydroxybutyrate were measured daily for 4 weeks and thereafter weekly. Plasma glucose, total, and conjugated bilirubin were measured twice weekly.

‡ Synthamin 17® contains per liter: leucine, 47.3 mmol; isoleucine, 36.6 mmol; valine, 39.2 mmol; lysine, 31.8 mmol; phenylalanine, 37.5 mmol; methionine, 38.9 mmol; tryptophan, 8.8 mmol; threonine, 35.3 mmol; arginine, 59.7 mmol; histidine, 28.4 mmol; alanine, 233.5 mmol; glycine, 277 mmol; proline, 36.5 mmol; and tyrosine, 2.2 mmol.

MATERIALS AND METHODS

Venous blood samples were collected just prior to the administration of the intravenous nutrition. Blood samples were also collected from 12 normal male subjects (aged 22–43 years) for the assay of plasma carnitine fractions.

Plasma and urinary carnitines were measured by the radio-enzymic method of Parvin and Pande,[10] with modifications as suggested by Pande and Parvin[11] and Snoswell and Henderson,[12] following separation of the plasma into various carnitine fractions according to the method of Brass and Hoppel.[13] Plasma glucose, total, and conjugated bilirubin were measured, using a sequential multiple analyser with computer (Technician Instruments Corporation, Tarrytown, NY). Total cholesterol was measured by the method of Richmond,[14] triglycerides were measured by the method of Bucolo and David,[15] and 3-hydroxybutyrate was measured by the method of Williamson et al.[16] Results were analyzed for statistical significance using Student's *t*-test.

RESULTS

The results shown in Table I indicate that prior to carnitine administration the patient had low plasma carnitine concentrations in comparison with plasma carnitine concentrations we estimated from 12 normal male subjects of 38.9 ± 6.7, 3.9 ± 1.8, 10.4 ± 2.6, and 52.0 ± 6.7 μmol/liter (mean ± SD) for free, short chain acyl, long chain acyl, and total carnitine, respectively. The values for plasma carnitine concentrations were also low, with respect to total acid-soluble carnitine (free plus short chain acyl) published for normal male patients, ie 57 ± 13 (SD) μmol/liter when assayed by a similar radiochemical method.[17] Also, the daily excretion of free carnitine of 44 μmol/day (Table II) during the period was considerably less than the value of 175 ± 81 (SD) μmol/day reported for normal males.[18]

Following the daily administration of 400 mg of L-carnitine, plasma carnitine concentrations plateaued at higher values after 3 days (Fig. 1), indicating that this

TABLE II
Urinary excretion of carnitine in the patient

	Normal values[a]	Before L-carnitine administration	L-carnitine administration		
			400 mg/day[b]	40 mg/day	60 mg/day
			μmol/24 hr		
Free carnitine	175 ± 81[c]	44 ± 10 (3)	1170 ± 79 (4)	104 ± 15 (3)	141 ± 29 (2)
Short chain acyl-carnitine		77 ± 13 (3)	363 ± 66 (4)	101 ± 4 (3)	178 ± 18 (2)
Total acid-soluble carnitine	239 ± 56[e]	120 ± 7 (3)	1530 ± 52	205 ± 15 (3)	319 ± 47 (2)

[a] The figures shown are means ± SEM with the number of observations in brackets.
[b] The samples at 400 mg/day were collected between the 4th and 7th days of carnitine administration.
[c] From Reference 18.
[d] Total acid-soluble carnitine, free + short chain acyl-carnitine.
[e] From Reference 17.

time may have been required to saturate the extra cellular tissue space in the body. Subsequent administration of a maintenance dose of 40-mg L-carnitine/day resulted in the establishment of plasma carnitine concentrations nearer the normal values (Fig. 1). However, the daily excretion of total acid-soluble carnitine of 205 ± 15 μmol/day (Table II) was still slightly below the normal daily excretion for males of 239 ± 56 (SD) μmol/day.[17] The maintenance dose was therefore increased to 60 mg/day for 3 weeks and maintained at that level thereafter. This intake value is very similar to the mean daily excretion value of 59.3 mg reported for a larger population of males.[19] At this maintenance dose the total plasma carnitine concentration of 44 μmol/liter (Table 1) was in the normal range, although the concentration of the long chain acyl fraction of 14.4 μmol/liter was somewhat elevated with respect to normal values reported here and by Genuth and Hoppel.[20]

The administration of carnitine resulted in a reduction in the elevated plasma total and conjugated bilirubin to normal levels, although the serum 3-hydroxybutyrate, triglyceride, and total cholesterol levels remained unchanged (Table I).

With the maintenance dose of carnitine the plasma glucose also remained in the normal range and was significantly above that prior to carnitine administration (Table 1). Furthermore, the patient had no symptomatic episodes of hypoglycemia with cessation of his daily TPN infusion. The low plasma 3-hydroxybutyrate concentrations in this patient, both before and after carnitine administration, may be correlated with the elevated long chain acyl carnitine fraction (Table I) and may indicate some enzymic deficiency in the liver of the patient. During the first week of L-carnitine administration the patient began to improve clinically. He became ambulant and spent less and less time in bed throughout the day, gaining in strength and noting a sense of wellbeing that he had not had during the previous 12 months. This improved clinical status has now continued for 13 months on the maintenance carnitine infusion of 60 mg L-carnitine/day.

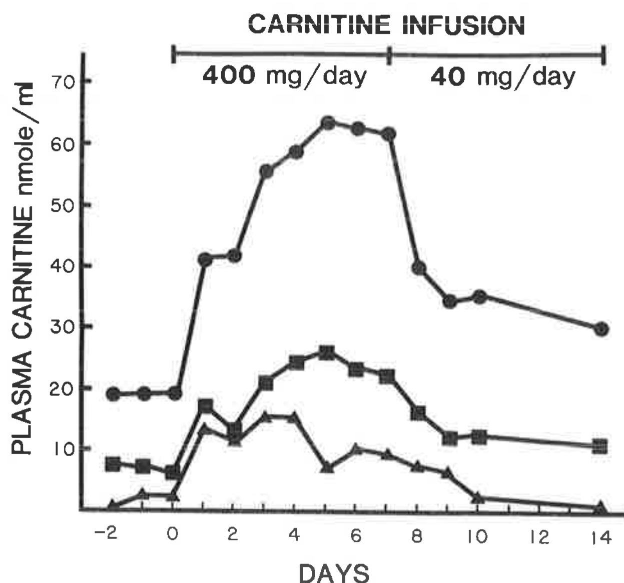


FIG. 1. The effect of L-carnitine administration on plasma carnitine concentrations in the patient. ●——●, free carnitine; ■——■, long chain acylcarnitine; ▲——▲, short chain acylcarnitine.

DISCUSSION

A defect in lipid metabolism with hepatic fatty infiltration and abnormal liver function tests often occurs in patients receiving parenteral nutrition.[5] Fundamentally the disturbance is due to an increased production, diminished utilization or defect in secretion of hepatic lipids, and each of these defects have been reported with intravenous alimentation. For example, extensive production of lipid occurs with excessive glucose administration, even in patients with high calorific requirements.[21] Furthermore, when glucose is administered continuously, the lipid so formed is unable to be mobilized easily, due to the persistent effect of insulin promoting lipogenesis and inhibiting lipolysis.[22, 23] Defective secretion of hepatic lipids has also been described with deficiencies of essential fatty acids,[24] choline and methionine deficiencies,[25] bacterial toxins,[26] abnormal metabolism of bile salts[27] and toxic effects of amino-acids and their metabolites.[28, 29] Diminished utilization of fatty acids for energy and ketone production are further insulin-induced effects.[30]

Since the transport of long chain fatty acids from the sites of activation in the cytoplasm to the sites of β-oxidation in the mitochondria requires carnitine and since there is competition between the pathways of β-oxidation and triglyceride formation for cytosolic-free fatty acids, carnitine deficiency enhances hepatic lipogenesis.[31, 32]

Carnitine deficiency is unlikely to occur in healthy individuals as 16–20 mg can be synthesized endogenously

daily in man and 60–75 mg is present in the normal diet.[33] Apart from the rare patient with a genetically determined biochemical defect of carnitine metabolism, and the patient with chronic renal failure with excessive loss of carnitine during hemodialysis, acquired carnitine deficiency has mainly been reported in patients with severe nutritional protein deficiency and malnourished cirrhotic patients, due to a defective endogenous synthesis and low dietary intake of carnitine.[2]

As synthesis of carnitine by intestinal microflora has not yet been described,[34] patients receiving TPN have their dietary intake of carnitine reduced essentially to zero. If they have hepatic dysfunction as well, then they may be unable to produce adequate amounts of carnitine for normal fatty acid metabolism. Moreover, if carnitine deficiency exists, then the hepatic dysfunction may be exacerbated due to excessive triglyceride deposition within the hepatocytes.

Low levels of carnitine have been reported in neonates and infants receiving TPN.[7,8] In experimental studies, rats fed parenterally have a reduced incidence of hepatic steatosis and an improved N_2 balance when their nutritional regimen is supplemented with carnitine.[35] In the patient we describe, the hepatic failure may have been induced by gross abdominal sepsis and TPN. However, the continuing presence of abnormal liver function, generalized weakness, and severe reactive hypoglycemia suggested an additional disorder.

Carnitine deficiency was suspected by the presence of low plasma and urinary levels and although plasma carnitine levels may not necessarily correlate with tissue carnitine levels,[36] severely depressed plasma levels observed in our patient were certainly suggestive of depletion. Plasma values were corrected by L-carnitine administration, initially 400 mg/day for 7 days and subsequently 60 mg/day continuously. The addition of carnitine was associated with a return to normal of plasma bilirubin levels, absence of reactive hypoglycemia and improved muscle strength. We did not observe any evidence of cardiomyopathy,[37,38] acidosis,[2,39] or, with carnitine repletion, change in plasma lipids[40] or ketones,[19] as has been previously reported.

In patients requiring TPN hepatic dysfunction may develop, diminishing endogenous synthesis of carnitine. If carnitine deficiency is present it will lead to hepatic steatosis, which will, in turn, exacerbate the presence of hepatic dysfunction. Thus, it would seem that for patients requiring long-term TPN, carnitine addition may be necessary to ensure normal fatty acid metabolism.

ACKNOWLEDGMENTS

We are grateful to Otsuka Pharmaceuticals, Osaka, Japan, and to Sigma-tau Pharmaceuticals, Rome, Italy, for providing L-carnitine.

REFERENCES

1. Fritz IB: Carnitine and its role in fatty acid metabolism. Adv Lipid Res 1:285–334, 1963
2. Engel AG: Possible causes and effects of carnitine deficiency in man. IN Carnitine Biosynthesis, Metabolism, and Functions Frenkel RA and McGarry JD (eds). Academic Press, New York, 1980, pp. 271–285
3. Bohmer T, Bergrem H, Eiklid K: Carnitine deficiency induced during intermittent haemodialysis for renal failure. Lancet 1:126–128, 1978
4. Khan L, Bamji MS: Tissue carnitine deficiency due to dietary lysine deficiency: Triglyceride accumulation and concomitant impairment in fatty acid oxidation. J Nutr 109:24–31, 1979
5. Sheldon GF, Petersen SR, Sanders R: Hepatic dysfunction during hyperalimentation. Arch Surg 113:504–507, 1978
6. Dudrick SJ, MacFadyen BV, van Buren CT, et al: Parental hyperalimentation: Metabolic problems and solutions. Ann Surg 176:259–264, 1972
7. Schiff D, Chan G, Seccombe D, et al: Plasma carnitine levels during intravenous feeding of the neonate. J Pediatr 95:1043–1046, 1979
8. Penn D, Sommerfeld ES, Pascu F: Decreased tissue carnitine concentrations in newborn infants receiving total parenteral nutrition. J Pediatr 98:976–978, 1981
9. Tao RC, Yoshimura NN: Carnitine metabolism and its application in parenteral nutrition. JPEN 4:469–486, 1980
10. Parvin R, Pande SV: Microdetermination of (−) carnitine and carnitine acetyl-transferase activity, Anal Biochem 79:190–201, 1977
11. Pande SV, Parvin R: Carnitine-acylcarnitine translocase catalyzes an equilibrating unidirectional transport as well. J Biol Chem 255: 2994–3001, 1980
12. Snoswell AM, Henderson GD: Carnitine and metabolism in ruminant animals. IN Carnitine Biosynthesis, Metabolism, and Functions (Frenkel RA, McGarry JD (eds). Academic Press, New York, 1980, pp. 191–204
13. Brass EP, Hoppel CL: Carnitine metabolism in the fasting rat. J Biol Chem 253:2688–2693, 1978
14. Richmond W: Preparation and properties of a cholesterol oxidase from nocardia SP and its application to the enzymatic assay of total cholesterol in serum. Clin Chem 19:1350–1356, 1973
15. Bucolo G, David M: Quantitative determination of serum triglycerides by the use of enzymes. Clin Chem 14:476–482, 1973
16. Williamson DH, Mellanby J, Krebs HA: Enzymic determination of D(−)-β-hydroxybutyric acid and acetoacetic in blood. Biochem J 82:90–96, 1962
17. Cederblad G: Plasma carnitine and body composition. Clin Chim Acta 67:207–212, 1976
18. Cederblad G, Lindstedt S: Excretion of L-Carnitine in man. Clin Chim Acta 33:117–123, 1971
19. Maebashi M, Kawamara N, Sato K, et al: Urinary excretion of carnitine in man. J Lab Clin Med 87:760–766, 1976
20. Genuth SM, Hoppel CL: Plasma and urinary carnitine in diabetic ketosis. Diabetes 28:1083–1087, 1979
21. Wolfe RR, O'Donnell TF, Stone MD, et al: Investigation of factors determining the optimal glucose infusion rate in total parenteral nutrition. Metabolism 29:892–900, 1980
22. Maini B, Blackburn GL, Bistrian BR, et al: Cyclic hyperalimentation: An optimal technique for preservation of visceral protein. J Surg Res 20:515–525, 1976
23. Page CP, Clibon V: Man the meal-eater and his interaction with parenteral nutrition. JAMA 244:1950–1953, 1980
24. Wene JD, Connor WE, Denbesten L: The development of essential fatty acid deficiency in healthy men fed fat-free diets intravenously and orally. J Clin Invest 56:127–134, 1975
25. Truswell AS, Hansen JDL, Watson CE: Relation of serum lipids and lipoproteins to fatty liver in kwashiorkor. Am J Clin Nutr 22:568–576, 1969
26. Royle GT, Kettlewell MGW: Liver function tests in surgical infection and malnutrition. Ann Surg 192:192–194, 1980
27. Rager R, Finegold MJ: Cholestasis in immature newborn infants: Is parenteral alimentation responsible? J Pediatr 86:264–269, 1975
28. Cohen ME: Changes in hepatic function. IN Intravenous Nutrition in High Risk Infants. Winters RW, Hassellmeyer C (eds). John Wiley & Sons Inc., New York, 1975, pp. 293–313
29. Grant JP, Cox CE, Kleinman LM, et al: Serum hepatic enzymes and bilirubin elevations during parenteral nutrition. Surg Gynecol Obstet 145:573–580, 1977
30. McGarry JD, Foster DW: Regulation of hepatic fatty acid oxidation and ketone production. Annu Rev Biochem 49:395–420, 1980
31. Bremer J: Carnitine in intermediary metabolism. The biosynthesis of palmitoylcarnitine by cell subfractions. J Biol Chem 238:2774–

