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ASPECTS OF THE METABOLIC ROLE AND BIOSYNTHESIS OF CARNITINE

A Thesis
submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

by

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January 1977

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My love and thanks go to my wife, Robyn, who gave me encouragement and inspiration when I needed it, drive when I needed it, and love, warmth and friendship always.

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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.

NICK DIMITRI COSTA

PUBLICATIONS

Part of the work presented in this thesis has been reported elsewhere:

N.D. Costa and A.M. Snoswell

Acetylcarnitine hydrolysis in liver. *Proc. Aust. Biochem. Soc.* (1974) 7, 36.

N.D. Costa and A.M. Snoswell

Conversion of acetyl-CoA to acetate by the combined action of carnitine acetyltransferase and acetylcarnitine hydrolase. *Proc. Aust. Biochem. Soc.* (1975) 8, 51.

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

Endogenous acetate production by the liver in lactating ewes. *Proc. Aust. Biochem. Soc.* (1975) 8, 51.

N.D. Costa and A.M. Snoswell

Enzymic hydrolysis of acetylcarnitine in liver from rats, sheep and cows. *Biochem. J.* (1975) 152, 161-166.

N.D. Costa and A.M. Snoswell

Acetyl-Coenzyme A hydrolase, an artifact? *Biochem. J.* (1975) 152, 167-172.

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

Production of endogenous acetate by the liver in lactating ewes. *Aust. J. Biol. Sci.* (1976) 29, 33-42.

N.D. Costa and A.M. Snoswell

Biosynthesis of carnitine in hepatocytes isolated from rats and sheep. *Proc. Aust. Biochem. Soc.* (1976) 9, 35.

N.D. Costa, J.G. McLean and A.M. Snoswell

On the role of carnitine and acetylcarnitine in the liver of lactating and non-lactating cows. Submitted for publication.

PREFACE

Abbreviations approved by the Biochemical Journal (1976) for use without explanation are used as such throughout this thesis. Chemical compounds, their sources and degrees of purity are described in the text.

The following enzymes are referred to by name only:

Acetate thiokinase	EC 6.2.1.1
Acetyl-CoA hydrolase	EC 3.1.2.1
Acetyl-CoA synthetase	EC 6.2.1.1
Adenylate kinase	EC 2.7.4.3
Alanine dehydrogenase	EC 1.4.1.1
4-aminobutyric acid - glutamate transaminase	EC 2.6.1.19
ATP-citrate lyase	EC 4.1.3.8
Carboxylic ester hydrolase	EC 3.1.1.1
Carnitine acetyltransferase	EC 2.3.1.7
Carnitine palmitoyltransferase	EC 2.3.1.a
Catalase	EC 1.11.1.6
Cholinesterase	EC 3.1.1.8
Citrate synthase	EC 4.1.3.7
Glucose-6-phosphatase	EC 3.1.3.9
Glutamate decarboxylase	EC 4.1.1.15
Glutamate dehydrogenase	EC 1.4.1.2
L-glycerol-3-phosphate dehydrogenase	EC 1.1.1.8
3-hydroxybutyrate dehydrogenase	EC 1.1.1.30
Lactate dehydrogenase	EC 1.1.1.27
Monoamine oxidase	EC 1.4.3.4
Phosphotransacetylase	EC 2.3.1.8

Protein methylase III	EC 2.1.1.25
Rotenone-insensitive NADH-cytochrome *c* reductase	EC 1.6.99.3
Succinate dehydrogenase	EC 1.3.99.1
Succinic semialdehyde dehydrogenase	EC 1.2.1.16
4-trimethylaminobutyric acid hydroxylase	EC 1.14.11.1

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SUMMARY

1. Carnitine, via the carnitine acetyltransferase reaction, is involved in the transfer of acetyl groups from acetyl-CoA across the inner mitochondrial membrane to form acetylcarnitine.

2. The enzymic utilization of *O*-acetyl-L-carnitine other than via carnitine acetyltransferase was investigated in liver homogenates from rats, sheep and dry cows. An enzymic utilization of *O*-acetyl-L-carnitine via hydrolysis of the ester bond to yield stoichiometric quantities of acetate and L-carnitine was demonstrated; 0.55, 0.53 and 0.30 μmol of acetyl-L-carnitine were utilized/min per g wet weight of liver homogenates from rats, sheep and dry cows respectively. The acetylcarnitine hydrolysis was not due to a non-specific esterase or non-specific cholinesterase. *O*-acetyl-D-carnitine was not utilized. The activity was associated with the enriched outer mitochondrial membrane fraction from rat liver. Isolation of this fraction resulted in an eight-fold purification of acetylcarnitine hydrolase activity. The Km for acetylcarnitine was 2 mM and 1.5 mM for rat and sheep liver acetylcarnitine hydrolase (determined in homogenates) respectively. There was a significant increase in acetylcarnitine hydrolase activity in rat liver on starvation and a significant decrease in the liver of sheep that were severely alloxan-diabetic. Acetylcarnitine hydrolase activity was not detected in kidney, heart and skeletal muscle homogenates from rats and sheep.

3. The nature of the acetyl-CoA hydrolase reaction in rat and sheep liver homogenates was investigated. The activity determined in an incubated system was 5.10 and 3.28 nmol/min per mg of protein for rat and sheep liver homogenates respectively. This activity was not affected by the addition

of L-carnitine, but was decreased by the addition of D-carnitine. No acetyl-CoA hydrolase activity could be detected in rat or sheep liver homogenates first treated with Sephadex G-25. This treatment decreased the carnitine concentrations of the homogenates to about one-twentieth. Subsequent addition of L-carnitine, but not D-carnitine, restored the apparent acetyl-CoA hydrolase activity. Sephadex treatment did not affect acetylcarnitine hydrolase activity of the homogenates, which was 5.8 and 8.1 nmol/min per mg of protein respectively for rat and sheep liver. Direct spectrophotometric assay of acetyl-CoA hydrolase, based on the reaction of CoA released with 5,5'-dithiobis-(2-nitrobenzoic acid), clearly demonstrated that after Sephadex treatment no activity could be measured. Carnitine acetyltransferase activity measured in the same assay system in response to added L-carnitine was very low in normal rat liver homogenates, owing to the apparent high acetyl-CoA hydrolase activity, but was increased markedly after Sephadex treatment. The Vmax for this enzyme in rat liver homogenates was increased from 3.4 to 14.8 nmol/min per mg of protein, whereas the Km for L-carnitine was decreased from 936 to 32 μM after Sephadex treatment. Acetyl-CoA hydrolase activity could be demonstrated in disrupted rat liver mitochondria but not in separated outer or inner mitochondrial membrane fractions. Activity could be demonstrated after recombination of outer and inner mitochondrial membrane fractions. The outer mitochondrial membrane fraction showed acetylcarnitine hydrolase activity and the inner mitochondrial membrane fraction showed carnitine acetyltransferase activity. The results presented demonstrate that acetyl-CoA hydrolase activity in rat and sheep liver is an artifact and the activity is due to the combined reactions of carnitine acetyltransferase and acetyl carnitine.

4. The role of carnitine and acetylcarnitine in the diversion of acetyl groups of acetyl-CoA away from ketone production was investigated in liver

of four non-lactating and four lactating cows. The carnitine and acetylcarnitine concentrations in liver of lactating and non-lactating cows were not significantly different. The concentration of carnitine in liver of non-lactating cows was 6.5 nmol/g wet weight and acetylcarnitine was 23.9 nmol/g wet weight. The concentration of carnitine and acetylcarnitine in liver of lactating cows was 14.8 and 11.2 nmol/g wet weight respectively. The acetyl-CoA concentration in liver increased significantly in lactating as compared to non-lactating cows. There was no increase in 3-hydroxybutyrate concentration in lactating cow liver as compared to non-lactating cow liver. 3-Phosphoglycerate concentration in liver increased from 128 nmol/g wet weight in non-lactating cows to 231 nmol/g wet weight in lactating cows. The acetylcarnitine and 3-phosphoglycerate concentrations in cow liver were negatively related. The liver activity of both acetylcarnitine hydrolase and carnitine acetyltransferase increased significantly (three- and two-fold respectively) in lactating as compared to non-lactating cows. The results presented demonstrated a role for carnitine acetyltransferase and acetylcarnitine hydrolase in the relief of 'acetyl pressure' in the liver of lactating and non-lactating cows, resulting in the production of acetate as an end-product of these two combined enzyme reactions.

5. The production of endogenous acetate by the liver was investigated in lactating ewes using animals with indwelling arterial, and portal and hepatic venous cannulae. The capacity of the liver to produce acetate from acetyl-CoA *in vitro* was also examined using homogenates prepared from liver biopsy samples. Mean arterial, portal and hepatic venous blood acetate concentrations in four ewes at 4 weeks lactation were 0.40, 1.00 and 1.46 mM respectively. The mean exogenous and endogenous acetate production rates were 56 and 54 nmol/h respectively, giving a total of 110 mmol/h. The mean portal-hepatic venous difference in free fatty acid concentration was 81 μM. Converting this uptake of free fatty acids by

the liver (based on palmitate as a standard) to 2-carbon equivalents, the acetate produced accounted for 70% of the free fatty acid uptake. The correlation coefficient (r^2) between uptake of free fatty acids and production of acetate by the liver was 0.83 ($P < 0.01$).

Calculation of the net acetate production *in vivo* gave a mean value for the production of acetate of 0.75 mmol/min. Calculation of the *in vitro* enzymic capacity of the liver to produce acetate from acetyl-CoA gave a mean of 0.94 mmol/min. These results indicate that the enzymic production of acetate from acetyl-CoA, via carnitine acetyltransferase and acetylcarnitine hydrolase, can adequately account for the substantial production of acetate by the liver of lactating ewes.

6. The uptake of acetate by the mammary gland was investigated in lactating ewes using animals with arterial and mammary venous cannulae. The activities of carnitine acetyl- and palmitoyltransferases and acetate thiokinase in the mammary gland homogenates from the lactating ewes were also determined. Mean arterial and mammary venous blood acetate concentrations in three ewes at 4 weeks lactation were 0.32 μmol/ml and 0.17 μmol/ml respectively. The mean acetate extraction ratio was 47%. The supply of acetate (as measured by hepatic venous - arterial acetate concentrations) and the utilization of this acetate (as measured by hepatic venous - mammary venous acetate concentrations) were directly related [a correlation coefficient (r^2) of 0.97]. The activities of carnitine acetyltransferase, carnitine palmitoyltransferase and acetate thiokinase were 0.61, 0.75 and 1.47 μmol/min per g wet weight respectively. Carnitine acetyltransferase activity was present both in the 100,000*g* pellet and in the supernatant, which indicated that enzyme may be involved in the mitochondrial oxidation of acetate in the mammary gland.

7. The site of carnitine biosynthesis in rats and sheep was investigated. Using the criterion of detectable 4-trimethylaminobutyric acid hydroxylase

activity, the liver was the only site of carnitine biosynthesis in the rat (liver activity detected was 0.49 nmol/min per mg of protein). 4-Trimethylaminobutyric acid hydroxylase activity in sheep was detected in liver (0.20 nmol/min per mg of protein), kidney cortex (0.17 nmol/min per mg of protein), skeletal muscle (0.10 nmol/min per mg of protein) and heart (0.05 nmol/min per mg of protein). 4-Dimethyl- and 4-monomethyl-aminobutyric acids (but no 4-aminobutyric acid) were also hydroxylated to some extent by rat liver homogenates. S-Adenosylmethionine (0.3 mM) significantly stimulated the hydroxylation of these compounds by rat liver homogenates.

8. The precursors of the carbon skeleton and the source of the methyl groups of carnitine were investigated using hepatocyte suspensions isolated from rats and sheep. The morphological integrity of the hepatocytes was established prior to use. The metabolic integrity of the hepatocytes was established using the criterion of glucose formation from lactate and propionate in the case of rats and sheep respectively. Radioactivity from 4-aminobutyric acid, via successive 4-amino-methylation and 3-hydroxylation was incorporated into carnitine in the hepatocyte suspensions from both rats and sheep. Radioactivity from [U - ^{14}C]- but not [1 - ^{14}C]-L-lysine was incorporated into carnitine by rat and sheep hepatocyte preparations. Radioactivity from methionine was also incorporated into carnitine by rat and sheep hepatocytes. The rate of synthesis of carnitine from 4-aminobutyric acid was five- to six-fold faster in hepatocyte suspensions isolated from alloxan-diabetic as compared to normal rats. The rate of synthesis of carnitine from 4-aminobutyric acid was only 1½-fold faster in hepatocyte suspensions isolated from alloxan-diabetic sheep 1 day withdrawn from insulin after insulin-stabilization as compared to insulin-stabilized alloxan-diabetic sheep. The rate of synthesis of carnitine from L-lysine was four-fold faster in alloxan-

diabetic as compared to normal rat hepatocyte suspensions. The rate of synthesis of carnitine from L-lysine was 2¼ times faster in insulin-stabilized, 1 day withdrawn from insulin, alloxan-diabetic sheep as compared to insulin-stabilized, alloxan-diabetic sheep hepatocyte suspensions. In both rat and sheep hepatocyte suspensions prepared from animals in the above physiological states, the rates of synthesis of carnitine from lysine was almost two- to five-fold faster than the corresponding rates of synthesis from 4-aminobutyric acid. The biosynthesis of carnitine from both lysine and 4-aminobutyric acid, their relative importance and possible biosynthetic pathway with possible controls are discussed.

9. The work in this thesis is presented in three parts, each part introduced and discussed separately. A general discussion correlates the different facets of the study and outlines the inter-relationships between the metabolic role of carnitine and its biosynthesis.

PART I: UTILIZATION OF ACETYLCARNITINE IN ANIMAL TISSUES

1. Introduction

1.1 General Introduction

Carnitine (4-trimethylamino-3-hydroxybutyric acid) was independently discovered about 70 years ago by two groups of investigators (Gulewitsch and Krimberg, 1905; Kutscher, 1905). Although carnitine is widely distributed in tissues of animals, plants and microorganisms, with highest concentrations in muscles from vertebrates and invertebrates (Fraenkel and Friedman, 1957; Fritz, 1963; Pearson and Tubbs, 1967; Snoswell and Henderson, 1970; Snoswell and Koundakjian, 1972), no physiological role for this compound became apparent for many years. Despite close resemblance in structure to choline, carnitine, as well as its acetyl derivative, has negligible activity on neuro-muscular preparations (for reviews see Fraenkel and Friedman, 1957; Fritz, 1963) and also cannot substitute for choline in the diet (Fraenkel and Friedman, 1957; Fritz and DuPont, 1957; Adams *et al.*, 1960; Corredor *et al.*, 1967) except in two insect species of the order Diptera (Fraenkel *et al.*, 1955; Hodgson *et al.*, 1960).

Friedman and Fraenkel (1955) made the significant discovery that pigeon and sheep liver extracts contained the enzyme, carnitine acetyltransferase, which catalyses the reaction:

acetyl-S-CoA + L-carnitine $\rightleftharpoons$ CoA + acetyl-L-carnitine.

Fritz (1961) reported that carnitine stimulated fatty acid oxidation in liver homogenates and subsequently this finding was extended to many other tissues. It is now accepted that carnitine is involved in the

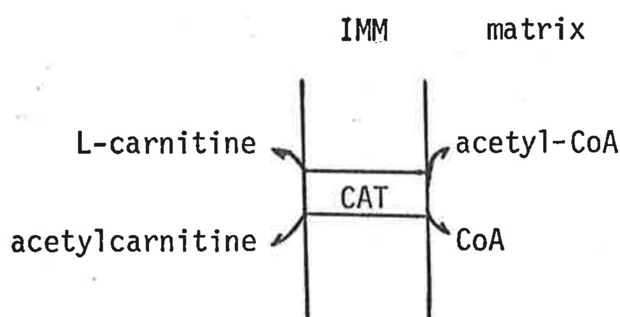
transport of fatty acyl groups across the inner mitochondrial membrane (Fritz, 1963; Fritz and Yue, 1963; Bremer, 1962*a*). This study is concerned with the role of carnitine in the transport of short-chain fatty acyl (mainly acetyl) groups across the inner mitochondrial membrane.

1.2 Production and utilization of *O*-acetyl-L-carnitine

The only known pathway to date for the production and utilization of *O*-acetyl-L-carnitine is via the enzyme, carnitine acetyltransferase (Friedman and Fraenkel, 1955; Bremer, 1962*a*; Fritz *et al.*, 1963).

The intracellular distribution and mode of action of carnitine acetyltransferase have been examined by several groups of investigators. Carnitine acetyltransferase has been suggested to be associated with the mitochondria of the cell (Beenakkers and Klingenberg, 1964; Beenakkers and Henderson, 1967; Barker, Fincham and Hardwick, 1968; Brdickza *et al.*, 1969; Snoswell and Koundakjian, 1972; Edwards *et al.*, 1974). A wider distribution extended to peroxisomal and lipid-rich membranous fractions has also been suggested (Markwell *et al.*, 1973). The investigations of Edwards *et al.* (1974) using various tissues and Snoswell and Koundakjian (1972) using sheep liver suggested that the mitochondrial carnitine acetyltransferase was associated entirely with the inner mitochondrial membrane but Beenakkers and Henderson (1967) using locust's flight muscle and Brdickza *et al.* (1969) using rat liver suggested that there was also an 'outer pool' of carnitine acetyltransferase. Experiments using rat liver mitochondria showed that carnitine could only penetrate the same mitochondrial space as sucrose (Yates and Garland, 1966; Brosnan and Fritz, 1971) and that swelling did not occur when mitochondria were

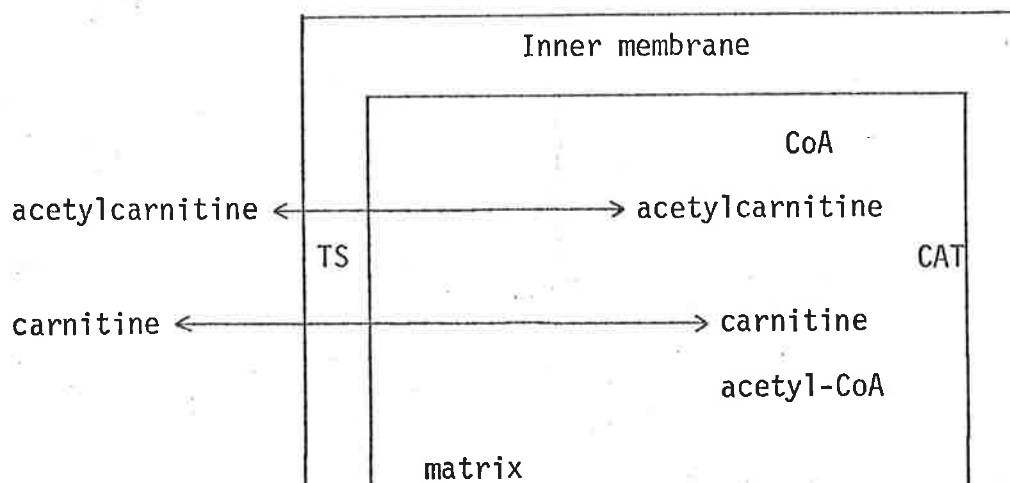
suspended in an iso-osmotic carnitine solution (Levitsky and Skulachev, 1972). Also acetyl-CoA and CoA were shown to be unable to penetrate the inner mitochondrial membrane (Spencer and Lowenstein, 1962; Fritz and Yue, 1963). These findings, together with the fact that isolated rat liver mitochondria contain less than 0.1 nmol of L-carnitine per mg of protein, have led to the hypothesis that the 'inner pool' of carnitine acetyltransferase is vectorially mounted (Yates and Garland, 1966) and catalyzes a direct transfer of acetyl groups between external carnitine and matrix CoA, which may be schematically represented:



IMM = inner mitochondrial membrane

CAT = carnitine acetyltransferase

However, experiments using rat and ox heart mitochondria (containing 1.2 and 2.4 nmol of L-carnitine per mg of protein respectively) showed that there was a carnitine-acylcarnitine translocase system operating in these preparations (Ramsay and Tubbs, 1974; Pande, 1975; Ramsay and Tubbs, 1975). Further, carnitine-acylcarnitine exchange was suggested as the mechanism of acetyl-group transport across the inner mitochondrial membrane (Ramsay and Tubbs, 1975), which may be schematically represented:



TS = translocase system

CAT = carnitine acetyltransferase

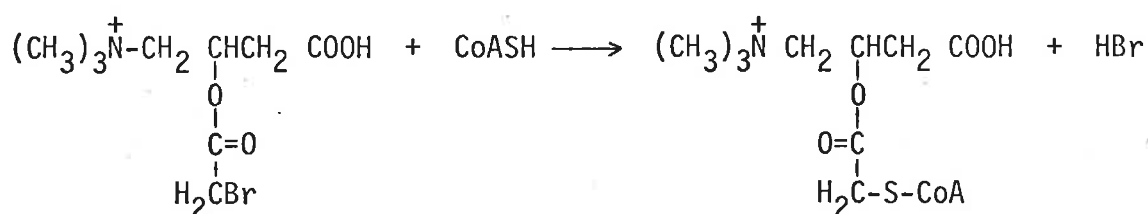
The concentration of acetylcarnitine produced via carnitine acetyltransferase within the liver has been shown to increase during metabolic stress, e.g. starvation and alloxan-diabetes, in the rat (Pearson and Tubbs, 1967) and sheep (Snoswell and Henderson, 1970; Snoswell and Koundakjian, 1972). Indeed, in sheep liver, the concentration of free L-carnitine also increases in these stress states (Snoswell and Henderson, 1970; Snoswell and Koundakjian, 1972).

The cellular distribution and mode of action of carnitine acetyltransferase, together with the changes in acetylcarnitine concentration during metabolic stress, have led to the hypothesis that the physiological role of carnitine acetyltransferase is to provide an 'acetyl sink' whereby the acetylation state of matrix CoA may be buffered by transporting acetyl groups to an extramitochondrial 'store' of acetylcarnitine (Pearson and Tubbs, 1967; Snoswell and Henderson, 1970; Snoswell and Koundakjian, 1972; Edwards *et al.*, 1974).

However, in the framing of the above hypotheses there was no consideration given to alternate utilization of acetylcarnitine.

Lowenstein (1964) reported a low incorporation of the acetyl group of acetylcarnitine into fatty acids in rat liver. A possible hydrolysis to yield acetate was suggested as the simplest interpretation of this observation. During investigations of the reversibility of the carnitine acetyltransferase reaction in rat liver, Bremer (1962*a*) reported some alternative utilization of acetylcarnitine. Mahadevan and Sauer (1969), using rat liver microsomal preparations, established an enzymic hydrolysis of C_6-C_{18} acylcarnitines but did not detect any hydrolysis of C_2-C_4 acylcarnitines. Thus, a more specific investigation of the enzymic utilization of acetylcarnitine seemed warranted.

In none of the above reports was the utilization of acetylcarnitine by carnitine acetyltransferase prevented. Carnitine acetyltransferase is competitively inhibited by D-carnitine (Ki, 2.3 x 10^{-3} M) and acetyl-D-carnitine (Ki, 2.5 x 10^{-4} M) (Fritz and Schultz, 1965) and is inactivated in the presence of bromoacetyl-L-carnitine and CoA (Chase and Tubbs, 1969). Under the appropriate conditions, 1 mole of bromocetylcarnitine completely inactivated 1 mole of enzyme. Chase and Tubbs (1969) postulated the following mechanism:



$$(CH_3)_3\overset{+}{N}\text{-}CH_2\,CH(O\text{-}C(=O)\text{-}CH_2Br)CH_2\,COOH + CoASH \longrightarrow (CH_3)_3\overset{+}{N}\,CH_2\,CH(O\text{-}C(=O)\text{-}CH_2\text{-}S\text{-}CoA)CH_2\,COOH + HBr$$

whereby the S-carboxymethyl bridge between the CoA and carnitine remained associated with the enzyme as an inactive complex. In this investigation bromoacetyl-L-carnitine was used to completely inhibit the carnitine acetyltransferase activity.

1.3 The relationship between carnitine acetyltransferase and acetyl-CoA hydrolase in the utilization of acetyl-CoA

The initial report describing the activity of an acetyl-CoA hydrolase in a pig heart preparation only implied the presence of such an enzyme (Gergely *et al.*, 1952), but on the basis of that report the enzyme was classified and numbered. The enzyme was reported to be widely distributed in bovine tissues (Quraishi and Cook, 1972) and in rat and sheep tissues (Knowles *et al.*, 1974). In each investigation a high activity was reported in the liver, with the activity predominantly localized in the mitochondria. From experiments in rat liver, Murthy and Steiner (1973) reported that the enzyme activity was associated with the inner mitochondrial membrane.

Both acetyl-CoA hydrolase and carnitine acetyltransferase therefore would be competing for the same matrix pool of acetyl-CoA. Carnitine acetyltransferase has a Km for acetyl-CoA of approximately 0.04 mM (Fritz *et al.*, 1963; Chase, 1967), whereas the Km for acetyl-CoA of the acetyl-CoA hydrolase is approximately 0.7 mM (Knowles *et al.*, 1974). The concentration of acetyl-CoA in rat and sheep liver is in the order of 0.04-0.08 mM (Allred and Guy, 1969; Snoswell and Henderson, 1970).

Since it is difficult to conceive of a physiological function for the acetyl-CoA hydrolase from these findings, the nature and characteristics of the acetyl-CoA hydrolase reactions were investigated in relation to the carnitine acetyltransferase activity.

2. Enzymic hydrolysis of *O*-acetyl-L-carnitine in liver from rats, sheep and cows

2.1 Materials and Methods

2.1.1 Animals

The rats used were hooded Wistar males weighing 250-300 g and were fed on a pelleted rat diet (Charlicks, Adelaide, S. Austral., Australia). Food was withheld from the starved rats for 48 hours.

The sheep used were 4-year-old Merino wethers, weighing between 38 and 48 Kg. These animals were fed *ad libitum* on lucerne-hay chaff. Tissue from Merino sheep was also obtained from the abattoirs directly after slaughter.

Non-lactating cows were predominantly Jersey and were all approximately 3 years of age. The cows were grazed on irrigated perennial pasture consisting mainly of white clover, cocksfoot, paspallum and rye grass. No supplementary feed was given.

2.1.2 Alloxan-diabetic animals

Alloxan-diabetes was produced in adult Merino wethers by injecting a sterile saline solution (0.9% NaCl) containing alloxan (60 mg/Kg body weight) into the jugular vein. The animals which spontaneously decreased their food intake were killed 5-6 days later.

2.1.3 Tissue preparations and homogenates

Rats were killed by cervical dislocation and exsanguination. The sheep were killed by severing the neck. Liver

tissue from non-lactating cows was obtained by Dr. J.G. McLean by biopsy as described by Baird and Heitzman (1970). Fresh samples of liver, kidney, heart and skeletal muscle (M. biceps femoris) from rats and sheep were collected directly into 0.25 M sucrose containing 23 mM potassium phosphate (pH 7.4). The fresh tissues were homogenized with a Potter-Elvehjem homogenizer in 0.25 M sucrose containing 23 mM potassium phosphate (pH 7.4) as the homogenizing buffer (40%, w/v).

2.1.4 Tissue fractionation

Rat liver was collected directly into cold 0.25 M sucrose containing 23 mM potassium phosphate (pH 7.4). Homogenates (10%, w/v) were then prepared in the same sucrose-phosphate solution by gentle action with a Potter-Elvehjem homogenizer with a tight-fitting Teflon pestle. These homogenates were then centrifuged at 700 *g* to remove unbroken cells and nuclei. The supernatant fractions were centrifuged at 10,000 *g* for 10 minutes to sediment mitochondria. The supernatant fraction from the 10,000 *g* centrifugation was further centrifuged at 100,000 *g* for 1 hour. The supernatants from this centrifugation were designated 'cytosol fractions' and the pellets were resuspended in sucrose-phosphate medium to give 'microsomal fractions'. The mitochondrial pellets were washed twice in the sucrose-phosphate medium and recentrifuged at 13,000 *g* for 10 minutes.

2.1.5 Mitochondrial fractionation

Rat liver mitochondria were isolated in 0.44 M sucrose-10 mM triethanolamine (pH 7.6)-2 mM EDTA by the method of Brdiczka *et al.* (1969). A slight modification of the method of Hoppel and Tomec (1972) was used to separate the mitochondrial fractions in that all pellet

fractions were resuspended in 0.25 M sucrose-23 mM potassium phosphate, (pH 7.4).

2.1.6 Instrumentation

All assays were performed on a Zeiss PMQ II spectrophotometer fitted with an automatic sample changer and TE converter (Carl Zeiss, Oberkochen, Germany) connected to a Rikadenki model B140 recorder (Rikadenki Kogyo Co., Tokyo, Japan). The instrument was fitted with a temperature-controlled cell-holder and the temperature was maintained at 37°C for all assays.

2.1.7 Enzyme assays

2.1.7.1 Carnitine acetyltransferase

Liver homogenates (20%, w/v) from rat, sheep and cows were homogenized in 0.025 M sucrose-2.3 mM potassium phosphate (pH 7.4)-0.1% Triton X-100, then frozen in liquid nitrogen and thawed. The homogenates were then centrifuged at 8,000 *g* for 3 minutes in an Eppendorf centrifuge model 3200 (Eppendorf, Gerateban, Netheter and Hinz, GmbH, Hamburg, Germany) and the supernatants used for assay. The assay system was similar to that described by Solberg *et al.* (1972) and contained 400 mM Tris-HCl (pH 8.0), 100 μM 5,5'-dithiobis-(2-nitrobenzoic acid), 100 μM acetyl-CoA and enzyme fraction in a total volume of 1.0 ml. The reaction was started by the addition of 3.3 mM L-carnitine. The inhibition of carnitine acetyltransferase by bromoacetyl-L-carnitine was investigated in the same assay system. The carnitine acetyltransferase was preincubated with bromoacetylcarnitine (1-20 μM).

2.1.7.2 Enzymic utilization of *O*-acetyl-L-carnitine

Homogenates (40%, w/v) homogenized in 0.25 M sucrose-23 mM potassium phosphate (pH 7.4) were used. Activities were measured by determining the rate of acetylcarnitine utilization and L-carnitine and acetate formation. The incubation series for each tissue consisted of a boiled-homogenate reaction, zero-time reaction, and two tubes to determine acetyl-L-carnitine utilization in the presence and absence of bromoacetyl-L-carnitine. Eppendorf tubes contained, in a total volume of 1.0 ml, 0.3 M Tris-HCl (pH 8.0), 70 μM bromoacetyl-L-carnitine, 5 mM acetyl-L-carnitine, and tissue homogenate. The tubes were incubated for 0, 5, 10, 15, 20 and 30 minutes at 37°C and the reaction was stopped with 200 μl of 15% (w/v) perchloric acid. After centrifugation at 8,000 *g* for 2-5 minutes, the supernatant was neutralized with 3M KOH. The enzyme activity was linear, with respect to time, for 20 minutes and therefore a 20 minute incubation was routinely used. Utilization of *O*-acetyl-D-carnitine, *O*-propionyl-L-carnitine and *O*-butyryl-L-carnitine was determined in the same manner as utilization of *O*-acetyl-L-carnitine.

2.1.7.3 Rotenone-insensitive NADH-cytochrome *c* reductase

This was assayed in the digitonin-treated submitochondrial fractions of rat liver by a system similar to that described by Sottocasa *et al.* (1967). The spectrophotometric assay was performed at 550 nm and the assay system contained 240 mM potassium phosphate (pH 7.6), 5 mM EDTA, 20 μM rotenone, 0.08 mM cytochrome *c* and submitochondrial fraction in a final volume of 1.0 ml. The reaction was started with 10 μl of 10 mM NADH.

2.1.7.4 Adenylate kinase

This was assayed in the submitochondrial fractions from rat liver by the spectrophotometric assay at 340 nm. The assay system contained, in a final volume of 1.0 ml, 50 mM triethanolamine buffer (pH 7.6), 5 mM EDTA, 8 mM $MgSO_4$, 75 mM KCl, 0.22 mM NADH, 3 mM ATP, 0.8 mM phosphoenolpyruvate 10 μg pyruvate kinase, 50 μg lactate dehydrogenase, and enzyme fraction. The reaction was started with 3 mM AMP.

2.1.7.5 Succinate dehydrogenase

This was assayed in the submitochondrial fractions from rat liver by spectrophotometric assay at 550 nm. The assay system contained, in a total volume of 1.0 ml, 100 mM potassium phosphate (pH 7.6), 0.1 mM phenazine methosulphate, 0.08 mM cytochrome c, 5 mM EDTA, 20 μM rotenone, and enzyme fraction. The reaction was started with 5 mM succinate.

2.1.7.6 Glutamate dehydrogenase

This was assayed in the subcellular fractions from rat liver by spectrophotometric assay at 340 nm. The assay system contained, in a total volume of 1.0 ml, 100 mM potassium phosphate (pH 7.4), 5 mM 2-oxoglutarate, 0.2 mM NADH, 1.5 mM ADP, 0.3 mM EDTA, 10 μM rotenone, and tissue fraction. The reaction was started with 25 mM ammonium chloride.

2.1.7.7 Lactate dehydrogenase

This was assayed in the subcellular fractions from rat liver by spectrophotometric assay at 340 nm. The assay system

contained, in a final volume of 1.0 ml, 200 mM Tris-HCl (pH 7.4), 0.2 mM NADH, 10 μM rotenone, and tissue fraction. The reaction was started by addition of 1 mM pyruvate.

2.1.7.8 Glucose-6-phosphatase

This was assayed in subcellular fractions from rat liver by measuring the amount of inorganic phosphate released after incubation with glucose-6-phosphatase. The assay procedure was as described by Baginski *et al.* (1974).

2.1.8 Metabolite assays

Acetylcarnitine was measured by the method of Pearson and Tubbs (1964), and free carnitine by the method of Marquis and Fritz (1964). Free acetate was determined by the kinetic method of Knowles *et al.* (1974).

Proteins were determined by the biuret method.

2.1.9 Preparation of bromoacetyl-L-carnitine

The bromoacetyl derivative of L-carnitine was prepared by modification of several methods (Fraenkel and Friedman, 1957; Zeigler *et al.*, 1967; Chase and Tubbs, 1969). Two grams (15.3 mmol) of bromoacetic acid (recrystallized) were reacted with 2.4 ml (26.4 mmol) of bromoacetyl bromide for 3 hours at 75°C. One gram (5.9 mmol) of L-carnitine was then added and the mixture reacted for a further hour. The mixture was then cooled and extracted eight times with ice-cold diethyl ether. The residue was dried in a stream of N_2 gas. The dried residue was dissolved in ethanol:70% w/v perchloric acid (4:3). Bromoacetyl-L-carnitine perchlorate was recrystallized from isopropyl alcohol. The

resultant crystals were stored frozen *in vacuo*. A sample of bromoacetyl-L-carnitine perchlorate was also kindly supplied by Dr. P.K. Tubbs, Department of Biochemistry, University of Cambridge, Cambridge, U.K.

2.1.10 Chemicals

L-carnitine hydrochloride and *O*-acetyl-L-carnitine chloride were generously supplied by Otsuka Pharmaceuticals, Osaka, Japan, and were recrystallized from ethanol. Propionyl-L-carnitine, butyryl-L-carnitine chloride and *O*-acetyl-D-carnitine were prepared by the method of Bohmer and Bremer (1968). Acetyl-CoA was prepared by the method of Stadtman (1957). CoA was obtained from P-L Biochemicals Inc., Milwaukee, Wisconsin, U.S.A.; enzymes were from C.F. Boehringer und Soehne GmbH, Mannheim, Germany.

2.2 Results

2.2.1 Enzymic utilization of *O*-acetyl-L-carnitine by normal sheep liver homogenates

The results in Table 1 show a small amount of chemical hydrolysis of acetylcarnitine at the pH of the incubation. In addition to the chemical hydrolysis of acetylcarnitine, there is a significantly greater ($P < 0.001$) enzymic utilization of acetylcarnitine. This acetyl-carnitine utilization and carnitine production may proceed via the carnitine acetyltransferase reaction. In the presence of bromoacetyl-carnitine, a potent specific inhibitor of carnitine acetyltransferase (Chase and Tubbs, 1969), there is a small decrease in acetylcarnitine utilization ($P < 0.05$) proceeding via carnitine acetyltransferase. The carnitine acetyltransferase activity present in these sheep liver homogenates is completely inhibited by the concentration of bromoacetyl-

TABLE 1

Enzymic utilization of O-acetyl-L-carnitine by normal sheep liver homogenates

Liver samples were obtained from sheep killed at the abattoirs. Incubations of acetyl-L-carnitine with sheep liver homogenates were performed in the presence and absence of bromoacetyl-L-carnitine as described in Part I, 2.1.7.2. Results are the means ± S.E.M. for six animals.

	Activity (μmol/min per g of wet liver tissue)	
	Acetylcarnitine utilized	Carnitine produced
Normal - inhibitor	0.715 ± 0.05	0.680 ± 0.10
Normal + inhibitor	0.550 ± 0.05	0.515 ± 0.05
Boiled homogenate	0.155 ± 0.025	0.100 ± 0.020

carnitine used in the incubation (i.e. 70 μM). This was verified by an independent spectrophotometric assay of carnitine acetyltransferase inhibition by bromoacetylcarnitine as described in Part I, 2.1.7.1. In subsequent assays of acetylcarnitine utilization, this concentration of bromoacetylcarnitine was included in all incubations. There remains, however, a significant ($P < 0.001$) and substantial utilization of acetylcarnitine of 0.55 μmol/min per g of fresh liver tissue. Thus, the enzymic utilization of acetylcarnitine and production of L-carnitine can proceed via an alternative enzymic mechanism to carnitine acetyltransferase.

2.2.2 Enzymic hydrolysis of acetylcarnitine by homogenates of normal rat, sheep and cow liver

Results in Table 2 demonstrate an overall balance between acetylcarnitine utilized and L-carnitine and acetate produced in incubations of liver homogenates from these species. Within each species there is no significant difference with respect to the use of any of the three compounds for assay of acetylcarnitine hydrolysis activity. Although the amount of acetylcarnitine utilized varies between the different species, this balanced relationship holds throughout. These results establish a stoichiometric relationship between acetylcarnitine utilized and acetate and carnitine produced in the absence of carnitine acetyltransferase activity. Thus, the enzymic utilization of acetylcarnitine in the presence of bromoacetylcarnitine in liver homogenates from these species would appear to proceed via the hydrolytic cleavage of the ester bond of acetylcarnitine.

2.2.3 Enzymic specificity of acetylcarnitine hydrolysis

The hydrolysis of the ester bond of acetylcarnitine could be catalysed by a non-specific esterase. The main non-specific esterase present in liver in various species is carboxylic ester hydrolase

TABLE 2

Enzymic hydrolysis of acetylcarnitine by homogenates from normal rat, sheep and cow liver

Homogenates were prepared and assayed as described in the text in Part I, 2.1.7.2. The values shown are means ± S.E.M., with the number of animals used in parentheses.

	Activity (μmol/min per g of fresh liver tissue) as per method of assay		
	Acetylcarnitine utilized	Carnitine produced	Acetate produced
Rat	0.50 (4) ± 0.10	0.50 (4) ± 0.05	0.45 (4) ± 0.10
Sheep	0.55 (6) ± 0.05	0.52 (6) ± 0.05	N.A.*
Dry cow	0.30 (3) ± 0.05	0.30 (3) ± 0.10	0.30 (3) ± 0.05

*N.A. - not assayed

(Shibko and Tappet, 1964; Chow and Ecobichon, 1973; Ecobichon, 1973). This enzyme is inhibited by 1 mM iodoacetamide (Shibko and Tappet, 1964), but addition of this concentration of iodoacetamide had no effect on the rate of utilization of acetylcarnitine by liver homogenates. Purified preparations of acylcholine acylhydrolase did not hydrolyse acetylcarnitine to any significant extent. Liver homogenates from the rat, sheep and cow did not hydrolyse the unnatural acetyl-D-carnitine as measured by production of acetate. Thus, acetylcarnitine hydrolysis appears to proceed via a stereospecific enzyme, subsequently referred to as acetylcarnitine hydrolase.

2.2.4 Substrate specificity of acetylcarnitine hydrolase

The acetylcarnitine hydrolase from rat liver also hydrolysed propionyl and butyryl esters of L-carnitine. The rate of utilization of propionyl-L-carnitine by acetylcarnitine hydrolase was 9.0 nmol of carnitine liberated/min per mg tissue protein, which was greater than the rate of utilization of acetyl-L-carnitine (5.8 nmol/min per mg tissue protein). In contrast, the activity of acetylcarnitine hydrolase with butyryl-L-carnitine as substrate was 2.8 nmol/min per mg tissue protein.

2.2.5 Michaelis-Menton constants for acetylcarnitine hydrolase from rat and sheep liver

The pH optimum for acetylcarnitine hydrolase was 8.0. The kinetic constants for acetylcarnitine hydrolase from the livers of rats and sheep were determined using crude homogenates from these two sources. The Km for acetylcarnitine of rat liver acetylcarnitine hydrolase, as shown in Figures 1 and 3, lies in the range 1.5-2.0 mM. The Km for acetylcarnitine of sheep liver acetylcarnitine hydrolase, as shown in

FIGURE 1

The Michaelis-Menton kinetics of acetylcarnitine hydrolase in rat liver homogenates

The homogenates were prepared and assayed as described in Part I, 2.1. Acetylcarnitine concentration varied between 0.25 mM and 10 mM. The results are means of triplicate assays from 2 rats.

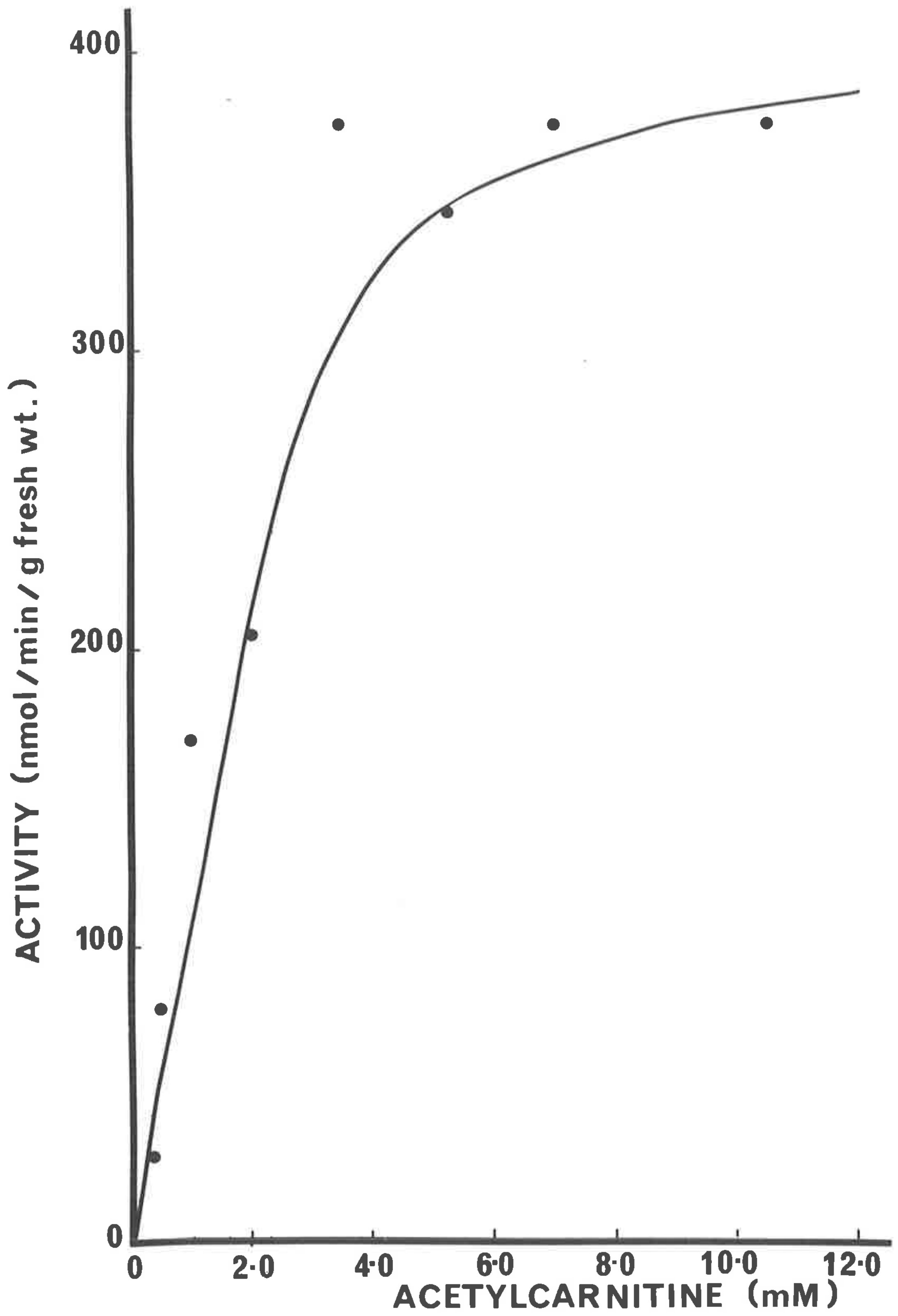


Figure 1

FIGURE 2

The Michaelis-Menton kinetics of acetylcarnitine hydrolase in sheep liver homogenates

The homogenates were prepared and assayed as described in Part I, 2.1. Acetylcarnitine concentration varied between 0.25 mM and 7 mM. The results are means of triplicate assays from 2 sheep.

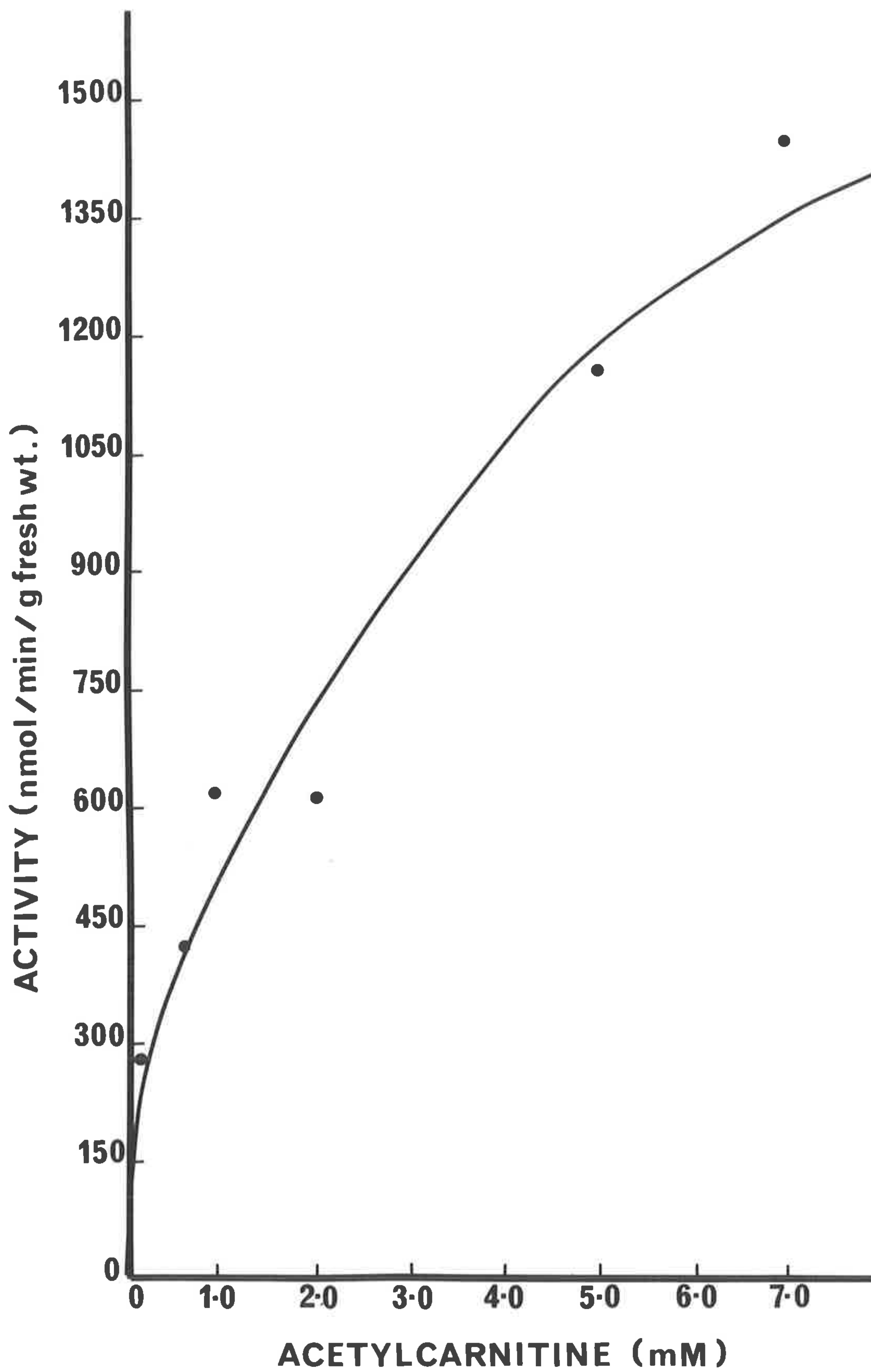


Figure 2

FIGURE 3

Lineweaver-Burk plot for acetylcarnitine hydrolase in homogenates from rat and sheep liver

The homogenates from rat and sheep liver were prepared and assayed as described in Part I, 2.1. Experimental data were weighted for accuracy by the statistical method of Wilkinson (1961) by using an appropriate computer program.

