LIPID METABOLISM IN SHEEP:

A STUDY OF THE METABOLISM OF KETONE BODIES AND CARNITINE IN VARIOUS TISSUES OF THE SHEEP

A Thesis

presented in fulfilment of the requirements
for admission to the degree of

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by

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CONTENTS

DECLARATION

I hereby declare that the work presented in this thesis has been performed by myself, except where otherwise stated in the text, and that it has not been submitted in any previous application for a degree.

Patricia Koundakjian

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PUBLICATIONS

Part of the work described in this thesis has been published:

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Ketone body and fatty acid metabolism in sheep tissues. 3-Hydroxybutyrate dehydrogenase, a cytoplasmic enzyme in sheep liver and kidney. Biochem. J. (1970) 119, 49.

A.M. SNOSWELL and PATRICIA P. KOUNDAKJIAN

Relationships between carnitine and coenzyme A esters in tissues of normal and alloxan-diabetic sheep. Biochem. J. (1972) 127, 133.

PATRICIA P. KOUNDAKJIAN and A.M. SNOSWELL

3-Hydroxy acid dehydrogenases in sheep tissues. Biochem. J. (1972) 127, 449.

PREFACE

Abbreviations approved by the Biochemical Journal (1972) for use without explanation are used as such throughout this thesis.

Some chemical compounds, their source and degree of purity are described in the text. 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. Alloxan was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., enzymes from C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany and CoA from Calbiochem, Los Angeles, Calif., U.S.A. Acetyl-CoA was prepared from free CoA and redistilled acetic anhydride by the method of Stadtman (1957). Other compounds used were obtained from the usual commercial sources and were of the highest purity available. Solvents were redistilled prior to use.

The following enzymes are referred to by name only:

Acetate thiokinase, EC 6.2.1.1

Acyl-CoA synthetase, EC 6.2.1.2

Arylamine acetyltransferase, EC 2.3.1.5

ATPase, EC 3.6.1.3

Carnitine acetyltransferase, EC 2.3.1.7

Carnitine palmitoyltransferase, EC 2.3.1.a

Catalase, EC 1.11.1.6

Choline acetyltransferase, EC 2.3.1.6

Choline kinase, EC 2.7.1.32

Citrate synthase, EC 4.1.3.7

Fructose-1-6-diphosphatase, EC 3.1.3.11

Glucose-6-phosphatase, EC 3.1.3.9

Glutamate decarboxylase, EC 4.1.1.15

Glutamate dehydrogenase, EC 1.4.1.3

L-gulonate-NAD^+ oxidoreductase, EC 1.1.1.45

D(-)-3-hydroxybutyrate-NAD^+ oxidoreductase (3-hydroxybutyrate dehydrogenase), EC 1.1.1.30

HMG-CoA lyase, EC 4.1.3.4

HMG-CoA synthase, EC 4.1.3.5

Isocitrate dehydrogenase, EC 1.1.1.41

Lactate dehydrogenase, EC 1.1.1.27

Malate dehydrogenase, EC 1.1.1.37

Monoamine oxidase, EC 1.4.3.4

NADH oxidase, EC 1.6.99.3

3-oxo acid CoA transferase (succinyl-CoA:3-oxo acid transferase), EC 2.8.3.5

Phosphoenol-pyruvate carboxykinase, EC 4.1.1.32

Phosphotransacetylase, EC 2.3.1.8

Pyruvate carboxylase, EC 6.4.1.1

Pyruvate dehydrogenase, EC 1.2.4.1

SYNOPSIS

1. Both ketone bodies and carnitine are involved in lipid metabolism, upon which ruminants are almost exclusively dependent for their energy requirements.

2. Ketone body concentrations in the blood were measured enzymically, and it was found that the blood of normal sheep has a high [3-hydroxybutyrate]/[acetoacetate] ratio compared with that of non-ruminants. 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 refeeding.

3. Ketone body concentrations were measured in blood taken from indwelling hepatic and portal vein cannulae in normal and alloxan-diabetic sheep. With normal sheep fed on lucerne the net production of ketone bodies by the liver consisted only of 3-hydroxybutyrate. With the alloxan-treated animals, when ketone body production was high, 20 to 30% of the net hepatic ketone body output was acetoacetate.

4. During the oxidation of palmitoyl-L-carnitine by sheep liver mitochondria, acetoacetate production was found to account for 63% of the observed oxygen uptake (cf. 60% for rat liver mitochondria). When the incubation mixtures were allowed to remain under anaerobic conditions for 10 minutes, about half of the acetoacetate was reduced to 3-hydroxybutyrate by rat liver mitochondria, but no 3-hydroxybutyrate was produced by sheep liver mitochondria.

5. The distribution and activity of 3-hydroxybutyrate dehydrogenase were studied in various tissues of the sheep using DL-3-hydroxybutyrate as substrate. Kidney cortex was found to have the highest activity (twice that of rat kidney cortex), but the activity of sheep liver was found to be less than 2% of that in rat liver. The enzyme activity of sheep kidney cortex and liver was found to be predominantly in the cytosol, in contrast to the rat where the activity is mitochondrial.

6. The soluble cytoplasmic 3-hydroxybutyrate dehydrogenase activity of sheep kidney cortex was shown by other workers to be that of a non-specific L-3-hydroxy acid dehydrogenase. The activities of L- and D-3-hydroxybutyrate dehydrogenases and their intracellular distribution were examined in various sheep tissues. The soluble enzyme activity of the cytosol was found in all cases to be that of L-3-hydroxy acid dehydrogenase. D-3-hydroxybutyrate dehydrogenase activity of all the sheep tissues examined was associated with the mitochondria, and was very low in liver and kidney cortex.

7. Oxidation of laurate, myristate, palmitate and stearate by sheep liver mitochondria was found to be completely dependent on the presence of L-carnitine under conditions where the free fatty acids are readily oxidised by rat liver mitochondria. Free butyrate and crotonate were slowly oxidised by sheep liver mitochondria, but acetate oxidation appeared also to depend on the presence of L-carnitine.

8. The concentrations of free carnitine, carnitine esters, free Co-enzyme A (CoA) and acetyl-CoA were measured in several tissues of normal and alloxan-diabetic sheep. The total acid-soluble carnitine concen-

trations of four tissues from normal sheep showed a wide variation not reported for other species. In the sheep tissues examined there was found to be a reciprocal relationship between the concentrations of total acid-soluble carnitine and of free CoA plus acetyl-CoA. No such relationship between these two classes of compounds has been shown or can be inferred for any other species.

In alloxan-diabetes, the concentration of acetylcarnitine was increased in all tissues examined, and the total acid-soluble carnitine concentration was increased sevenfold in liver and twofold in kidney cortex.

The concentration of acetyl-CoA was equal to that of free CoA in the tissues of both normal and alloxan-diabetic sheep, but the concentration of free CoA plus acetyl-CoA in liver increased approximately twofold in alloxan-diabetes.

9. The subcellular distributions of carnitine, CoA, carnitine acetyltransferase and acetate thiokinase were studied in tissues taken from normal sheep. Carnitine was confined to the cytosol in all tissues examined. CoA was equally distributed between the mitochondria and the cytosol of liver, approximately 25% was present in the cytosol of kidney cortex and there was virtually none in this fraction of heart and skeletal muscle. Carnitine acetyltransferase was solely mitochondrial in all tissues, and 90% of the activity was latent. Acetate thiokinase was predominantly (90%) present in the cytosol of liver, but less than 10% was found in the cytosol of heart and skeletal muscle.

10. The biosynthesis and degradation of carnitine were studied in liver and skeletal muscle of sheep. With neither of these tissues were experiments using homogenates successful. However, using liver slices taken from starved sheep it was shown that carnitine accumulated when the slices were incubated with 4-amino-butyric acid. It has so far proved impossible to present unequivocal qualitative evidence for the biosynthesis of carnitine from labelled precursors by sheep liver slices.

The degradation of labelled carnitine by sheep liver slices and homogenates has been briefly examined, and it appears to involve a complete breakdown of the molecule.

11. Each section of the work is introduced and discussed separately. A general discussion correlates the different facets of the study and outlines the interrelationships thought to exist between carnitine, CoA and the ketone bodies in sheep. The importance of these compounds, and the enzymes involved in the metabolism of them, in the overall metabolism of the sheep is emphasized.

GENERAL INTRODUCTION

The major part of the energy requirement of sheep, and other ruminants, is met by the metabolism of the volatile fatty acids which result from microbial breakdown of ingested food within the rumen. Reid (1968) estimates that at least 60% of the digestible energy of a maintenance roughage ration fed to adult sheep will be absorbed as volatile fatty acids. Leat and Ford (1966) indicate that in sheep with plasma free fatty acid concentrations above 100 μ-equiv./l (which is usually the case in normal, fed animals) the contribution of long-chain fatty acids to the overall CO_2 output is about 12%.

Sheep absorb only minute quantities of carbohydrate from the gut as practically all of the ingested carbohydrate is broken down by the rumen microflora. But, of course, glucose is still required as it is the major energy source for a number of tissues, brain and red blood cells in particular. Sheep are therefore almost entirely dependent upon gluconeogenesis to supply those tissues needing glucose as a primary source of energy. The major precursor of glucose in ruminants is propionic acid - one of the three major volatile fatty acids absorbed from the rumen. As well as being adapted to high rates of gluconeogenesis, principally by regulation of enzyme activity, sheep are also able to "spare" glucose by utilizing the volatile fatty acids for such things as lipogenesis (from acetate). Ballard *et al.* (1969) have reviewed gluconeogenesis and lipogenesis in ruminants and make comparisons with the situations pertaining in non-ruminants. It is obvious from the above summary that high rates of fatty acid metabolism and

gluconeogenesis are of extreme importance to the well-being of sheep even under normal conditions. Conditions of stress such as exposure to cold, starvation, pregnancy, parturition and lactation (and alloxan-diabetes in the laboratory) can reasonably be expected to impose some strain on a system already highly adapted to extensive fatty acid metabolism and gluconeogenesis. Indeed, two metabolic disorders of domestic ruminants which are of significant economic importance are pregnancy toxaemia of sheep and acetonaemia (bovine ketosis) of cattle. Both conditions involve apparent perturbations in fatty acid metabolism, ketone body production and/or utilization, gluconeogenesis and glucose utilization. Kronfeld (1970) has compared these two conditions and Reid (1968) discussed in detail the toxaemia of under-nourished pregnant sheep.

Although a great deal of work has been done on ruminant ketosis most of the early investigations have been made in vivo using whole animals. More recently the activities and distribution in ruminant tissue of some of the enzymes involved in ketogenesis (see Part I, Introduction) have been studied in detail. One of the purposes of the present study was to examine certain aspects of ketone body metabolism by sheep under normal and stressed conditions at three levels: the whole animal, the tissues and at the subcellular level, in order to try and gain a better understanding of the ready susceptibility of ruminants to ketosis.

As with earlier work on ruminant ketosis, most of the experiments on fatty acid oxidation in ruminants have been done on whole animals. Thus, it is perhaps not surprising that the involvement of carnitine in

ruminant metabolism was not investigated at all until very recently (Snoswell and Henderson, 1970). Carnitine is a quaternary ammonium compound and the only function unequivocally established for it is the transport of long-chain fatty acyl groups across mitochondrial membranes (via the carnitine acyltransferase systems) for subsequent β-oxidation within the mitochondria. The role of carnitine in fatty acid metabolism has been reviewed by Fritz (1963) and more recent work on this subject is discussed in Part II (Introduction). The results reported by Snoswell and Henderson (1970) for the activities of the enzymes carnitine acetyltransferase and carnitine palmitoyltransferase, and for the concentrations of various carnitine and acyl-carnitine fractions, of sheep liver indicate that the metabolism of carnitine by sheep is very different from that of rats. These findings led to other investigations which are reported in this thesis: viz. an examination of the effect of carnitine on fatty acid oxidation by isolated sheep liver mitochondria was studied. The variations in carnitine and acyl-carnitine ester concentrations in several tissues of sheep, under different metabolic conditions, were also evaluated. Similar studies on CoA and acetyl-CoA concentrations were also carried out as it became increasingly obvious that, at least in sheep, the metabolism of carnitine and CoA compounds is very closely integrated.

The other major work reported here is an investigation of the biosynthesis of carnitine using sheep tissue preparations. It seemed likely that sheep tissues could provide an experimental system for such a study, as it was found that the carnitine concentrations in the tissues varied markedly and rapidly when the animals were subjected to stress.

The results of the experimental work, and some of the points arising from them, are summarised in the Synopsis.

PART I

The metabolism of ketone bodies by normal and stressed sheep

I. Introduction

As was mentioned in the General Introduction, the production of ketone bodies in sheep has been investigated extensively since these animals are more prone to ketosis than non-ruminants. However, until recently the majority of investigations have been made *in vivo*, with whole animals. Also, it has been assumed in most cases that the various enzyme reactions and the activities and distribution of these enzymes are similar in ruminants to those occurring in the rat, the animal most frequently used in detailed studies on ketone body metabolism (see Pearce, 1960, for a review of early studies on bovine ketosis). An obvious exception to the above generalization is the relatively long-standing recognition that in sheep, as in other ruminants, ketone bodies are produced in two main tissues - the rumen epithelium and the liver (Pennington, 1952). This is in contrast to the situation in the rat, and other monogastrics, where the liver is the main site of ketone body formation. The mucosa of the large gut of rabbits has been shown to be capable of forming ketone bodies from butyrate (Henning and Hird, 1972), but it is considered that such ketogenesis is not extensive *in vivo*.

Regulation of hepatic ketogenesis in monogastric animals

In the liver cell, acetoacetate is produced in the mitochondria from acetyl-CoA, derived from the oxidation of fatty acids, via the hydroxymethylglutaryl-CoA (HMG-CoA) pathway (Lynen *et al.*, 1958). The rate of hepatic ketone body production is increased under conditions where high concentrations of free fatty acids prevail, as in starvation. The subject of hepatic ketogenesis and what was known of its regulation

at the time (in the liver of the monogastric animal) was critically reviewed by Wieland (1968). The general conclusions on the factors controlling hepatic ketogenesis at this time were that increased hepatic fatty acid oxidation resulted in an increased $[NADH]/[NAD^+]$ ratio in the mitochondria. This in turn decreased the [oxaloacetate]/[malate] ratio by increasing the proportion of malate. A relatively lower concentration of oxaloacetate led to less condensation with acetyl-CoA (in the citrate synthase reaction), which was being produced in larger amounts from the fatty acids. The "excess" acetyl-CoA was diverted to ketone body formation. This diversion would be pushed further towards ketone body production, since (in the monogastric animal) conditions of high hepatic ketogenetic rates are invariably associated with increased rates of gluconeogenesis, so providing a further drain on the oxaloacetate (Krebs, 1966). To reinforce this theory was the finding that the activity of the Krebs cycle was depressed in acute ketosis. However, in the review of Wieland (1968) the evidence for Krebs cycle depression is based on calculations of the amount of oxygen used for ketogenesis, which was then subtracted from the total oxygen consumption to give the amount of oxygen said to be primarily used in citric acid cycle oxidations. Although the depression of citric acid cycle oxidation was apparently rather large in acute ketosis (86% of the total oxygen consumption of liver slices from normal animals was involved in Krebs cycle oxidations compared with 25% with slices from pancreatectomized animals) it must be remembered that the errors involved in chemical methods of measuring ketone body concentrations could lead to very large errors in secondary calculations of this kind.

Williamson, D.H. et al. (1968) confirmed that the HMG-CoA pathway was the operative one for hepatic ketogenesis, and they studied the intracellular distribution of the enzymes concerned and the variations exerted on the enzyme patterns by starvation, alloxan-diabetes and fat-feeding. Although increases in enzyme activities were observed with alloxan-diabetes and fat-feeding (for HMG-CoA synthase) and fat-feeding (for HMG-CoA lyase) they concluded that variations in these enzyme concentrations played no major role in the hepatic regulation of ketogenesis in starvation and alloxan-diabetes, but that increased ketogenesis is the result of an increased flow rate through the HMG-CoA cycle arising from increased fatty acid concentrations in the blood.

The more recent review of McGarry and Foster (1972) on the regulation of hepatic ketogenesis includes a detailed discussion on the competition between the pathways of β-oxidation and triglyceride synthesis for the incoming fatty acids as a means of controlling ketogenesis. The commonly held belief is that the rate of oxidation of fatty acids in the liver is a function of the rate of triglyceride formation, so that in normal liver, where delivery of substrate (fatty acids) to the liver is low most of the fats are used for triglyceride formation. In starvation, substrate delivery to the liver is increased and triglyceride synthesis is presumed to be depressed, so that more fatty acids are oxidised leading to the overproduction of acetyl-CoA, which in turn increases ketone body production. This theory was extended to cover the situation pertaining in diabetes, where triglyceride accumulates in the liver, by postulating that the esterifying pathway is not depressed in this state, but is saturated by the greater

input of fatty acids, so that a lot of fatty acid is still available for β-oxidation. On the basis of their studies with rat liver perfused with octanoic and oleic acids and various antiketogenic agents McGarry and Foster (1971b) suggest that instead of the rate of esterification of fatty acids being thought of as depressed in starvation, it would be better to think in terms of the rate of β-oxidation being depressed in the normal liver, and in the starved liver β-oxidation being increased at the expense of triglyceride formation. The accumulation of triglycerides in the diabetic liver can then be accounted for by the saturation of the β-oxidation pathway leaving excess fatty acid for esterification.

The reasoning behind this hypothesis is that McGarry and Foster (1971b) found no effect of antiketogenic agents (which did not affect uptake of fatty acids or make any significant difference to the amount of CO_2 formed) on ketogenesis from octanoic acid (which is not esterified), but with oleic acid plus antiketogenic agents a great increase in triglyceride formation was observed at the expense of ketogenesis. With livers taken from starved rats, the incorporation of oleic acid into triglycerides was reduced, and the proportion of the infused fatty acid going to ketone bodies was increased, though not at the expense of CO_2 production. Using octanoic acid, the increase in ketogenesis observed when livers of starved animals were used was not as great as that seen when oleic acid was the substrate (see also McGarry and Foster, 1971a), and the major part of the increase could be accounted for by the decrease in lipogenesis (from octanoate via 2C fragments) observed in livers of starved animals. Diagrams illustrating the two hypotheses are to be

found in the paper by McGarry and Foster (1972).

The effect of antiketogenic agents on sn-glycero-3-phosphate synthesis was varied, and the concentration of this compound was higher in the livers of fasted than of fed rats (McGarry and Foster, 1971b). Thus, the decrease in triglyceride synthesis cannot be accounted for by lack of sn-glycero-3-phosphate.

The figures for CO_2 production from both octanoate and oleate indicate that there was very little depression of Krebs cycle activity in livers from ketotic animals (McGarry and Foster, 1971a, b).

Thus, McGarry and Foster (1972) conclude that the regulation of ketogenesis is not simply controlled by the rate of delivery of free fatty acids to the liver, but that control is exerted at two main sites: (a) the entry to the β-oxidation pathway and (b) the utilization of acetyl-CoA. The findings outlined above for octanoate, which does not require carnitine for its oxidation in rat liver, indicate that the control at the site of entry to the β-oxidation pathway might be at the level of long-chain-acyl carnitine transferase (McGarry and Foster, 1972). The utilization of acetyl-CoA depends on the activity of the pathways of lipogenesis and of the Krebs cycle. McGarry and Foster (1971a, b, 1972) found that the Krebs cycle is only marginally depressed in ketosis, but that fatty acid synthesis is markedly reduced in both starvation and diabetes.

In the latest paper from McGarry's group (McGarry et al., 1973) the effects of starvation and refeeding of rats on carbohydrate metabolism, fatty acid oxidation and esterification and their

relationship to ketogenesis in the liver have been studied at much shorter time intervals after treatment. The results obtained indicate that responses to starvation and refeeding can take place very rapidly (within 6 h), with the capacity of the perfused livers of starved animals for ketogenesis and gluconeogenesis being markedly increased within this period, after an apparently slow rise in the first 5 h. The response of the liver to refeeding took place almost immediately, with a very dramatic fall in the rate of ketogenesis by the perfused liver. In these experiments it was also found that the uptake of fatty acids by the perfused liver was virtually the same regardless of the nutritional status of the donor rat, and the findings outlined above for fatty acid incorporation into either ketone bodies or triglycerides were confirmed. The hypothesis that control might be exerted at the level of long-chain acyl carnitine transferase was strengthened by the observation that blocking the oxidation of fatty acids by adding (+)-decanoylcarnitine to the perfusion fluid switched the metabolism of oleic acid from ketogenesis to triglyceride synthesis. The finding of increased carnitine palmitoyltransferase activity in the livers of fat-fed, alloxan-diabetic and starved rats (compared with normal rats) by Norum (1965), which was not due to de novo enzyme synthesis, is also indicative of control at this point.

REVIEW

Thus the present knowledge on the control of hepatic ketogenesis in starved rats can be summarised as follows: starvation leads to increased mobilisation of fats from the depots so that plasma free fatty acid concentrations rise. Uptake of fatty acids by the liver may then be raised and the fatty acids are directed to β-oxidation by an increase

in the activity of long-chain acyl carnitine transferase (with a concomitant fall in triglyceride formation). The increased flow through the oxidation pathway results in large amounts of acetyl-CoA being formed. Because lipogenesis is reduced, Krebs cycle activity unchanged and gluconeogenesis increased, the "excess" acetyl-CoA is directed to ketogenesis. This direction is probably also influenced by the change in the [oxaloacetate]/[malate] ratio brought about by the increased [NADH]/[NAD^+] ratio which occurs when β-oxidation rates are increased. In alloxan-diabetes the only apparent differences are that more fatty acids are taken up by the liver, so that even the greater rate of β-oxidation cannot accommodate the full amount of substrate provided. The excess fatty acid is directed to the still fully-functional triglyceride synthesising pathway.

The theory of actively enhanced hepatic β-oxidation in starvation is more attractive than that of actively depressed triglyceride synthesis in view of the results referred to above - particularly the effects of antiketogenic agents and a potent inhibitor of carnitine palmitoyltransferase ((+)- decanoylcarnitine), the increased activity of carnitine palmitoyltransferase and the maintained or increased concentrations of sn-glycero-3-phosphate. This theory also more readily encompasses the situation pertaining in diabetic ketosis, since one does not now have to propose a de-repression of the triglyceride synthesising pathway in order to explain the accumulation of hepatic triglyceride in this condition. The recent work of Ontko (1972) on factors affecting the partition between esterification and oxidation of fatty acids in isolated rat liver cells further substantiates the

hypothesis of actively enhanced β-oxidation in starvation.

Regulation of hepatic ketogenesis in ruminants

With ruminants, the enzymes of hepatic acetoacetate formation in bovines have been shown to be the same as, and of similar activity to those of rat liver (Baird et al., 1970) i.e. acetoacetate is formed from acetyl-CoA via the HMG-CoA pathway, with HMG-CoA synthase being the apparent rate-limiting enzyme. There were no differences in the activities of the HMG-CoA pathway enzymes in the livers of ketotic cows compared with normal animals. (For the rat, Williamson, D.H. et al. (1968) found some increases with alloxan-diabetic animals, but concluded that only increased flow through the cycle was responsible for its increased output). It is reasonable to assume that the same pathway for ketogenesis exists in sheep liver.

In sheep, as in rats, the main source of hepatic ketone bodies is plasma free fatty acids (Katz and Bergman, 1969a), and there is only limited hepatic ketogenesis in the normal, fed animal.

The subject of the control of hepatic ketogenesis in ruminants has had very little attention compared with that given to the rat, and because of the great differences in the overall metabolism of the two groups of animals it would be dangerous to make direct extrapolations from one (rat) to the other (ruminants). In the fed sheep hepatic ketogenesis can certainly be assumed to be limited by the relative lack of supply of long-chain free fatty acids, and it is possible that, as with the rat, triglyceride synthesis is quantitatively more important than β-oxidation under these circumstances. Another possible control

factor in the fed ruminant is propionate, one of the volatile fatty acids produced during rumen fermentation. It has been shown by Bush and Milligan (1971a, b) that propionate could inhibit hepatic ketogenesis in two ways: by the formation of oxaloacetate from propionate, or by the demonstrated inhibition of HMG-CoA synthase by propionyl-CoA. When other Krebs cycle intermediates were used as sources of oxaloacetate the inhibition of ketogenesis was not as great as that seen with propionate, so it was concluded that the major effect of propionate was that on HMG-CoA synthase. Such a control factor would, of course, be removed in starvation but in the feeding alloxan-diabetic animal it would presumably still be present. The relative importance of propionate as a regulator of ruminant hepatic ketogenesis can probably only be accurately assessed by using perfused livers taken from sheep in various metabolic states. It is possible that the antiketogenic effect of propionate is mediated in the same way as those of the antiketogenic agents used in perfused rat livers (McGarry and Foster, 1971b) i.e. by diverting fatty acids from β-oxidation to triglyceride synthesis. Ciman et al. (1972) suggest that the antiketogenic effect of propionate (in rat liver mitochondria) can be attributed mainly to inhibition of fatty acid activation, by decreasing the [ATP]/[AMP] ratio leading to inhibition of ATP-dependent acyl-CoA synthetase.

Little is known of the relative rates of β-oxidation and triglyceride synthesis in the ruminant liver in various metabolic states. However, one way in which the starved sheep differs markedly from the starved rat is that the liver of starved non-pregnant sheep can often be quite fatty (personal observation). This can be interpreted in two

ways, both of which assume that, as is suggested for the rat (above), there is no actual blocking of triglyceride biosynthesis during starvation. The rate of uptake of fatty acids by the liver of starved sheep could be relatively much greater than in the rat, so assuming that the relative rates of β-oxidation and triglyceride synthesis are the same, there is simply a saturation of the β-oxidation system and the overflow is accommodated by esterification. Alternatively, it is possible that even in starvation the rate of β-oxidation by sheep liver is relatively low.

The situation in alloxan-diabetes in sheep seems to be perfectly analogous to that in rats (above).

In the rat ketotic conditions are invariably associated with increased hepatic gluconeogenesis, so that assessments of the effects of gluconeogenesis on ketogenesis apply in all cases. The situation is more difficult in ruminants where high rates of hepatic gluconeogenesis pertain in the fed animal, and the rate of gluconeogenesis falls in starvation as precursor supplies diminish. Baird et al. (1972) showed that the activity of the key gluconeogenic enzyme, phosphopyruvate carboxylase (phosphoenolpyruvate carboxykinase) of bovine liver fell significantly on starvation, and there was no change in the activities of other specifically gluconeogenic enzymes. This is in direct contrast to the situation in the rat where hepatic gluconeogenic enzyme activities rise on starvation (Filsell et al., 1969). Using liver from spontaneously ketotic cows the activities of the gluconeogenic enzymes were found to be no different from those of normal animals (Baird et al., 1968). In bovine ketosis there is an increased demand

for gluconeogenesis to meet the needs of lactation, and the findings of Baird et al. (1968) - above, plus their observation of reductions in the hepatic concentrations of glucogenic amino acids and oxoacids (particularly oxaloacetate) led them to conclude that lack of oxaloacetate is the primary cause of the condition. It is suggested that the gluconeogenic enzymes could be allosterically affected by changed metabolite concentrations (Baird et al., 1968). Using starved cows, Baird et al. (1972) found that metabolite concentration changes in the liver were similar to those seen in spontaneously ketotic cows, and despite the recorded fall in the activity of hepatic phosphoenolpyruvate carboxykinase in starved animals (above), they conclude that the ketosis of starvation in lactating cows has a similar origin to that of "acetonaemia", i.e. a shortage of glucose precursors. Acetonaemia occurs at a time when the demand for gluconeogenesis is increased by lactation. Presumably then, under these circumstances as in starvation, more fat is mobilised from the body depots to meet the energy requirement, which is in excess of that provided by the food intake, and because oxaloacetate in particular is depleted, acetyl-CoA arising from fatty acid oxidation is diverted to ketogenesis. Ballard, Hanson and Kronfeld (1968) demonstrated markedly reduced malate dehydrogenase activity in the livers of spontaneously ketotic cows which could lead to a reduction in the synthesis of oxaloacetate, which could therefore also result in a decreased oxaloacetate concentration. There is some evidence that in bovine liver the activity of the Krebs cycle may be diminished in starvation since Baird et al. (1972) have shown that liver citrate concentrations fall to 28% of the control values ($P < 0.001$) and hepatic

citrate synthase activity is decreased to 82% of control values (though this was not statistically significant) at the same time as oxaloacetate concentrations become limiting. In rat liver the evidence is against any reduction in Krebs cycle activity (see above) and there is controversy concerning changes in citrate concentration (Baird et al., 1972).

Much less is known about the metabolite concentrations of sheep liver, and since the aetiology of pregnancy toxaemia is different from that of bovine ketosis it would be presumptuous to assume that what occurs in the ketotic cow will also occur in the ketotic sheep. In pregnancy toxaemia the drain on blood glucose is provided by the foetus(es) - which is analogous to that provided by lactation in bovine acetonaemia. However, pregnancy toxaemia is nearly always complicated by the anorexia which almost invariably precipitates the clinical condition, particularly with fat ewes. Anorexia can be caused simply by the fact that the foetus(es) is taking up so much abdominal space that there is not room for the rumen either to fill adequately or to have unhindered motility. Another means by which anorexia is often induced is by sudden inclement weather, e.g. snow, which cuts off the food supply. In bovine ketosis anorexia is rarely a cause, but may be an effect, of the condition.

The concentrations of the nicotinamide coenzymes in livers of normal, fasted and toxaemic pregnant sheep have been measured by Kronfeld and Raggi (1966). It was found that the hepatic concentration of total nicotinamide coenzymes fell significantly ($P < 0.001$) in both fasted and toxaemic sheep. The concentrations of all fractions fell

significantly in the livers of toxaemic sheep, and those of NAD^+ and NADH in starved, pregnant sheep. The $[NADH]/[NAD^+]$ ratio did not change significantly with either form of stress, and if anything showed a slight tendency to fall. This is in marked contrast to the situation in the livers of diabetic rats where there was no change in total nicotinamide coenzyme concentration, but the proportion of reduced coenzymes was markedly increased (Kronfeld and Raggi, 1964). Thus it is not possible to argue for sheep liver, as one can for rat liver, that the increased $[NADH]/[NAD^+]$ ratio can alter the [oxaloacetate]/[malate] ratio in favour of malate, so leading to a relative decrease in oxaloacetate concentration. However, as was mentioned previously, in bovine liver there is a definite fall in oxaloacetate concentration in ketosis and starvation (Baird et al., 1968, 1972), but in this case it is most likely to be due to a deficiency of precursors rather than the alteration of the redox state of the cell. This is almost certainly true of the liver of the anorexic sheep. (Ballard et al. (1968)* found only one significant change in hepatic nicotinamide coenzyme concentrations when livers from starved and spontaneously ketotic cows were compared with those from normal animals - this was a rise in the NADH concentration in the livers of starved cows. If this rise occurred in the mitochondria at the expense of NAD^+, then the altered redox state theory could apply in this case. The results of Baird et al. (1972) show that the NAD^+ concentration of whole liver of starved lactating cows fell to 48% of control values.) It is difficult to assess the pertinence of the results of Kronfeld and Raggi (1966) in regard to control of ketogenesis, gluconeogenesis or lipogenesis since the values

* Ballard, Hanson, Kronfeld and Raggi (1968)

obtained for nicotinamide coenzyme concentrations are for whole liver, particularly as there was no change in the ratio of NADH to NAD^+ (there was a significant fall in the $[NADPH]/[NADP^+]$ ratio in the liver of fasted pregnant sheep). It seems that under such circumstances one could expect to find decreases in rates of fatty acid oxidation, gluconeogenesis, pentose phosphate cycle activity and/or lipogenesis, or in all four! Until data are available on the intracellular distribution of the changes in nicotinamide coenzyme concentrations it will not be possible to assess how these changes affect the metabolism of the cell (or cell compartment). Williamson et al. (1967) emphasize that the ratios of oxidised and reduced dinucleotides in cytoplasm and mitochondria are vastly different and do not necessarily move in parallel when changes occur in the metabolic state of the liver.