2779, 1963

32. Bressler R: Fatty acid oxidation. IN Comprehensive Biochemistry Lipid Metabolism. Vol. 18, Florkin M, Stotz EM (eds). Elsevier, New York, 1970, p. 331
33. Rudman D, Sewell EW, Ansley JD: Deficiency of carnitine in cachectic cirrhotic patients. J Clin Invest 60:716–723, 1977
34. Mackowiak PA: The normal microbial flora. N Engl J Med 307:83–93, 1982
35. Tao RC, Peck GK, Yoshimura NN: Effect of carnitine on liver fat and nitrogen balance in intravenously fed growing rats. J Nutr 111:171–177, 1981
36. Cederblad G, Lindstedt S, Lundholm K: Concentration of carnitine in human muscle tissue. Clin Chim Acta 53:311–321, 1974
37. Tripp ME, Katcher ML, Peters HA, et al: Systemic carnitine deficiency presenting as familial endocardial fibroelastosis: A treatable cardiomyopathy. N Engl J Med 305:385–390, 1981
38. Hart ZM, Chang CH, Dimauro S, et al: Muscle carnitine deficiency and foetal cardiomyopathy. Neurology 28:147–151, 1978
39. Karpati G, Carpenter S, Engel AG, et al: The syndrome of systemic carnitine deficiency clinical, morphologic, biochemical, and pathophysiologic features. Neurology 25:16–24, 1975
40. Casciani CU, Caruso U, Cravotto E, et al: Effect of L-carnitine on lipid pattern in haemodialysis. Lancet 2:1309–1310, 1980

Biochem. J. (1979) **180**, 559–565
Printed in Great Britain

The Low Availability of Dietary Choline for the Nutrition of the Sheep

By A. R. NEILL,* D. W. GRIME, A. M. SNOSWELL,† A. J. NORTHROP, D. B. LINDSAY and R. M. C. DAWSON
Biochemistry Department, A.R.C. Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

(*Received* 29 *November* 1978)

1. Choline, which is present in the diet of the sheep either in the non-esterified form or combined in phospholipids, is rapidly degraded in the rumen. The ultimate product formed from the *N*-methyl groups is methane. 2. Analysis of the non-esterified choline and the phosphatidylcholine in ruminal and abomasal digesta indicate that the phospholipid is the main vehicle for the passage of choline to the lower digestive tract. 3. The concentration of phosphatidylcholine in abomasal digesta is lower than that of ruminal digesta, which is in line with a selective retention of protozoa in the rumen as observed by others. 4. On defaunation of the rumen to remove ciliated protozoa the concentration of phosphatidylcholine in ruminal digesta falls markedly and becomes lower than that in abomasal digesta. 5. Calculation shows that the adult sheep obtains at most only about 20–25 mg of effective choline per day from its diet (0.002–0.0025% of dietary total dry-weight intake). This is some fifty times less than the minimum required to avoid pathological lesions and death in other species investigated (0.1%+ of dietary dry-weight intake). 6. Sheep liver can synthesize choline from [^{14}C]ethanolamine both *in vitro* and *in vivo*, but the synthesis of choline per kg body weight is many times less than it is in the rat. 7. The intact sheep oxidizes an injected dose of [1,2-^{14}C]choline to CO_2 at a rate that is several times less than that observed for the rat. This could help to explain the apparent minimal requirement of sheep for dietary choline.

Choline present in the diet of a sheep largely as the phosphatidylcholine of higher plant membranes is rapidly liberated and degraded in the rumen by microbes. The *N*-methyl groups form trimethylamine and ultimately methane (Neill *et al.*, 1978). The only choline escaping such degradation appears to be a small percentage that is incorporated into the membranes of ciliated protozoa as phosphatidylcholine (Broad & Dawson, 1976). There is evidence that protozoa are selectively retained on the passage of digesta out of the rumen (Weller & Pilgrim, 1974; Bauchop & Clarke, 1976; Harrison, 1979). Moreover, sheep whose rumens have been defaunated to remove ciliated protozoa remain, in our experience, completely healthy for 6 months or longer and show no signs of choline deficiency. Thus it is important to know how much choline is passing from the rumen in both normal and defaunated sheep. Analysis of abomasal digesta indicates that the dietary choline made available for absorption in the lower digestive tract is minimal. In other species a dietary intake at an equivalent low concentration would cause pathological lesions and death.

Experiments have been carried out to try to ascertain the reason for the lack of sensitivity of the sheep to supply of exogenous choline. The main synthetic organ in mammals, the liver, appears to be less effective at synthesizing choline via phosphatidylethanolamine than does rat liver. On the other hand, whole-animal experiments show that choline appears to be oxidized to CO_2 less readily in the sheep than in the rat.

Experimental

Collection of digesta samples and preparation for analysis

Unless otherwise indicated, Clun Forest wether sheep (*Ovis aries*) were used. They were housed indoors and fed once daily (09.00 h) a ration of 1 kg of hay/chaff and 100 g of oats. Defaunation of the rumen was carried out as described by Dawson & Kemp (1969), and checked periodically for the absence of ciliate protozoa by microscopic examination of the rumen fluid.

Animals were fitted with a standard cannula (Jarrett, 1948) in a rumen fistula. Abomasal cannulae were constructed of polythene tubing (15 cm × 1 cm)

* Present address: Animal Research Institute, Department of Primary Industries, Yeerongpilly, Queensland 4105, Australia.

† Present address: Waite Agricultural Research Institute, University of Adelaide, South Australia, Australia.

to which a polythene flange was heat-sealed about 2cm from the end. The cannula was inserted near the pylorus and exteriorized about 10–12cm dorsal to the midline.

Fluid was collected through the rumen fistulae by gentle suction through a wide-bore tube; fluid from the abomasum cannulae ran out under gravity. Representative portions (5ml) were dried to constant weight at 110°C. A further portion (20ml) from the same sample of digesta was mixed with 50ml of methanol and then with 25ml of chloroform and homogenized by blending. A further 25ml of chloroform and 25ml of water was then added and the mixture reblended. The upper aqueous layer was removed and was used to determine the concentration of non-esterified choline. The lower chloroform layer was filtered through a small glass-wool pad and the filtrate evaporated to dryness *in vacuo* at 50°C. The residue was taken up in chloroform (5ml).

Determination of choline

In the initial stages of the present investigation, non-esterified choline was determined in ethanolic extracts of digesta by the frog-rectus-muscle assay of Eadie *et al.* (1973).

Choline was later determined by using a choline acetyltransferase method (Eadie *et al.*, 1973), either directly for non-esterified choline in the aqueous layer (see above) or after hydrolysis of the phospholipids in the solvent layer. The specificity of the method was improved by using controls with choline esterase (EC 3.1.1.8) and acetyldephospho-CoA added (S. P. Mann & A. M. Snoswell, unpublished work). To liberate choline from the phospholipids, a suitable portion of the chloroform solution of the phospholipids was evaporated to dryness at 50°C, and treated with 0.08ml of carbon tetrachloride, 0.75ml of ethanol, 0.07ml of water and 0.025ml of 1M-NaOH. After incubating the mixture for 20min at 37°C to deacylate the phosphatidylcholine and its lyso analogue (Dawson *et al.*, 1962), 0.23ml of 5M-HCl was added and the unstoppered tubes heated at 100°C for 15min to release choline from glycerophosphocholine.

Determination of phosphatidylcholine

The lipid extracts of digesta samples were highly coloured with partially degraded plant pigments and rich in neutral lipids, mainly fatty acids. Consequently a second run of solvent on t.l.c. was necessary to obtain satisfactory resolution of the phosphatidylcholine spot. A portion of the chloroform solution of digesta lipids (0.5–1.0ml) was evaporated to near-dryness under N_2 at 50°C and quantitatively applied to a silica-gel t.l.c. plate (Merck 60F 254) that had previously been chromatographically washed with diethyl ether. The plate was first developed to the end in chloroform/methanol (9.1, v/v). After drying it was again developed with chloroform/methanol/water/acetic acid solvent (60:60:5:1, by vol.) in the same direction by using a saturation chamber for both separations. The dried plate was sprayed to detect phospholipids (Vaskovsky & Kostetsky, 1968), and the phosphatidylcholine spot located with the help of markers, scraped off and its phosphorus content determined (Bartlett, 1959). On some occasions the lipids were run as a strip, the phosphatidylcholine was located with iodine vapour, and the appropriate strip eluted and analysed for phosphorus and choline.

Respiratory-gas analysis

The head of the animal was enclosed in a chamber connected to a respiratory-pattern analyser developed in this Department by A. Northrop and D. B. Lindsay and similar to that described by Young *et al.* (1975). Respiratory gases were assayed continuously by using i.r. analysers for CO_2 and methane [Hartmann and Braun (U.K.) Ltd., Moulton Park, Northampton, U.K.], and the total radioactivity monitored with a Cary–Tolbert ionization chamber and Cary 401 amplifier (Cary Instruments, Monrovia, CA, U.S.A.). Radioactivity in CO_2 was determined by passing the expired air through barium hydroxide.

Studies using [^{14}C]ethanolamine as substrate

After overnight food deprivation for 18h, sheep were maintained under anaesthesia by continual injections of pentobarbitone sodium via a catheterized jugular vein. The mesenteric region was exposed surgically and a catheter inserted in the portal vein. [2-^{14}C]Ethanolamine hydrochloride in 1.25ml of sterile 0.9% NaCl was injected slowly via this catheter followed by the injection of a further 1.5ml of 0.9% NaCl. The liver was biopsied hourly over a period of 4h. Excessive bleeding was prevented by clamping the lesions. Liver samples were homogenized and extracted as described above.

In a separate experiment, sheep liver obtained at slaughter was immediately perfused with Krebs–Ringer bicarbonate buffer, pH 7.4, at 4°C and kept on ice while preparing tissue slices (0.5mm thick). Tissue samples (0.5g wet wt.) were incubated at 37°C under O_2/CO_2 (19:1) in 2.5ml of Krebs–Ringer bicarbonate buffer, pH 7.4, containing [2-^{14}C]-ethanolamine hydrochloride, 2mM-L-asparagine, 2mM-L-glutamine and 2mM-propionic acid with or without 0.67M-L-methionine. After 3h the incubation flasks were immersed in boiling water for 7min. Lipids were extracted with chloroform/methanol (2.1, v/v), washed and analysed as described below.

Lipid extracts were analysed by t.l.c. on activated silica gel. The plates were developed in diethyl ether to remove neutral lipids, dried and redeveloped in

chloroform/methanol/aq. NH_3 (sp.gr. 0.88)/water (110:50:3:5, by vol.). Individual components were located by using markers and radioautography. Areas of silica gel containing radioactivity were scraped into vials for liquid-scintillation counting. Lipid phosphorus in the total extracts and in the separated phospholipids was determined by the method of Bartlett (1959).

Radioactivity determinations

Aqueous samples (0.1–1.0ml) were mixed with 10ml of Unisolve (Koch–Light, Colnbrook, Bucks., U.K.) for liquid-scintillation counting. The solvent was removed from the samples of the lipid extract before adding the Unisolve. $^{14}CO_2$ was trapped as insoluble barium carbonate, dried and suspended in Cab-O-Sil (Koch–Light) for liquid-scintillation counting. Lipid bound to silica gel was counted similarly.

Materials

[*Me*-^{14}C]Choline and [2-^{14}C]ethanolamine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and [1,2-^{14}C]choline from New England Nuclear, Dreieichenheim, Germany. U-^{14}C-labelled grass was prepared in the chamber described by Dawson & Hemington (1974).

Results and Specific Discussion

Free choline in digesta

The hay used as fodder contained 524nmol of non-esterified choline/g wet wt., whereas the concentration of lipid choline was only 64nmol/g. (Similar results have been obtained by Snoswell *et al.*, 1978.) By contrast, the oats that were used as a minor component of the diet contained 467nmol of lipid choline/g, whereas the non-esterified choline concentration was only 53nmol/g. Calculation from these values shows that the experimental sheep on the standard diet (1000g of hay plus 100g of oats per day) would be receiving approx. 529μmol of non-esterified choline per day plus any liberated from the dietary phosphatidylcholine by the combined action of the phospholipases and phosphodiesterases of rumen micro-organisms (Dawson, 1959; Hazlewood & Dawson, 1975). In spite of this, the concentration of non-esterified choline in the digesta passing into the abomasum is minimal. Nine determinations on such digesta samples removed from three different sheep gave a mean value of 1.07 ± 0.19 (S.E.M.) nmol of choline/ml, with no apparent variation with the time after feeding. Assuming a maximum daily flow rate of the digesta of 9 litres (Faichney & Weston, 1971; Faichney, 1972), the maximum amount of non-esterified choline passing through the abomasum (9.6μmol/day) is only a few per cent of the total choline in the diet.

The explanation for this loss is presumably the active microbial destruction of choline in the rumen to produce trimethylamine and eventually methane (Neill *et al.*, 1978). To investigate this further we injected non-esterified [*Me*-^{14}C]choline into the rumen of sheep and observed the liberation of radioactivity in the expired respiratory gases. Radioactivity rapidly appeared in the expired gases, and Fig. 1 shows that there was a close relationship between this radioactivity and the methane released. This methane was released in bursts, presumably appearing each time the sheep eructated (belched). The CO_2 expired was collected in alkali and contained negligible quantities of $^{14}CO_2$.