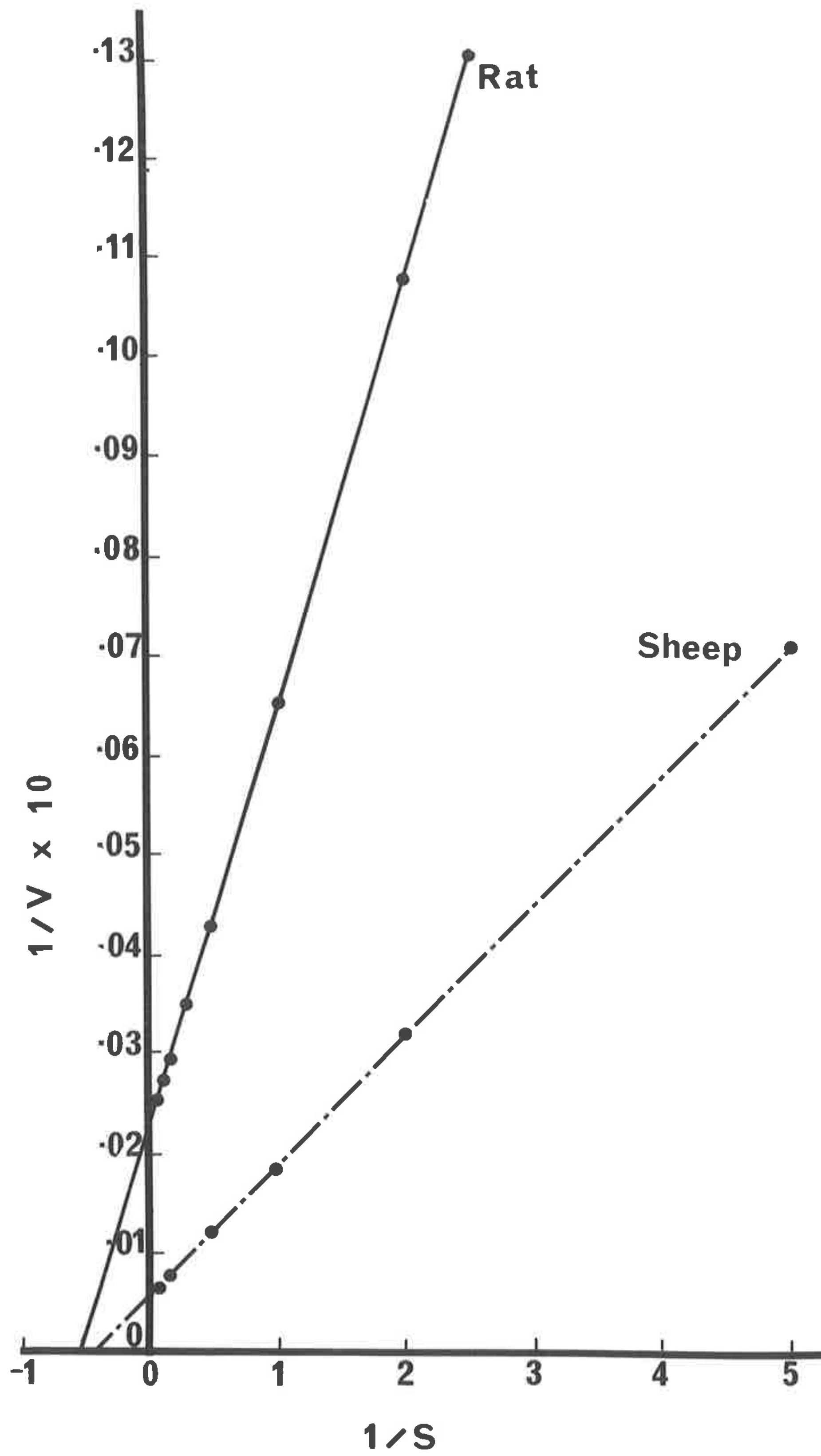


Figure 3

Figures 2 and 3, lies in the range 0.72-1.5 mM. The values shown in Figure 3 were determined from Lineweaver-Burk plots by a statistical weighting method for $\frac{1}{V}$ described by Wilkinson (1961).

2.2.6 Intracellular localization of acetylcarnitine hydrolase in rat liver

Preliminary fractionation of rat liver homogenates into mitochondrial, microsomal and cytosolic fractions and subsequent analysis of these fractions for acetylcarnitine hydrolase activity showed the activity to be localized in the mitochondrial fraction. Submitochondrial fractions were then prepared by using the modifications described in the text. Rotenone-insensitive NADH-cytochrome *c* reductase was chosen as the marker enzyme for the outer-membrane fraction. Ernster and Kuylensteirna (1968) demonstrated that contamination by microsomal rotenone-insensitive NADH-cytochrome *c* reductase could be significantly decreased after a few washings of the mitochondrial preparations. This was verified by glucose-6-phosphatase assay. The advantages of using this marker enzyme instead of the more commonly used monoamine oxidase were greater ease and sensitivity of measurement due to the much higher activity of rotenone-insensitive NADH-cytochrome *c* reductase.

There was difficulty in preparing a rigidly pure outer-membrane fraction, as the results in Table 3 indicate. Solberg (1974), using a similar method of preparation, has also reported difficulty in preparing rigidly pure outer-membrane fractions. Thus, the preparation was rather an enrichment of the outer-membrane fraction. The activity of acetyl-carnitine hydrolase measured in this fraction represented an eight-fold purification over the activity in the crude homogenates from rat liver.

TABLE 3

Submitochondrial localization of acetyl-L-carnitine hydrolase activity within rat liver mitochondria

Submitochondrial fractions were prepared and assayed as described in Part I, 2.1. The values are means ± S.E.M. of three experiments.

	% of total activity in submitochondial fractions		
	Outer membrane	Inter-membranal space	Inner-membrane and matrix
Rotenone-insensitive cytochrome *c*-succinate dehydrogenase	13 ± 6	4 ± 1	85 ± 7
Adenylate kinase	12 ± 6	88 ± 6	N.D.*
Rotenone-insensitive cytochrome *c*-NADH reductase	64 ± 8	35 ± 8	N.D.*
Acetylcarnitine hydrolase	72 ± 10	23 ± 10	N.D.*

*N.D. - not detectable

TABLE 4

Enzymic hydrolysis of acetyl-L-carnitine by homogenates of rat and sheep liver under various conditions

Liver homogenates were prepared and assayed as described in the text in Part I, 2.1. The results are means ± S.E.M., with the number of animals in parentheses. Statistical difference was determined by the student's t-test.

Animal	Physiological state	Activity of acetylcarnitine hydrolase (μmol/min per g fresh tissue)	
Rat	Normal	0.55 (4) ± 0.10	$P < 0.05$
	Starved	0.95 (2) ± 0.25	
Sheep	Normal	0.55 (6) ± 0.05	$P < 0.01$
	Alloxan-diabetic	0.35 (2) ± 0.10	

2.2.7 Tissue distribution of acetylcarnitine hydrolysis in rat and sheep

There was no detectable enzymic hydrolysis of acetyl-carnitine in homogenates of heart, kidney cortex or skeletal muscle from rats and sheep.

2.2.8 Effect of various physiological states on enzymic hydrolysis of acetyl-L-carnitine by homogenates of rat and sheep liver

The results in Table 4 show a significant ($P < 0.05$), almost two-fold, increase in acetylcarnitine hydrolase activity in liver from rats starved for 48 hours over liver from normal rats. In contrast to these results, in liver from alloxan-diabetic sheep there was a significant ($P < 0.01$) decrease in acetylcarnitine hydrolase activity when compared with the activity in normal sheep liver. All values for acetyl-carnitine hydrolase activity shown in Table 4 are determined by overall average of measurement of acetylcarnitine utilized and L-carnitine and acetate produced.

3. Carnitine-dependent transfer of acetyl groups from acetyl-CoA across the inner mitochondrial membrane of liver from rats and sheep

3.1 Materials and methods

3.1.1 Animals

The rats used were hooded Wistar females (150-200 g) which had been fed on a pelleted rat diet (Charlicks, Adelaide, S. Austral., Australia).

The sheep used were 9-month-old Merino wethers which had been fed on a lucerne-chaff diet.

3.1.2 Tissue preparations

) The rats and sheep were killed by severing the necks and samples of liver were collected into 0.25 M sucrose containing 23 mM potassium phosphate (pH 7.4).

3.1.3 Homogenates

Homogenates (20%, w/v) were prepared in 0.025 M sucrose containing 2.3 mM potassium phosphate (pH 7.4)-0.1% Triton X-100 buffer with a Potter-Elvehjem homogenizer. These homogenates were then centrifuged at 8,000 *g* for 3 minutes and the supernatants used for assay. In addition, 2 ml fractions of supernatant were passed down a column (22.5 cm x 1.5 cm) of Sephadex G-25 (coarse) in 25 mM potassium phosphate (pH 7.4). The protein peak and total acid-soluble carnitine peak were monitored. Only fractions at the beginning of the protein peak were taken for enzyme assay (to ensure that there was minimal contamination with endogenous carnitine).

3.1.4 Subcellular fractions

Mitochondria were prepared from rat liver by the method described by Snoswell and Koundakjian (1972). The mitochondria were suspended in 0.44 sucrose-10 mM triethanolamine (pH 7.6)-2 mM EDTA as described by Brdiczka *et al.* (1969). The method of Hoppel and Tomec (1972) was used to separate the submitochondrial fractions, which were resuspended in 0.25 M sucrose-23 mM potassium phosphate (pH 7.4). The integrity of each fraction was established by using the marker enzymes, as described in Part I, 2.1.7.

3.1.5 Enzyme assays

All assays were determined by the instrumentation described in Part I, 2.1.6.

3.1.5.1 Carnitine acetyltransferase

The activity of this enzyme was assayed in homogenates from sheep and rat liver both before and after treatment with Sephadex G-25 and also in submitochondrial fractions from rat liver. The reaction was monitored spectrophotometrically at 412 nm, as described in Part I, 2.1.7.1. The reaction kinetics of carnitine acetyltransferase before and after Sephadex G-25 treatment were investigated over the range 2 μM-4.5 mM L-carnitine.

3.1.5.2 Acetylcarnitine hydrolase

The activity of this enzyme was assayed in homogenates from sheep and rat liver both before and after treatment with Sephadex G-25 and also in submitochondrial fractions derived from rat liver. The enzyme assay system was the same as described in Part I, 2.1.7.2.

3.1.5.3 Acetyl-CoA hydrolase

The activity of this enzyme was assayed in homogenates from sheep and rat liver both before and after Sephadex G-25 treatment and also in submitochondrial fractions from rat liver. The activity was determined by two independent methods. The spectrophotometric assay conducted at 412 nm contained 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 400 mM Tris-HCl (pH 8.2), 0.1 mM acetyl-CoA, and enzyme preparation

in a total volume of 1.0 ml. The 5,5'-dithiobis-(2-nitrobenzoic acid) was added first and the rate allowed to become linear before the addition of acetyl-CoA. The activity was also assayed in an incubated system similar to that described by Knowles *et al.* (1974). L- or D-carnitine (3.3 mM) was added to this system. The reaction was started by adding 4.0 mM acetyl-CoA.

3.1.5.4 Rotenone-insensitive NADH-cytochrome *c* reductase

The activity of this enzyme was assayed in the digitonin-treated submitochondrial fractions of rat liver as described in Part I, 2.1.7.3.

3.1.5.5 Adenylate kinase

The activity of this enzyme was assayed in the submitochondrial fractions from rat liver as described in Part I, 2.1.7.4.

3.1.5.6 Succinate dehydrogenase

The activity of this enzyme was assayed in the submitochondrial fractions from rat liver as described in Part I, 2.1.7.5.

3.1.5.7 Glutamate dehydrogenase

The activity of this enzyme was assayed in the submitochondrial fractions from rat liver as described in Part I, 2.1.7.6.

3.1.6 Metabolite assays

Total acid-soluble carnitine was measured by the method of Pearson and Tubbs (1967) and free carnitine by the method of Marquis and Fritz (1964).

Acetate was determined by the kinetic method of Knowles *et al.* (1974).

Protein was determined by the biuret method.

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 by phosphotransacetylase, by the method of Michal and Bergmeyer (1963). However, as this method only assays reduced CoA, the standards were preincubated for 10 minutes at 25°C with 2 μl of 0.2 M dithiothreitol to ensure all the CoA was in the reduced form.

3.1.7 Chemicals

These were the same as described in Part I, 2.1.10.

3.2 Results

3.2.1 Effect of stereoisomers of carnitine on acetyl-CoA hydrolase activity in normal and Sephadex G-25-treated homogenates of rat and sheep liver

The results in Tables 5 and 6 indicate that, after the passage of homogenates of both rat and sheep liver down columns of Sephadex G-25, no acetyl-CoA hydrolase activity could be detected. This is in contrast to the appreciable activity measured in the untreated homogenates from both species. The total acid-soluble carnitine concentrations in the

TABLE 5

Effect of stereoisomers of carnitine on acetyl-CoA hydrolase activity in normal and Sephadex G-25-treated homogenates of rat liver

Homogenates from rat liver were prepared and assayed as described in Part I, 3.1. The values are means ± S.E.M. of three experiments.

Additions	Acetyl-CoA hydrolase activity in rat liver (nmol/min per mg of protein)	
	Normal	Sephadex G-25-treated
None	5.10 ± 0.98	N.D.*
L-carnitine	4.87 ± 0.87	5.98 ± 1.26
D-carnitine	3.23 ± 1.00	N.D.*

*N.D. - not detectable

TABLE 6

Effect of stereoisomers of carnitine on acetyl-CoA hydrolase activity in normal and Sephadex G-25-treated homogenates of sheep liver

Homogenates from sheep liver were prepared and assayed as described in Part I, 3.1. The values are means ± S.E.M. of three experiments.

Additions	Acetyl-CoA hydrolase activity in sheep liver (nmol/min per mg of protein)	
	Normal	Sephadex G-25-treated
None	3.28 ± 0.28	N.D.*
L-carnitine	3.27 ± 0.14	11.56 ± 1.26
D-carnitine	2.39 ± 0.15	N.D.*

*N.D. - not detectable

homogenates initially were 40 and 24 nmol/ml for rat and sheep liver respectively. These concentrations were decreased to approximately one-twentieth of these values by Sephadex treatment.

Addition of L-carnitine to untreated homogenates of either rat or sheep liver had no effect on the acetyl-CoA hydrolase activity but resulted in appreciable apparent acetyl-CoA hydrolase in the Sephadex-treated homogenates (Table 5 and 6). In fact, the activity in the Sephadex-treated sheep liver homogenates supplemented with L-carnitine was considerably higher than the activity in the untreated homogenate (Table 5). The reason for this is not clear.

Addition of D-carnitine significantly ($P < 0.01$) decreased the activity of acetyl-CoA hydrolase in untreated homogenates of sheep liver (Table 6). The decrease in activity of acetyl-CoA hydrolase in rat liver homogenates on the addition of D-carnitine was not significant (Table 5). In marked contrast with the addition of L-carnitine, there was no detectable activity of acetyl-CoA hydrolase in the Sephadex-treated homogenates on addition of D-carnitine (Tables 5 and 6).

The acetylcarnitine hydrolase activity determined at the same (pH 8.2) as the acetyl-CoA hydrolase activity was 5.8 and 8.1 nmol/min per mg of protein for the rat and sheep liver homogenates respectively. Significantly, these activities were not affected by the Sephadex G-25 treatment. The carnitine acetyltransferase activities determined after Sephadex treatment were 14.8 and 22.2 nmol/min per mg tissue protein for the rat and sheep liver homogenates respectively.

3.2.2 Spectrophotometric assay of acetyl-CoA hydrolase and carnitine acetyltransferase in normal and Sephadex-treated homogenates from rat liver

All the acetyl-CoA hydrolase activities expressed in

Tables 5 and 6 were determined on the basis of acetyl-CoA utilized and acetate and free CoA released in an incubation system; in each assay there was stoichiometric balance between acetyl-CoA utilized and acetate and free CoA produced.

The results presented in Figure 4 indicate clearly that there is no detectable acetyl-CoA hydrolase activity in rat liver homogenates previously treated with Sephadex G-25 (compare Fig. 4c with 4a). Figure 4b shows a much more dramatic inhibition by added D-carnitine on acetyl-CoA hydrolase activity with normal homogenates than is apparent from the activities shown in Table 5. In the spectrophotometric assay system the ratio of added D-carnitine to endogenous L-carnitine is considerably greater than in the incubated system.

3.2.3 The Michaelis-Menton kinetics of acetyl-CoA hydrolase and carnitine acetyltransferase reactions in rat liver homogenates before and after treatment with Sephadex G-25

The acetyl-CoA hydrolase reaction in untreated rat liver homogenates has a Km for acetyl-CoA of 0.2±0.05 mM. This Km was determined from a Lineweaver-Burk plot of experimental data computer-fitted to a straight line by the statistical weighting method of Wilkinson (1961) (Figure 5). The results from Figure 4c clearly indicate that Michaelis-Menton reaction kinetics for apparent acetyl-CoA hydrolysis can only be determined in untreated rat liver homogenates.

The results in Figure 4a indicate that in rat liver homogenates the activity of carnitine acetyltransferase is very low due to the high rate of apparent acetyl-CoA hydrolysis which is deducted from the rate in the presence of L-carnitine. However, there is a substantial carnitine

FIGURE 4

Spectrophotometric assay of acetyl-CoA hydrolase and carnitine acetyltransferase in normal and Sephadex-treated homogenates from rat liver

Recording of E_{412} is shown; a downward deflection represents an increase in absorbance. The reaction was started by adding 5,5'-dithiobis-(2-nitrobenzoic acid) and the recordings shown were obtained after this initial reaction became linear. Homogenates were prepared and assayed as described in Part I, 3.1. Acetyl-CoA (0.1 mM) and L- or D-carnitine (3.3 mM) were added as indicated.

(a) and (b) Normal homogenate

(c) Homogenate that had first been passed down a Sephadex G-25 column (1.5 cm x 22.5 cm) equilibrated with potassium phosphate (pH 7.4). The initial tubes of peak protein concentration were assayed.

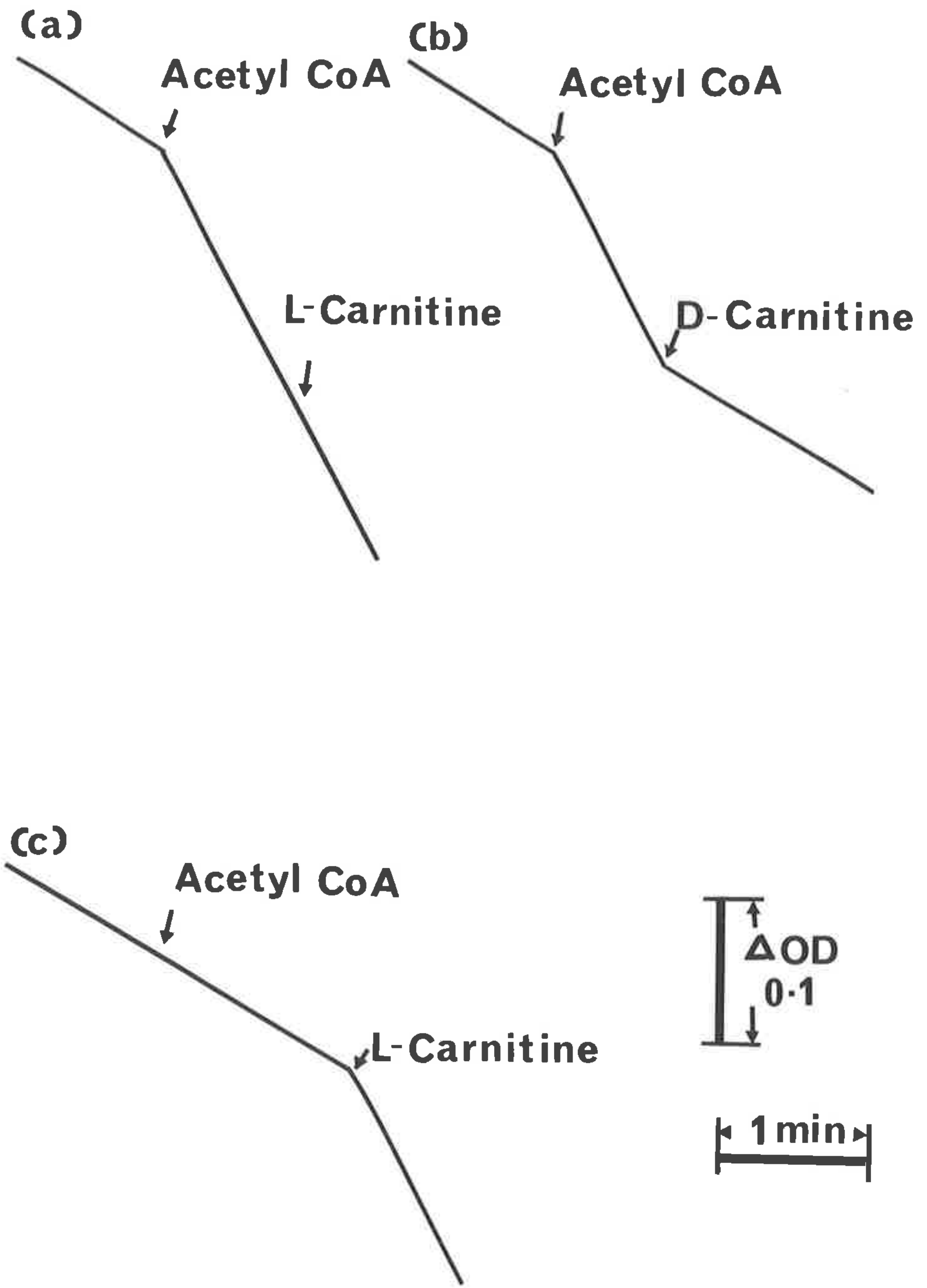


Figure 4

FIGURE 5

Lineweaver-Burk plot for acetyl-CoA hydrolase in rat liver homogenates

Homogenates were prepared and assayed as described in Part I, 3.1. Acetyl-CoA concentration varied between 11 μM and 0.5 mM.

●——● Experimental data weighted for accuracy by the statistical method of Wilkinson (1961) by using an appropriate computer program.

o Experimental data.

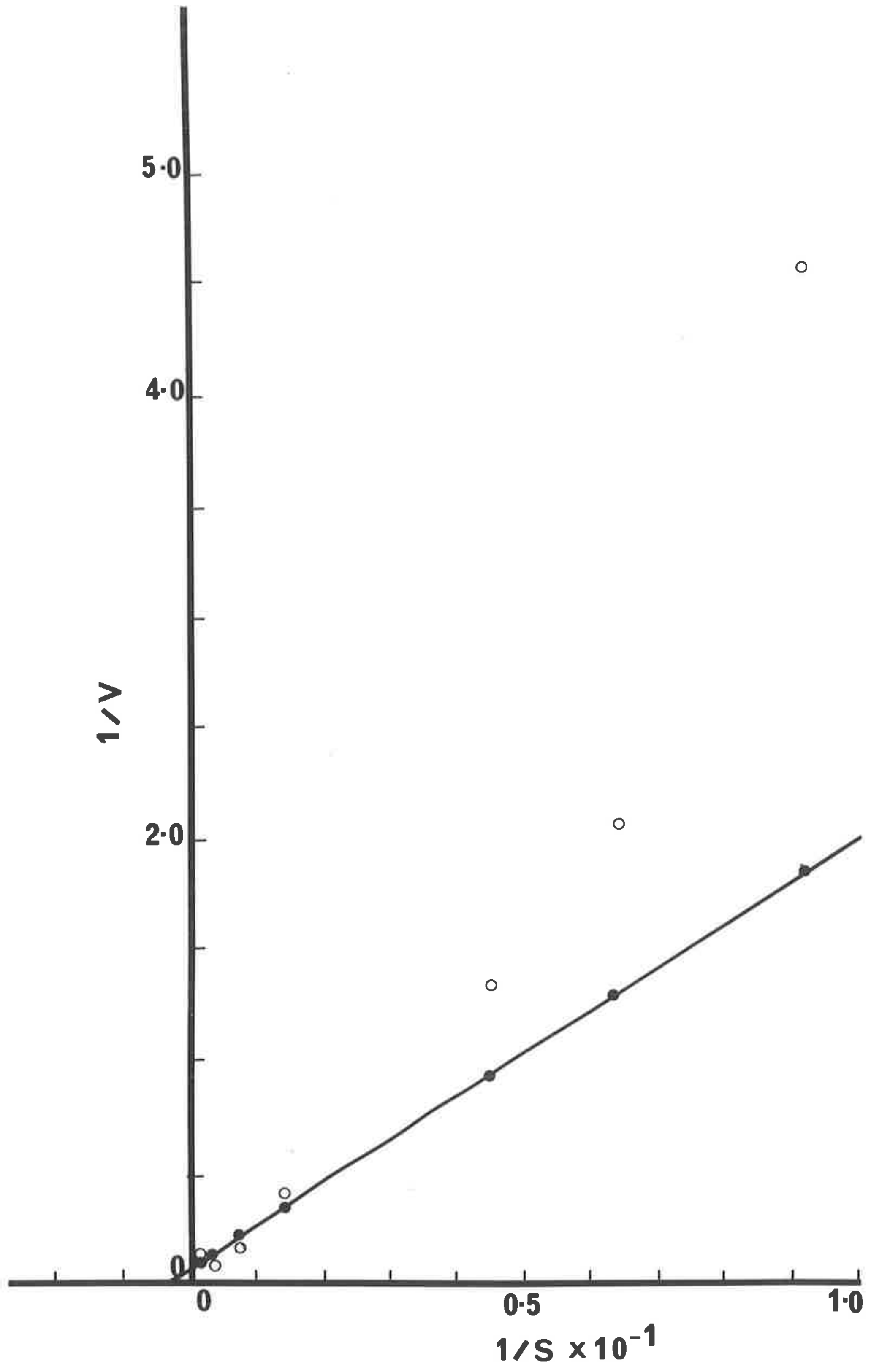


Figure 5

FIGURE 6

The Michaelis-Menton reaction kinetics for carnitine acetyltransferase in normal and Sephadex G-25-treated rat liver homogenates

The normal and Sephadex G-25-treated homogenates were prepared as described in Part I, 3.1. Acetyl-CoA concentration was 0.1 mM and L-carnitine concentration varied between 2 μM and 6.7 mM for normal homogenates and 2 μM and 4.5 mM for Sephadex G-25-treated homogenates.

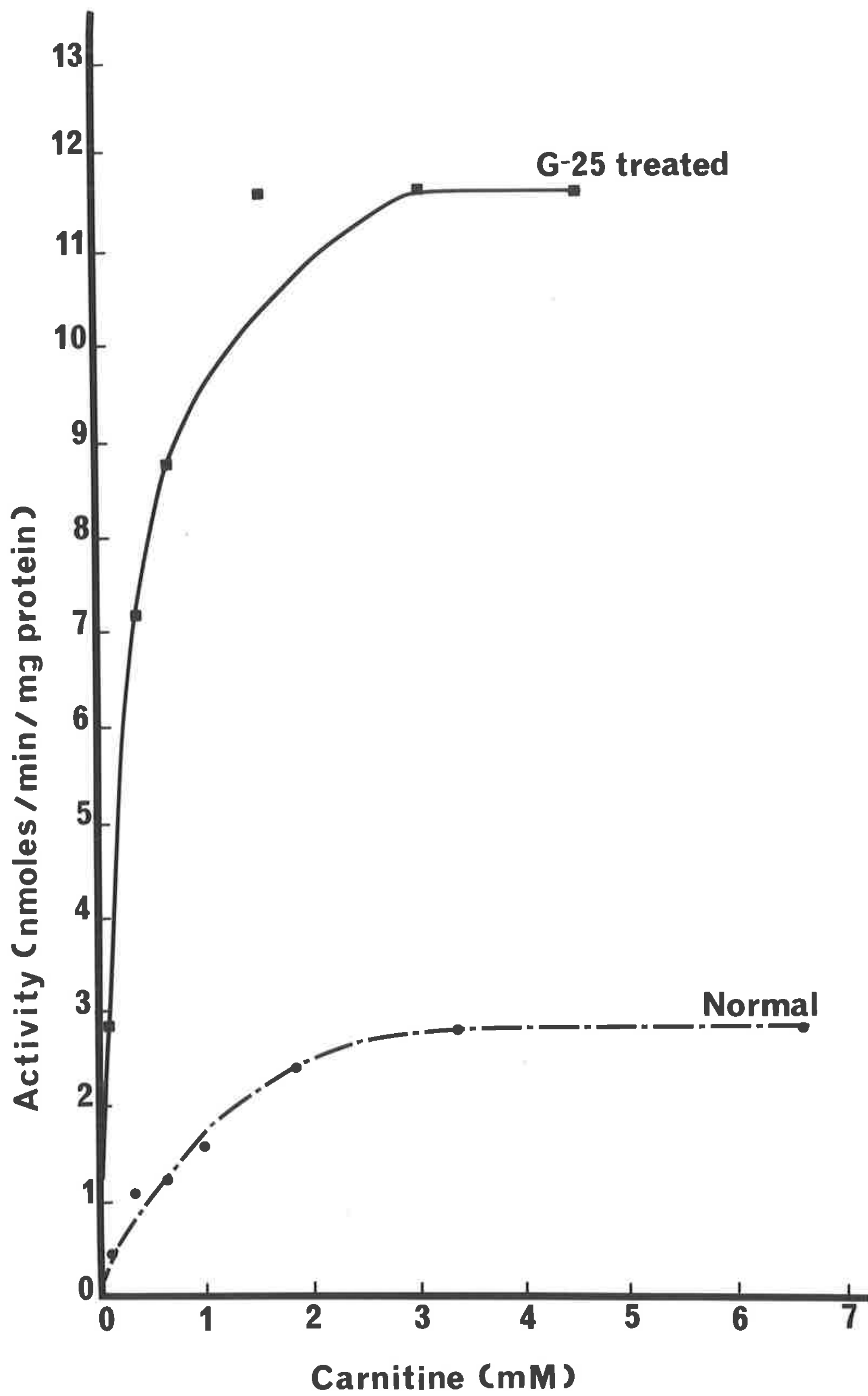


Figure 6

FIGURE 7

Lineweaver-Burk plot for the carnitine acetyltransferase reaction in normal and Sephadex G-25-treated rat liver homogenates

Homogenates and conditions of assay were as described in Figure 6. Experimental data were weighted for accuracy by the statistical method of Wilkinson (1961) by using an appropriate computer program.

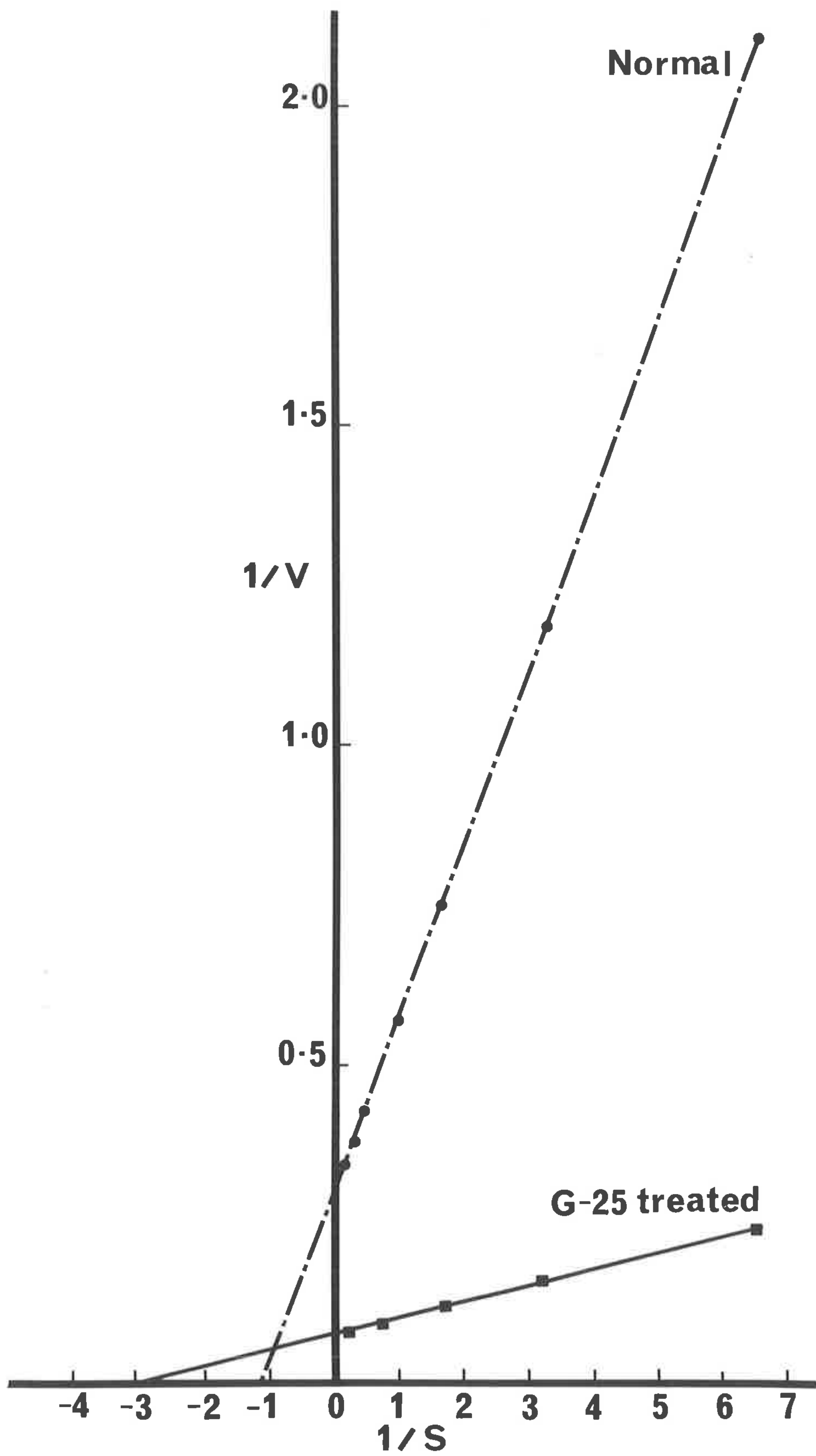


Figure 7

acetyltransferase activity detectable after prior treatment of the rat liver homogenate with Sephadex G-25 (Figure 4c). This effect on carnitine acetyltransferase is dramatically illustrated in Figure 6, which shows the saturation kinetics, and in Figure 7, which shows Lineweaver-Burk plots of computer-fitted experimental data before and after Sephadex treatment. The Vmax of the carnitine acetyltransferase reaction was increased from 3.4±0.24 nmol/min per mg protein to 13.0±0.82 nmol/min per mg protein, and the Km for carnitine was substantially decreased from 0.94±0.20 mM to 0.032±0.01 mM after Sephadex G-25 treatment.

3.2.4 Acetyl-CoA hydrolysis in rat liver mitochondria and in inner and outer mitochondrial membrane fractions

The results presented in Figure 8 show the spectrophotometric assay of acetyl-CoA hydrolase activity in rat liver mitochondria. No acetyl-CoA hydrolase activity was detected in other subcellular fractions.

Appreciable acetyl-CoA hydrolase activity was measured in disrupted whole mitochondria (Figure 8a). However, no activity could be detected in either the inner mitochondrial membrane fraction (Figure 8b) or the outer mitochondrial membrane fraction (Figure 8c). When these two membrane fractions were recombined (in ratio of 1:1 of volume), acetyl-CoA hydrolase activity could again be detected (Figure 8d).

There was no detectable acetyl-CoA hydrolase activity in the other two submitochondrial fractions, the intermembranal space and the matrix fraction.

The results from Table 7 show that the carnitine acetyltransferase activity is associated with the inner membrane-matrix fraction and the acetylcarnitine hydrolase activity is associated with the outer-membrane fraction. The activity of carnitine acetyltransferase in the inner

FIGURE 8

Acetyl-CoA hydrolase activity in rat liver mitochondria and in inner and outer mitochondrial membrane fractions

Mitochondrial and submitochondrial fractions were prepared as described in Part I, 2.1. Acetyl-CoA hydrolase was assayed as described in Part I, 3.1.5.3. Acetyl-CoA (0.1 mM) was added as indicated.

(a) disrupted rat liver mitochondria

(b) inner mitochondrial membrane fraction

(c) outer mitochondrial membrane fraction

(d) recombined inner and outer membrane fractions (1 : 1).

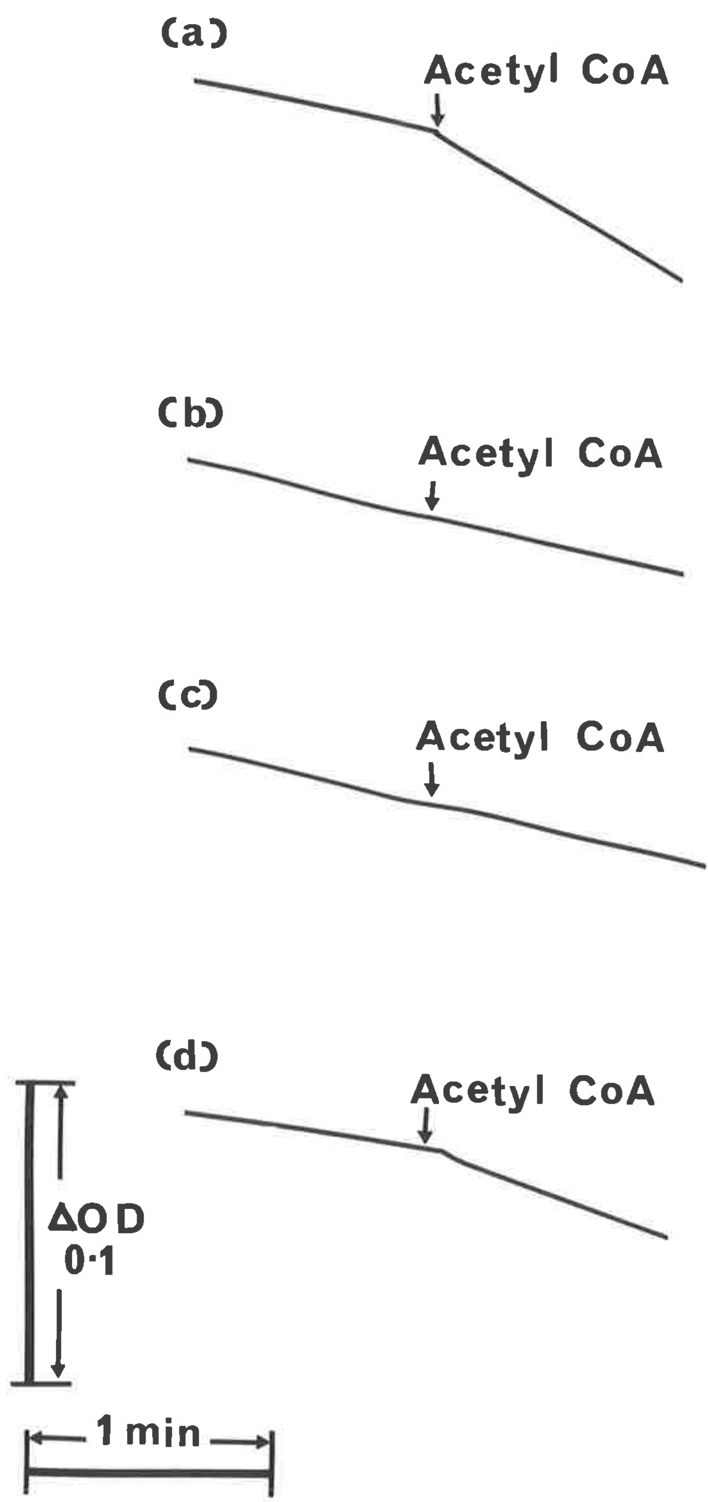


Figure 8

TABLE 7

Submitochondrial distribution of carnitine acetyltransferase and acetylcarnitine hydrolase in rat liver

Submitochondrial fractions were prepared and assayed as described in Part I, 3.1. The values are means of two experiments.

	% of total activity in submitochondrial fractions		
	Outer-membrane	Intermembranal space	Inner-membrane and matrix
Adenylate kinase	4	91	5
Rotenone-insensitive NADH-cytochrome *c* reductase	71	29	N.D.*
Glutamate dehydrogenase	N.D.*	15	85
Succinate dehydrogenase	N.D.*	N.D.*	100
Carnitine acetyltransferase	N.D.*	N.D.*	100
Acetylcarnitine hydrolase	85	15	N.D.*

*N.D. - not detectable

membrane-matrix fraction was 147 nmols/min per mg protein and the activity of acetylcarnitine hydrolase in the outer-membrane fraction was 28 nmol/min per mg protein.

4. Discussion

The results presented in Part I, 2.2.1 clearly indicate that utilization of acetylcarnitine within the assay incubation system from sheep liver homogenates is mainly enzymic rather than chemical. This enzymic utilization was demonstrated with the liver homogenates from three different species and the stoichiometry of acetylcarnitine utilization is consistent with a concept of hydrolysis of the ester bond to yield equimolar proportions of acetate and L-carnitine. These findings are not entirely inconsistent with the observations of Bremer (1962*a*) who, using rat liver mitochondria, found some alternative utilization of acetylcarnitine other than via the carnitine acetyl-transferase reaction. Also, Bremer and Davis (1974) reported acetyl-carnitine concentrations lower than expected in their experiments with rat liver mitochondria, which is again consistent with an alternative utilization of acetylcarnitine.

The hydrolysis of acetylcarnitine is stereospecific for the L-isomer since acetyl-D-carnitine was not hydrolyzed. This finding is also indicative of an enzymic rather than a chemical utilization. Acetyl-carnitine was hydrolyzed in the presence of iodoacetamide but was not hydrolyzed by non-specific cholinesterase, indicating that hydrolysis of acetyl-L-carnitine is due to a specific enzyme entity rather than a non-specific esterase. This specific hydrolase activity in rat liver cells is associated with the outer mitochondrial membrane. Thus, attempts by Mahadevan and Sauer (1969) to establish hydrolysis of short-chain

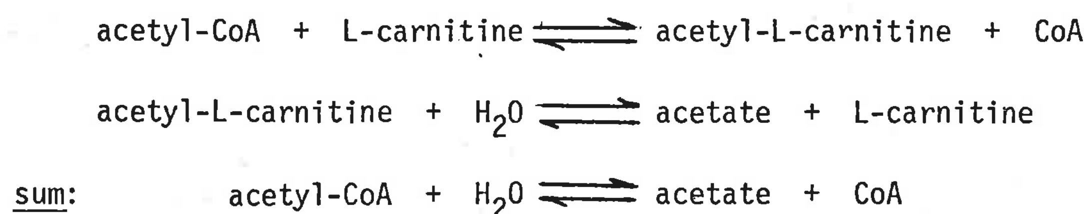
carnitine esters in enriched microsomal fractions from rat liver were unlikely to have been successful. The isolation of an enriched outer mitochondrial membrane fraction also resulted in an eight-fold purification of acetylcarnitine hydrolase activity over that present in the crude rat liver homogenate. Further attempts to purify this enzyme met with difficulty due to its lability. Low concentrations of bromoacetyl-L-carnitine (at which carnitine acetyltransferase was completely inactivated) did not inhibit acetylcarnitine hydrolase but inhibited the acetyl-CoA hydrolase activity. This result suggests that acetyl-CoA hydrolase activity might be due to the combined action of carnitine acetyltransferase and acetylcarnitine hydrolase.

The results presented in Part I, 3.2.1 and 3.2.2 clearly indicate that in both rat and sheep liver the measurement of acetyl-CoA hydrolase activity depends on the presence of endogenous carnitine. When this was substantially removed by passage through a Sephadex G-25 column, no acetyl-CoA hydrolase activity was observed. Also, addition of D-carnitine, a competitive inhibitor of carnitine acetyltransferase (Fritz and Schultz, 1965), decreased acetyl-CoA hydrolase activity in both rat and sheep liver homogenates, particularly in the spectrophotometric assay with normal rat liver homogenate, where the ratio of D-carnitine to endogenous L-carnitine was greater than in the incubated system for enzymic assay. Conversely, when L-carnitine was added to homogenates from which endogenous L-carnitine was previously removed, then the apparent hydrolase activity was restored.

The results presented in Part I, 3.2.4 also demonstrated that, although acetyl-CoA hydrolase activity could be measured in whole rat liver mitochondria, no acetyl-CoA hydrolase activity could be measured either in the inner mitochondrial membrane, which contained carnitine acetyltransferase activity (Table 7), or in the outer mitochondrial

membrane, which contained acetylcarnitine hydrolase activity (Table 7). However, on combination of these two mitochondrial membrane fractions, acetyl-CoA hydrolase activity could again be measured.

These results clearly show that, in rat and sheep liver, acetyl-CoA hydrolase is an artifact and the activity measured is due to the combined action of two enzymes, carnitine acetyltransferase present in the inner mitochondrial membrane and acetylcarnitine hydrolase present in the outer mitochondrial membrane. The sum of these two reactions does lead to an overall acetyl-CoA hydrolase reaction as indicated:



This finding explains the anomalous situation referred to in the Introduction of having two enzymes, namely carnitine acetyltransferase and acetyl-CoA hydrolase, present in the inner mitochondrial membrane both utilizing matrix acetyl-CoA.