In starved non-pregnant sheep the hepatic activities of three of the key gluconeogenic enzymes (glucose-6-phosphatase, fructose-1-6-diphosphatase and pyruvate carboxylase) were increased compared with those of normal sheep, while the activity of phosphoenolpyruvate carboxykinase was unchanged (Filsell et al., 1969). This is in contrast to the situation in bovine liver where there was no change in the activity of glucose-6-phosphatase, while that of phosphoenol-pyruvate carboxykinase was reduced on starvation of lactating cows (Baird et al., 1972). Ballard et al. (1968)* found increased pyruvate carboxylase activity in starved lactating cows, but also found no change in phosphoenolpyruvate carboxykinase activity. Filsell et al. (1969) found similar changes in the livers of fasted pregnant sheep to those above, but when using sheep made diabetic either with alloxan

* Ballard, Hanson, Kronfeld and Raggi (1968)

or by removing the pancreas the activity of phosphoenolpyruvate carboxykinase was also increased. (Using spontaneously ketotic cows, Baird et al. (1968) found no changes in any of these enzyme activities). With rat liver all four enzymes are increased when the animals are fasted, though the increases are significant only for glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (Filsell et al., 1969). These findings in sheep liver contrasted with those in rat liver led Filsell et al. (1969) to postulate that there is no increase in gluconeogenesis via this pathway in fasted sheep, whereas in the rat there is. The work of Bergman et al. (1968) also indicates that hepatic gluconeogenesis is reduced in fasted sheep, and that a much larger proportion of glucose is provided from glycerol under stressed conditions, including hypoglycaemia of pregnant animals - though glucose production by the livers of normal pregnant sheep is higher than that of normal fed sheep (Katz and Bergman, 1969a).

Thus it appears that in the fasting sheep the primary cause of increased hepatic ketogenesis from free fatty acids is probably a decreased oxaloacetate concentration caused by the lack of propionate. In the pregnant toxaemic sheep this may be the initial cause of increased ketogenesis too, and in this case the system is probably strained, before excess ketogenesis is manifest, by the demands of the foetus(es) for glucose. Reid and Hogan (1959) found that blood citrate concentrations of sheep were increased after feeding and were highly correlated with blood glucose concentrations. In fasted pregnant sheep blood citrate concentrations fell as did glucose while ketone body concentrations rose. If blood citrate concentrations

reflect those of the liver (as found for cattle by Baird et al., 1972) then it appears that Krebs cycle activity could be depressed in ketotic sheep. As with cattle, no predictions can be made for sheep concerning the relative roles of β-oxidation and esterification of the free fatty acid by the liver in the control of ketogenesis.

No mention has been made of hormonal control of hepatic ketogenesis in either monogastrics or ruminants as it is considered that this aspect is beyond the scope of this thesis. It is obvious that hormones play a major role in the onset of ketosis and in the state of the animal during and after the expression of the clinical condition. However, the parameters which would be affected by hormones are those already discussed above.

Ketogenesis in the rumen epithelium

Katz and Bergman (1969a) in their studies with sheep having implanted cannulae in the hepatic and portal veins showed that the portal drained viscera is the primary site of ketone production in fed sheep, and it is well known that the main site of this ketogenesis is the rumen epithelium (see e.g. Annison and Lewis, 1959). The main source of ketone bodies in rumen epithelium is butyrate (Leng and West, 1969), and although they claim that acetoacetate is the main ketone body released by this tissue, it is now known that 3-hydroxybutyrate is in fact virtually the only ketone body released by the rumen epithelium (e.g. Katz and Bergman, 1969a, and this thesis).

The actual pathway for the production of acetoacetate from acetoacetyl-CoA in rumen epithelium is still in doubt. The work of

Hird and Symons (1961) indicated that the HMG-CoA pathway is the operative one for acetoacetate formation in this tissue. Baird et al. (1970) also maintained that this is the major route for ketogenesis in rumen epithelium, having found in their studies only very little acetoacetyl-CoA deacylase activity in this tissue even with an assay system using much higher concentrations of acetoacetyl-CoA than could be expected to be found in the tissue. Bush and Milligan (1971b) found approximately equal activities of HMG-CoA synthase and acetoacetyl-CoA deacylase in their preparations from rumen epithelium, though the activity of deacylase they found was 50% and the synthase activity only 10% of those reported by Baird et al. (1970). The concentration of acetoacetyl-CoA used by Bush and Milligan (1971a, b) in measuring synthase activity is up to 225,000 times that which can be expected to be found in the rat liver cell (Williamson, D.H. et al., 1968; Lee and Fritz, 1972), and is up to five times greater than that used by Baird et al. (1970). Stewart and Rudney (1966) have shown that acetoacetyl-CoA inhibits the HMG-CoA synthase reaction at low concentrations - the highest concentration they used (154 μM) is less than that (approximately 230 μM) calculated to have been used by Bush and Milligan (1971a, b). Thus, it is likely that the much lower HMG-CoA synthase activity observed by these workers (cf. Baird et al., 1970) was an artefact of their methodology. No details of the assay system used for deacylase activity are given in either of the papers by Bush and Milligan (1971a, b), but assuming that they used similarly high acetoacetyl-CoA concentrations as for the synthase assays, it is unlikely that the low activity detected could contribute to acetoacetate synthesis in vivo.

Bush and Milligan (1971b) found that addition of succinate to the assay system used for acetoacetyl-CoA deacylase activity of rumen epithelial extracts resulted in much greater deacylation of acetoacetyl-CoA. Since they were able to detect succinyl-CoA in the mixture of reaction products, they concluded that the enzyme responsible for the deacylation was 3-oxo acid CoA transferase. It is suggested that this enzyme is responsible for the major part of the acetoacetate production of rumen epithelium partly because no inhibition of ketogenesis by propionate was demonstrated in rumen epithelium, whereas propionyl-CoA was shown to inhibit the HMG-CoA synthase of liver (Bush and Milligan, 1971a), and partly on the grounds that the transferase pathway would apparently yield more high energy bonds than the HMG-CoA pathway.

The lack of inhibition by propionate of ketogenesis in rumen epithelium can probably be fairly simply explained by the fact that propionate or propionyl-CoA might never be found at the HMG-CoA synthase site in rumen epithelium. Whether or not propionate or propionyl-CoA has any effect on rumen epithelial HMG-CoA synthase could perhaps be checked by using subcellular fractions of the tissue (which are difficult to prepare) or by using partially purified enzyme from rumen epithelium.

The arguments in favour of transferase on energetic grounds (Bush and Milligan, 1971b) are not really very strong. Since 3-hydroxybutyrate is the major ketone body released by the rumen epithelium, there would be a net yield of only one high energy bond per butyrate converted (from the resynthesis of succinate from succinyl-CoA) compared

with none for the HMG-CoA synthase pathway. However, the yielding of energy is not the main purpose of the 3-hydroxybutyrate formation - which, primarily, is to get the butyrate out into the bloodstream in a form which can bypass utilization in the liver but which can be readily utilized by the extra-hepatic tissues.

Bush and Milligan (1971b) state that the transferase enzyme could participate "to a significant extent in ketogenesis in rumen epithelium, in a manner similar to that discussed recently for rat kidney cortex (Weidemann and Krebs, 1969b)". However, Weidemann and Krebs (1969b) emphasize that the enzyme is present in rat kidney cortex as part of an acetoacetate utilizing system, and that the ketogenic capacity of the tissue is likely to be evident only when the concentration of acetyl-CoA is relatively high. It is considered unlikely that conditions of renal ketogenesis occur often in vivo (Weidemann and Krebs, 1969b). A low [succinyl-CoA]/[acetoacetyl-CoA] ratio would tend to favour ketogenesis, but in view of the extremely low tissue concentrations of acetoacetyl-CoA (Williamson, D.H. et al., 1968; Lee and Fritz, 1972), and the relatively high Km of the pig heart enzyme for acetoacetyl-CoA (Hersh and Jencks, 1967), it seems unlikely that favourable conditions could prevail for acetoacetate formation in vivo, since Km values for the enzyme from other tissues are likely to be at least of the same order. Also, the synthesis of ketone bodies from butyrate via such a pathway would not involve an increased concentration of acetyl-CoA (Weidemann and Krebs, 1969b), and in the rumen epithelium of a fed animal there is not likely to be any excessive build up of this metabolite.

Most of the available information indicates that the succinyl-CoA: 3-oxo acid transferase activity of tissues is associated with utilization of ketone bodies (e.g. Weidemann and Krebs, 1969b; Tildon et al., 1971 and Bässler et al., 1973) and the kinetics of the reaction coupled with what is known of tissue concentrations of the metabolites concerned would appear to substantiate this view. It seems unlikely that the concentration of acetoacetyl-CoA in rumen epithelium is about 100,000 times greater than that of other tissues. And the Km of the HMG-CoA synthase for acetoacetyl-CoA of rat liver mitochondria has been calculated to be of the same order as the concentration of the metabolite (Lee and Fritz, 1972). It is reasonable to assume then that the Km of the HMG-CoA synthase for acetoacetyl-CoA of rumen epithelium would also be very low, and that it is the HMG-CoA pathway which predominates in rumen epithelial ketogenesis as it does in liver.

Concentrations of ketone bodies in blood

The introduction of specific enzymic methods for the measurement of D-3-hydroxybutyrate and acetoacetate (Williamson et al., 1962) has meant that accurate assessments of the concentrations and ratios of these two metabolites can be made and valid comparisons made between animal treatments and between the results of different workers. In the past the chemical methods of ketone body analysis have led to controversial findings, particularly with regard to the ratios of 3-hydroxybutyrate to acetoacetate. For instance Leng and West (1969) found that the main ketone body released from the rumen epithelium was acetoacetate. It is now known that 3-hydroxybutyrate constitutes the major ketone body released by this tissue (Katz and Bergman, 1969a and

this thesis). The results published for the [3-hydroxybutyrate]/[acetoacetate] ratio of sheep blood range from 1:4 (Procos, 1962) to 2:1 (Reid, 1960) and Leng and Annison (1963) implied that in normal sheep 3-hydroxybutyrate represents about 85% of the blood ketone bodies. In view of these facts it is surprising to find that chemical methods are still used for ketone body assays by many research groups, though the expense of the enzyme precludes its routine use in clinical laboratories.

Preliminary work of Snoswell (1968), using the enzymic assay methods, indicated that the [3-hydroxybutyrate]/[acetoacetate] ratio of ruminant blood was much higher than that of non-ruminants, and that, in contrast to non-ruminants, this ratio fell when the animal was starved. Baird et al. (1968) also found a high ratio in the blood of cattle. Katz and Bergman (1969a) using chemical methods, found that 3-hydroxybutyrate was virtually the only ketone body produced by both the portal-drained viscera and the liver of fed sheep, but that on fasting acetoacetate comprised 20 to 30% of the blood ketone bodies. In the present study the concentrations of 3-hydroxybutyrate and acetoacetate were measured enzymically in blood taken from fed, starved and re-fed sheep, and hepatic ketogenesis was studied in sheep during the development of alloxan diabetes. The production of ketone bodies during the oxidation of palmitoyl-L-carnitine by isolated sheep liver mitochondria was also measured.

3-hydroxybutyrate dehydrogenase

3-hydroxybutyrate dehydrogenase, the enzyme which catalyses the interconversion of 3-hydroxybutyrate and acetoacetate, is regarded as a

classical "marker enzyme" for the inner mitochondrial membranes (e.g. see Tubbs and Garland, 1968). Attempts to use this enzyme to identify mitochondrial membrane fractions prepared from sheep liver failed as little or no activity was detected. This finding, plus the fact that Baird et al. (1968) mention unpublished work in which it was found that the 3-hydroxybutyrate dehydrogenase activity of bovine liver was less than one-thirtieth of that of rat liver, led to an investigation of the intracellular distribution of this enzyme in liver and other tissues of the sheep. Following publication of this work (Koundakjian and Snoswell, 1970), Williamson and Kuenzel (1971) showed that the "soluble" 3-hydroxybutyrate dehydrogenase activity of sheep kidney cortex described by Koundakjian and Snoswell (1970) is, in fact, that of an L-3-hydroxy acid dehydrogenase (probably L-gulonate-NAD^+ oxidoreductase) not D-3-hydroxybutyrate dehydrogenase as was implied (Koundakjian and Snoswell, 1970). Smiley and 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 and Ashwell, 1961). In order to ascertain the contribution of L-3-hydroxy acid dehydrogenase to the overall 3-hydroxybutyrate dehydrogenase activity of sheep tissues, the activity of dehydrogenases of L- and D-3-hydroxybutyrate was examined in various tissues of the sheep.

The correlations between the blood concentrations of 3-hydroxybutyrate and acetoacetate, the known sites of ketone body production and the tissue distribution of the D- and L-3-hydroxybutyrate dehydrogenases are discussed.

I. Experimental and Results

I.A. Preparation of 3-hydroxybutyrate dehydrogenase (D(-)-3-hydroxybutyrate-NAD^+ oxidoreductase) from Rhodopseudomonas spheroides

I.A.1. Purification procedure

Culture of Rhodopseudomonas spheroides. A freeze-dried sample of R. spheroides was obtained from The National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland (N.C.I.B. number 8253). The basic culture medium used was as described by Williamson et al. (1962) except that only one-tenth the amount of potassium hydrogen phosphates was used. The salt solution was sterilized by autoclaving for 20 min at 12 lb/in^2. The vitamin solution, sterilized with a Millipore filter (0.2 μ), was added to the salt solution under aseptic conditions. For preliminary culture of the bacteria 0.2% (w/v) yeast extract was added to 250 ml of the basic medium. The preliminary cultures were incubated aerobically at 30°C in the dark for 24 h. A 250 ml preliminary culture was used to inoculate each 8 l batch of medium in 10 l aspirators fitted with sintered glass air distributor tubes. These cultures were grown aerobically at 30°C in the dark for 48 h. The cells were collected in a Sorval centrifuge (RC_2B, fitted with a continuous flow rotor) and washed twice with a total volume of 2 l of 10 mM-potassium phosphate buffer, pH 7.4. The cells were weighed, suspended in the above buffer (1 g/1 ml) and the suspension stored in the deep-freeze at -15°C for 48 h.

The suspension was thawed and the cells sonically disrupted with a large titanium probe on an ultrasonic disintegrator operating at 20 kHz/s. Total sonication time was 20 min (in 1 min periods) at 0°C.

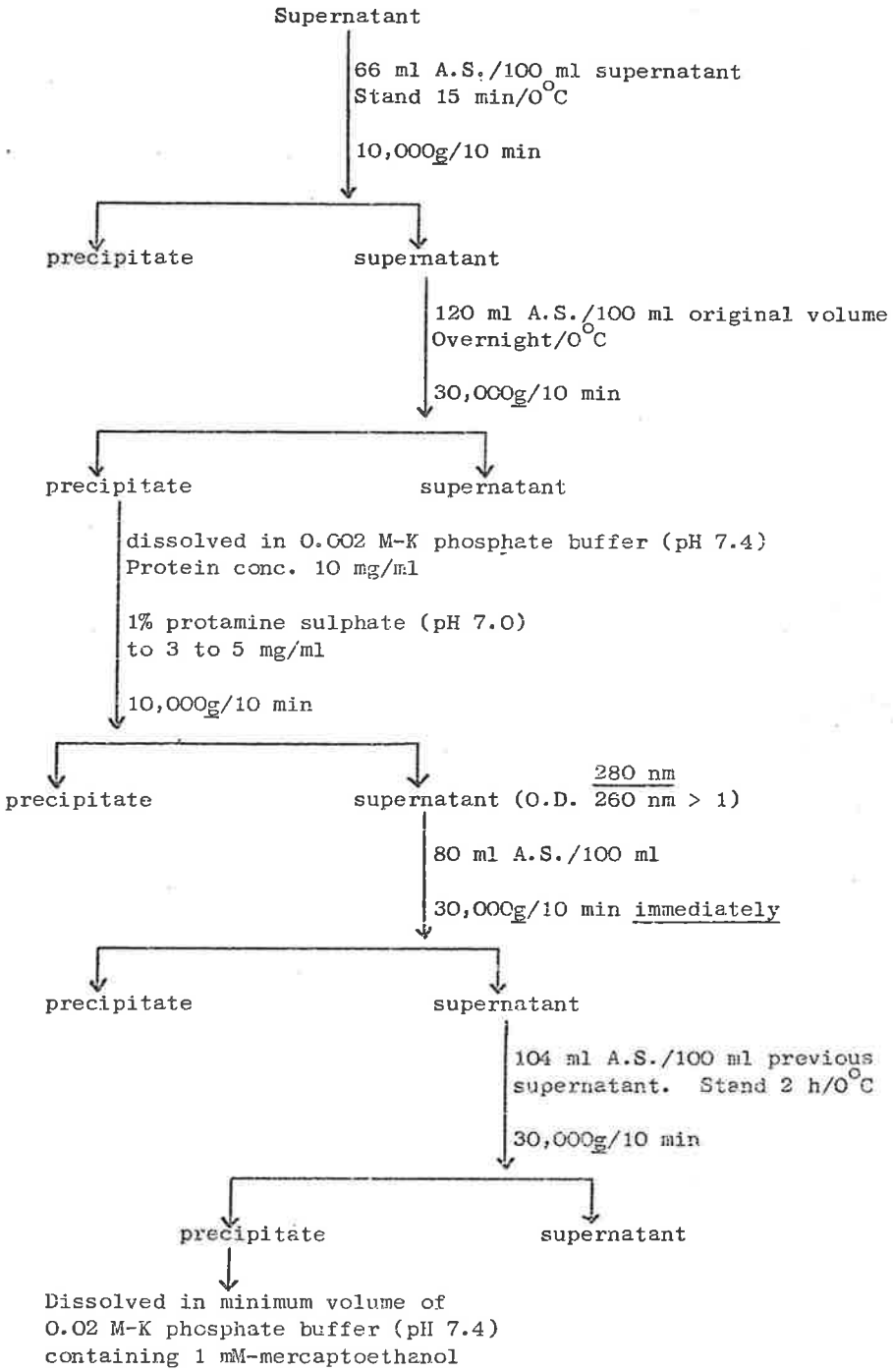
The sonicated suspension was centrifuged at 30,000g for 10 min. Ammonium sulphate and protamine sulphate fractionations of the supernatant were made as shown in Fig. 1. The final ammonium sulphate precipitate was dissolved in the minimum volume of 0.02 M-potassium phosphate buffer (pH 7.4) containing 1 mM-mercaptoethanol. The solution was passed through a small column (2 x 15 cm) of coarse Sephadex G-25 which was equilibrated in the same buffer. The sulphate-free protein fraction was collected and further purified on an anion-exchange column of DEAE-Sephadex, as suggested by Williamson et al. (1967). The solution was applied to a column (2 x 25 cm) of DEAE-Sephadex A-50 prepared according to Bergmeyer et al. (1967), and equilibrated with 0.02 M-potassium phosphate buffer (pH 7.4). Elution was started with 0.02 M-potassium phosphate buffer (pH 7.4) containing 0.2 M-NaCl and continued until the protein concentration of the eluate was low. A linear gradient of NaCl was then applied using buffer solutions with 0.2 M- and 0.4 M-NaCl. The eluate was collected in 5 ml fractions using a fraction collector (Paton Industries, South Australia, Australia). The active fractions were combined and the protein precipitated with solid ammonium sulphate (50 g/100 ml).

The enzyme was stored in the refrigerator as a suspension in 50% neutral ammonium sulphate at a concentration of about 35 units/ml.

3-hydroxybutyrate dehydrogenase activity was determined by the method of Bergmeyer et al. (1967) throughout the purification procedure. Protein concentrations were determined by the biuret method (Gornall et al., 1949). If the protein solution was deeply coloured the total absorption at 540 nm was noted and the biuret was then decolourised

FIG. 1

Ammonium sulphate fractionation of sonicated supernatant of R. spheroides



A.S. = saturated ammonium sulphate (pH 7.6)

with solid KCN so that absorption due to the pigment could be measured and appropriate corrections made.

The results of a typical purification procedure are summarised in Table 1 and Fig. 2.

Recovery of enzyme activity was generally between 25% and 30% with a specific activity of about 10 units/mg protein, representing a 50 to 100-fold purification. Contamination with lactate and malate dehydrogenases was negligible.

I.A.2. Ketone body assays using the purified enzyme

Assays. The assay systems used for acetoacetate and 3-hydroxybutyrate were essentially those described by Williamson et al. (1962). Bergmeyer et al. (1967) found that the addition of $CaCl_2$ (1 μmol/ml final volume) stabilised the full activity of the enzyme for at least 30 min at 37°C. (In the absence of added cations activity fell to 16% of the initial value in 30 min). $CaCl_2$ was therefore used routinely in all assays at a final concentration of 1 μmol/ml final volume.

As mitochondrial 3-hydroxybutyrate dehydrogenase has a known requirement for added phospholipid in order to obtain maximum activity (see e.g. Sekuzu et al., 1963), the effect of asolectin (purified soya phosphatides) on the purified bacterial enzyme was investigated. Oxidation of 3-hydroxybutyrate was found to be enhanced by 37% in the presence of 0.75 mg/ml (final volume) of asolectin.

Dithiothreitol at a final concentration of 20 mM was also found to enhance oxidation of 3-hydroxybutyrate: with both asolectin and

TABLE 1

Isolation of 3-hydroxybutyrate dehydrogenase from R. spheroides: a typical purification table

Wet weight of cells - 101 g.

	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Sonicate supernatant	1378	6273	0.22	100
1st A.S. supernatant	1146	2747	0.42	83
2nd A.S. precipitate	802	1210	0.66	58
P.S. supernatant	640	1032	0.62	46
3rd A.S. supernatant	758	1341	0.57	55
4th A.S. precipitate	758	653	1.15	55
Sephadex G-25 eluate	752	552	1.36	55
DEAE-Sephadex eluate	436	41	10.63	32
5th A.S. precipitate	387	-	-	28

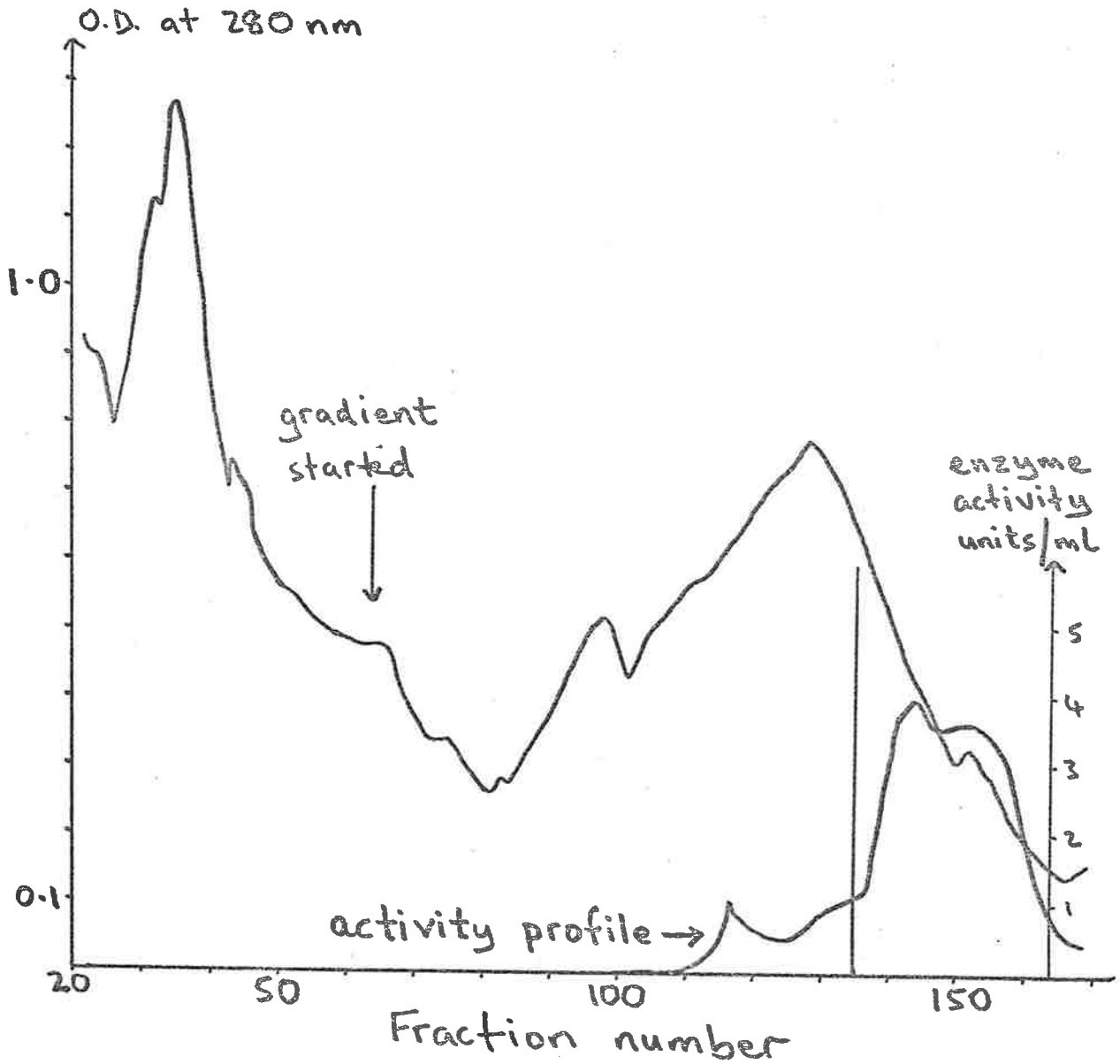
A.S. = ammonium sulphate; P.S. = protamine sulphate

Units = μmol/min at 25°C

FIG. 2

Elution profile of DEAE-Sephadex A-50 column used in the purification of 3-hydroxybutyrate dehydrogenase

Protein was eluted with 0.02 M-K phosphate buffer (pH 7.4) with 0.2 M-NaCl until the point marked "gradient started". Elution was continued with a linear gradient of 0.2 M-NaCl to 0.4 M-NaCl in the same buffer. Fraction volume was 5 ml. The fractions between the vertical lines contained most of the activity and were pooled, and the enzyme precipitated with ammonium sulphate.



dithiothreitol, activity was 80% more than that obtained in the basic incubation mixture. Thus both compounds were used in assays of 3-hydroxybutyrate.

As the reduction of acetoacetate proceeds very readily no additions other than $CaCl_2$ were made to the incubation mixture as described by Williamson et al. (1962).

I.B. Ketone-body concentrations in the blood of various species including fed and starved sheep

I.B.1. Materials and methods

Animals. The sheep used were 4 year-old Merino or Merino x Dorset Horn wethers weighing between 35 and 45 kg. 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 in the Results section. 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 (250-300 g); the pigeons were of mixed strain. Both the rats and pigeons were fed on a pelleted diet suitable for small animals (Barastoc and Co., Melbourne, Vic., Australia).

Blood 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. 2.0 ml samples of whole blood were immediately added to 1.0 ml of ice-cold 30% (w/v) $HClO_4$ with the sheep and lambs and 0.2-0.3 ml blood samples to 2.0 ml of 3% (w/v) $HClO_4$ with the other species.

Assay procedure. An aliquot of the clear supernatant obtained on centrifuging the $HClO_4$ extract of blood was neutralized with 3N-KOH, allowed to stand on ice for 30 min and the $KClO_4$ precipitate removed

by centrifuging. Acetoacetate and 3-hydroxybutyrate were determined on aliquots of the neutral supernatant by the specific enzymic methods developed by Williamson et al. (1962) with modifications as indicated in Section I.A.2. The enzyme used was 3-hydroxybutyrate dehydrogenase isolated from Rhodopseudomonas spheroides as detailed in Section I.A.1. Semi-micro cuvettes were used for the reaction, which was initiated by adding the enzyme. Extinction values were monitored with recording equipment attached to an Eppendorf photometer fitted with an automatic sample changer (Eppendorf, Netheler und Hinz, Hamburg, Germany). The temperature was 25°C, and the wavelength 340 nm for acetoacetate and 366 nm for 3-hydroxybutyrate. A correction was applied for any absorption changes in a control cell which contained all reagents except the unknown sample.

Recovery of 3-hydroxybutyrate and acetoacetate added to blood. Known amounts (approximately equivalent to 0.5 or 1.0 μmol) of standardised 3-hydroxybutyrate and acetoacetate solutions were added to blood samples of which duplicates were taken for determination of initial ketone-body concentrations. The deproteinization, neutralization and assay procedures were exactly as described above. After correcting for initial concentrations, the percentage recovery of D(-)-3-hydroxybutyrate was 98.9 ± 2.3 (S.E.M.) and of acetoacetate 100.3 ± 4.1 (S.E.M.).

Chemicals. Lithium acetoacetate was prepared by the method of Hall (1962).

I.B.2. Results

In Table 2 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 x Dorset Horn wethers (see Table 3). Also, differences in the 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 et al. (1965). Eggleston and 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 2 for the blood ketone-body concentration in lambs indicate [3-hydroxybutyrate]/[acetoacetate] ratios similar to those of non-ruminant species. The lambs were one day-old and thus there was no active rumen fermentation. Knodt et al. (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 2

Acetoacetate and 3-hydroxybutyrate in the blood of various species

The smaller group of sheep were Merino x Dorset Horn wethers fed on pelleted sheep cubes. The larger group were Merino wethers fed on a mixture of lucerne and wheaten chaff. Acetoacetate and 3-hydroxybutyrate were determined as described in the text. 0.002 μmol/ml was the lowest detectable concentration. The results shown are means ± S.E.M.

Species	No. of animals	Acetoacetate (μmol/ml)	3-Hydroxybutyrate (μmol/ml)	$\frac{[\text{3-Hydroxybutyrate}]}{[\text{acetoacetate}]}$ 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

* Samples from these animals were collected and assayed by Dr. J.G. McLean and Dr. I.W. Caple of The Sub-Department of Veterinary Biochemistry, University of Melbourne, Melbourne, Vic., Australia.

TABLE 3

Acetoacetate and 3-hydroxybutyrate in the blood of normal, starved and re-fed sheep

Each group of four animals was stabilised 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 x 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 the text. The values are the means ± S.E.M. for four animals in each group.

Sheep	Dietary status	Acetoacetate (μmol/ml)	3-Hydroxybutyrate (μmol/ml)	$\frac{[\text{3-Hydroxybutyrate}]}{[\text{acetoacetate}]}$ 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 x 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 3 show that the [3-hydroxybutyrate]/[acetoacetate] ratio in sheep blood falls on starvation and increases again on re-feeding. Total 3-hydroxybutyrate plus acetoacetate 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 cross-bred sheep than in the pure Merinos, the former sheep showing a higher [3-hydroxybutyrate]/[acetoacetate] ratio in the blood under normal feeding conditions.

I.C. Ketone-body production by the livers of normal and alloxan-diabetic sheep

I.C.1. Materials and methods

Animals. Eighteen-month-old Merino wethers weighing 26 to 29 kg were kept in metabolism crates. They were fed either on lucerne-hay chaff or on 75% wheaten-hay chaff, 25% lucerne-hay chaff. Prior to surgery the animals were starved for 24 h.

Surgery. Surgical placement of cannulae into the portal and hepatic veins was carried out by Dr. G. McIntosh (CSIRO, Division of Nutritional Biochemistry, Adelaide, S. Australia) essentially according to the method of Katz and Bergman (1969b). The cannulae were kept patent by flushing daily with heparin in 0.9% saline (100 units heparin/ml). The animals were allowed to return to full food intake before blood sampling was started. This normally took 4 to 5 days.

Alloxan treatment. Before alloxan administration the animals were allowed to stabilise on full food intake for 5 to 7 days, during which time blood samples were taken daily from both cannulae. Alloxan (60 mg/kg body weight) was dissolved in sterile saline (0.9%) and immediately injected into a jugular vein.

Assays. Blood samples were extracted with $HClO_4$ and the neutralized extracts assayed for 3-hydroxybutyrate and acetoacetate as described in Section I.B.1. All assays were done in duplicate.

I.C.2. Results

The implanted cannulae were successfully used for blood sampling over periods of two to three weeks. Any blockages were cleared either with a small bolus of air or with heparinized-saline.