In a further experiment of this type, an animal was fed before and during the experimental procedure to slow the formation of methane from the trimethylamine formed from choline (Neill *et al.*, 1978). The animal expired 76% of the [*Me*-^{14}C]choline radioactivity injected into the rumen as methane over 6h. The animal was then killed and the radioactivity in the liver, rumen wall and blood plasma assessed. Of

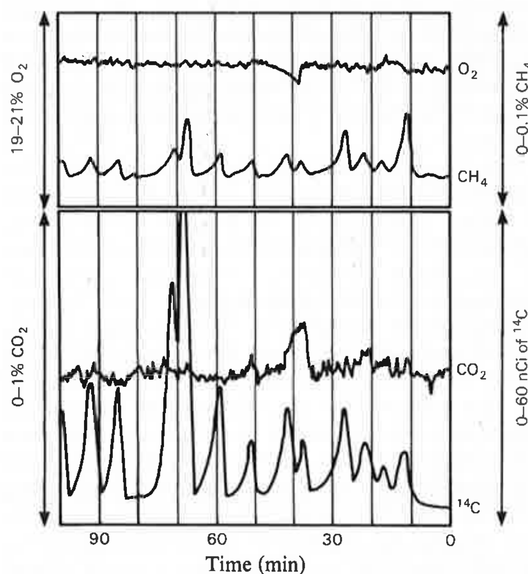


Fig. 1. *Recorder traces of the concentrations of respiratory gases and gaseous radioactivity in expired air from a sheep after injection into the rumen of 20μCi of [Me-^{14}C]choline*

[*Me*-^{14}C]Choline was injected through a rumen cannula in a sheep deprived of food for 24h. The sheep's head and neck were confined in a chamber through which outside air was drawn at about 30 litres/min. Part of the outflow passed to a gas-analysis system that continuously monitored the gas concentrations by means of two twin-channel recorders. The results were reproducible in two additional experiments of this type.

the 0.07% of the administered ^{14}C dose found in the whole liver, the major portion was present as lipid; the other tissues examined contained only traces of radioactivity. It can be calculated from the results of Neill *et al.* (1978) that under these conditions a minimum of 15% of the ^{14}C administered would have remained in the rumen as trimethylamine. Clearly therefore only a very small percentage of the non-esterified choline that enters the rumen, or is produced in this organ, is absorbed as such and becomes incorporated into the tissue lipid.

Lipid choline in digesta

Choline analysis of the abomasum contents showed that, although these contained negligible amounts of non-esterified choline, there were appreciable quantities of lipid choline. Samples taken at random showed 11–44nmol of lipid choline/g wet wt., and this amounted to 81–96% of the total choline in the sample. T.l.c. revealed that this lipid choline was almost exclusively phosphatidylcholine, although traces of lysophosphatidylcholine were occasionally observed. A sample of the phospholipid that chromatographed as phosphatidylcholine was isolated and identified by (*a*) co-chromatography with authentic phosphatidylcholine, (*b*) its chemical degradation to glycerophosphocholine (Dawson *et al.*, 1962), and (*c*) its analysis giving a choline/P molar ratio of 0.95:1 (authentic phosphatidylcholine gave a molar ratio of 0.94:1 by the same methods of analysis).

The question therefore arose as to whether this phosphatidylcholine present in the abomasum was of dietary origin, and if so, whether it contributed substantially to the nutritional choline requirements of the sheep. When U-^{14}C-labelled grass was introduced into the rumen of sheep, the [^{14}C]phosphatidylcholine contained in it was initially rapidly broken down, but eventually the radioactivity in the rumen phosphatidylcholine remained constant (Fig. 2). This can possibly be attributed to the ruminal protozoa requiring choline for growth, so that some of the dietary choline of the sheep is incorporated into protozoal phosphatidylcholine and thus spared from bacterial degradation (Broad & Dawson, 1976). This ruminal preservation of phosphatidylcholine is virtually eliminated when the rumen is free of ciliated protozoa (Broad & Dawson, 1976).

Studies have suggested that ruminal protozoa are selectively retained in the rumen (Weller & Pilgrim, 1974; Bauchop & Clarke, 1976). To investigate the throughput of phosphatidylcholine to the abomasum we measured the concentration of this phospholipid in the rumen and in the abomasum at various times after feeding. The rumen concentration of phosphatidylcholine proved to be very variable. This can possibly be attributed, at least in part, to the difficulty of obtaining a representative sample of fluid from

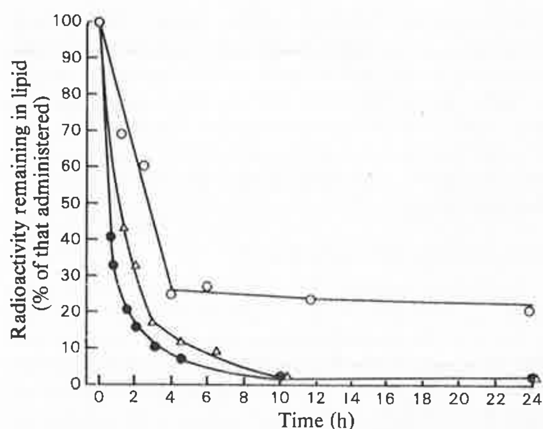


Fig. 2. *Degradation of grass phosphatidylcholine in the rumen*

U-^{14}C-labelled grass was introduced into the rumen via a fistula in a pasture-fed animal at zero time. The animal was then allowed to continue eating pasture. Rumen contents were removed at various times, the lipids extracted, separated on columns and by t.l.c. as described by Dawson & Hemington (1974). The activity in the phosphatidylcholine obtained from each sample was compared with that of the non-metabolized β-carotene, as described by Dawson *et al.* (1977). The change in the ratio of activities of these two lipids represents the proportion of the phosphatidylcholine radioactivity lost in a given time. For comparison, included on the same graph is the disappearance of radioactivity from two grass galactolipids, which are totally metabolized and where little recycling of the radioactivity into microbial galactolipids occurs. Essentially similar results were obtained in three additional experiments using two experimental animals. ○, Phosphatidylcholine; ●, monogalactosyldiacylglycerol; △, digalactosyldiacylglycerol.

the organ through a narrow fistula. Even samples removed in rapid succession tended to show considerable variation in dry weight and in phosphatidylcholine concentration. Nevertheless, even with this analytical limitation, at all times after feeding the concentration of phosphatidylcholine in the rumen was appreciably higher than that in the abomasum on a digesta-dry-weight basis (Fig. 3). The suggestion of an increase in the ruminal concentration some hours after feeding could reflect the conversion of the non-esterified choline of the diet into protozoal phosphatidylcholine, also perhaps because these same protozoa are much easier to remove from the rumen during sampling than the recently ingested large pieces of leaf material. The mean concentration of phosphatidylcholine in rumen contents was $28.4 \pm 2.46\,\mu g$ of P/g dry wt. of digesta for 49 observations made at all times after

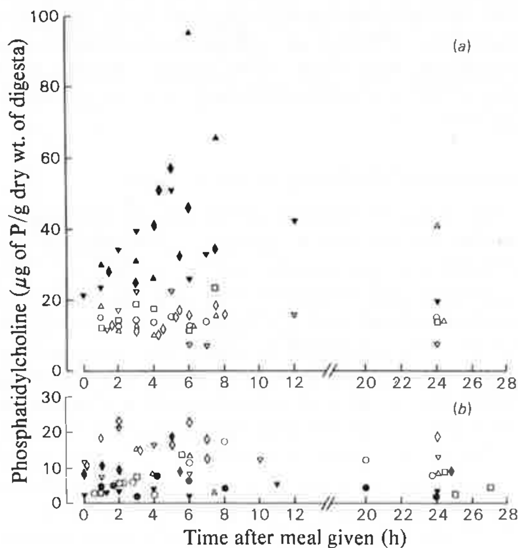


Fig. 3. *Phosphatidylcholine content of digesta removed from the rumens and abomasums of normal and rumen-defaunated sheep*

The digesta were removed through fistulae in the rumen and abomasum at various times after the presentation of a meal of 1000g of hay/chaff and 100g of oats. This was usually consumed within 2h, but in some defaunated animals the food ingestion was over a longer period. Each symbol represents a different experiment on an individual sheep. Open symbols, abomasal digesta; closed symbols, rumen digesta; (*a*) normal sheep; (*b*) rumen-defaunated sheep.

feeding, whereas the corresponding value for abomasal digesta was $14.3 \pm 0.69\,\mu g$ of P/g dry wt. (68 observations), the difference being significant. [$P = 0.0005$ (Student's *t* test)]. No evidence was observed of any change in abosmasal phosphatidylcholine concentration at various times after feeding (Fig. 3).

When the rumens of sheep were defaunated, the amount of ruminal phosphatidylcholine decreased dramatically (Fig. 3), 23 observations showing a mean value of $6.4 \pm 0.90\,\mu g$ of P/g dry wt. of digesta, which was significantly lower than that in the abomasal contents [$11.4 \pm 1.11\,\mu g$ of P/dry wt., 37 observations; $P = 0.0022$ (Student's *t* test)]. This marked decrease in ruminal phosphatidylcholine on defaunation is to be expected, since the ciliated protozoa that contain phosphatidylcholine as the major component of their membrane phospholipids (Dawson & Kemp, 1967) are eliminated. By contrast, the bacterial membranes contain little phosphatidylcholine (Dawson & Kemp, 1969). The origin of the ruminal phosphatidylcholine in the defaunated rumen is not obvious. It could represent non-hydrolysed plant phospholipid, it could arise from flagellated protozoa, which are known to proliferate after defaunation, or it could come from sources within the sheep tissues, e.g. secretions or sloughed-off rumen epithelial cells. The higher concentration of phosphatidylcholine in the abomasal digesta compared with that in the rumen might be due to a number of possibilities, e.g. a selected throughput of material that contains phosphatidylcholine from the rumen digesta, a selective digestion and absorption of materials that do not contain phosphatidylcholine, alternatively an enrichment of the abomasal digesta with phosphatidylcholine-containing material from the secretions into it or by regurgitation from the lower digestive tract, e.g. bile.

Calculations based on the phosphatidylcholine concentration of abomasal digesta and the known throughput of 7.5–10 litres per day (Faichney & Weston, 1971; Faichney, 1972) indicate that at maximum the sheep can receive only 15–20mg of dietary choline per day through the passage of phosphatidylcholine and 2–3mg through the non-esterified base (a maximum of 0.25mg/kg body wt.). Since the sheep receives only these limited amounts of choline from its diet, experiments were initiated to see whether this species avoided the pathological results of choline deficiencies (so well documented in other species) either by a greatly increased synthesis in the liver by *N*-methylation of phosphatidylethanolamine or alternatively by a decreased oxidation through the choline oxidase system.

Choline synthesis in sheep liver

It is to be expected, by analogy with other mammals, that maximum synthesis of choline would occur in the liver (Bremer & Greenberg, 1961). In preliminary experiments it has been found that, when [2-^{14}C]ethanolamine was injected intravenously into a sheep, there was a definite incorporation of label into the phosphatidylcholine pool of the liver in biopsy samples taken at times up to 4h after isotope administration (Table 1). Assuming a liver-to-body-weight ratio of 1.9:100, it can be calculated from the results that the sheep's liver would be able to synthesize approx. 9mg of choline/day per kg body wt. This is very considerably more than that available through dietary sources. When sheep liver slices were incubated with [^{14}C]ethanolamine in the absence of methionine, an equivalent calculation gave a maximum of 11.3mg of choline formed per kg body wt., which agreed well with the results obtained *in vivo*. As is to be expected, the addition of methionine to the incubation medium stimulated the synthesis of choline (Table 1).

The rate of conversion of [^{14}C]ethanolamine into choline in the sheep appears to be much lower than that reported from similar experiments with [^{14}C]-ethanolamine in the intact rat (Bjornstad &

Table 1. *Incorporation of* [2-^{14}C]*ethanolamine into sheep liver lipids*

In Expt. (*a*) a Soay ram (19.5kg body wt.) deprived of food overnight was anaesthetized with pentobarbitone sodium. [2-^{14}C]Ethanolamine hydrochloride (250μCi; 55mCi/mmol) was injected in the portal vein and the liver biopsied hourly for 4h. In Expt. (*b*) sheep liver slices (500mg wet wt.) were incubated in Krebs–Ringer bicarbonate buffer, pH7.4, at 37°C under O_2/CO_2 (19:1) with 1.6μCi of [2-^{14}C]ethanolamine hydrochloride, L-asparagine (2mM), L-glutamine (2mM) and sodium propionate (2mM) with or without L-methionine (0.67mM). Total medium volume was 2.5ml. Incubations were stopped after 3h. The lipids were extracted from tissues and analysed as described in text. The results are the specific radioactivities (nCi/μmol of P) in total phospholipid (TP), phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

Expt. (*a*)

Sampling time (h)	TP	PC	PE
1	3.6	0.2	10.7
2	3.8	0.4	10.9
3	4.2	0.3	12.3
4	4.4	0.5	12.8

Expt. (*b*)

Incubation conditions	TP	PC	PE
−Methionine	76.6	9.8	214.0
−Methionine	71.2	7.7	199.8
+Methionine	67.3	14.5	175.7
+Methionine	83.8	23.8	214.4

Table 2. *Production of* $^{14}CO_2$ *from* [1,2-^{14}C]*choline in the intact sheep and rat*

Rats were injected with 5–10μCi of [1,2-^{14}C]choline and sheep with 40–50μCi, together with choline carrier in proportion to the body weight of sheep or rat. The output of $^{14}CO_2$ was monitored and integrated over a 5h period with the animal's head in a chamber connected to a respiratory-pattern analyser.

Animal	Injection route	Percentage of [^{14}C]choline oxidized to $^{14}CO_2$ in 5h
Sheep	Intraperitoneal	1.6
	Intravenous	2.1
	Intravenous	1.5
		1.73 ± 0.19 (mean ± S.E.M.)
Rat	Intraperitoneal	7.5
	Intra-arterial	2.7
	Intravenous	5.5
	Intravenous	5.3
	Intravenous	5.1
		5.22 ± 0.76 (mean ± S.E.M.)