The results presented in Part I, 3.2.3 indicate that use of the spectrophotometric method for the assay of carnitine acetyltransferase, based on the reaction of CoA liberated with 5,5'-dithiobis-(2-nitrobenzoic acid) at 412 nm after L-carnitine addition, underestimates the activity of the enzyme. The initial rate of acetyl-CoA breakdown is attributed to acetyl-CoA hydrolase activity, but in fact is due to carnitine acetyltransferase reacting with endogenous carnitine. This is particularly so with rat liver. It has been assumed in the past that in this type of assay the small amount of endogenous carnitine present would be insufficient to allow any appreciable measurement of carnitine acetyltransferase. However, these results indicate that the Km of the rat liver enzyme for L-carnitine, i.e. 32 μM, is considerably less than the 120 μM

reported for pigeon breast-muscle enzyme (Chase, 1967) or the 310 μM for pig heart enzyme (Fritz *et al.*, 1963). Thus, previous reports of low carnitine acetyltransferase activity in rat liver (Barker *et al.*, 1968; Snoswell and Henderson, 1970; Solberg *et al.*, 1972) appear to be underestimates. This also raises some doubts about various activities of carnitine acetyltransferase and short-chain acyltransferases obtained with this assay procedure after various treatments. Increased carnitine acetyltransferase activity has been reported in rat liver after treatment of the rats with clofibrate (chlorophenoxyisobutyrate), a drug which lowers plasma lipids (Solberg *et al.*, 1972; Kähönen and Ylikahri, 1974). This increase in activity may arise from variations in the concentrations of endogenous carnitine in rat liver which could lead to considerable differences in the assayed carnitine acetyltransferase activity. Thus, it would be of interest to determine the effect of clofibrate on the concentrations of carnitine in rat liver.

The precise physiological role of an acetylcarnitine hydrolase within rat liver in the normal, fed and starved state is difficult to delineate, compounded in part by the lack of a precise role for carnitine acetyltransferase. In liver from normal rats, carnitine acetyltransferase would be competing with citrate synthase for mitochondrial matrix acetyl-CoA. Inhibition of citrate cleavage enzyme in rat liver by its competitive inhibitor (-)-hydroxycitrate (Watson and Lowenstein, 1970; Lowenstein, 1971) or by an antibody (Daikuhara *et al.*, 1968) only inhibited fatty acid synthesis or formation of extramitochondrial acetyl-CoA by 80-85%, suggesting an additional pathway for the transfer of acetyl groups. This additional acetyl transfer may be mediated via carnitine acetyltransferase and acetylcarnitine hydrolase in normal rats. The total concentration of acetyl-CoA within rat liver is approximately 40 nmol per g wet tissue (Allred and Guy, 1969). The Km for acetyl-CoA of carnitine acetyltrans-

ferase is 4 x 10^{-5} M (Fritz *et al.*, 1963; Chase, 1967) and of citrate synthase 1.6 x 10^{-5} M (Shepherd and Garland, 1969) in rat liver. The Vmax for carnitine acetyltransferase in rat liver is 13 nmol/min per mg protein (Part I, 3.2.3) and for citrate synthase 32 nmol/min per mg protein (Srere, 1967). Calculations from these constants for carnitine acetyltransferase and citrate synthase would indicate a greater role for carnitine acetyltransferase in acetyl transfer than is suggested by the inhibition of citrate cleavage enzyme cited above.

Thus, in view of the points outlined above, acetyl transfer via carnitine acetyltransferase in normal liver may be regulated primarily by the pool of free carnitine in the liver rather than the pool of acetyl-CoA. The study of carnitine turnover in rat tissues by Brooks and McIntosh (1975) showed that carnitine turnover within rat liver fitted a two compartment model by mathematical reasoning. The larger compartment (89% of liver carnitine) had a turnover time of 1.3 hours and the smaller compartment (11% of liver carnitine), 903 hours. The mathematical model assumed steady-state conditions, where excretion of carnitine was measured at 10 μmols/day. Extrapolating from this model, the smaller compartment may reflect the size of the metabolic pool of carnitine in the liver (the larger compartment rapidly exchanging with the blood carnitine, representing the transportable pool of carnitine) and thus the pool of carnitine available for the role of acyl transfer would be of the order of 10-30 nmols (Pearson and Tubbs, 1967; Brooks and McIntosh, 1975).

A possible role for acetylcarnitine hydrolase may be to maintain the availability of free carnitine via hydrolysis of acetylcarnitine formed. This role would be regulated by the concentration of acetyl-carnitine in the liver and the high Km (1.5 to 2.0 mM) for acetylcarnitine of the acetylcarnitine hydrolase. This Km value was determined from a crude preparation and as such there must be some reservation as to its

absolute accuracy. The concentration of acetylcarnitine in normal rat liver is 41±9 nmols per g wet weight (Pearson and Tubbs, 1967) which is not transported from the liver in the blood (no detectable acetylcarnitine in blood) and may be compartmented in the mitochondria (Bremer and Davis, 1974). Thus, there may be high local concentrations of acetylcarnitine within the mitochondria, which approach the Km for acetylcarnitine of the acetylcarnitine hydrolase in liver. The increase is acetylcarnitine concentration (95±16 nmols/g wet weight; Pearson and Tubbs, 1967) and acetylcarnitine hydrolase activity in the starved rat is consistent with the hypothesis of the short-chain carnitine ester system buffering 'acetyl pressure' (Pearson and Tubbs, 1967) generated by β-oxidation of fatty acids during starvation. The increase in activity allows a faster rate of carnitine recycling through carnitine acetyltransferase and acetylcarnitine hydrolase and also maintains free carnitine for complexing with long-chain fatty acids via carnitine palmitoyltransferase. The enzyme couple of carnitine acetyltransferase and acetylcarnitine hydrolase would divert 'acetyl pressure' from ketone body formation to acetate production. The energy lost in production of acetate from acetyl-CoA would be equivalent to that lost in the condensation of acetyl-CoA to form acetoacetyl-CoA. The findings of Seufert *et al.* (1974) showed that the livers from both normal and starved rats, when perfused with hexanoate, not only produced more ketones but also produced significantly more acetate.

The high activity of an acetylcarnitine hydrolase in the presence of a high carnitine acetyltransferase activity in normal sheep liver may ease a high ratio of acetyl-CoA/CoA (1:1 from Snoswell and Henderson, 1970) with the resultant bonus of producing acetate, a major metabolic fuel in peripheral tissues in sheep (Blaxter, 1962; Annison and Armstrong, 1970). In severely alloxan-diabetic sheep there is a significant decrease

in acetylcarnitine hydrolase activity in the liver. This would firstly decrease the amount of L-carnitine recycled through the carnitine acetyl-transferase and acetylcarnitine hydrolase couple which may be reflected in the need to synthesize more carnitine as a compensating measure, and seondly result in an accumulation of acetyl-L-carnitine (see Snoswell and Koundakjian, 1972). In the severely alloxan-diabetic sheep, Snoswell and McIntosh (1974) reported a twenty-fold increase in total acid-soluble carnitine and a thirty-fold increase in acetyl-L-carnitine. The physiological role of acetylcarnitine hydrolase in dry dairy cow liver may indeed be similar to that outlined in normal sheep liver. Further investigations of the role of acetylcarnitine hydrolase in ruminant liver are presented in Part II.

PART II: ASPECTS OF ACETATE METABOLISM IN THE RUMINANTS

1. Introduction

1.1 General Introduction

Volatile fatty acids (VFA), predominantly acetic, propionic and butyric acids, are the major end-products of microbial fermentation in the ruminant digestive tract (Masson and Phillipson, 1951). The estimates of Seeley *et al.* (1969) showed VFA comprised 60-80% of the daily metabolizable energy intake in a wide range of forage rations given to sheep. Of the three major volatile fatty acids, propionate is removed by the liver and converted to glucose (Bergman *et al.*, 1966) and butyrate is largely converted to 3-hydroxybutryate by the rumen epithelium, both *in vitro* (Stevens, 1970) and *in vivo* (Katz and Bergman, 1969*a*). The small amounts of butyrate that appear in portal blood are removed by the liver (Bergman and Wolff, 1971).

Acetate is the only volatile fatty acid to occur in peripheral blood in concentrations of 1-2 mmol/l (Annison, 1954; Bergman and Wolff, 1971). Blaxter (1962) proposed that a large proportion of the energy requirements of herbivorous animals was derived from the oxidation of acetate. The adaptation of the technique of isotope-dilution to the study of ruminant metabolism by Annison and Lindsay (1961) provided the first physiological determinations of the rate of entry of acetate into the circulation. Subsequently many investigators have used this technique to estimate the rates of entry of acetate into the total body pool of ruminants. These estimates have been collated in a review of volatile fatty acid metabolism by Annison and Armstrong (1970). From these studies it was established that the total rate of acetate production consisted of

two discrete components, an exogenous component absorbed from the alimentary tract and produced mainly in the rumen and an endogenous component.

1.2 Significance of endogenous acetate production in the ruminant

Entry rates of acetate determined in the whole animal represented the total acetate entry and gave only a limited indication of the contribution of endogenous acetate to the total entry of acetate. Thus, exogenous and/or endogenous acetate production must be determined independently of the total acetate entry to gauge the significance of the endogenous acetate production in a given physiological state.

Annison and White (1962) attempted to establish both the concept and significance of endogenous acetate production by comparing total acetate entry in fed sheep to the acetate entry in sheep where the rumen contents had been replaced with a solution of salts approximating in composition to the saliva. The total acetate entry in the fed sheep was 3.5 to 4.6 mg acetate/min per Kg body weight, while the entry in sheep with rumen contents removed was 1.6 to 2.0 mg acetate/min per Kg body weight. Calculated from these estimates, the contribution of endogenous acetate was 43-46% of the total acetate entry in the fed sheep. A solution of labelled acetate was placed in the emptied rumen of a sheep starved for 24 hours and, by comparing the specific activity of blood acetate and rumen acetate, Annison and White were able to estimate directly the contribution of the entry of endogenous acetate. At high rumen acetate concentrations (150 mM and greater), concentrations of blood acetate were 1-2 mM, similar to those found in fed sheep, and the ratios of specific activity indicated that entry of endogenous acetate accounted for 25% of total entry. The percentage contribution of endogenous acetate entry increased as the acetate concentration of blood declined. Annison and

White suggested from these observations that exogenous production of acetate regulated the endogenous entry of acetate. Although the designs of these experiments were not strictly physiological, Annison and White were the first workers to demonstrate and quantify the endogenous entry of acetate.

A more physiological approach to estimating endogenous acetate entry was reported by Annison and Armstrong (1970). A comparison was made of the rates of entry of acetate into the total body pool and the rates of production of acetate in the rumens of sheep fed at hourly intervals. These rates were measured in separate experiments, with each animal serving as its own control. The ruminal acetate production rates accounted for 50-60% of the total acetate entry. Annison and Armstrong suggested that endogenous entry of acetate would constitute the majority of the difference since post-ruminal acetate production was shown to be low by Annison and Lewis (1959).

Exogenous and endogenous entry of acetate in these reports was not determined coincidentally with the total entry of acetate in the whole animal. Also, ruminal acetate production or concentration was related directly to total acetate turnover without defining the extent to which acetate was metabolized by the liver and gut itself.

Bergman and Wolff (1971), attempting to resolve these deficiencies, added to the technique of isotopic-dilution the refinement of indwelling cannulae placed in the major splanchnic blood vessels. Thus, net and actual rates of portal absorption and liver metabolism of acetate could be measured simultaneously with total entry rates of acetate. However, rumen acetate concentration was not measured directly by these workers. The portal absorption rates reported varied (depending on the food intake of the sheep) between 22 and 76% of the total acetate turnover. Net acetate appearance in the portal blood averaged 50% of the total acetate

turnover. These observations of Bergman and Wolff supported those of Annison and White (1962) in that both groups found the contribution of endogenous acetate entry to total acetate entry was highest, when arterial blood concentrations of acetate were low (less than 0.5 mmol/l).

However, Jarrett, Filsell and Ballard (1976) reported that low arterial acetate concentrations observed during starvation were insufficient to sustain uptake of acetate by the hind limb of sheep. These workers found that acetate accounted for 2% or less of the oxygen uptake by the limb during exercise or starvation. Since direct oxidation of acetate was minimal during starvation, Jarrett *et al.* (1976) disputed the significance of endogenous acetate production in these sheep. Unfortunately Jarrett *et al.* did not quantitate acetate synthesis directly or indirectly by the use of labelled acetate, which would have supplied direct evidence in support of their argument. Nonetheless, important questions have been raised by these workers concerning endogenous acetate metabolism and also about the precise nature of the utilization of acetate by the ruminant.

1.3 Origins of endogenous acetate production in the ruminant

Several ruminant tissues have been shown to release acetate, as determined by the criterion of increased venous blood acetate concentrations.

The results of Bergman and Wolff (1971) showed that 20% of the endogenous acetate production in fed sheep was attributable to liver production of acetate. This production of acetate was detected in 2 out of 4 experiments with fed sheep and overall there was no net release of acetate due to utilization of acetate within the liver itself. The liver from sheep fasted for 3 days did in fact release acetate, but again this acetate production represented only 17% of the total endogenous acetate production. Bergman and Wolff suggested that the majority of the

endogenous acetate was released into the blood by general body tissues, the design of their experiments not allowing them to be more specific.

In contrast to the findings of Bergman and Wolff, 1971; Baird, Symons and Ash (1974,1975) reported a substantial net production of acetate by the liver from lactating dairy cows. The hepatic production observed by Baird *et al.* (1974) was 31.68 mmol/min which was almost of the same order as the acetate production from the gut of 34.75 mmol/min. Further observations made over extended sampling periods (Baird *et al.*, 1975) showed that the hepatic acetate release was variable and averaged approximately 50% of the gut production. The total endogenous acetate production was not determined and so the contribution of hepatic production to total endogenous production of acetate could not be calculated. Also, the net hepatic production of acetate by liver from non-lactating dairy cows was not determined and thus direct comparisons of hepatic acetate production between non-lactating and lactating dairy cows were not made by these workers. Nevertheless, in conjunction with the findings of Bergman and Wolff (1971) in non-lactating sheep, Baird, Symons and Ash (1975) postulated that the large net hepatic production of acetate by the lactating dairy cow was a feature of the lactation state in that animal.

Annison and Linzell (1964) reported that in the lactating mammary gland of goat there was a simultaneous uptake of acetate, as measured by arterio-venous differences, and release of acetate, as indicated by lower specific activity of labelled acetate in mammary venous blood than in arterial blood. The mammary gland of lactating dairy cows was also shown to release acetate by the same criterion (Annison, Bickerstaffe and Linzell, 1974). The quantity of acetate released from the mammary gland of lactating dairy cows was shown to be dependent on the amount of roughage in the diet of these animals. There were no reports of comparative observations of acetate release from non-lactating mammary gland.

Annison and Armstrong (1970) reported that the head of the cow could release acetate, as indicated by lower specific activity of labelled acetate in venous blood than in arterial blood.

1.4 Precursors of endogenous acetate in the ruminant

Fatty acids, certain amino acids and ketones which were catabolized via acetyl-CoA could all be potential contributors to endogenous acetate.

The entry of endogenous acetate in 24 hour starved sheep with emptied rumens was reduced markedly by infusion of glucose and was also reduced in the initial stages of acetate infusion (Annison and White, 1962). Since administration of glucose or acetate was known to lower the concentrations of free fatty acids in sheep plasma (Lindsay, 1959; Annison, 1960), Annison and White postulated that free fatty acids could be a source of endogenous acetate. West and Annison (1964) reported labelling of plasma acetate during the infusion of ^{14}C-labelled palmitate into fed sheep. The specific activity of plasma acid, expressed as a percentage of the specific activity of plasma palmitate, varied between 1.77 and 2.18 depending on the ^{14}C-label position of the palmitate. Palmquist (1972) also reported transfer of ^{14}C-label from infused [1-^{14}C]-palmitate to plasma acetate in fed sheep. The transfer quotients of the ^{14}C-label were 1.96 and 4.42 in two experiments. The transfer quotients in sheep starved for 48 hours increased to 16.00. Palmquist calculated that the estimates of endogenous acetate from palmitate oxidation in the fasted sheep were in close agreement with the values derived from isotope transfer by Annison and White (1962). However, Palmquist reported a much lower transfer of label from palmitate to acetate in lactating dairy cows. The transfer quotients in repeats of an experiment with one Jersey cow were 0.77 and 1.52 and in one experiment with an Holstein cow the transfer quotient was 0.00. The Holstein cow had been fed a high grain-low roughage

diet which had been shown to depress plasma free fatty acid concentration and increase plasma glucose concentration, (Palmquist and Conrad, 1971). Also, this type of diet was shown to lower production of endogenous acetate by the lactating mammary gland of dairy cows (Annison, Bickerstaffe and Linzell, 1974). Unfortunately, in this regard, Baird *et al.* (1975) did not determine uptake of free fatty acids by the liver in their experiments with lactating dairy cows. Palmquist's estimates of endogenous acetate entry from palmitate oxidation in fed sheep and lactating dairy cows could not account for the entries previously determined by Annison and White (1962) and Lee and Williams (1962) in these animals.

Annison *et al.* (1967) postulated that proteins in the fed ruminant could contribute substantially to endogenous acetate via amino acids which were not precursors of pyruvate. Leucine, isoleucine and lysine by this reasoning, would be the most important precursors of endogenous acetate. The combined liver uptake of these amino acids reported by Wolff, Bergman and Williams (1972) was 1.22 mmol/hr. The endogenous acetate production rate in fed sheep reported by Bergman and Wolff (1971) was 32 mmol/hr, of which the liver production contributed 21% or 6.72 mmol/hr. The combined contribution of these amino acids only accounted for approximately 15% of the hepatic endogenous acetate production, on the assumption that the two groups of fed sheep were comparable. The results from Baird *et al.* (1975) for dairy cows showed that the combined hepatic uptake of these amino acids was 1.54 mmol/min, while the net hepatic acetate production in the same animal was 27.99 mmol/min. Thus, in the fed sheep and lactating dairy cow, leucine, isoleucine and lysine did not contribute significantly to the endogenous acetate production rates observed.

The gluconeogenic amino acids metabolized via pyruvate could contribute, in some degree, to endogenous acetate production. Wolff and Bergman (1972) calculated that, in the fed sheep, conversion to glucose

accounted for 26% of the alanine, aspartate and glutamate turnover and only 5-7% of the glycine and serine turnover. Calculated from the results of Baird *et al.* (1975) in lactating dairy cows, the combined hepatic uptake of these amino acids (10.61 mmol/min ideally, assuming total conversion to acetate) accounted for only about 30% of the total hepatic acetate output of 27.99 mmol/min. Thus, in the lactating dairy cow, gluconeogenic amino acids would not contribute to a great extent to endogenous acetate production. The endogenous acetate production in the liver of fed sheep (calculated as 21% of the total endogenous acetate production) from Bergman and Wolff (1971) was 6.72 mmol/hr. The combined hepatic uptake of alanine, aspartate, serine and glycine in fed sheep reported by Wolff, Bergman and Williams was 8.28 mmol/hr. Glutamate was actually produced by the liver of fed sheep at a rate of 1.11 mmol/hr (Wolff *et al.*, 1972). Thus, in the fed sheep, gluconeogenic amino acids could contribute in some degree to the hepatic acetate production.

Ballard (1972) raised the possibility that utilization of ketones by extrahepatic tissues of the ruminant could give rise to endogenous acetate. No evidence was given in support of this suggestion. The experiments of West and Annison (1964) and Palmquist (1972) were not designed to differentiate transfer of label from ketones to acetate or from palmitate to acetate.

Ethyl alcohol could also be metabolized to acetate by ruminant tissues. However, hepatic uptake of ethyl alcohol in the lactating dairy cow, determined by Baird *et al.* (1975), accounted for only about 6% of the hepatic acetate production.

1.5 Enzymic production of acetate in the ruminant

The oxidation of long-chain fatty acids and the catabolism of

amino acids both produce acetyl-CoA within the mitochondrial matrix. Since the inner mitochondrial membrane is not freely permeable to acetyl-CoA, Lowenstein (1964) proposed three mechanisms whereby the acetyl moiety of acetyl-CoA is transported across the inner mitochondrial membrane barrier.

The first mechanism proceeded by the conversion of the acetyl group of intramitochondrial acetyl-CoA into citrate, a reaction catalyzed by mitochondrial citrate synthase (Ochoa, 1955). This was followed by the diffusion of citrate from the mitochondria. Extramitochondrial citrate was then cleaved to acetyl-CoA and oxaloacetate by ATP-dependent citrate lyase (Srere, 1967). However, Hanson and Ballard (1967) and Ballard, Hanson and Kronfeld (1969) showed that the activities of mitochondrial citrate synthase and ATP-citrate lyase in ruminant liver and adipose tissue were much lower than the corresponding activities assayed in the rat. These workers suggested that transfer of acetyl moieties from acetyl-CoA via citrate was of limited significance in ruminant tissues.

The second mechanism proposed by Lowenstein proceeds by the hydrolysis of intramitochondrial acetyl-CoA to acetate and subsequent diffusion of acetate from the mitochondria. Annison *et al.* (1967) suggested that this mechanism was of major significance in ruminant tissues for acetate production. Quraishi and Cook (1972) reported that there was demonstrable acetyl-CoA hydrolase activity in many Holstein steer tissues. The highest activities of acetyl-CoA hydrolase were associated with kidney tissue, followed by liver and brain tissue, while the lowest were associated with skeletal muscle (5-30% of the liver activity of 56.83 nmol of substrate reacting/min per mg protein. Knowles *et al.* (1974) reported a wide distribution of acetyl-CoA hydrolase activity in sheep tissues also. The liver activity of 10.0 nmol/min per mg protein was highest in sheep tissues, followed by muscle activity,

while kidney activity was very low (3.12 nmol/min per mg protein). The differences between relative tissue activities and absolute activities of acetyl-CoA hydrolase could reflect species variation or the fact that these groups used quite different assay procedures to estimate acetyl-CoA hydrolase activity. The findings reported in Part I of this thesis showed that acetyl-CoA hydrolase, proposed as a second mechanism by Lowenstein (1964) and measured by Quraishi and Cook (1972) and Knowles *et al.* (1974), was not a single enzyme entity.

The third mechanism proposed by Lowenstein proceeded by the transfer of the acetyl moiety of intramitochondrial acetyl-CoA to carnitine, yielding acetylcarnitine. This reaction is catalyzed by carnitine acetyltransferase (Friedman and Fraenkel, 1955; Bremer, 1962*a*; Fritz 1963; Fritz and Yue, 1963). The activity of carnitine acetyltransferase was forty five-fold greater in sheep liver and approximately thirty-fold greater in goat liver than the activities reported for rat liver (Barker, Fincham and Hardwick, 1968; Snoswell and Henderson, 1970). The carnitine acetyltransferase activity detected in sheep tissues was almost exclusively associated with the mitochondria (Snoswell and Koundakjian, 1972; Edwards *et al.*, 1974).

These observations are consistent with carnitine acetyltransferase playing a major role in acetyl moiety transfer in ruminant tissues, particularly the liver. The acetylcarnitine produced by this mechanism could be hydrolyzed, via acetylcarnitine hydrolase, to acetate in the liver of ruminants (Part I). This mechanism would propose a coupled system for producing acetate, viz. carnitine acetyltransferase and acetyl-carnitine hydrolase, which encompassed the second and third mechanisms for acetyl transfer proposed by Lowenstein (1964).

1.6 Acetate metabolism by the mammary gland

Acetate, because of its arterial concentration and high rate of uptake by the mammary gland of lactating goats (Popják *et al*, 1951; Annison and Linzell, 1964) and lactating dairy cows (McClymont, 1951; Bickerstaffe *et al*., 1974), is considered one of the most important metabolites in the mammary gland metabolism of the ruminant. The acetate taken up by the mammary gland of lactating ruminants serves at least two major functions.

Firstly, acetate, and to a lesser extent 3-hydroxybutyrate, are sources of carbons for the *de novo* synthesis of milk fatty acids up to chain length C_{14} and part of C_{16} (Popják *et al*., 1951; Annison *et al*., 1967; Linzell *et al*., 1967; Palmquist *et al*., 1969). These fatty acids form about 40% by weight of milk fatty acids in ruminants (Linzell, 1968). The mechanism of acetate incorporation into fatty acids has been reviewed by Bauman and Davis (1974). Briefly summarized, it consists of the activation of acetate to acetyl-CoA in the cytosol of the cell and incorporation into fatty acids via the malonyl-CoA pathway involving acetyl-CoA carboxylase and fatty acid synthetase. The remainder of the fatty acids of ruminant milk fat, consisting mainly of C_{16} and C_{18} fatty acids, are synthesized from plasma triglycerides (West *et al*., 1972; Bickerstaffe *et al*., 1974).

Secondly, acetate may be utilized via the tricarboxylic acid cycle for the generation of energy. Annison and Linzell (1964) and Annison *et al*. (1967) reported that, in lactating goats, 38-56% of the acetate taken up by the mammary gland was oxidized, contributing 24-29% of the total CO_2 produced from the mammary gland. Bickerstaffe, Annison and Linzell (1974) reported that, in lactating dairy cows, 20-39% of the acetate taken up by the mammary gland was oxidized, contributing 24-36%

of the total CO_2 produced from the mammary gland. However, Bickerstaffe *et al.* (1974) calculated that there was no significant difference between these species in acetate oxidation and contribution to CO_2 production in the mammary gland. Davis and Bauman (1974) suggested that there was a reduced entry of glucose carbons into the Krebs cycle as acetyl-CoA in ruminant mammary gland and on this basis proposed that acetate was possibly the most important metabolite in the mammary gland of ruminants in the generation of energy via the Krebs cycle.

1.7 Objectives of this study

The lactating ruminant served as a model for the investigation of endogenous acetate metabolism.

Since the lactating dairy cow was shown to release acetate from the liver (Baird *et al.*, 1975), the hepatic metabolism of lactating and non-lactating dairy cows was compared. This comparison centred on the determination of the activities of carnitine acetyltransferase and acetylcarnitine hydrolase to establish the capacity of this enzyme couple to release acetate from acetyl-CoA. Also the concentrations of metabolites in the liver related to these activities were determined to focus upon changes in the lactation state.

The hepatic acetate metabolism of lactating ewes was investigated using indwelling cannulae as described by Snoswell and McIntosh (1974). This permitted quantitation of exogenous production of acetate and possible hepatic production of acetate. Biopsy and post-mortem samples of liver were taken to estimate the *in vitro* capacity of carnitine acetyltransferase and acetylcarnitine hydrolase in the lactating ewes. Hepatic acetate release was compared to *in vitro* enzymic capacity to release acetate. Also, relationships between possible precursors of endogenous acetate

(e.g. free fatty acids and amino acids) and endogenous acetate production were investigated. The results of Bergman and Wolff (1971) served as a basis for comparison with non-lactating ewes.

The acetate metabolism of the mammary gland in lactating ewes was also investigated. Since Baird *et al.* (1975) postulated that the significant hepatic release of acetate was associated with the lactation state, possible relationships between hepatic acetate metabolism and mammary gland acetate metabolism were investigated in lactating ewes.

2. A role for carnitine in enzymic acetate production in liver from dry and lactating cows

2.1 Materials and methods

2.1.1 Animals

Non-lactating cows were predominantly Jersey and were all approximately 3 years of age. The lactating cows were all Jersey x Friesian animals in the fifth month of their second lactation. The cows were grazed on irrigated perennial pasture consisting mainly of white clover, cocksfoot, paspallum and rye grass. No supplementary feed was given. All cows used in this study were generously supplied by the Victorian Department of Agriculture.

2.1.2 Tissue preparation and homogenates

Liver tissue from non-lactating and lactating cows was obtained by biopsy by Dr. J.G. McLean as described by Baird and Heitzman (1970). Biopsy samples were both freeze-clamped and collected fresh into 0.25 M sucrose-23 mM potassium phosphate (pH 7.4) buffer.

Perchloric acid extracts of all freeze-clamped tissue were prepared as described by Snoswell and Henderson (1970).

Fresh liver tissue was homogenized with a Potter-Elveljen homogenizer in either 0.25 M sucrose-23 mM potassium phosphate (40%, w/v) or 0.025 M sucrose-2.3 mM potassium phosphate (pH 7.4) with 0.1% Triton X-100 (20%, w/v).

2.1.3 Enzyme assays

2.1.3.1 Carnitine acetyltransferase

This enzyme activity was assayed in homogenates prepared in 0.025 M sucrose-2.3 mM potassium phosphate (pH 7.4) with 0.1% Triton X-100 (20%, w/v) by the method described in Part I, 2.1.7.1.

2.1.3.2 Acetylcarnitine hydrolase

This enzyme activity was assayed in homogenates prepared in 0.25 M sucrose-23 mM potassium phosphate (pH 7.4) (40%, w/v) by the method described in Part I, 2.1.7.2.

2.1.4 Metabolite assays

The perchloric acid extracts of liver samples were adjusted to (pH 6.5) with 3 N KOH and centrifuged at 5°C to remove potassium perchlorate. These supernatants were used for assay.

Acetylcarnitine was determined by the method of Pearson and Tubbs (1964).

The total amount of acid-soluble carnitine was determined by the method of Pearson and Tubbs (1967).

Free carnitine was determined by the method of Marquis and Fritz (1964).

Acetyl-CoA and acetyl-CoA plus free CoA were determined by the kinetic method of Allred and Guy (1969), as described in Part I, 3.1.6.

Acetoacetate was determined by spectrophotometric assay at 37°C and 340 mM. The assay system contained 33 mM Tris-HCl (pH 7.0), 0.1 mM NADH, 1 mM $CaCl_2$ and neutralized extract, in a total volume of 1.0 ml. The assay was started with 2.5 µg 3-hydroxybutyrate dehydrogenase.

3-Hydroxybutyrate was determined by spectrophotometric assay at 37°C and 340 nm. The assay system contained 20 mM Tris-HCl (pH 8.5), 100 mM hydrazine buffer (pH 9.0), 0.4 mM NAD, 1 mM $CaCl_2$ and neutralized extract in a total volume of 1.0 ml. The assay was started with 2.5 µg 3-hydroxybutyrate dehydrogenase.

L-glycerol-3-phosphate was determined in the same cuvettes as 3-hydroxybutyrate and at the same temperature and wavelength. L-glycerol-3-phosphate dehydrogenase (10 µg) was added to the cuvettes after the 3-hydroxybutyrate assay had reached its end-point equilibrium.

2.1.5 Chemicals

Chemicals and enzymes were the same reagent grade and from the same sources as described in Part I, 2.1.10.

2.2 Results

2.2.1 Free carnitine, acetylcarnitine and total acid-soluble carnitine in livers from non-lactating and lactating cows

The results in Table 8 show that acetylcarnitine constitutes a relatively large proportion (approximately 36%) of the

TABLE 8

Free carnitine, acetylcarnitine and total acid-soluble carnitine in liver from non-lactating cows

Three-year-old predominantly Jersey cows that had been fed on irrigated pasture with no supplementary feed were used. Liver biopsy samples were immediately frozen with aluminium-faced tongs previously cooled in liquid nitrogen. Frozen tissue powders were extracted and assayed as described in Part II, 2.1.

	Concentration (nmol/g wet weight)		
Number of cow	Acetyl-carnitine	Free carnitine	Total acid soluble carnitine
13	12.7	30.9	54.5
59	29.1	13.8	54.9
63	34.6	31.7	82.9
86	19.1	29.7	77.0
Mean	23.9	26.5	67.3
S.E.M.	±4.9	±4.3	±7.4

TABLE 9

Free carnitine, acetylcarnitine and total acid-soluble carnitine in liver from lactating cows

Lactating cows were all Jersey x Friesian in the fifth month of their second lactation and were fed on irrigated pasture with no supplementary feeding. Tissue samples were prepared and assayed as described in Part II, 2.1.

	Concentration (nmol/g wet weight)		
Number of cow	Acetyl-carnitine	Free carnitine	Total acid-soluble carnitine
330	21.1	9.8	31.0
340	5.5	25.3	30.8
440	6.2	17.0	7.8
460	11.8	7.3	27.8
Mean	11.2	14.8	24.4
S.E.M.	±3.6	±4.0	±5.6

total acid-soluble carnitine in livers from non-lactating cows. The mean ratio of free carnitine/acetylcarnitine was 0.90 in these samples. From the results shown in Table 8, the individual animal values and the mean values for the sum of free and acetylcarnitine constituted approximately 75% of the total acid-soluble carnitine.

The results in Table 9 show that acetylcarnitine also constitutes a relatively large proportion (approximately 46%) of the total acid-soluble carnitine in livers from lactating cows. Although this represents a larger proportion than in the livers from non-lactating cows there was no significant difference (t_6 = 1.43; $.20 < P < .30$) in acetylcarnitine concentration between the two groups of animals. There was no significant difference in free and total acid-soluble carnitine between the non-lactating and lactating groups due in large part to the degree of animal variation. The mean ratio of free carnitine/acetylcarnitine was 0.76 in livers from lactating cows. In contrast to the non-lactating cows, the sum of acetyl and free carnitine constituted almost all of the total acid-soluble carnitine in the livers of lactating cows.

2.2.2 Acetyl-CoA, total CoA and the ratio of acetyl-CoA to total CoA in livers from non-lactating and lactating cows

The results in Tables 10 and 11 show that the mean total CoA concentrations in livers from both non-lactating and lactating cows are similar. In contrast, the mean acetyl-CoA concentration of 86.1±2.1 nmol/g wet weight in livers from lactating cows (Table 11) is significantly ($P < .01$) greater than the mean acetyl-CoA concentration of 60.1±3.7 nmol/g wet weight in livers from non-lactating cows (Table 10). Additionally the ratio of acetyl-CoA/total CoA of 0.93±0.03 in liver from lactating cows (Table 11) is significantly greater ($P < 0.001$) than

TABLE 10

Acetyl-CoA, total (acetyl and free) CoA and the ratio of acetyl-CoA to total CoA in livers from non-lactating cows

Animals and tissue preparation were the same as described in Table 8. Assays were performed as described in Part II, 2.1.4.

	Concentration (nmol/g wet weight)		
Number of cow	Acetyl-CoA	Total (Acetyl + free) CoA	Acetyl-CoA / (acetyl + free CoA)
13	57.0	59.8	0.95
59	53.9	96.8	0.56
63	58.8	140.8	0.42
86	70.8	80.1	0.88
Mean	60.1	94.4	0.70
S.E.M.	±3.7	±17.2	±0.13

TABLE 11

Acetyl-CoA, total CoA (acetyl-CoA and free CoA) and ratio of acetyl-CoA to total CoA in livers from lactating cows

Lactating cows and tissue preparation were the same as described in Table 9. Assays were performed as described in Part II, 2.1.4.

	Concentration (nmol/g wet weight)		
Number of cow	Acetyl-CoA	Total (Acetyl + free) CoA	Acetyl-CoA / (acetyl + free CoA)
330	80.1	85.6	0.94
340	89.0	87.0	1.02
440	88.4	97.7	0.90
460	87.1	100.7	0.86
Mean	86.1	92.8	0.93
S.E.M.	±2.1	±3.8	±0.03

the ratio of acetyl-CoA/total CoA of 0.70±0.13 in livers from non-lactating cows (Table 10).

2.2.3 3-Hydroxybutyrate concentration in liver from non-lactating and lactating cows

The results in Table 12 show that the mean 3-hydroxybutyrate concentration in liver samples from non-lactating cows was 352±47 nmol/g wet weight. The mean 3-hydroxybutyrate concentration in liver samples from lactating cows was 292±52 nmol/g wet weight (Table 12). Although the 3-hydroxybutyrate concentration was lower in the livers from lactating cows, this difference was not significant ($t_6 = 0.79$; $0.40 < P < 0.50$).

2.2.4 L-glycerol-3-phosphate concentration in liver from non-lactating and lactating cows

The results in Table 13 show that there is an increase in the mean L-glycerol-3-phosphate concentration from 128±31 to 231±64 nmol/g wet weight in livers from non-lactating versus lactating cows respectively. This increase in L-glycerol-3-phosphate concentration is not significant ($t_6 = 1.20$; $0.10 < P < 0.20$). The results in Table 13 also indicate the large variability in L-glycerol-3-phosphate concentration between individual animals that underlies the lack of significant difference between the mean concentrations of the two groups.

2.2.5 Relationship between acetylcarnitine and L-glycerol-3-phosphate concentration in liver from cows

The results in Figure 9 indicate that there is a

TABLE 12

3-hydroxybutyrate concentration in liver from non-lactating and lactating cows

Animals and tissue preparation were the same as described in Tables 8 and 9. Assays were performed as described in Part II, 2.1.4.

	3-hydroxybutyrate concentration (nmol/g wet weight)		
Number of cow	Non-lactating cows	Number of cow	Lactating cows
13	297.9	330	429.7
59	249.5	340	256.2
63	454.6	440	300.8
86	405.2	460	181.8
Mean	352		292
S.E.M.	±47		±52

TABLE 13

L-glycerol-3-phosphate concentration in liver from non-lactating and lactating cows

Animals and tissue preparation were the same as described in Tables 8 and 9. Assays were performed as described in Part II, 2.1.4.

	L-glycerol-3-phosphate concentration (nmol/g wet weight)		
Number of cow	Non-lactating cows	Number of cow	Lactating cows
13	43.1	330	125.3
59	189.0	340	200.3
63	158.7	440	415.4
86	120.9	460	181.8
Mean	128		231
S.E.M.	±31		±64

FIGURE 9

Relationship between acetylcarnitine and L-glycerol-3-phosphate concentrations in cow liver

Acetylcarnitine and 3-phosphoglycerate concentrations were determined in cow liver biopsy samples as described in Part II, 2.1.4. The values shown were obtained from 3 non-lactating and 3 lactating dairy cows. The regression line fitted the equation $y' = 258.1 - 6.93x$ with a correlation coefficient (r^2) of 0.94.

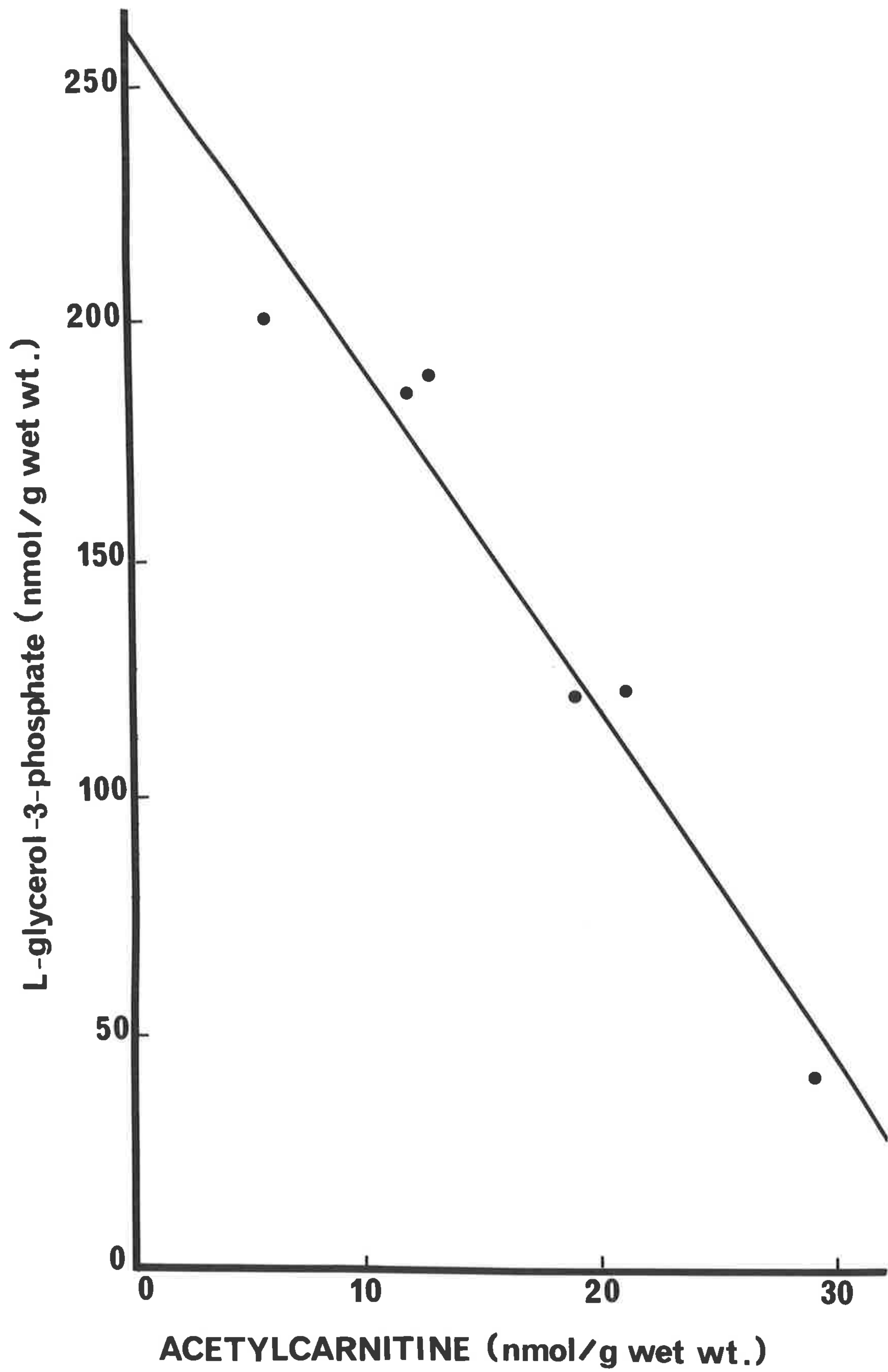


Figure 9

significant negative correlation (P 0.01) between L-glycerol-3-phosphate concentration and acetylcarnitine concentration in liver from these cows. The regression line is expressed by the equation $y = 258.1-0.93x$ with a correlation coefficient (r^2) of 0.94. The liver concentrations of cows 63 and 440 were omitted from this regression for the following reasons. Firstly, there was no statistical significance between the lactating and non-lactating groups for either L-glycerol-3-phosphate or acetylcarnitine, and secondly, these two values contained a standard error of estimate of y on x (Sy.x) of greater than 3. Thus, the results in Figure 9 indicate an inverse relationship between L-glycerol-3-phosphate and acetylcarnitine.

2.2.6 Carnitine acetyltransferase and acetylcarnitine hydrolase activity in liver from non-lactating and lactating cows

The results in Table 14 show that the mean activity for carnitine acetyltransferase in liver homogenates from non-lactating cows was 1.35±0.12 μmol/min per g wet weight. The mean activity for carnitine acetyltransferase in liver homogenates from lactating cows was 2.65±0.32 μmol/min per g wet weight (Table 14). This represents a significant increase ($P < 0.001$) in the activity of carnitine acetyltransferase in liver homogenates from lactating cows.

The results in Table 15 show that the mean activity for acetylcarnitine hydrolase in liver homogenates from non-lactating cows was 0.34±0.04 μmol/min per g wet weight. The mean activity for acetylcarnitine hydrolase in liver homogenates from lactating cows was 0.93±0.04 μmol/min per g wet weight (Table 15). Consistent with the increase in carnitine acetyltransferase activity in liver from lactating versus non-lactating cow, there is also a significant increase ($P < 0.001$) in acetylcarnitine hydrolase activity.

TABLE 14

Carnitine acetyltransferase activity in liver from non-lactating and lactating cows

Homogenates from cow liver were prepared and assayed as described in Part II, 2.1. Results for individual animals are means ± S.E.M. of triplicate assays.

	Carnitine acetyltransferase activity (μmol/min per g wet weight)
Non-lactating cow Number	
13	1.51 ± 0.40
59	1.42 ± 0.08
63	1.50 ± 0.07
86	0.95 ± 0.14
Mean ± S.E.M.	1.35 ± 0.12(12)
Lactating cow Number	
330	3.90 ± 0.19
340	1.89 ± 0.14
440	2.21 ± 0.28
460	2.00 ± 0.12
Mean ± S.E.M.	2.65 ± 0.32(12)*

*($P < 0.001$)

TABLE 15

Acetylcarnitine hydrolase activity in liver homogenates from non-lactating and lactating cows

Homogenates from cow liver were prepared and assayed as described in Part II, 2.1. Results for individual animals are means of determination of acetylcarnitine utilized, carnitine produced and acetate produced.

	Acetylcarnitine hydrolase activity (μmol/min per g wet weight)
Non-lactating cow Number	
13	0.37 ± 0.06
59	0.28 ± 0.05
63	0.22 ± 0.04
86	0.48 ± 0.07
Mean ± S.E.M.	0.34 ± 0.04(12)
Lactating cow Number	
330	1.04 ± 0.09
340	0.82 ± 0.08
440	0.97 ± 0.03
460	0.93 ± 0.03
Mean ± S.E.M.	0.93 ± 0.04(12)*

*($P < 0.001$)

3. Production of endogenous acetate by the liver in lactating ewes

3.1 Materials and methods

3.1.1 Animals

The sheep used were 5-year-old Merino ewes weighing approximately 50 Kg. One ewe was a Dorset x Merino with twin lambs. The animals were chosen so that, at the time of intensive sampling, they were at the height of lactation (see Snoswell and Linzell, 1975). The lambs were left with the ewes to maintain active lactation. The sampling was repeated on one ewe after 8 weeks of lactation. Under these conditions, milk yields after 8 weeks fall to 1.0 l/day compared with 2.9 l/day after 4 weeks lactation (Snoswell and Linzell, 1975). The animals were maintained in pens and fed chaffed lucerne hay *ad libitum*.

3.1.2 Surgical cannulation of blood vessels

Anaesthesia was induced with thiopental sodium (25 mg/Kg body weight) and maintained with Fluothane and oxygen. The sheep was laid on its left side and was clipped and sterilized with nitromersol (5 mg/ml) over the right flank and thorax. Access was gained to the liver via a paracostal incision 3-4 cm behind the last rib and extending from the sternum to the midflank region. The ventral lobe of the liver was retracted from the diaphragm to expose the posterior vena cava where it passes through the diaphragm. The entrance of the hepatic vein to the vena cava could be palpated through the wall of the vena cava and the hepatic vein was followed as it passed centrally down the ventral lobe of the liver. The hepatic vein was cannulated from the visceral surface about 5 cm from the ventral border by making a stab incision with a large bore (No. 13) hyperdermic needle attached to a suitable handle. The bevel of

the needle faced toward the liver surface. A polyvinyl tube (1.5 mm i.d., 2.5 mm o.d. and 60 cm in length) was passed through the lumen of the needle into the vein and the needle withdrawn while keeping the tube in place. Correct positioning of the tube at this stage was indicated by a free flow of blood through the tube. The tube was then passed further into the hepatic vein until it could be palpated within the lumen of the vena cava. The tip was subsequently withdrawn into the hepatic vein until it was 2 cm from the vena cava (as determined by palpation). The tube was then fixed to the surface of the liver with tissue adhesive (methyl α-cyanoacrylate) and exteriorized.

The portal vein was cannulated near its entry to the liver by means of a stab incision, after a purse-string suture had first been placed in a position to stop subsequent haemorrhaging. A similar cannula to that used for the hepatic vein was then passed 4-5 cm towards the liver and fixed in position with tissue adhesive. This cannulae was also exteriorized and both cannulae were attached to the skin with sutures (size 0 braided silk) and taken up onto the back of the sheep for each of sampling. To ensure that the exteriorized cannulae were not displaced by the sheep they were contained in a thick rubber envelope (15 cm x 15 cm).

The operation was completed in 1.5 hours and the sheep returned to the cage. The animals normally took 4-5 days to return to full food intake and then blood sampling commenced.

Two further cannulae were also inserted under local anaesthesia on the morning of the sampling. One cannula was inserted into the femoral artery via the saphenous branch. Access to the vessel was gained via a skin incision on the inside of the hind limb near the pelvic symphysis. The artery was dissected free from the surrounding tissues and ligated. The cannula (1.0 mm i.d., 1.5 mm o.d.) was introduced through an incision and passed for several centimetres up into the deep femoral vessels. The

cannula was tied into place with ligatures, the skin incision closed with metal clips and the cannula taken up behind the leg and tied to the wool on the back of the sheep. A further cannula (polyvinyl tube 1.0 mm i.d., 1.5 mm o.d.) was also inserted through a 13-gauge needle into the superficial mammary vein, which was clearly visible on the abdomen anterior to the udder. The cannula was fixed in place with metal clips. All cannulae were kept patent by flushing with heparin-saline mixture (100 i.u. heparin/ml).