Before alloxan treatment. The concentrations of acetoacetate and 3-hydroxybutyrate and the ratios of [3-hydroxybutyrate]/[acetoacetate] for both groups of sheep are shown in Table 4. The ketone body concentrations in both hepatic and portal venous blood, and the ratios in hepatic venous blood are similar to those in the jugular blood of lucerne-fed Merino sheep (Table 3). However, both groups of sheep had significantly ($P < 0.05$) higher concentrations of acetoacetate in the portal blood than in the hepatic blood (Tables 4 and 5). The [3-hydroxybutyrate]/[acetoacetate] ratios of the portal blood reflected this higher acetoacetate concentration in that they were lower than those in the hepatic blood (Table 4) but only in the lucerne-fed sheep was this difference significant ($P < 0.05$).

3-hydroxybutyrate was the only ketone body produced by the livers of both groups of sheep (Table 5) and there was an uptake of acetoacetate. The production of 3-hydroxybutyrate by the livers of lucerne-fed sheep was about 3 times that of the wheat/lucerne-fed sheep, while acetoacetate uptake was virtually the same for both groups (Table 5).

Three days after alloxan treatment. The increase in total ketone body concentration (acetoacetate + 3-hydroxybutyrate) in both hepatic and portal blood 3 days after alloxan treatment was approximately

TABLE 4

Acetoacetate and 3-hydroxybutyrate in the hepatic and portal venous blood of normal and alloxan-diabetic sheep

Eighteen-month-old Merino wethers were surgically treated, sampled and given alloxan (60 mg/kg body weight) as described in the text. Duplicate blood samples were assayed for ketone bodies as described in Section I.B.1. The figures shown are the means ± S.E.M. for three animals in each group.

Diet	Treatment	Hepatic			Portal			[3-hydroxybutyrate] / [acetoacetate] ratio	
		Acetoacetate (μmol/ml)	3-hydroxybutyrate (μmol/ml)	Total* (μmol/ml)	Acetoacetate (μmol/ml)	3-hydroxybutyrate (μmol/ml)	Total* (μmol/ml)	Hepatic	Portal
100% lucerne-hay chaff	Before alloxan	0.016 ± 0.003	0.317 ± 0.012	0.332 ± 0.010	0.033 ± 0.006	0.207 ± 0.008	0.241 ± 0.014	24.1 ± 6.0	6.8 ± 1.2
	3 days after alloxan	1.050 ± 0.165	4.54 ± 0.82	5.56 ± 0.94	0.921 ± 0.150	4.27 ± 0.77	5.19 ± 0.88	4.3 ± 0.3	4.8 ± 0.6
75% wheaten-hay chaff, 25% lucerne-hay chaff	Before alloxan	0.027 ± 0.004	0.302 ± 0.033	0.302 ± 0.041	0.047 ± 0.008	0.286 ± 0.040	0.296 ± 0.033	14.1 ± 3.4	6.0 ± 0
	3 days after alloxan	0.646 ± 0.175	2.64 ± 0.77	3.28 ± 0.85	0.600 ± 0.166	2.43 ± 0.77	3.03 ± 0.84	4.3 ± 1.1	4.3 ± 1.3

* Total = [acetoacetate + 3-hydroxybutyrate]

TABLE 5

Differences in ketone-body concentrations of hepatic and portal venous blood from normal and alloxan-diabetic sheep

Ketone body concentration differences (hepatic-portal) were computed from the data used for Table 4. Most of the figures shown are the means ± S.E.M. Those figures without S.E.M. are the means of groups comprised of both positive and negative differences. There were three animals in each group.

Diet	Treatment	Acetoacetate (μmol/ml)	3-hydroxybutyrate (μmol/ml)	Total* (μmol/ml)
100% lucerne-hay chaff	Before alloxan	-0.016 ± 0.003	0.109 ± 0.014	0.092 ± 0.016
	3 days after alloxan	0.130 ± 0.015	0.270	0.367
75% wheaten-hay chaff, 25% lucerne-hay chaff	Before alloxan	-0.021 ± 0.008	0.036	0.019
	3 days after alloxan	0.047 ± 0.013	0.210 ± 0.020	0.256 ± 0.026

* Total = [acetoacetate + 3-hydroxybutyrate]

20-fold in the lucerne-fed sheep but only about 10-fold in the wheat/lucerne-fed sheep (Table 4). The increase in concentration was largest for acetoacetate in the hepatic blood of both groups (66-fold for lucerne-fed sheep and 24-fold for the sheep on the mixed diet). Acetoacetate concentration also increased more in the portal blood than did the concentration of 3-hydroxybutyrate (Table 4). Thus there was a very marked fall in the [3-hydroxybutyrate]/[acetoacetate] ratio in hepatic blood, and a somewhat lesser decrease in the ratio in the portal blood of both groups. The ratio in both hepatic and portal blood of all sheep was about the same ([3-hydroxybutyrate]/[acetoacetate] ≃ 4) three days after alloxan treatment (Table 4).

The production of ketone bodies by the liver was markedly increased in the alloxan-diabetic condition (Table 5). In this state of high hepatic ketone body production a fairly large proportion of the net hepatic output was acetoacetate: approximately 30% of the total output for lucerne-fed sheep, and about 20% for the animals on the wheat/lucerne diet (Table 5). This is in direct contrast with the situation in normal sheep liver where there was a net uptake of acetoacetate (Table 5).

Because of two negative hepatic/portal differences for 3-hydroxybutyrate it is difficult to assess the actual extent by which total ketone body production was increased, but it is probable, from the results given in Table 5, that the increase was greater for total ketone bodies (3-hydroxybutyrate + acetoacetate) in the sheep fed on the lucerne-hay chaff diet.

I.D. Ketone-body production by isolated sheep liver mitochondria

I.D.1. Materials and methods

Animals. The sheep used were 4-year-old Merino wethers weighing between 35 and 48 kg. They were pen-fed on lucerne-hay chaff. The rats were males of the Wistar strain (250-300 g) fed on a pelleted diet for small animals (Barastoc and Co., Melbourne, Vic., Australia).

Tissue samples. The sheep were killed by severing the necks. Rats were killed by stunning followed by exsanguination. Liver samples were collected into ice-cold 0.25 M-sucrose immediately after slaughter.

Preparation of mitochondria. Homogenates of the liver were prepared (5%, w/v) in 0.25 M-sucrose at 0°C with the aid of a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 700*g* for 5 min to remove cell debris and nuclei. The supernatant fractions were centrifuged at 7,000*g* for 10 min to sediment the mitochondria. The mitochondrial pellets were washed in 0.25 M-sucrose and recentrifuged at 13,000*g* for 10 min and finally suspended in 0.25 M-sucrose.

The mitochondria prepared from sheep liver 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 isolated mitochondria were not extensively damaged.

Assay procedures. Oxygen uptake was measured polarographically with a small Clark-type oxygen electrode (Titron Instrument Co., Sandringham, Vic., Australia) as described by Snoswell (1966). The incubation mixture and assay procedures are described in Table 6.

I.D.2. Results

During the oxidation of palmitoyl-L-carnitine by sheep liver mitochondria 84 nmol of acetoacetate was formed with a concomitant uptake of 235 nmol of oxygen (Table 6). Thus acetoacetate formation accounts for 63% of the oxygen uptake observed, based on the equation:

$$\text{Palmitate} + 7O_2 \longrightarrow 4 \text{ acetoacetate} + 4H_2O$$

Similarly, acetoacetate production accounted for approximately 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 6). Similar results with rat liver mitochondria have been reported by Portenhauser et al. (1969). In contrast no 3-hydroxybutyrate was produced in the experiments with sheep liver mitochondria. This difference 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,

TABLE 6

Oxidation of palmitoylcarnitine by sheep and rat liver mitochondria and the associated production of ketone bodies

The standard mixture contained 80 mM-KCl, 20 mM-tris-chloride buffer (pH 7.4), 2 mM-$MgCl_2$, 1 mM-EDTA, 1.25 mM-AMP, 1.25 mM-sodium-potassium phosphate (pH 7.4), and 25 mg of bovine serum albumin (free from fatty acids), in a total volume of 2.5 ml. The substrate was 45 μM-palmitoyl-L-carnitine and the reaction was started by the addition of 5-7 mg of protein of sheep or rat liver mitochondria. Oxygen uptake was measured polarographically at 25°C. 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 0.2 ml of 30% $HClO_4$. Acetoacetate and 3-hydroxybutyrate in the neutralized supernatants were measured by the enzymic method of Williamson et al. (1962) with modifications as indicated in Section I.A.2. 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 1 nmol under the conditions of assay.

	Sheep			Rat		
Incubation conditions	Oxygen uptake (nmol)	Acetoacetate formed (nmol)	3-Hydroxybutyrate formed (nmol)	Oxygen uptake (nmol)	Acetoacetate formed (nmol)	3-Hydroxybutyrate formed (nmol)
Standard	235 ± 3(3)	84 ± 1(3)	N.D.(3)	180 ± 4(3)	62 ± 2(3)	5 ± 1(3)
Plus 10 min subsequent anaerobic incubation	239 ± 3(3)	87 ± 1(3)	N.D.(3)	183 ± 4(3)	32 ± 2(3)	33 ± 2(3)
*Plus 3 nmol 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)

* 10 mM-malonate and 10 μM-fluorocitrate were added to the standard incubation medium.

resulted in the production of 3-hydroxybutyrate as the main end product with rat liver mitochondria (Table 6). Again, there was no 3-hydroxybutyrate produced with sheep liver mitochondria (Table 6).

I.E. 3-hydroxybutyrate dehydrogenase activity in various tissues of the sheep and the rat

I.E.1. Materials and methods

Animals. The animals used were described in Section I.D.1.

Tissue samples. The animals were killed as described in Section I.D.1. Samples of liver, kidney, heart, rumen wall, skeletal muscle, spleen, brain and small intestine of sheep were collected into ice-cold 0.25 M-sucrose immediately after slaughter. Liver and kidney samples from rats, and cattle (at the local abattoirs), were treated similarly. Adipose tissue from sheep was collected into 0.25 M-sucrose at 37°C.

Homogenates and subcellular fractions. Homogenates (5%, w/v) of all tissues except adipose tissue and rumen epithelium were prepared in 0.25 M-sucrose at 0°C with the aid of a Potter-Elvehjem homogenizer. Mitochondria were prepared from liver and kidney-cortex homogenates as described in Section I.D.1. Using the same criteria as were applied to sheep liver mitochondria the kidney-cortex mitochondria were judged to have not been extensively damaged. Microsome and supernatant (cytosol) fractions of the liver and kidney-cortex of sheep were prepared by centrifuging at 100,000g for 30 min the supernatants obtained after sedimentation of the mitochondria. The microsomal pellet was resuspended in 0.25 M-sucrose and the supernatant represented the cytosol fraction. Homogenates of other tissues were centrifuged directly at 100,000g to give cytosol and particulate fractions.

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 0.25 M-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 0.25 M-sucrose at 37°C.

Assay procedures. 3-Hydroxybutyrate dehydrogenase activities were determined on all particulate fractions before and after a total of 2 min sonication (in 30 s periods) in an MSE ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., London S.W.1, U.K.) at 20 kHz and 1.5 A.

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 3 min with glass beads in the presence of 1% (w/v) digitonin; addition of 5% (w/v) Tween 80; exposure to 0.125 M-, 0.05 M- and 0.025 M-sucrose for 15 min at 0°C.

The 3-hydroxybutyrate dehydrogenase activity was measured in the sonicated fractions (see above) by a modification of the method of Lehninger et al. (1960). The assay mixture consisted of 33 mM-tris-HCl

buffer (pH 8.5), 1.8 mM-NAD, 50 mM-nicotinamide, 20 mM-dithiothreitol, 1 mM-$CaCl_2$, 1 mM-KCN, 0.75 mg of asolectin (Associated Concentrates Inc., New York, N.Y., U.S.A.) and 22 mM-sodium DL-3-hydroxybutyrate in a final volume of 1.0 ml. 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 366 nm 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.

I.E.2. Results

Kidney cortex was found to have the highest 3-hydroxybutyrate dehydrogenase activity of the various sheep tissues examined (Table 7). The activity in sheep kidney cortex was approximately the same as in rat kidney cortex (Table 8). However, the activity of this enzyme in sheep liver was less than 2% of that in rat liver (Table 8). 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 the activity of homogenates of liver prepared in this way was the

TABLE 7

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 text. 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

TABLE 8

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 text. 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.

	Rat		Sheep	
Tissue fraction	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

same as that obtained by the direct homogenization of fresh tissue.

The 3-hydroxybutyrate dehydrogenase activity in sheep liver and kidney cortex was found predominantly in the cytosol fraction, whereas the activity in rat liver and kidney cortex is mainly in the mitochondria (Table 8). 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 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 7), 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.

In the course of other experiments using starved sheep, some tissue samples were taken for assay of 3-hydroxybutyrate dehydrogenase activity. No differences in activity from those of normal sheep were observed.

I.F. Characterization of the 3-hydroxybutyrate dehydrogenases of sheep tissues

I.F.1. Materials and methods

Animals. The sheep used were 3-year-old Merino wethers weighing about 40 kg and pen-fed on lucerne-hay chaff.

Tissue samples and fractionation. The sheep were killed by severing the necks. Samples of liver, kidney cortex, heart, skeletal muscle and rumen epithelium were collected, and homogenates, subcellular fractions and sonicated fractions were prepared as described in Section I.E.1. except that the medium used was 0.25 M-sucrose in 23 mM-potassium phosphate buffer, pH 7.2. Phosphate-buffered sucrose was used because recovery of glutamate dehydrogenase activity is impaired in the absence of phosphate (Walter and Anabitarte, 1971).

Assay procedures. 3-Hydroxy acid dehydrogenase activities were measured as described for 3-hydroxybutyrate dehydrogenase in Section I.E.1. with either 22 mM-sodium DL-3-hydroxybutyrate or 11 mM-sodium L-3-hydroxybutyrate as substrate. Enzyme activity was also measured in the reverse direction, the reaction mixture consisting of (final concentrations) 33 mM-tris-HCl buffer (pH 7.0), 0.2 mM-NADH, 50 mM-nicotinamide, 1 mM-$CaCl_2$ and 10 mM-sodium acetoacetate in a final volume of 1.0 ml. All activities recorded were corrected for blank values.

Glutamate dehydrogenase was assayed by the method of Barker et al. (1968), but 1.5 mM-ADP was used to ensure maximal activity,

10 μM-rotenone replaced 3 mM-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. 3 ml), 33 mM-tris-HCl buffer (pH 7.0), 0.2 mM-NADH, 67 mM-nicotinamide, 1 mM-$CaCl_2$, 10 μM-rotenone and 3.28 mM-sodium acetoacetate; for 3-hydroxybutyrate oxidation (final vol. 3 ml), 33 mM-tris-HCl buffer (pH 8.5), 1.8 mM-NAD^+, 67 mM-nicotinamide, 1 mM-$CaCl_2$, 20 mM-dithiothreitol, 1 mM-KCN, 2.25 mg of asolectin (purified soya phosphatides - to meet the known requirement of mitochondrial 3-hydroxybutyrate dehydrogenase for phospholipid) and either 22 mM-sodium DL-3-hydroxybutyrate or 11 mM-sodium L-3-hydroxybutyrate. A 0.2 ml portion of tissue fraction was used in each assay and the reaction was started by the addition of substrate. After 1 h at 20°C, 0.3 ml 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) with modifications as indicated in Section I.A.2. All values presented were corrected for zero-time ketone-body concentrations.

Chemicals. The L-isomer of 3-hydroxybutyric acid was prepared from the DL-acid by the method of McCann and Greville (1962). This preparation of L-isomer contained only 1.2% of D-isomer. A

sufficiently pure sample of D-isomer could not be obtained either with this method (McCann and Greville, 1962) or with several others tried.

I.F.2. Results

The results presented in Table 9 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 activity of skeletal muscle fractions indicated extensive mitochondrial breakdown (see Section II. Appendix for a discussion of the difficulties involved in fractionating sheep skeletal muscle), so results obtained for this tissue have been omitted.

The dehydrogenase activities detected with DL-3-hydroxybutyrate as substrate (Table 9) were similar to those reported in Section I.E.2. 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 9). 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

TABLE 9

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 Section I.F.1. 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 with		
		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

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 10. 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 10) 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

TABLE 10

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 Section I.F.1. 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.

		Liver			Kidney cortex			Heart		Rumen epithelium	
Substrate	Reactant determined	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

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 used, but the amounts produced by the cytosol fractions were virtually the same (Table 10). 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 10). This is in accord with the facts that the kidney cortex has been shown to have the highest D-3-hydroxybutyrate-NAD^+ oxidoreductase activity of sheep tissues (see Section I.E.2.) and that since rumen epithelium produces D(-)-3-hydroxybutyrate from butyrate in the fed animal (Annison and Lewis, 1959) it is likely to have much higher D(-)-3-hydroxybutyrate-NAD^+ oxidoreductase activity than is readily demonstrable.

I. Discussion

The introduction of the enzymic method for the determination of blood acetoacetate and 3-hydroxybutyrate concentrations (Williamson et al., 1962) has led to renewed interest in the use of ketone body concentrations and ratios as parameters of the metabolic status of an animal or experimental system (see e.g. Mayor et al., 1967; Stephens et al., 1971; Bailey et al., 1971; Bailey and Horne, 1972). The values reported in this thesis for sheep blood appear to be the first obtained by the enzymic methods for this species.

The results presented in Section I.B.2. 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 and Robertson (1952) found, using chemical methods, that 3-hydroxybutyrate was the only ketone body that could be detected in the blood of normal dairy cattle. As was mentioned in the Introduction to this section, the results published for individual ketone body concentrations, and thus, also for the ratios, in sheep blood have been variable (Procos, 1962; Reid, 1960; Leng and Annison, 1963).

The high [3-hydroxybutyrate]/[acetoacetate] ratio in the blood of sheep and cattle reported here is probably a general characteristic of normal adult 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 in Section I.E.2. 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 (Annison and Lewis, 1959). Oxidation of fatty acids leads to a low $[NAD^+]/[NADH]$ ratio within rat heart mitochondria (Klingenberg and 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, since the enzyme is almost certainly associated with the mitochondria of the tissue. Baird et al. (1972) also found the [3-hydroxybutyrate]/[acetoacetate] ratio in the blood of normal, lactating cows to be high (9.7, more than twice the value of approximately 4 found in rats). The high values for the ratio found in ruminant blood are undoubtedly a reflection of the fact that the rumen epithelium contributes a large amount of 3-hydroxybutyrate to the blood of fed animals, while the contribution of acetoacetate from either the rumen epithelium or the liver (of fed animals) is relatively low (Katz and Bergman, 1969a).

In starved sheep it was found that the [3-hydroxybutyrate]/[acetoacetate] ratio in the blood falls markedly, particularly in the cross-bred animals used. This is in direct contrast with the results for the rat where the blood ratio rises on starvation (see e.g. Berry et al., 1965). In the rat this increase in the ratio is attributed to increased fatty acid oxidation in the liver (the major site of ketogenesis) and the consequent effect of the concentration of NADH

in the mitochondria of the hepatic cells (Williamson and Wilson, 1965). Katz and Bergman (1969a) have shown that in the sheep there is a close correlation between the concentration of free fatty acids in the blood and ketone body production by the liver, particularly in starvation. The results presented in Sections I.E.2. and I.F.2. show that sheep liver has very low D-3-hydroxybutyrate dehydrogenase activity, and in Section I.D.2. it is shown 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, as suggested previously, the normal high ratio is mainly due to the production of 3-hydroxybutyrate in the rumen epithelium; this production will diminish greatly on starvation. Secondly, on starvation the main site of ketone body production is the liver (Katz and Bergman, 1969a) and sheep liver mitochondria produce acetoacetate as the major ketone body. Once again, the fall in the ratio observed on starvation of sheep may well be a general response of ruminants. Baird et al. (1972) found a similar reduction in the [3-hydroxybutyrate]/[acetoacetate] ratio in the blood of starved cows. The fall in the ratio in the blood on starvation is also a reflection of a reduction in the hepatic ratio for both sheep (Snoswell, 1968) and cattle (Baird et al., 1972).

The results presented in Tables 4 and 5 (Section I.C.2.) for sheep fitted with cannulae in the portal and hepatic veins show that there is an uptake of acetoacetate by the liver of the normal sheep, and an output of 3-hydroxybutyrate. Katz and Bergman (1969a) obtained similar

results using fed sheep. This uptake of acetoacetate by the liver and its reduction to 3-hydroxybutyrate indicates that, at low levels of ketone body production, the activity of hepatic D-3-hydroxybutyrate dehydrogenase, although very low (see Section I.F.2.) must be sufficient to effect this conversion. The higher concentration of acetoacetate in the portal venous blood of both groups of sheep may have arisen in part from the breakdown of amino acids in the rumen. The reasons for the apparently higher rates of hepatic ketone body output by the sheep fed on lucerne compared with those of animals fed on the wheat/lucerne diet (Table 5) are not clear, but it could be that the lucerne diet results in more ketogenic amino acids being presented to the liver via the portal blood.

In marked contrast to the results obtained with normal animals, those obtained from animals three days after alloxan treatment, when hepatic ketone body production was greatly increased, show that in this condition the net hepatic ketone body output consisted of both acetoacetate and 3-hydroxybutyrate. This is indicated by the much greater depression in the [3-hydroxybutyrate]/[acetoacetate] ratio in the hepatic venous blood compared with the portal blood (Table 4). The percentages of acetoacetate in the total hepatic ketone body output (30% for lucerne-fed sheep and 20% for the sheep on a mixed diet) are similar to those reported by Katz and Bergman (1969a) for starved and ketotic sheep. Thus it seems that at high rates of hepatic ketogenesis in sheep the low activity of D-3-hydroxybutyrate dehydrogenase (see Section I.F.2.) limits the rate of conversion of acetoacetate to 3-hydroxybutyrate. The limited conversion of acetoacetate to

3-hydroxybutyrate cannot be attributed to a lowered redox-state in the sheep liver mitochondria of diabetic animals as this remains the same as in normal sheep (A.M. Snoswell, unpublished work). Moreover, the limitation is unlikely to be due to decreased activity of the D-3-hydroxybutyrate dehydrogenase in the diabetic animal as Watson and Lindsay (1972) found no significant difference in the activity of this enzyme in the livers of ketonaemic sheep compared with those of normal sheep.

The results presented in Section I.E.2. show that there is a profound difference in the activity of DL-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 DL-3-hydroxybutyrate dehydrogenase in the liver of various species although no ruminant species were examined. Baird et al. (1968) report that the activity of this enzyme in bovine liver is less than one-thirtieth of that in rat liver.

Of the tissues of the sheep examined, DL-3-hydroxybutyrate dehydrogenase activity was located predominantly in the particulate fractions of rumen epithelium, skeletal muscle, heart and brain, probably in the mitochondria. The activity in kidney cortex mitochondria was low (less than 6% of the total activity detected using

DL-3-hydroxybutyrate as substrate), while that of sheep liver mitochondria was virtually negligible (Section I.E.2.). The absence of this enzyme in liver mitochondria of sheep is particularly unusual as this organ under certain conditions produces ketone bodies in large quantities (see Katz and Bergman, 1969a) and ketone bodies are known to be produced in mitochondria (Lehninger, 1964). The experiments reported in Section I.B. 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 maximal rate by the addition of malonate and fluorocitrate to block oxidation in the tricarboxylic acid cycle and an increase in reducing potential was provided (by the addition of rotenone or by anaerobic 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 isolated sheep liver mitochondria internally generated acetoacetate is not reduced to 3-hydroxybutyrate even under reducing conditions. These results thus confirm the virtual absence of 3-hydroxybutyrate dehydrogenase from sheep liver mitochondria. Nielsen and Fleischer (1969) also showed that ruminant liver mitochondria are virtually devoid of 3-hydroxybutyrate dehydrogenase.

In the assay system for 3-hydroxybutyrate dehydrogenase the substrate used was DL-3-hydroxybutyrate (Section I.E.1.), and when activity was found in the cytosol of various tissues, notably kidney, it was assigned to D-3-hydroxybutyrate dehydrogenase (Koundakjian and Snoswell, 1970). This assumption was apparently further substantiated

by finding that isolated sheep liver mitochondria produced only acetoacetate during the oxidation of palmitoyl-L-carnitine, but when an aliquot of sheep liver cytosol was added to the incubation mixture, D-3-hydroxybutyrate was detected as one of the reaction products (Section I.D.2 and Koundakjian and Snoswell, 1970). However, as stated in the Introduction, Williamson and Kuenzel (1971) pointed out that the soluble enzyme activity of sheep kidney cortex is in fact that of an L-3-hydroxy acid dehydrogenase. The results presented in Section I.F.2. for the distribution of the two enzymes in various sheep tissues indicate that 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 ones 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.

It can only be assumed that, in the work referred to above, where the addition of sheep liver cytosol to sheep liver mitochondria resulted in the apparent formation of D-3-hydroxybutyrate during fatty acid oxidation, the subtraction of control values from the test values was omitted since sheep liver cytosol has been found (Koundakjian and

Snoswell, unpublished work) to have concentrations of D-3-hydroxybutyrate sufficiently high to account for the published results of Koundakjian and Snoswell, (1970).

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 and Kuenzel (1971) have shown that the Km value of the kidney-cortex enzyme for acetoacetate is high.

The results of Watson and Lindsay (1972) on the distribution of the D- and L-3-hydroxybutyrate dehydrogenases in sheep tissues confirm that the soluble enzyme of kidney cortex is an L-3-hydroxy acid dehydrogenase, and Zawierucha et al. (1972) have partially purified the enzyme from sheep kidney cortex and showed that it reacts only with DL-3-hydroxybutyrate and D-gluconate, not with D-3-hydroxybutyrate. However, the results of Watson and Lindsay (1972) suggest that the soluble enzyme activity of sheep liver and rumen epithelium is that of D-3-hydroxybutyrate dehydrogenase. This seems unlikely in view of the fact that they found no stimulation of the activity of this enzyme by phosphatidylcholine whereas the enzyme as purified from bacteria (this thesis), beef heart mitochondria (Sekuzu et al., 1963) or rat liver mitochondria (Gotterer, 1967) has a definite requirement for added phospholipid in order to obtain maximum activity. As stated

in Section I.E.2., phospholipid was routinely included in the assay mixture of all tissue fractions used in this study, but it was found that the cytosol dehydrogenase did not in fact require this addition for maximal activity. It is possible that the D-3-hydroxybutyrate used by Watson and Lindsay (1972) was more heavily contaminated with the L-isomer than they detected.

Another soluble and relatively non-specific L-3-hydroxy acid dehydrogenase has been purified from Drosophila (Borack and Sofer, 1971). This enzyme was found to react with L-3-hydroxybutyrate and L-gulonate, but not with the corresponding D-isomers. It seems probable that all soluble L-3-hydroxybutyrate dehydrogenases can be equated with the L-gulonate dehydrogenase discovered in pig kidney cytosol by Smiley and Ashwell (1961) i.e. they are 3-hydroxy acid dehydrogenases for which the only known specificity criterion is that the hydroxyl group is in the L-configuration.

The activities reported by Dhand et al. (1970) for DL-3-hydroxybutyrate dehydrogenase in homogenates of the livers of adult Dorset Horn sheep are up to four times as high as those reported for Merino sheep (this study and Watson and Lindsay, 1972) and Clun Forest wethers (also in Watson and Lindsay, 1972). These higher apparent activities could be due in part to the higher temperature used for assay (37^{o}C as compared with 20^{o}C in this study and 30^{o}C used by Watson and Lindsay, 1972), though the activities reported by Watson and Lindsay (1972) for liver were only fractionally different from those found in this study despite a temperature difference of 10^{o}C. The assay systems used in all three studies were essentially the same. Thus, it is possible

that the differences in activity are a reflection of the breed difference, though whether the activity differences are those of D- or L-3-hydroxybutyrate dehydrogenase, or of both, cannot be assessed from the published results.

The question of the inducibility of D-3-hydroxybutyrate dehydrogenase (activity) by different metabolic states in non-ruminants has not been satisfactorily solved. The brain enzyme has been most studied as Owen et al. (1967) have shown that the human brain is able to oxidise ketone bodies in place of some glucose during prolonged fasting. Adult rat brain 3-hydroxybutyrate dehydrogenase activity has been shown to increase to a maximum on fasting for 72 h, though the activity falls again by 120 h (Smith et al., 1968), and also to be unchanged by starvation (Williamson et al., 1971; Pull and McIllwain, 1971). Thaler (1972) using pregnant rats found no change in the activity of maternal rat brain 3-hydroxybutyrate dehydrogenase on starvation, but the activity of the foetal brains was increased. These different results cannot be attributed to strain differences of the rats used since all studies except that of Williamson et al. (1971) used Sprague-Dawley rats. Assay conditions were comparable in all cases.

The situation is not much clearer with rat liver - Williamson et al. (1971) found little change in 3-hydroxybutyrate dehydrogenase activity of liver on starvation (a very slight rise was observed in the mean value of two animals). Studies of Schäfer and Nägel (1968) suggest that insulin could increase the activity of the enzyme in rat liver mitochondria and those of Boveris et al. (1969) that removal of the pancreas causes a fall in activity. However, the latter two

studies used different techniques for assessment of enzyme activity and so are probably not strictly comparable with that of Williamson et al. (1971). But Roldan et al. (1971), using dogs, found that removal of the pancreas led to a 50% fall in hepatic 3-hydroxybutyrate dehydrogenase activity, which, together with the blood parameters, was restored to normal by insulin. Growth hormone administration caused increased blood ketone body, glucose and free fatty acid concentrations with no alteration in the activity of the enzyme under discussion. In this study (Roldan et al., 1971) the method used for assaying enzyme activity was similar to that of Lehninger et al. (1960), which is the method used by the majority of workers. Thus it is possible that the liver enzyme of non-ruminants is inducible and could be controlled by insulin concentrations or by the concentrations of metabolites affected by insulin.

In sheep tissues there is no change in enzyme activity induced by starvation in wethers (this thesis) or by starvation of pregnant sheep (Watson and Lindsay, 1972). It is therefore apparent that increased ketogenesis and ketone body utilization in starved sheep are functions of increased substrate availability, as suggested by Williamson et al. (1971) for ketotic rats.

D-3-hydroxybutyrate dehydrogenase is associated only with the inner mitochondrial membrane, at least in rat liver (Schnaitman et al., 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 and activity 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 activity in this tissue is extremely low. Ballard, Hanson and 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 activity is very low.

The utilization of ketone bodies by the extrahepatic tissues is, of course, very important in the ketotic animal, and the rate of utilization could well be expected to have an effect on the apparent degree of "ketosis" observed. Krebs (1966) stated that the ketone bodies form a readily oxidisable fuel to be used when the supply of glucose is restricted, and that the mild ketosis of e.g. starvation is a physiological process designed to maintain caloric homeostasis.

Recently there has been renewed interest in ketone body utilization, and in the enzymes involved, in both ruminants and non-ruminants. Williamson et al. (1971) after studying changes in two key enzymes of acetoacetate utilization in various rat tissues, concluded that the rate of ketone body utilization is a function of substrate concentration rather than changes in enzyme activities. This implies that ketone body utilization rate should increase as ketogenesis is

increased. Whilst this may be true during mild ketosis, the system seems to break down in severe ketosis. Bässler et al. (1972) showed that, with no change in renal elimination, the blood concentration of acetoacetate of normal rats rose slowly and linearly when acetoacetate was infused at rates of 10 to 150 μmoles/min per kg, while that of streptozotocin-diabetic rats rose linearly up to infusion rates of 50 μmoles/min per kg and exponentially thereafter. Chronic insulin treatment restored a normal pattern. These findings imply that utilization of ketone bodies is impaired in diabetes and that the impairment is due to lack of insulin. Later work of Bässler et al. (1973) showed that acetoacetate oxidation by skeletal muscle mitochondria was reduced when the animal was made diabetic. Insulin administration to the donor animals raised the oxidation rate, but it did not reach control values. Bässler et al. (1973) found that the 3-oxo acid CoA-transferase activity of skeletal muscle was reduced in starved and diabetic animals, while the activity in heart and kidney was unaffected (though the activity in brain rose on starvation) and it is suggested that this loss of activity in skeletal muscle mitochondria is responsible for the phenomena observed by Bässler et al. (1972) - above. Unfortunately, Williamson et al. (1971) did not examine the transferase activity of skeletal muscle from alloxan-diabetic rats, but they did find a significant rise in this activity in the skeletal muscle of rats starved for 48 h (Bässler et al., 1973, starved their rats for 6 to 8 days). The rise in activity at 48 h reported by Williamson et al. (1971) could mean that tissue (skeletal muscle) utilization of ketone bodies is enhanced early in starvation

ketosis, but it may later be inhibited as suggested by Bässler et al. (1973). Why and how the enzyme activity could be inhibited is not known, and this aspect should be examined in more detail. It is also obvious that the time course of events regarding ketone body utilization in rat tissues should be studied more closely.