Bremer, 1966). From these results it can be calculated that male rat liver can synthesize about 100mg/day per kg body wt., and the value is about 25% higher for female animals. Bremer & Greenberg (1961) found that isolated microsomal fractions from sheep liver were less effective at synthesizing choline when compared with those from species known to be susceptible to choline deficiency, e.g. rat, mouse and chicken.

Oxidation of choline by the intact sheep

To obtain a measure of the rate of oxidation of choline in the whole sheep, [1,2-^{14}C]choline was injected into the animal and the output of $^{14}CO_2$ monitored and integrated by using the respiratory-pattern analyser over a 5h period. This showed that 1.5–2% of the injected radioactivity was expired as $^{14}CO_2$ during this time (Table 2). Corresponding values for the rat were on average three times higher ($P=0.012$; Student's *t* test), suggesting that the rate of oxidation of choline was much more rapid in this species. This assumes that the values obtained are not distorted by any large differences in the rate at which the non-esterified choline of tissues (particularly liver, where choline oxidase is concentrated) comes into equilibrium with the body pool.

Discussion

The present measurements clearly show that, in a sheep fed a diet of hay/oats, the extensive microbial hydrolysis of phospholipids, and the degradation of the *N*-methyl groups of any non-esterified or released choline into trimethylamine and eventually methane in the rumen, leads to only minimal passage of choline, mainly as phosphatidylcholine, to the lower digestive tract. Calculation based on the assumption that all the choline present in the abomasum is of dietary origin suggests that no more than 20–25mg/day survives destruction in the rumen, and it is very possible that this is an overestimate. This represents an amount of choline equivalent to about 0.002–0.0025% of the total dietary intake of dry matter, and is a concentration that would certainly produce severe pathological lesions (e.g. fatty liver, haemorrhagic kidney) and death in many non-ruminant species. For example, the choline requirements for mice, rats, guinea pigs, pigs and poultry have been found to be 0.1% or more of the dry-matter intake (Reid, 1955; Lucas & Ridout, 1967).

It would appear, therefore, that the adult sheep is less sensitive to a low choline supply from the diet. Few studies have been carried out on the choline requirements of ruminant animals, although Johnson *et al.* (1951) showed that the neonatal calf rapidly deteriorated if fed an artifical diet milk replacer devoid of choline. However, two animals given the same diet when a few weeks of age developed no sign of choline deficiency. This could indicate that sensitivity to choline deficiency in ruminants decreases with age.

Reasons for the apparent normal health of sheep receiving such limited amounts of dietary choline are not fully established. Clearly choline can be synthesized by the animals, presumably by using methionine as methyl donor for the methylation of phosphatidylethanolamine. Calculation based on our limited observations indicates, however, that this formation of choline is many times less on a body-weight basis than is the choline synthesis observed in rat liver (Bjornstad & Bremer, 1966). This would therefore in no way help to explain the resistance of the sheep to low choline intake compared with the rat. Furthermore, since methionine is the first limiting amino acid in the nutrition of sheep (Chalupa, 1972; Reis *et al.*, 1973), its supplies for transmethylation reactions are likely to be limited.

Within the limitations of the experimental technique, the demonstration of a severalfold decrease in the oxidation of choline in the intact sheep compared with the rat could help to explain the decreased requirement of the ruminant for choline. However, although rat liver is known to be very rich in choline oxidase, there appears to be no direct relationship between the activity of hepatic choline oxidase and susceptibility to choline deficiency throughout a variety of species (Lucas & Ridout, 1967).

For economic reasons it was not possible to determine the rates of oxidation of choline after the total body pool of lipid choline had equilibrated with the injected [^{14}C]choline. If such an exchange is slower in the sheep than in the rat (which is likely in a situation where conservation of a vital nutrient is essential), then it could be that the real difference in the rate of oxidation of choline between the two species is even greater than that measured. In this respect, Henderson (1978) found that, in sheep hepatocyte preparations, the ratio of incorporation of label from [1,2-^{14}C]choline into betaine as compared with phosphatidylcholine for a 1 h incubation was 0.84:1. A similar ratio for rat hepatocytes calculated from the data published by Sundler & Akesson (1975) is 26:1. If these values can be directly compared, they indicate that sheep liver cells can use available choline more effectively for membrane maintenance than can the cells of rat liver. Also, once encaptured in the pool of phosphatidylcholine in the liver, the very low concentrations of glycerophosphinicocholine diesterase (EC 3.1.4.2) in sheep liver (Dawson, 1956) may prevent the ready release of non-esterified choline as a substrate for choline oxidase.

A. R. N. was the recipient of an Australian Meat Research Committee Overseas Study Award, and A. M. S. received a grant from The Royal Society (London) and a Nuffield Foundation Commonwealth Bursary.

References

Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468

Bauchop, T. & Clarke, R. T. J. (1976) *Appl. Environ. Microb.* **32**, 417–422

Bjornstad, P. & Bremer, J. (1966) *J. Lipid Res.* **7**, 38–45

Bremer, J. & Greenberg, D. M. (1961) *Biochim. Biophys. Acta* **46**, 205–216

Broad, T. E. & Dawson, R. M. C. (1976) *J. Gen. Microbiol.* **92**, 391–397

Chalupa, W. (1972) *Fed. Proc. Fed Am. Soc. Exp. Biol.* **31**, 1152–1164

Dawson, R. M. C. (1956) *Biochem. J.* **62**, 689–693

Dawson, R. M. C. (1959) *Nature (London)* **183**, 1822–1823

Dawson, R. M. C. & Hemington, N. (1974) *Br. J. Nutr.* **32**, 327–340

Dawson, R. M. C., & Kemp, P. (1967) *Biochem. J.* **105**, 837–842

Dawson, R. M. C. & Kemp, P. (1969) *Biochem. J.* **115**, 351–352

Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962) *Biochem. J.* **84**, 497–501

Dawson, R. M. C. Hemington, N. & Hazlewood, G. P. (1977) *Br. J. Nutr.* **38**, 225–232

Eadie, I., Hebb, C. & Mann, S. P. (1973) *J. Neurochem.* **20**, 1499–1502

Faichney, G. J. (1972) *Aust. J. Agric. Res.* **23**, 859–869

Faichney, G. J & Weston, R. H. (1971) *Aust. J. Agric. Res.* **22**, 461–468

Harrison, D. G. (1979) *Br. J. Nutr.* in the press

Hazlewood, G. P. & Dawson, R. M. C. (1975) *J. Gen. Microbiol.* **89**, 163–174

Henderson, G. D. (1978) Ph.D. Thesis, University of Adelaide

Jarrett, I. G. (1948) *J. Counc. Sci. Ind. Res. Aust.* **21**, 311–315

Johnson, B. C., Mitchell, H. H., Pinkos, J. A. & Morrill, C. C. (1951) *J. Nutr.* **43**, 37–48

Lucas, C. C. & Ridout, J. H. (1967) *Prog. Chem. Fats Other Lipids* **10**, 1–150

Neill, A. R., Grime, D. W. & Dawson, R. M. C. (1978) *Biochem. J.* **170**, 529–535

Reid, M. E. (1955) *J. Nutr.* **56**, 215–229

Reis, P. J., Tunks, D. A. & Sharry, L. F. (1973) *Aust. J. Biol. Sci.* **26**, 635–644

Snoswell, A. M., Henderson, G. D. & Dawson, R. M. C. (1978) *Proc. Aust. Nutr. Soc.* in the press

Sundler, R. & Akesson, B. (1975) *J. Biol. Chem.* **250**, 3359–3367

Vaskovsky, V. E. & Kostetsky, E. Y. (1968) *J. Lipid Res.* **9**, 396

Weller, R. A. & Pilgrim, A. F. (1974) *Br. J. Nutr.* **32**, 341–351

Young, B. A., Kerrigan, B. & Christopherson, R. J. (1975) *Can. J. Anim. Sci.* **55**, 17–22

Biochimica et Biophysica Acta, 710 (1982) 236–241
Elsevier Biomedical Press

BBA 51034

STUDIES OF LIVER LIPIDS IN NORMAL, ALLOXAN-DIABETIC AND PREGNANCY-TOXAEMIC SHEEP

GRAHAM D. HENDERSON, LEANNA C. READ * and ALAN M. SNOSWELL

Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia, 5064 (Australia)

(Received August 17th, 1981)

Key words: Lipid composition; Alloxan diabetes; Pregnancy toxemia; (Sheep liver)

Triacylglycerols were the major lipid class in the fatty livers from alloxan-diabetic sheep and those suffering from pregnancy toxaemia, with the concentrations increased by 15- and 25-fold, respectively, compared with the normal state. Analysis of the fatty acid composition of total liver triacylglycerols in these animals showed a significant decrease in the proportion of saturated fatty acids, 16:0 and 18:0, and increase in the proportion of polyunsaturated fatty acids (18:2ω6, 18:3ω3 and 20:4ω6), particularly in those with pregnancy toxaemia. In contrast, total liver phospholipids showed a significant increase in the proportion of 18:0 in ewes with pregnancy toxaemia and a significant decrease in a range of polyunsaturated fatty acids in both the diabetic and toxaemic animals. Also, although the concentration of both phosphatidylcholine and phosphatidylethanolamine increased in the diabetic livers the ratio of phosphatidylcholine/phosphatidylethanolamine fell significantly, from 2.22 in the control animals to 1.59. The data suggest that, following the large influx of plasma fatty acids into the ovine liver in diabetes and pregnancy toxaemia, there is a diversion of polyunsaturated fatty acids from phospholipids to triacylglycerols. In diabetic sheep these changes may in turn affect phosphatidylcholine synthesis via the methylation pathway in liver. These changes in lipid composition may, in part, explain the degenerative changes in membrane and subcellular organelle structure and the failure of liver function observed both in advanced diabetes and in severe pregnancy toxaemia.

Introduction

In sheep under normal conditions, lipid accounts for only about 5% of the fresh weight of the liver [1]. However, in both starvation [2] and severe diabetes [3] there is a marked accumulation of lipid (predominantly triacylglycerols), giving rise to a fatty liver. A similar fatty infiltration of the liver is one of the most characteristic features of pregnancy toxaemia in the sheep [4] and both in this condition and in diabetes there are marked changes in the structure of hepatocellular organelles [5] and impairment of hepatic function.

The lipid composition of ovine liver has been examined by a number of workers [1,6–9], but these studies have related mainly to normal animals. Little data is available on the lipid composition of liver from alloxan-diabetic sheep and, more particularly, from ewes suffering from pregnancy toxaemia and thus we have examined liver lipid composition in these conditions.

Methods and Materials

The sheep used in these experiments were 2-year-old Merino wethers (approx. 40–45 kg)

* Present address: C.S.I.R.O. Division of Human Nutrition, Kintore Avenue, Adelaide, South Australia, 5000, Australia.
Abbreviation: VLDL, very low density lipoproteins.

0005-2760/820000–0000/$02.75 © 1982 Elsevier Biomedical Press

maintained in pens on 1000g of lucerne chaff per day. Diabetes was induced by an intravenous injection of alloxan (50 mg/kg body wt.) 5 days prior to killing. At slaughter, blood glucose was greater than 12 mM and ketone bodies greater than 7 mM. Merino ewes suffering from pregnancy toxaemia were field cases and were in a moderate-to-severe stage of the disease as judged by clinical symptoms (see Ref. 4).

Livers from all animals were freeze-clamped within 20 s of slaughter as described previously [10]. Water content of the liver was determined by weighing duplicate samples of frozen liver powder before and after drying at 80°C for 48 h.

Lipids were extracted from the frozen powdered liver samples into chloroform/methanol mixture (2:1, v/v) containing the antioxidant 4-methyl-2, 6-di-tert-butyl-phenol (50 mg/l), essentially by the method of Folch et al. [11] but with the modifications described by Henderson [12]. Concentrated lipid extracts (20–80 mg/lipid per ml) were stored under N_2 at −14°C prior to analysis. Total lipid concentration was determined by weighing an aliquot of the lipid extract after drying at 60°C for 2 h.

TLC procedures used for separation of the various lipids were those of Skipski and Barclay [13] but with the modifications described by Henderson [12]. Authentic standards were applied to each plate and were detected subsequently under ultraviolet light after spraying the outside lanes with 2′,7′-dichlorofluorescein. Sample areas corresponding to triacylglycerols and phospholipids were scraped from the plates and extracted twice with chloroform/methanol (2 : 1, v/v). The triacylglycerol fraction was saponified [14] and the glycerol determined enzymically [15].

The total phospholipid extracts were rechromatographed on 200×200×0.5 mm plates of washed silica gel H (type 60) and developed in chloroform/methanol/acetic acid/water (25:15:4:2, v/v). A standard mixture, PL-3, containing phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and ceramide phosphocholine from the Hormel Institute, Austin, MN, U.S.A., was run in outside lanes. The various phospholipid classes were detected by charring and the areas corresponding to phosphatidylcholine and phosphatidylethanolamine were removed. These classes and total phospholipids were quantified by determination of phosphorus.

Samples of total triacylglycerol and phospholipid were methylated without prior removal of silica gel by reacting with 5 ml of 5% sulphuric acid in methanol at 60°C for 3 h. Methyl esters of the fatty acids were then extracted into petroleum ether (40–70°C, b.p.), concentrated and stored under nitrogen. The methyl esters were separated by gas-liquid chromatography on 6-ft. columns of both 10% EGSS-X and 10% EGSS-Y on Gas-chrom.P solid support. Separations were monitored in a Perkin Elmer (Model 801) gas chromatograph with a flame ionization detector. The gas flow rate was 35 cc/min at gas pressures of 60, 40 and 20 lbs/inch^2 for nitrogen, air and hydrogen, respectively. The methyl esters were identified by relation to retention times for standards 18:0, 18:1, 18:2, 18:3, 20:0, 20:4 and 22:6 (Nucheck Prep. Inc., Elysian, MN, U.S.A.) and to tables of equivalent chain lengths [16,17].

Statistics. Significant differences were determined by the use of Students *t*-test.

Chemicals. These were as follows: silica gel G (type 60) and silica gel H (type 60) from E. Merck A.E., Darmstadt, F.R.G.; alloxan from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; 4-methyl-2,6-di-tert-butylphenol from Calbiochem, Los Angeles, CA, U.S.A. Lipid standards were obtained from Koch-Light Laboratories, Sigma chemical Co., St. Louis, MO, U.S.A. and standard PL-3 from the Hormel Institute, Austin, MN, U.S.A.