3.1.3 Measurement of hepatic blood flow

Total hepatic venous blood flow through the liver was assessed using the bromosulphophthalein (BSP) clearance technique as described by Shoemaker (1964) and Katz and Bergman (1969*b*). A priming dose of 100 mg BSP was administered intravenously, followed by a constant infusion of 10 mg/min into the jugular vein over a period of 2.5 hours. Thirty minutes were allowed for equilibration of the dye with the blood, after which simultaneous portal and hepatic venous samples (5 ml) were withdrawn into heparinized syringes every 20 minutes for the following 2 hours. BSP concentrations were determined on plasmas which were prepared on the same day. Packed cell volumes were determined on the blood samples and a sample of hepatic venous blood was drawn prior to commencing the infusion, as an analytical blank. Plasma BSP concentrations were determined by the method of Varley (1963).

Blood flow determinations were made midway through the experimental blood sampling periods, on either one or two occasions, depending on the length of the sampling period. In all blood flow determinations (with one exception), blood levels of BSP were constant over the period of analysis. There was no evidence of changing packed cell volumes, as might be expected if excessive removal of blood was occurring. Arterial samples

drawn together with portal venous samples were shown to contain equivalent BSP concentrations.

3.1.4 Tissue preparations

The sheep were killed by severing the necks, and samples of liver were immediately freeze-clamped with aluminium-faced tongs previously cooled in liquid nitrogen (Wollenberger *et al.*, 1960). The perchloric acid extracts of frozen tissue were prepared as described by Snoswell and Henderson (1970). Fresh liver samples were collected into cold 0.25 M sucrose-23 mM potassium phosphate (pH 7.4) both from biopsy sampling at time of operation and at post-mortem. Mammary tissue was also collected in the same buffer at post-mortem.

3.1.5 Blood samples

Blood samples for metabolite assays were drawn from all four cannulae (mammary venous, femoral arterial, portal and hepatic venous) simultaneously at 0.5 or 1 hour intervals for 12 samples. Blood fractions of 2.0 ml were immediately added to 2.0 ml of 15% perchloric acid. In addition 1.0 ml fractions were added to a mixture of 20 ml chloroform plus 6.625 ml of 0.1 M sodium phosphate buffer (pH 6.2) for the determination of free fatty acids.

3.1.6 Subcellular fractionation of mammary gland tissue

The preparation of subcellular fractions from mammary gland tissue, like heart or muscle tissue, was much more difficult than the subcellular fractionation of tissue such as liver (see Snoswell and Koundakjian, 1972) since such direct preparation of subcellular fractions was not possible due to extensive loss of the mitochondrial-matrix

marker enzyme, citrate synthase, into the cytosol fractions. Thus, 'press fractions' of mammary tissue were prepared by the method described by Snoswell and Koundakjian (1972) for heart tissue.

3.1.7 Instrumentation

The instrumentation for the enzyme and metabolite assays was as described in Part I, 2.1.6, except for free fatty acid assays, which were determined in the Auto Analyzer (Technicon Instruments Corporation Ardsley, N.Y., U.S.A.).

3.1.8 Enzyme assays

3.1.8.1 Carnitine acetyltransferase

This enzyme activity was assayed in the supernatant fractions obtained by centrifuging at 8,000 *g* for 3 minutes frozen and thawed homogenates (20%, w/v) of liver prepared in hypotonic 0.025 M sucrose containing 2.3 mM potassium phosphate (pH 7.4) and 0.1% Triton X-100. The enzyme activity was also measured in mammary gland tissue prepared as for the liver homogenates and in homogenates (20%, w/v) prepared in 0.25 M sucrose containing 23 mM potassium phosphate (pH 7.4). The reaction mixture and mode of assay were as described in Part I, 2.1.7.1.

3.1.8.2 Carnitine palmitoyltransferase

This enzyme activity was measured in homogenates of liver and mammary gland tissue prepared as described for the carnitine acetyltransferase assay. The reaction mixture consisted of 200 mM Tris-HCl (pH 8.2), 100 μM palmitoyl-CoA, 330 μM L-carnitine, 100 μM

5,5'-dithiobis(2-nitrobenzoic acid) and tissue homogenate (2 mg wet weight of tissue) in a total volume of 1.0 ml. The reaction was monitored spectrophotometrically by the increase in absorbance at 412 nm due to the yellow 5-thio-2-nitrobenzoic acid anion. There was an increase in absorbance with time in the absence of L-carnitine, due to the palmitoyl-CoA hydrolase reaction. The reaction assay was started by additon of L-carnitine. The difference in the two rates of absorbance increase at 412 nm was the truecarnitine palmitoyltransferase activity.

3.1.8.3 Enzymic capacity to produce acetate from acetyl-CoA

The acetate production capacity was assayed in homogenates of liver prepared in 0.025 M sucrose containing 2.3 mM potassium phosphate (pH 7.4) and 0.1% Triton X-100. The results from Part I, 3.2 showed that acetyl-CoA hydrolase activity is really due to the coupled activity of carnitine acetyltransferase and acetylcarnitine hydrolase. Thus, the acetyl-CoA hydrolase activity is termed acetate production capacity via the coupled enzyme system. The assay system used to measure the coupled enzyme system contained, in a final volume of 1.0 ml, 400 mM Tris-HCl (pH 8.0), 100 μM 5,5'-dithiobis(2-nitrobenzoic acid), and 2 mg wet weight of liver homogenate. The assay was monitroed spectrophotometrically by the increase in absorbance at 412 nm after addition of 100 μM acetyl-CoA. This spectrophotometric assay gave equivalent results to the incubation assay system described in Part I, 3.1.5.3 and proved more convenient to use.

3.1.8.4 Acetylcarnitine hydrolase

The acetylcarnitine hydrolase activity was assayed in homogenates of mammary gland tissue prepared in 0.25 M sucrose

containing 23 mM potassium phosphate (pH 7.4). The assay system used was as described in Part I, 2.1.7.2.

3.1.8.5 Acetate thiokinase

The acetate thiokinase activity was assayed in the supernatant fractions (obtained by centrifuging at 8,000 *g* for 3 minutes) from liver and mammary gland homogenates prepared both in hypotonic 0.025 M sucrose containing 2.3 mM potassium phosphate (pH 7.4) and 0.1% Triton X-100 and in normal 0.25 M sucrose containing 23 mM potassium phosphate (pH 7.4) and 'press fractions' from the mammary gland. The assay mixture contained 45 mM Tris-HCl buffer (pH 8.2), 100 mM potassium acetate, 1 mM magnesium chloride, 40 mM DL-malate, 0.3 mM CoA, 0.5 mM NAD^+, 10 mM glutathione, 5 µl malate dehydrogenase (5,500 u/ml), 5 µl citrate synthase (220 u/ml) and 4 mg fresh weight of tissue, in a final volume of 1.0 ml. The assay mixture was preincubated for 10 minutes at 37°C and the assay monitored spectrophotometrically at 340 nm. The reaction was started by the addition of 10 mM ATP.

3.1.8.6 Citrate synthase

The citrate synthase activity was measured in the same 'press fractions' as described for the acetate thiokinase activity. The assay was monitored spectrophotometrically at 412 nm by the method described by Shepherd and Garland (1969).

3.1.9 Metabolite assays

The perchloric acid extracts of blood and frozen tissue samples were adjusted to (pH 6.5) with 3 M KOH and then centrifuged at 5°C to remove the potassium perchlorate.

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

Free fatty acids were estimated in the chloroform extracts of the blood samples according to the method described by Dalton and Kowalski (1967).

Acetylcarnitine was measured in the neutralized extracts of blood and liver by the method of Pearson and Tubbs (1964).

Free carnitine was measured in the neutralized extracts of blood and liver by the method of Marquis and Fritz (1964).

Alanine was measured in neutralized blood extracts. The assay system contained 20 mM sodium carbonate (pH 10.0), 5 mM NAD^+, 25 µg alanine dehydrogenase, and neutralized extract, in a final volume of 1.0 ml. Reduction of NAD^+ was monitored spectrophotometrically at 340 nm after additon of enzyme.

3.1.10 Calculation of results

Acetate production rates were determined by the method of Bergman and Wolff (1971) using the following equations:

Net appearance in portal blood in mmol/hr

$$= F_{pv}(C_{pv} - Ca) \qquad (1)$$

Net hepatic production in mmol/hr

$$= F_{pv}(C_{hv} - C_{pv}) + Fa(C_{hv} - Ca) \qquad (2)$$

% net hepatic production per circulation (or production ratio)

$$= 100(\text{net hepatic production})/(FaCa + F_{pv}C_{pv}) \quad (3)$$

Net hepatic artery production in mmol/hr

$$= Fa(C_{pv} - Ca) \quad (4)$$

Actual liver production in mmol/hr

= Net hepatic production — net hepatic artery production

$$= F_{pv}C_{hv} - F_{hv}C_{pv} \quad (5)$$

Where F_{pv}, F_{hv} and Fa are the rates of whole blood flow (litres/hr) for the portal vein, hepatic vein and hepatic artery respectively. C_{pv}, C_{hv} and Ca are the concentrations of acetate (mM) in the portal vein, hepatic vein and hepatic artery vessels respectively.

3.1.11 Chemicals

L-carnitine chloride and *O*-acetyl-L-carnitine were generously supplied by Otsuka Pharmaceuticals, Osaka, Japan. CoA and palmitoyl-CoA were obtained from P-L Biochemicals Inc., Milwaukee, Wisonsin, U.S.A., and enzymes from C.F. Boehringer und Soehne GmbH, Mannheim, Germany. Heparin was obtained from Evans Medical Australia Pty. Ltd., Boronia, Vic., Australia. Acetyl-CoA was prepared from free CoA and redistilled acetic anhydride by the method of Stadtman (1957). All other chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

3.2 Results

3.2.1 Acetate concentrations in arterial and in portal and hepatic venous blood from lactating ewes

The results in Figures 10 and 11 show the blood acetate

FIGURE 10

Half-hourly acetate concentrations in arterial, portal and hepatic venous blood of ewe number 4 at 4 weeks lactation

Acetate was estimated in duplicate as described in Part II, 3.1.9. The twin lambs of the lactating ewe were permitted to suckle at will during the sampling period. The ewe was at 4 weeks lactation.

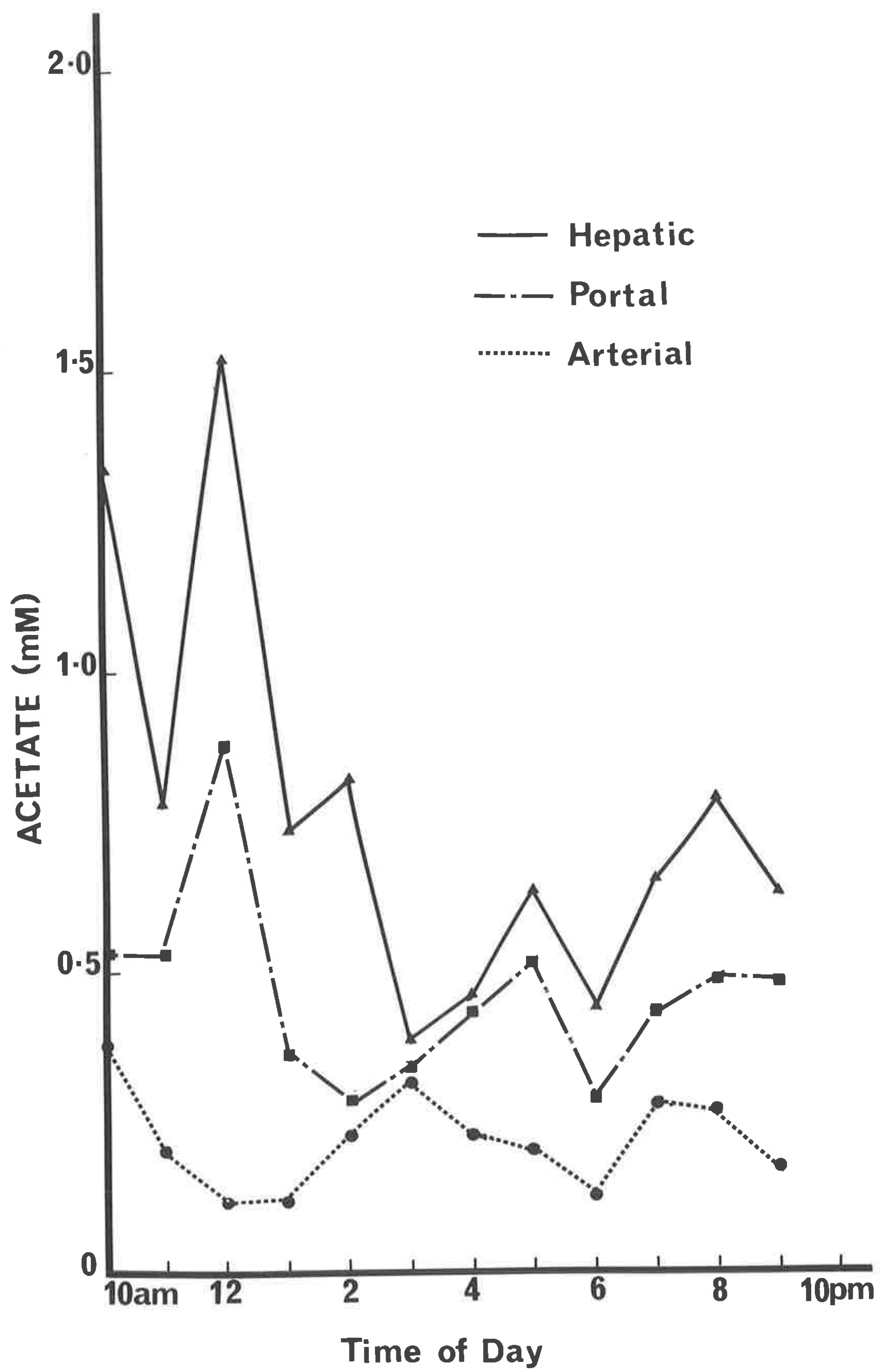


Figure 10

FIGURE 11

Half-hourly differences in acetate concentration of portal and arterial, and of hepatic venous and portal blood in ewe number 4 at 4 weeks lactation

Acetate concentrations and conditions during the sampling period were as described in Figure 10.

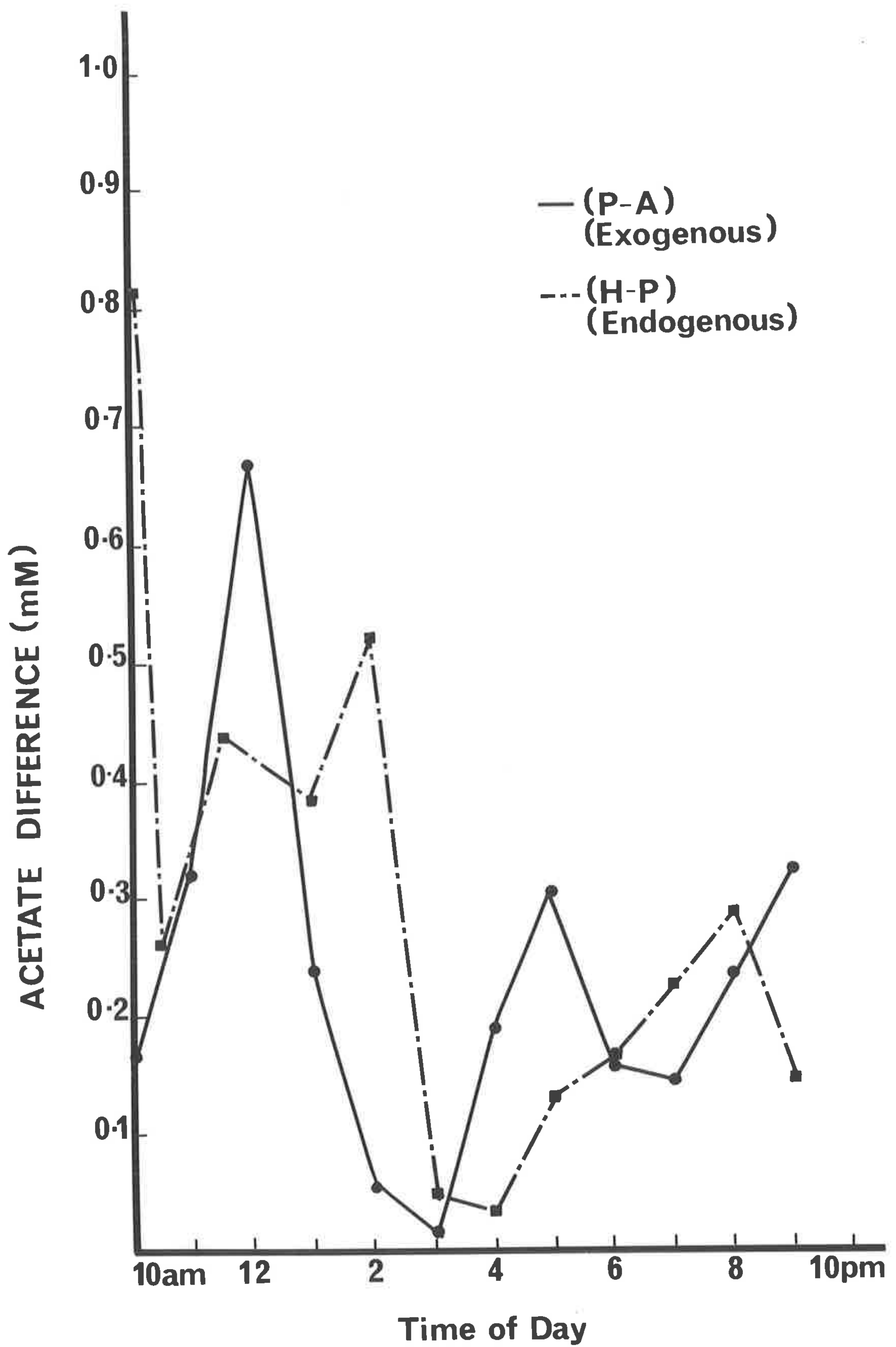


Figure 11

TABLE 16

Acetate concentrations in arterial, portal and hepatic venous blood of lactating ewes

Blood samples were removed simultaneously from indwelling arterial, portal and hepatic venous cannulae, generally at hourly intervals for 12 hours. The four ewes were sampled at approximately 4 weeks after the onset of lactation and for ewe number 4 the sampling was repeated after 8 weeks of lactation. 2 ml fractions of blood were added to 2 ml of 15% (w/v) perchloric acid and acetate estimated in duplicate as described in Part II, 3.1., on each neutralized perchloric extract. The figures shown are means ± S.E.M. with the number of blood samples assayed in brackets. The significance of the differences between hepatic-portal and portal-arterial samples, as determined by paired t-test, is also indicated.

	Blood acetate concentration (μmol/ml)				
Ewe	Hepatic(H)	Portal(P)	Arterial(A)	H - P	P - A
1	2.70 ± 0.18(6)	1.82 ± 0.19(6)	0.95 ± 0.17(6)	0.88 ± 0.24(6) $P < 0.01$	0.88 ± 0.17(6) $P < 0.01$
2	1.08 ± 0.08(6)	0.66 ± 0.11(6)	0.20 ± 0.04(6)	0.42 ± 0.06(6) $P < 0.01$	0.46 ± 0.05(6) $P < 0.05$
3	1.31 ± 0.13(11)	1.05 ± 0.13(11)	0.21 ± 0.05(11)	0.26 ± 0.06(11) $P < 0.01$	0.82 ± 0.16(11) $P < 0.001$
4a	0.76 ± 0.10(12)	0.46 ± 0.05(12)	0.22 ± 0.02(12)	0.29 ± 0.06(12) $P < 0.01$	0.23 ± 0.05(12) $P < 0.001$
Mean	1.46	1.00	0.40	0.46	0.60
4b	0.46 ± 0.02(12)	0.40 ± 0.03(12)	0.24 ± 0.02(12)	0.07 ± 0.02(12) $P < 0.001$	0.16 ± 0.03(12) $P < 0.001$

4a - sampled after 4 weeks lactation
4b - the same ewe resampled after 8 weeks lactation

concentrations and net differences in the same ewe at 4 weeks lactation respectively. These results clearly demonstrate the variability in acetate concentration of the arterial, portal and hepatic venous blood. Thus, for animals fed once a day, a number of samplings over an extended time period (e.g. 12-24 hours) would be required to obtain a pattern of acetate concentrations.

The results in Figures 12a and b, again from the same ewe but at 8 weeks lactation to allow comparison within the same animal, show that over the time period indicated there is always a net output of acetate, as judged on the basis of (H-P) acetate concentration, from the liver and at no time during the sampling period did the liver appear to take-up acetate.

The results in Table 16 show the concentration of acetate in the portal, hepatic venous and arterial blood of four ewes. As with acetate concentrations within an animal shown in Figures 10 and 11, these results show considerable variation in the concentration of acetate between these four ewes. However, in each animal there is a significant ($P < 0.01$) mean production of acetate by the liver of 0.46 μmol/ml as judged by hepatic-portal differences in blood acetate. Also, as would be expected, there is a significant ($P < 0.001$) mean contribution from the alimentary tract of 0.60 μmol/ml as judged by portal-arterial differences in blood acetate.

The results in Table 16 also show that the mean hepatic-portal (endogenous) difference in acetate concentration is 43% of the mean total (hepatic-portal) and (portal-arterial) difference of acetate concentration. The results from ewe number 4 (Table 16) show that the hepatic-portal difference in acetate concentration decreased significantly ($P < 0.001$) from 56%, at four weeks lactation, to 30% at eight weeks lactation, of the total difference in acetate concentration.

3.2.2 Free fatty acid concentration in portal and hepatic venous blood from lactating ewes

The portal blood concentration of free fatty acids exceeded that in the hepatic venous blood (Table 17). In three of the five samplings from the 4 ewes, the portal-hepatic difference (i.e. uptake of free fatty acid by the liver) was significant. The mean portal-hepatic difference for the 4 ewes was 81 nmol/ml. The portal and hepatic venous blood concentrations of free fatty acids were significantly less ($P < 0.01$ and $P < 0.05$ respectively) for ewe Number 4 at 8 weeks lactation than those determined at 4 weeks lactation (Table 17). The free fatty acid concentrations are based on the use of palmitic acid as a standard, so that the uptake of fatty acid from the blood across the liver is equivalent to 8 x 81 or 648 nmol/ml potential 2-carbon units. Thus, acetate produced by the liver, viz. 460 nmol/ml (Table 16), represents 70% of the 2-carbon potential of the free fatty acids taken up by the liver. The portal-hepatic difference in the concentration of free fatty acids in the blood varied considerably over the total sampling periods as was the case with acetate concentrations (Table 16). Indeed it was found essential to take a considerable number of blood samples over an extended period to obtain statistically significant results.

3.2.3 The relationship between portal-hepatic venous differences in blood free fatty acid and hepatic venous-portal differences in blood acetate

The relationships between time of sampling and (portal-hepatic venous) difference in blood free fatty acid and (hepatic venous-portal) difference in blood acetate are shown in Figures 13 and 14. These results are taken from ewe Number 4 where after 4 weeks lactation the mean

FIGURE 12

12(a) *Half-hourly acetate concentrations in arterial, portal and hepatic venous blood of ewe number 4 at 8 weeks lactation*

Acetate was estimated in blood samples in duplicate as described in Part II, 3.1.9. The twin lambs were permitted to suckle at will during the sampling period.

12(b) *Half-hourly differences in acetate concentrations of portal and arterial, and of hepatic venous and portal blood in ewe number 4 at 8 weeks lactation.*

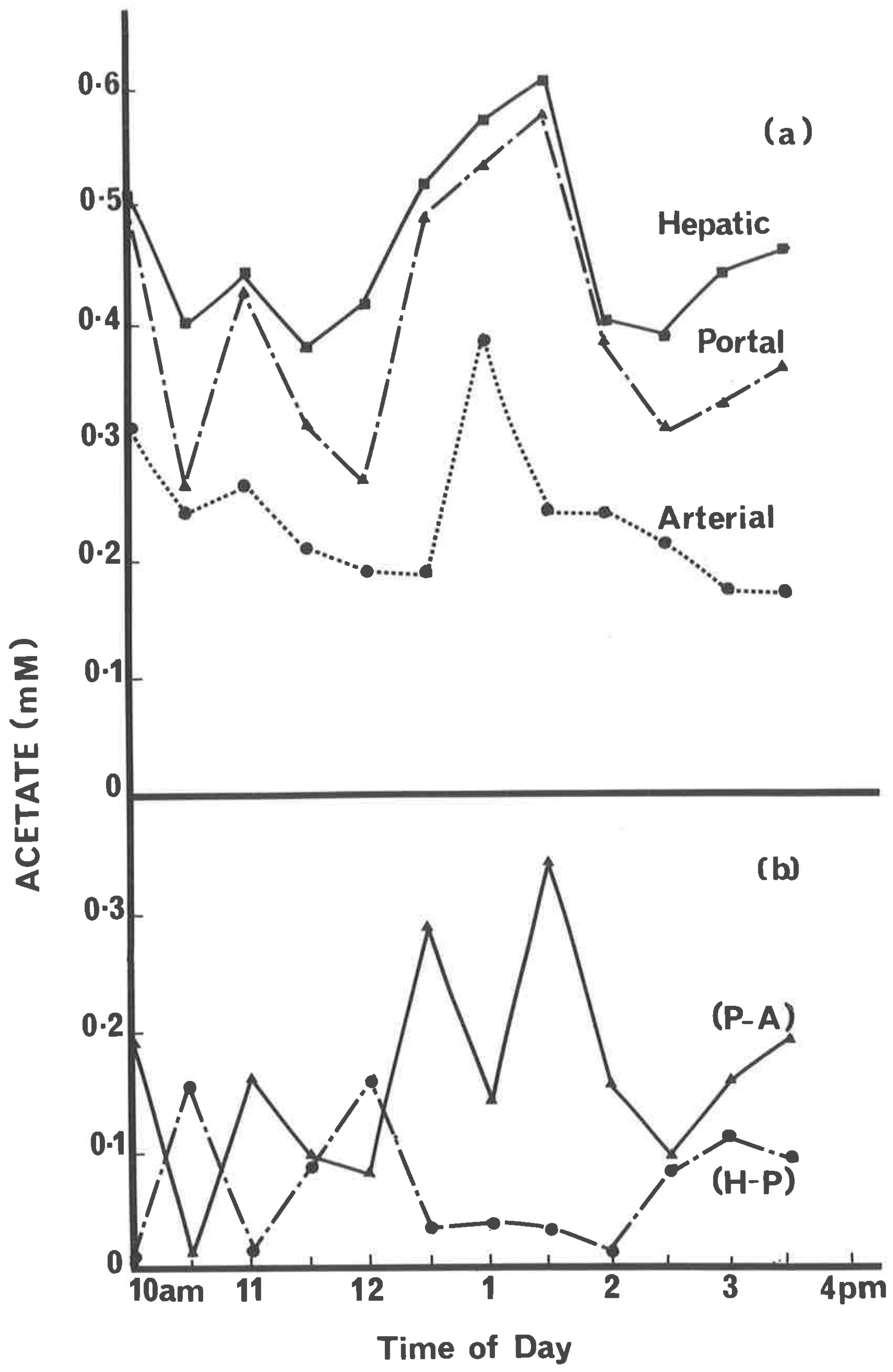


Figure 12

TABLE 17

Free fatty acid concentrations in hepatic and portal venous blood of lactating ewes

The same blood samples described in Table 1 were used. 1.0 ml fractions of blood were added to 20 ml chloroform plus 6.63 ml 0.01 M sodium phosphate buffer (pH 7.4). Free fatty acids were determined in the chloroform extracts as described in Part II, 3.1. The results are the means S.E.M. with the number of blood samples in brackets. The significance of the differences between portal and hepatic concentrations were determined by paired t-test.

Free fatty acid concentration in blood (nmol/ml)				
Ewe	Hepatic(H)	Portal(P)	P - H	
1	251 ± 51(4)	314 ± 67(4)	63 ± 33(4)	N.S.
2	251 ± 29(9)	365 ± 66(9)	114 ± 41(9)	$P < 0.05$
3	346 ± 53(10)	403 ± 73(10)	57 ± 24(10)	N.S.*
4a	448 ± 16(10)	535 ± 25(10)	87 ± 26(10)	$P < 0.01$
Mean	324	404	81	
4b	365 ± 24(11)	394 ± 20(11)	29 ± 8(11)	$P < 0.01$

4a - sampled after 4 weeks lactation
4b - the same ewe resampled after 8 weeks lactation
*N.S. - not significant

FIGURE 13

Half-hourly differences in hepatic venous and portal blood acetate concentrations and portal and hepatic venous blood free fatty acid concentrations in ewe number 4 at 4 weeks lactation

Acetate and free fatty acids were estimated in duplicate in portal and hepatic venous blood samples as described in Part II, 3.1.9.

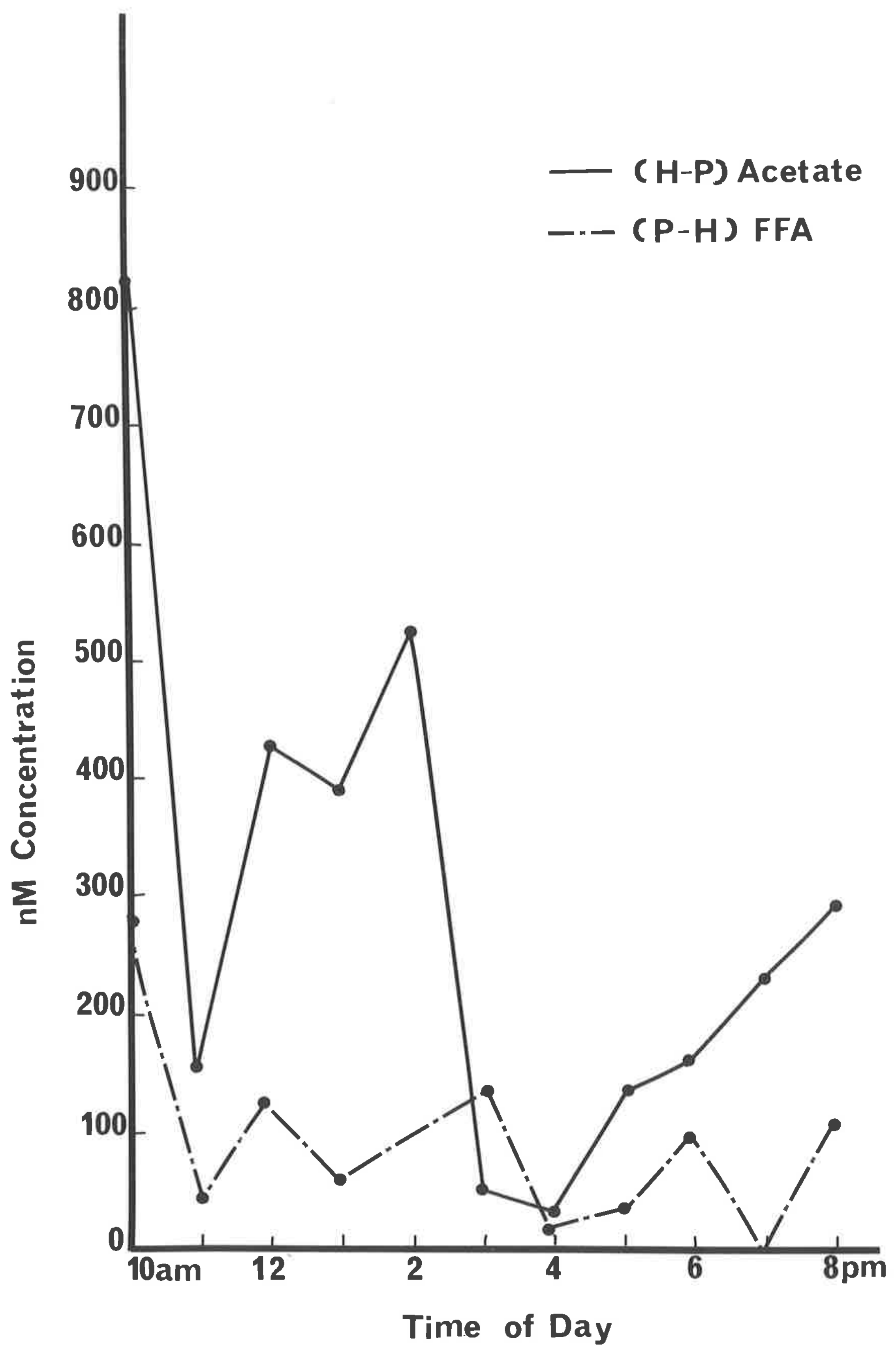


Figure 13

FIGURE 14

Half-hourly differences in hepatic venous and portal blood acetate concentrations and portal and hepatic venous blood free fatty acid concentrations in ewe number 4 at 8 weeks lactation

Acetate and free fatty acids were estimated in duplicate in portal and hepatic venous blood samples as described in Part II, 3.1.9.

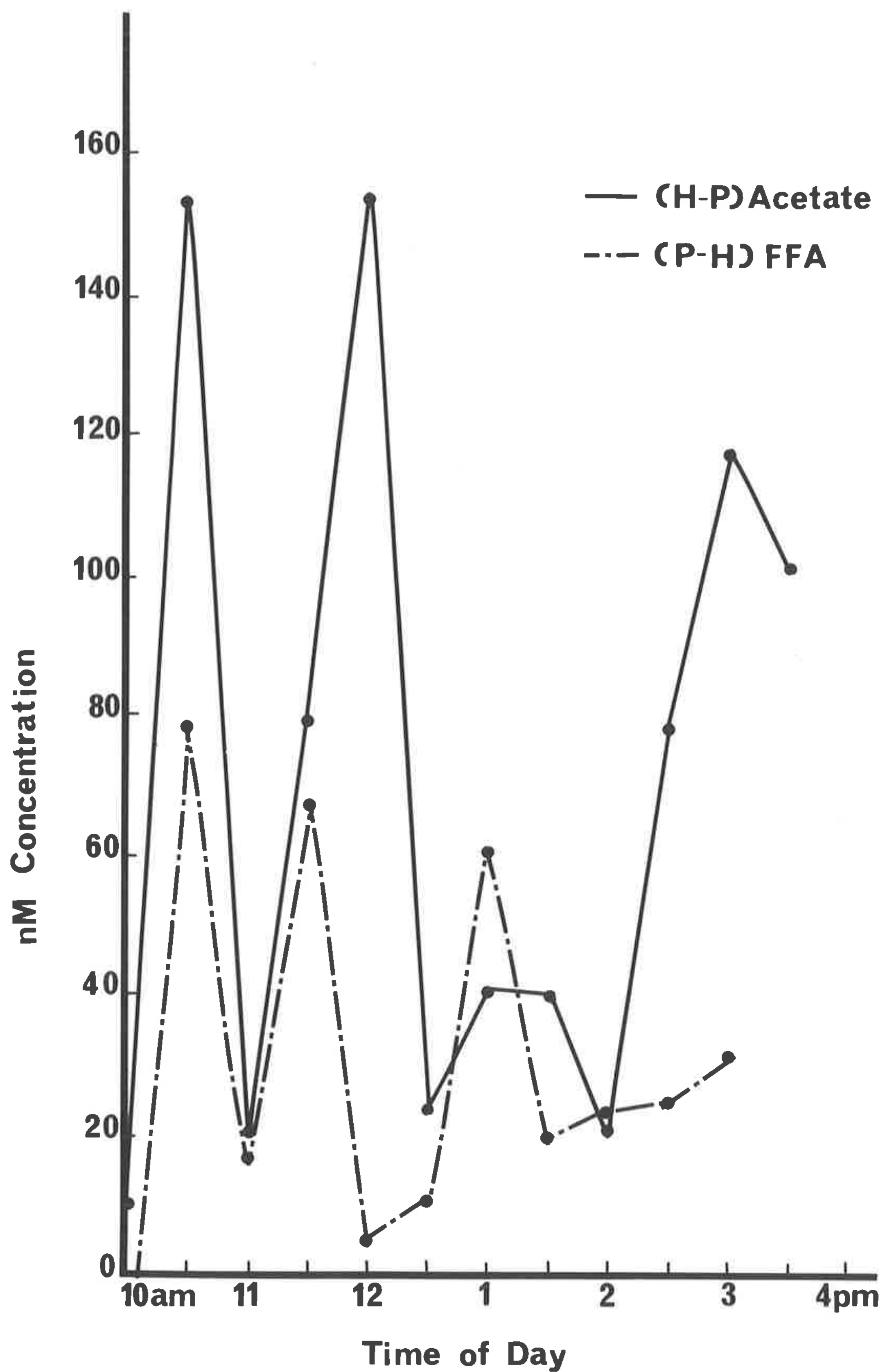


Figure 14

portal-hepatic difference in free fatty acid concentration was 87 nmol/ml while the hepatic venous-portal difference in acetate concentration was 0.29 μmol/ml, but after 8 weeks lactation these differences had fallen significantly ($P < 0.01$) to 29 nmol/ml and 0.07 μmol/ml respectively.

The results shown in Figure 15 indicate a significant correlation ($P < 0.01$) between the (portal-hepatic venous) difference for free fatty acids and (hepatic-portal) difference in blood acetate concentrations with a correlation coefficient (r^2) of 0.83 after 4 weeks of lactation. At the later stage of 8 weeks lactation there was no significant correlation between free fatty acid uptake and acetate production (coefficient of correlation (r^2) of 0.41).

3.2.4 Concentration of alanine, acetylcarnitine and free carnitine in portal and hepatic venous blood

The concentration of L-alanine in portal blood was 32.2±2.7 nmol/ml, and in hepatic venous blood 16.4±4.3 nmol/ml. This represents a significant ($P < 0.05$) portal-hepatic venous difference (or hepatic uptake) of 15.8±1.6 nmol/ml.

There was no detectable acetylcarnitine in portal or hepatic venous blood.

The concentration of free carnitine in portal blood was 33.0±2.9 nmol/ml and in hepatic venous blood, 25.4±5.0 nmol/ml. This difference in concentration for free carnitine was not significant.

3.2.5 The activity of carnitine acetyltransferase, carnitine palmitoyltransferase and enzymic conversion of acetyl-CoA to acetate in liver homogenates

The activity of carnitine acetyltransferase in liver

FIGURE 15

The relationship between hepatic venous - portal differences in blood acetate concentrations and portal - hepatic venous differences in blood free fatty acid concentrations

The acetate and free fatty acids were determined in duplicate in hepatic venous and portal blood samples taken from ewe number 4 at 4 weeks lactation. The regression line was expressed by the equation $y = 26.52 + 0.25x$ with a correlation coefficient (r^2) of 0.83.

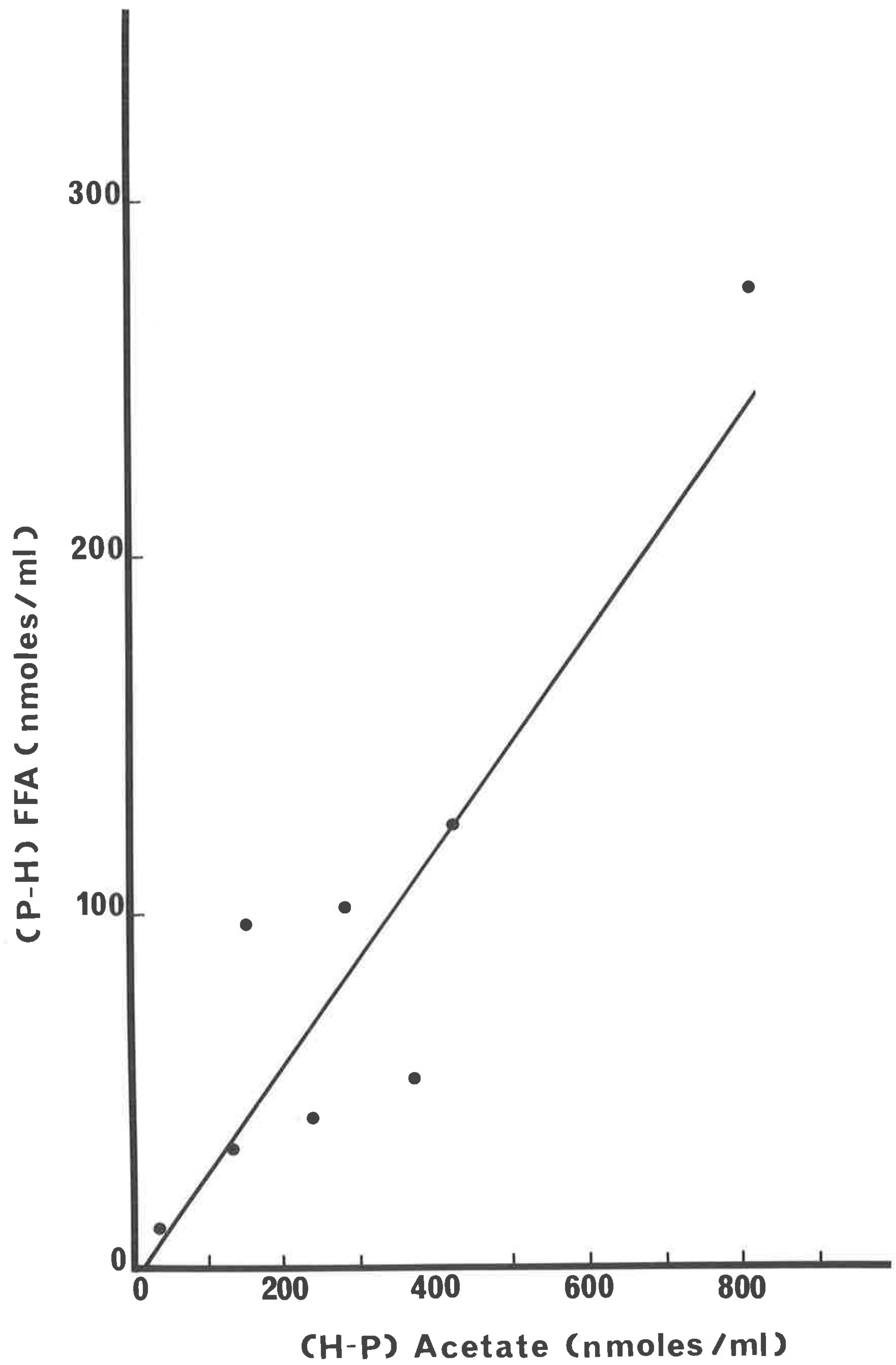


Figure 15

homogenates from these lactating ewes was 2.2 μmol/min per g wet weight (Table 18), which is comparable to that in normal wethers as reported previously (Snoswell and Koundakjian, 1972). The activity of carnitine palmitoyltransferase was 4.1 μmol/min per g wet weight (Table 18). In contrast to the carnitine acetyltransferase activity, the CPT is much greater than that reported previously for wethers (Snoswell and Henderson, 1970).

The mean enzymic activity for conversion of acetyl-CoA to acetate in these liver homogenates was 1.1 μmol/min per g wet weight (Table 18). This activity represents the capacity of the carnitine acetyltransferase and acetylcarnitine hydrolase reactions to produce acetate from acetyl-CoA (see Part II, 3.1.8.3).

There was no detectable acetate thiokinase activity in these liver homogenates.

The concentrations of free carnitine and acetylcarnitine in freeze-clamped liver samples from these lactating ewes were 117±25 and 95±31 nmol/g wet weight respectively (means±S.E.M. for six samples). The concentration of acetylcarnitine was much greater than that reported by Snoswell and Koundakjian (1972) for normal wethers fed on a similar diet.

3.2.6 Comparison of the actual *in vivo* production rate of acetate by the liver with the *in vitro* enzymic capacity of the liver to convert acetyl-CoA to acetate as determined on biopsy samples from lactating ewes

The mean total enzymic capacity of the liver to produce acetate from acetyl-CoA for the 4 ewes at 4 weeks lactation, calculated by the product of the *in vitro* enzymic capacity and the total liver weight, was 56.4 mmol/hr (Table 19). The ewe at 8 weeks lactation showed an

TABLE 18

The activity of carnitine acetyltransferase, carnitine palmitoyltransferase and enzymic conversion of acetyl-CoA to acetate in liver biopsies from lactating ewes

Liver biopsy samples were removed at the time of surgical insertion of the cannulae and again at autopsy. The activities shown are the means of assays on these two samples. Activities were measured as described in Part II, 3.1., on the supernatant fractions obtained by centrifuging (8,000 *g* for 3 min) frozen and thawed homogenates (1 in 5 w/v) of liver prepared in 0.025 M sucrose contain 23 mM potassium phosphate (pH 7.4) and 0.1% Triton X-100. The mean values ± S.E.M. are those for all assays on 4 ewes. The number of assays is shown in brackets.

	Liver enzyme activities (μmol/min/g wet tissue)		
Ewe	Carnitine acetyltransferase	Carnitine palmitoyltransferase	Acetate production from acetyl-CoA
1	2.1	4.3	0.7
2	3.0	4.2	1.2
3	2.4	5.3	1.5
4a	1.5	2.2	1.3
4b	2.4	4.2	1.1
Mean	2.2 ± 0.23(10)	4.1 ± 0.33(8)	1.1 ± 0.14(10)

4a - sampled after 4 weeks lactation
4b - the same ewe resampled after 8 weeks lactation

TABLE 19

In vitro enzymic capacity of the liver to convert acetyl-CoA to acetate as determined on biopsy samples from lactating ewes

Liver samples were collected and assayed as described in Table 18.

Ewe	Enzymic acetate production rate (μmol/min per g wet weight)	Total liver wet weight (g)	Total enzymic acetate production capacity (mmol/hr)
1	0.7	914	38.4
2	1.2	840	60.5
3	1.5	818	76.8
4a	1.3	620*	48.6
Mean	1.2	798	56.4
4b	1.1	620	40.8

4a - sampled after 4 weeks lactation
4b - the same ewe resampled after 8 weeks lactation
*liver weight after 8 weeks lactation used, at 4 weeks the weight may have been greater

in vitro enzymic capacity to produce acetate of 40.8 mmol/hr. The liver weight determined at 8 weeks of lactation was substantially less than that determined at 4 weeks lactation viz. 620 g as against 857 g respectively.

Hepatic venous blood flow averaged 1.74 l/min (Table 20). This rate is not significantly different from the rates previously reported by Bergman and Wolff (1971). Portal venous whole blood flow, calculated at 90% of hepatic venous flow (see Bergman and Wolff, 1971), averaged 1.57 l/min (Table 20). Hepatic arterial blood flow was calculated as the difference between hepatic venous and portal venous blood flow and as such averaged 0.17 l/min.

Mean net appearance of acetate in portal blood (or acetate produced from the alimentary tract) was 56.5 mmol/hr for ewes at 4 weeks lactation (Table 21). After 8 weeks lactation the net appearance of acetate in portal blood was 15.0 mmol/hr. Mean net hepatic production of acetate at 4 weeks lactation was 54.0 mmol/hr (Table 21). This acetate production rate is a measure of both the liver enzymic production and the contribution of the hepatic artery. The mean production ratio (or % net hepatic production per circulation) of acetate was 54.9% (Table 21). The mean hepatic artery production can be calculated to be 6.1 mmol/hr (Table 21). By difference the mean liver enzymic production of acetate was 47.9 mmol/hr of the mean net hepatic production of 54.0 mmol/hr (Table 21).

Since net hepatic production of acetate represents total endogenous production (hepatic artery and liver enzymic input) and net appearance in the portal blood total exogenous production (alimentary tract input) the mean total acetate production was 110.5 mmol/hr for the ewes at 4 weeks lactation (from Table 21).