Contrary to the unlikely claim of Bässler et al. (1972) that the rate of renal elimination of ketone bodies by rats is unchanged in diabetes (for which no reference is given), Galvin et al. (1968) found, using human subjects, that although the [3-hydroxybutyrate]/[acetoacetate] ratio in the blood remained constant as ketosis developed, during the progress of the condition more 3-hydroxybutyrate was excreted in the urine than acetoacetate, the rate of increase of extraction of 3-hydroxybutyrate being exponential above a certain plasma concentration while that of acetoacetate remained linear. In minimal ketosis more acetoacetate was excreted than 3-hydroxybutyrate. As there was no change in creatinine clearance, these results were interpreted as meaning that renal reabsorption of both compounds can occur, with that for acetoacetate being maximal in ketosis. In view of the maintained [3-hydroxybutyrate]/[acetoacetate] ratio in the blood of the subjects in this study it is possible that extrahepatic tissue metabolism of acetoacetate is much greater than metabolism of 3-hydroxybutyrate. The relative activity of 3-hydroxybutyrate dehydrogenase could be a regulatory factor in this case.

Balasse and Havel (1971) have used dogs made ketonaemic by acetoacetate infusion in comparison with pancreatectomized dogs in order to investigate the effect of insulin on peripheral utilization of

ketone bodies. Their results indicate that extrahepatic ketone body utilization is increased by insulin and that impaired utilization in the absence of insulin is a major factor in the development of severe ketosis.

Because of the variety of monogastric animal species used in studies on the control of ketone body utilization and the comparative sparsity of such studies it is difficult to come to any definite conclusions concerning this topic. It is apparent that the metabolism of the brain adapts to increased ketone body utilization, though whether or not this adaptation involves increases in enzyme activity or concentration is not clear. As far as the other extrahepatic tissues are concerned it is evident that insulin plays a major role in the control of ketone body oxidation, and that renal excretion may play an important part in the regulation of the amounts of each ketone body available for oxidation. At relatively low rates of ketogenesis the rate of utilization in the tissues can apparently be increased, and the increased rate is probably simply due to a rise in the flow rate through the pathway. But once plasma ketone body concentrations reach a critical level the system breaks down and severe ketonaemia can develop unhindered.

In bovine ketosis the situation regarding ketone body utilization is complicated by the fact that the ketone bodies may contribute to the synthesis of milk fat. The work of Palmquist et al. (1969) indicates that 3-hydroxybutyrate can contribute up to 8% of the total fatty acid carbon of milk in normal cows, and both Palmquist et al. (1969) and Smith and McCarthy (1969) found that 3-hydroxybutyrate is mainly incorporated as a 4C unit in ruminants. 3-hydroxybutyrate has also

been shown to be able to contribute to milk fat in goats (Hawkins and Williamson, 1972). In non-ruminants incorporation is via 2C units (Smith and McCarthy, 1969; Kinsella, 1970). It is possible that, at least during the early stages of bovine ketosis, the utilization of 3-hydroxybutyrate by the udder could be increased, though as milk yield and fat content tend to fall dramatically in severe bovine ketosis it is doubtful that this route for 3-hydroxybutyrate removal is operative under these conditions.

Leng (1965) showed that for sheep, the entry rate of 3-hydroxybutyrate was linear with the blood concentration up to a critical value, after which small increases in entry rate led to very large increases in blood concentration. Since he found that the contribution of 3-hydroxybutyrate to expired CO_2 was raised in starved pregnant sheep compared with non-pregnant sheep, it was concluded that the raised blood ketone concentration of pregnant ketotic sheep was due only to increased ketogenesis, not to impaired utilization. In later work, Leng (1966) measured excretion rates of 3-hydroxybutyrate as well as entry rates, and the difference between the two was taken to represent the rate of utilization. From this study it was concluded that there might in fact be some inhibition of tissue utilization of 3-hydroxybutyrate in starved pregnant sheep, as infusion of insulin or glucose plus insulin led to stimulation of utilization (Leng, 1966).

As 3-hydroxybutyrate is the major circulating ketone body in ruminants, and since it must be oxidised to acetoacetate before being utilized for energy-producing purposes, it is highly likely that the 3-hydroxybutyrate dehydrogenase activity of the extrahepatic tissues (except perhaps rumen epithelium) is involved in ketone body

utilization. Thus, the results presented in this thesis for D-3-hydroxybutyrate enzyme activities of sheep tissues indicate that kidney cortex is probably a major tissue for oxidation of ketone bodies in the sheep. Leng and 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 and Krebs (1969b) have shown that ketone bodies are a preferred metabolic fuel for rat kidney. Other sheep tissues found to have relatively high D-3-hydroxybutyrate dehydrogenase activities (this study) are skeletal muscle, brain and heart. The fact that brain can adapt to oxidising ketone bodies has already been discussed, and rat heart has been shown to preferentially utilize acetoacetate in the presence of other substrates (Williamson and Krebs, 1961). The skeletal muscle represents the largest tissue mass responsible for ketone body utilization, and it has been suggested that this tissue of rats is subject to control of ketone body oxidation by insulin (Bässler et al., 1973). Although the activity of 3-hydroxybutyrate dehydrogenase of rat skeletal muscle is low compared with rat liver (Lehninger et al., 1960), the activity of sheep muscle (this study) is higher than that of rat (38 v 23 μmol/h per g of tissue) and it is second in activity to kidney cortex (excluding rumen epithelium). Thus, it is obvious that sheep skeletal muscle has, on this basis, an enormous capacity for oxidation of 3-hydroxybutyrate. It would be interesting to examine the ability of this tissue to oxidise ketone bodies in vitro, from animals in various metabolic states, to evaluate the changes which might occur.

The only enzyme of ketone body utilization to have been studied in ruminants in different metabolic states is 3-hydroxybutyrate dehydrogenase of sheep tissues, and the activity of this does not change with ketonaemia (Watson and Lindsay, 1972) or starvation (this study). As normal ruminants are likely to be well adapted to ketone body utilization it is probably not surprising to find that there is no enzyme adaptation to metabolic stress by these animals (as far as is known), and it is likely that one of the causes of the severe ketonaemia of bovine ketosis and pregnancy toxaemia is relatively diminished tissue utilization of ketone bodies even though the contribution to expired CO_2 is increased. The controlling factor might also be insulin in these animals, and it seems probable that the control is exerted at some stage after the oxidation of 3-hydroxybutyrate. In bovines, the situation could also be influenced by changes in the amount of 3-hydroxybutyrate used by the mammary gland.

The causes of the physiological and neurological effects of pregnancy toxaemia and bovine ketosis are still the subject of controversy. Kronfeld (personal communication to A.M. Snoswell) argues strongly in favour of hypoglycaemia being the cause of the clinical manifestations of acetonaemia and of the early stages of pregnancy toxaemia. Recent work by Heitzman et al. (1972), with cows made ketotic by thyroxine administration, shows that cows can show clinical signs of ketosis whilst having normal blood glucose concentrations. Kronfeld (personal communication) made the observations that the 3-hydroxybutyrate concentration in the blood of cows is always (relatively) high and bears no relationship to clinical signs, but

that a rise in blood acetoacetate concentration is often, but not always associated with such signs. His work (Kronfeld et al., 1968) showing that the mammary gland of lactating cows can take up 3-hydroxybutyrate and release an equivalent amount of acetoacetate into the blood stream; and the work reported in this thesis showing that the [3-hydroxybutyrate]/[acetoacetate] ratio in the blood of ruminants falls on starvation or in alloxan diabetes, might be important in this respect. If the ruminant kidney behaves as the human kidney (Galvin et al., 1968) in excreting 3-hydroxybutyrate while reabsorbing acetoacetate at high plasma concentrations, this also could influence the ratio of the ketone bodies in the blood. Heitzman et al. (1972) found that it was possible to induce clinical ketosis with thyroxine only in cows having elevated 3-hydroxybutyrate concentrations in the blood prior to treatment, but that the appearance of clinical signs was accompanied by a 16-fold increase in blood acetoacetate concentration, with little change in the concentration of 3-hydroxybutyrate. It is suggested that the finding of raised 3-hydroxybutyrate concentrations in the blood of cows might provide a means of screening for animals susceptible to clinical ketosis, but that the clinical syndrome appears to be associated with the acetoacetate concentration (Heitzman et al., 1972).

The statement of Behnke (1964) that the accumulation of acetoacetate depresses the functioning of the central nervous system in ketosis, as the acidosis of 3-hydroxybutyrate accumulation is readily compensated, could not be substantiated on correspondence with him. However, if it is true that acetoacetate is the causative agent in the brain depression of ketosis, it could be argued that the 3-hydroxy-

butyrate dehydrogenase of liver (and rumen epithelium) is part of a detoxifying process for what is otherwise a great potential source of energy. The relative lack of this enzyme in ruminant liver may be one reason why these animals are more prone to severe ketosis than non-ruminants.

PART II

The metabolism of carnitine and coenzyme A in various tissues of normal and alloxan diabetic sheep

II. Introduction

Carnitine was discovered by two independent groups in 1905 (Gulewitsch and Krimberg, 1905; Kutscher, 1905), but it was not until 1952 that any function was assigned to it (below). Some details of the chemistry and history of carnitine are given in the Introduction to Part III, and the subject has been reviewed by Fraenkel and Friedman (1957). Carter (1952) established the identity of carnitine with vitamin B_T, a nutritional requirement of the meal worm Tenebrio molitor (Fraenkel, 1948; Fraenkel et al., 1950); and it has since been shown that carnitine is a vitamin for the larvae of several members of the family Tenebrionidae (see Fraenkel and Friedman, 1957). This requirement of some larvae led to the development of biological assays for carnitine which resulted in the examination of a large number of plant and animal materials for carnitine content. Fraenkel and Friedman (1957) list the carnitine concentrations of several tissues of plants and animals; the highest amounts were found in the skeletal muscles of vertebrates, with beef muscle extract having the greatest carnitine concentration.

The possible involvement of carnitine in fat metabolism

At the time of the review by Fraenkel and Friedman (1957) it was suggested that carnitine might be involved in fat metabolism. This suggestion was based partly on the discovery of an enzyme in pigeon liver which catalysed the formation of acetylcarnitine (Friedman and Fraenkel, 1955). Also, Bhattacharyya et al. (1955) showed that 4-butyrobetaine was the only carnitine analogue which inhibited

carnitine utilization by Tenebrio larvae (4-butyrobetaine differs from carnitine only in lacking the 3-hydroxyl group, which is the point of acetylation). The work of Fritz (1955) demonstrated that the effect of the factor present in muscle extracts which caused increased oxidation of palmitic acid by liver slices and homogenates was identical with that of carnitine. The involvement of carnitine in fatty acid metabolism has since then been fully substantiated and is discussed in some detail below.

Other functions assigned to carnitine

The wide distribution of carnitine in biological materials has, over the years, led to its implication in many physiological phenomena. Fraenkel and Friedman (1957) reviewed attempts made to establish the pharmacological activity of carnitine. Bender and Adams (1962) investigated a number of the physiological functions assigned to carnitine, which by this date were numerous and included stimulation of appetite, growth, and secretion of salivary, gastric, pancreatic and bile juices. (References to these and several other suggested functions can be found in the paper of Bender and Adams, 1962). These workers investigated the effects of carnitine on the growth rate of rats and on the stimulation of protein synthesis and gastric secretion. They also looked for carnitine in "lipocaic" (an extract of pancreas) and in muscle and liver phospholipids, as these had been reported to contain carnitine (Binon and Deltour, 1956a,b). No stimulatory effects of carnitine were found on growth, protein synthesis or gastric secretion, and it seemed that the "carnitine" identified by chemical

methods in lipocaic and phospholipids was almost certainly choline (Bender and Adams, 1962).

More recently, it has been shown that carnitine can be utilized by the larvae of the blowfly, Phormia regina (which do not require carnitine as a vitamin). As a result of this metabolism of carnitine some of the tissue phospholipids contain 3-methylcholine, the decarboxylation product of carnitine, instead of choline (Bieber et al., 1963). In these experiments carnitine was fed in place of choline in the diet, so it seems extremely unlikely that such a usage of carnitine would normally occur. It is interesting, however, that these animals can utilize carnitine in place of choline, and it would be of further interest to know whether the carnitine decarboxylating enzyme is normally present (active) or whether it is induced by carnitine feeding.

All other functions postulated for carnitine are involved in fat metabolism and fall into three main groups. Firstly, those in which carnitine directly stimulates fatty acid oxidation and this itself has an effect on the parameter observed (e.g. increased hepatic gluconeogenesis or ketogenesis). The subject of carnitine stimulation of fatty acid oxidation is discussed in some detail below.

Secondly, those systems in which carnitine "spares" CoA by acylcarnitine formation. For instance, Hülsmann et al. (1966) showed that the stimulation by carnitine of acetoacetate oxidation in mitochondria from kidney and heart was due to a CoA sparing effect. Also Weidemann and Krebs (1969a) reported that the stimulatory effects of carnitine on gluconeogenesis from propionate in rat kidney cortex

are due in part to sparing of extramitochondrial CoA for propionate activation.

The third main category of the functions of carnitine in fat metabolism is that of acyl-group transfer into the mitochondria, for purposes other than oxidation, or out from the mitochondria. In the study of Weidemann and Krebs (1969a), it was shown that carnitine also stimulates gluconeogenesis from propionate by facilitating the transfer of propionate, as propionoylcarnitine, into the mitochondria for carboxylation of the re-formed propionoyl-CoA to methylmalonoyl-CoA. The stimulation by carnitine of fatty acid synthesis from precursors other than acetate is attributed to the removal of intramitochondrial acetyl-CoA, arising from metabolism of these precursors, from the mitochondria as acetylcarnitine (Bressler and Katz, 1965). Whether or not such a system would contribute much to fatty acid synthesis *in vivo* in tissues with active citrate transfer and cleavage is doubtful. The complex subject of carnitine and long-chain acylcarnitine involvement in fatty acid synthesis from acetate and citrate in rat liver preparations has been extensively studied by Marquis *et al.* (1967).

Carnitine, carnitine palmitoyltransferase and long-chain fatty acid oxidation

The function of carnitine in fatty acid oxidation has been reviewed several times in recent years (e.g. Fritz, 1961; 1963 and 1967; Garland *et al.*, 1969 and Bressler, 1970a,b), and details of the discovery that carnitine stimulates ketogenesis and CO_2 production from long-chain fatty acids *in vitro* are to be found in the paper by Fritz (1955). That carnitine was also effective in stimulating

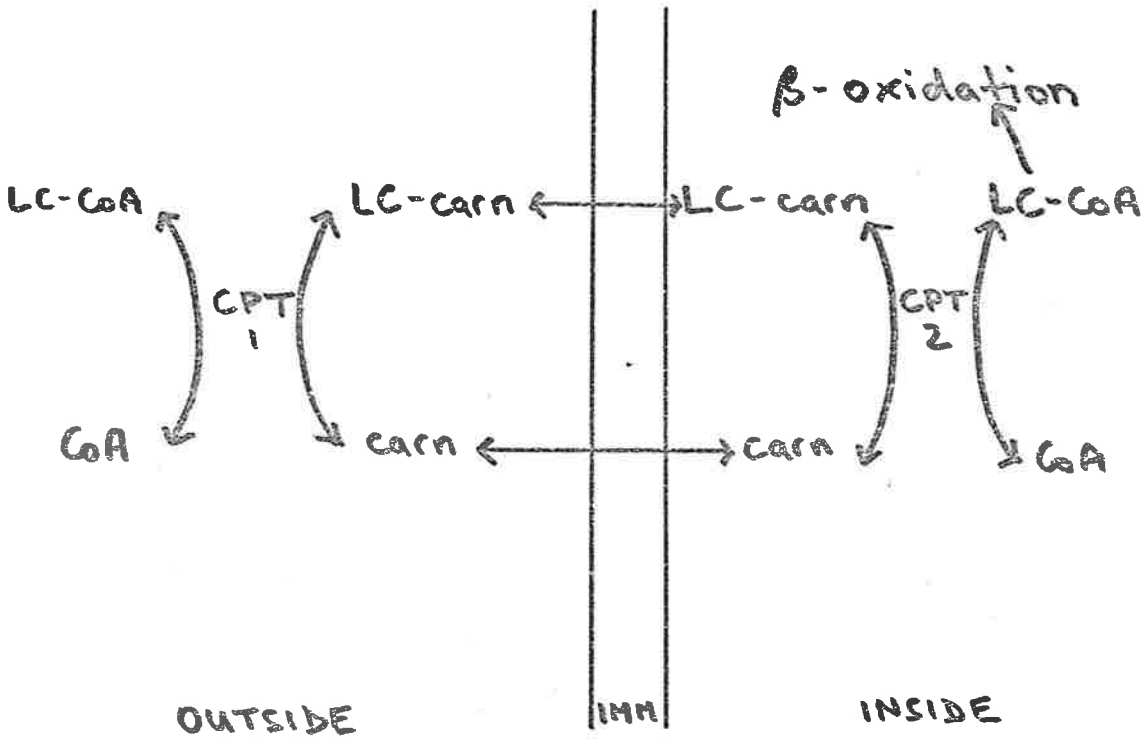
long-chain fatty acid oxidation *in vivo* was shown by Miller and Krake (1962), but at that time the site of carnitine action was still unknown. Although the enzyme carnitine acetyltransferase was discovered by Friedman and Fraenkel in 1955, it could not be directly implicated in the carnitine-induced stimulation of fatty acid oxidation since this was confined mainly to long-chain fatty acids. Fritz *et al*. (1963) showed that carnitine acetyltransferase would not utilize palmitoyl-CoA, though the stimulation of long-chain fatty acid oxidation by acetylcarnitine was postulated to involve this enzyme (Fritz, 1963). The above observations and others (such as the failure of carnitine to stimulate the conversion of palmitoyl-CoA to CO_2 in lysed liver particles, the demonstration of palmitoyl-CoA formation from palmitoyl-carnitine, and the discovery that fatty acyl-CoA esters are not oxidised by intact mitochondria) led Fritz and Yue (1962) to conclude that there must be an enzyme which could catalyse the reaction:

$$\text{palmitoylcarnitine} + \text{CoA} \rightleftharpoons \text{palmitoyl-CoA} + \text{carnitine}$$

Fritz and Yue (1963) later showed that this enzyme did exist in rat heart preparations, and they proposed that such a transferase could effect the transfer of palmitate, as the palmitoyl-carnitine ester, from the extramitochondrial site across the acyl-CoA-barrier to an intramitochondrial site for oxidation. It was suggested that two carnitine transacylase sites would be required, one for acylcarnitine formation at the acyl-CoA-barrier, and one within the barrier for re-formation of acyl-CoA. The scheme is illustrated in Fig. 3. Bremer (1963) provided further evidence for the existence of a carnitine

FIG. 3

A postulated mode of activity for carnitine palmitoyltransferase



IMM = inner mitochondrial membrane
LC = long-chain fatty acyl group
CPT_1 = outer carnitine palmitoyltransferase
CPT_2 = inner carnitine palmitoyltransferase

palmitoyltransferase and detected activity in the microsomes and mitochondria of rat liver, and Norum (1964) purified the enzyme from calf-liver mitochondria.

The scheme of Fritz (1963), illustrated in Fig. 3, used to explain the effect of carnitine on fatty acid oxidation was widely adopted and is included in very recent reviews (e.g. Bressler, 1970a,b) with little alteration, even though Fraenkel (1954) had shown that muscle sarcosomes contain little or no carnitine, and Garland and Yates (1967) found that the permeable space of rat liver mitochondria is the same for carnitine as it is for acyl-CoA. These findings are incompatible with transfer of acylcarnitine to the mitochondrial matrix and the subsequent release of carnitine via an intramitochondrial carnitine acyltransferase. Yates and Garland (1966) suggested the possibility of the enzyme being vectorially mounted in the inner mitochondrial membrane, so that carnitine and acylcarnitine would be confined to the outer mitochondrial region, in order to account for the results of permeability studies. They also proposed that an enzyme thus situated would be latent towards added palmitoyl-CoA and carnitine, but not to palmitoylcarnitine nor to endogenous palmitoyl-CoA plus added carnitine. If this was the case, then there must be a second carnitine palmitoyltransferase located outside the inner mitochondrial membrane which would not be latent to added palmitoyl-CoA and carnitine. Their results (Yates and Garland, 1966) apparently substantiated these predictions, although no marker enzyme studies were included, and showed that most of the palmitoyl-transferase activity was associated with the inner mitochondrial membrane (about 90%) and the remainder was "soluble". The fact that

the enzymes from the two sites had different K_m values for carnitine and K_i values for palmitoyl-D-carnitine suggests that they might be two distinct enzymes.

A scheme involving a vectorially mounted transferase also necessarily involves postulating two mitochondrial pools of CoA. A mechanism for carnitine participation in long-chain fatty acid oxidation involving a vectorial transferase and only one CoA pool was suggested by Garland et al. (1969). This involved a hypothetical vectorially mounted palmitoylcarnitine ligase (ATP specific) and a palmitate acceptor (AH) other than free CoA. However, such a scheme does not take account of the fact that acyl-CoA is formed extramitochondrially and ignores the existence of the non-latent carnitine palmitoyltransferase (which was first demonstrated by the same research group).

Recent work using inhibitors and more sensitive assay methods has proved that carnitine palmitoyltransferase exists as two enzymes at two locations. Yates and Garland (1970) showed that only one-fifth of the total carnitine palmitoyltransferase of intact rat liver mitochondria was assayable, and they found that this enzyme was inhibited by 2-bromostearoyl-CoA, whereas the latent enzyme was not. Chase and Tubbs (1972) showed that carnitine-dependent oxidation of palmitate or palmitoyl-CoA was inhibited by 2-bromopalmitoyl-CoA, and the oxidation of palmitoylcarnitine in intact rat liver mitochondria was unaffected. The oxidation of all three substrates was inhibited by 2-bromopalmitoyl-carnitine in intact mitochondria and by 2-bromopalmitoyl-CoA in broken mitochondria. These results were interpreted as indicating that there are two distinct carnitine palmitoyltransferases,

the outer one being susceptible to inhibition by the 2-bromoacyl esters of both CoA and carnitine, and the inner one inhibited only by the carnitine ester.

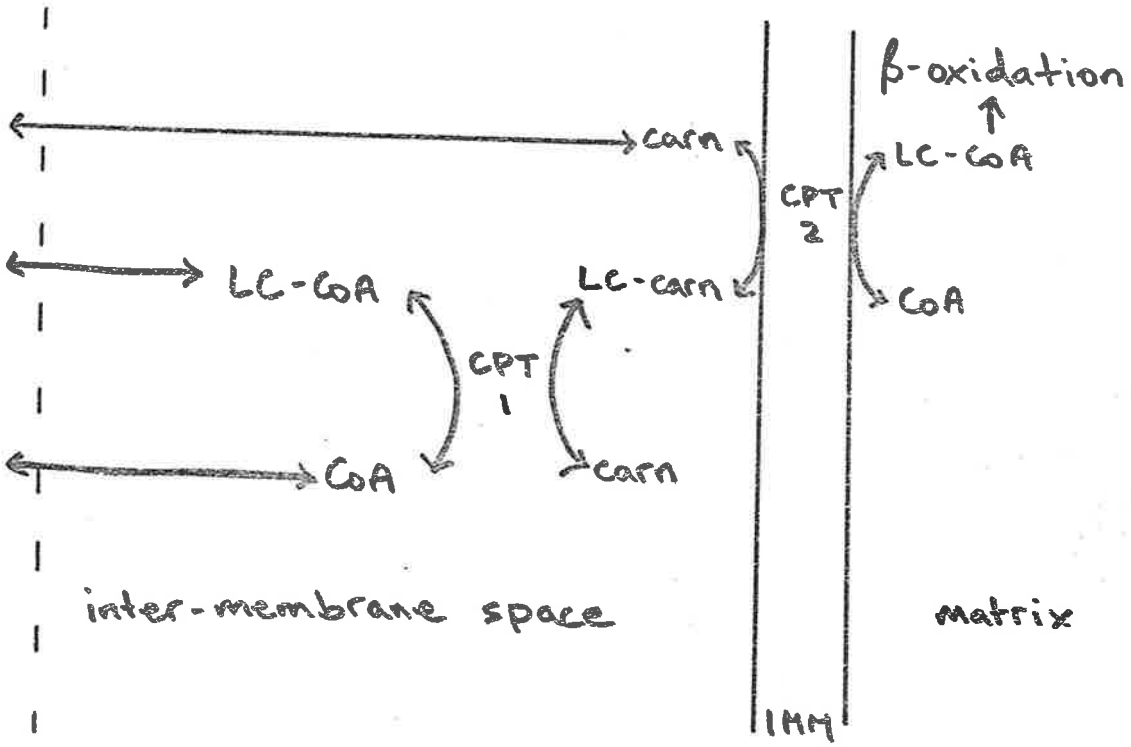
Thus the pathway of carnitine-stimulated long-chain fatty acid oxidation may be outlined as follows: long-chain acyl-CoA is formed extramitochondrially using ATP as the energy source and enters the intermembrane space of the mitochondrion. The carnitine ester is formed from the CoA ester *via* the outer carnitine palmitoyltransferase. The vectorially mounted transferase of the inner mitochondrial membrane then catalyses the production of acyl-CoA within the matrix for β-oxidation. The scheme is illustrated in Fig. 4. This system seems to be operative for long-chain fatty acid oxidation in several rat tissues, and presumably the recently discovered carnitine octanoyltransferase (Solberg, 1971 and 1972) functions in a similar manner for carnitine-dependent oxidation of medium-chain fatty acids.

Carnitine-independent oxidation of long-chain fatty acids

The oxidation of long-chain fatty acids by rat liver mitochondria can be studied under conditions which render the process independent of added carnitine (Greville and Tubbs, 1968). Rossi *et al.* (1967) showed that this occurs while endogenous ATP or GTP is still available. The GTP-dependent activating system is intramitochondrial, and studies with inhibitors showed that there are two mitochondrial ATP-dependent systems (Rossi *et al.*, 1967). Van Tol and Hülsmann (1970) also demonstrated two ATP-dependent fatty acid activating systems in rat liver mitochondria, in a study making extensive use of marker enzymes;

FIG. 4

A postulated mode of activity for carnitine palmitoyltransferase having the enzyme vectorially mounted



CPT 1 = outer carnitine palmitoyltransferase
CPT2 = inner carnitine palmitoyltransferase
IMM = inner mitochondrial membrane
carn = L-carnitine
LC = long-chain fatty acyl group

and they found that 90% of the activity was associated with the outer mitochondrial membrane and was dependent on carnitine for oxidation of the formed acyl-CoA. The other 10% was associated with the inner mitochondrial membrane and carnitine-independent fatty acid oxidation. Carnitine-independent oxidation of fatty acids by isolated mitochondria is always associated with the use of endogenous CoA, while carnitine-dependent oxidation utilizes exogenous CoA (Yates et al., 1966).

It is not known whether the carnitine-dependent or the independent pathway is the one functional in vivo, or perhaps both could be functional, the one to be used at any given time being directed by the metabolic state of the tissue. Carnitine palmitoyltransferase activity has been shown to increase in the livers of fasted, fat-fed and diabetic rats (Norum, 1965) and the long-chain fatty acylcarnitine content of rat tissues has been shown to increase (as does long-chain acyl-CoA) in conditions where fatty acid oxidation is increased, such as alloxan diabetes and starvation (Pearson and Tubbs, 1964a and 1967; Bøhmer et al., 1966). Williamson, J.R. et al. (1968) showed that the oxidation of oleate by perfused liver could be inhibited by the carnitine palmitoyltransferase inhibitor, decanoyl-(+)-carnitine, though the formation of oleoyl-CoA was unaffected. Thus it seems likely that long-chain fatty acid oxidation in vivo is dependent on carnitine and carnitine palmitoyltransferase.

However, this conclusion still leaves a number of questions concerning the role of carnitine unanswered. For instance, the original function established for carnitine was, as mentioned previously, as a vitamin for the larvae of Tenebrio molitor. Fritz (1961 and 1962)

found that carnitine-deficient Tenebrio larvae were able to oxidise labelled palmitate to CO_2 equally as well as control animals, and that whole larval homogenates or larval muscle homogenates showed no requirement for added carnitine in order to obtain maximal rates of palmitate oxidation when prepared from either deficient or control larvae.

Carnitine acetyltransferase

Another unanswered question concerns the function of carnitine acetyltransferase. As short-chain fatty acids are of minor importance in monogastrics, and since these fatty acids, if present, could be oxidised independently of carnitine, it seems unlikely that the enzyme is directly involved in fatty acid oxidation. Fritz and Yue (1964) did show that added carnitine increased the rate of oxidation of external acetyl-CoA by isolated rat heart mitochondria, but not that of free acetate. However carnitine stimulation of acetyl-CoA oxidation is unlikely to occur in the intact tissue, as the acetate activating enzyme of rat heart is mainly associated with the mitochondria (Ballard, 1972).

It appears to be generally agreed that carnitine acetyltransferase is, like carnitine palmitoyltransferase, associated mainly with the mitochondria. As with carnitine palmitoyltransferase, it was at first difficult to pinpoint the exact location of the enzyme within the mitochondria. The work of Beenakkers and Klingenberg (1964) suggested that the enzyme was "soluble", while Brdiczka et al. (1969) found in a study making extensive use of marker enzymes with pig kidney and rat

liver mitochondria that 25% of the activity was in the outer mitochondrial compartment and 75% in the inner compartment. They suggested a dual localization for carnitine acetyltransferase (as had been proposed by Beenakkers and Henderson, 1967), in order to explain the way in which carnitine and the enzyme enable acetyl groups to cross the inner mitochondrial membrane, which is impermeable to acetyl-CoA. This scheme involves a matrix-situated carnitine acetyltransferase and also the presence of carnitine and acetylcarnitine within the mitochondrial matrix. The scheme is therefore untenable because of the impermeability of the mitochondrial matrix to carnitine and the failure to detect carnitine within mitochondria (as was detailed above for a similar scheme proposed for carnitine palmitoyltransferase). Barker et al. (1968) showed that the carnitine acetyltransferase of guinea-pig liver may be largely or completely membrane bound (to the inner mitochondrial membrane) and that only about 7% of the total activity of disrupted mitochondria was detectable in intact mitochondria. Proportions of up to 15% of the total activity were detected in intact liver and mammary gland mitochondria from guinea-pig, goat and sheep. Because the enzyme activity of rat liver and mammary gland was comparatively very low, activity measured in intact mitochondria was considered to be unreliable (30% of the total activity for liver and 11% for mammary gland). Barker et al. (1968) concluded from their results and those of Tubbs and Chase (1966) using the inhibitors bromoacetyl-CoA and bromoacetyl-carnitine, that only a small proportion of the enzyme is available to external acetyl-CoA, and that the two enzymes (overt and latent) are localized so that the overt activity is

outside the inner membrane, while the latent enzyme is probably bound to the inner membrane, in much the same way as shown for carnitine palmitoyltransferase.

The localization of enzyme activity does not, however, provide much insight into the function of the enzyme. Because the catalysed reaction is freely reversible, it is conceivable that the enzyme could be used equally well for transport of acetyl groups out from the mitochondria as for transport in the opposite direction. Indeed, this function has been proposed as a means of generating extramitochondrial acetyl-CoA for fatty acid synthesis (see e.g. Bressler and Katz, 1965) from precursors which give rise to intramitochondrial acetyl-CoA, as was mentioned previously. However, the work of Lowenstein (1963) and Kornacker and Lowenstein (1965) indicates that, in liver, the citrate cleavage pathway is almost certainly the major route of acetate production for lipogenesis, and for rat mammary gland Lowenstein (1963) showed that fatty acid synthesis from acetylcarnitine proceeded at only one-tenth the rate observed with acetate or citrate. As stated above, rat liver and mammary gland have relatively low carnitine acetyltransferase activity (Barker et al., 1968), though that of goat mammary gland, which synthesises fatty acids principally from acetate, is relatively high. It seems likely that the wide variation in activity of carnitine acetyltransferase in different tissues and different species (see e.g. Beenakkers and Klingenberg, 1964; Barker et al., 1968) could indicate multiple roles for this enzyme.