Results

The amount of total lipid in sheep liver increased 3-fold and 4–5-fold in diabetic animals and those suffering from pregnancy toxaemia, respectively, when expressed on a fat-free dry weight basis (Table I). Subsequent separation of the lipid classes by thin-layer chromatography showed that triacylglycerol was the major lipid fraction, although there was also a small increase in the cholesterol ester fraction in the toxaemic ewes (not shown). The triacylglycerol fraction increased 15-fold and 25-fold in the diabetic and toxaemic animals, respectively, (Table I). Although the total amount of phospholipid did not vary significantly

TABLE I

LIPID FRACTIONS IN LIVER FROM NORMAL, ALLOXAN-DIABETIC AND PREGNANCY-TOXAEMIC SHEEP

Figures are means ± S.E., with the number of animals given in brackets. Values are mg/g dry fat-free tissue. For triacylglycerols an average molecular weight of 885, and for phospholipids of 760 is assumed.

State	Total-lipid	Triacylglycerols	Phospholipids
Normal	227 ± 7 (5)	15 ± 3 (4)	166 ± 16 (5)
Alloxan-diabetic	681 ± 42 (5)	434 ± 35 (5)	152 ± 14 (5)
Pregnancy-toxaemic ewes	1 040 ± 61 (5)	743 ± 71 (5)	121 ± 4 (4)

from normal in the metabolic stress states (Table I), when expressed as a percentage of the total lipid, it fell from 73% in the normal to 22% in the diabetic sheep and to 12% in the toxaemic animals. Because of the significant differences in liver volume in the three states, due essentially to changes in water and lipid content and because of the inverse correlation between lipid and moisture content of the liver (Fig. 1), the results in Table I are expressed per g dry weight of fat-free tissue for meaningful comparisons.

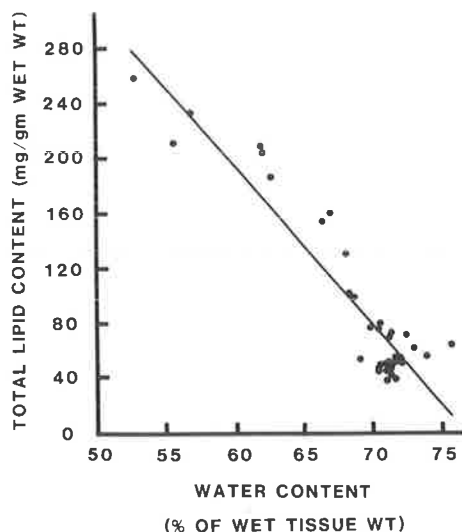


Fig. 1. Relationship between moisture content and total lipid content of sheep liver. The data collected from 39 sheep ranging from normal ewes and wethers to alloxan-diabetic wethers and field cases of ewes suffering from pregnancy toxaemia. The regression line, which exhibits the linear regression of total lipid content on moisture content was $y = 887.8 - 11.57x$. The coefficient of the determination (r^2) was 0.87.

Analysis of the fatty acid composition of total liver triacylglycerols showed that in the normal animals the principle fatty acids were 16:0, 18:0 and 18:1ω9 (Table II), as observed by others [6]. Livers from diabetic animals showed a significant decrease in the contributions of 16:0 and 18:0 while the relative proportions of 18:1ω9, 18:2ω6, 18:3ω3 and 20:4ω6 increased significantly. Similar, but more pronounced changes were seen in the toxaemic livers (Table II). Differences between the two stress states were seen in that 18:1ω9 did not differ from the normals in pregnancy toxaemia and that 18:4ω3 and 22:5ω3 also rose significantly.

In contrast, almost opposite changes occurred in the proportions of fatty acids in the total liver phospholipids from stressed animals with respect to the saturated fatty acids and polyunsaturated fatty acids (Table III). In the toxaemic animals the proportion of 18:0 and 18:1ω9 increased significantly while a range of polyunsaturated fatty acids (from 18:3ω3 to 22:6ω3) decreased significantly in proportion when compared to the normal livers (Table III). In the diabetic livers only 18:1ω9 increased and the range of polyunsaturated fatty acids which decreased was not quite as extensive.

Separation of the phospholipids into individual classes showed that the concentration of both the phosphatidylcholine fraction and the phosphatidylethanolamine fraction increased significantly in the livers from diabetic animals (Table IV) although the increase in the phosphatidylethanolamine fraction was relatively greater so that the ratio phosphatidylcholine/phosphatidylethanolamine fell significantly in the diabetic state (Table IV).

TABLE II

FATTY ACID COMPOSITION OF TOTAL LIVER TRIACYLGLYCEROLS FROM SHEEP UNDER VARIOUS CONDITIONS

Fatty acids, as methyl esters, were separated by GLC on both EGSS-X and EGSS-Y columns and identified as described in the text. Figures are the means ± S.E. for three sheep in each in each group. The figures marked with an asterisk show significant difference (at least $P<0.05$) from the normal figures.

Fatty acid (% of total fatty acid)	Condition		
	Normal	Alloxan-diabetic	Pregnancy-toxaemic
12:0	0.4±0.1	0.4±0.2	0.4±0.2
14:0	0.9±0.2	0.6±0.1	0.5±0.2
16:0	24.5±1.4	19.5±1.2 *	16.5±1.5 *
16:1ω9+17:0	5.2±0.6	4.7±0.6	4.5±0.7
18:0	16.5±1.5	13.4±0.9 *	10.1±1.2 *
18:1ω9	33.3±1.4	39.4±2.1 *	36.3±2.5
18:2ω6	7.3±0.5	8.7±0.7 *	12.7±1.4 *
18:3ω3	2.7±0.1	3.9±0.1 *	8.0±0.2 *
18:4ω3	0.8±0.1	1.0±0.1	1.9±0.2 *
20:4ω6	1.0±0.1	1.5±0.1 *	1.7±0.2 *
20:4ω3	1.0±0.1	0.8±0.1	0.8±0.1
20:5ω3	1.1±0.1	0.8±0.1	0.8±0.1
22:0	07.±0.1	0.6±0.1	0.6±0.1
22:4ω6	0.6±0.1	0.6±0.1	0.6±0.1
22:5ω6	0.6±0.1	0.5±0.1	0.6±0.1
22:5ω3	2.9±0.1	2.9±0.2	3.3±0.2 *
22:6ω3	0.5±0.1	0.4±0.1	0.5±0.1
24:0	0.2±0.1	0.2±0.1	0.2±0.1

TABLE III

FATTY ACID COMPOSITION OF TOTAL LIVER PHOSPHOLIPIDS FROM SHEEP UNDER VARIOUS CONDITIONS

Fatty acid separations as in Table II. Figures and the means ± S.E. for three sheep in each group. The figures marked with an asterisk show significant differences (at least $P<0.05$) from the normal figures.

Fatty acid (% of total fatty acid)	Condition		
	Normal	Alloxan-diabetic	Pregnancy-toxaemic
<16:0	0.5±0.2	trace	trace
16:0	8.5±1.0	9.2±0.2	7.5±1.0
16:1+17:0	0.8±0.5	2.2±0.2 *	1.3±0.3
18:0	18.9±1.5	20.2±1.2	23.9±0.6 *
18:1ω9	12.5±0.7	20.7±0.3 *	19.9±1.5 *
18:2ω6	8.2±0.7	8.8±0.5	9.3±0.6
18:3ω3	3.5±0.6	3.7±0.2	2.6±0.2 *
18:4ω3	0.6±0.1	0.9±0.2	0.4±0.2
20:2ω6	1.0±0.4	1.0±0.1	1.0±0.2
20:3ω6	1.5±0.1	1.0±0.1 *	0.5±0.3 *
20:4ω6	11.1±1.0	9.6±0.6	9.9±0.7
20:5ω3	5.3±0.7	2.9±0.3 *	3.1±0.5 *
22:4ω6	0.8±0.1	0.8±0.1	0.6±0.1 *
22:5ω6	0.7±1.1	0.5±0.1 *	0.3±0.1 *
22:5ω3	8.5±0.7	6.8±0.8 *	6.8±0.7 *
22:6ω3	12.6±1.5	7.4±1.2 *	6.4±0.9 *
23:0	1.7±0.1	1.7±0.2	1.7±0.4
24:0	1.0±0.1	1.0±0.1	1.0±0.3
>22:6	2.4±1.1	3.6±1.4	3.8±1.5

TABLE IV

PHOSPHOLIPIDS IN LIVER FROM NORMAL AND ALLOXAN-DIABETIC SHEEP

Figures are means± for three sheep in each group. Values are μmol/g dry weight fat-free tissue.

	Phosphatidylcholine	Phosphatidylethanolamine	Ratio phosphatidylcholine/ phosphatidylethanolamine
Normal	74.4±2.1	35.8±1.9	2.22±0.10
Diabetic	92.4±3.9	58.8±5.4	1.59±0.12
P	<0.001	<0.02	<0.02

Discussion

Triacylglycerols were found to be the major lipid fraction in the liver of alloxan-diabetic sheep and those suffering from pregnancy toxaemia. Although this lipid class is the one which usually accumulates in the liver in a variety of species in various physiological and pathological conditions, ruminants are particularly prone to the development of fatty livers. This probably reflects their very limited ability to transport triacylglycerols out of the liver as VLDL [18]. The inherent inability to synthesize VLDL may be associated with the low rate of fatty acid synthesis in sheep liver [19,20] as the rate of triacylglycerol release from perfused rat livers correlated highly with the rate of fatty acid synthesis [21]. However, this may be only part of the reason as high concentrations of plasma free fatty acids are available in the stress states studied here and triacylglycerol release from the liver is also correlated with incoming fatty acid concentration, at least in perfused rat liver [22]. A more likely reason may be due to the presence of a continuous basal lamina in the hepatic sinusoids of sheep [23,24] and other ruminants, which may impede the movement of large molecules, such as VLDL, from the parenchymal cells across the space of Disse to the sinusoids. Because of this limitation the triacylglycerol content of sheep liver may be very sensitive to the concentration of incoming fatty acids from the plasma. The sensitive nature of this state may also be reflected in the way in which the carnitine content of sheep liver increased markedly in the diabetic state [25,26] and in pregnancy toxaemia [27], presumably to increase the flux of oxidation of fatty acids which would otherwise be limited if the carnitine content remained at normal concentrations [27].

The main feature of the results presented here is the diversion of polyunsaturated fatty acids from phospholipids into triacylglycerols in the liver of the diabetic and toxaemic animals. This diversion of polyunsaturated fatty acids away from phospholipids is apparently due to their increased utilization in the synthesis of the large amounts of triacylglycerol which accumulate in these stress states. This is an apparently unique feature observed in sheep liver and raises important questions about the control of triacylglycerol and phospholipid synthesis in this species.

The reduced content of polyunsaturated fatty acids in the phospholipids of sheep liver in the diabetic state and in animals suffering from pregnancy toxaemia might well be expected to have a number of metabolic consequences. It might be anticipated that choline synthesis in the liver would be depressed because the methylation pathway for phosphatidylcholine synthesis from phosphatidylethanolamine (the only pathway of choline synthesis) in most active for tetra and polyenoic classes of phosphatidylethanolamine [28]. We found that the ratio of phosphatidylcholine/phosphatidylethanolamine was depressed significantly in the diabetic sheep. A reduced phosphatidylcholine/ phosphatidylethanolamine ratio has been interpreted as evidence for impaired choline biosynthesis in rat liver [29]. Further, isolated hepatocytes from an alloxan-diabetic sheep showed a marked depression of ^{3}H incorporation from L-[*methyl*-^{3}H]methionine into phosphatidylcholine when compared with hepatocytes from an insulin-stabilized, alloxan-diabetic sheep [12]. The amount

of choline available from the diet at the abomasum [30] would also be depressed with the decreased food intake which occurs in the diabetic and toxaemic states.

Thus, the effects outlined above, particularly in relation to phosphatidylcholine, may well be responsible for some of the pronounced membrane changes seen in the electron micrographs of the liver of the alloxan-diabetic sheep [5] and, in turn, on total liver function.

Acknowledgements

The authors are indebted to Dr. A.R. Sinclair for assistance and advice on the separation of the fatty acids, to Dr. D.L. Topping for help with the manuscript and to R. Fishlock for general assistance. The work was supported by a grant from the Australian Research Grants Committee. G.D.H. was supported by a Commonwealth Postgraduate Award and L.C.R. by an R.K. Morton Memorial Scholarship and Barker Scholarship from the University of Adelaide.