The ewes at 4 weeks lactation had a mean total liver enzymic acetate

TABLE 20

Whole blood flow through the liver of lactating ewes

Hepatic venous blood flows are the means of two determinations measured by BSP extraction as described in Part II, 3.1.3. Portal venous blood flow was calculated at 90% of hepatic venous blood flow and hepatic arterial blood flow by difference.

Ewe	Hepatic venous blood flow (l/min)	Portal venous blood flow (l/min)	Hepatic artery blood flow (l/min)
1	1.35	1.22	0.13
2	1.74	1.57	0.17
3	2.06	1.85	0.21
4a	1.81	1.63	0.18
Mean	1.74	1.57	0.17
4b	1.73	1.56	0.17

4a - sampled after 4 weeks lactation
4b - the same ewe sampled after 8 weeks lactation

TABLE 21

In vivo production of acetate in lactating ewes

These values were calculated as described in Part II, 3.1.10.

Ewe	Net appearance in portal blood (mmol/hr)	Net hepatic production (mmol/hr)	Hepatic artery production (mmol/hr)	Actual liver production (mmol/hr)	Production ratio (%)
1	64.4	78.4	6.9	71.5	55.6
2	43.3	48.8	1.6	47.2	78.8
3	91.0	41.3	10.3	31.0	34.7
4a	22.5	34.2	2.5	31.7	72.2
Mean	56.5	54.0	6.1	47.9	54.9
4b	15.0	9.0	1.6	7.4	22.5

4a - sampled after 4 weeks lactation   4b - the same ewe sampled after 8 weeks lactation

production capacity of 56.4 mmol/hr (Table 19) and had an actual liver acetate production rate of 47.9 mmol/hr (Table 21). The total enzymic acetate production capacity of ewe Number 4 did not decrease markedly at 8 weeks lactation (40.8 mmol/hr) as compared to 4 weeks lactation (48.6 mmol/hr) (Table 19). However, both the actual liver production of acetate decreased markedly from 31.7 mmol/hr to 7.4 mmol/hr at 4 and 8 weeks lactation respectively (Table 21) and the production ratio decreased from 72.2% to 22.5% at 4 and 8 weeks respectively (Table 21).

3.2.7 Acetate concentration in arterial and mammary venous blood of lactating ewes

The results in Figures 16 and 17 show the acetate concentrations of arterial and mammary venous blood in ewe Number 4 at 4 and 8 weeks lactation respectively. Increases in acetate concentrations in both arterial and mammary venous blood after suckling are shown in Figure 16a. At both 4 and 8 weeks lactation there was a constant uptake of acetate as shown in Figures 16b and 17b respectively.

The results in Table 22 show that there is a substantial and significant ($P < 0.01$) uptake of acetate by the mammary gland of ewes at 4 weeks lactation. The percentage extraction of acetate per circulation, calculated by the equation $(A - V)/A \times 100$ for ewes at 4 weeks lactation, was in the narrow range of 45-49% with a mean extraction of 47±1%. At 8 weeks lactation the percentage extraction of acetate by the mammary gland of ewe Number 4 had risen significantly ($P < 0.01$) to 74%.

The results in Figure 18 indicate a significant correlation ($P < 0.001$) between the hepatic venous-mammary venous difference and the hepatic venous-arterial difference in blood acetate concentrations, with a correlation coefficient (r^2) of 0.97.

FIGURE 16

16(a) *Half-hourly acetate concentrations in arterial and mammary venous blood in ewe number 4 at 4 weeks lactation*

Acetate was estimated in duplicate as described in Part II, 3.1.9. ↓ indicates when lambs suckled.

16(b) *Half-hourly differences in acetate concentrations of arterial - mammary venous blood.*

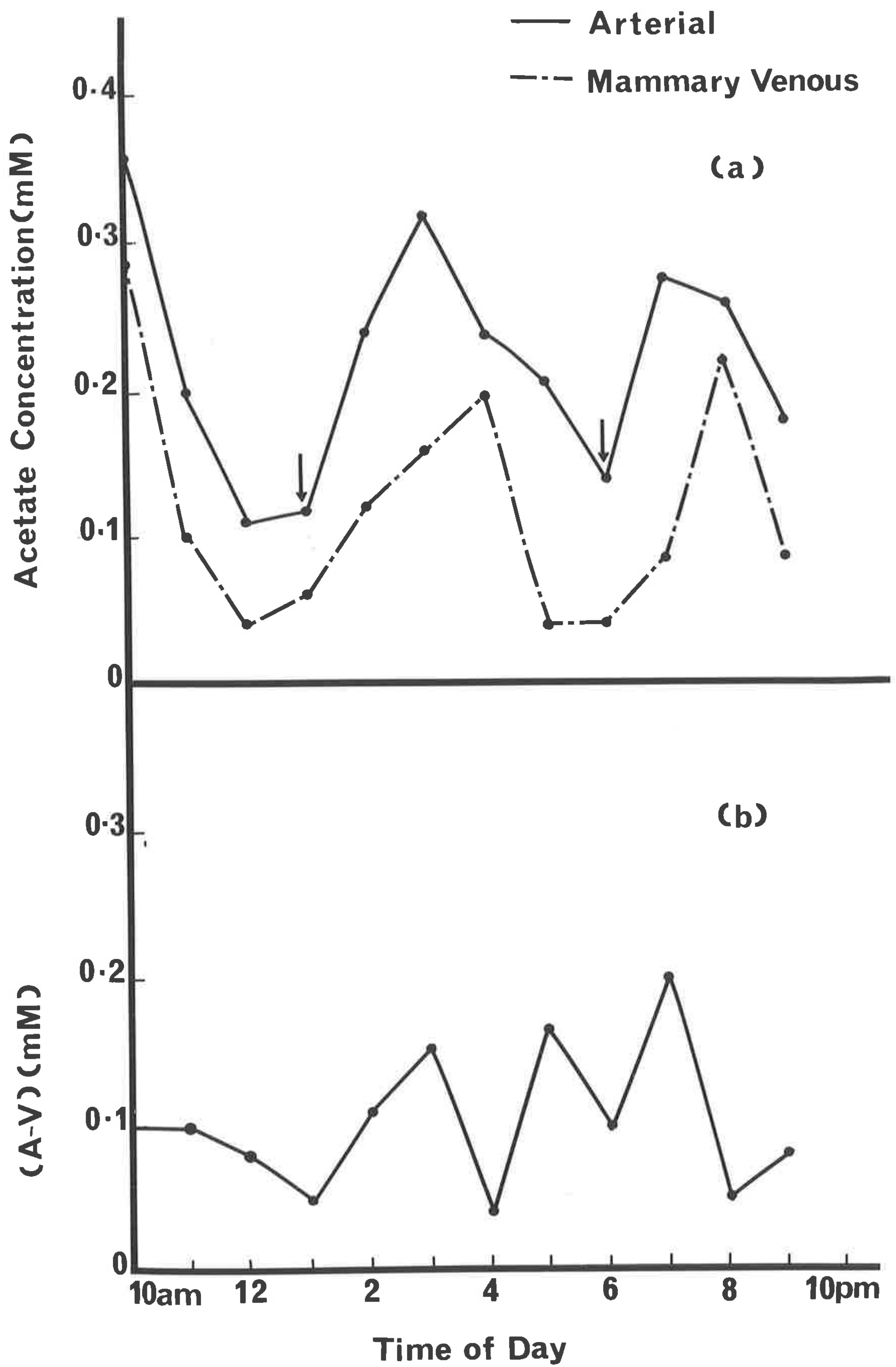


Figure 16

FIGURE 17

17(a) *Hourly acetate concentrations in arterial and mammary venous blood in ewe number 4 at 8 weeks lactation*

Acetate was estimated in duplicate as described in Part II, 3.1.9.

17(b) *Hourly differences in acetate concentrations of arterial - mammary venous blood.*

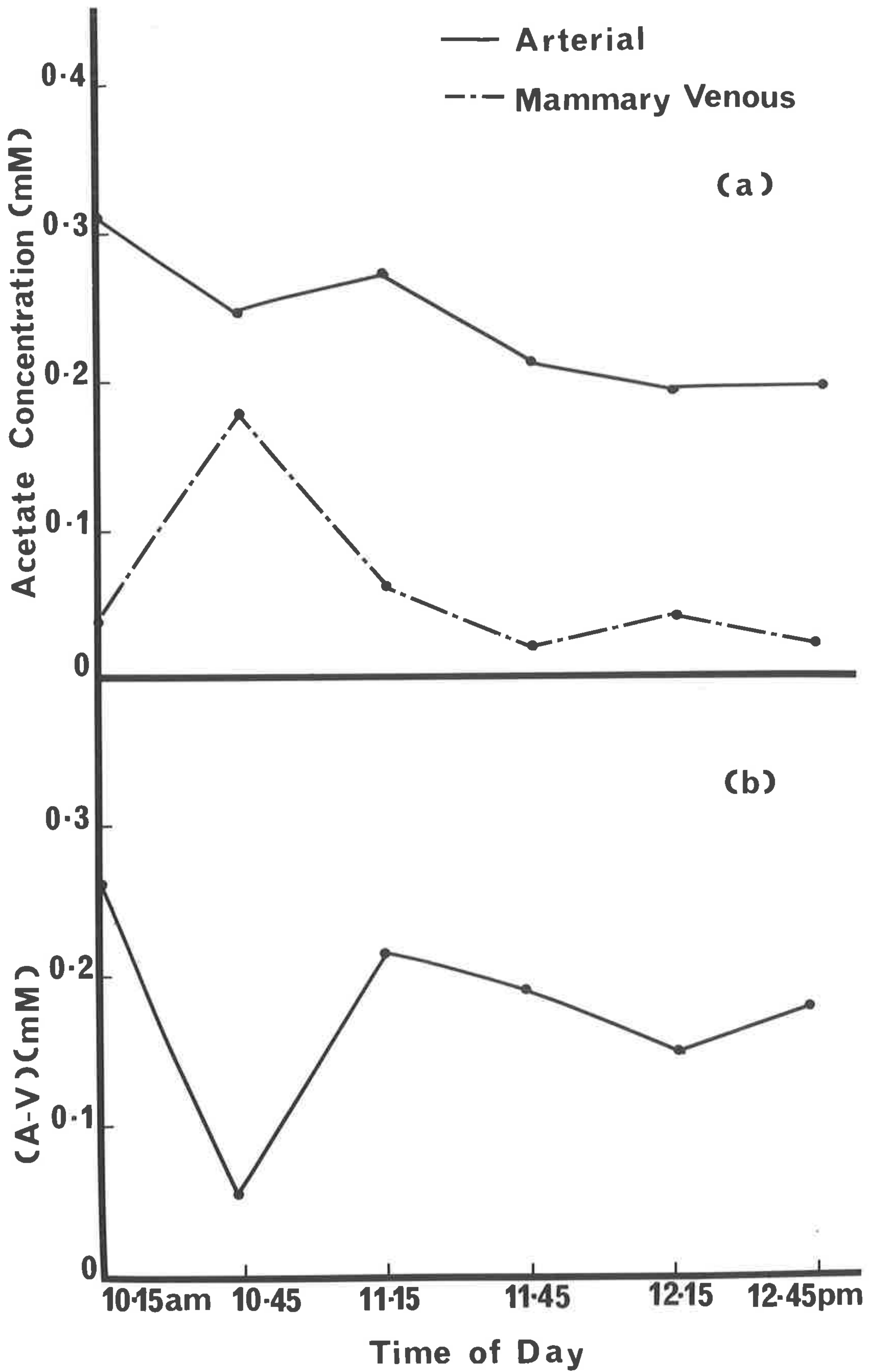


Figure 17

TABLE 22

Acetate concentrations in arterial and mammary venous blood of lactating ewes

Acetate was estimated in duplicate as described in Part II, 3.1. The values shown are the means ± S.E.M. with number of blood samples assayed given in parentheses. The significance of the differences between arterial and mammary venous samples, as determined by paired t-test, is also indicated.

	Blood acetate concentration (μmol/ml)		
Ewe	Arterial(A)	Mammary Venous(V)	A - V
2	0.47 ± 0.10(6)	0.23 ± 0.05(6)	0.23 ± 0.05 $P < 0.05$
3	0.27 ± 0.07(6)	0.15 ± 0.05(6)	0.12 ± 0.02 $P < 0.01$
4a	0.22 ± 0.02(12)	0.12 ± 0.02(12)	0.10 ± 0.01 $P < 0.001$
Mean	0.32	0.17	0.15
4b	0.24 ± 0.02(6)	0.06 ± 0.02(6)	0.18 ± 0.07 $P < 0.001$

FIGURE 18

The relationship between (hepatic venous - arterial) acetate concentration and (hepatic venous - mammary venous) acetate concentration in ewe number 4 at 4 weeks lactation

Acetate was estimated in duplicate as described in Part II, 3.1.9. The regression line was expressed by the equation $y = 131 + 0.96x$ with a correlation coefficient (r^2) of 0.97.

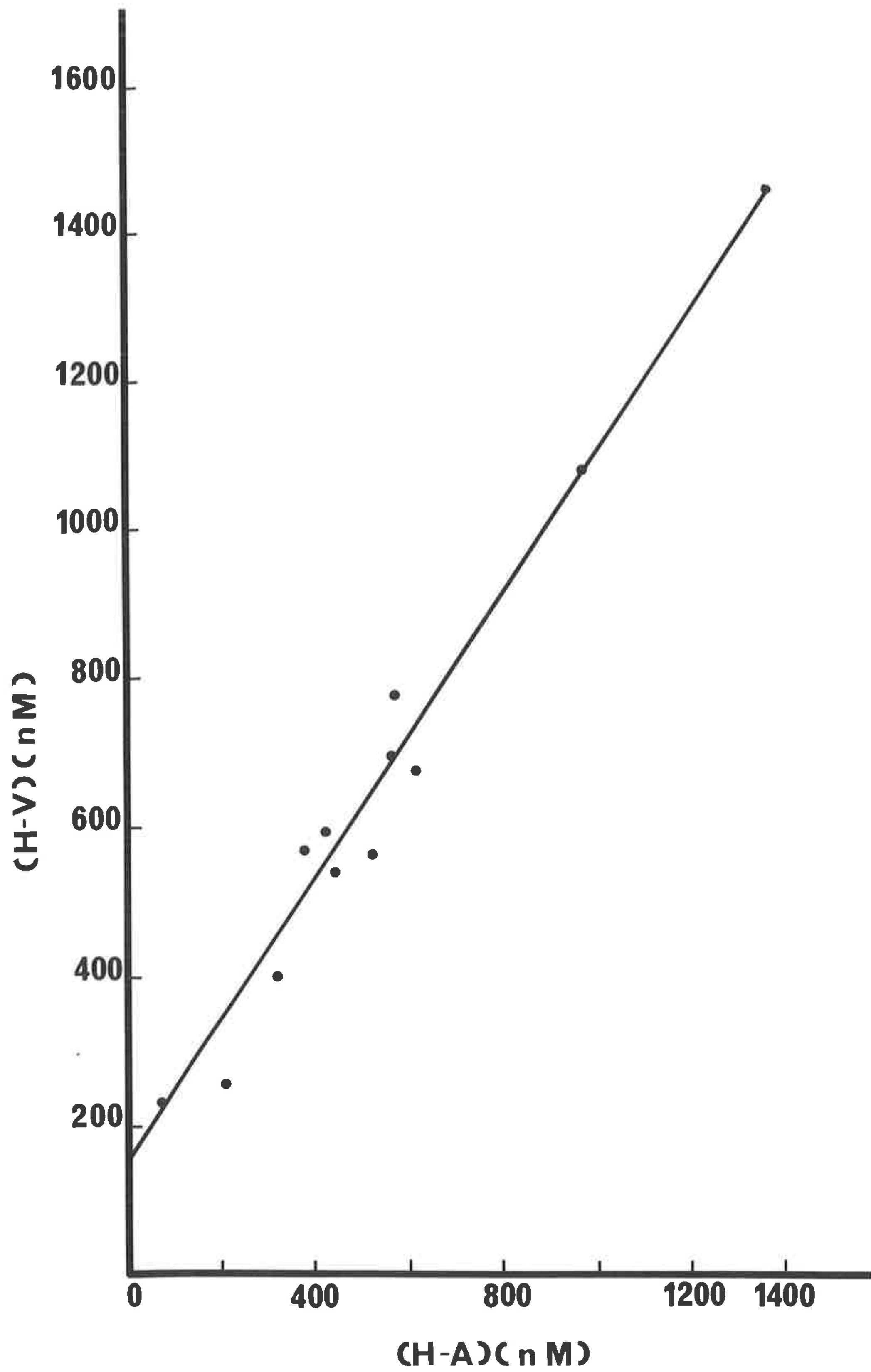


Figure 18

3.2.8 Enzymic activities in mammary tissue from lactating ewes

The activity of carnitine acetyltransferase in mammary tissue homogenates from lactating ewes was 0.61 μmol/min per g wet weight (Table 23) which is comparable to that previously reported for sheep liver and kidney (Snoswell and Koundakjian, 1972). Some 50% of the activity detected in homogenates treated with Triton X-100 was found in homogenates prepared with 0.25 M sucrose-23 mM potassium phosphate suggesting that only half the carnitine acetyltransferase activity was 'latent', or membrane bound.

The activity of carnitine palmitoyltransferase in mammary tissue homogenates from lactating ewes was 0.75 μmol/min per g wet weight (Table 23). In contrast to the carnitine acetyltransferase activity, 85% of the carnitine palmitoyltransferase activity was found to be 'latent' by the same criterion of isotonic versus hypotonic homogenates.

The activity of acetate thiokinase in mammary tissue homogenates was 1.47 μmol/min per g wet weight (Table 23) whereas thiokinase activity could not be detected in liver homogenates from these ewes. The acetate thiokinase in mammary tissue from these ewes would appear to be predominantly located in the cytoplasm since a 'press' (Snoswell and Koundakjian, 1972) or cytosolic fraction contained virtually the same activity detected in a whole tissue homogenate. The same 'press fraction' contained only 2% of the mitochondrial matrix marker enzyme, citrate synthase, when compared to a whole tissue homogenate, indicating that the 'press fraction' was almost free of mitochondrial contamination.

Acetylcarnitine hydrolase activity was not detected in these mammary tissue homogenates (Table 23) indicating that acetylcarnitine could not be cleaved to acetate and L-carnitine as in liver homogenates from these animals (Table 18).

TABLE 23

Enzyme activities in mammary tissue from lactating ewes

Homogenates were prepared and assayed as described in Part II, 3.1. The values are means ± S.E.M. for 5 samples from 3 animals.

Enzyme	Activity (μmol/min per g wet weight)
Carnitine acetyltransferase	0.61 ± 0.12
Carnitine palmitoyltransferase	0.75 ± 0.22
Acetate thiokinase	1.47 ± 0.54
Acetylcarnitine hydrolase	N.D.*

*N.D. - not detectable

4. Discussion

The results presented in Part II, 3.2 clearly indicate a substantial net production of acetate by the liver of lactating ewes. The net hepatic production of endogenous acetate was almost equivalent to the exogenous acetate production. Actual liver production of acetate accounted for the greater portion (approximately 87%) of the net hepatic production than did the contribution of acetate via the hepatic artery. The hepatic-portal differences in acetate presented in Table 20 were similar to those reported by Baird *et al.* (1975) in lactating dairy cows. However, the hepatic production of acetate is much lower in lactating ewes than in lactating dairy cows due mainly to the greater hepatic venous and portal venous blood flows in the lactating cows (Baird *et al.*, 1975). There is continual hepatic release of acetate over an extended period of sampling in lactating ewes which contrasts with the intermittent hepatic production of acetate in lactating cows reported by Baird *et al.* (1975).

The net hepatic production of 54 mmols of acetate per hour in lactating ewes contrasts markedly with the net hepatic production of 1 mmol of acetate per hour in fed, non-lactating sheep and 4 mmols of acetate per hour in 3-day fasted sheep reported by Bergman and Wolff (1971). However, in the 3-day fasted sheep the total acetate turnover was considerably reduced. The total acetate production (i.e. exogenous and endogenous) in lactating ewes was 110 mmol/hr which is considerably higher than the total acetate production in fed sheep of 75 mmol/hr reported by Bergman and Wolff (1971). The endogenous acetate production of approximately 50% of the total acetate production supports the observations of Annison and Armstrong (1970) in continuously fed, non-lactating sheep. The hepatic acetate production in the ewe samples at 8 weeks lactation was 9 mmol/hr compared with 34.2 mmol/hr in the same ewe at 4 weeks lactation. Thus, the

net hepatic production of acetate in these ewes does appear to be related to the lactation state.

The net exogenous acetate production in lactating ewes was 56 mmol/hr compared with 74 mmol/hr reported by Bergman and Wolff (1971) in non-lactating, fed sheep. Acetate concentrations in portal venous and arterial blood were lower in lactating ewes than in non-lactating sheep. Also, as shown by Baird *et al.* (1975), portal and arterial acetate concentrations were lower in lactating versus non-lactating cows. The relative rates in which acetate, propionate and butyrate were absorbed from the rumen in lactating dairy cows were 9:2.5:1 while in non-lactating dairy cows they were 38:9:1 respectively. The proportions for these VFA's reported by Bergman and Wolff (1971) in non-lactating fed sheep were 34:9:1 respectively. Baird *et al.* (1975) suggested from the evidence of the absorption of these VFA's that an effect of lactation in the dairy cow may be to cause a relative increase in the quantity of butyrate being absorbed from the rumen. However, it is tempting to suggest that an effect of lactation in the ruminant may be to cause a relative deficiency in the amount of acetate being absorbed from the portal-drained viscera.

The results presented in Part II, 3.2 also indicated that there was a significant hepatic uptake of free fatty acids in lactating ewes. The free fatty acid concentration of hepatic venous and portal venous blood in lactating ewes was similar to the concentrations reported by Katz and Bergman (1969*a*) in non-pregnant and twin-pregnant ewes. However, the hepatic uptake of free fatty acids appeared to be greater in the ewes at 4 weeks lactation versus the ewe at 8 weeks lactation (Table 17) and also the non-lactating ewes (Katz and Bergman, 1969*a*). This evidence suggests that the increased hepatic uptake of free fatty acids may also be related to the lactation state. On the basis of 2-carbon equivalents, the hepatic acetate produced accounted for 70% of the free fatty acid

uptake. Further, when the uptake of free fatty acids and the output of acetate by the liver for each successive blood sample were plotted (see Figure 15), there was a significant correlation ($r^2 = 0.83$) between the hepatic free fatty acid uptake and acetate production. With the reservation that correlation does not imply causation, it would appear likely that the free fatty acids could be the major precursors of the hepatic acetate production. These results do not establish free fatty acids as the source of the endogenous acetate as this could only be determined by infusing labelled free fatty acids. But, in this regard, both Palmquist (1972) and West and Annison (1964) have demonstrated that significant incorporation of label from [^{14}C]-palmitate into plasma acetate occurs in the sheep.

The production of endogenous acetate from other precursors is also possible. There was a significant portal-hepatic difference in alanine concentration of 15.8 nmol/ml in ewe Number 1, which is comparable with the difference of 22 to 27 nmol/ml reported by Baird *et al.* (1975) in lactating cows. However, the hepatic-portal difference in acetate in the same ewe was 880 nmol/ml. Thus, alanine could contribute a maximum of only 1.8% of the acetate produced by the liver in this ewe. Other amino acids may be utilized to produce acetate but there is still no evidence in the literature or from this study that amino acids could be significant precursors of endogenous acetate.

Since the reports of Baird *et al.* (1974,1975) have shown that ethanol could contribute less than 5% of the endogenous acetate produced in lactating dairy cows, hepatic uptake of ethanol in lactating ewes was not determined. There was no detectable acetylcarnitine in hepatic venous blood from lactating ewes indicating that it is unlikely that acetate produced in the liver is transported as an acetylated compound to the extrahepatic tissues.

The results presented in Part II, 2.2 and 3.2 provide convincing evidence that the enzyme couple, carnitine acetyltransferase and acetylcarnitine hydrolase, is the major mechanism whereby acetate can be produced from intramitochondrial acetyl-CoA in ruminant liver. However, there are differences in the adaptation of this mechanism to the lactation state in the dairy cow and ewe.

The results presented in Part II, 2.2 show that there are significant metabolic changes in the liver of lactating, as compared to non-lactating dairy cows. The hepatic concentrations of acetyl-CoA increase significantly in lactating dairy cows but there was no change in hepatic concentrations of total CoA, which were similar to those reported by Baird, Heitzman and Snoswell (1972) in lactating dairy cows. The ratio of hepatic [free CoA]:[acetyl-CoA] decreased from 0.3:1 in non-lactating cows to 0.07:1 in lactating dairy cows. Baird *et al.* (1972) reported a ratio of 0.2:1 in lactating dairy cows. In contrast Snoswell and Henderson (1970) reported ratios of 1:1 in normal sheep and 0.34:1 in starved sheep, while Allred and Guy (1969) reported a ratio of 4:1 in normal rats.

The ratio of [free CoA]:[acetyl-CoA] has an important controlling effect upon metabolism generally (Wieland and Weiss, 1963; Tubbs and Garland, 1964) and upon pressure to form aceto-acetate and 3-hydroxybutyrate in particular (Greville and Tubbs, 1968).

The formation of acetylcarnitine in rat liver (Pearson and Tubbs, 1967) and increase in carnitine and acetylcarnitine in sheep liver (Snoswell and Koundakjian, 1972) have been suggested as mechanisms whereby 'acetyl pressure' in these animals can be diverted from ketone body synthesis. However, in liver from lactating cows, there was no increase in either acetylcarnitine or carnitine concentrations and indeed these concentrations were lower than those reported in both rat and sheep liver

(Pearson and Tubbs, 1967; Snoswell and Henderson, 1970; Snoswell and Koundakjian, 1972). Although there was an increase in 'acetyl pressure' there was no increase in hepatic concentrations of acetoacetate and 3-hydroxybutyrate in lactating as compared to non-lactating cows. Thus, the results presented in Part II, 2.2 indicate that there was a diversion of 'acetyl pressure' away from ketone body formation which was not reflected by changes in hepatic concentrations of acetylcarnitine and free carnitine.

The hepatic activities of both carnitine acetyltransferase and acetylcarnitine hydrolase increased significantly in lactating as compared to non-lactating cows. These increases in activity may be a specific response to increased acetyl-CoA concentrations in the lactating dairy cow. The results presented in Part II, 2.2, together with the reports of Baird *et al.* (1974,1975) of hepatic production of acetate in the lactating dairy cow, suggest that there is an increased flux of acetyl groups from acetyl-CoA to acetate via carnitine acetyltransferase and acetylcarnitine hydrolase in the lactating dairy cow. Thus, in response to the increased 'acetyl pressure' acetate is produced as an alternative to production of acetoacetate and 3-hydroxybutyrate. It remains to be shown whether the enzyme couple carnitine acetyltransferase and acetylcarnitine hydrolase in the liver of lactating dairy cows has the capacity to account for the hepatic production of acetate.

In contrast to these findings in the lactating dairy cows, there was no increase in carnitine acetyltransferase activity in the liver of lactating as compared to non-lactating sheep(1.9 μmol/min per g wet weight). The mean enzymic capacity of the liver of lactating ewes to produce acetate from acetyl-CoA, as determined *in vitro* using homogenates of biopsy samples, was 56.4 mmol/hr, compared with an actual mean liver acetate production rate of 47.9 mmol/hr. The actual liver production may

be an underestimate of the total acetate produced within the liver since some of the acetate may have been utilized by the liver itself. This could be demonstrated by infusion experiments with labelled acetate. In spite of this reservation, it would appear that the enzymic capacity of the liver to produce acetate, as measured *in vitro*, could account for the hepatic production of acetate observed in the lactating ewes. The enzymic measurement of the conversion of acetyl-CoA to acetate determined in the lactating ewes involves a combination of the two enzymes, carnitine acetyltransferase and acetylcarnitine hydrolase. Thus, this coupled enzyme system adequately accounts for the breakdown of mitochondrial acetyl-CoA leading to the release of acetate in liver of lactating ewes.

The reason why liver from dairy cows and ewes should produce considerable quantities of acetate during lactation is of interest. Acetate may be produced to relieve an intramitochondrial build-up of acetyl-CoA derived from fatty acid oxidation. Sheep liver mitochondria oxidize fatty acids at a relatively slow rate (Koundakjian and Snoswell, 1970) and this appears to be due to a lesser ability to oxidize Krebs cycle intermediates. The high activity of carnitine palmitoyltransferase in the livers of lactating ewes (Table 18), together with the significant uptake of fatty acids and hepatic acetate production *in vivo*, suggests that the limitation on total fatty acid oxidation is not due to a limitation on the conversion of fatty acids to acetyl-CoA but rather on the subsequent oxidation of the acetyl-CoA. Baird and Heitzman (1970) reported significantly reduced concentrations of a number of Krebs cycle intermediates in livers of lactating as compared to non-lactating dairy cows, suggesting a decreased turnover of the Krebs cycle in bovine liver during lactation. If Krebs cycle activity in sheep and cow liver is depressed during lactation, the fate of excess acetyl-CoA would largely be determined by

the relative activities of carnitine acetyltransferase and of the ketone body-producing enzymes and their affinities for acetyl-CoA. In this respect the activity of carnitine acetyltransferase in the liver of both lactating dairy cows (2.65 μmol min^{-1} per g wet weight tissue; see Table 14) and lactating ewes (2.22 μmol min^{-1} per g wet weight tissue; see Table 18) is considerable. Acetate production from intra-mitochondrial acetyl-CoA would appear to be an energetically wasteful process, yet it is no more wasteful than ketone body production and is proposed as an alternative process which produces an important metabolite in the lactating ruminant. The mechanisms which control the hepatic acetate production obviously require further investigations.

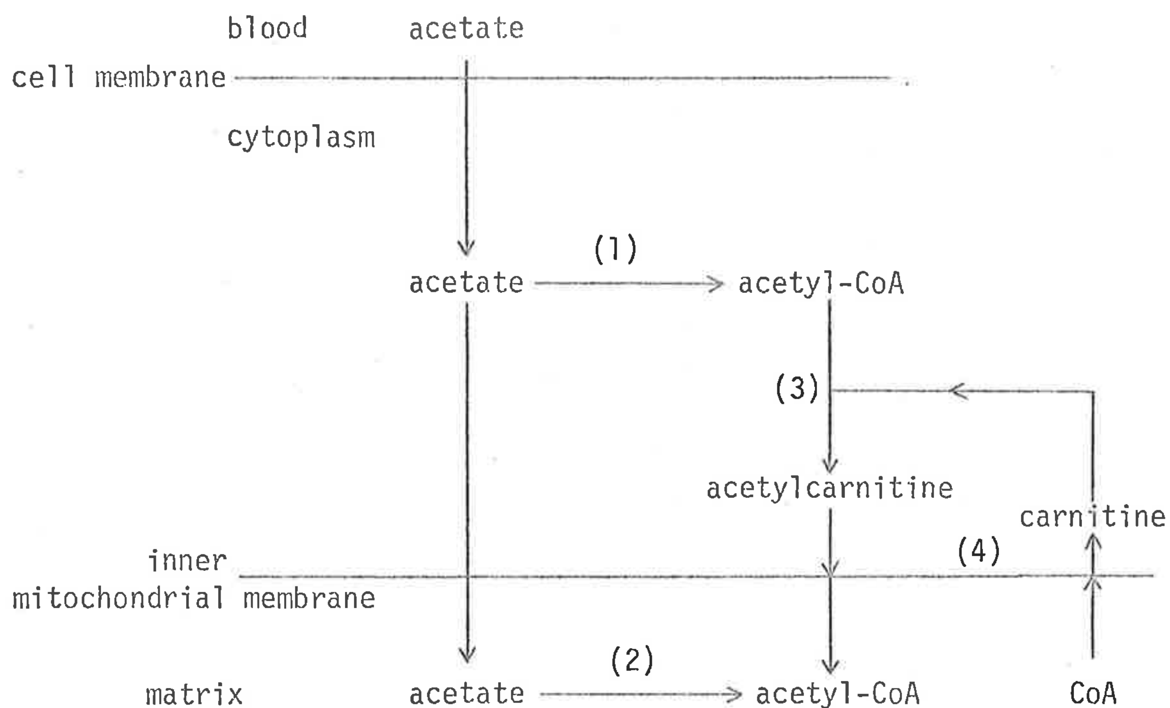
The results presented in Part II, 3.2.7 clearly indicate that there was an uptake of acetate by the mammary gland of lactating ewes. Uptake of acetate by lactating mammary gland has been shown in diary cows (McClymont, 1951; Bickerstaffe, Annison and Linzell, 1974) and in goats (Popják *et al*, 1951; Annison and Linzell, 1964) but there have been no previous reports in lactating ewes. The extraction (i.e. arteriovenous difference/arterial concentration x 100) of acetate by the mammary gland of lactating ewes was 47%, which is lower than the acetate extraction of 63% in goats (Linzell, 1968) and of 61% in dairy cows (Bickerstaffe *et al*., 1974). However, these differences may be due to the samplings being performed at relatively different stages of lactation in each species as the extraction of acetate in ewe number 4 had risen significantly ($P < 0.01$) to 74% at 8 weeks lactation. The concentration of acetate at the hepatic vein (i.e. exogenous and endogenous production) directly influenced the acetate metabolism of the mammary gland in lactating ewes. There was a significant correlation ($r^2 = 0.97$) between net total production of acetate and net uptake of acetate as measured at the mammary vein in the lactating ewes (see Figure 18).

Although it is statistically improper to imply direct cause and effect from this correlation, the wider evidence presented here suggests interesting consequences of this correlation. The mammary gland of lactating ewes has a specific requirement for acetate to synthesize milk fatty acids up to C_{14} and part of C_{16} (see Part II, 1.6). The total hepatic production of acetate during lactation may be greater than in normal, fed sheep in order to sustain uptake and utilization of acetate for fatty acid synthesis by the lactating mammary gland. Thus, increased liver production of acetate from endogenous sources during lactation may in part be a response to supplement the exogenous production of acetate to maintain high total acetate production in the lactating ewes. This would appear to require a 'futile' cycle where free fatty acids are converted to acetate by the liver and this acetate utilized by the mammary gland for the synthesis of fatty acids in milk. Jarrett *et al.* (1976), in studies with oxidation of substrates by sheep hind limb, suggested that sheep tissues converted acetate to free fatty acids, triglycerides and ketones instead of directly oxidizing acetate and that these nutrients served as acetate stores during periods of acetate oxidation e.g. fasting or exercise. However, Bickerstaffe *et al.* (1974) has shown that free fatty acids are not taken up by the lactating mammary gland of cow. Thus, the acetate store provided by free fatty acids, as proposed by Jarrett *et al.* (1976) must be converted to acetate by the liver before acetate can be utilized by the lactating mammary gland for fatty acid synthesis and energy. Of course this remains pure speculation until further evidence can be provided. The infusion of labelled acetate into pregnant sheep would presumably label the free fatty acid stores and the metabolism of these labelled fatty acids during lactation could be investigated to provide part of the required evidence.

Approximately half of the acetate taken up by the lactating mammary

gland in ruminants is oxidized to provide energy (Annison and Linzell, 1964; Bickerstaffe *et al.*, 1974; Davis and Bauman, 1974). The activity and distribution of carnitine acetyltransferase and carnitine palmitoyl-transferase in mammary tissue from lactating ewes suggest that carnitine is an essential co-factor in the oxidation of both acetate and long-chain fatty acids in mammary tissue. The concentration of total-acid-soluble carnitine in mammary gland from lactating goats (324 nmol/g wet weight) was comparable to the liver concentration (Snoswell and Linzell, 1975). Also the activity and distribution of acetate thiokinase in mammary tissue from lactating ewes suggest that this enzyme could provide acetyl-CoA for fatty acid synthesis in the cytoplasm. This result is in agreement with the findings of Bauman *et al.* (1973,1974) in sheep and Quraishi and Cook (1972) in cows. Since the activity of acetate thiokinase is predominantly distributed in the cytoplasm [less than 20% of total activity is mito-chondrial in bovine tissues (Quraishi and Cook, 1972)], the significance of its role in oxidation of acetate is of some interest.

Acetyl-CoA formed by cytoplasmic acetate thiokinase must be trans-ported across the permeability barrier of the inner mitochondrial membrane before it can be oxidized via the Krebs cycle. The distribution of carnitine acetyltransferase and the concentration of carnitine in mammary gland tissue suggests that it may be involved specifically in this transfer of acetyl-CoA to the oxidative compartment of the cell. Acetate may also free diffuse across the inner mitochondrial membrane and be activated to acetyl-CoA by the mitochondrial acetate thiokinase for subsequent oxidation to CO_2. These two pathways may be represented schematically:



(1) cytoplasmic acetate thiokinase
(2) mitochondrial acetate thiokinase
(3) 'external pool' of carnitine acetyltransferase
(4) mitochondrial pool of carnitine acetyltransferase

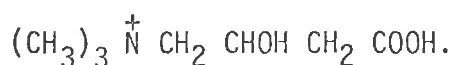
The relative significance of the two pathways in oxidation of acetate by lactating mammary gland in ruminants bears further investigation.

PART III: BIOSYNTHESIS OF CARNITINE IN ANIMAL TISSUES

1. Introduction

1.1 General Introduction

Carnitine (3-hydroxy-4-trimethylaminobutyric acid) was discovered by Gulewitsch and Krimberg (1905) in muscle extracts, 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 (Krimberg, 1907; Engeland, 1909; Fischer and Göddertz, 1910; Engeland, 1921). The identification of carnitine as 3-hydroxy-4-trimethylaminobutyric acid was firmly established by Tomita (1926) and Tomita and Sendju (1927); the formula is now generally written as:



$$(CH_3)_3 \overset{+}{N} CH_2 \ CHOH \ CH_2 \ COOH.$$

Subsequent to this finding there was only sporadic interest in carnitine until 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 *et al.*, 1952). Using this biological assay, Fraenkel (1952) demonstrated the biosynthesis of carnitine in the developing chick embryo. Fraenkel (1953) postulated that carnitine is synthesized in all organisms which do not require it in the diet. Yue and Fritz (1962) found that injected tritiated carnitine could be taken up from the plasma by several tissues of dogs and rats and on this basis 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. Strength, Yu and Davis (1965) found that dietary supplementation of carnitine did not effect the carcass carnitine concentration or the growth rate of rats. Grüner *et al.* (1966) found that [^{15}N]carnitine fed to humans was metabolized and excreted as trimethylamine oxide, apparently without any mixing with endogenous tissue carnitine. However, Tanphaichitr *et al.* (1976) found that carnitine supplementation given to rats fed diets containing no detectable carnitine, and limiting in threonine and lysine, resulted in significantly higher concentrations of liver and skeletal muscle carnitine.

1.2 Turnover of carnitine

The evidence available in the literature suggests that the turnover of carnitine in animals is very slow.

Wolf and Berger (1961), investigating the decrease in specific activity of the carnitine excreted in the urine of rats over a period of 10 days after administration of labelled carnitine, reported a half-life of 67 days and a body pool of carnitine of 47.0 mg in 130 g rat. However, in order to assure adequate radioactivity for their measurements, they had to inject 37.1 mg of ^{14}C-DL-carnitine. Khairallah and Mehlman (1965), using a much higher specific activity of DL-carnitine (1 mg, 6.0 μCi), reported turnover times of carnitine of 6-10 and 12-14 days in female and male rats respectively. Stress conditions of starvation, choline-deficiency, alloxan-diabetes and cold-acclimatization in male rats all lowered the turnover time (4-5 days on average) and body pool of carnitine (Therriault and Mehlman, 1965; Mehlman *et al.*, 1969; Mehlman *et al.*, 1971). Tsai *et al.* (1974) suggested that there are in fact two pools of carnitine in male rats with turnover times of 4.6 and 24.5 days.

1.3 4-Trimethylaminobutyric acid hydroxylase

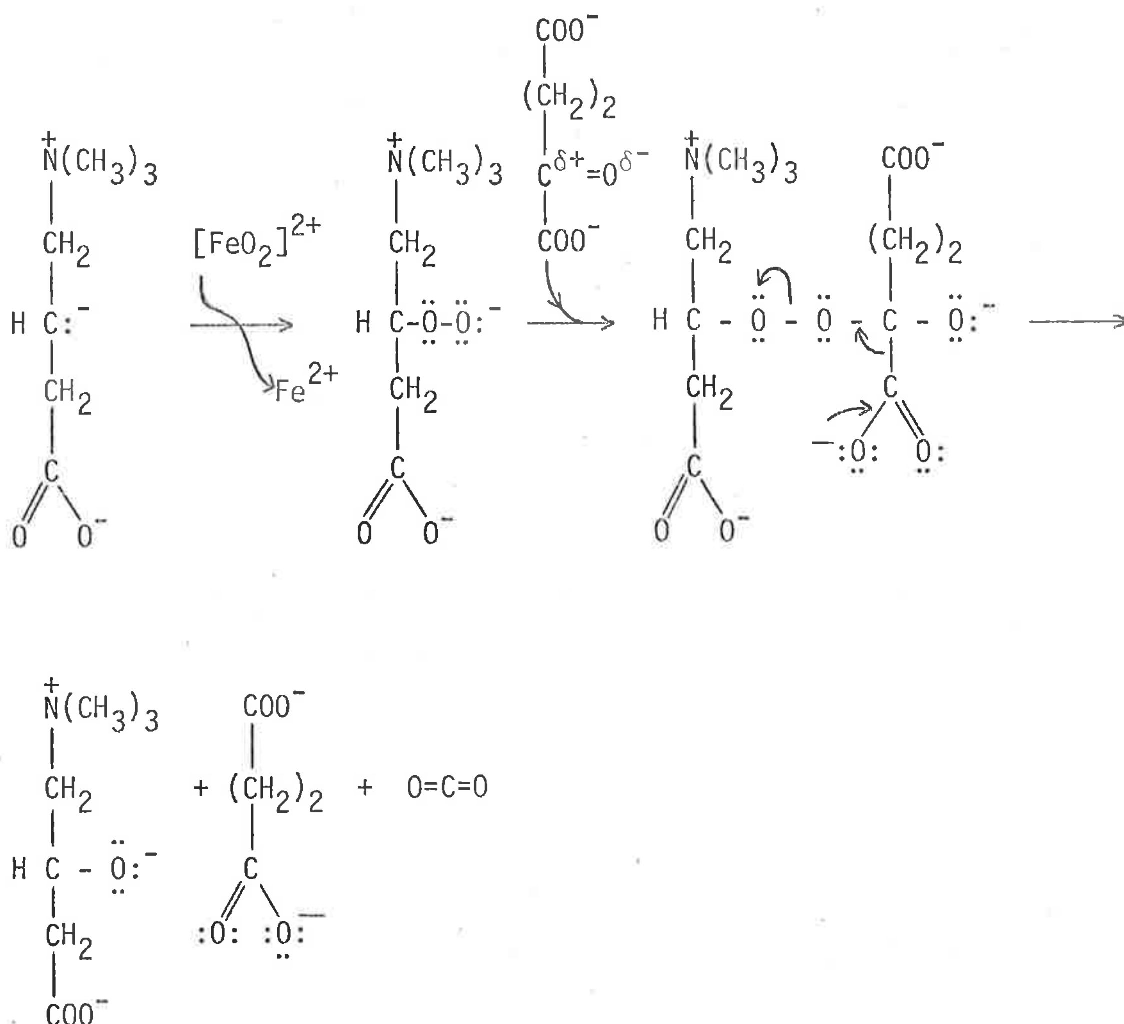
It is now generally accepted that 4-trimethylaminobutyric acid is the immediate precursor in the biosynthetic pathway for carnitine formation.

Linnewah (1929), in experiments involving the injection of 4-trimethylaminobutyrate into dogs, found an increase in carnitine excretion in the urine which led him to suggest that 4-trimethylamino-butyrate could be converted to carnitine by β-oxidation via the intermediate crotonbetaine (4-trimethylaminobut-2,3-ene). Lindstedt and Lindstedt (1961), Bremer (1962*b*) and Lindstedt and Lindstedt (1965) found that ^{14}C-labelled 4-trimethylaminobutyrate was efficiently converted to carnitine but could find no evidence in support of the hypothetical intermediate, crotonbetaine.

Lindstedt (1967) and Lindstedt *et al.* (1967), from studies in rat liver and *Pseudomonas sp* AK1 respectively, found a soluble enzyme protein which hydroxylated 4-trimethylaminobutyrate to carnitine. The enzyme, 4-trimethylaminobutyrate hydroxylase, was subsequently partially purified from these two sources (Lindstedt and Lindstedt, 1970; Lindstedt *et al.*, 1970). The enzyme reaction required molecular oxygen (Linblad *et al.*, 1969), ferrous ions and 2-oxoglutarate (Lindstedt *et al.*, 1967) and was stimulated by ascorbate and catalase (Lindstedt *et al.*, 1970).

From these requirements and the stoichiometric relationships shown by Holme *et al.* (1968), Lindstedt and Lindstedt (1970) suggested a mixed-function, dioxygenase mechanism of reaction whereby 4-trimethylamino-butyrate is hydroxylated to carnitine simultaneously with the oxidative decarboxylation of 2-oxoglutarate in a reaction sequence involving the intermediate formation of a peroxide of the two substrates. Ferrous ions acted as an oxygen-activating agent and ascorbate and catalase maintained

the ferrous ions and free sulphydryl groups (also necessary for enzyme activity) in the reduced state. This can be represented schematically:



This conversion of 4-trimethylaminobutyrate to carnitine via 4-trimethylaminobutyrate hydroxylase has been used as a key to unlock the sites of carnitine biosynthesis in animal tissues. Lindstedt (1967), from investigations in rat tissues, found 4-trimethylaminobutyric acid hydroxylase activity in liver but not in kidney or muscle. Bohmer (1974), studying the fate of intravenously injected [methyl-^{3}H]-4-trimethylaminobutyrate in normal rats and rats where the liver had been excluded from the circulation, concluded that the liver was the only organ which coverted 4-trimethylaminobutyrate to carnitine and that the carnitine was subsequently rapidly transported to other organs. In contrast, Erfle (1975) found that, in sheep tissues, 4-trimethylaminobutyric acid

hydroxylase activity was present in kidney cortex and muscle as well as liver. Although the hydroxylase activity was highest in the liver, Erfle suggested the low muscle hydroxylation could contribute significantly to carnitine formation by virtue of the large mass of skeletal muscle in the sheep. However, Snoswell and McIntosh (1974) found a net hepatic production of carnitine as measured by hepatic venous-portal venous difference in severely alloxan-diabetic sheep (a metabolic state where carnitine production is greatly increased above normal) but no significant change in skeletal muscle carnitine concentration.

1.4 Possible precursors of the carbon skeleton of carnitine

Investigations into the origin of the carbon chain of carnitine have centred around three possible precursors, choline, 4-aminobutyrate and lysine.

Choline, because of its structural similarity to carnitine, was proposed as a possible precursor by Strack *et al.* (1935). The proposed route of synthesis involved in aldol condensation between choline aldehyde and acetaldehyde and, following the discovery of the condensation between oxaloacetate and acetyl-CoA to form citrate, this proposal gained some popularity (Fraenkel and Friedman, 1957; Cantoni, 1960; Strack *et al.*, 1960). However, Bremer (1961) demonstrated that there was no synthesis of carnitine from 1,2-^{14}C-choline in rats.

Engeland and Kutscher (1910) proposed that the 'α-oxy-γ-butyrobetaine' found in muscle extracts might be formed from 4-aminobutyrate by a process involving exhaustive methylation. Hosein *et al.* (1962) incubated 4-aminobutyrate and s-adenosylmethionine with brain and muscle homogenates from rats and rabbits and succeeded in isolating an acid reinekate precipitate identified as 4-trimethylaminobutyrate. However, Fritz (1963)

calculated that the percentage conversion to 4-trimethylaminobutyrate reported by Hosein *et al.* (1962) was in fact negligible (0.0075%).