Another function postulated for the acetyltransferase is the provision of an "acetyl buffer" system (Pearson and Tubbs, 1967) so

that changes in the [acetylcarnitine]/[free carnitine] ratio damp down what would otherwise be excessive changes in acetyl-CoA concentration. This point is further elaborated upon in the Discussion (Part II).

Variations in the concentrations of carnitine and its esters with changes in metabolic state

The distribution of carnitine, acetylcarnitine and carnitine acetyltransferase in several tissues of normal rats was reported by Marquis and Fritz (1965). Bøhmer (1967) showed that fasting and corn oil feeding led to increased long-chain acylcarnitine and acetylcarnitine concentrations, at the expense of free carnitine, in rat liver, and similar changes were seen in adipose tissue on fasting or alloxan treatment. The carnitine and carnitine ester concentrations of rat kidney and heart responded to alloxan diabetes in the same way (Bøhmer et al., 1966; Pearson and Tubbs, 1964a). Pearson and Tubbs (1964b) had shown earlier that acetylcarnitine concentrations were increased in rat heart and liver in alloxan diabetes. They also showed that perfusion of the normal rat heart with various compounds led to significant changes in the acetylcarnitine concentration (e.g. perfusion with glucose and insulin led to a marked fall in acetylcarnitine, while addition of acetate to the glucose/insulin perfusion resulted in acetylcarnitine concentrations more than twice as high as in the controls). Further work of Pearson and Tubbs (1967) on rat heart showed that perfusion with propionate prevented the rise in acetylcarnitine concentration which was otherwise seen on perfusion with pyruvate, 3-hydroxybutyrate and fatty acids with an even number of

carbon atoms. Similarly, the rise in acid-insoluble carnitine concentration on perfusion with palmitate was blocked by propionate. These inhibitions were not due to extensive formation of propionoyl-CoA or propionoylcarnitine, and since propionate is an important metabolite in ruminants, it was considered important to study carnitine and carnitine-ester concentrations in tissues of sheep on different diets and in starvation (Snoswell and Henderson, 1970). Pearson and Tubbs (1967) also found that starvation of rats led to increases in acid-insoluble carnitine in heart, and in liver, to increases in acetylcarnitine as well. Fat-feeding caused similar changes, but also caused a fall in the acetylcarnitine content of the heart. They observed that changes in the acylation state of carnitine could occur very rapidly (there was little change in the total carnitine concentration due to different metabolic states in any tissue).

Therriault and Mehlman (1965) showed that the total carnitine content of cold-acclimatized rats was eight times that of rats kept at 25°C, with the ratio of lipid bound to free carnitine being unaltered. The increased carnitine content was not due to retention, as the half-life and turnover time of carnitine were reduced in the cold rats, and the excretion rate was increased 8-fold. The metabolism of carnitine in alloxan diabetic rats was also studied (Mehlman et al., 1969) and it was found that, as with the cold-acclimatized rats, the half-life and turnover time were reduced compared with normal animals, but in contrast the body pool and excretion rate of carnitine were reduced. The carnitine concentration of the gastrocnemius muscle was found to have fallen to half that of

controls, but this could be restored by treatment with insulin. Thus there are quite different responses to two situations, under both of which one would expect to find increased fat mobilization and oxidation. Mehlman et al. (1969) suggest that, since only about 70% of the urinary excretion of radioisotope after administration of labelled carnitine was identified as carnitine in alloxan diabetic rats, the rest being 3-methylcholine, the lowered body pool of carnitine in these rats is due to the decreased turnover time and an increased rate of metabolism of carnitine. In the cold-acclimatized rats 3-methylcholine accounted for only 10% of the excreted carnitine (Therriault and Mehlman, 1965) and insulin treatment of alloxan diabetic rats reduced the excretion of 3-methylcholine (Mehlman et al., 1969). Therefore, it seems likely that the differences in the responses might be attributable to differences in the plasma insulin concentrations of the animals. Perhaps a lack of insulin results in greater rates of carnitine metabolism, or in reduced carnitine synthesis, in rats.

Unfortunately, none of the studies on the carnitine fraction concentrations in various tissues of rats under different metabolic conditions included the whole spectrum of tissue types most commonly used in such work. It can be inferred from the available data that, in alloxan diabetic rats, the total carnitine content of the skeletal muscle is reduced (Mehlman et al., 1969). That of liver, kidney, adipose tissue and heart is little changed though in these tissues there are marked changes in the relative amounts of the acylcarnitine fractions (e.g. Bøhmer et al., 1966; Pearson and Tubbs, 1964a).

Using guinea-pigs Erfle and Sauer (1967) found that fasting ketosis resulted in a significant fall in the free carnitine concentration of liver, with a non-significant rise in the acetylcarnitine fraction. The concentrations of both fractions were reduced in skeletal muscle.

The only other animal tissue to have been studied is sheep liver (Snoswell and Henderson, 1970). They found that dietary factors markedly affected the [free carnitine]/[acetylcarnitine] ratio and that proportion of the total acid-soluble carnitine which is probably propionoylcarnitine (Bøhmer and Bremer, 1968). The total acid-soluble carnitine of sheep liver was found to increase about 5-fold on starvation (a very much greater increase than was noted for rat liver by Pearson and Tubbs, 1967), and, in contrast to the rat, the free carnitine fraction was also increased on starvation. The situation in sheep is considered further below and in the Discussion (Part II).

Variations in the concentrations of CoA and its esters in animal tissues under different metabolic conditions

Very little work has been reported on this subject. The concentrations of CoA in different tissues of several normal animals are tabulated by Glock (1961), and Allred and Guy (1969) give total CoA and acetyl-CoA concentrations obtained by more reliable methods for some tissues of normal rats. Starvation of rats has been shown to result in elevated concentrations of both long-chain acyl-CoA and acetyl-CoA in liver (Bortz and Lynen, 1963), as might be expected; and Tubbs and Garland (1964) showed that the total CoA concentration in rat liver is

increased following starvation.

In contrast, it appears that alloxan diabetes results in a decrease in the total CoA content of rat liver (Tubbs and Garland, 1964) though there is an increase in the CoA esters (Wieland and Weiss, 1963; Tubbs and Garland, 1964). In rat heart, alloxan diabetes is reported to result in increased long-chain acyl-CoA concentrations, though no figures are given (Tubbs and Garland, 1964); and Pearson and Tubbs (1967) found no change in the acetyl-CoA content of rat heart in either starved or diabetic animals. A communication by Sauer and Erfle (1966) claims to report attempts made "to correlate changes in concentration of free CoA and acetyl-CoA with the induction of ketosis" in rat and guinea-pig liver. The method by which ketosis was induced is not made clear and neither is it obvious whether the figures quoted are for normal or ketotic animals or for both. In a later publication (Erfle and Sauer, 1967) it was shown that the acetyl-CoA content of guinea-pig liver was doubled when pregnant animals were fasted and the free CoA concentration was unchanged. In skeletal muscle, acetyl-CoA concentrations also rose but no free CoA results are presented for this tissue (Erfle and Sauer, 1967).

In sheep liver the [free CoA]/[acetyl-CoA] ratio was found to vary with dietary variations, but no change in the sum of CoA plus acetyl-CoA was observed (Snoswell and Henderson, 1970). In confirmation of the work of Jarret and Filsell (1964), Snoswell and Henderson (1970) also found that the free CoA plus acetyl-CoA concentration of sheep liver is less than that of rat liver.

Objectives of this study

It is perhaps to be expected that the metabolism of carnitine (and CoA) in sheep tissues would differ quantitatively from that of rat tissues since sheep are so much more dependent on fatty acid metabolism for their energy requirements. The work of Snoswell and Henderson (1970) suggests that the differences between the two species in carnitine and CoA metabolism in liver are extremely marked, and that large variations in the concentration of carnitine in sheep liver could be rapidly induced by metabolic stress.

A detailed examination of carnitine and CoA metabolism in various sheep tissues taken from both normal and stressed animals appeared to be necessary in view of these results (Snoswell and Henderson, 1970) and those of Mayfield et al. (1966) who showed that acetate, a major fuel of ruminants, is metabolized mainly in the extrahepatic tissues. Also, in order to understand more fully the role of carnitine acetyltransferase in the interconversions of acetylcarnitine, CoA, acetyl-CoA and carnitine, the intracellular distributions of carnitine acetyltransferase, carnitine, CoA and acetate thiokinase have been examined.

Alloxan diabetes was chosen for use as the metabolically stressed state because Snoswell and Henderson (1970) found that this condition caused a dramatic increase in the total acid-soluble carnitine content of sheep liver. In contrast, no change in concentration is observed in rat liver after alloxan treatment (e.g. Bøhmer, 1967) and the total acid-soluble carnitine concentration of rat skeletal muscle is reduced in alloxan diabetes (Mehlman et al., 1969).

The study on sheep was extended to include normal young (pre-ruminant) lambs for comparison with adult sheep.

This study is the first in which a co-ordinated examination of variations in the concentrations of carnitine and CoA and their acetyl-esters has been undertaken in several tissues of one animal species in different metabolic states. Such a study might provide some insight into the relationships between these metabolites and into their possible roles in the control of fatty acid metabolism at all levels from the mitochondrion to the whole animal.

II. Experimental and Results

II.A. Fatty acid oxidation by isolated sheep liver mitochondria

II.A.1. Materials and methods

Animals. The animals used are described in Section I.D.1.

Tissue samples. Animals were killed, liver samples taken and mitochondria prepared as described in Section I.D.1.

Assay procedure. Oxygen uptake was measured as described in Section I.D.1. Oxidation rates with free fatty acids and fatty acyl carnitine derivatives were measured in a medium described by Bode and 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 used in the oxidation experiments 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 (Biochemistry Dept., S.G.S., A.N.U., Canberra) from the corresponding acid chlorides and L-carnitine hydrochloride using the method of Bremer (1968).

II.A.2. Results

The results presented in Table 11 indicate that long-chain fatty acids were not oxidised by sheep liver mitochondria. Subsequent

TABLE 11

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 and 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.

Chain length of saturated fatty acid	Oxygen uptake (ng-atoms/min per mg of mitochondrial protein)			
	Sheep liver mitochondria		Rat liver mitochondria	
	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	

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 oxidised slowly. Acetate was not oxidised but acetyl-L-carnitine was oxidised slowly (Table 11). The L-carnitine esters of the long-chain fatty acids were oxidised 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 oxidising the same substrate (Table 11).

In contrast, the results presented in Table 11 also show that free long-chain fatty acids, especially laurate and myristate, were oxidised 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 11.

II.B. Free carnitine, carnitine esters, free CoA and acetyl-CoA in the tissues of sheep in various metabolic states

II.B.1. Materials and methods

Animals. Two groups of adult Merino wethers were used: one group was of animals approximately 4 years old and weighing between 35 and 45 kg which were fed on lucerne-hay chaff. The others were about eighteen months old, weighing 26 to 29 kg and were kept in metabolism crates. They were fed on either lucerne-hay chaff or on 75% wheaten-hay chaff, 25% lucerne-hay chaff as indicated in the Results section. The lambs used were Merinos aged between 5 and 16 days. They were bottle-fed on reconstituted skim-milk powder with a vitamin supplement.

Alloxan treatment. Alloxan diabetes was induced by injecting a sterile solution of alloxan in isotonic saline (60 mg/kg body weight) into the jugular vein. The 4-year-old animals were killed 3 days later. (Results are presented only for those animals in which the blood glucose concentration had risen to 200 mg/100 ml). The eighteen-month-old wethers were killed daily from 4 days to 7 days after treatment.

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 and Henderson, 1970).

Blood samples were taken from the jugular vein of normal and alloxan-treated adult sheep and urine samples were obtained by fastening a collecting vessel under the wethers. Aliquots (2 ml) of whole blood (heparinized) or urine were extracted with 2 ml of ice-cold 30% (w/v) $HClO_4$. The supernatants of the extracts were neutralized and assayed for total acid-soluble carnitine and acetylcarnitine by the methods outlined below.

Metabolite assays. Acetylcarnitine was measured by the method of Pearson and Tubbs (1964b), and free carnitine by the method of Marquis and 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 pH 8.5 with 1 M-tris base and heating at 90°C for 5 min before assay, as suggested by Marquis and Fritz (1964). Pearson et al. (1969) suggest that significant hydrolysis of short-chain carnitine esters may occur under these conditions. No breakdown of a 0.2 mM solution of O-acetyl-L-carnitine in 20 mM-tris-HCl (pH 8.5) could be detected after 15 min heating at 90°C. 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 and Tubbs (1967).

Acetyl-CoA and acetyl-CoA plus free CoA were measured by the kinetic method of Allred and 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 by the method of Michal and Bergmeyer (1963). However, as this method only assays reduced CoA, the standards were preincubated for 10 min at 25°C with 2 μl of 0.2 M-dithiothreitol to ensure that all the CoA was in the reduced form.

Instrumentation was as described by Snoswell and Henderson (1970).

II.B.2. Results

II.B.2.1. Normal adult sheep

The results presented in Table 12 show that acetylcarnitine constitutes a relatively small portion of the total acid-soluble carnitine in all tissues shown except the heart, where it represents approximately 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 12). The concentrations of the various carnitine fractions in sheep liver recorded in Table 12 are comparable with those in the liver of sheep fed on lucerne ad libitum, as reported by Snoswell and Henderson (1970).

TABLE 12

Free carnitine, carnitine esters, free CoA and acetyl-CoA in sheep tissues

Four-year-old 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)	1320 ± 478	9860 ± 1380	12900 ± 880	17 ± 5	1.0 ± 0.1	1.3 ± 0.2

The concentrations of acetyl-CoA are approximately equal to the concentrations of free CoA in all four sheep tissues (Table 12) and the free CoA plus acetyl-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 free CoA plus acetyl-CoA and total acid-soluble carnitine/g wet wt. respectively, whereas the same muscle from Suffolk sheep (see Section II.Appendix) 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 free CoA plus acetyl-CoA and total acid-soluble carnitine respectively (Table 12). These results, together with those for M. biceps femoris for Merino lambs (Table 13), suggest an inverse relationship between CoA and carnitine concentrations in sheep muscles.

II.B.2.2. Lambs

The concentration of total acid-soluble carnitine in the liver of lambs (Table 13) is comparable with that of adult sheep (Table 12) 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 13 with Table 12), which undoubtedly reflects the milk diet of the lambs. The total acid-soluble carnitine content of skeletal muscle in the lamb (Table 13) is significantly ($P < 0.01$) less than that of the adult sheep (Table 12), being only about 40% of the adult value. The concentrations of

TABLE 13

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 the text. 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

acetyl-CoA and free CoA in the lamb liver (Table 13) are very similar to those of the adult sheep (Table 12), but the total concentration of free CoA plus acetyl-CoA in lamb skeletal muscle is about 2.5 times ($P < 0.05$) that of the adult sheep.

II.B.2.3. Four-year-old alloxan-diabetic sheep (3 days after alloxan)

The amounts of acetylcarnitine in the liver, kidney cortex, heart and skeletal muscle of alloxan-diabetic sheep (Table 14) are considerably greater than in the same tissues of normal sheep (Table 12). Also, although the total acid-soluble carnitine concentrations of heart and skeletal muscle are virtually the same in alloxan-diabetic sheep and normal sheep, the concentration of this carnitine fraction is approximately 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 sheep that was previously stabilised by continuous intravenous infusion of insulin. Samples of liver were removed surgically. The first one contained 66 nmol/g wet wt. of total acid-soluble carnitine, but 24 h after the withdrawal of insulin this value, measured in a second sample of liver, was 2210 nmol/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

TABLE 14

Free carnitine, carnitine esters, free CoA and acetyl-CoA in tissues of alloxan-diabetic sheep

Four-year-old Merino wethers were given an intravenous injection of alloxan (60 mg/kg body wt.) 3 days before slaughter. Tissue samples and assays were as described in the text. 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	Free 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 (M. biceps femoris)	4530 ± 471	7200 ± 574	12800 ± 820	34 ± 11	2.3 ± 0.6	1.7 ± 0.6

alloxan-diabetic sheep (Table 14), 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 14). The concentration of free CoA plus acetyl-CoA in the liver of alloxan-diabetic sheep (Table 14) is approximately twice ($P < 0.01$) that of the same tissue in the normal sheep (Table 12).

II.B.2.4. Eighteen-month-old alloxan-diabetic sheep (4 days to 7 days after alloxan)

Comparison of the results presented in Table 15 with those in Table 14 shows that more prolonged exposure to alloxan caused no further major change in total acid-soluble carnitine concentrations in any of the tissues examined. Neither was there any significant change in acetylcarnitine concentrations, except in heart where the observed increase might be significant but large differences in the sample variances impede a statistical analysis of the results.

The concentrations of free carnitine have apparently risen quite markedly in all four tissues following longer exposure to alloxan (compare Table 15 with Table 14). However, the figures for free carnitine concentrations in Table 15 are misleading since they were obtained by difference. (At the time that this work was done $KHCO_3$ was substituted for KOH as the neutralizing agent for the $HClO_4$ extract of tissue. This change of procedure resulted in considerable hydrolysis of acetylcarnitine when the extract was heated at pH 8.5

TABLE 15

Free carnitine, acetylcarnitine, free CoA and acetyl-CoA in tissues of sheep following prolonged exposure to alloxan

Eighteen-month-old Merino wethers were given an intravenous injection of alloxan (60 mg/kg body wt.) 4, 5, 6 or 7 days before slaughter. Tissue samples and assays were as described in the text. Results are from single animals for days 4, 5 and 6, and for day 7 are means ± S.E.M. for three animals.

Days after alloxan	Tissue	Concentrations (nmol/g wet wt.) Acetylcarnitine	*Free carnitine	Total acid-soluble carnitine	Acetyl-CoA	Free CoA
4	Liver	283	484	767	56	72
	Kidney cortex	316	754	1070	39	65
	Heart	892	1738	2630	6.4	9.8
	Skeletal muscle	1370	9330	10700	3.0	3.0
5	Liver	296	375	671	133	7
	Kidney cortex	733	677	1410	87	27
	Heart	1630	1870	3500	8.3	6.9
	Skeletal muscle	1120	12080	13200	1.2	2.7
6	Liver	484	686	1170	97	25
	Kidney cortex	737	533	1270	57	25
	Heart	1780	1840	3620	6.2	4.7
	Skeletal muscle	2290	10610	12900	1.9	1.4
7	Liver	305 ± 47	562 ± 227	893 ± 137	65 ± 7	31 ± 4
	Kidney cortex	553 ± 101	594 ± 86	987 ± 208	53 ± 11	35 ± 1
	Heart	2281 ± 276	2287 ± 523	4569 ± 751	7.8 ± 0.1	7.2 ± 0.9
	Skeletal muscle	4606 ± 821	8672 ± 714	13278 ± 312	0.8 ± 0.1	0.9 ± 0.2

* Free carnitine concentrations obtained by difference (see text).

to destroy endogenous free thiol groups prior to assaying for free carnitine. Unfortunately it took some time to discover this fact. Subsequently, free carnitine was assayed without heating after leaving the neutralized extracts at 4°C overnight).

Free CoA and acetyl-CoA concentrations also showed no major changes between 3 and 7 days exposure to alloxan (compare Table 15 with Table 14). The falls in the concentrations of acetyl-CoA in skeletal muscle and of CoA in liver may be significant, but again large differences in the sample variances rendered statistical analysis unreliable.

The only unequivocally significant result to emerge from this section of the work is the fall in [free CoA plus acetyl-CoA] concentration ($P < 0.01$) seen in the liver of sheep exposed to alloxan for 7 days compared with those treated for only 3 days (Table 16), and the fall in the [free CoA]/[acetyl-CoA] ratio may be significant ($P = 0.05$).

II.B.2.5. Eighteen-month-old sheep undergoing spontaneous starvation after treatment with alloxan

Spontaneous starvation after alloxan treatment has been a fairly rare occurrence in the animals studied, and at the time of writing only the data from two sheep were available. One of these animals was fed on a wheaten-hay/lucerne hay (75/25) diet and the other on lucerne-hay chaff. The concentrations of the various carnitine and CoA fractions in the liver of normal, eighteen-month-old sheep fed on lucerne-hay chaff were the same as those for 4-year-old animals (Table 12). In the livers of normal animals fed on wheaten-hay chaff/lucerne-hay chaff the concentration of total acid-soluble carnitine

TABLE 16

[Free CoA + acetyl-CoA] and the [free CoA]/[acetyl-CoA] ratio in the tissue of 3 day- and 7 day-alloxan diabetic sheep

The results presented were computed from the raw data which was used for the relevant columns of Tables 14 and 15. Results are means ± S.E.M. for three animals.

	3 days exposure to alloxan		7 days exposure to alloxan	
Tissue	*[Free CoA + Acetyl-CoA]	$\frac{[\text{Free CoA}]}{[\text{Acetyl-CoA}]}$ ratio	*[Free CoA + Acetyl-CoA]	$\frac{[\text{Free CoA}]}{[\text{Acetyl-CoA}]}$ ratio
Liver	169 ± 11	1.17 ± 0.40	96 ± 3	0.51 ± 0.10
Kidney cortex	86 ± 10	0.80 ± 0.17	89 ± 11	0.71 ± 0.12
Heart	19 ± 3	0.87 ± 0.24	15 ± 1	0.91 ± 0.11
Skeletal muscle	4 ± 1	0.70 ± 0.25	1.7 ± 0.3	1.07 ± 0.24

* CoA concentrations in nmol/g wet wt.

was the same as that of the lucerne-fed sheep (≃ 150 nmol/g wet wt.), but the proportion of acetylcarnitine was greater (≃ 58 nmol/g wet wt. compared with ≃ 4 nmol/g wet wt. for lucerne fed sheep). No figures are available for the metabolite concentrations in the other tissues for normal eighteen-month-old sheep, but for the lucerne-fed animals it can reasonably be assumed that they would be the same as for the 4-year-old sheep (Table 12), and for the animals on the mixed diet at least the total acid-soluble carnitine concentrations can be assumed to be similar to these (Table 12).

Comparing the results in Table 17 with those in Table 12 (and with the figures given above) it is clear that the combination of alloxan-diabetes and spontaneous starvation causes even more dramatic increases in both total acid-soluble carnitine and acetylcarnitine concentrations in liver than does alloxan diabetes alone (also compare Table 17 with Table 15 - 7 days column). The degree of starvation is apparently directly related to the amount by which the metabolite concentration rises - sheep number A1.742, which ate nearly 300g of food on day 7 showed an 18-fold increase in acetyl-carnitine concentration and a 16-fold increase in total acid-soluble carnitine (compare Table 17 with the figures given above for normal sheep on the wheat/lucerne diet). These increases were 2-fold and 2½-fold respectively compared with non-starved alloxan diabetic sheep (compare Table 17 with Table 15 - 7 days column). The concentrations of these metabolites increased 800-fold and 38-fold in the liver of sheep number 1.128 (which ate nothing on day 7) above the concentrations found in the livers of normal lucerne-fed sheep (compare Table 17

TABLE 17

Free carnitine, acetylcarnitine, free CoA and acetyl-CoA in tissues of two sheep undergoing spontaneous starvation after treatment with alloxan

Eighteen-month-old Merino wethers were given an intravenous injection of alloxan (60 mg/kg body wt.) 7 days before slaughter. 1000 g of either lucerne-hay chaff (sheep no. 1.128) or 75% wheaten-hay chaff, 25% lucerne-hay chaff (sheep no. A1.742) was offered daily and the residue weighed. Tissue samples and assays were as described in the text.

Sheep Number	Food intake (g)								Tissue	Concentrations (nmol/g wet wt.)				
	Before alloxan (average)	Days after alloxan 1	2	3	4	5	6	7		Acetylcarnitine	*Free carnitine	Total acid-soluble carnitine	Acetyl-CoA	Free CoA
A1.742	740	570	620	620	575	365	465	287	Liver	1090	1190	2280	-	-
									Kidney cortex	222	878	1100	-	-
									Heart	1150	3010	4160	-	-
									Skeletal muscle	3070	9730	12800	-	-
1.128	900	880	820	440	345	250	20	0	Liver	3313	2066	5379	67	23
									Kidney cortex	865	920	1785	36	23
									Heart	883	2972	3855	5.2	8.1
									Skeletal muscle	2017	13233	15250	1.6	5.4

* Free carnitine concentrations obtained by difference (see text).

with Table 12), and both metabolite concentrations were increased 6-fold compared with those of non-starved alloxan diabetic animals (compare Table 17 with Table 15 - 7 days column). Somewhat lesser increases in the concentrations of acetylcarnitine and total acid-soluble carnitine were apparent in kidney cortex, and only in the kidney cortex of sheep number 1.128 were the increases greater than those seen in non-starved alloxan-diabetic sheep. There were no obvious changes in either CoA or acetyl-CoA concentrations in liver and kidney cortex (compare Table 17 with Table 12 and Table 15 - 7 days column).

In contrast, in neither sheep was there any real change in the concentration of acetylcarnitine in either heart or skeletal muscle and the total acid-soluble carnitine concentration rose only slightly in the skeletal muscle of sheep number 1.128. There was also no change in the CoA or acetyl-CoA concentrations in heart (Table 17) compared with those for non-starved alloxan-diabetic sheep (Table 15 - 7 days column), but there was an apparent 50% fall in the concentrations of both these metabolites compared with those of normal sheep (Table 12). In skeletal muscle the concentration of acetyl-CoA may have increased compared with normal and non-starved alloxan diabetic animals, and the concentration of free CoA increased about 5-fold (compare Table 17 with Table 15 - 7 days column, and Table 12).

II.B.2.6. Blood and urine

The concentration of total acid-soluble carnitine in the jugular blood of normal, lucerne-fed wethers is about 50 nmol/ml (Table 18). Within 24 h of alloxan administration the concentration fell to an

TABLE 18

Concentrations of total acid-soluble carnitine in jugular blood and amounts excreted in urine of normal and alloxan diabetic sheep

Eighteen-month-old Merino wethers were treated and sampled, and the samples assayed for total acid-soluble carnitine, as described in the text. All assays were done in duplicate. n = number of samples. Results are given as means ± S.E.M. where applicable.

Days after alloxan	Blood (Concentrations in nmol/ml)				Urine (Amount excreted in µmol/24 h)	
		n*	Sheep no. 1.128	n		n**
Pre-alloxan	48 ± 3	8	46 ± 2	2	73 ± 13	9
1	38 ± 3	4	33	1	185 ± 44	3
2	46 ± 4	4	35	1	102 ± 19	3
3	51 ± 4	4	45	1	70 ± 14	3
4	60 ± 4	4	65	1	110 ± 16	3
5	64 ± 3	3	62	1	136 ± 7	2
6	81 ± 28	2	109	1	169	1
7	119	1	119	1	-	

* Includes sheep number 1.128

** Excludes sheep number 1.128

average of about 40 nmol/ml, which may be significantly different from normal ($P = 0.05$). In individual animals the decrease in concentration was much greater, but rather high variances make it difficult to assess whether there is any real significance in this result. (Results presented in Section III.D.2. for the total acid-soluble carnitine concentrations in portal and hepatic venous blood suggest that the fall in concentration is a significant effect of alloxan treatment). By 48 h after alloxan treatment the concentration of total acid-soluble carnitine in the jugular blood was similar to normal values, and by 4 days after treatment it was significantly higher ($P < 0.05$ at 4 days and $P < 0.01$ at 5 days) than the pre-treatment concentration (Table 18).

The results presented from sheep number 1.128, which underwent spontaneous starvation after alloxan treatment, show that this situation makes little difference to the general pattern of events (Table 18). The immediate post-alloxan drop in carnitine concentration may have been greater than was seen for the whole group of sheep, and the rise was by day 6 obviously higher than those seen in the blood of sheep which retained their appetites.

The excretion rate for total acid-soluble carnitine is about 70 μmol per day for normal sheep fed on lucerne (Table 18), and it rose quite dramatically within 24 h of alloxan treatment to 185 μmol/day. Urinary excretion rates then fell to levels not significantly different from normal for 3 days, but by 5 days after alloxan administration excretion rates were double the normal rate ($P < 0.01$).

The excretion rates of total acid-soluble carnitine and free carnitine for the spontaneously starved sheep (number 1.128) have been separately tabulated (Table 19). The average excretion rate of total acid-soluble carnitine over the three days before alloxan administration was about the same as that of the group of sheep (Table 18): 78 μmol/24 h for sheep number 1.128 compared with 73 ± 13 μmol/24 h. As with the group of animals the excretion rate increased markedly in the first 24 h after alloxan treatment, but unlike the excretion rates of the group those of sheep number 1.128 remained at levels which would probably be significantly higher than the average pre-alloxan levels (Table 19). By day 7 over 500 μmol of total acid-soluble carnitine were being excreted in 24 h by this animal.

The proportion of free carnitine excreted by sheep number 1.128 (Table 19) rose from an average of 24% (before alloxan) to an average of 46% after treatment ($P < 0.01$). Excretion rates for acetylcarnitine by sheep number 1.128 are available for only 2 days (Table 19), but on both days the sum of free-plus acetylcarnitine fell markedly short of the total acid-soluble carnitine. Thus it is obvious that some metabolite of carnitine other than free or acetylcarnitine is excreted in fairly large quantities by alloxan-diabetic sheep. With two other sheep the urinary excretion of free carnitine rose from about 27% of the total acid-soluble carnitine (before alloxan) to 43% by three days after alloxan treatment. Acetylcarnitine was not detectable in one of these samples, nor in three others tested (on days 3 and 4 after alloxan administration). No figures are available at present for acetylcarnitine excretion rates by normal sheep.

TABLE 19

Amounts of total acid-soluble carnitine and free carnitine excreted in the urine of sheep number 1.128 before and after alloxan administration

Sheep number 1.128 was an eighteen-month-old Merino wether which underwent spontaneous starvation after treatment with alloxan. Urine samples were collected and assayed (in duplicate) as described in the text.

		Amount excreted (μmol/24 h)		
Date	Days after alloxan	Total acid-sol. carnitine	Free carnitine	Free carnitine (Percent)
2/8	-	73	13	17.8
3/8	-	109	32	29.4
4/8	-	51	13	25.5
5/8	1	193	98	50.7
6/8	2	140	91	65.0
7/8	3	120	32	26.7
8/8	4	106	43	40.6
9/8	5	129*	59	45.7
10/8	6	218*	105	48.2
11/8	7	518	251	48.5

* Amounts of acetylcarnitine excreted were 37 and 25 μmol/24 h on days 5 and 6 (respectively) after alloxan.

APPENDIX TO SECTION II, EXPERIMENTAL AND RESULTS

All the work reported in this Appendix

was done by Dr. A.M. Snoswell while on study-leave

at the University of Bristol, U.K.

II. Appendix. Subcellular distribution of carnitine, CoA, carnitine acetyltransferase and acetate thiokinase in sheep tissues

II. Appendix.1. Materials and methods

Animals. Suffolk sheep were slaughtered at the abattoirs at Bristol.

Tissues. Samples of liver and kidney cortex were collected directly into ice-cold 0.25 M-sucrose containing 23 mM-potassium phosphate (pH 7.2). Heart and skeletal muscle (M. sternothyreoidus) samples were collected in the electrolyte buffer described by Chappell and Perry (1954).

Homogenates and subcellular fractions. Homogenates (10%, w/v) of liver and kidney cortex were prepared in the phosphate-buffered sucrose (above) with a Potter-Elvehjem homogenizer. These homogenates were then centrifuged at 700g to remove cell debris and nuclei. The supernatant fractions were centrifuged at 10000g for 10 min to sediment mitochondria. The mitochondrial pellets were washed twice in the sucrose-phosphate medium and re-centrifuged at 13000g for 10 min. Microsomal and supernatant (cytosol) fractions were prepared by centrifugation (for 1 h at 100000g) 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 of Chappell and Perry (1954) 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 and Hülsmann, 1970). Thus fractionation methods involving the use of a proteinase were not used. 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 fractions 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 4 h (skeletal muscle) or 33000*g* for 1½ h (heart) in a procedure similar to that described by Amberson *et al.* (1964). The small volumes of supernatants thus obtained were designated "press" fractions.