References

1 Peters, J.A. and Smith, L.M. (1964) Biochem. J. 92, 375–385
2 Patterson, D.S.P. (1966) Res. Vet. Sci. 7, 484–492
3 Jarrett, I.G., Potter, B.J. and Packham, A. (1956) Aust. J. Exp. Biol. Med. Sci. 34, 133–142
4 Reid, R.L. (1965) Adv. Vet. Sci. 12, 163–238
5 Taylor, P.H., Wallace, J.C. and Keech, D.B. (1971) Biochim. Biophys. Acta, 237, 179–191
6 Dawson, R.M.C. (1960) Biochem. J. 75, 45–53
7 Scott, T.W., Setchell, B.P. and Bassett, J.M. (1967) Biochem. J. 104, 1040–1047
8 Getz, G.S., Bartley, W., Lurie, D. and Notton, B.M. (1968) Biochim. Biophys. Acta, 152, 325–339
9 Noble, R.C., Steele, W. and Moore, J.H. (1971) Lipids, 6, 926–929
10 Snoswell, A.M. and Henderson, G.D. (1970) Biochem. J. 119, 59–65
11 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
12 Henderson, G.D. (1978) Ph.D. thesis, University of Adelaide
13 Skipski, V.P. and Barclay, M. (1969) Methods Enzymol. 14, 530–598
14 Denton, R.M. and Randle, P.J. (1967) Biochem. J. 104, 416–422
15 Pinter, J.K., Hayashi, J.A. and Watson, J.A. (1967) Arch. Biochem. Biophys. 121, 404–414
16 Christie, W.W. (1975) Lipid Analysis, pp. 106–110, Pergamon Press, London
17 Jamieson, G.R. (1975) J. Chromatog. Sci. 13, 491–497
18 Coleman, R. (1973) Biochim. Biophys. Acta Library 3, 345–375
19 Ingle, D.L., Bauman, D.E. and Garrigus, U.S. (1972) J. Nutr. 102, 609–616
20 Ingle, D.L., Bauman, D.E. and Garrigus, U.S. (1972) J. Nutr. 102, 617–624
21 Windmueller, H.G. and Spaeth, A.E. (1967) Arch. Biochem. Biophys. 122, 362–369
22 Mayes, P.A. and Felts, J.M. (1967) Nature, London, 215, 716–718
23 Grubb, D.J. and Jones, A.L. (1971) Anat. Record 170, 75–80
24 Gemmell, R.T. and Heath, T. (1972) Anat. Record 172, 57–70
25 Snoswell, A.M. and Koundakjian, P.P. (1972) Biochem. J. 127, 133–141
26 Snoswell, A.M. and McIntosh, G.H. (1974) Aust. J. Biol. Sci. 27, 645–650
27 Snoswell, A.M. and Henderson, G.D. (1980) in Carnitine Biosynthesis, Metabolism and Functions (Frenkel, R.A. and McGarry, J.D., eds.), pp. 191–204, Academic Press, New York
28 MacDonald, G. and Thompson, W. (1975) Biochim. Biophys. Acta, 398, 424–432
29 Lyman, R.L., Giotas, C., Medwadowski, B. and Miljanich, P. (1975) Lipids 10, 157–167
30 Neill, A.R., Grime, D.W., Snoswell, A.M., Northrop, A.J., Lindsay, D.B. and Dawson, R.M.C. (1979) Biochem. J. 180, 559–565

Biochem. J. (1984) **217**, 399–408
Printed in Great Britain

Uptake and output of various forms of choline by organs of the conscious chronically catheterized sheep

Brenton S. ROBINSON,* Alan M. SNOSWELL,* William B. RUNCIMAN† and Richard N. UPTON†
**Department of Animal Sciences, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064, Australia, and*
†Department of Anaesthesia and Intensive Care, Flinders Medical Centre, The Flinders University of South Australia, Bedford Park, South Australia 5042, Australia

(*Received 2 June 1983/Accepted 14 September 1983*)

1. The net uptake and output of plasma unesterified choline, glycerophosphocholine, phosphocholine and lipid choline by organs of the conscious chronically catheterized sheep were measured. 2. There was significant production of plasma unesterified choline by the upper- and lower-body regions and the alimentary tract and uptake by the liver, lungs and kidneys. The upper- and lower-body regions drained by the venae cavae provided the bulk (about 82%) of the total body venous return of plasma unesterified choline. Production of plasma unesterified choline by the alimentary tract was approximately balanced by the plasma unesterified choline taken up by the liver, and was almost equal to the amount of choline secreted in the bile. 3. There was a considerable amount of glycerophosphocholine in the liver and there was production of plasma glycerophosphocholine by the liver and uptake by the lungs and kidneys. Glycerophosphocholine was higher in the plasma of sheep than in that of rats. 4. Plasma phosphocholine was produced by the alimentary tract and kidneys. 5. There was production of plasma lipid choline by the upper- and lower-body regions drained by the venae cavae. 6. The results suggest that the sheep synthesizes substantial amounts of choline in extrahepatic tissues and has the capacity for extensive retention and recycling of bile choline. These observations, coupled with a slow turnover of the endogenous choline body pool, explain the low requirement of sheep for dietary choline in contrast with non-ruminant species.

Sheep derive less than 0.1 mmol of choline/day from the diet, as there is rapid microbial breakdown of dietary choline to trimethylamine and methane in the alimentary tract (Neill *et al.*, 1979; Dawson *et al.*, 1981). This is about 100 times less on a body-weight basis than the minimum intake required to avoid pathological lesions (e.g. fatty liver, haemorrhagic kidney) and death in many non-ruminant species. In the sheep more than 99% of the choline body pool is of endogenous origin, and this is in contrast with rats, where 18–54% of the body choline is of dietary origin (Dawson *et al.*, 1981). Sheep liver is less effective at synthesizing choline through the methylation of phosphatidylethanolamine to phosphatidylcholine than is rat liver (Bremer & Greenberg, 1961; Henderson, 1978; Neill *et al.*, 1979). Sheep appear to survive on a low choline intake owing to a slower turnover of the choline body pool (Dawson *et al.*, 1981).

It can be calculated from the data of Neill *et al.* (1979) and Dawson *et al.* (1981) that hepatic synthesis and dietary supply of choline only account for 29% of the daily choline requirement of the adult sheep. This suggests that extrahepatic tissues of sheep may be capable of substantial choline production in order to maintain the balance of the endogenous body pool. This would be in contrast with rats, where choline synthesis by extrahepatic tissues is of minor importance (Bremer & Greenberg, 1961; Bjørnstad & Bremer, 1966).

Mammals secrete considerable amounts of choline in the form of phosphatidylcholine into the intestinal lumen through the bile (Van Golde & Van den Bergh, 1977). Balint *et al.* (1967) and Treble *et al.* (1970) have shown that bile phosphatidylcholine is preferentially synthesized from unesterified choline in rat liver. Bile phosphatidyl-

choline synthesis and secretion in rats is influenced by dietary choline (Robins, 1974; Robins & Armstrong, 1976). In rats (Robins, 1975) and humans (Saunders, 1970) low amounts of the choline derived from bile phosphatidylcholine are re-incorporated into bile. Sheep secrete about 13mmol of phosphatidylcholine in bile per day (Adams & Heath, 1963; Noble, 1978). Thus, since sheep receive a minimal dietary choline intake, there must be extensive enterohepatic recirculation and re-utilization of bile choline.

A conscious chronically catheterized sheep preparation has been developed whereby regional blood flow, O_2 utilization, and drug and metabolite disposition may be directly measured in conscious unrestrained animals (Mather *et al.*, 1982; Runciman, 1982). This preparation has been used to measure the net uptake and output of various forms of choline by organs of the sheep. This experimental approach has allowed the origin of the bulk of the endogenous choline body pool and the potential for retention and recycling of bile choline in sheep to be examined.

Experimental

Animals

Merino wethers, aged 1–2 years and weighing 30–45kg, were used for conscious chronically catheterized sheep preparations, and wethers, approx. 2 years old and weighing 35–45kg, were used for obtaining tissue samples. Sheep were provided with water *ad libitum* and a daily ration of 1kg of lucerne chaff. Before surgery the animals were starved for 24h.

Hooded Wistar male rats weighing 250–300g were used. They were given water and a pelleted rat diet (Charlicks, Adelaide, South Australia, Australia) *ad libitum.*

Chronically catheterized sheep

Five sheep were chronically catheterized with a combination of descending aorta, pulmonary artery, renal vein, hepatic vein, superior vena cava, inferior vena cava and portal vein catheters and maintained as described by Runciman (1982) and Mather *et al.* (1982). The superior vena cava catheter was placed caudal to the jugular, subclavian and brachiocephalic veins, and the inferior vena cava catheter caudal to the renal veins. The techniques for securing the catheters and maintaining their patency have allowed data collection for up to 18 weeks. Each sheep was allowed to stabilize on full food intake for 5–7 days post-operatively before blood samples were taken.

Measurement of blood flow rates in chronically catheterized sheep

Cardiac output was routinely determined by the thermodilution method by using 10ml of cold (0°C) 5% glucose and corrected for thermal losses as calibrated by dye dilution measurements (Runciman, 1982). Renal blood flow was measured by the Fick method, with sodium [^{125}I]iodohippurate as the indicator and assay by gamma scintillation counting (Runciman, 1982). Liver blood flow was measured by the Fick method with bromosulphophthalein as the indicator and assayed by a high-pressure liquid-chromatographic technique (Runciman, 1982). Portal blood flow was determined by an indicator dilution method with sodium [^{125}I]iodohippurate (Runciman, 1982).

Collection and treatment of blood samples for choline determination

Blood samples (10ml) were withdrawn simultaneously from respective catheters of conscious chronically prepared sheep at hourly intervals for at least 4h. Blood samples (5ml) were drawn from the descending aorta of rats (anaesthetized with diethyl ether) after abdominal incision. The blood samples were immediately transferred into ice-cold heparinized glass centrifuge tubes and centrifuged at 1200g for 10min at 4°C. The plasma was removed and, in some cases, the cells were resuspended to the original blood volume with ice-cold NaCl solution. It was necessary to maintain and centrifuge blood at 4°C after sampling, as there was a rapid rise in unesterified choline in plasma and cells if the blood remained at room temperature. In whole blood, plasma and cells maintained at 4°C, no increase in unesterified choline could be detected in a time interval of 3h.

Approx. 3ml of plasma was immediately transferred to a membrane cone (Centriflo CF 25; Amicon Corp., Lexington, MA, U.S.A.; mol.wt. >25000 excluded) and centrifuged at 1000g for 20min at 4°C (Eckernäs & Aquilonius, 1977). The clear ultrafiltrates of plasma (pH7.5, approx. 0.5ml) were stored at −80°C until assayed for unesterified choline. They were stable at −80°C for at least 3 months and stable at 22°C for at least 24h.

Plasma or resuspended cells (1ml in each case) were immediately extracted with 20vol. of ice-cold chloroform/methanol (20:1, v/v) containing the antioxidant 2,6-di-t-butyl-4-methylphenol (50mg/l), essentially by the method of Folch *et al.* (1957) but with modifications described by Henderson (1978). The lower chloroform layer containing lipid choline was evaporated to dryness *in vacuo* at 40°C and the residue redissolved in 10ml of chloroform. Of the chloroform extract 2ml was

evaporated to dryness under N_2 and the lipid choline hydrolysed to unesterified choline with 3ml of 6M-HCl at 110°C for 24h in a sealed test tube. The fraction was taken to dryness and stored in a vacuum desiccator containing KOH pellets to remove traces of acid. The residue was redissolved in a 5mM-sodium phosphate buffer (pH7.8) and assayed for free choline. The upper aqueous layer containing glycerophosphocholine, phosphocholine and unesterified choline was evaporated to dryness *in vacuo* at 40°C and the residue redissolved in 2ml of water and extracted three times with 1ml of diethyl ether. The pH of the sample was adjusted to 8–9 with NH_3 vapour and applied to a column (0.8cm × 10cm) of Dowex AG 50W (X8; 100–200 mesh; H^+ form) cation-exchange resin and successively washed with 25ml of water, 30ml of 0.4M-HCl and 25ml of 3M-HCl. These three fractions contained glycerophosphocholine, phosphocholine and unesterified choline respectively (Webster & Cooper, 1968; Illingworth & Portman, 1972). The glycerophosphocholine and phosphocholine fractions were taken to dryness *in vacuo* at 40°C. The glycerophosphocholine residue was hydrolysed to unesterified choline in 5ml of 1M-HCl at 100°C for 30min. The phosphocholine residue was hydrolysed to unesterified choline in 5ml of 3M-HCl at 123°C for 24h in a sealed test tube. All fractions were taken to dryness *in vacuo* and stored in a desiccator containing KOH pellets. The residues were redissolved in 5mM-sodium phosphate buffer (pH7.8) and assayed for unesterified choline. The specificity of this technique was checked by applying a concentrated portion of each column fraction with corresponding standards on 0.2mm pre-coated silica gel 60 plates (E. Merck, Darmstadt, Germany) and developing in the solvent systems methanol/0.6% NaCl/NH_3 (sp.gr. 0.88) (10:10:1, by vol.), methanol/acetone/11M-HCl (45:5:2, by vol.) and chloroform/methanol/NH_3 (sp.gr. 0.88) (6:3:1, by vol.). The compounds were detected with I_2 vapour, removed from the plates and quantified by determination of choline.

Collection and treatment of sheep tissues for choline determination

Sheep were killed by severing the necks, and samples of liver, kidney cortex, heart, skeletal muscle (musculus biceps femoris) and small intestine (jejunum) were rapidly excised and freeze-clamped with aluminium-faced tongs previously cooled in liquid N_2. The frozen tissue samples were powdered in a stainless-steel mortar with a heavy stainless-steel pestle. Powdered tissue (1g) was extracted and the various forms of choline were separated and hydrolysed to unesterified choline as described for blood samples.

Determination of choline

Unesterified choline was assayed by a modification of the radioenzymic method with choline acetyltransferase and labelled acetyl-CoA, described by Shea & Aprison (1973) and Hebb *et al.* (1975). Choline acetyltransferase was purified from sheep brain caudate nuclei by the method of Ryan & McClure (1979). [^{3}H]Acetyl-CoA was prepared from CoA and [^{3}H]acetic anhydride by the method of Stadtman (1957) and assayed as described by Decker (1974). The following components were incubated for 15min at 37°C in stoppered plastic centrifuge tubes (Eppendorf, Hamburg, Germany) in a final volume of 50µl: 50mM-sodium phosphate buffer (pH7.8); 0.2mM-eserine (physostigmine) salicylate salt; 1mM-EDTA (pH7.8); 0.5mg of bovine serum albumin/ml; 0.4mM-[^{3}H]acetyl-CoA (100000d.p.m./nmol); 0–0.04mM sample or standard choline chloride; enzyme solution (pH7.2, activity 0.1µmol/min per ml). The incubation mixture consisted of 20µl of reaction mixture, 20µl of sample or standard, 5µl of [^{3}H]acetyl-CoA and 5µl of enzyme solution. The reaction was started by the addition of enzyme solution and stopped with 550µl of 100mM-sodium phosphate buffer (pH7.8), followed immediately with 300µl of heptan-2-one containing sodium tetraphenylboron (25mg/ml). The tubes were shaken for 10min and centrifuged for 8min in a Zentrifuge 3200 (Eppendorf, Hamburg, Germany). Of the heptan-2-one layer 150µl was added to 3.5ml of scintillation fluor [7g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/litre of toluene and Triton X-100 (2:1, v/v)], and radioactivity determined in a liquid-scintillation counter (Packard Tri-Carb 460 CD; Packard Instrument Co. Inc., IL, U.S.A.). All samples were determined in duplicate.

Chemicals

These were obtained as follows: choline chloride, glycerophosphocholine ($CdCl_2$ complex), phosphocholine (calcium salt) and eserine salicylate salt from Sigma Chemical Co., St. Louis, MO, U.S.A.; 2,6-di-t-butyl-4-methylphenol from Calbiochem, Los Angeles, CA, U.S.A.; Dowex AG 50W (X8; 100–200 mesh; H^+ form) from Bio-Rad Laboratories, Richmond, CA, U.S.A.; sodium tetraphenylboron from BDH Chemicals Australia Pty. Ltd., Pt. Fairy, Vic., Australia, and [^{3}H]acetic anhydride (sp. radioactivity 500Ci/mol) from Amersham Australia Pty. Ltd., Sydney, N.S.W., Australia.