Bremer (1962*b*) and Lindstedt and Lindstedt (1965) investigated the possible conversion of ^{14}C-labelled 4-aminobutyrate to carnitine in rats and mice respectively. Bremer reported that 11 hours after injection there was a very small incorporation of ^{14}C-label into carnitine but 94% of the injected activity was excreted in the respiratory carbon dioxide during the first 5 hours. Lindstedt and Lindstedt could not detect any conversion of 4-aminobutyrate to carnitine in mice after a 10-20 hour period and explained this on the basis that 4-aminobutyric acid was rapidly metabolized and there could possibly be permeability barriers to the 4-aminobutyrate. In addition to 4-aminobutyrate, both groups investigated possible conversion of 4-dimethylaminobutyrate to carnitine, as methylation of the 4-amino groups blocks the transamination of 4-aminobutyrate to succinic semialdehyde and subsequent oxidation in the TCA cycle. However, the incorporation of labelled 4-dimethylaminobutyrate into carnitine in rats *in vivo* was small (about 0.1%) but a considerable amount was converted to a compound tentatively identified as 3-hydroxy-4-dimethylaminobutyrate (Bremer, 1962*b*). Lindstedt and Lindstedt (1965) reported similar findings in mice and also detected demethylation to 4-methylaminobutyrate. When labelled 3-hydroxy-4-dimethylaminobutyrate was used in mice, only 0.003% of the dose given was converted to carnitine (Lindstedt and Lindstedt, 1965). No conversion to carnitine of peptide-bound 4-aminobutyric acid (homocarnosine; an oligopeptide of 4-aminobutyric acid and L-histidine) could be demonstrated in mice (Lindstedt and Lindstedt, 1965). These authors suggested that, if 4-aminobutyric acid is a precursor of carnitine, the initial methylating steps must be rate-limiting, since 3-hydroxylation of 4-trimethylaminobutyrate and 4-dimethylaminobutyrate occurs readily.

The source of the 4-aminobutyric acid proposed as a carnitine precursor remains to be elucidated. Until recently it was thought that 4-aminobutyric acid was present only in nervous tissues, where it functioned as a neuro-inhibitor (see Albers, 1960; Roberts and Eidelberg, 1960). With the development of more sophisticated biochemical assay methods, 4-aminobutyric acid was detected in low concentrations in several tissues of man and other mammals, with human kidney having the highest concentration of the non-neural tissues (0.5 μmol/g wet weight, see Zachmann *et al.*, 1966). 4-Aminobutyric can be produced in rat kidney cortex by glutamate decarboxylase, which differs from the brain enzyme in that anions (e.g. Cl^-) and carbonyl-trapping agents (e.g. semicarbazide and aminooxyacetic acid) stimulate kidney enzyme activity but inhibit brain enzyme activity (Haber *et al.*, 1970; Lancaster *et al.*, 1973). Vavatsi-Manos *et al.* (1973) suggested that 4-aminobutyric acid present in rat kidney cortex may have a role in ammoniagenesis and acid-base regulation. Seiler and Eichentopf (1975) found concentrations of 4-aminobutyric acid of 31.5 ± 3 nmol/g wet weight in mouse liver but showed that the greater proportion arose from oxidation of putrescine (1,4-diaminobutane) by the liver rather than decarboxylation of glutamate.

4-Aminobutyric acid may be synthesized in one organ and taken up by others. There is no evidence that 4-aminobutyric acid synthesized in the brain can readily leave that organ and enter the blood, and it is known that the brain does not take up 4-aminobutyrate from the blood (Roberts and Eidelberg, 1960). However, other tissues have been shown to take up 4-aminobutyric acid from the blood pasma *in vivo*, and the liver in rats, mice and guinea pigs accumulated the most (van Gelder and Elliott, 1958; Elliott and Jasper, 1959; Tsukada *et al.*, 1960; Roberts and Eidelberg, 1960). Muscle and kidney also take up 4-aminobutyric acid from the plasma.

Lancaster *et al.* (1973) showed that, in rat kidney cortex slices, 4-aminobutyric acid was transported by a mediated process which appeared to be energy dependent, although uptake against a gradient was not observed. The chemical distribution ratio (concentration in intracellular fluid/concentration in extracellular fluid) for 4-aminobutyric acid in rat kidney cortex slices remained between 0.43 and 0.54 at extracellular fluid concentrations of 4-aminobutyric acid between 1 and 60 mM (Lancaster *et al.*, 1973). Orally administered 4-aminobutyric acid is also taken up by some tissues, which suggests that the diet may be another possible source of 4-aminobutyric acid. There are numerous reports of high concentrations of 4-aminobutyric acid in various plant tissues (Steward *et al.*, 1949; Synge, 1951; Albers, 1960) and thus there may be high dietary concentrations in herbivores.

L-lysine has also been proposed as the precursor of the carbon skeleton of carnitine in *Neurospora crassa* and rats.

Horne and Broquist (1973) conducted a series of isotopic labelling experiments with *Neurospora crassa* lysine auxotrophs. They found that radioactivity from DL-[6-^{14}C]-lysine and DL-[4,5-^{3}H]-lysine was incorporated into carnitine (average incorporation of 0.14% for both isotopes), whereas radioactivity from DL-[1-^{14}C]-lysine and DL-[2-^{14}C]-lysine was not incorporated into carnitine in significant amounts (incorporation of 0.006% and 0.01% respectively). In an experiment where *Neurospora crassa* was grown on [6-^{15}N]-lysine, Horne and Broquist found a 20.3% ^{15}N excess in carnitine while expecting a theoretical value of 44.8%, suggesting that the 6-N atom of lysine was incorporated into carnitine but that there was considerable dilution with ^{14}N in the medium during carnitine biosynthesis. However, Horne and Broquist reported that 6-N-[methyl-^{3}H] trimethyl-lysine was incorporated in high yield (16% and 19% in two experiments) into carnitine.

Wolf and Berger (1961) could not detect any conversion of 0.9 μCi [U-^{14}C]-lysine to carnitine 24 hours after injection into normal rats. Tanphaichitr and Broquist (1973) found 0.057% and 0.07% conversion of 246 μCi [6-^{14}C]-DL-lysine to carnitine in normal and lysine-deficient rats respectively, three days after injection. However, they did not detect any conversion of DL-[2-^{14}C]-lysine in lysine deficient rats. In agreement with the findings of Horne and Broquist (1973) in *Neurospora crassa*, Tanphaichitr and Broquist (1973) found 6-N-[methyl-^{3}H]trimethyl-L-lysine was converted in high yield to carnitine (15.1% and 22.6% of the administered dose per 100 g of skeletal muscle in normal and lysine-deficient rats respectively).

Cox and Hoppel (1973*a*) also detected the conversion of L-[U-^{14}C]-lysine to carnitine in rats, reporting recoveries in carcass carnitine from the administered dose of 0.005% in normal, 0.014% in choline-deficient and 0.026% in lysine-deficient rats. These workers did not detect any conversion of [1-^{14}C]- or [2-^{14}C]-DL-lysine to carnitine in rats. Further, Cox and Hoppel (1973*a*) showed that the lysine label in carnitine was not in the 4-N-methyl groups and therefore was part of the carbon chain.

Cox and Hoppel (1973*b*) detected a 17% conversion of 6-N-[methyl-^{14}C]-trimethyl-L-lysine into carcass and urine carnitine in lysine-deficient rats. The trimethyl-lysine was converted into at least four other metabolites *in vivo* (Cox and Hoppel, 1973*b*). A total of 8% was recovered as 4-trimethylaminobutyrate, mostly in the urine, and 2% in three minor metabolites (Cox and Hoppel, 1973*b*). The three minor components were subsequently indentified as 5-N-trimethylaminopentoate, 6-N-trimethyl-2-N-acetyl-lysine and 6-N-trimethyl-3-hydroxy-lysine (Hoppel *et al.*, 1976).

Haigler and Broquist (1974), from rat tissue slice investigations, reported that liver, kidney, muscle, heart and testis all formed 4-trimethylaminobutyrate from 6-N-trimethyl-lysine, but liver and testis also formed carnitine in about 7% and 1% yield respectively. Cox and Hoppel (1974) reported that liver slices from both normal and lysine-deficient rats converted 6-N-[methyl-^{14}C]-trimethyl-lysine into carnitine, 4-N-trimethylaminobutyrate and at least three other unidentified metabolites. However, neither group detected any conversion of L-[U-^{14}C]-lysine into carnitine under the same conditions in the liver slice systems.

The mechanism whereby 4-trimethylaminobutyrate is biosynthesized from 6-N-trimethyl-lysine remains to be delineated. Cox and Hoppel (1974), without experimental evidence, postulated a pathway where the 2-amino group of 6-N-trimethyl-lysine was removed by an ammonia lyase similar to histidase to yield 6-N-trimethylamino-2-hexenoate, which was subsequently hydrated, followed by β-oxidation and thiolytic cleavage of this compound to ultimately yield acetate and 4-trimethylaminobutyrate. Hochalter and Henderson (1976) administered 6-N-trimethyl-L-[1-^{14}C]-lysine together with sodium benzoate to rats and analysed the excreted hippuric acid for radioactivity. From 21 to 30% of the trimethyl-lysine-^{14}C metabolized was incorporated into hippuric acid. Hochalter and Henderson suggested from these findings that carbons 1 and 2 of 6-N-trimethyl-lysine were converted directly to glycine, possibly via aldol cleavage of 6-N-trimethyl-3-hydroxy-lysine intermediate.

1.5 Methylation of possible carbon skeleton precursors of carnitine

Methionine has been recognized as the penultimate source of the N-methyl groups of carnitine.

Wolf and Berger (1961) reported that after administration of 45 μCi

[methyl-^{14}C]-methionine to a 97 g rat, 2.92 x 10^{-3} μCi of this radioactivity was incorporated into the methyl groups of carnitine after 9 days. Bremer (1961) reported 0.01-0.02% conversion of 75 μCi of [methyl-^{14}C]-methionine into carnitine 24 hours after administration in rats. Strength *et al.* (1965) found maximum incorporation of radioactivity from [methyl-^{14}C]-methionine into carnitine of liver from rats fed a choline-deficient diet (3.10% of the dose) 3 hours after injection and that subsequently the radioactivity in carnitine was transferred to the carcass. Horne and Broquist (1973) reported a conversion of 0.23% of [methyl-^{3}H]-methionine into carnitine in *Neurospora crassa*, and Tanphaichitr and Broquist (1973) reported a conversion of 0.05% of [methyl-^{3}H]-methionine into skeletal muscle carnitine in lysine-deficient rats. However, in all these experiments the initial methyl acceptor was not determined.

Attempts by Bremer (1962*b*) and Lindstedt and Lindstedt (1965) to demonstrate that these methyl groups are introduced at the level of 4-aminobutyric acid met with limited success. Only conversion of 4-dimethylaminobutyric acid (in small amounts) to carnitine could be demonstrated but not 4-aminobutyric acid; 4-methylaminobutyric acid was not used by either group.

Cox and Hoppel (1973*a*), by injecting DL-[4,5-^{3}H]-lysine and [methyl-^{14}C]-methionine into choline-deficient rats and L-[U-^{14}C]-lysine and [methyl-^{3}H]-methionine into lysine-deficient rats, showed that label from both compounds was incorporated into carnitine, with 91% of the [methyl-^{14}C]- and 98% of [methyl-^{3}H]-methionine label present in the 4-N-methyl groups of carnitine. Tanphaichitr and Broquist (1973) and Cox and Hoppel (1973*b*), in order to reconcile the high efficiency of conversion of 6-N-[methyl-^{3}H or ^{14}C]-trimethyl-lysine to carnitine as compared to

labelled methionine, suggested that lysine is methylated by methionine to the level of 6-N-trimethyl-lysine which, rather than providing labile methyl groups, is converted *per se* to carnitine (see 1.4 of Introduction).

The mechanism whereby lysine is methylated to 6-N-trimethyl-lysine is a contentious matter. The discovery of 6-N-mono-, di-, and trimethyl-lysine residues in many proteins such as histones, cytochrome *c* and myosin from diverse sources (Paik and Kim, 1971,1975) has led to speculation that lysine destined for carnitine biosynthesis is methylated in the protein-bound form (Tanphaichitr and Broquist, 1973; Cox and Hoppel, 1973*b*,1974). Paik and Kim (1970) first demonstrated the presence in calf thymus nuclei of protein methylase III, which carries out the 6-N-methylation of lysine residues in histones but not free lysine. The enzymatic methylation of lysine residues in muscle proteins of chicks (Krzymik *et al.*, 1971) and chicken embryo nuclei (Greenaway and Levine, 1974) was subsequently demonstrated. Paik and Kim (1971) reported that in rats the specific activity of protein methylase III was higher in liver (1.07 pmol/min per mg protein) than muscle (0.05 pmol/min per mg protein) and heart (0.42 pmol/min per mg protein). Thus the proposed mechanism involves incorporation of lysine into proteins via protein synthesis, 6-N-methylation of the protein-bound lysine to give protein-bound 6-N-trimethyl-lysine, which is subsequently released via proteolysis to 6-N-trimethyl-lysine. Aronson *et al.* (1976) reported that the intravenously injected polypeptide, asialo-fetuin containing ^{14}C-labelled 6-N-trimethyl-lysine residues rapidly accumulated in rat liver and was hydrolyzed in the lysosomes to release free radioactive 6-N-trimethyl-lysine, which was subsequently converted to carnitine. However, they found that asialo-fetuin polypeptide containing ^{14}C-labelled 6-N-monomethyl- and dimethyl-lysine also accumulated in the rat liver and was hydrolyzed in a similar manner to the ^{14}C-trimethyl-lysine-asialo-fetuin, but no

radioactivity was incorporated into carnitine.

In contrast to these proposals, Rebouche and Broquist (1976) reported enzymatic 6-N-methylation of free lysine in *Neurospora crassa* both *in vivo* and *in vitro*. These workers showed that 6-N-monomethyl- and 6-N-dimethyl-lysine as well as free lysine were discrete intermediates in the biosynthesis of carnitine in *Neurospora crassa*, and that methionine *in vivo* and s-adenosyl-methionine *in vitro* acted as the source of methyl groups. Under optimal conditions *in vitro* the methylation of 6-N-di-methyl-lysine to 6-N-trimethyl-lysine proceeded at twice the rate of methylation of 6-N-monomethyl-lysine to 6-N-dimethyl-lysine and 10 times the rate of methylation of free lysine. Borum (1976) purified the s-adenosyl-L-methionine : L-lysine 6-N-methyltransferase from *Neurospora crassa* and showed that all three methylations were catalyzed by a single protein.

Free 6-N-trimethyl-lysine (Kakimoto and Akazawa, 1970) and free 6-N-methylated lysines (Lange *et al.*, 1973) have been isolated from human plasma and urine. The concentrations of the free 6-N-methylated lysines in human plasma were lysine, 216 nmol/ml; 6-N-methyl-lysine, 5.86 nmol/ml; 6-N-dimethyl-lysine, 0.64 nmol/ml and 6-N-trimethyl-lysine, 0.42 nmol/ml (Lange *et al.*, 1973). The renal clearance of 6-N-trimethyl-lysine in man was 100 times faster than lysine (47.3 μmol/4 hr) and clearance of 6-N-dimethyl-lysine was similar to 6-N-trimethyl-lysine; 6-N-methyl-lysine clearance was similar to lysine clearance (Lange *et al.*, 1973). Wolff, Bergman and Williams (1972) reported concentrations of 6-N-methyl-lysine of 110-131 μmol/l in sheep arterial blood (c.f. lysine arterial concentrations, 65-110 μmol/l) but found that the net hepatic metabolism was so small as to be undetectable.

1.6 Objectives of this study

The tissue site of carnitine biosynthesis was investigated in rats, sheep and goats using the criterion of the presence of 4-trimethylaminobutyric acid hydroxylase activity. The substrate specificity with respect to methylated forms of 4-aminobutyric acid was also investigated in both species.

The biosynthesis of carnitine was investigated in both rat and sheep liver, using hepatocyte suspensions from these sources. The hepatocyte suspensions offered several advantages over other *in vitro* procedures such as perfused organ, tissue slice and homogenate systems. Cellular integrity and permeability barriers are maintained in the isolated, intact, hepatocyte, in contrast to homogenates. The rate of metabolic processes, in general, is substantially greater in hepatocyte suspensions as compared to tissue slices from liver and a greater number of investigations can be performed on hepatocyte suspensions as compared to perfused liver, thus allowing different experiments to be performed on tissue from the same animal, thereby reducing between animal variation. As a specific advantage in investigations into carnitine biosynthesis (where rate of synthesis is very slow), high initial specific activity of substrates can be readily achieved.

The incorporation of radioactive label from 4-aminobutyric acid and L-lysine into carnitine was compared concurrently in hepatocyte suspensions prepared from both rats and sheep. The incorporation of radioactive label from methionine into carnitine was also investigated in the same hepatocyte suspensions. Hepatocytes were also prepared from alloxan-diabetic rats, where the turnover time of carnitine was reduced (Mehlman *et al.*, 1969). Alloxan-diabetic, insulin-stabilized and alloxan-diabetic insulin-withdrawn sheep were used as a result of the studies of Snoswell

and Koundakjian (1972) and Snoswell and McIntosh (1974), who reported significant increases (up to twenty-fold in severely alloxan-diabetic) in total acid-soluble carnitine in livers from alloxan-diabetic sheep and suggested that there may be a faster biosynthetic rate for carnitine production in these animals.

2. Tissue and substrate specificity of 4-trimethylaminobutyric acid hydrolase

2.1 Materials and methods

2.1.1 Animals

The rats used were hooded Wistar females weighing 150-200 g and were fed *ad libitum* on a pelletted rat diet (Charlicks, Adelaide, S. Austral., Australia).

The sheep used were 2-year-old Merino wethers, weighing between 35-40 Kg. These animals were fed *ad libitum* on lucerne-hay chaff. 1-day-old lambs were also obtained from the abbatoirs.

The goats used were 1-year-old Chamois cross males. These animals were fed *ad libitum* on lucerne-hay chaff.

2.1.2 Alloxan-diabetic animals

Alloxan-diabetes was produced in 2-year-old Merino wethers by injecting a sterile solution of alloxan (60 mg/Kg body weight) into the jugular vein. These animals spontaneously reduced their food intakes and were killed 5-6 days later. Blood total acid-soluble carnitine had doubled in 2 days prior to slaughter.

Alloxan-diabetes was produced in female rats by an intra-peritoneal injection of a sterile solution of alloxan (250 mg/Kg body weight in 0.9% NaCl). The rats were killed 24 hr after alloxan-injection at which time blood glucose concentrations were approximately 200 mg/100 ml.

2.1.3 Tissue preparations and homogenates

All animals were killed by cervical dislocation and exsanguination.

Samples of liver, kidney cortex, heart and skeletal muscle (M. biceps femoris) from all adult animals were collected directly into 0.25 M sucrose containing 23 mM potassium phosphate (pH 7.4). Samples of liver the skeletal muscle from 1-day-old lambs were collected directly into 0.25 M sucrose containing 23 mM potassium phosphate (pH 7.4). Homogenates (20%, w/v) were then prepared in the same sucrose-phosphate buffer using a Potter-Elvehjen homogenizer in the case of liver and kidney cortex samples and a Polytron Type PT10 203500 tissue homogenizer and sonicator fitted with a PCU-2 speed control (Kinematica GmbH, Luzern, Switzerland) in the case of heart and skeletal muscle samples.

2.1.4 Assay procedure

The hydroxylating activity was assayed by incubating radioactive substrate with tissue homogenates and cofactors. The assay system was a modification of the systems described by Lindstedt *et al.* (1970) and Holme *et al.* (1968). The incubation system contained 15 mM potassium phosphate buffer (pH 7.0), (3.6 mM; 0.71 μCi) [1-^{14}C]-2-oxoglutarate, 0.6 mM ferrous sulphate, 14 mM potassium ascorbate, 1 mg catalase, 1 mM sodium arsenite, 2 mM magnesium chloride, 0.7 mM potassium chloride, 2.9 mM 4-trimethylaminobutyrate and 5-7 mg tissue protein in a

total volume of 0.7 ml. The incubation mixture was reacted in tapered ½" centrifuge tubes fitted with Subaseal caps (No. 25) supporting plastic cups containing filter paper soaked in 0.1 ml 3 N potassium hydroxide. The assay was conducted in a PI reciprocating shaking water bath model RW1812 (Paton Industries, Adelaide, Australia), set at 160 OPM and 37°C for 45 minutes. After this time 1.0 ml of 10% (w/v) TCA was added through the Subaseal cap and $^{14}CO_2$ collected for 2 hours.

2.1.5 Specificity of 4-trimethylaminobutyric acid hydroxylase

The hydroxylation of 4-aminobutyrate, 4-monomethylaminobutyrate and 4-dimethylaminobutyrate was assayed in the same manner to the hydroxylation of 4-trimethylaminobutyrate. Possible methylation or effect of s-adenosylmethionine on the hydroxylation of these substrates was investigated by the addition of 0.3 mM s-adenosylmethionine to the assay incubation.

2.1.6 $^{14}CO_2$ counting

The filter papers soaked with 0.1 ml 3 N KOH were added to 10 ml of solution containing 7.0 g PPO (2,5-diphenyloxazole) and 0.3 g dimethyl POPOP (1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene) per litre of toluene and Triton X-100 (2:1) and also 0.9 ml water in standard glass vials (Packard Instument Co., Chicago, Illinois, U.S.A.). The samples were counted in a Packard Tricarb liquid scintillation spectrometer, model 3375. Channels-ratio quench and correction curves calculated using Packard Instruments Co. standards were used to determine counting efficiency.

2.1.7 Chemicals

4-Trimethylaminobutyrate (deoxycarnitine) and 4-methylaminobutyrate were obtained from Calbiochem, Los Angeles, California, U.S.A. 4-Dimethylaminobutyrate hydrochloride was a generous gift from Dr. J. Bremer (University of Oslo, Norway). s-Adenosylmethionine was obtained from C.F. Boehringer and Soehne GmbH, Mannheim, Germany. [1-^{14}C]-2-oxoglutarate was obtained from New England Nuclear, Boston, Massachusetts, U.S.A. 2-Oxoglutarate and other chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Alloxan was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K.

2.2 Results

2.2.1 Distribution of 4-trimethylaminobutyric acid hydroxylase activity in rat, sheep and goat tissues

4-Trimethylaminobutyric acid hydroxylase activity was detected in homogenates from rat liver but there was no detectable activity in homogenates from rat kidney, heart and skeletal muscle (Table 24). The activity of 4-trimethylaminobutyric acid hydroxylase in liver homogenates was 0.49 and 0.53 nmol CO_2 produced/min per mg protein in normal and alloxan-diabetic rats respectively (Table 24). The slight increase in activity in alloxan-diabetic as compared to normal rats was not significant. These activities in rat liver are higher than those reported by Lindstedt (1967) and Erfle (1975) (0.12 and 0.39 nmol/min per mg protein respectively).

In contrast to these results 4-trimethylaminobutyric acid hydroxylase activity was detected in homogenates of liver, kidney cortex, heart and

TABLE 24

The distribution of 4-trimethylaminobutyric acid hydroxylase activity in tissues from normal and alloxan-diabetic rats

Homogenates were prepared and assayed as described in Part III, 2.1. The values are means ± S.E.M. for triplicate samples from 3 animals in each group. Activity expressed in nmols CO_2 produced/min per mg protein.

Tissue	Normal	Alloxan-diabetic
Liver	0.49 ± 0.08	0.53 ± 0.07*
Kidney	N.D.**	N.D.**
Heart	N.D.**	N.D.**
Skeletal muscle	N.D.**	N.D.**

*not significant
**N.D. - not detectable

TABLE 25

The distribution of 4-trimethylaminobutyric acid hydroxylase activity in tissues from normal and alloxan-diabetic sheep, normal goats and 1-day-old lambs

Homogenates were prepared and assayed as described in Part III, 2.1. The values are means ± S.E.M. for triplicate samples from 2 animals in each case. The enzymic activity is expressed in nmol CO_2 produced/min per mg protein.

	Sheep			
Tissue	Normal	Alloxan-diabetic	Goats	Lambs
Liver	0.20 ± 0.05	0.24 ± 0.04	0.18 ± 0.03	0.21 ± 0.04
Kidney cortex	0.17 ± 0.02	0.20 ± 0.04	0.23 ± 0.03	N.A.*
Heart	0.05 ± 0.02	0.06 ± 0.02	0.10 ± 0.02	N.A.*
Skeletal muscle	0.10 ± 0.03	0.08 ± 0.02	0.20 ± 0.04	N.A.*

*N.A. - not assayed

skeletal muscle prepared from sheep and goats (Table 25). The activity of this enzyme in normal sheep tissues was highest in the liver (0.20 nmol/min per mg protein) followed by kidney cortex (0.17 nmol/min per mg protein), skeletal muscle (0.10 nmol/min per mg protein) and heart (0.05 nmol/min per mg protein) (Table 25). There were no significant changes in activity in tissues from alloxan-diabetic sheep where again the highest detectable activity was present in liver (0.24 nmol CO_2 produced/min per mg protein) followed by kidney cortex (0.20 nmol/min per mg protein), skeletal muscle (0.08 nmol/min per mg protein) and heart (0.06 nmol/min per mg protein) (Table 25). The activity of 4-trimethyl-aminobutyric acid hydroxylase in liver homogenates from 1-day-old lambs was 0.21 nmol CO_2 produced/min per mg protein (Table 25). The 4-trimethylaminobutyric acid hydroxylase activities in tissues from normal sheep (Table 25) are similar to those reported by Erfle (1975) with the exception that Erfle did not detect any activity in heart homogenates. The distribution of 4-trimethylaminobutyric acid hydroxylase in goat tissues was similar to that in sheep tissues (Table 25). However, in goat tissues, the kidney cortex activity (0.23 nmol CO_2 produced/min per mg protein) was highest followed by skeletal muscle (0.20 nmol/min per mg protein), liver (0.18 nmol/min per mg protein) and heart (0.10 nmol/min per mg protein) (Table 25).

2.2.2 Substrate specificity of 4-trimethylaminobutyric acid hydroxylase

The results in Table 26 show that 4-dimethylaminobutyric acid and 4-monomethylaminobutyric acid were hydroxylated by rat liver homogenates at rates of approximately 11% and 5% respectively, the rate of hydroxylation of 4-trimethylaminobutyric acid (0.56 nmol CO_2 produced/

TABLE 26

The specificity of 4-trimethylaminobutyric acid hydroxylase in rat liver

Homogenates were prepared and assayed as described in Part III, 2.1. The values are means ± S.E.M. for triplicate samples from 3 animals. The activities are expressed in nmol CO_2 produced/min per mg protein.

Substrate	Additions	Activity
4-trimethylamino butyric acid	None	0.56 ± 0.06
	0.3 mM s-adenosylmethionine	0.93 ± 0.04**
4-dimethylamino-butyric acid	None	0.06 ± 0.01
	0.3 mM s-adenosylmethionine	0.13 ± 0.01**
4-monomethylamino-butyric acid	None	0.03 ± 0.006
	0.3 mM s-adenosylmethionine	0.06 ± 0.02*
4-aminobutyric acid	None	N.D.†
	0.3 mM s-adenosylmethionine	N.D.†

**Significant $P < 0.01$
*Significant $P < 0.05$
†N.D. - not detectable

min per mg protein). 4-Aminobutyric acid was not hydroxylated (Table 26).

The hydroxylation of 4-trimethylaminobutyric acid was significantly ($P < 0.01$) stimulated in both rat liver (Table 26) and sheep liver by addition of 0.3 mM s-adenosylmethionine to the assay incubation.

The amount of carbon dioxide produced from 4-dimethylaminobutyric acid and 4-monomethylaminobutyric acid was significantly greater also ($P < 0.01$ and 0.05 respectively) upon addition of 0.3 mM s-adenosylmethionine to the assay incubations (Table 26). However, the conditions of the assay did not differentiate between direct stimulation of hydroxylation or conversion of these compounds to 4-trimethylaminobutyric acid by methylation via s-adenosylmethionine and subsequent hydroxylation.

3. Biosynthesis of carnitine in rat hepatocyte suspensions

3.1 Materials and methods

3.1.1 Animals

The rats used were hooded Wistar males weighing 200-300 g. Food was withheld from rats, subsequently referred to as normal, for 18-24 hours.

3.1.2 Alloxan-diabetic animals

Alloxan-diabetes was produced in adult male rats by an intraperitoneal injection of a sterile solution of alloxan (250 mg/Kg body weight in 0.9% NaCl). The rats were used 24 hours after alloxan injection, at which time blood glucose concentrations were approximately 200 mg/100 ml.

3.1.3 Buffers

Modified calcium and glucose-free Hank's buffer was made up in a stock solution of 80.0 g NaCl, 4.0 g KCl, 0.6 g $Na_2HPO_4.2H_2O$, 0.6 g KH_2PO_4 and 2.0 g $MgSO_4.7H_2O$ per litre and stored at 4°C. The stock solution was diluted 1:9 with H_2O and gassed for 30 minutes at 37°C with 95% O_2, 5% CO_2 before the addition of $NaHCO_3$ to a final concentration of 25 mM. Thirty minutes after the addition of $NaHCO_3$ the pH was adjusted to 7.4 with 1 M $NaHCO_3$.

Modified Krebs-Henseleit bicarbonate buffer consisted of (in parts by volume) 100 parts, 0.9% NaCl; 4 parts, 1.15% KCl; 1 part, 2.11% KH_2PO_4; 1 part, 3.80% $MgSO_4.7H_2O$ and 21 parts, 1.3% $NaHCO_3$. The buffer was gassed with 95% O_2, 5% CO_2 for 1 hour at room temperature.

Krebs-Henseleit bicarbonate buffer consisted of (in parts by volume) 100 parts, 0.9% NaCl; 4 parts, 1.15% KCl; 3 parts, 1.22% $CaCl_2$; 1 part, 2.11% KH_2PO_4; 1 part, 3.80% $MgSO_4.7H_2O$ and 21 parts, 1.3% $NaHCO_3$. The buffer was gassed with 95% O_2, 5% CO_2 for 1 hour at room temperature.

Krebs Improved I consisted of (in parts by volume) 80 parts, 0.9% NaCl; 4 parts, 1.15% KCl; 3 parts, 1.22% $CaCl_2$; 1 part, 2.11% KH_2PO_4; 1 part, 3.80% $MgSO_4.7H_2O$; 21 parts, 1.3% $NaHCO_3$; 4 parts, 1.76% Na pyruvate; 7 parts, 1.16% Na fumarate; 4 parts, 2.70% Na glutamate and 5 parts, 5.40% D(+)-glucose. The buffer was gassed with 95% O_2, 5% CO_2 for 1 hour at room temperature.

3.1.4 Isolation of hepatocytes

The male rats were anaesthetized with diethyl ether until there was no eye reflex and then placed on a dissecting board. Anaethesia was maintained throughout the surgery with diethyl ether. The

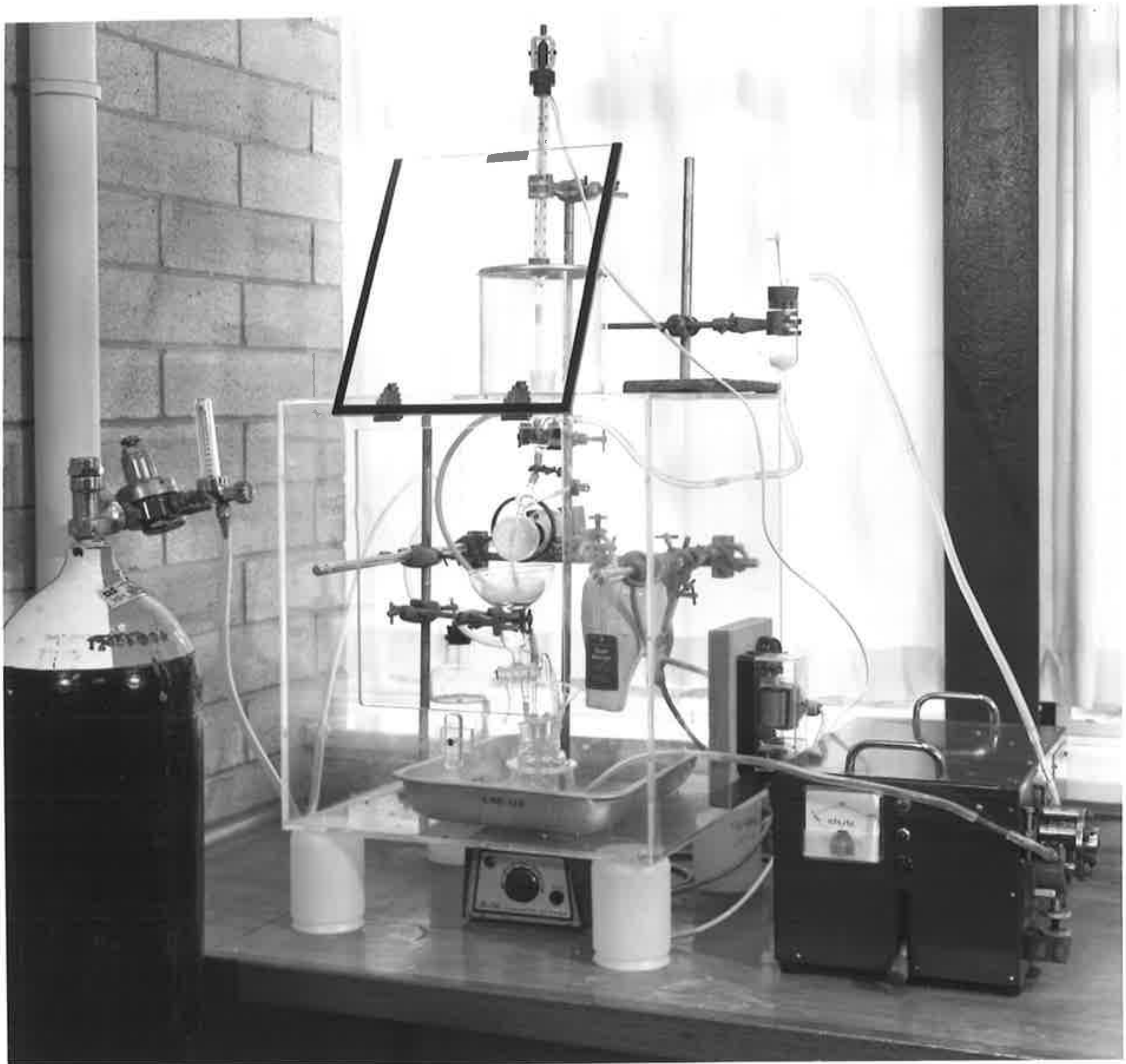
peritoneal cavity was opened by making two incisions laterally from the midline of the upper abdomen proceeding to the dorsal side. An incision was also made posteriorally along the midline and the resulting flaps of skin held back. The peritoneal cavity was then opened at the anterior end with an incision large enough to expose the viscera. The mesentery connected to the caudate lobe of the liver and the abdomen was carefully separated so as not to puncture the blood supply. The oesophagus and gastric vein were exposed, tied with two ligatures (braided silk 2/0), and then cut between the ligatures. Heparin (0.3 ml; 5000 U/ml) was injected through a 25 G, ½" needle into the posteria vena cava. A loose ligature (braided silk 2/0) was placed around the protal vein and a small incision made on the ventral surface of the portal vein behind the ligature. A small glass cannula connected to a reservoir of 50 ml of Krebs-Henseleit buffer (at 37°C), minus calcium, was rapidly inserted into the incision and secured by the ligature. The hepatic vein was cut and the liver flushed with buffer until it is cleared of blood (care was taken to exclude all air bubbles from the system). The liver was then dissected from the rat and placed in the perfusion apparatus (Figure 19).

The liver was perfused with 100 ml of modified Hank's buffer (minus calcium and glucose) for 15 minutes. 15 mg of collagenase (Sigma batch No. 94C-0019) was dissolved in 10 ml of perfusate and introduced into the medium. The liver was digested with collagenase for a further 10-13 minutes (dependent upon an even 'leaky' appearance). At the end of this period the liver was removed from the perfusion apparatus and minced gently in 50 ml of the perfusion buffer containing collagenase. The mixture was filtered through 1 mm terylene mesh with the aid of a teflon pestle. The resultant suspension was placed in a 250 ml plastic bottle (plastic apparatus was used throughout the procedure whenever possible),

FIGURE 19

Perfusion apparatus for preparation of hepatocytes from rats

The perfusion apparatus was contained in a perspex cabinet. The perfusion buffers and apparatus were maintained at 37°C (±1°C) by a hairdryer connected via a relay switch to a thermostat thermometer set at 37°C and mounted in the top buffer reservoir. A 20 cm head of pressure from the top level of buffer in the top reservoir to the perforated polythene dish contained in the perfusion bowl was maintained. A bottom reservoir (capacity of 150 ml) was fitted with a multi-socket lid through which carbogen gas (95% O_2, 5% CO_2) at 3 ℓ/min was introduced. Oxygenated buffer from the bottom reservoir was pumped to the top reservoir via a peristaltic pump (capacity of flow: 1 ℓ/min).



gassed for 1 minute with 'carbogen' (95% O_2, 5% CO_2) at 1.5 ℓ/min, rocked at 50 OPM at 37°C for 2 minutes and finally regassed under the same conditions. The suspension was then poured through 0.1 mm nylon mesh. The filtrate was centrifuged at 80 *g* for 75 seconds. The pellet was washed twice with Krebs-Henseleit buffer and finally resuspended in either Krebs-Henseleit or Krebs Improved I buffer. The final cell suspension routinely contained over 90% intact (i.e. trypan-blue excluding) cells and was not contaminated with non-parenchymal cells by more than 4% (see Figures 20 and 21). The cell suspensions were used as quickly as possible.

3.1.5 Synthesis of glucose from lactate, fructose and alanine by hepatocyte suspensions

These experiments were carried out at 37°C in a Paton-PI reciprocating water bath (Model RW1812, Paton Industries, Adelaide, Australia) set at 100 oscillations per minute. Incubations were performed in plastic scintillation vials and contained in a final volume of 1.0 ml; 0.3 ml cell suspension (in Krebs-Henseleit buffer), 10 mM calcium-L(+)-lactate, L-alanine or D-fructose, and Krebs-Henseleit buffer. The vials were flushed with 95% O_2, 5% CO_2 and sealed. The reactions were stopped with 1.0 ml of 1.8% perchloric acid at 30 minute intervals up to 2.5 hours. Glucose was assayed in the deproteinized supernatants according to the method of Bergmeyer and Bernt (1974). A standard curve for glucose was prepared on each occasion.

3.1.6 The conversion of labelled precursors to carnitine by hepatocyte suspensions

The incubations were carried out at 37°C in a Paton-PI

FIGURE 20

Hepatocytes from normal rats (magnification x 100) isolated as described in Part III, 3.1.4.

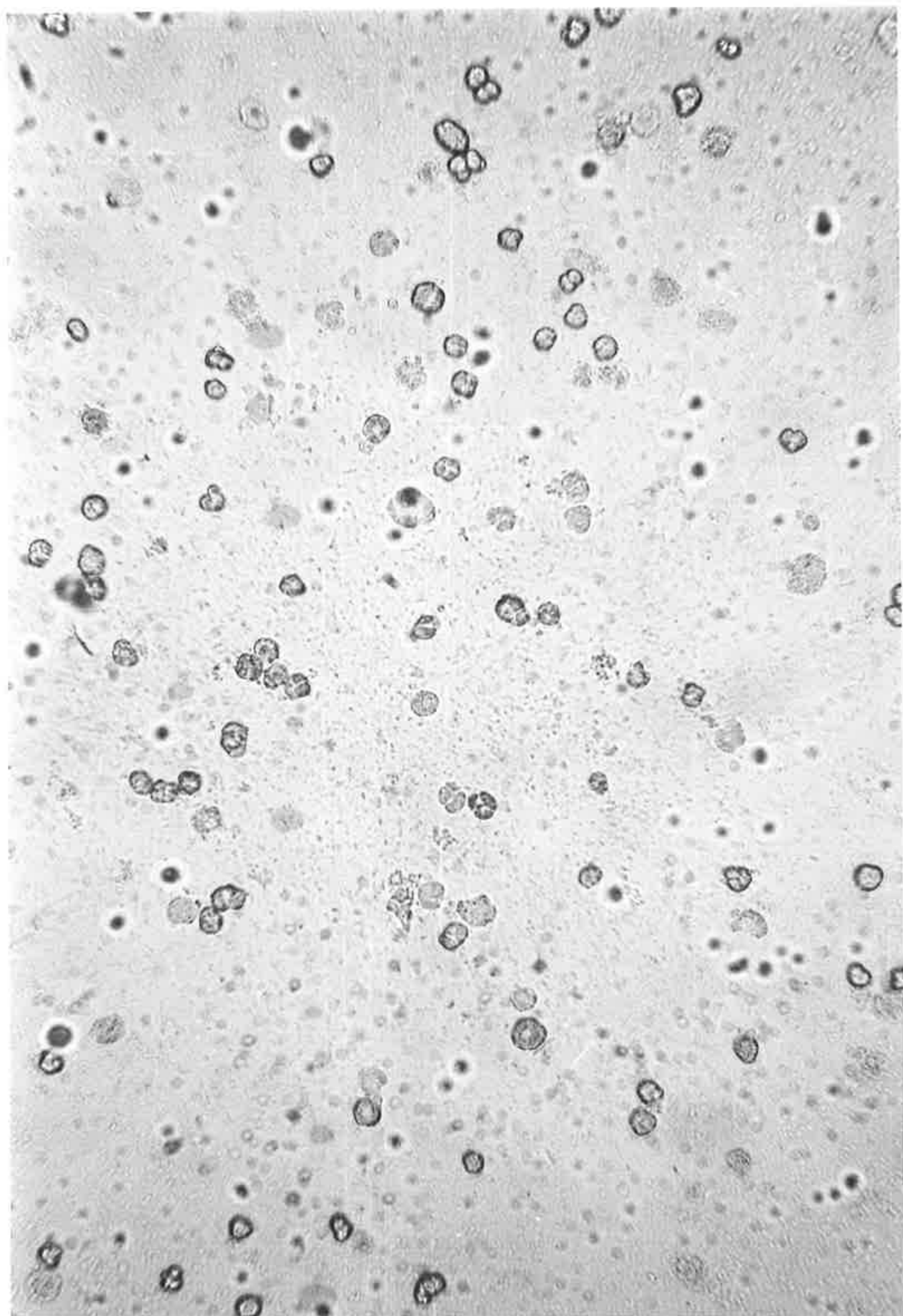
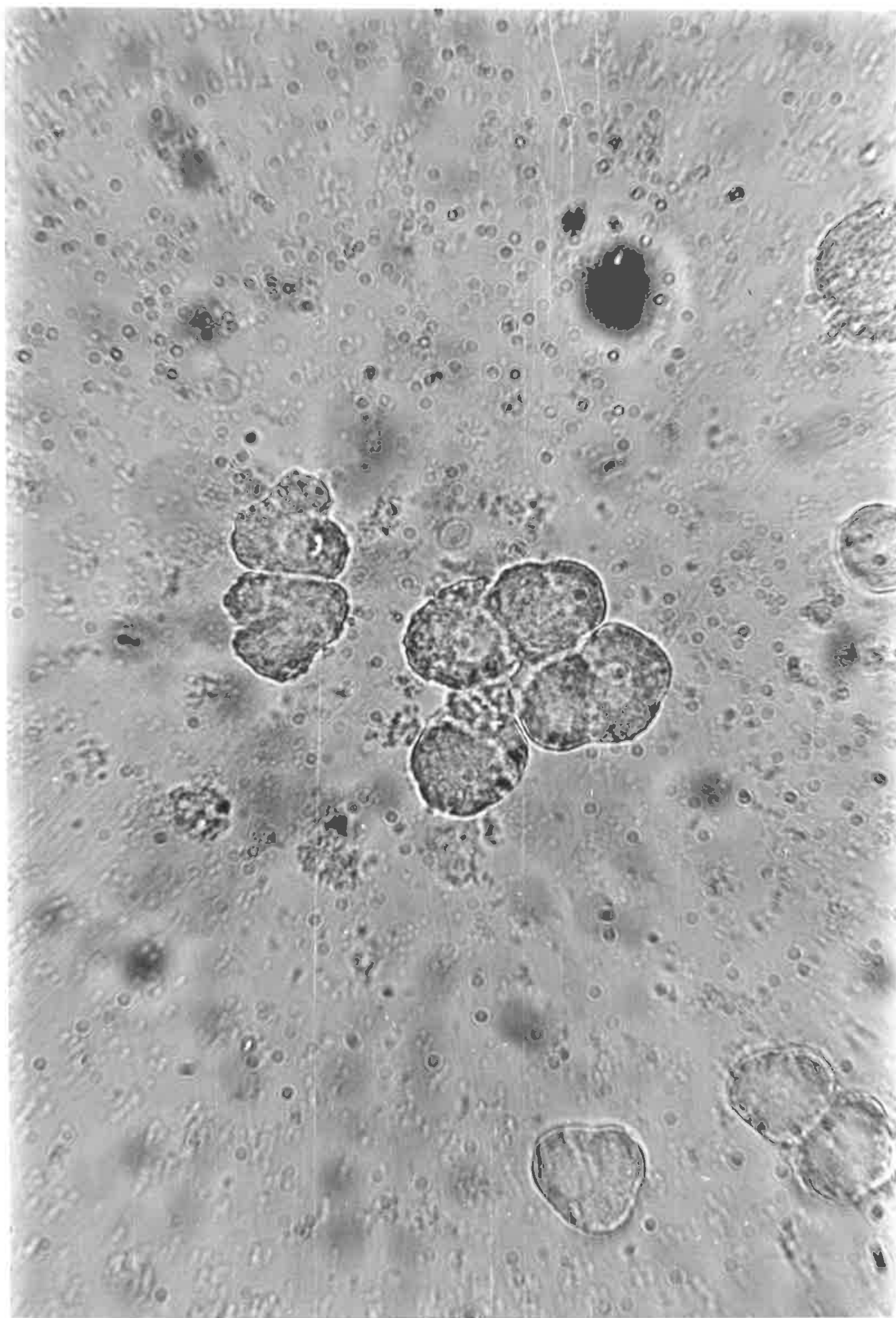


FIGURE 21

Hepatocytes from normal rats (magnification x 400) isolated by the method as described in Part III, 3.1.4.



reciprocating water bath set at 100 oscillations per minute. Plastic scintillation vials contained in a final volume of 1.0 ml; 0.8 ml hepatocyte suspension (mean 25 mg protein/ml) in Krebs Improved I buffer and the combination of substrates; (1 mM, 3.5 μCi) [U-^{14}C]-4-amino-butyrate and 3 mM L-methionine (1 mM, 3.5 μCi) [U-^{14}C]-4-aminobutyrate, 3 mM L-methionine and 1 mM iso-nicotinyl hydrazide (INH); (1 mM, 3 μCi) [1-^{14}C]-4-aminobutyrate and 3 mM L-methionine; (1 mM, 1.5 μCi) [U-^{14}C]-L-lysine and 3 mM L-methionine; (1 mM 1.5 μCi) [1-^{14}C]-L-lysine and 3 mM methionine; 1 mM 4-aminobutyrate, 1 mM L-lysine and (3 mM, 6 μCi) [^{3}H-methyl]-L-methionine. The vials were flushed with 95% O_2, 5% CO_2 and sealed with Subaseal caps (No. 33) supporting plastic cups containing filter paper soaked in 0.1 ml 3 N KOH in the case of ^{14}C-labelled substrates. The reactions were stopped with 1.0 ml 10% (w/v) TCA at 30 minute intervals up to 2.5 hours. $^{14}CO_2$ was collected for 2 hours.