Homogenates, mitochondrial and microsomal fractions were disrupted by sonic disintegration for 3 min at 0°C (in 15 s intervals with 15 s cooling in between) by using a Soniprobe (Dawe Instruments Ltd., London W.3, U.K.) at 20 kHz and 2 A. The preparations were

subsequently centrifuged at 20000g for 2 min and the supernatant fractions were used for the assay of enzymes.

Immediately after isolation, aliquots (1.5 ml) of the various homogenates and subcellular fractions were treated with 0.25 ml 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. This was assayed in sonic extracts of the various subcellular fractions by coupling the reaction with that catalysed by arylamine acetyltransferase and measuring the change in extinction at 460 nm caused by acetylation of (p-aminophenylazo)benzene-p-sulphonic acid in an assay system similar to that used for pyruvate dehydrogenase 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 (University of Bristol, U.K.). The assay system contained 100 mM-tris-HCl (pH 7.8), 0.5 mM-EDTA, 1 mM-$MgCl_2$, 5 mM-mercaptoethanol, 100 mM-potassium acetate, 10 mM-ATP and 0.3 unit of arylamine acetyltransferase plus the sample in a total volume of 1.0 ml. The reaction was started by the addition of 0.3 μmol of CoA and a linear rate of reaction was observed after 5 to 10 min. A molar extinction coefficient (substrate-acetylated substrate) at 460 nm 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 (0.025 M) plus 0.1% Triton X-100 for 30 min. Activity was also assayed in whole mitochondrial suspensions. The assay system was similar to that described by Barker et al. (1968) but included 0.18 M-sucrose, and the acetyl-CoA was added 5 min before adding 5,5'-dithiobis-(2-nitrobenzoic acid) to ensure maximum activity.

Citrate synthase. This was assayed spectrophotometrically by using dithiobis(nitrobenzoic acid) as described by Shepherd and Garland (1969). Enzyme activity was assayed in sonic extracts of the various subcellular fractions of heart and skeletal muscle.

Glutamate dehydrogenase. 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 1.5 mM-ADP to ensure maximum activity, 10 μM-rotenone in place of 3 mM-KCN and P_i in place of tris-HCl buffer. Recovery of the glutamate dehydrogenase present in homogenates and the various subcellular fractions could only be achieved if P_i was included in the homogenizing medium and in the assay medium (Walter and Anabitarte, 1971).

Lactate dehydrogenase. This was assayed in sonic extracts of the various subcellular fractions by spectrophotometric assay at 340 nm. The system contained 200 mM-tris-HCl (pH 7.4), 0.2 mM-NADH, 10 μM-rotenone and enzyme fraction in a final volume of 1.0 ml. The reaction was started by addition of 1 mM-pyruvate.

Metabolite assays. Total acid-soluble carnitine and acetyl-CoA plus free CoA were measured by the methods described in Section II.B.1.

II. Appendix.2. Results

The subcellular distribution of the measured metabolites and enzymes, together with the marker enzymes, glutamate dehydrogenase and lactate dehydrogenase, in sheep liver is shown in Table 20. 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.

Free CoA plus acetyl-CoA was distributed equally between the mitochondrial and cytosol fractions (Table 20) whereas the carnitine was present solely in the cytosol, as it was in all tissues examined (see also Tables 21 and 22). In kidney cortex only approximately 25% of the free CoA plus acetyl-CoA was present in the cytosol (Table 21).

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

TABLE 20

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		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 21

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.

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 and Bremer (1970) have shown that there is considerable loss of CoA from rat liver mitochondria that were incubated at 30°C for 20 min. 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 20) and in kidney cortex (Table 21). 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 0.025 M-sucrose containing 0.1% Triton X-100.

About 90% of the acetate thiokinase was present in the cytosol of sheep liver (Table 20) but only 30% was present in this fraction in kidney cortex (Table 21).

Owing to the difficulties in preparing subcellular fractions from sheep heart and skeletal muscle, as outlined above, 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 22). To express the results obtained with the "press" fractions quantitatively and to compare them with those for homogenates all concentrations and

TABLE 22

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 (nmol/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 (7)	0.169 (5)	0.503 (1)	15.8 (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 (7)	N.D.	0.084 (< 1)

activities were related to the amount of lactate dehydrogenase, the cytoplasmic marker enzyme. The results presented in Table 22 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 free CoA plus acetyl-CoA, acetate thiokinase or carnitine acetyltransferase in either sheep heart or skeletal muscle (Table 22). 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 approximately 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 and Garland, 1968) and it is thus assumed that the acetate thiokinase is present in the matrix fraction of the sheep muscle tissues.

II. Discussion

The subjects of carnitine-dependent and carnitine-independent long-chain fatty acid oxidation are reviewed in the Introduction (Part II). The experimental conditions chosen in the present study of the oxidation of long-chain fatty acids by isolated liver mitochondria are known (for the rat) to result in appreciable oxidation of these fatty acids independent of L-carnitine (see Introduction, Part II). Yet under these conditions it was found that the oxidation of long-chain fatty acids by sheep liver mitochondria was totally dependent on the presence of L-carnitine (Table 11). This could be indicative of a different system for the activation of fatty acids in sheep liver mitochondria from that operative in rat liver mitochondria (see Van den Bergh, 1967; Garland and Yates, 1967), though it seems unlikely that there is any major mechanistic difference. However, it is possible that the location and/or activity of either the fatty acid activating enzymes or the carnitine palmitoyltransferases could be different in sheep liver from those of rat liver.

Some preliminary experiments were carried out in an attempt to assess the activities of the various ATP-dependent acyl-CoA synthetases in subcellular and submitochondrial particles of sheep liver, and also of rat liver for comparison. The results obtained with rat liver preparations were in agreement with those of Van den Bergh et al. (1969), Skrede and Bremer (1970) and van Tol and Hülsmann (1970). Subfractions of sheep liver cells and mitochondria proved considerably more difficult to prepare satisfactorily, and contamination with

endogenous fat was a major problem. The distribution pattern of the synthetases of sheep liver was very similar to that of rat liver with the exception that a fairly high palmitic acid activation was detected in the high speed supernatant (free of microsomes) of the sheep liver homogenate. No such activity was detectable in the same fraction of rat liver, in agreement with Farstad et al. (1967). This observation does not provide an explanation for the carnitine-dependence of fatty acid oxidation by sheep liver mitochondria. It does, however, indicate that there may be fundamental differences in fatty acid activation at this level, between sheep and rat liver. A thorough investigation of the distribution and activities of the ATP- and GTP-dependent fatty acid activating enzymes and the carnitine palmitoyltransferases of sheep liver would be worthwhile.

The activity of carnitine palmitoyltransferase in sheep liver mitochondria is the same as that of rat liver mitochondria (Snoswell and Henderson, 1970) so the lower rates of fatty acid oxidation by sheep liver mitochondria (Table 11) cannot be directly attributed to this system. However, the intramitochondrial location and the distribution of the overt and latent forms of the enzyme (as discussed for rat liver, see Introduction) could be different in sheep liver mitochondria. The oxidative activity of sheep liver mitochondria using glutamate as substrate was also lower than in rat liver mitochondria (Table 11). Thus it is possible that there is simply a lower overall oxidative capacity in the sheep liver mitochondria.

More evidence that carnitine metabolism in sheep may differ greatly from that of non-ruminants is provided by the results presented in Section II.B. The wide range in the concentrations of total acid-soluble carnitine in the four sheep tissues examined is noteworthy (Table 12). 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 and 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 and Friedman, 1957). The total acid-soluble carnitine concentration of sheep skeletal muscle (Table 12) is about 20 times greater than that of rat skeletal muscle (Pearson and Tubbs, 1967). This difference is not due to the use of different assay procedures. The total acid-soluble carnitine content of rat liver and skeletal muscle was found to be 232 and 634 nmol/g wet wt. respectively (mean values), which is comparable with the mean values 296 and 627 nmol/g wet wt. respectively reported by Pearson and Tubbs (1967).

The concentration of free CoA plus acetyl-CoA is greatest in sheep liver and least in sheep skeletal muscle, of the tissues examined (Table 12); this contrasts 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 free CoA plus acetyl-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 concentrations of total acid-soluble carnitine and free CoA plus acetyl-CoA may be observed in a single sheep tissue: the skeletal muscle of the young lamb has only 40% of the total acid-soluble carnitine concentration of that of the adult sheep but 2.5 times the concentration of free CoA plus acetyl-CoA (Table 13). This inverse relationship between the concentrations of free CoA plus acetyl-CoA and total acid-scluble carnitine was also observed when various sheep muscles were compared. These results suggest that the synthesis and/or degradation of carnitine and CoA in these tissues might be closely integrated.

The relationship between carnitine and CoA in sheep tissues emphasizes the possible role of carnitine acetyltransferase, particularly in the muscle tissues. Also, the high concentrations of carnitine in sheep heart and skeletal muscle indicate 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). Carnitine acetyltransferase is localized in the mitochondria of all four sheep tissues examined and most of the activity is latent, i.e. released only after exposure of the mitochondria to hypo-osmotic solutions containing detergent (Section II, Appendix.2). The carnitine acetyltransferase activities of rat, guinea pig, goat and sheep liver mitochondria have also been shown to be largely latent (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 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 is much more likely that the role of carnitine acetyltransferase and carnitine is in a buffer system, as suggested by Pearson and Tubbs (1967). 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. The results presented in Table 22 indicate that the CoA of sheep muscle tissues is virtually confined to the mitochondria and carnitine to the cytoplasm. Yates and Garland (1966) have shown that the inner membrane of rat liver mitochondria is impermeable to carnitine. As mentioned in the Introduction (Part II) for carnitine palmitoyltransferase, the carnitine acetyltransferase located in the inner mitochondrial membrane appears to act as a vectorial transferase which reacts with CoA and acetyl-CoA on the inside and carnitine and acetylcarnitine on the outside. The net effect of such a system would be to remove acetyl groups from the mitochondria in times of increased "acetyl pressure", i.e. during increased fatty acid oxidation, and transfer them 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 3 g in a 50 kg sheep, if the total muscle mass is taken into account. In

support of this concept the results presented here for alloxan diabetic sheep (Tables 14 and 15) indicate a marked increase in the acetyl-carnitine concentration of skeletal muscle, but relatively little change in the ratio [acetyl-CoA]/[free CoA] compared with the normal animal (Table 12).

For an 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 pH 7.0 by using a partially purified enzyme from pig heart. From the results in Table 12, $K_{app.}$ = [acetyl-CoA][L-carnitine]/[acetyl-L-carnitine][CoA] = 1.9 for sheep heart and 4.2 for skeletal muscle. In alloxan diabetes (Table 14) the values are 1.3 and 2.2 respectively. Thus the reaction in these sheep muscles *in vivo* appears to be near equilibrium, assuming that the carnitine acetyl-transferase in these tissues is accessible to intramitochondrial CoA and acetyl-CoA and to cytoplasmic carnitine and acetylcarnitine. 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 administration of alloxan resulted in an approximately seven-fold increase in the total acid-soluble carnitine content of sheep liver in three days (Section II.B.2.3.). Marked increases in the individual fractions of the total acid-soluble carnitine, i.e. free carnitine and acetylcarnitine also occur (compare Table 14 with Table

12). A significant but less pronounced (twofold) rise in free CoA plus acetyl-CoA was also noted in sheep liver three days after alloxan treatment, in contrast with the rat, where there is little change (Tubbs and Garland, 1964). The [free CoA]/[acetyl-CoA] ratio in normal sheep liver is approximately 1:1 (Table 12), which is considerably lower than the ratio of 3-4:1 in rat liver (Allred and Guy, 1969; Bode et al., 1970). This ratio remains at approximately 1:1 in the liver of sheep three days after alloxan treatment (Table 16). By seven days after treatment the ratio falls to about 0.5:1 but is associated with a considerable reduction in the concentration of free CoA plus acetyl-CoA. 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.

Another 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 (Table 14). There was a significant increase in this fraction in the heart in alloxan diabetic sheep (compare Table 14 with Table 12), 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 and Tubbs, 1967).

Starvation has been shown to result in raised concentrations of free carnitine and acetylcarnitine in sheep liver (Snoswell and Henderson, 1970), and greater increases were observed in the livers of alloxan diabetic sheep (above). It was found that spontaneous starvation in alloxan diabetic sheep resulted in even more marked increases in the concentrations of these carnitine fractions in the liver. This reaction proved impossible to induce by deliberate starvation (either sudden or gradual) of alloxan diabetic sheep. The aetiology of the condition is not understood and it would obviously be of interest to investigate it further. However, since very few sheep undergo spontaneous starvation following alloxan administration such a study would necessarily be rather prolonged.

It has been suggested that carnitine may be synthesized in one tissue and transported to others. For example, Lindstedt (1967) suggests that synthesis may occur in rat liver and carnitine might then be transported to muscle and kidney (cf. Strength et al., 1965). This subject is discussed in more detail in Part III. The decline in carnitine concentration which occurs in the skeletal muscle of rats made diabetic with alloxan (Mehlman et al., 1969) could indicate that, in alloxan diabetes, carnitine is mobilised from muscle. Mobilisation and inter-tissue transfer of carnitine, if on a large enough scale, might be detectable by measuring changes in the concentration of carnitine in the blood, and correlating these with variations found in tissue concentrations. Results obtained for changes in the concentrations of a metabolite in the blood are meaningless without concurrent observations of changes in the excretion rate.

The results presented in Section II.B.2.6. show that alloxan treatment caused an early (within 24 h) fall in the concentration of total acid-soluble carnitine in the blood which was accompanied by increased urinary excretion of carnitine. By 7 days after alloxan administration, total acid-soluble carnitine concentrations were raised in both urine and blood. The increased output of carnitine in the urine in the first 24 h after treatment has been calculated to exceed by 2 to 3 times the amount by which the carnitine in the blood was reduced. Thus, it seems that an increase in the excretion rate of carnitine is one of the first obvious manifestations of alloxan diabetes in sheep, and that there must also be mobilisation from some tissue(s) during this early period.

It was found that the concentration of total acid-soluble carnitine in sheep skeletal muscle was marginally less (about 100 nmol/g wet wt.) 3 days after alloxan administration than it was in normal animals (compare Table 14 with Table 12). Such a difference, if a result of mobilisation rather than degradation of carnitine, would result in the addition of about 0.4 g of carnitine to that already circulating. However, the results of subsequent experiments appear not to support the hypothesis of mobilisation of carnitine from skeletal muscle: there was no detectable difference in the total acid-soluble carnitine concentrations of blood samples taken from the femoral artery and vein of normal sheep or of diabetic sheep at any time after alloxan was given (A.M. Snoswell, unpublished work). This finding also indicates, of course, that neither is there any uptake of carnitine by sheep skeletal muscle. It seems unlikely that carnitine

would be mobilised in an acid-insoluble form (i.e. as long-chain fatty acyl esters).

Results presented in Section III.D.2. for the total acid-soluble carnitine concentrations of portal and hepatic venous blood show that there is a net output of carnitine from the liver of spontaneously starved alloxan diabetic sheep. There is some indication of carnitine output by the livers of diabetic sheep which do not starve, but it is much less and is not statistically significant.

Experiments performed since the work reported in this thesis was completed (A.M. Snoswell, unpublished work) have established that the total acid-soluble carnitine concentration of sheep skeletal muscle definitely rises when the animals undergo spontaneous starvation after being given alloxan - values of up to 21000 nmol/g wet wt. have been found 7 days after treatment. It is still not known whether there is in fact any fall in the total acid-soluble carnitine content of sheep skeletal muscle (or any other tissue) in the first 24 to 48 h after alloxan is given. As has already been discussed, the rise in total acid-soluble carnitine concentration of sheep liver is evident and significant by 3 days after alloxan administration.

Thus it appears that, in sheep, alloxan diabetes causes increased biosynthesis of carnitine in both skeletal muscle and liver, with possible active mobilisation from the liver; but at present it is not known which, if any, organ or tissue might take up carnitine under these circumstances. In this regard, it is interesting to note that Yue and Fritz (1962) found that, of several dog tissues examined, all except brain took up tritiated carnitine from the plasma after

intravenous administration of the compound. Rat heart, skeletal muscle and liver also took up labelled carnitine from the blood plasma (Yue and Fritz, 1962). The increased total acid-soluble carnitine concentration of sheep kidney which occurs as a consequence of alloxan diabetes may be related to the apparently specific response of the excretory system for carnitine.

In contrast to the rise in carnitine excretion which occurs in sheep when they become alloxan diabetic, Mehlman et al. (1969) found that alloxan diabetic rats excrete only half as much carnitine in the urine as do normal rats. Excretion of the decarboxylation product of carnitine - 3-methylcholine - was detected with alloxan diabetic rats (and to a lesser extent in insulin-treated alloxan diabetic animals), and 3-methylcholine constituted a considerable proportion of the radioactivity in the urine of diabetic rats which had been injected with labelled carnitine (Mehlman et al., 1969). In the urine of alloxan diabetic sheep a large amount of the total acid-soluble carnitine cannot be accounted for. The unidentified carnitine metabolite could be another short-chain fatty acyl ester of carnitine (perhaps propionoylcarnitine) but it could be 3-methylcholine if this compound reacts with carnitine acetyltransferase.

The evidence presented in this thesis suggests that carnitine has an important quantitative role in the overall metabolism of sheep, as the skeletal muscle, which contains over 2 mg of carnitine per g wet wt., constitutes the largest tissue mass of the animal. Fraenkel and 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). The total acid-soluble carnitine concentration of the milk of a normal ewe has been found to be about 500 nmol/ml which is more than four times that of normal dairy cattle (A.M. Snoswell, unpublished work). Thus it would appear that carnitine may have a particularly important role in the metabolism of ruminant animals, especially under conditions of metabolic stress. This statement immediately suggests that further work which could obviously be of some economic importance is the examination of carnitine metabolism in pregnant and lactating ruminants.

PART III

The biosynthesis and degradation of carnitine in sheep tissues

III. Introduction

Carnitine (3-hydroxy-4-butyrobetaine) was discovered by Gulewitsch and Krimberg (1905) in extracts of muscle, and in the same year Kutscher (1905) found "novain" in meat extracts. Novain was later shown to be identical with carnitine (Krimberg, 1906). Several possible structures were proposed for carnitine, the main contentious factor being the position of the hydroxyl group. The identification of carnitine as 4-trimethylamino-3-hydroxybutyrate (3-hydroxy-4-butyrobetaine) was firmly established by Tomita (1926). The formula of carnitine is now generally written as:

$$(CH_3)_3N^+CH_2\,CHOH\,CH_2\,COOH$$

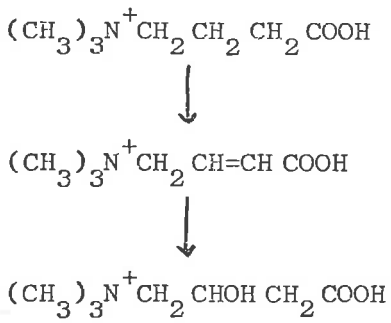
Interest in carnitine in the 25 years following its discovery was only sporadic, presumably because no function could be assigned to it. It was not until 1952 that its function as a vitamin for the larvae of some insects was established, and somewhat later the role of carnitine in fatty acid oxidation was elucidated. The functions of carnitine are discussed in detail in the Introduction, Part II. Another probable reason for the apparent lack of interest in carnitine may have been that the techniques available for measuring its concentration were tedious and inaccurate. The development of a biological assay for vitamin B_T utilizing the meal worm (Tenebrio molitor) by Fraenkel (1951) and the subsequent identification of vitamin B_T as carnitine (Carter, 1952) eventually led to the demonstration of biosynthesis of carnitine in the chick embryo (Fraenkel, 1952). Fraenkel (1953) postulated that carnitine is synthesized in all organisms which do not require it in

the diet. Yue and Fritz (1962) suggested that some of the tissue carnitine of meat-eating animals (which do not require carnitine as a vitamin) could be derived from the diet, as meat contains high concentrations of carnitine, because they found that injected tritiated carnitine could be taken up from the plasma by several tissues of dogs and rats. However, Grüner et al. (1966) found that [^{15}N]carnitine fed to humans was metabolised and excreted as trimethylamine oxide apparently without any mixing with endogenous tissue carnitine.

The first demonstration of any reaction of the biosynthetic pathway for carnitine was that of Lindstedt and Lindstedt (1961) who showed that 4-butyrobetaine is converted to carnitine by mice. It had previously been suggested that 4-butyrobetaine might be the immediate precursor of carnitine (Engeland, 1921; Linneweh, 1929). This reaction and the search for the precursor of the carbon skeleton of carnitine are discussed in detail below.

The hydroxylation of 4-butyrobetaine to form carnitine

It is beyond dispute that 4-butyrobetaine is an immediate precursor of carnitine in the rat, mouse and a strain of Pseudomonas (Lindstedt and Lindstedt, 1961; Bremer, 1962; Lindstedt and Lindstedt, 1965). As was mentioned above, Engeland (1921) and Linneweh (1929) had predicted this reaction, and Linneweh (1929) suggested that carnitine was formed by β-oxidation of the 4-butyrobetaine (as for oxidation of fatty acids by "Knoop's rule"). Crotonbetaine was postulated as an intermediate:



Lindstedt's group has established that the reaction is probably a mono-oxygenase reaction, and they have partially purified the 4-butyrobetaine hydroxylase of rat liver and of a strain of Pseudomonas (Lindstedt and Lindstedt, 1970; Lindstedt et al., 1970). Research has shown that the enzymes of both rat liver and Pseudomonas are soluble and have a requirement for molecular oxygen, ferrous ion and 2-oxoglutarate. Stimulation of activity occurs when ascorbate is added to the reaction mixture, and catalase prevents the reduction in activity otherwise seen on preincubation of the enzyme with ascorbate and ferrous ion. Addition of microsomes or of NADPH, isocitrate and isocitrate dehydrogenase had originally been found to stimulate activity, but this was later shown to be related to 2-oxoglutarate formation. Details of the work outlined above are to be found in the papers of Lindstedt et al. (1967), Lindstedt (1967), Holme et al. (1968), Lindstedt and Lindstedt (1970) and Lindstedt et al. (1970).

It was found that there is a stoichiometric relationship between the formation of CO_2 and succinate from 2-oxoglutarate and the formation of carnitine from 4-butyrobetaine. No free succinic semialdehyde could be detected, and succinic semialdehyde could not be used to replace 2-oxoglutarate. Indeed, with the partially purified 4-butyrobetaine

hydroxylase of rat liver, succinic semialdehyde inhibited activity, though no inhibition occurred with the enzyme from Pseudomonas. This was the only major difference between the enzymes from the two sources (see list of references, above).

In summary, Lindstedt and Lindstedt (1970) suggest that "4-butyrobetaine is hydroxylated to carnitine simultaneously with the oxidative decarboxylation of 2-ketoglutarate in a reaction sequence which involves the intermediate formation of a peroxide of the two substrates. Ferrous ion might act as an oxygen-activating agent. Free sulfhydryl groups are apparently necessary for enzymic activity and ascorbate and catalase probably act by maintaining these groups as well as ferrous ion in the reduced state".

Precursors of the carbon chain of carnitine

The origin of the carbon skeleton of carnitine is still unknown. Several compounds, including 4-aminobutyric acid, choline and lysine, have been examined in various experimental systems in efforts to detect their conversion to carnitine. The literature pertaining to these, and other possible precursors of the carbon chain of carnitine, is reviewed below.

Engeland and Kutscher (1910) proposed that the "α-oxy γ-butyrobetaine" found in muscle extracts might be formed from 4-aminobutyric acid by a process involving exhaustive methylation. Bremer (1961 and 1962) suggested that 4-aminobutyric acid, or a close derivative of it, could be the precursor of the carbon chain of carnitine. Hosein,

Smart, Hawkins, Rochon and Strasberg (1962) claimed to have detected the synthesis of 4-butyrobetaine from 4-aminobutyric acid and S-adenosylmethionine in simple homogenates of brain and skeletal muscle. The results of these experiments were not convincing and Fritz (1963) has calculated that the amount of incorporation of 4-aminobutyric acid into 4-butyrobetaine in the work of Hosein, Smart, Hawkins, Rochon and Strasberg (1962) was in fact negligible.

Pisano et al. (1960) refer to a personal communication from S. Lindstedt in which he reports that there was no significant conversion of 4-aminobutyric acid to carnitine detectable in the urine of rats which had been subjected to a single injection of the labelled acid with a carnitine carrier. Lindstedt and Lindstedt (1961) later confirmed that they were unable to detect carnitine formation from labelled 4-aminobutyric acid. Following further experiments, Lindstedt and Lindstedt (1965) stated that "at the present time one must conclude that there is no evidence for a biosynthetic pathway in the rat or mouse from 4-aminobutyric acid to carnitine". However, the results of Lindstedt and Lindstedt (1961 and 1965) could have been negative for reasons other than the non-existence of the pathway. The slow turnover of carnitine (about 13 days in normal male rats - see Khairallah and Mehlman, 1965), together with rapid metabolism and excretion of the administered 4-aminobutyric acid would affect the amount of conversion to carnitine detected in relatively short-term experiments. The possible existence of permeability barriers to 4-aminobutyric acid should be taken into account, too.

The origin of the 4-aminobutyric acid proposed as a carnitine precursor must also be considered. In their original paper on the subject of carnitine biosynthesis, Engeland and Kutscher (1910) suggested that the 4-aminobutyric acid was the "putrefaction product" of the glutamic acid of the muscle protein (cf. Ackermann and Kutscher, 1910 and Ackermann, 1910). With the development of more sophisticated biochemical assay methods came the general agreement that 4-aminobutyrate is present only in the nervous tissue, where its main function is as a neuro-inhibitor. Recently it has been shown that 4-aminobutyric acid can be detected in low concentration in several tissues of man and other mammals, with human kidney having the highest concentration of the non-neural tissues examined (see Lancaster et al., 1973, for references). In rat kidney cortex 4-aminobutyric acid is produced by glutamate decarboxylase, which differs markedly in a number of ways from the brain enzyme (Haber et al., 1970; Lancaster et al., 1973). One role for 4-aminobutyric acid in rat kidney cortex may be in ammoniagenesis and acid-base regulation (Vavatsi-Manos et al., 1973). Human liver has been reported to contain some 4-aminobutyric acid (Zachmann et al., 1966) and Tsukada et al. (1960) have shown that guinea pig liver is able to oxidise it to CO_2.

It is possible that a compound (in this case, 4-aminobutyric acid) is synthesized in one organ and taken up by others. There is no evidence that the 4-aminobutyric acid synthesized in the brain can readily leave that organ and enter the blood, and it is known that brain does not take up 4-aminobutyric acid from the blood (see Roberts

and Eidelberg, 1960, for discussion of these points). However, other tissues have been shown to take up 4-aminobutyric acid from the blood plasma *in vivo*, and it appears that in rats, mice and guinea pigs the liver probably accumulates the most (e.g. van Gelder and Elliott, 1958; Elliott and Jasper, 1959; Tsukada *et al.*, 1960; Roberts and Eidelberg, 1960). The same reports also show that muscle and kidney take up 4-aminobutyric acid from the plasma, and that tissues will also absorb the compound after oral administration, but to a lesser extent. The fact that orally administered 4-aminobutyric acid is taken up by some tissues does suggest that the diet might be another possible source of this compound, at least in herbivores. There are numerous reports of high concentrations of 4-aminobutyric acid in various plant materials (e.g. Steward *et al.*, 1949; Synge, 1951; Albers, 1960). Another potential source of 4-aminobutyric acid is the microflora of the gut - Jakoby (1960) showed that this metabolite can be both synthesized and utilized by *Pseudomonas*. Jakoby (1960) referred to work of his demonstrating that 4-aminobutyric acid may be formed from putrescine via the oxidation of 4-aminobutyraldehyde. The enzyme which oxidises 4-aminobutyraldehyde could not be demonstrated in human liver, kidney or brain, but it is stated that putrescine is known to be rapidly utilized by the normal flora of the gastrointestinal tract (Jakoby, 1960). In support of the concept of synthesis of 4-aminobutyric acid by the microflora of the gut is the fact that it can be detected in the faeces of 22% of normal dairy cattle (van Rheenen, 1963).

Derivatives of 4-aminobutyric acid

Carter et al. (1952) found that 3-hydroxy 4-aminobutyrate could replace carnitine in the diet of the carnitine-requiring Tenebrio larvae and suggested that these organisms could methylate this compound to form carnitine. In rats (Wolf and Berger, 1961) and mice (Lindstedt and Lindstedt, 1965) no conversion of 3-hydroxy 4-aminobutyrate to carnitine was detected in vivo.

If successive methylation of 4-aminobutyric acid is the pathway leading to 4-butyrobetaine, then 4-dimethylaminobutyrate could be an intermediate. As methylation blocks the pathway which so rapidly metabolises 4-aminobutyric acid (the formation of succinate via succinic semialdehyde) it was felt that the use of this derivative might be successful in delineating a further part of the biosynthetic route of carnitine (Bremer, 1962). However, the incorporation of labelled 4-dimethylaminobutyrate into carnitine by rats in vivo was negligible (about 0.1%) but a considerable amount was converted to a compound tentatively identified as 4-dimethylamino-3-hydroxybutyrate (Bremer, 1962). Lindstedt and Lindstedt (1965), with mice, obtained similar results when using 4-dimethylaminobutyrate as substrate, and they also detected the degradation products, 4-methylaminobutyrate and 4-aminobutyrate. When labelled 4-dimethylamino-3-hydroxybutyrate was used as a carnitine precursor in mice, only 0.003% of the dose given was converted to carnitine (Lindstedt and Lindstedt, 1965).

The results discussed above indicate that 4-dimethylaminobutyrate may be a precursor of carnitine, but it is obvious that the free compound is not readily utilized. It is possible that the dimethyl derivative exists only as an enzyme-bound intermediate, or perhaps as a CoA-ester. Hydroxylation in the 3-position evidently blocks the biosynthetic pathway.

These experiments can all be criticised in a similar way to those using 4-aminobutyric acid as the substrate in that they are all of short duration compared with the turnover time of carnitine, and there is also a high excretion rate of all the compounds used. Again, too, permeability barriers could present acute problems in work of this nature. A major point of criticism is that nearly all of the results referred to above were obtained from single experiments on single animals. In studies of this type it would be preferable to examine animals at different time intervals after the administration of the labelled compounds. The metabolic state of the animal could also influence the rate of carnitine biosynthesis, and a study utilizing e.g. starved, fat-fed or alloxan-treated animals might be rewarding.

Other possible precursors of carnitine

The structures of choline and carnitine are similar, so it is not surprising to find that choline has been proposed as a precursor for carnitine. Strack et al. (1935), without any experimental evidence, proposed a pathway involving an aldol condensation between choline aldehyde and acetaldehyde:

$$(CH_3)_3N^+CH_2\,CHO + CH_3\,CHO$$

$$\downarrow$$

$$(CH_3)_3N^+CH_2\,CHOH\,CH_2\,CHO$$

$$\downarrow + O$$

$$(CH_3)_3N^+CH_2\,CHOH\,CH_2\,COOH$$

Cantoni (1960) found this hypothesis attractive as it utilizes a pathway analogous to some already established biochemical reaction sequences. If acetaldehyde is replaced by acetyl-CoA, the reaction can be compared with the condensation of oxaloacetate with acetyl-CoA to form citrate. Cantoni (1960) depicted a scheme going from choline aldehyde plus acetyl-CoA to carnitine via the CoA-ester of carnitine. He cited a personal communication from Hosein, in which it was claimed that the CoA-ester of 4-butyrobetaine can be isolated from normal mammalian brain, as evidence in support of this pathway. Published work of Hosein, Proulx and Ara (1962) provides only somewhat vague indications for the existence of the CoA-esters of carnitine, 4-butyrobetaine etc. and they suggest themselves that the findings could be artefacts.