Results

Unesterified choline concentrations in blood plasma from various vessels of chronically catheterized sheep

The results in Table 1 show the plasma unesterified choline concentrations in seven different vessels of five chronically catheterized sheep. Sheep with blood haemoglobin type A showed lower plasma unesterified choline concentrations than sheep with blood haemoglobin type B in corresponding vessels. The values reported in the present paper are lower than those reported for non-ruminant species (Wang & Haubrich, 1975; Zeisel & Wurtman, 1981; Zeisel, 1981).

Differences in plasma unesterified choline concentrations between vessels can give a measure of the net uptake and output of choline by various organs. The values shown in Table 2 indicate a significant production of unesterified choline by the alimentary tract and the upper- and lower-body regions and uptake by the liver, lungs and kidneys of sheep. The production of unesterified choline by the alimentary tract was substantial and was particularly pronounced in sheep 3. Unesterified choline production by the alimentary tract was approximately balanced by hepatic uptake if a correction is made for the input into the liver via the hepatic artery (approx. 20% of the total liver blood flow) in addition to the main input via the portal vein. There was almost equal production of unesterified choline by the venae cavae-drained upper- and lower-body regions. The uptake of unesterified choline by the lungs and kidneys was relatively small.

Choline ester concentrations in blood plasma from various vessels of chronically catheterized sheep

The plasma concentrations of glycerophosphocholine, phosphocholine and lipid choline in different vessels of three chronically catheterized sheep are shown in Table 3. Lipid choline constitutes the major form of choline in plasma and the values shown are in the range reported for sheep by Lindsay & Leat (1975). There was significant production of lipid choline by the venae cavae-drained upper- and lower-body regions of sheep 5. No uptake or output of lipid choline was detected across any other organs of the three animals.

The data in Table 3 appear to be the first accurate values published for glycerophosphocholine and phosphocholine in plasma from the use of a highly sensitive radioenzymic choline assay. Hinton & Setchell (1980) measured glycerophosphocholine and phosphocholine in rat plasma using a relatively insensitive chemical phosphorus assay, and could merely suggest that the levels were less than 100 nmol/ml. Table 3 indicates significant production of glycerophosphocholine by the liver of sheep 2 and uptake by the lungs and kidneys of sheep 2 and 3 respectively. There was significant production of phosphocholine by the alimentary

Table 1. *Unesterified choline concentrations in blood plasma from various vessels of chronically catheterized sheep*

Blood samples were collected simultaneously from different vessels of conscious chronically catheterized Merino wethers. Unesterified choline was determined in plasma ultrafiltrates in duplicate as described in the text. The values shown are means ± S.E.M. with the numbers of blood samples given in parentheses.

Sheep	Blood haemoglobin type	Unesterified choline concentration in blood plasma (nmol/ml)						
		Portal vein	Descending aorta	Hepatic vein	Pulmonary artery	Renal vein	Superior vena cava	Inferior vena cava
1	B	10.4±1.9 (7)	6.3±1.3 (7)					
2	B	8.9±1.3 (4)	4.6±0.2 (4)	3.7±0.2(4)	6.0±0.3 (4)			
3	B	12.8±0.9 (5)	3.7±0.2 (5)	2.8±0.2 (5)		2.7±0.2 (5)		
Mean		10.7	4.9	3.3	6.0	2.7		
4	A		3.0±0.4 (4)	1.6±0.3 (4)	3.4±0.4 (4)	2.9±0.8 (4)	4.2±0.6 (4)	4.2±0.7 (4)
5	A		2.4±0.2 (4)	1.2±0.4 (4)	2.7±0.3 (4)	2.1±0.3 (4)	3.4±0.3 (4)	3.7±0.3 (4)
Mean			2.7	1.4	3.1	2.5	3.8	4.0

Table 2. *Differences in plasma unesterified choline concentration across various organs of chronically catheterized sheep*

The values shown are means ± S.E.M. for the numbers of blood samples given in parentheses. + indicates net output and − net uptake by an organ. The significance of the differences across organs, as determined by paired *t* test, is indicated. Abbreviations used: N.S., not significant; P, portal vein; A, descending aorta; HV, hepatic vein; PA, pulmonary artery; RV, renal vein; SVC, superior vena cava; IVC, inferior vena cava. See Table 1 for absolute plasma unesterified choline concentrations in various vessels.

		Differences in plasma unesterified choline concentration (nmol/ml)						
			Liver					
Sheep	Blood haemoglobin type	Alimentary tract (P−A)	(HV−P)	(HV−A)	Lung (A−PA)	Kidney (RV−A)	Upper body (SVC−A)	Lower body (IVC−A)
1	B	$+4.0 \pm 1.4$ (7) $P<0.05$						
2	B	$+4.3 \pm 1.2$ (4) $P<0.05$	-5.2 ± 1.4 (4) $P<0.05$	-0.9 ± 0.3 (4) N.S.	-1.4 ± 0.4 (4) $P<0.05$			
3	B	$+9.1 \pm 0.9$ (5) $P<0.001$	-10.0 ± 0.8 (5) $P<0.001$	-0.9 ± 0.3 (5) $P<0.05$		-1.0 ± 0.3 (5) $P<0.05$		
Mean		5.8	−7.6	−0.9	−1.4	−1.0		
4	A			-1.4 ± 0.4 (4) $P<0.05$	-0.5 ± 0.2 (4) N.S.	-0.1 ± 0.4 (4) N.S.	$+1.3 \pm 0.3$ (4) $P<0.02$	$+1.2 \pm 0.4$ (4) $P<0.05$
5	A			-1.1 ± 0.3 (4) $P<0.05$	-0.4 ± 0.1 (4) $P<0.05$	-0.3 ± 0.1 (4) $P<0.05$	$+1.1 \pm 0.1$ (4) $P<0.01$	$+1.4 \pm 0.1$ (4) $P<0.01$
Mean				−1.3	−0.5	−0.2	+1.2	+1.3

Table 3. *Choline ester concentrations in blood plasma from various vessels of chronically catheterized sheep*

Blood samples were collected as described in Table 1. Plasma samples were extracted with chloroform/methanol and glycerophosphocholine (Gro*P*Cho), phosphocholine (*P*Cho) and lipid choline (LC) were fractionated and determined in duplicate as described in the text. The values shown are means ± S.E.M. for the numbers of blood samples given in parentheses. Significant plasma concentration differences across organs, as determined by paired *t* test, are also indicated. Sheep 2 and 3 were of blood haemoglobin type B and sheep 5 was of blood haemoglobin type A. Other abbreviations are defined in the legend to Table 2.

		Choline ester concentration in blood plasma (nmol/ml)						
Sheep	Choline ester	Portal vein (P)	Descending aorta (A)	Hepatic vein (HV)	Pulmonary artery (PA)	Renal vein (RV)	Superior vena cava (SVC)	Inferior vena cava (IVC)
	Gro*P*Cho	2.4±0.3 (4)	2.1±0.3 (4)	4.3±0.7 (4)*	2.8±0.6 (4)†			
2	*P*Cho	1.7±0.1 (4)	1.5±0.2 (4)	2.4±0.4 (4)	1.2±0.1 (4)			
	LC	409.8±47.7 (4)	390.9±44.4 (4)	375.8±20.0 (4)	389.3±17.7 (4)			
	Gro*P*Cho	3.3±0.5 (5)	3.0±0.2 (5)	3.5±0.3 (5)		2.4±0.1 (5)‡		
3	*P*Cho	0.3±0.0 (5)§	0.1±0.0 (5)	0.3±0.1 (5)		0.4±0.1 (5)‖		
	LC	452.3±8.3 (5)	450.6±7.3 (5)	455.2±11.6 (5)		451.1±17.1 (5)		
	Gro*P*Cho		2.5±0.2 (4)				2.3±0.3 (4)	3.0±0.3 (4)
5	*P*Cho		0.4±0.1 (4)				0.3±0.1 (4)	0.2±0.1 (4)
	LC		387.1±16.7 (4)				435.1±15.2 (4)¶	414.8±20.2 (4)**

* Net output by liver: (HV−P); $P<0.05$; (HV−A), $P<0.02$.
† Net uptake by lung: (A−PA), $P<0.05$.
‡ Net uptake by kidney: (RV−A), $P<0.02$.
§ Net output by alimentary tract: (P−A), $P<0.001$.
‖ Net output by kidney: (RV−A), $P<0.01$.
¶ Net output by upper body: (SVC−A), $P<0.05$.
** Net output by lower body: (IVC−A), $P<0.05$.

tract and kidneys of sheep 3. No uptake or output of glycerophosphocholine and phosphocholine was observed across the upper- and lower-body regions drained by the venae cavae of sheep 5.

Blood flow rates measured in chronically catheterized sheep

The results of cardiac output, liver blood flow and kidney blood flow measurements in five chronically catheterized sheep are presented in Table 4 and are consistent with those obtained by Hales (1973) and Katz & Bergman (1969). Sheep with blood haemoglobin type A showed significantly higher cardiac output and regional blood flow rates than those with blood haemoglobin type B ($P<0.01$; Student's t test). This phenomenon is almost certainly related to the lower O_2-carrying capacity of haemoglobin type A compared with haemoglobin type B in sheep blood (Blunt & Huisman, 1975). The higher blood flow rates in the haemoglobin type A sheep may be responsible for the lower plasma unesterified choline concentrations in the various vessels of these animals, as shown in Table 1.

It was not possible to obtain reliable measurements of portal blood flow by using the sodium [^{125}I]iodohippurate indicator dilution method, as streaming occurred consistently in the portal vein. Examination of results obtained by others also provides evidence of streaming (Katz & Bergman, 1969) with the apparent relative contribution of the portal flow to hepatic flow being subject to wide variation. However, pooling weighted-mean sodium [^{125}I]iodohippurate values from 140 measurements in six sheep yielded an average portal fraction of 0.80 of total liver blood flow (Runciman, 1982). This value is similar to that reported for several species (Richardson & Withrington, 1981) and is used by others working with sheep (Katz & Bergman, 1969; Thompson *et al.*, 1978).

Concentrations of various forms of choline in sheep tissues

The concentrations of free choline and choline esters in adult sheep tissues are presented in Table 5. Lipid choline constitutes the major form of choline in all tissues examined, with the highest

Table 4. *Blood flow rates measured in chronically catheterized sheep*

Blood flow rates were determined as described in the text. The values are means ± S.D. for the numbers of measurements given in parentheses. Abbreviation used, n.d., not determined. Sheep 1, 2 and 3 were of blood haemoglobin type B and sheep 4 and 5 were of blood haemoglobin type A.

Sheep	Blood haemoglobin type	Blood flow rate (litre/min) Cardiac output	Liver blood flow	Kidney blood flow
1	B	n.d.	2.16 (1)	n.d.
2	B	3.91 ± 0.27 (6)	1.36 ± 0.08 (6)	0.74 ± 0.03 (6)
3	B	3.75 ± 0.36 (6)	1.50 ± 0.38 (6)	0.62 ± 0.06 (6)
Mean		3.83	1.67	0.68
4	A	6.26 ± 0.50 (6)	1.80 ± 0.19 (6)	n.d.
5	A	6.35 ± 0.42 (6)	2.14 ± 0.11 (6)	1.41 ± 0.18 (3)
Mean		6.31	1.97	1.41

Table 5. *Concentrations of various forms of choline in sheep tissues*

Merino wethers were killed and tissue samples immediately frozen with aluminium-faced tongs previously cooled in liquid N_2. The frozen tissue powders were extracted with chloroform/methanol and the various forms of choline fractionated and determined in duplicate as described in the text. The values shown are means ± S.E.M. for three adult sheep.

Tissue	Concentration (μmol/g wet wt.) Glycerophosphocholine	Phosphocholine	Unesterified choline	Lipid choline
Liver	2.65 ± 0.15	1.48 ± 0.02	0.48 ± 0.18	21.06 ± 0.96
Kidney cortex	0.59 ± 0.14	0.86 ± 0.09	0.15 ± 0.05	5.50 ± 0.10
Skeletal muscle (musculus biceps femoris)	0.16 ± 0.02	0.10 ± 0.01	0.56 ± 0.12	2.45 ± 0.15
Heart	0.28 ± 0.02	0.15 ± 0.01	0.49 ± 0.01	7.45 ± 0.15
Small intestine (jejunum)	0.61 ± 0.03	0.38 ± 0.04	1.25 ± 0.01	4.03 ± 0.63

Table 6. *Concentrations of various forms of choline in plasma and cells of sheep and rat blood*

Blood plasma and cells were extracted with chloroform/methanol and the various forms of choline fractionated and determined in duplicate as described in the text. Plasma unesterified choline was determined in ultrafiltrates. The values shown are means±S.E.M. for two blood samples taken from each species.

Species	Blood fraction	Concentration (nmol/ml)			
		Glycerophosphocholine	Phosphocholine	Unesterified choline	Lipid choline
Sheep	Plasma	4.3±0.1	0.4±0.1	8.4±0.1	452.7±45.2
	Cells	12.0±0.7	6.5±0.7	8.8±0.2	2751.8±95.0
Rat	Plasma	1.4±0.1	0.5±0.1	9.6±0.7	1045.1±73.9
	Cells	17.6±1.8	11.2±1.8	12.2±0.5	3516.8±54.9

concentration in the liver. The lipid choline values are comparable with those of other species reported by White (1973). The concentration of glycerophosphocholine is high in sheep liver (Table 5) and is about 11 times greater than the level reported in rat liver (Dawson, 1955*a*). Other workers have also observed a high concentration of glycerophosphocholine in sheep and bovine liver (Schmidt *et al.*, 1952, 1955; R. M. C. Dawson, personal communication). The other sheep tissues examined did not show the same high level of this derivative as the liver. The results in Table 5 indicate that the concentration of phosphocholine is relatively low in most tissues except the liver and kidney cortex. The phosphocholine values for sheep liver and small intestine in Table 5 are lower than those determined for the rat by Dawson (1955*b*). Conversely, the level of phosphocholine observed in sheep kidney appears to be higher than that of rat kidney (Dawson, 1955*b*). The results obtained for unesterified choline in sheep tissues are higher than those reported in corresponding guinea-pig and rat tissues (Haubrich *et al.*, 1975, 1976). The highest levels of unesterified choline were found in the small intestine and skeletal muscle of sheep (Table 5).