3.1.7 $^{14}CO_2$ counting

The radioactivity contained in the KOH soaked filter paper was counted as described in Part III, 2.1.6.

3.1.8 Separation of labelled products using thin-layer chromatography

The deproteinized supernatants from the incubations were extracted 4-5 times with diethyl ether to remove the TCA. The supernatants were then freeze-dried and brought to a final volume of 50 μl, which was chromatographed.

4-Aminobutyrate and L-carnitine and 4-trimethylaminobutyrate were separated on a 0.2 mm Silica Gel 60 analytical plates (Art 5533, Merck, Damstadt, West Germany) developed to a height of 15 cm with methanol/

concentrated ammonia (75:25). Resolution was increased by the use of 100 nmols of unlabelled 'carrier' standard for each compound. Rf values are shown in Table 27.

The N-methylated forms of 4-aminobutyric acid were not adequately resolved using Silica Gel G, so 4-aminobutyrate, 4-monomethylaminobutyrate and 4-dimethylaminobutyrate were separated on 0.2 mm cellulose analytical plates (Merck, Damstadt, West Germany) developed to a height of 15 cm with methanol/dioxan/concentrated ammonia (30:45:25). Resolution was increased by the use of 50 nmols of unlabelled 'carrier' standard for each compound. Rf values are shown in Table 27.

L-lysine and L-carnitine and 4-trimethylaminobutyrate were separated on 0.2 mm cellulose analytical plates developed to a height of 15 cm with methanol/dioxan/concentrated ammonia (30:45:25). 50 nmols of unlabelled 'carrier' standard was used. Rf values are shown in Table 28.

L-Methionine, L-carnitine and 4-trimethylaminobutyrate were separated on 0.2 mm Silica Gel analytical plates developed to a height of 15 cm with methanol/concentrated ammonia (75:25). Rf values are shown in Table 27.

3.1.9 Measurement of radioactivity

After visualization with iodine, appropriate spots were scraped from the plates and radioactivity eluted with 1.0 ml water. The 1.0 ml of water was then added to glass scintillation vials containing 10 ml of scintillation fluid consisting of the same Toluene:Triton X-100 mixture described in Part III, 2.1.6. The vials had routinely been background counted, and vials showing greater than 60 cpm were rejected. The samples were counted in a Packard Tricarb liquid scintillation

TABLE 27

Separation and identification of 4-aminobutyric acid, N-methylated products and methionine by thin-layer chromatography

Compound	Rf values ($\ell = 15$ cm)	
	methanol:dioxan:ammonia 30 : 45 : 25 0.2 mm cellulose plates	methanol:ammonia 75 : 25 0.2 mM silica gel G60 plates
4-aminobutyric acid	0.46	0.42
4-monomethylamino-butyric acid	0.56	0.45
4-dimethylamino-butyric acid	0.71	0.48
4-trimethylamino-butyric acid	0.42	0.17
L-carnitine	0.46	0.19
L-methionine		0.83

TABLE 28

Separation and identification of L-lysine, and L-carnitine by thin-layer chromatography

Compound	Rf values (ℓ = 15 cm) methanol:dioxan:ammonia (30:45:25) 0.2 mm cellulose plates
L-lysine	0.36
L-carnitine	0.46
5-hydroxyl-L-lysine	0.21
1,5-diaminopentane	0.72

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spectrometer, Model 3375. Channels-ratio and quench correction curves calculated using Packard Instruments Co. standards were used to determine counting efficiency.

3.1.10 Chemicals

L-Lysine, L-methionine and 4-aminobutyric acid were recrystallized from products obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Collagenase Type 1 (Batch No. 94C-0019) was obtained from Sigma Chemical Co. L-carnitine was generously supplied by Otsuka Pharmaceuticals, Osaka, Japan. 4-Dimethylaminobutyrate hydrochloride was generously supplied by Dr. J. Bremer (University of Oslo, Norway). 4-Trimethylaminobutyrate and 4-methylaminobutyrate were obtained from Calbiochem, Los Angeles, California, U.S.A. Alloxan was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. 4-[U-^{14}C]-aminobutyric acid, L-[U-^{14}C]-lysine, L-[^{3}H-methyl]-methionine, DL-[1-^{14}C]-lysine were obtained from the Radiochemical Centre, Amersham, England. 4-[1-^{14}C]-aminobutyric acid was obtained from ICN, Cleveland, Ohio, U.S.A.

3.2 Results

3.2.1 Synthesis of glucose by hepatocytes isolated from rats

The technique described in Part III, 3.1.4 yielded a high percentage of morphologically intact, isolated liver cells as judged by the criteria of visual intactness (Figures 20 and 21) and exclusion of trypan-blue (>90% exclusion).

As pointed out by Hems *et al.* (1966), a stringent test of the metabolic integrity of the liver cell is its ability to synthesize glucose from lactate, since this anabolic process involves both the

TABLE 29

Synthesis of glucose by hepatocytes isolated from rat liver

Hepatocytes were incubated at 37°C in Krebs-Henseleit medium.

Substrate was added at a final concentration of 10 mM.

Incubation time (min)	Wet weight of cells (mg)	Substrate	Rate of glucose formation (μmols/min per g wet weight)	
			24 hr starved rats	alloxan-diabetic rats
150	17.39	fructose	3.70	
		lactate	3.11	
180	12.95	alanine	2.18	
		lactate	2.64	
180	9.99	lactate		10.51
180	12.21	lactate		8.22
180	14.80	lactate		7.23

mitochondrial and cytoplasmic fractions of the cell working in unison. Accordingly, the synthetic ability of isolated hepatocytes was examined. For the study of glucose synthesis, cells were prepared from the livers of 24 hour starved and alloxan-diabetic rats.

The results in Table 29 show that hepatocytes isolated from both 24 hour starved and alloxan-diabetic rats synthesized glucose from lactate for incubation periods up to 180 minutes. The rate of glucose formation from lactate in hepatocytes from 24 hour starved rats was 3.11 and 2.64 μmols/min per g wet weight of cells (Table 29). These rates of synthesis are higher (two-fold) than those reported by Cornell *et al.* (1974). There was a significant increase ($P < 0.01$) in the rate of glucose formation from lactate in hepatocytes from alloxan-diabetic rats (see Table 29). The rate of glucose formation from fructose in hepatocytes from 24 hour starved rats was greater than the glucose formation from lactate (see Table 29), in agreement with the findings of Berry and Friend (1969). The glucose synthesized from alanine was only slightly less than that from lactate in hepatocytes from 24 hour starved rats (Table 29).

3.2.2 The conversion of ^{14}C-labelled 4-aminobutyric acid to carnitine by hepatocyte suspensions

The results shown in Figure 22 indicate that 4-aminobutyric acid was taken up and metabolized to carbon dioxide by hepatocyte suspensions. Carbon dioxide production from 4-aminobutyric acid was greatly reduced in the presence of the transamination inhibitor, isonicotinyl hydrazide (Figure 22). The rate of carbon dioxide production was greater in hepatocytes isolated from alloxan-diabetic as compared to normal rats (Figure 22). The ^{14}C-labelled carbon dioxide produced

FIGURE 22

The rates of production of carbon dioxide from 4-aminobutyric acid taken up by hepatocytes isolated from rats

Hepatocytes were prepared and incubated as described in Part III, 3.1. The $^{14}CO_2$ produced was counted as described in Part III, 2.1.6. The values shown are means of duplicate samples for each time of incubation obtained from 3 normal and 3 alloxan-diabetic rats.

INH: isonicotinylhydrazide

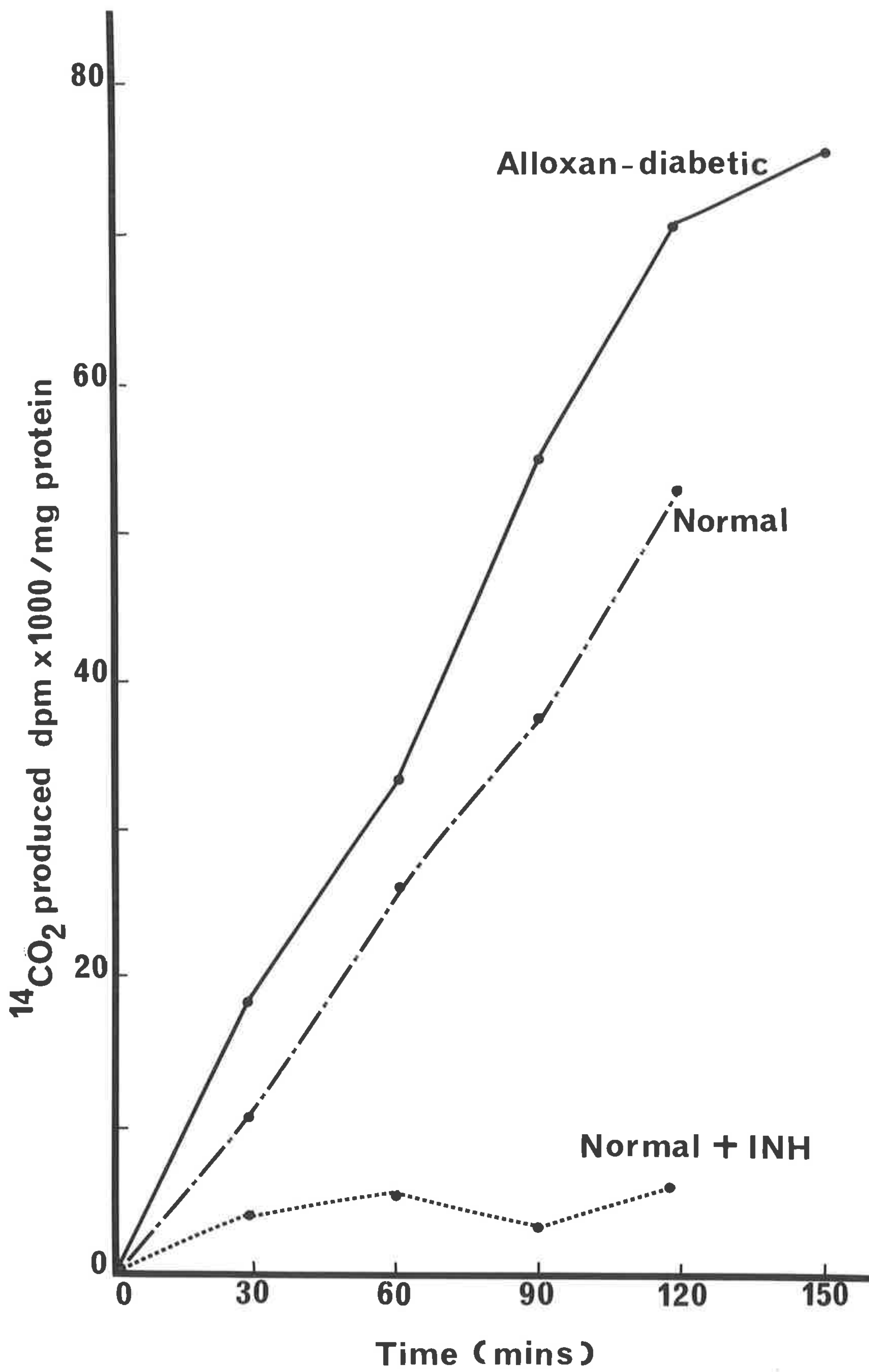


Figure 22

accounted for almost 22% and 26% of the initial radioactivity of the [U-^{14}C]-4-aminobutyric acid incubated with hepatocytes from normal and alloxan-diabetic rats respectively.

The 4-aminobutyric acid taken up by the hepatocytes suspensions for normal rats was also successively N-methylated to form 4-dimethyl-aminobutyric acid and L-carnitine (Figure 23). The N-methylation of 4-aminobutyric acid to form 4-dimethylaminobutyric acid appeared to proceed at a faster rate than the subsequent methylation and hydroxylation of 4-dimethylaminobutyric acid to form carnitine (Figure 23). However, the 4-dimethylaminobutyric acid may have been hydroxylated non-specifically by 4-trimethylaminobutyric acid hydroxylase to form 4-dimethylamino-3-hydroxybutyric acid (see Table 26) at a faster rate than it was methylated to form 4-trimethylaminobutyric acid. The chromatography system used (as described in Part III, 3.1.8) did not separate 4-dimethyl-aminobutyric acid and 4-dimethylamino-3-hydroxybutyric acid and indeed Bremer (1962*b*) and Lindstedt and Lindstedt (1965) reported great difficulty in attempting to separate these two compounds. The rates of synthesis of 4-dimethylaminobutyric acid from [U-^{14}C] and [1-^{14}C]-4-aminobutyric acid were similar in hepatocyte suspensions isolated from normal rats (Figure 24), as were the rates of carnitine synthesis (not shown).

4-Aminobutyric acid was N-methylated via 4-monomethyl and 4-dimethyl-aminobutyric acids to form 4-trimethylaminobutyric acid and carnitine at a faster rate in hepatocytes isolated from alloxan-diabetic rats (Figures 25 and 26) as compared to normal rats (Figure 23). 4-Monomethylamino-butyric acid was readily methylated to 4-dimethylaminobutyric acid in the hepatocyte suspensions isolated from alloxan-diabetic rats (Figures 25 and 26). 4-Dimethylaminobutyric acid was also readily methylated to 4-trimethylaminobutyric acid (and subsequent hydroxylation to carnitine) by hepatocyte suspensions from alloxan-diabetic rats (Figure 25) which

FIGURE 23

The rates of synthesis of 4-dimethylaminobutyric acid and carnitine from 4-aminobutyric acid in hepatocytes isolated from normal rats

The hepatocytes were prepared and incubated as described in Part III, 3.1. ^{14}C-labelled 4-dimethylaminobutyric acid and carnitine were separated from [U - ^{14}C] 4-aminobutyric acid (3.52 μCi) by thin-layer chromatography as described in Part III, 3.1.8 and counted as described in Part III, 3.1.9. The values shown were means of duplicate incubations obtained from 3 normal rats.

DMAB: 4-dimethylaminobutyric acid

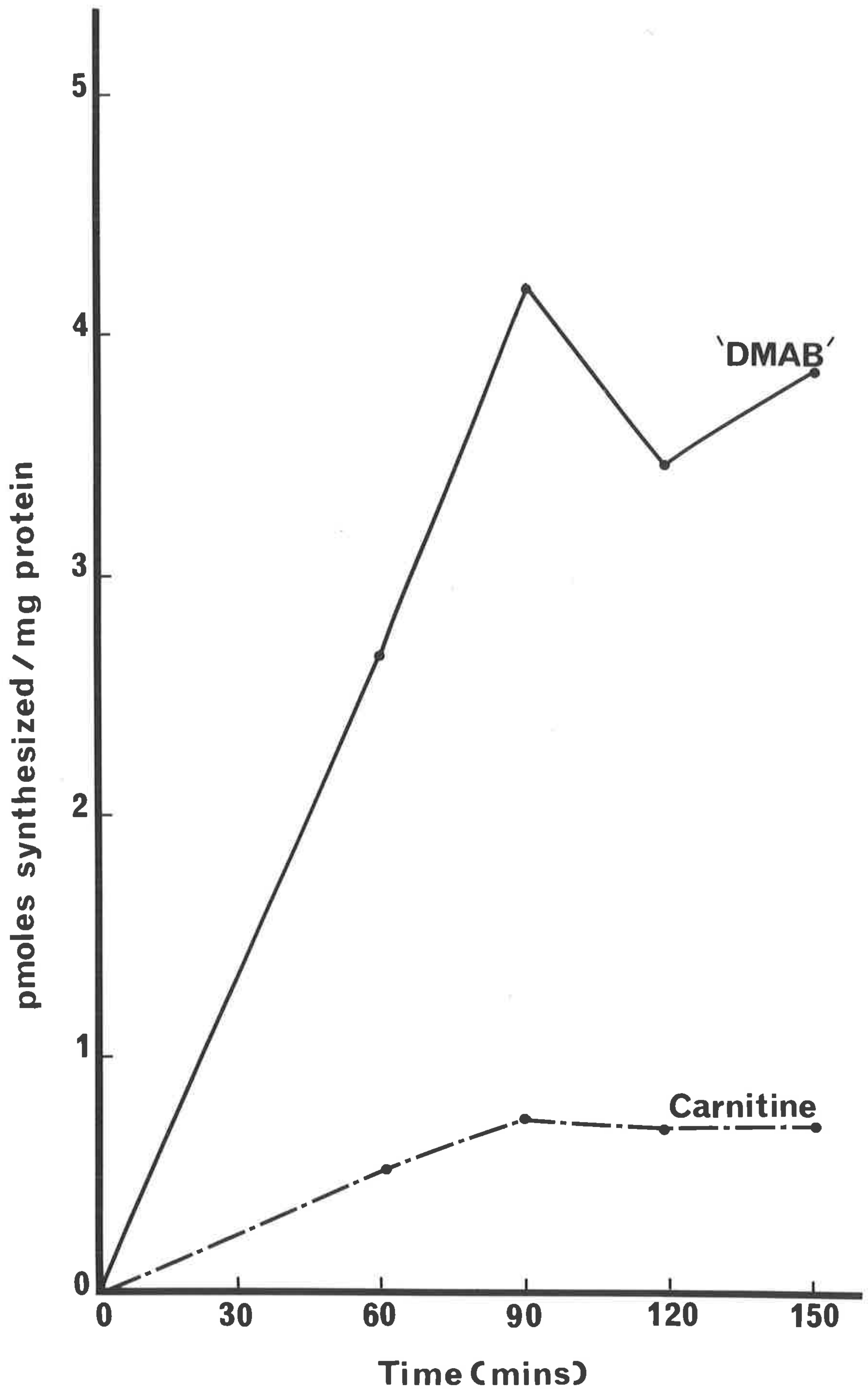


Figure 23

FIGURE 24

The rates of synthesis of 4-dimethylaminobutyric acid from [1 - ^{14}C]-and [U - ^{14}C]-4-aminobutyric acid in hepatocytes isolated from normal rats

Hepatocytes were prepared and incubated as described in Part III, 3.1, with [U - ^{14}C]-4-aminobutyric acid (3.52 μCi) and [1 - ^{14}C]-4-aminobutyric acid (3.0 μCi). ^{14}C-labelled 4-dimethylaminobutyric acid and 4-aminobutyric acid were separated by thin-layer chromatography as described in Part III, 3.1.8 and counted as described in Part III, 3.1.9. The values shown were means of duplicate incubations obtained from 2 normal rats.

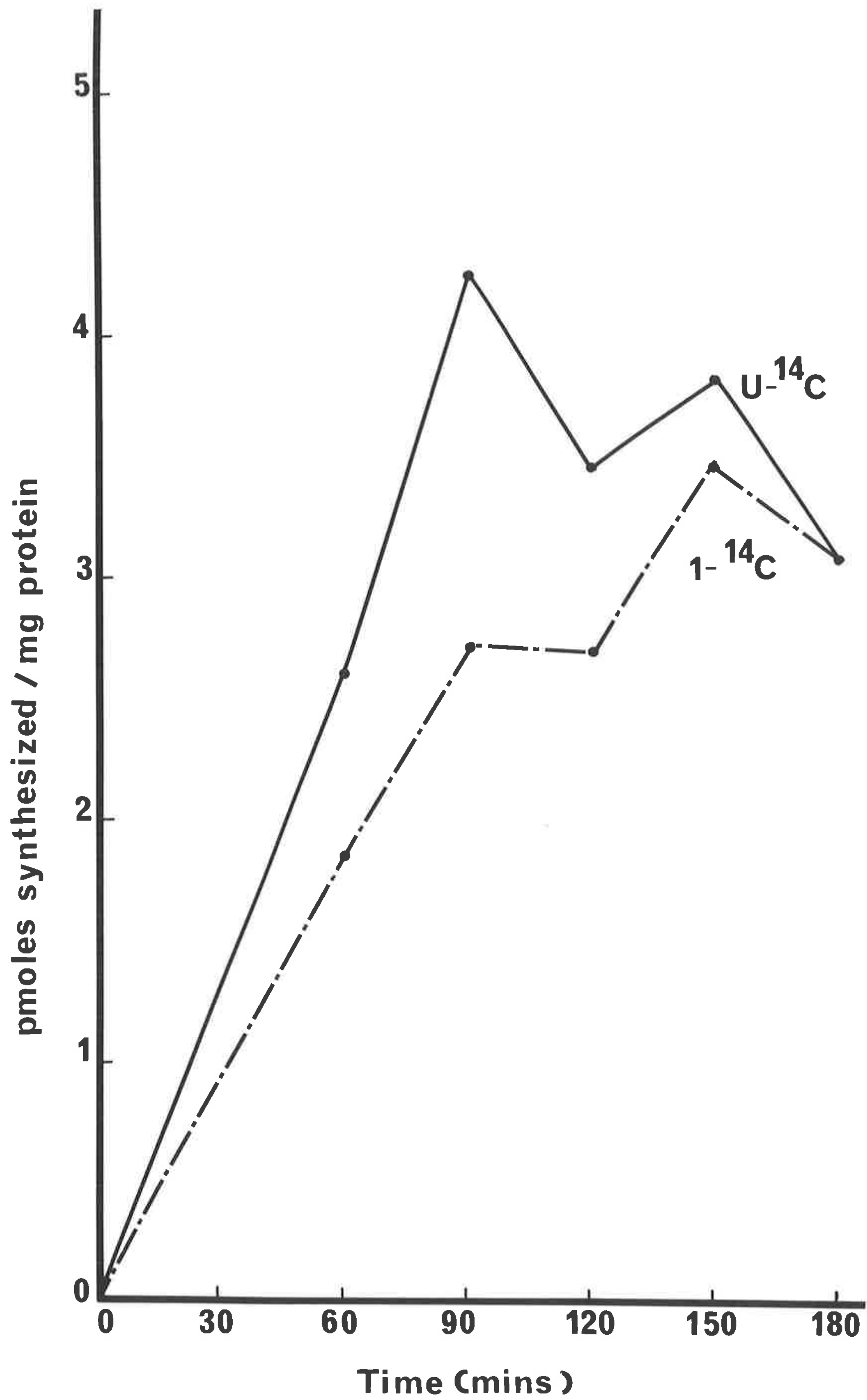


Figure 24

FIGURE 25

The rates of synthesis of 4-monomethylaminobutyric acid, 4-dimethylaminobutyric acid and carnitine from 4-aminobutyric acid in hepatocytes isolated from alloxan-diabetic rats

Hepatocytes were prepared and incubated with [U - ^{14}C] 4-aminobutyric acid (3.18 μCi) as described in Part III, 3.1. ^{14}C-labelled 4-monomethyl- and 4-dimethylaminobutyric acid and carnitine were separated from 4-aminobutyric acid by thin-layer chromatography as described in Part III, 3.1.8 and counted as described in Part III, 3.1.9. The values shown were means of duplicate samples obtained from 3 alloxan-diabetic rats.

MMAB: 4-monomethylaminobutyric acid

DMAB: 4-dimethylaminobutyric acid

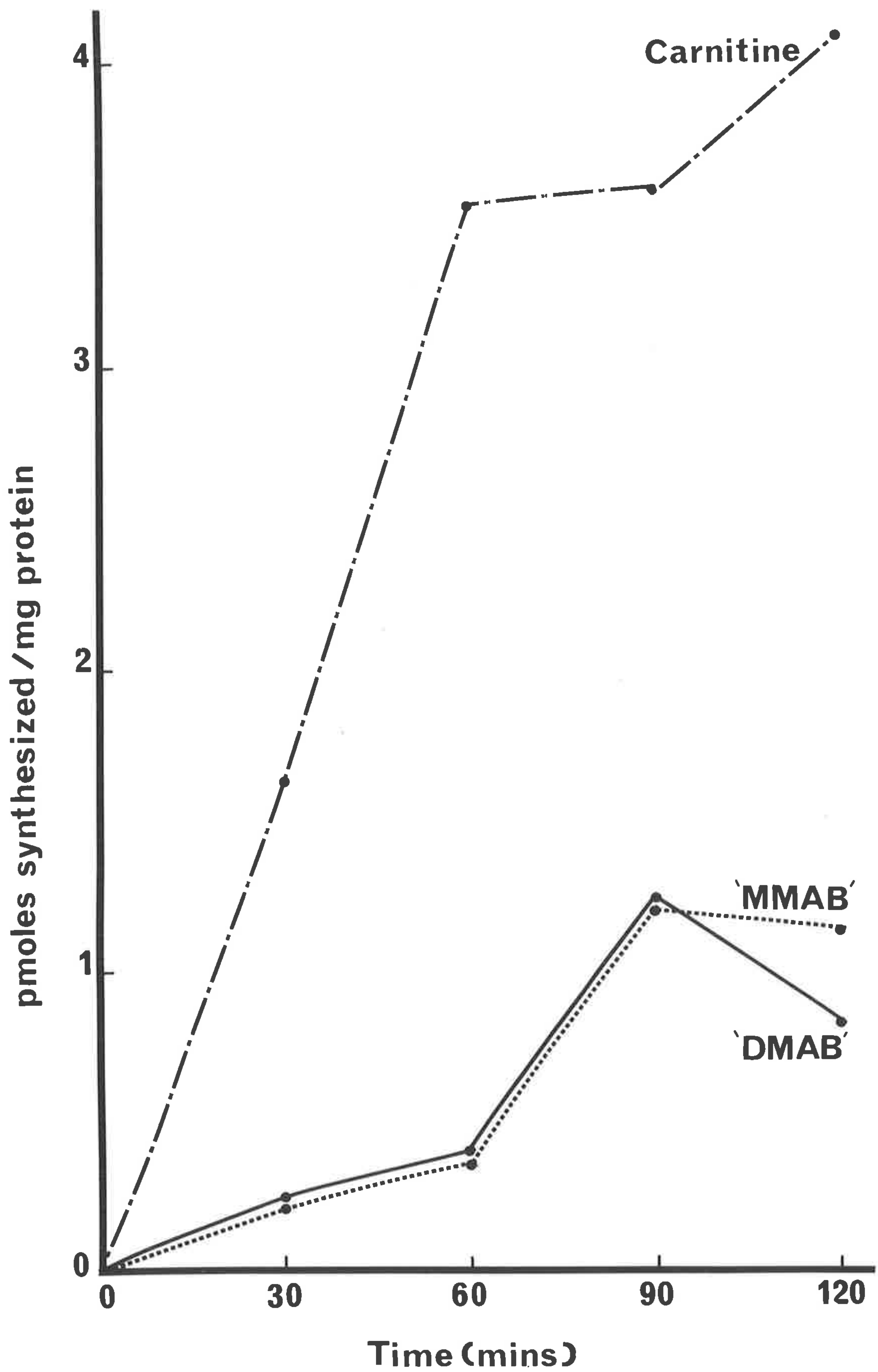


Figure 25

FIGURE 26

The rates of synthesis of 4-monomethyl- and 4-dimethylamino-butyric acid and carnitine from 4-aminobutyric acid in hepatocytes isolated from an alloxan-diabetic rat

Materials and methods were as described in Figure 25. The values shown were means of duplicate samples obtained from one alloxan-diabetic rat.

MMAB: 4-monomethylaminobutyric acid

DMAB: 4-dimethylaminobutyric acid

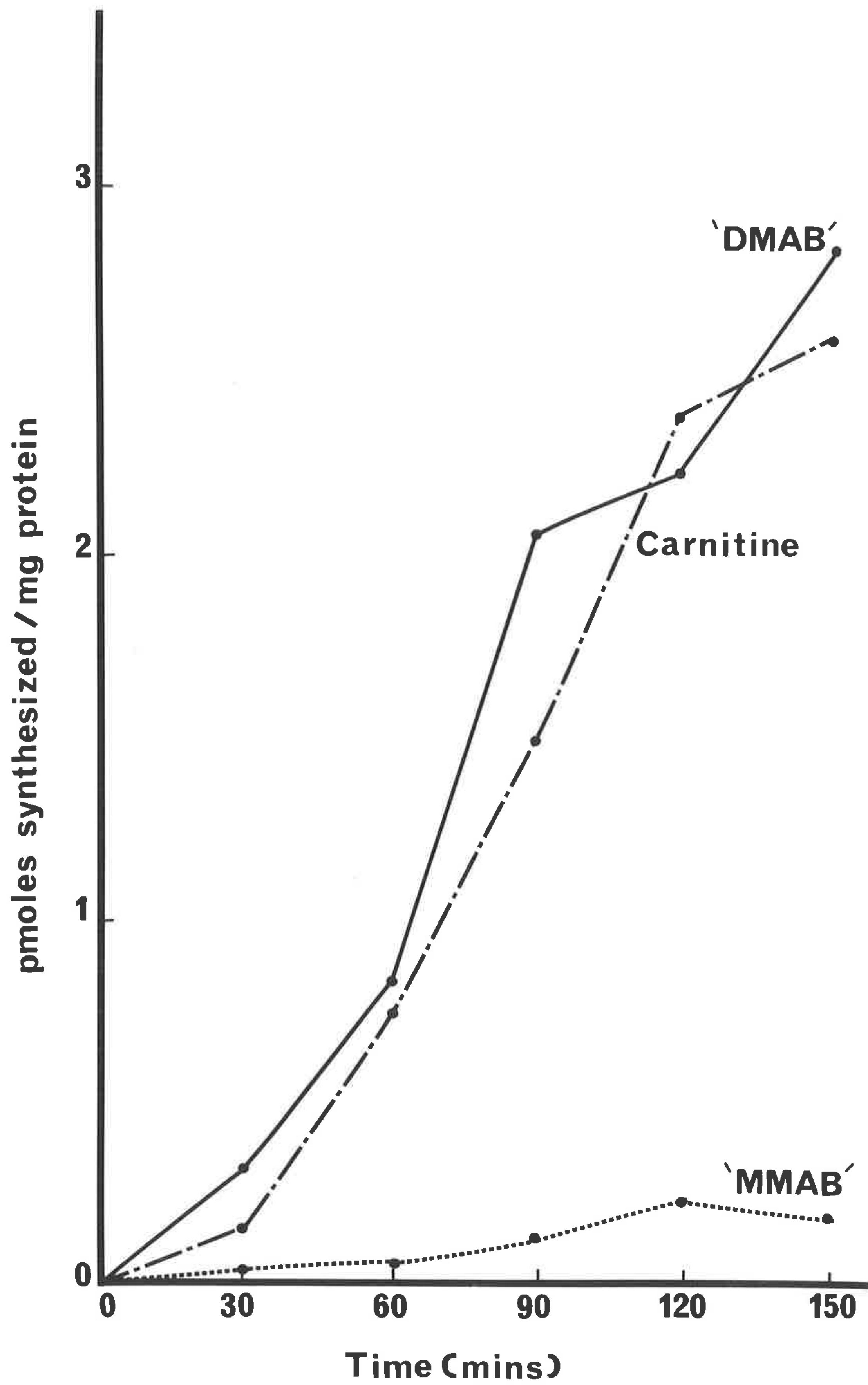


Figure 26

FIGURE 27

The rates of synthesis of carnitine from 4-aminobutyric acid in hepatocytes isolated from normal and alloxan-diabetic rats

Hepatocytes were prepared from normal and alloxan-diabetic rats as described in Part III, 3.1.4, and incubated with [U - ^{14}C] 4-aminobutyric acid as described in Figures 23 and 25 respectively. The values shown were means of duplicate samples obtained from 3 normal and 3 alloxan-diabetic rats.

A - D: alloxan-diabetic

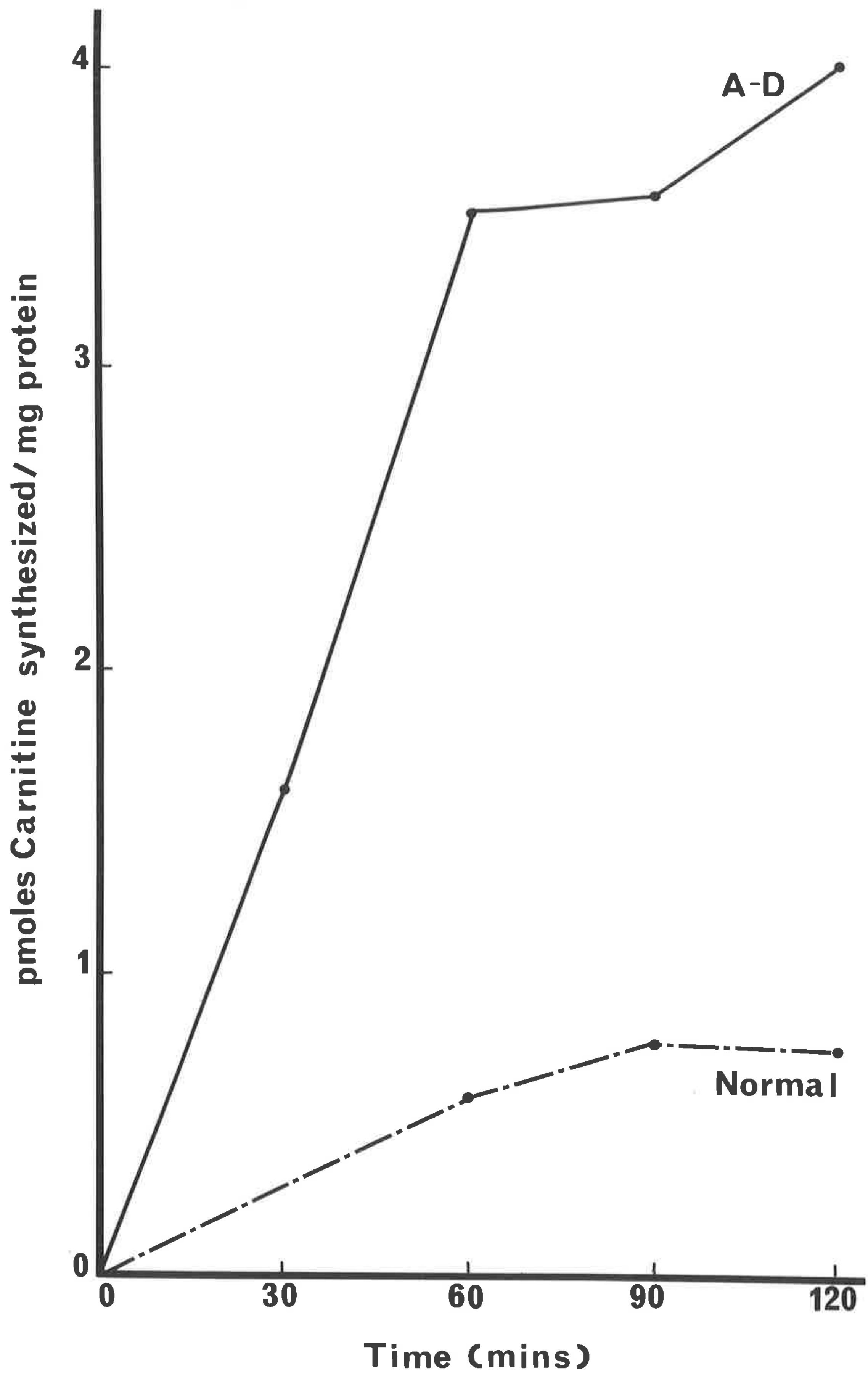


Figure 27

was in contrast to the results from normal rats (Figure 23). The results presented in Figure 26 were taken from one alloxan-diabetic rat where 4-dimethylaminobutyric acid was not as readily converted to carnitine and thus there was a significantly greater accumulation of 4-dimethylaminobutyric acid (and possibly 4-dimethylamino-3-hydroxybutyric) per mg protein in these incubations than in the hepatocytes incubations from the other alloxan-diabetic rats described in Figure 25. The rate of synthesis of carnitine from 4-aminobutyric acid was almost five- to six-fold greater in hepatocyte suspensions isolated from alloxan-diabetic as compared to normal rats (Figure 27).

3.2.3 The conversion of ^{14}C-labelled-lysine to carnitine by hepatocyte suspensions

L-[U-^{14}C]-lysine was taken up and metabolized to carbon dioxide by hepatocytes isolated from normal rats (Figure 28). L-[1-^{14}C]-lysine was also metabolized to carbon dioxide by normal rat hepatocytes. Approximately 25% of the L-[U-^{14}C]-lysine was converted to $^{14}CO_2$ after 150 minutes.

Radioactivity from L-[U-^{14}C]- but not DL-[1-^{14}C]-lysine was incorporated into carnitine by both normal and alloxan-diabetic rat hepatocytes.

The rate of synthesis of carnitine from L-[U-^{14}C]-lysine was almost five- to six-fold faster in hepatocytes isolated from alloxan-diabetic as compared to normal rats (Figure 29).

FIGURE 28

The rate of production of carbon dioxide from lysine in hepatocytes isolated from normal rats

The hepatocytes were prepared as described in Part III, 3.1.4, and incubated with L-[U - ^{14}C] lysine (1.57 μCi) as described in Part III, 3.1.6. $^{14}CO_2$ was counted as described in Part III, 3.1.7. The values shown were means obtained from 3 normal rats.

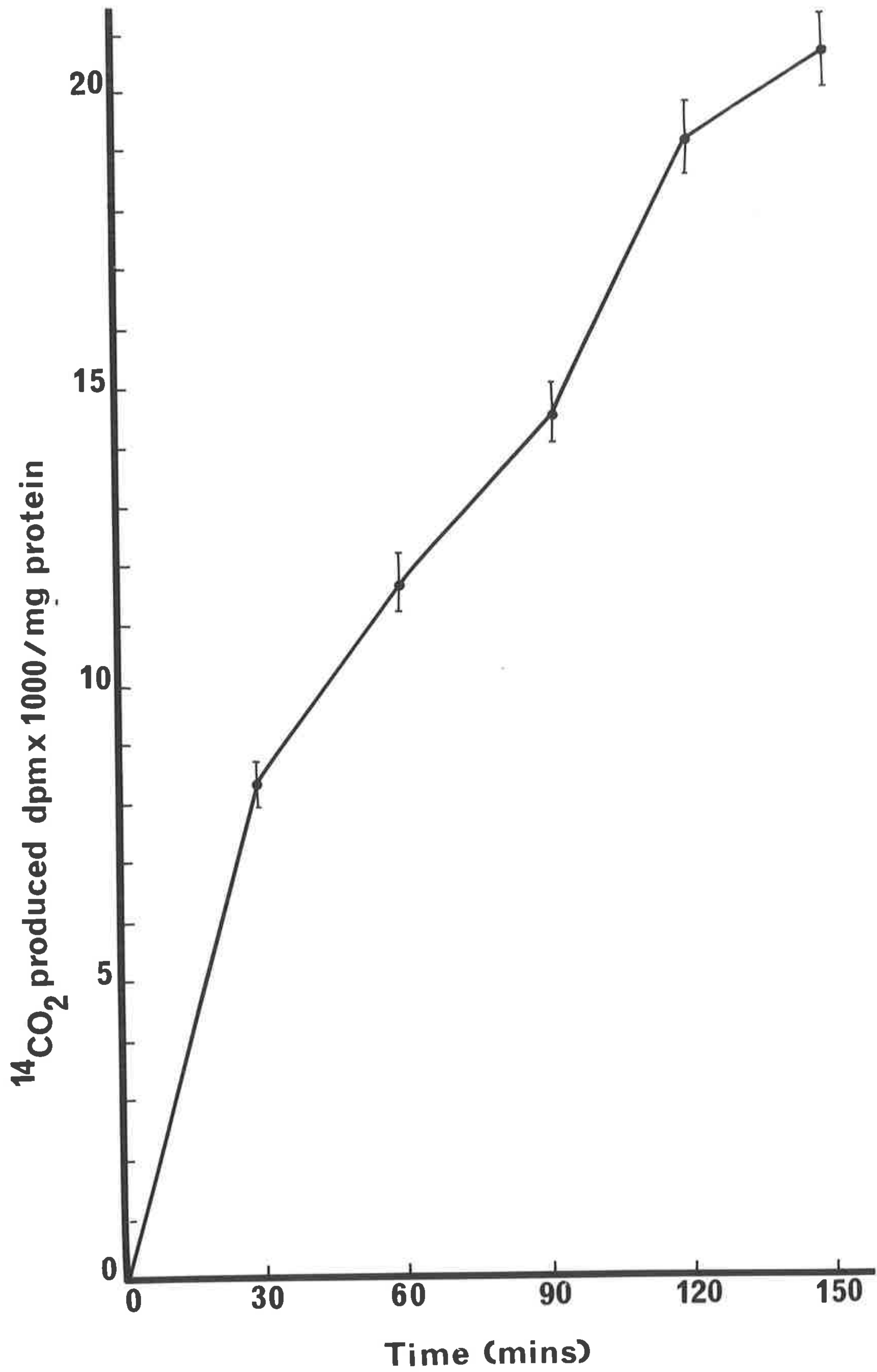


Figure 28

FIGURE 29

The rates of synthesis of carnitine from L-lysine in hepatocytes isolated from normal and alloxan-diabetic rats

Hepatocytes were prepared as described in Part III, 3.1.4, and incubated with L-[U - ^{14}C] lysine (1.57 μCi) as described in Part III, 3.1.6. ^{14}C-labelled carnitine was separated from L-lysine as described in Part III, 3.1.8 and counted as described in Part III, 3.1.9. The values shown were means obtained from 3 normal and 3 alloxan-diabetic rats.

A - D: alloxan-diabetic

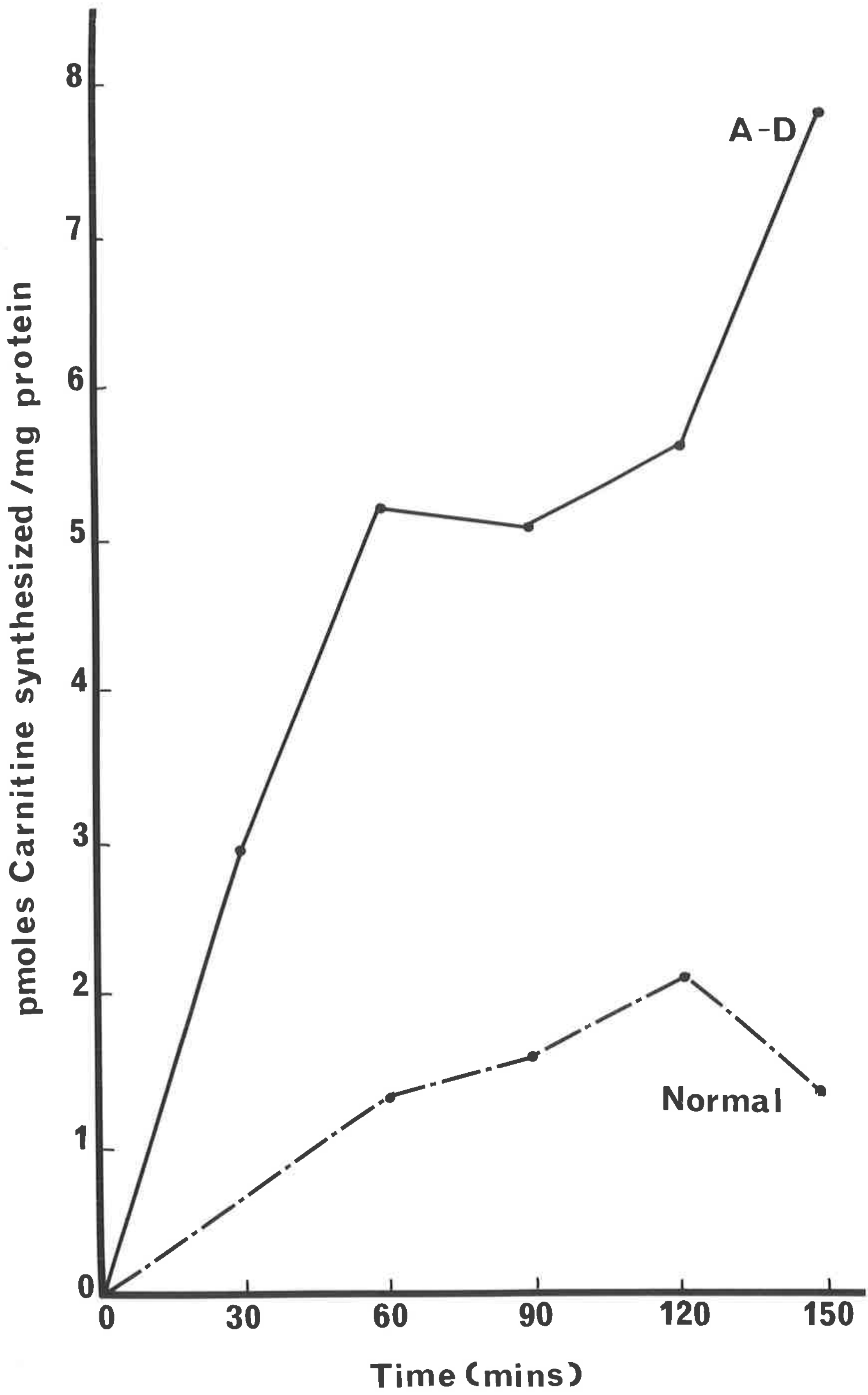


Figure 29

3.2.4 Comparison of the rates of synthesis of carnitine from L-[U-^{14}C]-lysine and [U-^{14}C]-4-aminobutyric acid by hepatocyte suspensions

L-[U-^{14}C]-lysine and [U-^{14}C]-4-aminobutyric acid were incubated in tandem in all hepatocyte preparations to minimize variation (between animals) and to allow direct comparison of these compounds as precursors of carnitine. The rate of synthesis of carnitine from lysine was almost three-fold faster than the corresponding rate from 4-aminobutyric acid in normal rat hepatocytes. In alloxan-diabetic rat hepatocytes the rate of synthesis of carnitine from lysine was two-fold greater than the corresponding rate from 4-aminobutyric acid (Figure 30).

3.2.5 The incorporation of ^{3}H from L-[methyl-^{3}H]-methionine into carnitine by hepatocyte suspensions

^{3}H-label from L-[methyl-^{3}H]-methionine was incorporated into carnitine at a rate of 2.9 pmols/methyl group of carnitine per pmol of carnitine per mg protein after 150 minutes incubation in hepatocytes isolated from alloxan-diabetic rats (Figure 31). L-[methyl-^{3}H]-methionine was incubated in tandem with L-[U-^{14}C]-lysine and [U-^{14}C]-4-aminobutyric acid in all hepatocyte preparations from alloxan-diabetic rats. The L-[methyl-^{3}H]-methionine was incubated with both 4-aminobutyric acid and L-lysine which resulted in a rate of carnitine synthesis of 8 pmols/mg protein after 120 minutes as compared to a combined rate of 9.23 pmols/mg protein for 4-aminobutyric acid and L-lysine after 120 minutes.

FIGURE 30

Comparison of the rates of synthesis of carnitine from 4-aminobutyric acid and L-lysine in hepatocytes isolated from alloxan-diabetic rats

The values shown were obtained from Figures 27 and 29.