Fritz (1955) has also looked into the question of choline as a possible precursor for carnitine. He suggested that the findings of Artom (1953), who showed that choline deficiency in rats on a low protein diet led to lower rates of hepatic long-chain fatty acid oxidation in vitro, could be indicative of carnitine-deficiency secondary to the choline deficiency. Fritz (1957) repeated and expanded upon Artom's work and showed, as Artom (1953) did, that the impaired

long-chain fatty acid oxidation *in vitro* with livers from choline-deficient rats fed a low protein diet, could be relieved by choline administration *in vivo*, but that choline had no effect *in vitro*. In contrast, carnitine was found by Fritz (1957) to enhance hepatic fatty acid oxidation *in vitro* regardless of whether the donor animal was on a choline-deficient or a choline-supplemented diet. Also, the effect of carnitine *in vitro* was to increase ketogenesis from fatty acid, whilst the stimulatory effect of choline given *in vivo* was on the production of CO_2 *in vitro*. Fritz (1957) concluded that choline and carnitine influence different parameters of fatty acid oxidation. This conclusion was further substantiated by the discovery by Fritz and Du Pont (1957) that the development of fatty livers by choline-deficient rats could not be prevented or reversed with dietary carnitine supplements, but addition of choline to the diet resulted in the loss of the excess fat from the liver.

Bremer (1961) injected rats with [1,2-^{14}C]choline and analysed the carnitine of skeletal muscle 24 h later. No radioactivity was found and it was concluded that choline is not an intermediate in carnitine biosynthesis.

There are reports that choline deficiency is accompanied by reduced carnitine concentrations in rat tissues (e.g. Strength *et al.*, 1965; Corredor *et al.*, 1967). The implication that this effect of choline deficiency is due to choline being a precursor of carnitine is not necessarily valid. Choline deficiency would, of course, cause changes in the metabolism of the affected animal in order to overcome

the effects of the condition. Since choline and carnitine are so similar in structure it is highly likely that they have some precursors in common, and that these precursors would be directed into choline biosynthesis at the expense of carnitine biosynthesis in animals fed a choline deficient diet. This is almost certainly true of the methyl groups which, for both compounds, arise from methionine via S-adenosylmethionine (see below). It has been shown that the carnitine concentration in the tissues of choline deficient animals can be increased by methionine (Strength et al., 1965; Corredor et al., 1967) or S-adenosylmethionine (Corredor et al., 1967) as well as by choline administration.

It is apparent from this review of the literature that choline is not a likely precursor of the carbon skeleton of carnitine. The structural similarity of the two compounds, the facts that both are involved in fat metabolism and that the methyl groups of both arise from the same source have all been contributory factors to the implication that choline may be a carnitine precursor. It is almost certain that a change of metabolic state which severely affects the tissue concentrations of one of these metabolites will have an effect on the other. This aspect is considered further in the Discussion, below.

Lysine has recently been proposed as a precursor of carnitine in Neurospora crassa and in rat tissues. Horne et al. (1971a) reported that a lysine auxotroph strain of Neurospora crassa incorporated label from [6-^{14}C]lysine and from [4,5-^{3}H]lysine into carnitine. However, using the tritiated lysine, only 0.16% of the radioactivity was found

in the carnitine fraction, which, when using tritiated compounds would hardly be a significant amount of incorporation. Using [6-^{14}C]lysine, 0.2% of the label was detected in carnitine (Horne et al., 1971a) and the specific activity of the labelled carnitine seems to be impossibly high. In a later paper Horne et al. (1971b) reported a specific activity for the carnitine which was higher than that of the administered lysine.

Horne et al. (1971b) found no incorporation of label into carnitine by Neurospora crassa when lysine labelled in carbon atoms 1 and 2 was used. On the basis of these results it was postulated that lysine is cleaved between carbon atoms 2 and 3 for conversion to carnitine. It was also suggested that the 6-N-trimethyl-5-hydroxy derivative of lysine could be the precursor, as this has a structural similarity to carnitine and has been found in biological materials:

$(CH_3)_3N^+CH_2\,CHOH\,CH_2\,COOH$

carnitine

$(CH_3)_3N^+CH_2\,CHOH\,CH_2\,CH_2\,CHNH_2\,COOH$

6-N-trimethyl-5-hydroxy lysine

Later studies of Horne and Broquist showed that the label of 6-N-[methyl-^{3}H]trimethyllysine was incorporated by Neurospora crassa into carnitine (the amount incorporated was 16% in one and 19% in a second experiment). They suggested a biosynthetic pathway involving the hydroxylation of 4-butyrobetaine as the last step. As has already been discussed (above) the hydroxylation of 4-butyrobetaine to

carnitine is a well recognised reaction in several species. It is significant to note that in the one experiment done with Neurospora crassa using labelled 4-butyrobetaine as the precursor of carnitine, only 9.4% incorporation was detected (Horne and Broquist, 1973). These results seem to indicate that the apparent synthesis of carnitine from 6-N-trimethyllysine labelled in the methyl groups is more likely to be simply transfer of the intact methyl groups. It would be of interest to examine other compounds, which at some stage of their biosynthesis are acceptors for methyl groups, in order to establish whether they, too, would incorporate label from methyl-labelled 6-N-trimethyllysine.

Broquist and his co-workers have also examined the possibility that lysine is a carnitine precursor in rats. Tanphaichitr et al. (1971) found that the carnitine concentration of the skeletal muscle and heart were reduced in lysine deficient rats, but that of liver was higher than normal. Administration of [6-^{14}C]lysine to a single lysine deficient rat resulted in the incorporation of 0.074% of the label into "total liver and muscle carnitine". No label could be detected in carnitine after giving [2-^{14}C]lysine to another lysine deficient rat (Tanphaichitr et al., 1971). The experiments reported by Tanphaichitr and Broquist (1973) indicate that in rats, as in Neurospora crassa there was much more incorporation into carnitine of label from 6-N-trimethyllysine tritiated in the methyl groups than from labelled lysine. The percentage incorporation was little different in normal rats from that of lysine-deficient rats. With rats, the incorporation of label into carnitine was highest when labelled

4-butyrobetaine was the precursor (Tanphaichitr and Broquist, 1973). Because label-incorporation into carnitine from methionine was low, it was claimed that the results with methyl-labelled 6-N-trimethyllysine were therefore not simply an expression of methyl transfer. However, similarly low figures were obtained with methionine using Neurospora crassa (Horne and Broquist, 1973) and were attributed to dilution by endogenous methionine. This would appear to be probable in the rat system too.

Many other compounds have been examined as possible carnitine precursors, without success. Wolf and Berger (1961) used acetate, formate, glycine, glucose, ergothionine, hydroxyproline, lysine, threonine, phenylalanine and histidine, all in short-term experiments. A long-term experiment using [1-^{14}C]glycine showed that some of the carbon chain of carnitine was derived from this source (Wolf and Berger, 1961). Lindstedt and Lindstedt (1965) used 5-trimethylaminovaleric acid, 6-trimethylaminocaproic acid (the higher homologues of 4-butyrobetaine), 4-trimethylaminobutan-1-ol, homocarnosine and ornithine.

Origin of the methyl groups of carnitine

The work of several groups has established that the methyl groups of carnitine are derived from methionine. Extensive labelling of carnitine following the administration of methyl-labelled methionine to rats has been found by Wolf and Berger (1961), Bremer (1961) and Strength et al. (1965). Wolf and Berger (1961) found that, of the radioactivity incorporated into carnitine from methionine 93% was in the

methyl groups, and Bremer (1961) obtained similar results. The activated form of methionine (S-adenosylmethionine) appears to be the actual methyl donor for carnitine (Corredor et al., 1967) as it is for choline (Bremer and Greenberg, 1961).

The site of carnitine biosynthesis

Carnitine has been found in a wide range of animal and plant tissues (see e.g. Fraenkel and Friedman, 1957; Broekhuysen et al., 1965 and this thesis, Part II) and it is possible that biosynthesis occurs in all carnitine-containing tissues of all organisms which do not require carnitine as a vitamin. There have been many suggestions that carnitine is synthesized in one tissue and transported to others. Strength et al. (1965) and Lindstedt (1967) proposed that carnitine might be produced in rat liver and transported to other tissues. The possibility of transport from the skeletal muscle to the blood and thence, perhaps, to other tissues is examined in the Discussion, Part II (above). That tissues can take up and retain carnitine from the blood stream was demonstrated by Yue and Fritz (1962), brain and red blood cells being the only tissues examined which failed to take up carnitine. The tritium concentration in rat liver declined fairly rapidly from a peak reached 3 h after administration of the labelled carnitine while that of other tissues continued to rise slowly (Yue and Fritz, 1962). This could indicate transport of carnitine from the liver.

Lindstedt and Lindstedt (1961) showed that 4-butyrobetaine hydroxylase activity is present in homogenates of liver and kidney of rats.

Hydroxylation of 4-butyrobetaine may also occur in the skeletal muscle and heart of lysine deficient rats (Tanphaichitr and Broquist, 1973).

Catabolism of carnitine

Various degrees of carnitine degradation have been found under different conditions, ranging from no detectable breakdown of tritiated carnitine given to dogs (Yue and Fritz, 1962) to complete catabolism of the molecule by living cells of a strain of Pseudomonas (Lindstedt et al., 1967). Lindstedt and Lindstedt (1961a) found that 3% of [methyl-^{14}C] carnitine and 8% of [carboxyl-^{14}C]carnitine was converted to respiratory $^{14}CO_2$ in 24 h after the injection of the labelled compounds into rats.

Several breakdown products of carnitine have been postulated and demonstrated. These include trimethylaminoacetone, $(CH_3)_3N^+CH_2\,CO\,CH_3$, (Strack et al., 1962; Grüner et al., 1966; Lindstedt et al., 1967); glycine betaine, $(CH_3)_3N^+CH_2\,COOH$, (Lindstedt et al., 1967); 3-dehydrocarnitine, $(CH_3)_3N^+CH_2\,CO\,CH_2\,COOH$, (Schöpp, 1969) and the decarboxylation product, 3-methylcholine, $(CH_3)_3N^+CH_2\,CHOH\,CH_3$.

The decarboxylation pathway of carnitine metabolism has been fairly extensively studied as it has been shown that 3-methylcholine can replace the choline of the phospholipids in housefly larvae when they are reared in the presence of carnitine, 3-methylcholine, 4-butyrobetaine or trimethylaminoacetone (Newburgh et al., 1962; Bieber et al., 1961; Bieber et al., 1963; Bieber et al., 1969). The carnitine decarboxylase of Phormia regina has been studied by Habibullah and Newburgh (1969). The excretion of 3-methylcholine in the urine of metabolically stressed

rats is briefly discussed above (Introduction, Part II), and Khairallah and Wolf (1967) have examined the carnitine decarboxylase of several rat tissues.

It is obvious that carnitine catabolism is not via a readily identified route. The fact that carnitine is retained in the body for long periods with little or no apparent breakdown (Yue and Fritz, 1962; Lindstedt and Lindstedt, 1965) makes examination of breakdown products of carnitine difficult as they occur in only small quantities.

The use of sheep tissues to study carnitine biosynthesis and degradation

As was reported in Part II of this thesis, the concentration of carnitine in sheep liver can change dramatically when the metabolic state of the animal is altered. It therefore seemed likely that sheep tissues, particularly liver, might prove to be useful in the elucidation of the pathway of carnitine biosynthesis. The high concentration of carnitine in sheep skeletal muscle, and the report of a fall in the carnitine concentration in the skeletal muscle of rats when they become alloxan diabetic (Mehlman et al., 1969) led to an examination of sheep skeletal muscle too, as a possible site of carnitine biosynthesis.

On the basis of all the work reported above on compounds proposed as precursors of carnitine, it was decided that the most likely source of the carbon chain of carnitine is 4-aminobutyric acid. A pathway involving stepwise methylation followed by hydroxylation seemed the most attractive, as 4-butyrobetaine has been shown to be readily converted to carnitine while 3-hydroxy compounds are not (see above).

Carnitine biosynthesis was studied in homogenates of sheep liver and skeletal muscle and in sheep liver slices. Insulin, long-chain fatty acid and serum taken from sheep in different metabolic states were used as additives to the reaction mixture in order to try and gain some understanding of the control of carnitine biosynthesis. Using animals having hepatic and portal venous cannulae the possibility of carnitine production and output by the liver was also examined. The degradation of [carboxyl-^{14}C]carnitine and [methyl-^{14}C]carnitine was also studied.

III. Experimental and Results

III.A. Carnitine biosynthesis in homogenates of sheep liver and skeletal muscle

III.A.1. Materials and methods

Animals. Normal, starved (5 days) and alloxan-diabetic (a solution in sterile, isotonic saline of 60 mg alloxan/kg was given intravenously 7 days prior to slaughter) 4-year-old Merino wethers, weighing about 40 kg were used.

Tissues. The animals were killed by severing the necks. Samples of liver and skeletal muscle (M. biceps femoris) were quickly freeze-clamped for total acid-soluble carnitine determinations as described in Section II.B.1. Other samples of both tissues were removed and placed in an ice-cold solution of 0.9% NaCl in 0.05 M-tris-HCl, pH 7.2.

Homogenates. The tissues were rinsed with the buffered saline solution (above) and blotted free of excess liquid. Homogenates (20%, w/v) were prepared in the buffered saline at $0^{\circ}C$ with the aid of a Potter-Elvehjem homogeniser. Any whole cells and other debris remaining were removed by centrifuging at 700g for 5 min.

Incubations. Incubations were carried out in 5 x ½" test-tubes which were mechanically agitated in a water-bath at $38^{\circ}C$ for 1 h. The basic incubation mixture consisted of the following in the phosphate-buffered saline (above): 20 µmol $MgCl_2$; .2 µmol 2-oxoglutarate; 30 µmol

ascorbate; 2 μmol $FeSO_4$; 1 μmol NADPH and 2 mg catalase. To this were added the following substrates in various combinations: 30 μmol ATP; 30 μmol L-methionine; 7.5 μmol S-adenosyl-L-methionine; 6 μmol 4-aminobutyric acid; 6 μmol 4-methylaminobutyric acid or 6 μmol 4-trimethylaminobutyric acid (4-butyrobetaine). Some incubations had no added substrates. The volume was adjusted with buffered saline to a final volume of 2 ml.

The incubation mixture and homogenates were warmed in the water-bath for 5 min, and 1 ml aliquots of homogenate were added to the incubation tubes. The contents of the tubes were thoroughly mixed and air was bubbled through them during incubation to meet the requirement of 4-butyrobetaine hydroxylase for molecular oxygen. The reaction was stopped with 0.5 ml of 30% (w/v) $HClO_4$ and the tubes refrigerated. Zero-time controls had $HClO_4$ included in the incubation mixture. All tests were done in duplicate.

Total acid-soluble carnitine was measured in the protein-free supernatants of the reaction mixtures as described in Section II.B.1.

III.A.2. Results

Except when 4-butyrobetaine was the substrate no consistent results were obtained with liver or skeletal muscle either within an experiment (even the duplicate tests gave far from similar final carnitine concentrations, although duplicate zero-time controls were satisfactory) or between experiments. There was also no evidence obtained of consistent trends in the results of experiments using tissues from animals in

different metabolic states.

With 4-butyrobetaine as substrate, liver homogenates were found to produce carnitine at a rate of 220 ± 28 nmol/g wet wt per h (mean of 5 experiments ± S.E.M.). No evidence for 4-butyrobetaine hydroxylation to carnitine in sheep skeletal muscle was obtained, but the high concentrations of carnitine present in this tissue might mask a low rate of carnitine biosynthesis (see Discussion).

III.B. Carnitine biosynthesis in slices of sheep liver

III.B.1. Materials and methods

Animals. 4-year-old Merino wethers weighing about 40 kg were starved for 5 days.

Tissues. The sheep were slaughtered by severing the necks. A portion of the liver was rapidly freeze-clamped for measurement of the total acid-soluble carnitine concentration as described in Section II.B.1. Other samples of liver were placed in 0.9% NaCl at $0^{\circ}C$.

Preparation of tissue slices. Connective tissue was removed from the liver samples and slices approximately 1 cm^2 and 1 mm thick were cut by hand using a single-edged razor blade. These slices were used to prepare 10 x 1 mm slices, 0.4 mm thick using a McIlwain-Buddle tissue slicer (The Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.). The slices were placed in a beaker containing a small amount of 0.9% NaCl at $0^{\circ}C$.

Incubations. The basic medium used was a bicarbonate buffer (Krebs and Henseleit, 1932) which consists of (by volume) 100 parts 0.154 M-NaCl; 4 parts 0.154 M-KCl; 3 parts 0.11 M-$CaCl_2$; 1 part 0.154 M-KH_2PO_4; 1 part 0.154 M-$MgSO_4$ and 21 parts 0.154 M-$NaHCO_3$. The $NaHCO_3$ solution was gassed with 95% O_2/5% CO_2 for 1 h at $0^{\circ}C$ before use. The mixture was then gassed for 10 min at $0^{\circ}C$ and then stoppered and stored at $0^{\circ}C$ until required.

50 ml Erlenmeyer flasks were used as incubation vessels. The empty flasks were placed on ice and solutions of substrates and possible effectors (below) put into them. 2 ml of 30% (w/v) $HClO_4$ was added to the zero-time control flasks and bicarbonate buffer mixture was added to adjust the volume in each flask to 3 ml.

The liver slices were quickly blotted as dry as possible and 500 mg of slices were added to each flask. A further 5 ml of bicarbonate buffer was added, the flasks filled with 95% O_2/5% CO_2 and sealed with a Subaseal cap. They were mechanically agitated in a water bath at 38°C for 1 h. The reaction was stopped by adding 2 ml 30% (w/v) $HClO_4$ through the cap.

The substrates used were 300 μmol 4-aminobutyric acid; 300 μmol 4-butyrobetaine; 150 μmol L-methionine and 150 μmol L-lysine in various combinations. The effects of the following additions were studied: 10 μmol palmitic acid plus 0.1 g of bovine serum albumin (fat-free); 40 units of insulin and 1 ml of serum taken from normal, starved or alloxan diabetic sheep.

All incubations were done in duplicate.

Measurement of carnitine. The contents of the flasks were homogenised with the aid of a Potter-Elvehjem homogeniser, and the homogenate volume adjusted to 25 ml with water. The protein precipitate was removed by centrifugation, and total acid-soluble carnitine measured in an aliquot of the supernatant as described in Section II.B.1.

III.B.2. Results

Preliminary experiments with slices of rat liver showed that recovery of carnitine in the prepared slices was about 87%. With sheep liver, recovery varied from less than 50% to nearly 100% at first, depending on the amount of liver processed. Loss of carnitine from liver slices is to be expected as carnitine is confined to the cytosol (see Section II. Appendix.2.) and so can easily be leached out. Analysis of the NaCl solution in which liver slices had been kept showed that it can contain up to 20 nmol of carnitine per ml. This loss of carnitine could be kept to a minimum by rapid processing of the tissue and by storage of the slices in as small a volume of saline as possible. Results are presented only for experiments in which the carnitine concentration of slices in zero-time controls was 85% or more of that found in the freeze-clamped liver.

The results of experiments using L-methionine and 4-aminobutyrate or L-lysine as substrates are shown in Table 23. It is apparent that incubation with L-methionine plus 4-aminobutyrate results in carnitine accumulation in the liver slices which is in excess of that found in the absence of substrates. The addition of normal sheep serum was found to enhance the accumulation, but serum taken from starved or alloxan diabetic sheep had no effect. Insulin additions caused very variable amounts of carnitine accumulation (sometimes even an apparent loss of carnitine occurred) and in no case was an accumulation seen which was greater than that with no substrate. When lysine was used as substrate the carnitine accumulation was the same as when no substrate was added

TABLE 23

Carnitine accumulation by sheep liver slices

Slices of sheep liver were prepared, incubated and analysed as described in the text. The figures are the amount by which the total acid-soluble carnitine content of the liver slices was found to have increased above zero-time control values. They are expressed as means ± S.E.M. for 4 experiments.

Substrate	Effector*	Increase in carnitine concentration (nmol/g wet wt per h)
None	None	43.5 ± 9.5
4-aminobutyrate + L-methionine	None	69.0 ± 11.3
Ditto	NS	102.0 ± 19.5
Ditto	SS	67.3 ± 24.5
Ditto	DS	66.0 ± 37.6
Ditto	FA	56.5 ± 20.3
Ditto	Insulin	Mean = -6
L-lysine + L-methionine	None	39.3 ± 21.9

* NS = serum from normal sheep
SS = serum from starved sheep
DS = serum from alloxan diabetic sheep
FA = palmitic acid plus bovine serum albumin

(Table 23).

Further experiments have shown that it is the 4-aminobutyric acid which is primarily responsible for the increase in carnitine content of sheep liver slices in this system (Table 24). The effect of normal sheep serum is probably either to enhance endogenous carnitine production, or to provide an alternative substrate (Table 24). The carnitine content of the serum itself is not the source of the increased carnitine as zero-time control values were subtracted.

Results obtained when 4-butyrobetaine was used as substrate were about the same as those with 4-aminobutyrate alone (60 and 55 nmol/g wet wt per h in two experiments). It is possible that permeability barriers exist for this compound, as much higher rates of apparent incorporation of 4-butyrobetaine into carnitine were observed using liver homogenates (above).

TABLE 24

Carnitine accumulation by sheep liver slices

Slices of sheep liver were prepared, incubated and analysed as described in the text. "Serum" is blood serum of normal sheep. The figures are the amount by which the total acid-soluble carnitine content of the liver slices was found to have increased above zero-time control values. They are expressed as the means of two results, with the individual figures given in parenthesis.

Addition to incubation mixture	Increase in carnitine concentration (nmol/g wet wt per h)
None	37.5 (35, 40)
4-aminobutyrate, L-methionine	71.5 (60, 83)
4-aminobutyrate, L-methionine and serum	133.5 (160, 107)
4-aminobutyrate	55.5 (51, 60)
L-methionine	-1.5 (2, -5)
Serum	66.0 (80, 52)
4-aminobutyrate, serum	51.0 (53, 49)
L-methionine, serum	63.0 (55, 71)

III.C. Carnitine biosynthesis from radioactive precursors using sheep liver slices

III.C.1. Materials and methods

Animals, tissues, preparation of tissue slices and incubation conditions. These were all as described in Section III.B.1. Homogenates of liver (10%, w/v) were prepared as described in Section III.A.1.

^{14}C-labelled compounds. Uniformly labelled [^{14}C]4-aminobutyric acid (4.6 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks, U.K. [Carboxyl-^{14}C]DL-carnitine HCl (8.11 mCi/mmol) and [methyl-^{14}C]CL-carnitine HCl (22.5 mCi/mmol) were obtained from International Chemical and Nuclear Corp., Irvine, Calif. 92664, U.S.A. [6-^{14}C]DL-lysine HCl (48 mCi/mmol) was obtained from Schwarz/Mann, Orangeburg, N.Y. 10962, U.S.A. [Carbonyl-^{14}C]creatinine HCl (5.15 mCi/mmol) was also from The Radiochemical Centre. All compounds were found to be pure as judged by thin layer chromatography.

Substrates. 1.2 μCi per incubation of the uniformly ^{14}C-labelled 4-aminobutyric acid (above) was diluted with unlabelled 4-aminobutyric acid to a final concentration of 200 nmol/incubation. Also added were 300 μmol L-methionine and 1 ml of normal sheep serum to each flask. Zero-time and unlabelled controls were set up with each experiment. All incubations were done in duplicate.

Preparation of reaction mixture for analysis. The reactions were stopped with 1 ml 30% (w/v) $HClO_4$ and the flask contents homogenised and

diluted to 25 ml (as in Section III.B.1.). The homogenates were centrifuged, and aliquots of the supernatants neutralized with saturated $KHCO_3$. The $KClO_4$ precipitate was removed by centrifuging. The supernatant was dried under a gentle stream of air and the residue taken up in the smallest possible volume of water.

Thin layer chromatography. The separation of 4-aminobutyric acid, its methyl derivatives and carnitine was required.

Thin layers of silica gel G (0.4 mm thick) were prepared on glass plates, and were activated at 105°C for 30 min just prior to use. Solvent systems I, II and III of Eneroth and Lindstedt (1965) were examined. Unlabelled "carriers" were added to the solutions under test, and chromatography was performed at 25°C. Standards were included in each set of plates, and creatinine was used as a reference point on all plates.

Column chromatography. Desalting of reaction mixtures and separation of 4-aminobutyric acid from carnitine (and any intermediates) were attempted by two methods, both of which are described by Lindstedt (1967). The resins used were Dowex AG 50W-X8 (-400 mesh, H^+) and the ion retardation resin AG IIA8. Column preparation and elution were as described by Lindstedt (1967). The columns were characterised using aqueous solutions of the pure compounds both separately and together. Separations on these columns were also examined using labelled compounds added to liver homogenates prepared in the reaction mixture used for liver slice incubations and treated with $HClO_4$ etc. as described above.

Measurement of radioactivity. Liquid samples were added to 10 ml of a solution of 4 g PPO (2,5-diphenyloxazole) and 0.1 g dimethyl POPOP (1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene) per litre of toluene in standard glass vials (Packard Instrument Co., Chicago, Illinois, U.S.A.). When large volumes of aqueous solution had to be analysed it was sometimes necessary to "dissolve" the sample in ethanol. Counting was done in a Packard Tricarb liquid scintillation spectrometer, model 3375. Channels-ratio quench-correction curves calculated using Packard Instrument Co. standards were used to determine counting efficiency.

Thin-layer chromatograms were scanned in a Packard Radiochromatogram scanner, model 7201, fitted with a recording rate meter. The efficiency for ^{14}C was about 10%.

III.C.2. Results

Accumulation of total acid-soluble carnitine in liver slices incubated with the lower concentration of 4-aminobutyric acid used here (200 nmol here cf. 300 μmol in Section III.B.) was less than that previously observed, but still exceeded that seen when no substrate was present. The accumulation was 48.0 ± 4.7 nmol/g wet wt per h (3 experiments) when 4-aminobutyric acid was the substrate compared with 36.3 ± 8.2 nmol/g wet wt per h (3 experiments) with no substrate. The difference is approximately 12 nmol/g wet wt per h compared with a difference of 26 nmol/g wet wt per h reported in Section III.B.2.

Separation of 4-aminobutyric acids, its methyl derivatives, carnitine and 3-methylcholine was achieved most satisfactorily in the

solvent system 75% methanol/25% ammonium hydroxide. Detection of the spots with ninhydrin and iodine vapour was good, and bands of radioactive standards were easily identified using the radiochromatogram scanner.

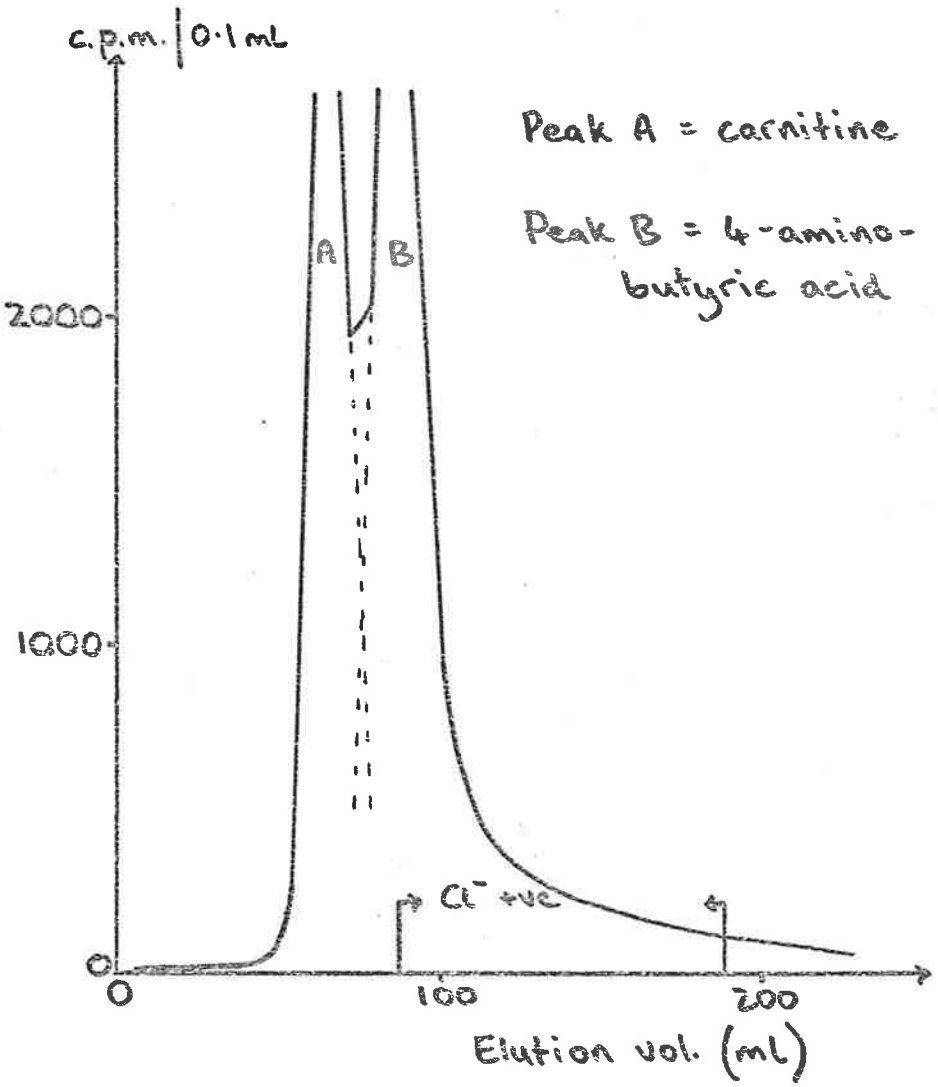
The application of "test" samples to the thin-layer chromatography plates proved very difficult. As the radioactive products were likely to be present in very small amounts, rather large aliquots of the final tissue extract had to be applied to the plates. It was found to be impossible to apply samples satisfactorily prior to desalting. Desalting on Dowex AG 50W-X8 columns was easily achieved, but detection of the carnitine and 4-aminobutyric acid in the eluate was difficult unless an amount of carrier was added which was too great to be applied to the thin-layers when the eluate was concentrated. These columns could not be used to resolve a mixture of carnitine and 4-aminobutyric acid.

Retardion AG IIA8 columns were characterised using radioactive 4-aminobutyric acid and/or carnitine added to liver homogenates (see above) and gave excellent results. Desalting ahead of the 4-aminobutyric acid peak was easily achieved, and 4-aminobutyric acid and carnitine were separated as sharp peaks, though there was a little "tailing" and some overlap between the peaks (and some overlap of the peaks when large amounts of both radioactive compounds were used), see Fig. 5. Thin-layer chromatography of the eluate corresponding to the peaks showed that the order of elution was carnitine first, then 4-aminobutyric acid plus salts.

FIG. 5

The elution profile of carnitine and 4-aminobutyric acid from a Retardion column

A column (2.2 x 29 cm) of Retardion (AG IIA8, 50-100 mesh) was prepared with water. A liver homogenate was prepared and [U-^{14}C] 4-aminobutyric acid (about 0.6 μCi), [methyl-^{14}C]DL-carnitine (about 0.8 μCi) added, and the unlabelled compounds (100 μmol of each). Preparation of the homogenate for chromatography is described in the text. The extract was applied to the column and eluted with water at a flow rate of approximately 1 ml/min. 5 ml fractions were collected. The radioactivity in 0.1 ml aliquots of the eluate was measured as described in the text. Chloride was detected with a solution of silver nitrate.



The results of an experiment in which a very small quantity of [methyl-^{14}C]carnitine was added to a liver homogenate plus the amount of [U-^{14}C]4-aminobutyric acid used in incubations are depicted in Fig. 6. It is apparent that good separation is possible in such a case.

It is not known where the methyl derivatives of 4-aminobutyric acid would be eluted as none of these were available in the radioactive form. Time did not permit an examination of the system using unlabelled compounds combined with an examination of the elution profile by thin-layer chromatography.

The results of one of two experiments (which gave essentially the same result) in which tissue slices were incubated with [U-^{14}C]4-aminobutyric acid are illustrated in Fig. 7. It is clear that there is some radioactivity present which is not that of 4-aminobutyric acid. This appears earlier than the 4-aminobutyric acid peak as a "shoulder". No such shoulder was seen during the characterisation of these columns. It is possible that this shoulder represents methyl derivatives of 4-aminobutyric acid, and maybe carnitine as well. Thin-layer chromatography of concentrated samples taken from both the shoulder region and the peak has established that the peak is 4-aminobutyric acid but has failed to resolve the identity of the components of the shoulder as the number of counts in this area is very low, and the efficiency of the scanner for $^{14}CO_2$ is only 10%.