Concentrations of various forms of choline in plasma and cells of sheep and rat blood

Table 6 shows that the concentrations of the various forms of choline are higher in the cells than in the plasma of sheep and rats. The ratio of unesterified choline concentration in the cells compared with that in plasma is slightly greater than 1 for both species examined, as previously found for humans (Hanin *et al.*, 1979; Barclay *et al.*, 1982). The plasma concentration of glycerophosphocholine was approx. 3 times greater in sheep than in rats (Table 6). In all other cases the rat showed higher levels of the various choline derivatives than did the sheep in both cells and plasma.

Discussion

Glycerophosphocholine is an intermediate in the catabolism of phosphatidylcholine to unesterified choline (Dawson, 1955*a*), and the concentration in sheep liver reported here is several times higher than that in rat liver (Dawson, 1955*a*; R. M. C. Dawson, personal communication). The activity of glycerophosphocholine phosphodiesterase (EC 3.1.4.2), which liberates unesterified choline from glycerophosphocholine, is negligible in sheep liver and high in rat liver (Dawson, 1956; R. M. C. Dawson, personal communication). These observations in sheep liver suggest a mechanism for conserving the choline moiety of phosphatidylcholine by preventing the ready release of unesterified choline as a substrate for oxidation to betaine and CO_2. The present work also shows that in sheep a portion of the liver glycerophosphocholine is transported to the lungs and kidneys via the plasma, which would help to supply the choline pool of these tissues. There appears to be production of phosphocholine by the alimentary tract and kidneys of the sheep. The latter observation fits in with the relatively high concentration of phosphocholine found in sheep kidney, and may be related to the low amounts of choline secreted in the urine (Luecke & Pearson, 1945). Plasma unesterified choline is taken up by the liver, lungs and kidneys of sheep, as shown in Table 2, and is likely to be used efficiently for lipid choline synthesis via the CDP-choline pathway. Uptake of unesterified choline by the lungs may be used in the synthesis of (dipalmitoyl) phosphatidylcholine, a principal pulmonary surfactant (Frosolono, 1977; Zeisel, 1981).

Dawson *et al.* (1981) calculated that sheep require approx. 17.3 mmol of choline/day in order to maintain the choline body pool, which is almost entirely of endogenous origin. Sheep derive less than 0.1 mmol of choline from the diet per day, as there is almost complete microbial destruction of dietary choline in the alimentary tract (Neill *et al.*, 1979; Dawson *et al.*, 1981). Neill *et al.* (1979)

showed that the sheep liver can synthesize a maximum of 0.1 mmol of choline/day per kg body wt. by the methylation of phosphatidylethanolamine to phosphatidylcholine (i.e. 5.0 mmol of choline/day for a 50 kg sheep). Thus these two sources of choline provide only 29% of the daily choline requirement, which implies that the bulk of the endogenous choline body pool is maintained by substantial choline synthesis in extrahepatic tissues of sheep. The data for chronically catheterized sheep 4 and 5, reported here, support this conclusion. The mean total body venous return of plasma unesterified choline for the two sheep is 15.1 μmol/min, on the basis of the mean hepatic venous plasma unesterified choline return of 2.8 μmol/min, and the mean superior and inferior venae cavae plasma unesterified choline return of 12.3 μmol/min, representing the rest of the body (taking the systemic venous blood flow as approximately the mean cardiac output minus mean hepatic and renal vein flows). The mean total body venous return of plasma unesterified choline is almost equal to the mean total body arterial output of 15.6 μmol/min, if corrections are made for uptake of unesterified choline by the liver via the hepatic artery (approx. 20% of the total mean liver blood flow) and uptake by the lungs and kidneys. Thus this whole-body calculation indicates that only 18% of the total body unesterified choline return is provided by the liver, and the major contribution (82%) is by the vanae cavae draining the upper- and lower-body regions of the sheep.

The upper- and lower-body regions drained by the venae cavae of sheep 5 produced substantial amounts of lipid choline in addition to unesterified choline. Since there was significant production of lipid choline and no uptake or output of glycerophosphocholine and phosphocholine in the plasma across the upper and lower body, it eliminates the possibility that unesterified choline production is derived from choline ester breakdown as the blood flows through these regions. Presumably the source of the lipid choline and unesterified choline is from net synthesis of phosphatidylcholine by the methylation of phosphatidylethanolamine in upper- and lower-body tissues of sheep, e.g. brain, endocrine glands, lymph nodes, endothelium of veins, skeletal muscle, bone marrow. Choline may also originate from bile and net synthesis in the mucosa of the intestine, reaching the venae cavae in lymph via the thoracic duct. In rats choline synthesis is of quantitative significance in the liver but not in extrahepatic tissues (Bremer & Greenberg, 1961; Bjørnstad & Bremer, 1966), and a substantial part of the choline body pool is of dietary origin (Dawson *et al.*, 1981).

Sheep secrete about 13 mmol of phosphatidylcholine into the intestinal lumen through bile per day (Adams & Heath, 1963; Noble, 1978). Balint *et al.* (1967) and Treble *et al.* (1970) have demonstrated that bile phosphatidylcholine is synthesized in liver from unesterified choline via the CDP-choline and base-exchange pathways, rather than by the methylation pathway. Since sheep receive only limited amounts of dietary choline, there must be efficient reabsorption and re-utilization of the bile choline from the intestine in order to maintain the balance of the endogenous choline pool and the daily rate of secretion in bile. The mean net plasma unesterified choline production by the alimentary tract of chronically catheterized sheep 1, 2 and 3 reported here is 11.2 mmol/day, on the basis of the mean portal minus arterial plasma unesterified choline difference and an average calculated portal blood flow (approx. 80% of the mean total liver blood flow). It is improbable that this unesterified choline arises from the hydrolysis of choline esters as the blood flows through the alimentary tract, since there was production of glycerophosphocholine and phosphocholine and no uptake or output of lipid choline in the plasma across this region. The amount of choline produced is much higher than that received from the diet (Dawson *et al.*, 1981) and is probably mainly derived from the phosphatidylcholine delivered in bile. The mean uptake of unesterified choline by the liver in the three sheep is 15.1 mmol/day [calculated from the equation of Bergman & Wolff (1971)] and is almost equal to the mean production of unesterified choline by the alimentary tract. Thus there appears to be considerable reabsorption and enterohepatic recirculation of the unesterified choline moiety of bile phosphatidylcholine from the intestine of the sheep. Presumably the unesterified choline taken up by the sheep liver is mainly re-utilized for bile phosphatidylcholine synthesis by the CDP-choline and base-exchange pathways. In non-ruminant species less than 10% of the choline moiety of bile lipid choline that enters the enterohepatic circulation is re-utilized for bile phosphatidylcholine synthesis in the liver (Saunders, 1970; Robins, 1975). This explains the dependence of bile phosphatidylcholine synthesis and secretion in rat liver on dietary choline (Robins, 1974; Robins & Armstrong, 1976).

It may be calculated from the data of Harrison & Leat (1972) and Christie (1978) that in sheep about 5 mmol of phosphatidylcholine is transported in thoracic duct lymph per day, most of which is supplied by the intestine. The total choline output by the intestine into the lymph and enterohepatic circulation is more than can be accounted for by bile and dietary sources. This suggests net synthesis of choline by the methylation pathway in the sheep intestine. Indeed, administration of

[^{14}C]ethanolamine to sheep clearly indicated that the small intestine is an active site for the synthesis of phosphatidylcholine (R. M. C. Dawson, personal communication). In contrast, the synthesis of choline by the methylation pathway is insignificant in the rat small intestine (Bremer & Greenberg, 1961; Wise & Elwyn, 1965; Bjørnstad & Bremer, 1966).

The use of chronically catheterized sheep has provided good evidence that the sheep synthesizes substantial amounts of choline in other tissues besides the liver and has the potential for the efficient retention and recycling of bile choline. These factors, coupled with a slow turnover of the endogenous choline body pool, explain the insensitivity of the sheep to a low dietary choline supply compared with many non-ruminant species.

We are indebted to Mr. M. J. Snoswell, Mr. R. C. Fishlock, Ms. R. Carapetis and Ms. T. U. Shepherd for their skilled technical assistance. Grants from the Davies Bequest Fund, the National Health and Medical Research Council of Australia and Astra Pharmaceuticals Pty. Ltd. are gratefully acknowledged. B. S. R. holds an Australian Wool Corporation Postgraduate Scholarship.

References

Adams, E. P. & Heath, T. J. (1963) *Biochim. Biophys. Acta* **70**, 688–690
Balint, J. A., Beeler, D. A., Treble, D. H. & Spitzer, H. L. (1967) *J. Lipid Res.* **8**, 486–493
Barclay, L. L., Blass, J. P., Kopp, U. & Hanin, I. (1982) *N. Engl. J. Med.* **307**, 501
Bergman, E. N. & Wolff, J. E. (1971) *Am. J. Physiol.* **221**, 586–592
Bjørnstad, P. & Bremer, J. (1966) *J. Lipid Res.* **7**, 38–45
Blunt, M. H. & Huisman, T. H. J. (1975) in *The Blood of Sheep: Composition and Function* (Blunt, M. H., ed.), pp. 155–183, Springer-Verlag, Berlin, Heidelberg and New York
Bremer, J. & Greenberg, D. M. (1961) *Biochim. Biophys. Acta* **46**, 205–216
Christie, W. W. (1978) *Prog. Lipid Res.* **17**, 111–205
Dawson, R. M. C. (1955*a*) *Biochem. J.* **59**, 5–8
Dawson, R. M. C. (1955*b*) *Biochem. J.* **60**, 325–328
Dawson, R. M. C. (1956) *Biochem. J.* **62**, 689–693
Dawson, R. M. C., Grime, D. W. & Lindsay, D. B. (1981) *Biochem. J.* **196**, 499–504
Decker, K. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., vol. 4, pp. 1988–1993, Academic Press, New York and London.
Eckernäs, S.-Å. & Aquilonius, S.-M. (1977) *Scand. J. Clin. Lab. Invest.* **37**, 183–187
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509.
Frosolono, M. F. (1977) in *Lipid Metabolism in Mammals* (Snyder, F., ed.), vol. 2, pp. 1–38, Plenum Press, New York
Hales, J. R. S. (1973) *Pflügers Arch.* **344**, 119–132
Hanin, I., Merikangas, J. R., Merikangas, K. R. & Kopp, U. (1979) *N. Engl. J. Med.* **301**, 661–662
Harrison, F. A. & Leat, W. M. F. (1972) *J. Physiol. (London)* **225**, 565–576
Haubrich, D. R., Wang, P. F. L. & Wedeking, P. W. (1975) *J. Pharmacol. Exp. Ther.* **193**, 246–255
Haubrich, D. R., Wang, P. F. L., Chippendale, T. & Proctor, E. (1976) *J. Neurochem.* **27**, 1305–1313
Hebb, C., Mann, S. P. & Mead, J. (1975) *Biochem. Pharmacol.* **24**, 1007–1011
Henderson, G. D. (1978) Ph.D. Thesis, University of Adelaide
Hinton, B. T. & Setchell, B. P. (1980) *J. Reprod. Fertil.* **58**, 401–406
Illingworth, D. R. & Portman, O. W. (1972) *J. Chromatogr.* **73**, 262–264
Katz, M. L. & Bergman, E. N. (1969) *Am. J. Physiol.* **216**, 946–952
Lindsay, D. B. & Leat, W. M. F. (1975) in *The Blood of Sheep: Composition and Function* (Blunt, M. H., ed. pp. 45–62, Springer-Verlag, Berlin, Heidelberg and New York
Luecke, R. W. & Pearson, P. B. (1945) *J. Biol. Chem.* **158**, 561–566
Mather, L. E., Runciman, W. B. & Ilsley, A. H. (1982) *Reg.-Anaesth. Suppl.* **7**, S24–S33
Neill, A. R., Grime, D. W., Snoswell, A. M., Northrop, A. J., Lindsay, D. B. & Dawson, R. M. C. (1979) *Biochem. J.* **180**, 559–565
Noble, R. C. (1978) *Prog. Lipid Res.* **17**, 55–91
Richardson, P. D. I. & Withrington, P. G. (1981) *Gastroenterology* **81**, 159–173
Robins, S. J. (1974) *Gastroenterology* **67**, 823
Robins, S. J. (1975) *Am. J. Physiol.* **229**, 598–602
Robins, S. J. & Armstrong, M. J. (1976) *Gastroenterology* **70**, 397–402
Runciman, W. B. (1982) Ph.D. Thesis, Flinders University of South Australia
Ryan, R. L. & McClure, W. O. (1979) *Biochemistry* **18**, 5357–5365
Saunders, D. R. (1970) *Gastroenterology* **59**, 848–852
Schmidt, G., Hecht, L., Fallot, P., Greenbaum, L. & Thannhauser, S. J. (1952) *J. Biol. Chem.* **197**, 601–609
Schmidt, G., Greenbaum, L. M., Fallot, P., Walker A. C. & Thannhauser, S. J. (1955) *J. Biol. Chem.* **212**, 887–895
Shea, P. A. & Aprison, M. H. (1973) *Anal. Biochem.* **56**, 165–177
Stadtman, E. R. (1957) *Methods Enzymol.* **3**, 931–941
Thompson, G. E., Bassett, J. M. & Bell, A. W. (1978) *Br. J. Nutr.* **39**, 219–225
Treble, D. H., Frumkin, S., Balint, J. A. & Beeler, D. A. (1970) *Biochim. Biophys. Acta* **202**, 163–171
Van Golde, L. M. G. & Van den Bergh, S. G. (1977) in *Lipid Metabolism in Mammals* (Snyder, F., ed.), vol. 1, pp. 35–149, Plenum Press, New York
Wang, F. L. & Haubrich, D. R. (1975) *Anal. Biochem.* **63**, 195–201
Webster, G. R. & Cooper, M. (1968) *J. Neurochem.* **15**, 795–802
White, D. A. (1973) *BBA Libr.* **3**, 441–482
Wise, E. M., Jr. & Elwyn, D. (1965) *J. Biol. Chem.* **240**, 1537–1548
Zeisel, S. H. (1981) *Annu. Rev. Nutr.* **1**, 95–121
Zeisel, S. H. & Wurtman, R. J. (1981) *Biochem. J.* **198**, 565–570