Experiments with labelled lysine were not successful, as this compound proved impossible to elute from the Retardion column.

FIG. 6

The elution profile of a small amount of carnitine together with a large amount of 4-aminobutyric acid from a Retardion column

The column and the technique used are described in the legend to Fig. 5, except that about 1.2 μCi of [U-^{14}C]4-aminobutyric acid and about 0.008 μCi of [methyl-^{14}C]DL-carnitine were added.

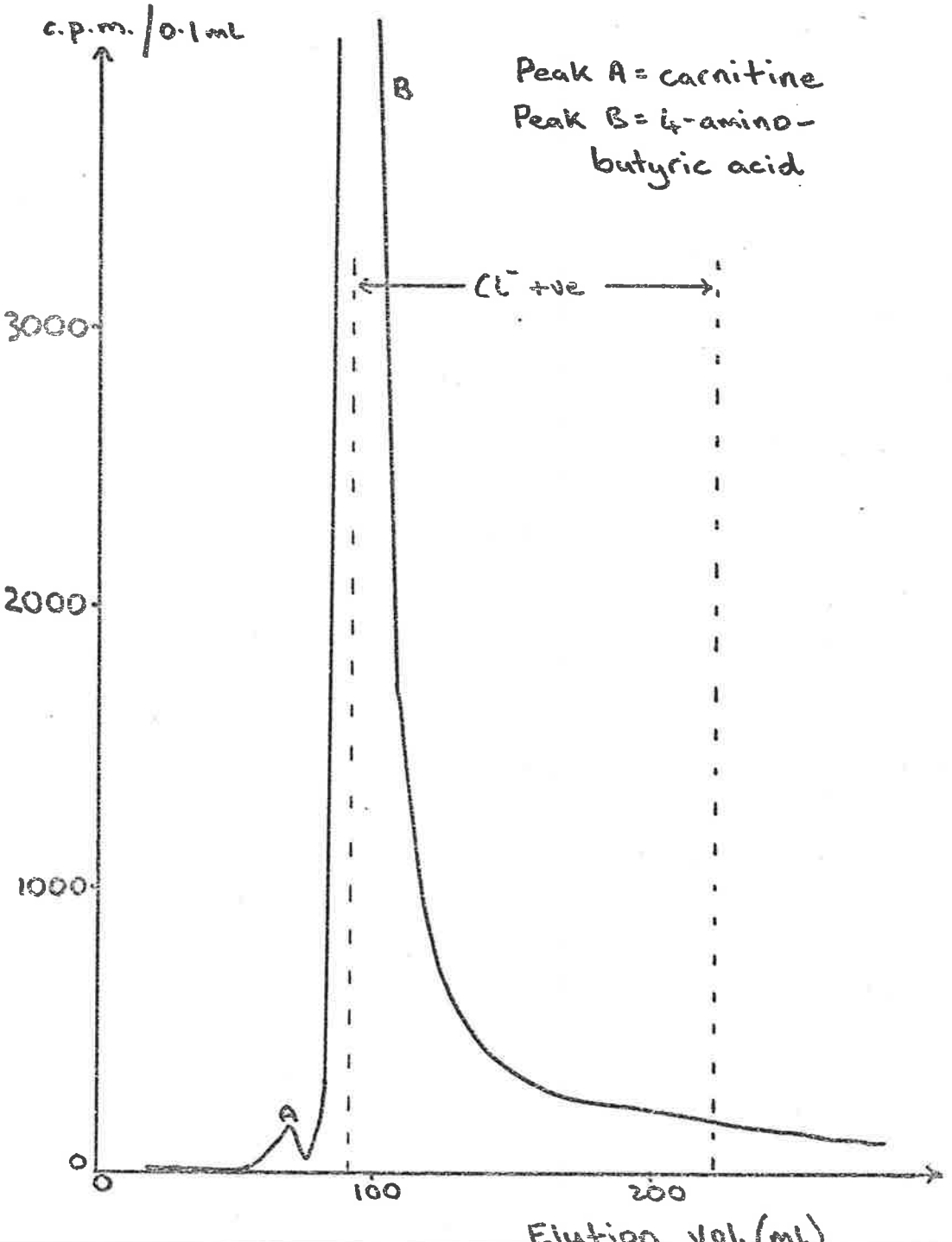
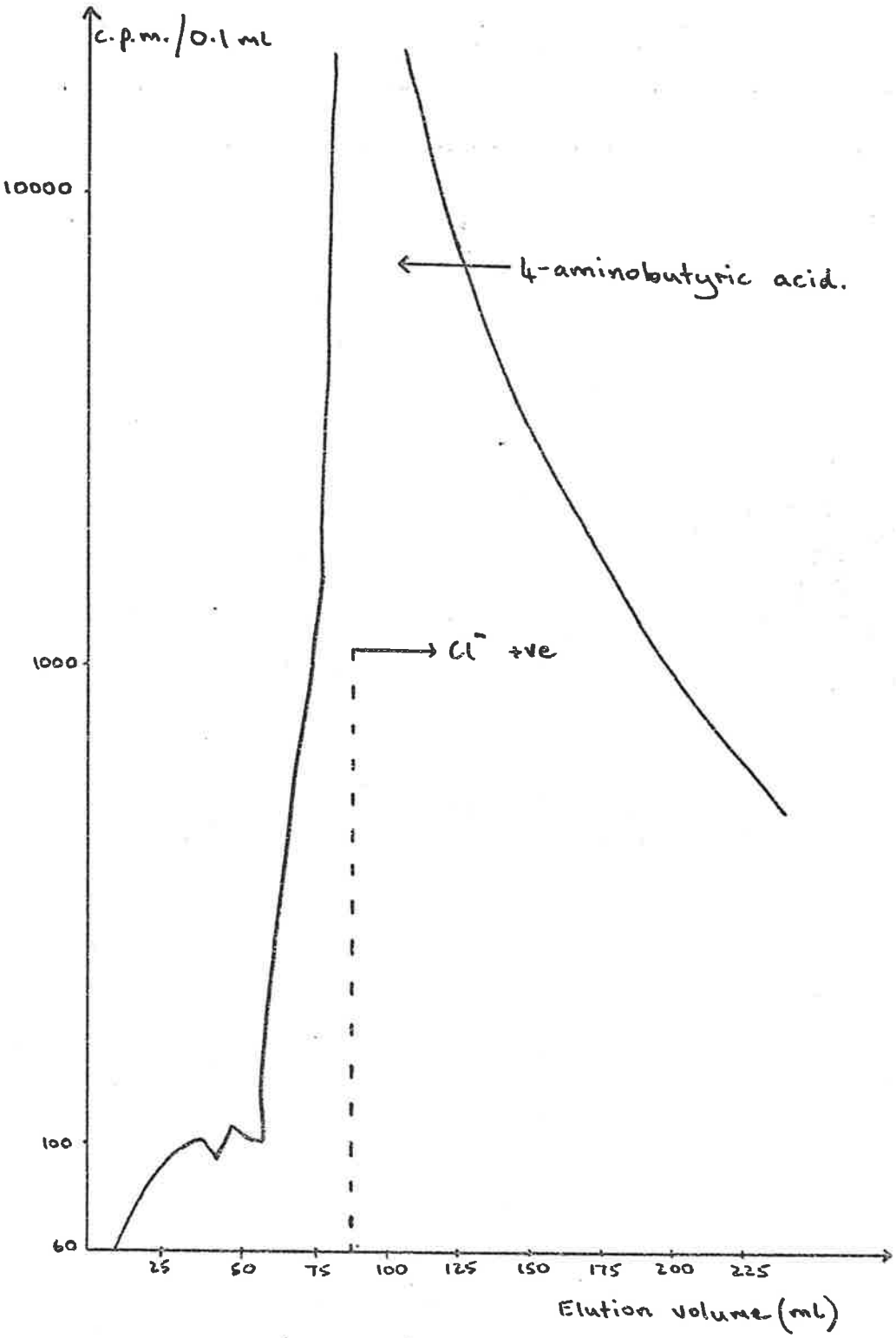


FIG. 7

The elution profile of an extract of liver slices which had been incubated with [U-^{14}C]4-aminobutyric acid

The column used is described in the legend to Fig. 5. Details of the incubation and extraction procedure are given in the text.



III.D. The production of carnitine by the skeletal muscle and liver of sheep, in vivo

III.D.1. Materials and methods

Animals and treatment. The sheep used and the surgical implantation of hepatic and portal venous cannulae are described in Section I.C.1. Cannulae were also inserted into the femoral artery and vein. Alloxan treatment was as described in Section I.C.1. The food intake of all animals remained at 750 g or more per day.

Blood samples. Blood samples from all cannulae were taken daily before and after alloxan treatment. Extraction and measurement of total acid-soluble carnitine were as described in Section II.B.2. All samples were taken and assayed in duplicate.

III.D.2. Results

The total acid-soluble carnitine concentrations of portal and hepatic venous blood taken from normal and alloxan treated sheep are shown in Table 25. It is obvious that neither in the normal sheep, nor at any stage in the alloxan treated sheep is there any significant difference between the concentrations in hepatic and portal venous blood. Only a few results are available for femoral arterial and venous blood of these sheep (the results are not tabulated). Again, there was no difference in the total acid-soluble carnitine concentrations of samples taken from the two blood vessels.

TABLE 25

Carnitine concentrations in the portal and hepatic venous blood of normal and alloxan-treated sheep

Blood samples were collected and assayed for total acid-soluble carnitine as described in the text. The figures shown are means ± S.E.M. for the number of samples shown in parentheses.

Days after alloxan	Total acid soluble carnitine (nmol/ml) Portal	Hepatic
0	39.3 ± 2.7 (18)	40.8 ± 2.8 (21)
1	35.8 ± 6.0 (4)	33.6 ± 3.3 (4)
2	31.3 ± 7.5 (3)	27.0 ± 5.2 (3)
3	40.8 ± 11.5 (3)	39.3 ± 8.1 (4)
4	44.2 ± 16.1 (4)	46.7 ± 13.4 (4)
5	58.4 ± 15.5 (3)	60.2 ± 15.4 (3)
6	59.4 ± 20.4 (4)	55.5 ± 20.7 (4)
7	72.6 ± 15.0 (4)	70.8 ± 14.1 (4)

The effect of alloxan administration on the total acid-soluble carnitine concentration of portal and hepatic venous blood was the same as that seen in jugular blood (Section II.B.2.6.). There was a fall in concentration in the first 24 to 48 h but by 7 days after treatment the concentration was higher than that in the blood of normal animals (Table 25).

III.E. Degradation of carnitine in sheep tissues

III.E.1. Materials and methods

Animals. The animals used are described in Section III.B.1.

Tissues. Liver samples were taken and slices prepared as described in Section III.B.1. Liver homogenates (10%, w/v) were prepared as described in Section III.A.1.

Radioactive compounds. The carboxyl- and methyl-labelled carnitine were described in Section III.C.1. The positions of the labels were checked by the method of Wolf and Berger (1961).

Incubations. Incubations using tissue slices were carried out in 25 ml Erlenmeyer flasks fitted with Subaseal caps which had plastic cups, containing 0.3 ml of 50/50 (v/v) mixture of 2-ethoxyethanol and ethanolamine, suspended from them. The basic incubation buffer was the bicarbonate buffer described in Section III.B.1. To 2 ml of this in the flasks was added either methyl- or carboxyl-labelled carnitine, each equivalent to approximately 5×10^6 cpm (in DL-carnitine), unlabelled DL-carnitine to give a final concentration of L-carnitine of 21 µmol/incubation and 0.2 g of liver slices. The flasks were gassed with 95% O_2/5% CO_2, stoppered and incubated for 1 h at 38°C in a shaking waterbath. The reaction was stopped with 1 ml 3N-HCl and shaking continued at 38°C for 1 h. Control flasks contained 1 ml 3N-HCl from the beginning of the incubation.

Homogenates (0.25 ml unless otherwise stated) were incubated in a similar fashion except that the incubation mixture consisted of 100 µmol potassium phosphate buffer, pH 7.4, 10 µmol ATP and 30 µmol $MgCl_2$ in water to a final volume of 1 ml (cf. Khairallah and Wolf, 1967).

$^{14}CO_2$ counting. The cups containing the CO_2 absorbant were dropped into 10 ml of a mixture of toluene and 2-ethoxyethanol (2:1, v/v) with 0.5% PPO and 0.03% dimethyl POPOP in standard glass vials (see Section III.C.1.). Counting and quench corrections were as described in Section III.C.1.

III.E.2. Results

Only a few results are available, but from these it is apparent that carnitine decarboxylation takes place rapidly in liver homogenates, and occurs to a lesser extent in liver slices. With homogenates, 262 nmol/g wet wt per h of carnitine was decarboxylated (average of two), while liver slices decarboxylated 13.6 ± 1.7 nmol/g wet wt per h (mean ± S.E.M. for five experiments) when [carboxyl-^{14}C]carnitine was used as substrate. Increasing the amount of homogenate used resulted in a decrease in the rate of release of $^{14}CO_2$ from [carboxyl-^{14}C]carnitine. In one experiment, 849 nmol/g wet wt per h of carnitine was decarboxylated when 0.125 ml of homogenate was used, but only 267 nmol/g wet wt per h when 0.5 ml was used.

In two experiments where both of the labelled carnitine samples were used (in separate incubations) it was found that, in each case, almost the same amount of breakdown was detected with both substrates.

[Carboxyl-^{14}C]carnitine gave results of 55 nmol/g wet wt per h of carnitine degraded in one experiment with slices, and 261 nmol/g wet wt per h in the other, which was with homogenate. The corresponding results for [methyl-^{14}C]carnitine were 66 and 273 nmol/g wet wt per h respectively.

Attempts to identify [methyl-^{14}C]3-methylcholine in the reaction mixture after using methyl-labelled carnitine were unsuccessful.

III. Discussion

The results presented in Part II of this thesis, plus those of Snoswell and Henderson (1970), indicate that carnitine must be synthesized by some sheep tissues. Treatment of sheep with alloxan eventually caused higher than normal concentrations of total acid-soluble carnitine in all tissues examined as well as in blood and urine (Part II, Experimental). Since starvation of sheep resulted in elevated carnitine concentrations in liver (Snoswell and Henderson, 1970), and the occurrence of spontaneous starvation in alloxan diabetic sheep caused even higher tissue, blood and urine carnitine concentrations than alloxan diabetes alone (Part II, Experimental), it is obvious that the diet cannot be the source of the extra carnitine. It seems unlikely that either the microflora of the gut or any one tissue or organ could be producing all this carnitine. The fact that the blood carnitine concentration rises in alloxan diabetes does, however, imply that some tissue(s) is capable of adding more carnitine to that already circulating. Unpublished results of A.M. Snoswell and G. McIntosh, referred to below, show that the liver of sheep is indeed capable of releasing carnitine to the circulation.

At the time the work using sheep tissue slices commenced it was decided to use only animals which had been starved for 5 days. This was because it was felt that in the tissues of the normal animal one might fail to see evidence of carnitine biosynthesis if the normal turnover of carnitine is slow, as it is in the rat (Mehlman *et al.*, 1969). In tissues of sheep with established alloxan diabetes, the high

carnitine concentrations already present could result in feed-back inhibition of biosynthesis, or to masking of small changes in concentration which might occur. The argument of feed-back inhibition and difficulty in detection of small carnitine concentration changes applies to skeletal muscle of all adult sheep, regardless of treatment. Thus, only liver was used in tissue slice experiments.

The period of 5 days starvation was chosen as Snoswell and Henderson (1970) had shown that 7 days starvation causes a 5-fold increase in the total acid-soluble carnitine concentration of sheep liver, and it is known that food remains in the rumen for 3 to 4 days after starvation is started. It was felt that by 5 days after food withdrawal the effects of starvation would be starting to be obvious.

The results presented in Section III.B.2. show that there was accumulation of carnitine in liver slices which were incubated with 4-aminobutyrate, and that the mixture of 4-aminobutyrate, L-methionine and normal sheep serum gave the greatest carnitine accumulation. The results of experiments using labelled 4-aminobutyrate as substrate are very tentatively interpreted as indicating that label from this substrate might be incorporated into carnitine (see Section III.C.2.). Unfortunately, it has so far proved impossible to present unequivocal evidence of carnitine biosynthesis in this system. Assuming that carnitine biosynthesis does occur by this route in the system used, the failure to detect carnitine biosynthesis can be attributed mainly to difficulties experienced in utilizing the techniques available for the separations required.

As reported in Section III.A.2., no reliable results were obtained for carnitine accumulation when homogenates of liver were used, unlike the situation with liver slices (above). It is possible that the structural integrity of the tissue is a requirement of the biosynthetic pathway *in toto*, or that the greater rate of carnitine decarboxylation which apparently took place in tissue homogenates (compared with slices) was sufficient to account for any newly synthesized carnitine.

When proposing the biosynthesis of a compound, rather than simply its ingestion and absorption, the existence of metabolic control systems must be considered. As alloxan diabetes causes such marked increases in the carnitine concentration of some sheep tissues (see Sections II.B.2.3. and II.B.2.4.), it is obvious that insulin must be regarded as a possible control factor. Trenkle (1970, 1971 and 1972) has shown that the insulin concentration of sheep plasma decreases on fasting, which also substantiates a theory of insulin involvement in the control of carnitine biosynthesis. The effect of insulin in rats must be different from that in sheep, as Mehlman *et al.* (1969) reported that alloxan diabetes resulted in a 50% reduction in the carnitine content of rat skeletal muscle. Normal carnitine concentrations were re-obtained when the diabetic rats were treated with insulin (Mehlman *et al.*, 1969).

Free fatty acid concentrations of sheep plasma are, of course, also altered by starvation and alloxan diabetes. A rise in concentration occurs in each state (Gooden, 1969), and it is possible that this change could influence the rate of carnitine biosynthesis.

Glucose concentrations in sheep plasma are elevated in alloxan diabetes, but starvation was found to cause a reduction in the blood glucose concentrations of sheep (Gooden, 1969). As the response of blood glucose concentration in the two metabolic states is different, while that of tissue carnitine (in sheep) is the same, it was considered unlikely that glucose itself plays a regulatory role in the biosynthesis of carnitine.

The effects of insulin, palmitic acid and serum from normal, starved and alloxan diabetic sheep on carnitine accumulation by sheep liver slices were studied. It is apparent from the results (Section III.B.2.) that only serum from normal sheep has a stimulatory effect. It is suggested that the serum is either providing an alternative substrate to the 4-aminobutyrate offered, or that it enhances endogenous carnitine production. It is not known if serum contains any 4-aminobutyrate, but it is considered unlikely that this could be the stimulatory factor as large amounts of this compound are already present in the incubation mixture. That serum from normal sheep should stimulate carnitine accumulation while serum taken from animals in a state in which carnitine biosynthesis should be occurring had no effect, was surprising. At present, no explanation can be offered for this finding. The lack of effect on, or apparent inhibition of carnitine accumulation in liver slices by insulin is interesting, but the results of these experiments were extremely variable and the significance of this effect in the system used is doubtful. Palmitic acid was found to have no effect.

The results of the experiments on cannulated sheep were disappointing. Individual results often gave the impression that there was a net output of carnitine by the liver, but the differences were small and not consistently obtained. Since the work reported in this thesis was finished A.M. Snoswell and G. McIntosh (unpublished work) have found that, in severely diabetic sheep (10 to 14 days after alloxan administration), which spontaneously restricted their food intakes, it can be definitely shown that the liver does release carnitine into the blood stream. The results of this work are summarised in Table 26. Even with these animals, no arterio-venous differences were observed across the musculature of the leg (see Discussion, Part II for details).

Although the results presented here are not unequivocal, it would not be presumptuous to suggest that they, in conjunction with those of Snoswell and McIntosh (above), do indicate that sheep liver can synthesize carnitine and that 4-aminobutyrate is probably a precursor. More work is obviously needed on this system, and some suggestions for such work are made below.

The detection of carnitine biosynthesis from labelled 4-aminobutyrate, and of intermediates should be possible when the technique of separation on the Retardion columns is refined. A problem which will be more difficult to resolve is that of getting sufficient radioactivity incorporated into the newly synthesized carnitine (and intermediates) for detection on thin-layer chromatograms. However, if the column technique can be sufficiently refined this might obviate the need for further chromatography. A means by which more heavily labelled products

TABLE 26

The production of carnitine by the liver of spontaneously-starved, alloxan-diabetic sheep

Eighteen-month-old Merino wethers were fitted with cannulae in the portal and hepatic veins as described in the text. They were given alloxan (60 mg/kg) 10 to 14 days before slaughter. Blood samples were taken and analysed for total acid-soluble carnitine as described in the text. The figures are individual values obtained for duplicate samples on the three days prior to alloxan administration, and the values for the few days before slaughter (as indicated). The results are from unpublished work of G. McIntosh and A.M. Snoswell.

		Carnitine concentrations (nmol/ml)						
		Days before alloxan			Days before slaughter			
Sheep no.	Vein	2	1	0	3	2	1	0
A1.761	Hepatic	21	34	34	98	70	59	279
		22	32	33	97	68	59	273
	Portal	24	38	34	38	23	37	201
		22	34	32	34	21	35	193
A1.567	Hepatic	45	51	40	-	78	60	108
		46	46	39	-	76	56	102
	Portal	44	48	42	-	70	45	95
		44	43	40	-	69	43	94

could be obtained would be to use more active enzyme systems i.e. to find the condition in the animal which gives the highest rate of carnitine biosynthesis. It might be worthwhile investigating the system at various stages of the development of alloxan diabetes.

The skeletal muscle of 5- to 16-day-old lambs has a carnitine concentration which is only 40% of that of adult sheep (Section II.B.2.2.). Henderson and Snoswell (unpublished work) have found that the skeletal muscle of very young lambs has an even lower carnitine concentration, and that increase in concentration is rapid at least in the first few days of life: at 12 h the concentration of total acid-soluble carnitine in lamb skeletal muscle is only 740 nmol/g wet wt; by 24 h it is 1430 and at 72 h, 2100 nmol/g wet wt. Thus, it is possible that the skeletal muscle of very young lambs (or even foetal lambs) might provide good experimental material for an examination of carnitine biosynthesis. Also, with very young, and therefore relatively very small, lambs it might be feasible to perform some in vivo experiments using labelled precursors of carnitine.

The results of Snoswell and McIntosh (unpublished work, referred to above) from spontaneously-starved alloxan-diabetic sheep having hepatic and portal venous cannulae, indicate that an investigation of carnitine biosynthesis by sheep liver in vivo could be performed on such animals by adding precursors and possible effectors of the system to the portal blood.

The fact that feed-back inhibition of the biosynthesis of carnitine could be caused by the high concentrations of carnitine in the

liver of alloxan diabetic animals was discussed above. It is, of course, quite possible that feed-back inhibition could occur at fairly low carnitine concentrations. If this was so, then the precautions taken to minimise loss of carnitine from the liver slices as they were prepared were probably mistaken. Perhaps by leaching out of the endogenous carnitine of the tissue, biosynthesis might be encouraged.

In retrospect, it is obvious that a number of things were neglected which should have been examined during the course of this work. One of these is the measurement of 4-butyrobetaine hydroxylase activity in sheep tissues. Lindstedt and Lindstedt (1970) used one assay system which depends on the detection of labelled carnitine after reaction with [methyl-$^{14}C_3$]4-butyrobetaine which, when using a partially purified enzyme system is quite feasible. With crude preparations of sheep tissues having unknown activity, all the problems outlined above which are inherent in the identification of labelled carnitine would, one suspects, rapidly become apparent. Lindstedt et al. (1970) have described another assay method based on the release of $^{14}CO_2$ from [carboxyl-^{14}C]2-oxoglutarate (which is oxidatively decarboxylated during the hydroxylation of 4-butyrobetaine - see Introduction, Part III). Using this method for assay of 4-butyrobetaine hydroxylase activity A.M. Snoswell (unpublished work) has found that sheep liver cytosol is only about 20% as active as rat liver cytosol and that the activities of sheep liver, kidney cortex and heart cytosol are about the same. That of sheep skeletal muscle may be lower, but only one assay of this tissue has been done so far. This situation is in contrast to that in the rat

where muscle and kidney cytosol were found to have no butyrobetaine hydroxylase activity (Lindstedt, 1967), and the same results have been obtained with rat tissues in this laboratory (A.M. Snoswell, unpublished work). This finding emphasizes further the profound differences in carnitine metabolism found to exist between ruminants (sheep) and non-ruminants (rats).

Another experiment which should be done is an assessment of the amount (if any) of labelled 4-aminobutyric acid which is taken up by liver slices under the conditions employed in these incubations. There have been several reports that 4-aminobutyric acid is not taken up by slices of any tissue examined other than brain (e.g. Elliott and Van Gelder, 1958; Tsukada et al., 1960). More recently, Vavatsi-Manos et al. (1973) showed that kidney cortex slices do take up 4-aminobutyric acid; but kidney is now known to contain and utilize 4-aminobutyrate (see Introduction, Part III). Tsukada et al. (1960) found that liver slices of guinea pigs produce $^{14}CO_2$ from [1-^{14}C]4-aminobutyric acid, even though they did not accumulate the substrate from the medium. The results of experiments (not reported) where $^{14}CO_2$ output was measured when [1-^{14}C]4-aminobutyric acid was the substrate for carnitine biosynthesis have indicated that 4-aminobutyrate is metabolised (decarboxylated) by sheep liver slices. Whether any further, or different, metabolism of 4-aminobutyric acid occurs is not known, at least until more work is done on the possible incorporation of this compound into carnitine.

When this study of carnitine biosynthesis was started, one intention was to measure the concentration of 4-aminobutyrate which is

(or may be) normally found in sheep tissues (non-neural) and blood by the enzymic method of Jakoby (1962). Unfortunately, by the time it was learnt that the enzyme was no longer commercially available it was too late to start a project involving enzyme purification.

An examination of the effects of insulin, free fatty acid and sheep serum on the 4-butyrobetaine hydroxylase of sheep tissues could provide some insight into the control of carnitine biosynthesis. However, it is anticipated that any such control is probably exerted at an earlier point on the biosynthetic pathway, as there are no reports of 4-butyrobetaine accumulation in animal tissues, and labelled 4-butyrobetaine injected into mice is rapidly converted to carnitine (Lindstedt and Lindstedt, 1965).

The stimulatory effect of normal sheep serum on carnitine accumulation in liver slices has been discussed above. It would obviously be of interest to attempt the identification of the factor present in normal sheep serum which causes this stimulation, and to examine the serum of starved and diabetic sheep to establish whether the factor is absent from the serum of these animals or whether an inhibitory factor has been added.

Although the results obtained with lysine as a carnitine precursor in sheep tissues were negative, this compound should perhaps be examined more closely. It is felt that the work of Broquist's group on this subject is not convincing (see Introduction, Part III) but the fact that some incorporation of [6-^{14}C]lysine but not of [2-^{14}C]lysine into carnitine was detected may be significant. The implication that

6-N-trimethyllysine could be involved in carnitine biosynthesis from lysine in rats (Tanphaichitr et al., 1971) has some interest when sheep are considered as 6-N-methyllysine has been shown to occur in sheep plasma (Weatherall and Haden, 1969), and the concentration of it in sheep plasma is high compared with that of pigs and rats (Bergen and Potter, 1971). The satisfactory separation of lysine from carnitine will probably not be possible on the Retardion columns as the elution profile for [^{14}C]lysine shows a shallow shoulder preceding the peak and a tail of radioactivity which could not be completely removed from the column. Other methods for the removal of lysine from the reaction mixture will have to be investigated.

Bergen and Potter (1971) showed that the availability of methionine affected the regulation of 6-N-methyllysine synthesis and turnover in sheep. S-adenosylmethionine is the methylating agent for lysine as well as being the donor of the methyl groups of carnitine. It is obvious that any radical changes in the concentration of one compound which obtains methyl groups from S-adenosylmethionine could be reflected in the concentrations of others. In this regard, an examination of changes in 6-N-methyllysine concentrations in sheep plasma caused by starvation or alloxan diabetes would be of interest. Another compound which utilizes S-adenosylmethionine in its biosynthesis is choline (Bremer and Greenberg, 1961). An investigation of changes in the concentrations of both choline and the choline-containing phospholipids, which might occur with changes in the metabolic state of sheep, was intended to be part of this study. Several attempts were made to

purify choline kinase and choline acetyltransferase from various sources in order to develop an enzymic assay for free choline, without success. A particular problem was the affinity of the enzyme preparations for carnitine. Time did not permit an examination of the phospholipids of sheep tissues, but such a study has now been started by another worker in this laboratory. Preliminary results (G.D. Henderson, unpublished work) have shown that the relative proportion of choline-containing phospholipids of liver is, in fact, lower in alloxan diabetic sheep compared with normal sheep, i.e. when carnitine concentrations are raised, the concentration of choline-containing phospholipid is reduced.

Methionine metabolism in sheep has been extensively examined with regard to wool production, but little research seems to have been done on S-adenosylmethionine formation and methyl-group donation in sheep. This is now being examined in this laboratory.

The degradation of carnitine in homogenates of liver taken from starved sheep can be quite extensive (up to 850 nmol/g wet wt per h) but results using homogenates were extremely varied. More consistent and much lower figures were obtained when liver slices were used. With both homogenates and slices, methyl-labelled carnitine was broken down as rapidly as carnitine labelled in the carboxyl group, indicating that there is extensive degradation of the carnitine molecule. It would be interesting to know whether variations in the rate of carnitine catabolism occur with changes in the metabolic state of sheep.

GENERAL DISCUSSION

The results presented in this thesis and those of other workers referred to in the text clearly establish that carnitine is of extreme significance in the metabolism of sheep and other ruminants, and that great differences exist in carnitine, CoA, fatty acid and ketone body metabolism between ruminants and non-ruminants.

The [free CoA]/[acetyl-CoA] ratio is a major factor in the regulation of hepatic ketogenesis, and the results presented in this thesis indicate that, in sheep tissues, carnitine may play an important role in regulating the degree of acetylation of CoA which is observed. The inverse relationship found to exist for carnitine and CoA concentrations in sheep tissues indicates that the metabolism of these compounds is probably highly integrated, and that carnitine has a function in "protecting" CoA from esterification (particularly acetylation, see below) and thus ensures the ready availability of the relatively small amount of CoA for participation in further metabolic processes.

The hypothesis that carnitine acetyltransferase could function to remove "acetate" from the mitochondria rather than facilitate its inward transfer was proposed when the intracellular location and latency of the enzyme were established (Barker et al., 1968). It was suggested that an enzyme thus situated would not be available to extramitochondrial acetyl-CoA. It is now suggested herein that the transfer of acetyl groups out from the mitochondria is one of the main functions of the hepatic carnitine and carnitine acetyltransferase system in sheep.

It is proposed that acetylcarnitine is formed from intramitochondrial acetyl-CoA via the transferase reaction, and that acetylcarnitine is hydrolysed in the cytosol of sheep liver.

This proposition is supported by recent results of N. Costa and A.M. Snoswell (unpublished work) who have demonstrated that hydrolysis of acetylcarnitine is catalysed by a specific enzyme which occurs in the cytosol of sheep hepatocytes (and in other sheep tissues). The activity of the enzyme was reduced by starvation and alloxan diabetes in sheep - which finding seems to partially explain the tremendous increase in the acetylcarnitine concentration and proportion which occurs in sheep liver (and skeletal muscle) under these circumstances. Carnitine acetyltransferase activity was found to be increased in the livers of alloxan diabetic sheep (N. Costa and A.M. Snoswell, unpublished work). These results emphasize the role of carnitine in relieving "acetyl pressure" on the CoA system, and suggest that carnitine participates in the production of acetate in sheep liver for further metabolism by the liver and other tissues of the sheep.

It is also apparent that this system plays an important regulatory role in hepatic ketogenesis, since the activity of the enzyme is reduced in conditions where high rates of ketogenesis are observed. The increase in carnitine concentration which occurs in alloxan diabetes is evidently not sufficient to absorb all the "excess" acetate (acetyl-CoA) which is produced under these conditions, and this is diverted to ketone body formation.

The economic importance of pathological ketotic conditions in ruminants has been mentioned previously. It is obvious that a study of carnitine and CoA metabolism in pregnant and lactating ruminants could greatly assist in elucidating the aetiology of pregnancy toxaemia and bovine ketosis.

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