GABA: 4-aminobutyric acid

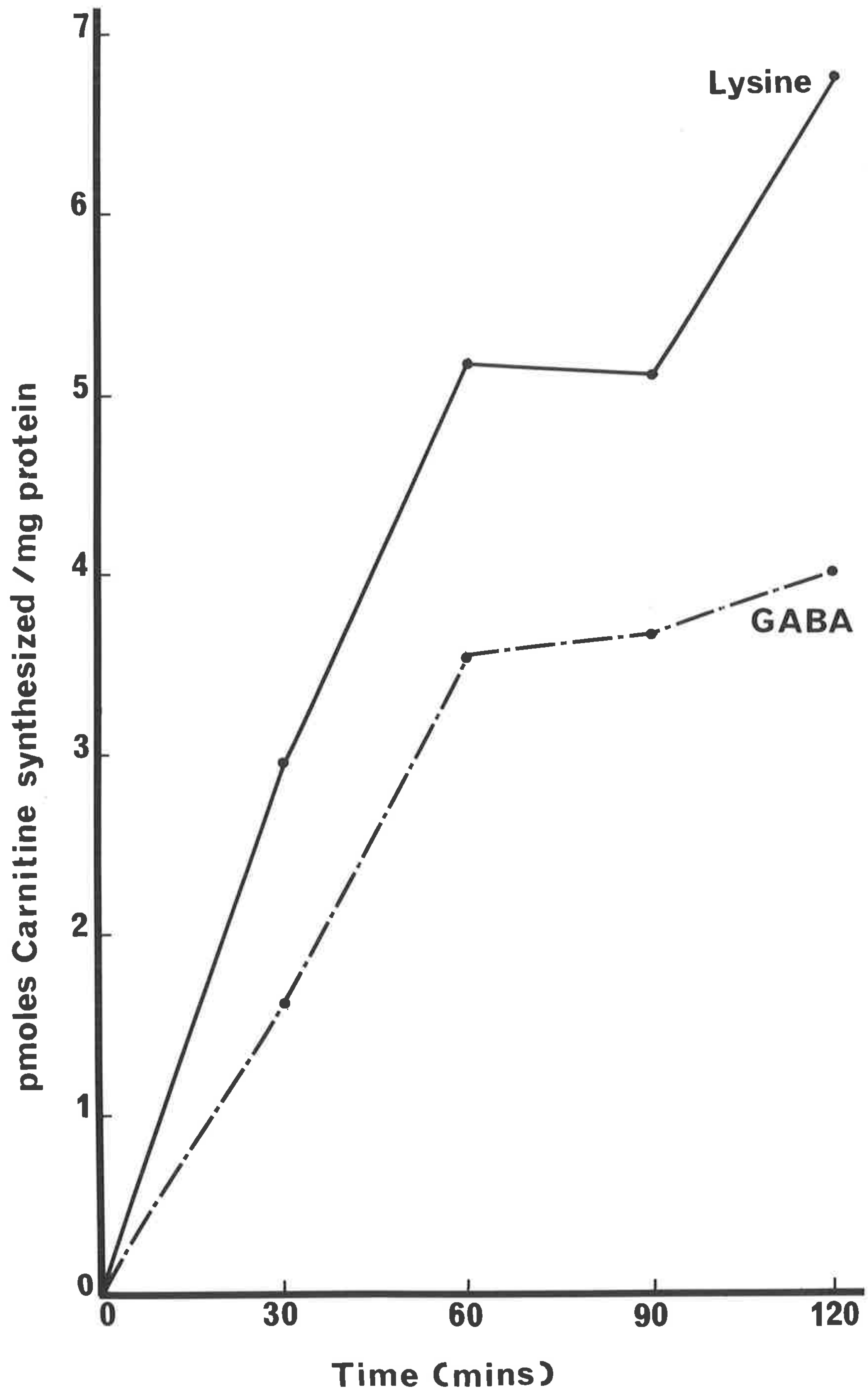


Figure 30

FIGURE 31

The rate of incorporation of methionine into carnitine by hepatocytes isolated from alloxan-diabetic rats

Hepatocytes were prepared as described in Part III, 3.1.4, and incubated with L-[methyl-^{3}H]-methionine (6 μCi) as described in Part III, 3.1.6. ^{3}H-labelled carnitine and methionine were separated as described in Part III, 3.1.8 and counted as described in Part III, 3.1.9. The values shown were rates of incorporation of methionine per methyl group of carnitine obtained from 3 alloxan-diabetic rats.

[CH_3]: Methyl group of carnitine

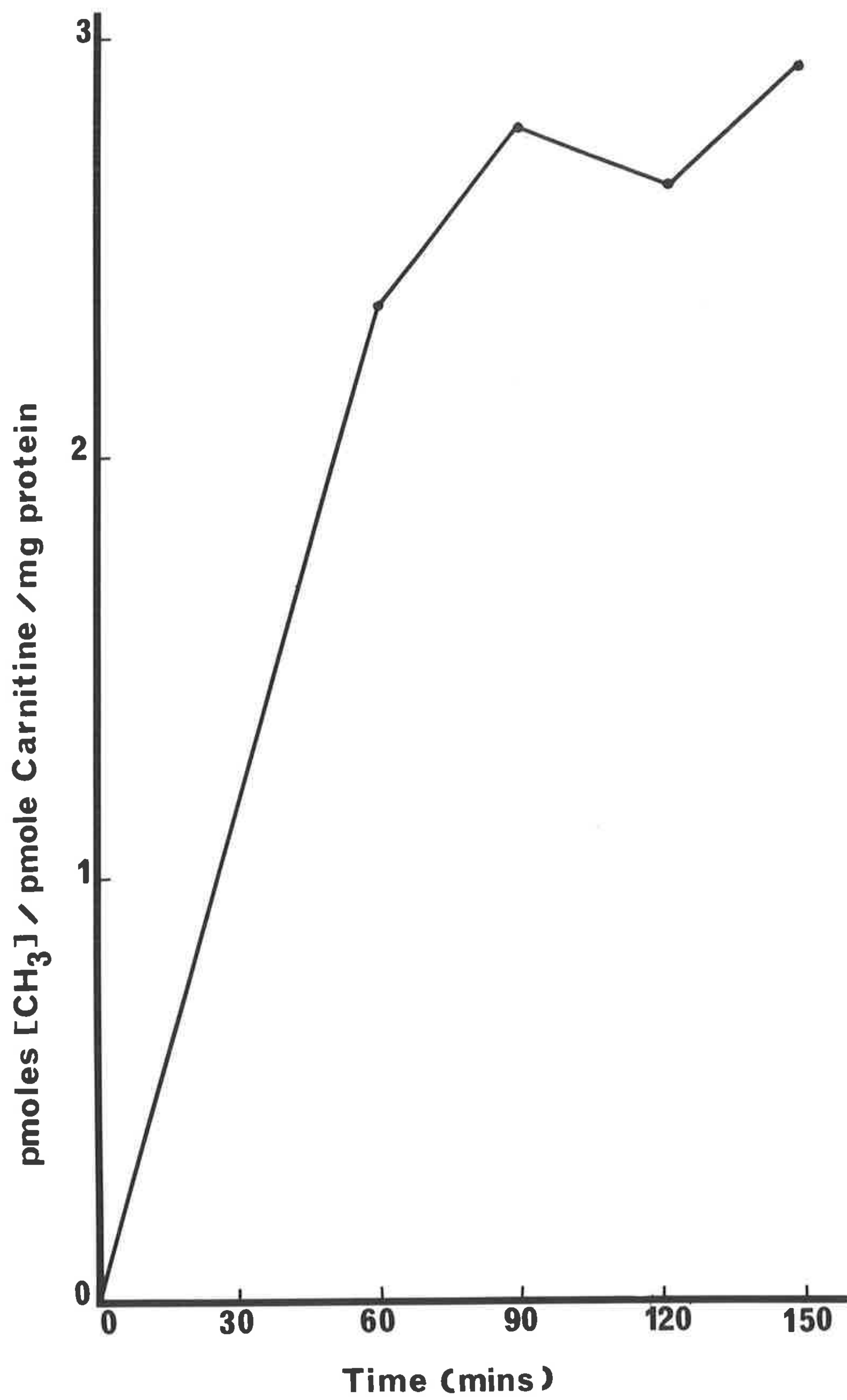


Figure 31

4. Biosynthesis of carnitine in sheep hepatocyte suspensions

4.1 Materials and methods

4.1.1 Animals

The sheep used were 2-year-old Merino wethers or non-pregnant ewes weighing between 35-40 Kg. Animals were fed *ad libitum* on lucerne-hay chaff.

4.1.2 Alloxan-diabetic animals

Alloxan-diabetes was produced by injecting a sterile solution of alloxan (60 mg/Kg body weight in 0.9% NaCl) into the jugular vein. The animals which spontaneously reduced their food intake were chosen for this study. When food intake had fallen to 300 g per day the animals were given 20 units Lente Novo Insulin (Insulin Zinc Suspension BP 40 U/ml, Novo Industrials, Copenhagen, Denmark) subcutaneously per day until food intake had increased to 1000 g per day. These animals are referred to in the text as alloxan-diabetic, insulin-stabilized or 'stabilized' animals and were slaughtered when food intake of 1000 g per day had been maintained for approximately 1 week. Other animals maintained at 20 units insulin per day and 1000 g food intake per day for 1 week were withdrawn from insulin 24 hours prior to slaughter. These animals are referred to in the text as alloxan-diabetic, insulin-withdrawn or 'withdrawn' animals.

4.1.3 Surgical cannulation of blood vessels

The portal vein, hepatic vein and carotid artery of 2-year-old Merino non-pregnant ewes were cannulated with polyvinyl tubes as described in Part II, 3.1.2.

4.1.4 Blood samples

Blood samples for metabolite assays were drawn from the cannulae simultaneously. Blood fractions of 2.0 ml were immediately added to 2.0 ml (15%, v/v) perchloric acid.

4.1.5 Buffers

A modified Krebs-Henseleit bicarbonate buffer was prepared essentially as described in Part III,3.1.3 but with the addition of a final concentration of 10 mM sodium propionate. The digestion buffer was basically a modified Kreb-Henseleit bicarbonate buffer as described in Part III,3.1.3 but with the addition of 10 mM sodium propionate and 2% defatted bovine serum albumin.

The resuspension buffer consisted of 2.25 g NaCl; 0.115 g KCl; .0527 g KH_2PO_4; 0.095 g $MgSO_4.7\ H_2O$; 0.682 g $NaHCO_3$ and 4.875 g gelatin in 325 mls water. The gelatin had replaced calcium chloride in supplying the physiological calcium concentration and had the advantage of reducing cell 'clumping' and adhesion (Katz *et al.*, 1975).

All buffers were gassed for 1 hour with 95% O_2, 5% CO_2 at room temperature.

4.1.6 Preparation and isolation of hepatocytes

The method of preparation and isolation of hepatocytes from sheep was modified from a method outlined by Dr. R. Ash (personal communication). The sheep were killed by severing the necks. The peritoneal cavity was opened as rapidly as possible and the liver removed. The 'capsule' was taken off the liver which was then bathed in oxygenated modified Krebs-Henseleit with 10 mM propionate. Slices of liver, varying

in thickness between 0.25-1.00 mm maximum, were cut by hand using siliconized skin graft knife blades (Eschmann, England).

Three g slices (approximately) were placed in 10 ml of oxygenated digestion buffer contained in 125 ml plastic pots. Collagenase (8-10 mg) and hyaluronidase (5 mg) were added to each pot and the pots were then flushed with 95% O_2, 5% CO_2 and sealed. Each pot (approximately 6 per experiment) was fixed in a PI reciprocating shaking water bath set at 37°C and 150 OPM and incubated for 20 minutes. At the end of this period $CaCl_2$ (to full strength of Krebs-Henseleit-bicarbonate buffer medium) was added, the pots reflushed with 95% O_2 and incubated for a further 20 minutes. The mixture was then filtered through 1 mm mesh terylene cloth and 0.1 mm nylon mesh. The filtrate was centrifuged at 40 *g* for 2.5 minutes and the pellet washed twice with resuspension Krebs-Henseleit-gelatin buffer. The cell suspension routinely contained over 90% intact (i.e. trypan-blue excluding) cells and was not contaminated with non-parenchymal cells by more than 4% (see Figures 32 and 33). Hepatocytes isolated from 'withdrawn' animals appeared to contain a lower percentage of intact cells and were generally more difficult to prepare. This finding appeared to be related to the degree of fat accumulation in the liver after withdrawal of insulin (see Figure 34). The cell suspensions were used as quickly as possible.

4.1.7 The conversion of propionate to glucose by hepatocyte suspensions

The incubations were carried out at 37°C in a PI reciprocating water bath set at 100 OPM. Plastic scintillation vials contained in a final volume of 1.0 ml; 0.3 ml cell suspension 10 mM sodium propionate and Krebs-Henseleit gelatin buffer. The vials were

FIGURE 32

Hepatocytes prepared from insulin-stabilized alloxan-diabetic sheep (magnification x 100) as described in Part III, 4.1.6.

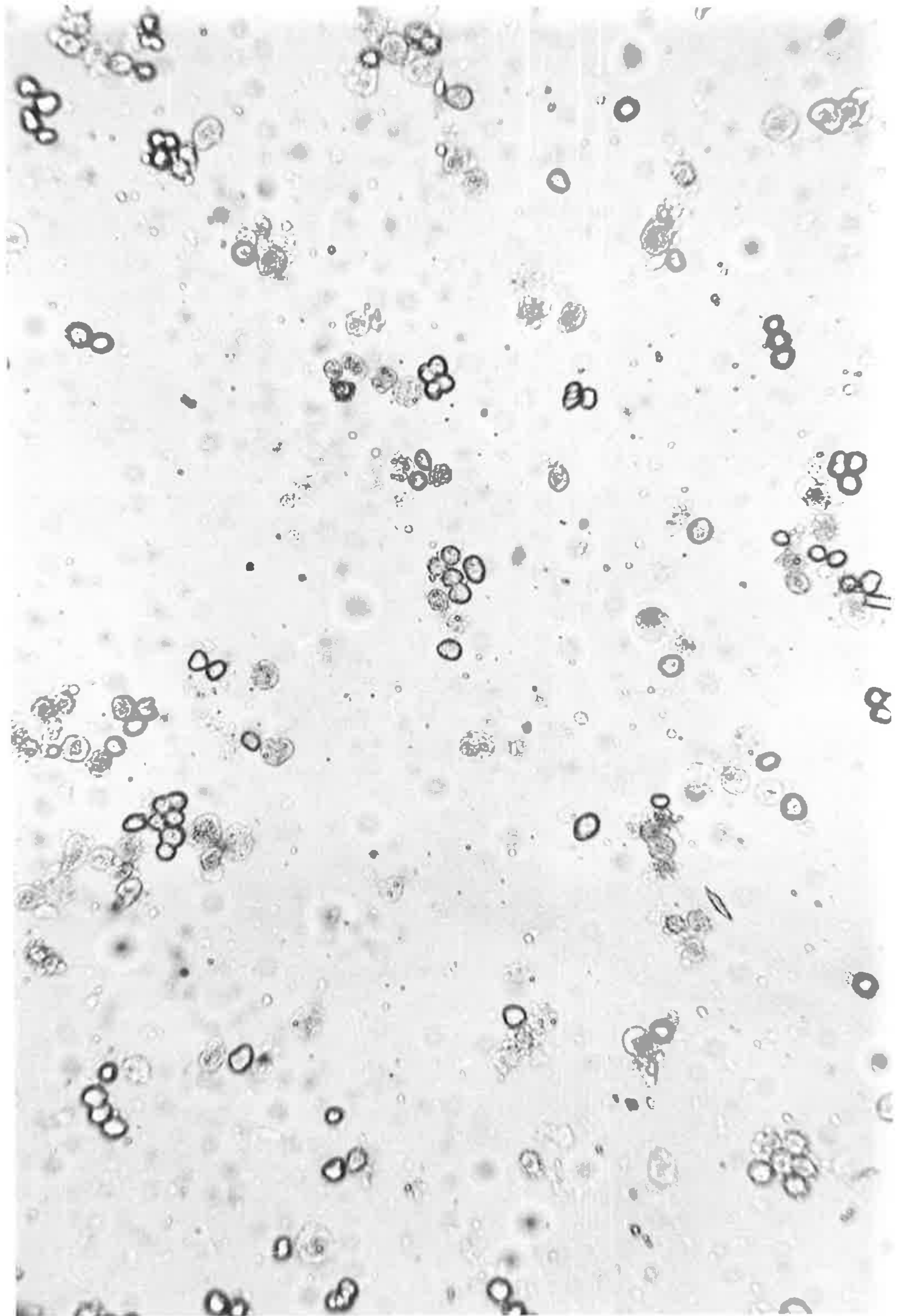


FIGURE 33

Hepatocytes prepared from alloxan-diabetic, insulin-stabilized sheep (magnification x 400) as described in Part III, 4.1.6.

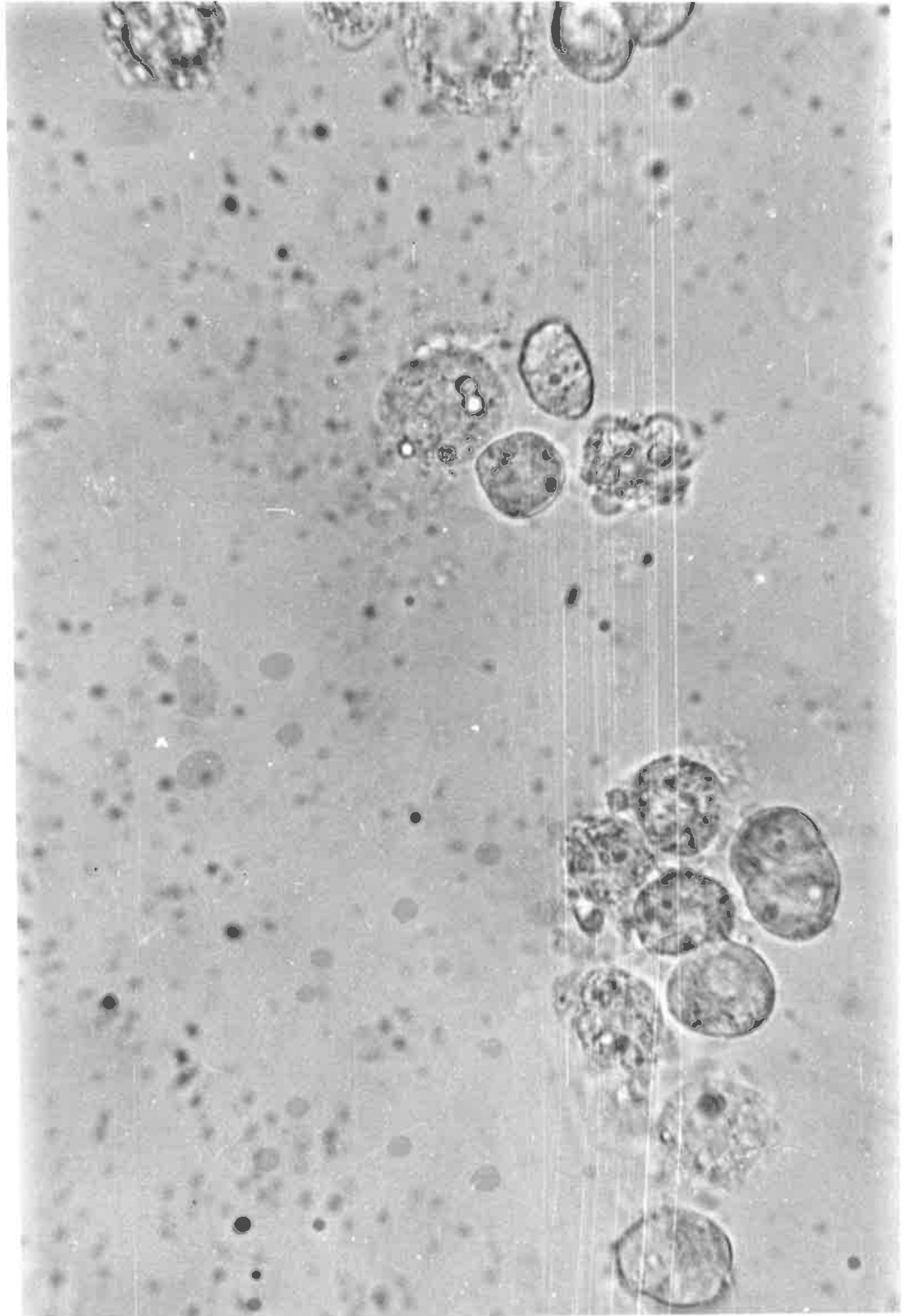


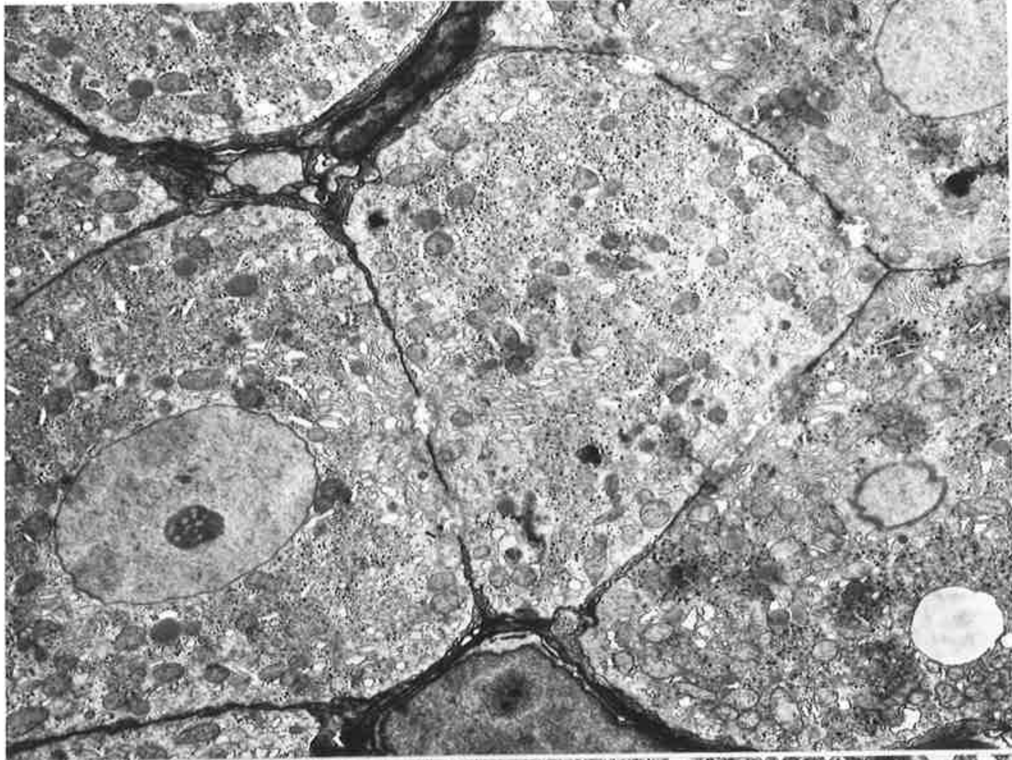
FIGURE 34

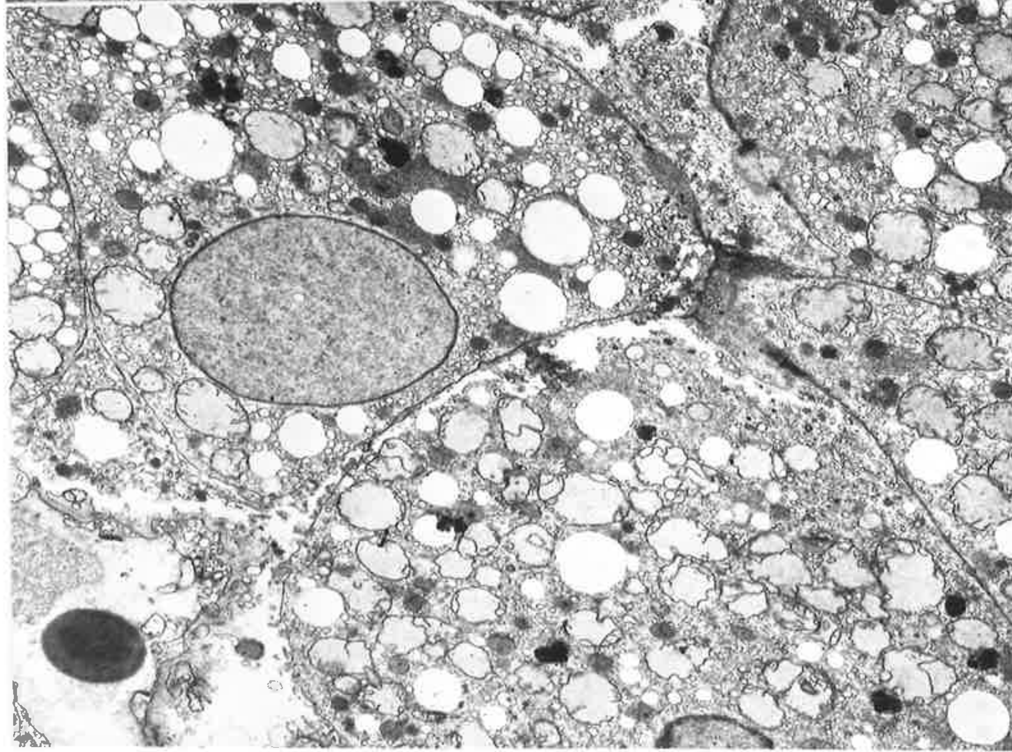
Electron-micrographs prepared from liver of

top: normal sheep

bottom: alloxan-diabetic sheep

(by courtesy of Dr. J. Wallace, Department of Biochemistry, University of Adelaide).





flushed with 95% O_2, 5% CO_2 and sealed. The reactions were stopped with 1.0 ml of 1.8% (v/v) perchloric acid at 30 minute intervals up to 2.5 hours. Glucose was assayed in the deproteinized supernatants according to the method of Bergmeyer and Bernt (1974). A standard curve for glucose was prepared on each occasion.

4.1.8 The conversion of ^{14}C-labelled precursors to carnitine by hepatocyte suspensions

The incubations were carried out at 37°C in a Paton PI reciprocating water bath set at 100 oscillations per minute. Plastic scintillation vials contained in a final volume of 1.0 ml; 0.5 ml hepatocytes suspension, 10 mM sodium pyruvate, 10 mM sodium fumarate, 10 mM sodium glutamate, Krebs-Henseleit-gelatin buffer and the combination of substrates; (1 mM, 3.6 μCi) [U-^{14}C]-4-aminobutyrate and 3 mM L-methionine; (1 mM, 2.59 μCi) L-[U-^{14}C]-lysine and 3 mM L-methionine; (1 mM, 1.5 μCi) DL-[1-^{14}C]-lysine and 3 mM L-methionine; 1 mM 4-amino-butyrate, 1 mM L-lysine and (3 mM, 2.5 μCi) L-[methyl-^{14}C]-methionine. The vials were flushed with 95% O_2, 5% CO_2 and sealed with Subaseal caps (No. 33) supporting plastic cups containing filter paper soaked with 0.1 ml 3 N KOH in the case of 4-[U-^{14}C]-aminobutyrate incubations. The reactions were stopped with 1.0 ml 10% (w/v) TCA at 30 minute intervals up to 2.5 hours. $^{14}CO_2$ was collected from the 4-aminobutyrate incubations for 2 hours.

4.1.9 $^{14}CO_2$ counting

The radioactivity contained in the KOH-soaked filter paper was counted as described in Part III, 2.1.6.

4.1.10 Separation of labelled products using thin-layer chromatography

The deproteinized supernatants from the incubations were extracted 4-5 times with diethylether to remove the TCA. The supernatants were then freeze-dried and brought to a volume of 50 µl which was chromatographed.

The separations of the various precursors and products was as described in Part III, 3.1.8 and the Rf values were the same as those reported in Tables 27 and 28.

4.1.11 Measurement of radioactivity

Radioactivity was measured as described in Part III, 3.1.9.

4.1.12 Assay of 4-aminobutyrate

4-Aminobutyrate was estimated in neutralized supernatants from blood samples by spectrophotometric assay at 340 nm. The assay system was similar to that described by Jackoby (1962) and contained in a final volume of 1.0 ml; 80 mM potassium phosphate buffer (pH 8.6), 0.2 mM dithiothreitol, 0.2 mM NADP, 2 mM 2-oxoglutarate neutralized extract and enzyme GABASE (30 µl). The reaction was started by addition of 2-oxoglutarate. Enzyme GABASE was a commercial preparation which consisted of a partially purified cell-free extract from *Pseudomonas flourescens* containing 4-aminobutyrate glutamic transaminase and succinic semialdehyde dehydrogenase.

4.1.13 Chemicals

Collagenase (Batch No. CLS45M[1] 81X) was obtained from Worthington Biochemical Corp., Freehold, New Jersey, U.S.A. and hyaluronidase Type 1 (Batch No. 24C-3290) from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Gelatin was obtained from Davis Gelatine (Aust) Pty. Ltd., Botany, N.S.W., Australia. GABASE was obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. [U-^{14}C]-4-aminobutyrate, L-[U-^{14}C]-lysine, DL-[1-^{14}C]-lysine and L-[methyl-^{14}C]-methionine were obtained from the Radiochemical Centre, Amersham, England. Alloxan was obtained from Koch-Light Laboratories, Colnbrook, Bucks., England.

4.2 Results

4.2.1 The conversion of propionate to glucose by sheep hepatocyte suspensions

Morphologically intact hepatocytes were difficult to prepare from both alloxan-diabetic, insulin-stabilized and withdrawn adult (2-year-old) sheep. The difficulty of preparation appeared to be associated with the fat content of the liver and indeed intact hepatocytes could not be prepared successfully from alloxan-diabetic sheep more than 24 hours withdrawn from insulin due to the accumulation of fat within the liver.

The anabolic process of glucose synthesis from propionate was used to assess the metabolic integrity of the hepatocyte preparations. Linear rates of glucose production from propionate were only sustained for periods of approximately 60 minutes. The rate of glucose production from propionate in hepatocytes prepared from insulin-stabilized sheep was 57 nmol/hr per mg protein. This was approximately one-third the rate

of 179 nmol/hr per mg dry weight in normal sheep hepatocytes reported by Ash *et al.* (1975). The rate of glucose production in hepatocytes prepared from 24 hour withdrawn sheep was 96 nmol/hr per mg protein.

4.2.2 The conversion of [U-^{14}C]-4-aminobutyric acid to carnitine by sheep hepatocyte suspensions

[U-^{14}C]-4-aminobutyric acid was taken up and metabolized to carbon dioxide by hepatocytes prepared from insulin-stabilized sheep (Figure 35).

[U-^{14}C]-4-aminobutyric acid was also converted to carnitine via N-methylated intermediates by hepatocytes prepared from insulin-stabilized sheep (Figure 36). The N-methylation of 4-aminobutyric acid to form 4-dimethylaminobutyric acid proceeded at a faster rate than the subsequent conversion of 4-dimethylaminobutyric acid to carnitine in these hepatocyte preparations (Figure 36).

The conversion of 4-aminobutyric acid to carnitine was approximately 1.5 times greater in hepatocytes prepared from 24 hour withdrawn as compared to insulin-stabilized, alloxan-diabetic sheep (Figure 37).

4.2.3 The conversion of L-[U-^{14}C]-lysine to carnitine by sheep hepatocyte suspensions

Radioactivity from L-[U-^{14}C]-lysine but not DL-[1-^{14}C]-lysine was incorporated into carnitine by sheep hepatocyte suspensions. The rate of synthesis of carnitine from L-[U-^{14}C]-lysine was approximately 2.24 times greater in hepatocytes prepared from 24 hour withdrawn as compared to insulin-stabilized, alloxan-diabetic sheep (Figure 38).

FIGURE 35

The rate of production of carbon dioxide from 4-aminobutyrate in hepatocytes isolated from insulin-stabilized, alloxan-diabetic sheep

Hepatocytes were prepared as described in Part III, 4.1.6, and incubated with 4-[U-^{14}C]-aminobutyric acid (3.6 μCi) as described in Part III, 4.1.8. $^{14}CO_2$ was counted as described in Part III, 3.1.7. The values shown were means obtained from 2 sheep.

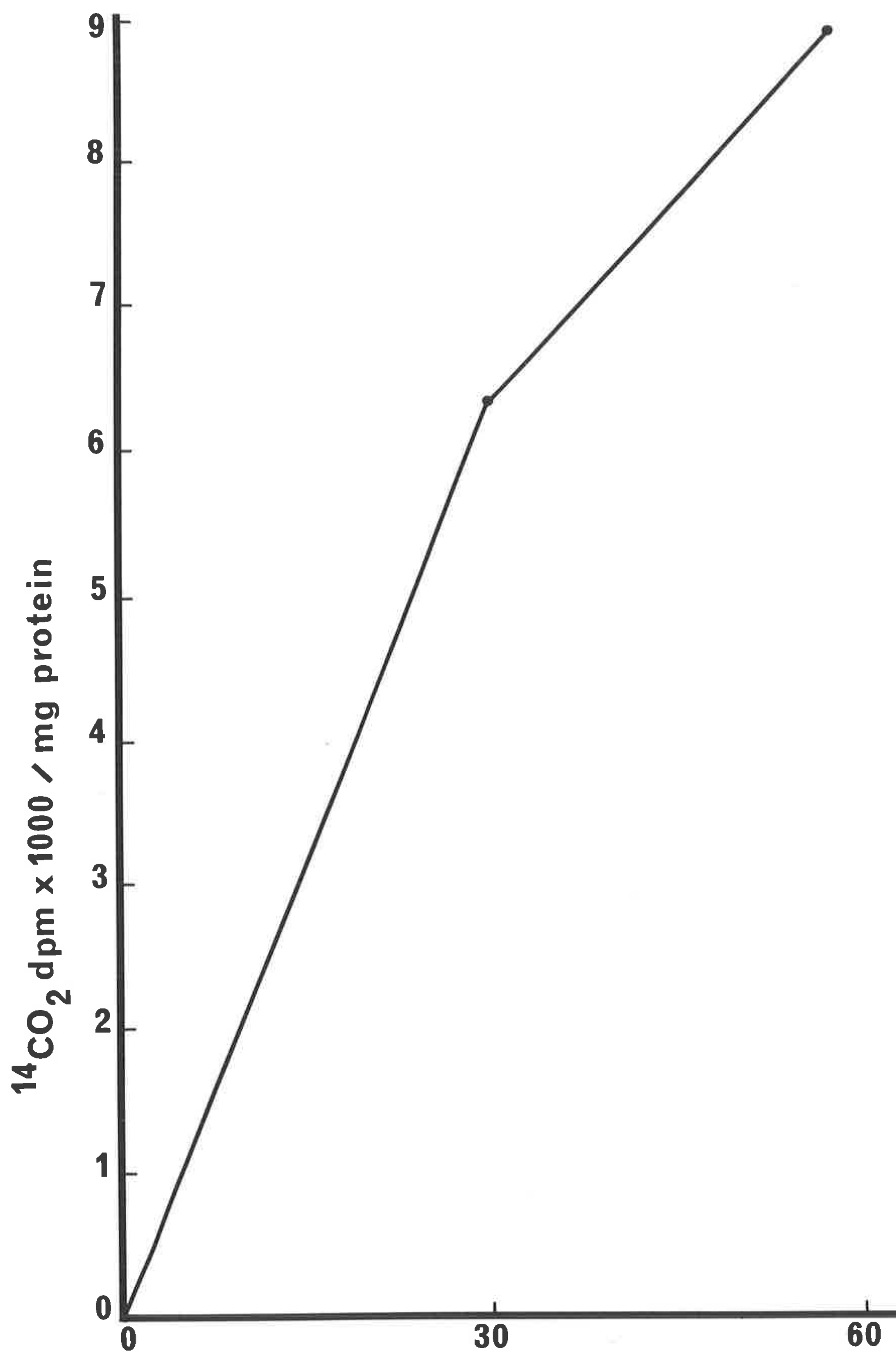


Figure 35

FIGURE 36

The rates of synthesis of 4-dimethylaminobutyric and carnitine from 4-aminobutyric acid in hepatocytes isolated from alloxan-diabetic, insulin-stabilized sheep

Hepatocytes were prepared and incubated as in Figure 35. The values shown were means obtained from 2 sheep.

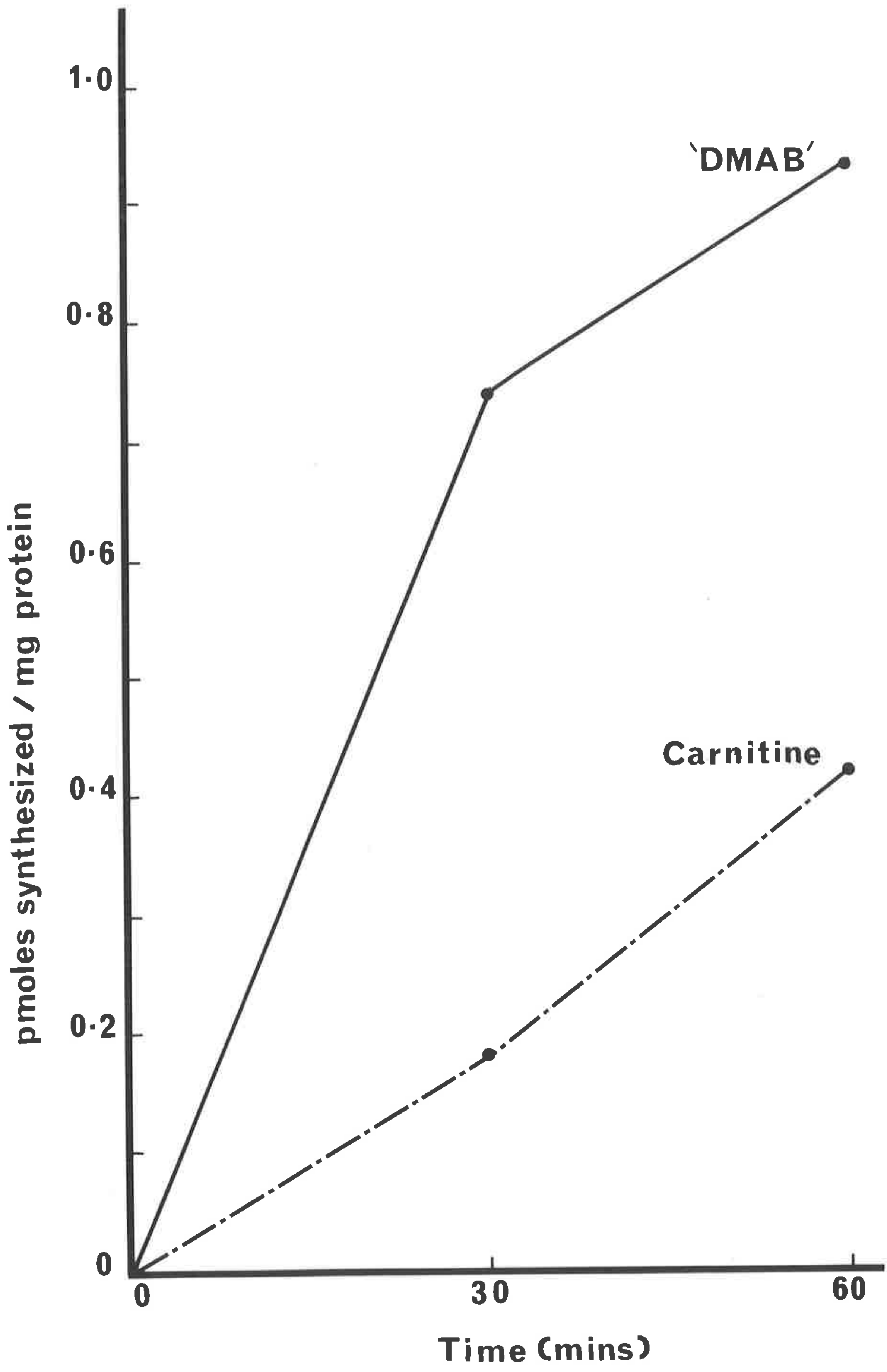


Figure 36

FIGURE 37

The rates of synthesis of carnitine from 4-aminobutyric acid in hepatocytes isolated alloxan-diabetic, insulin stabilized and one day withdrawn from insulin sheep

Hepatocytes were prepared and incubated as in Figure 35. The values shown are means obtained from 2 sheep in each case.

stabilized: alloxan-diabetic, insulin stabilized

withdrawn: alloxan-diabetic, one day withdrawn from insulin.

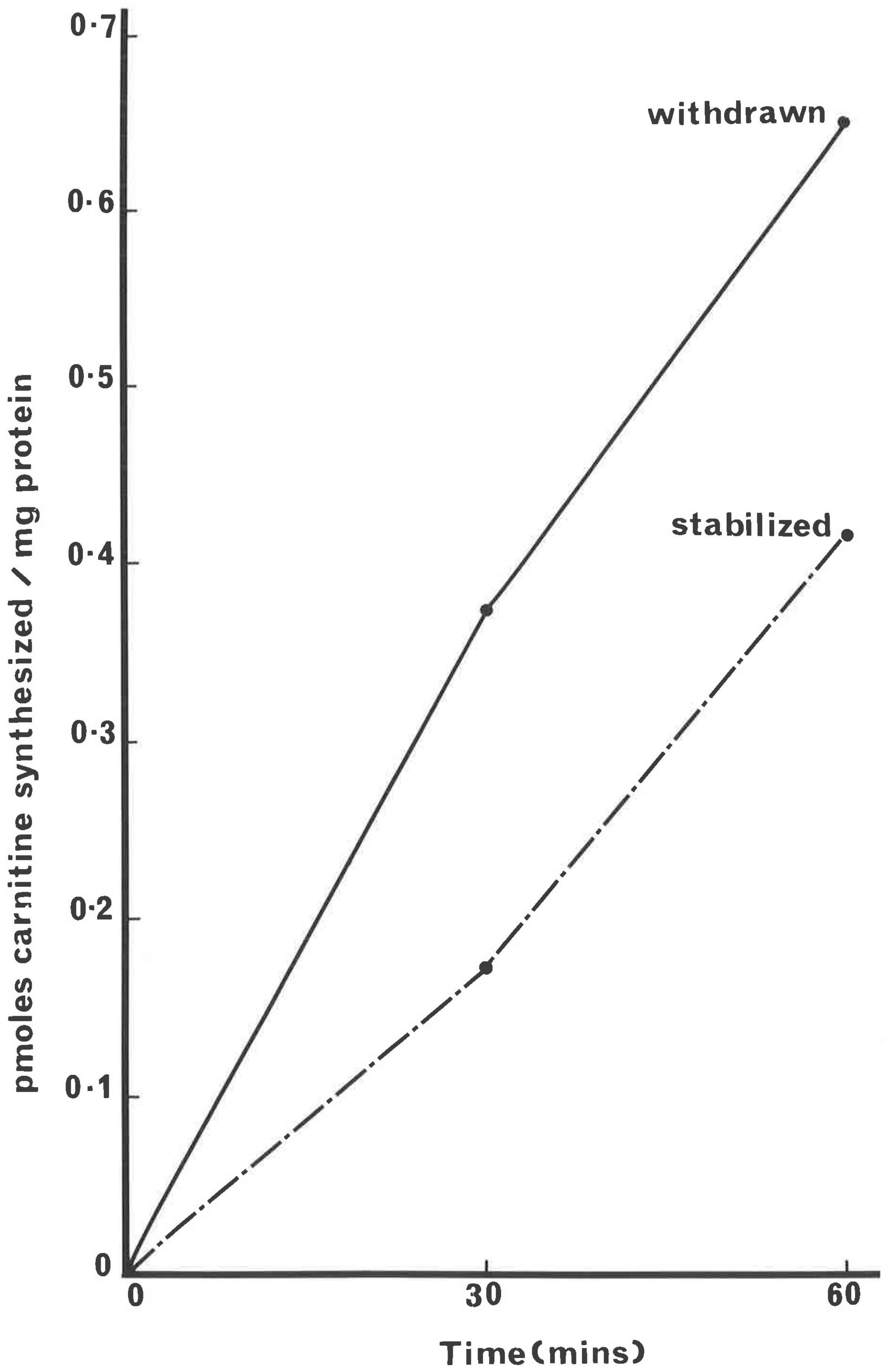


Figure 37

FIGURE 38

The rates of synthesis of carnitine from L-lysine in hepatocytes isolated from alloxan-diabetic, insulin-stabilized and one day withdrawn from insulin sheep

Hepatocytes were prepared as described in Part III, 4.1.6, and incubated with L-[U - ^{14}C]-lysine (2.59 μCi) as described in Part III, 4.1.8. ^{14}C-labelled lysine and carnitine were separated by thin-layer chromatography as described in Part III, 3.1.8. The values shown were duplicate samples obtained from 1 animal in each case.

stabilized: alloxan-diabetic, insulin-stabilized

withdrawn: alloxan-diabetic, one day withdrawn from insulin.

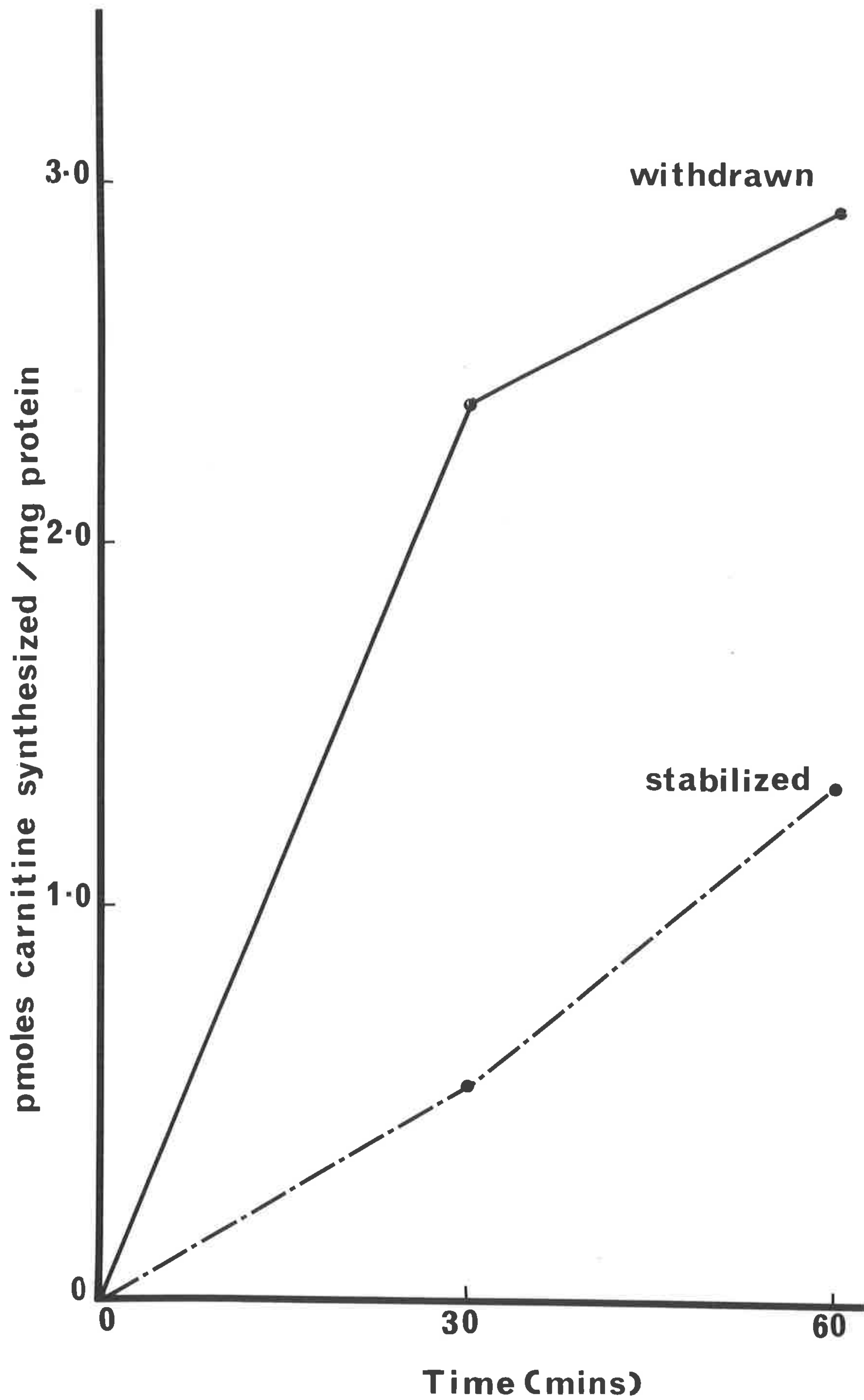


Figure 38

4.2.4 Comparison of the rates of synthesis of carnitine from lysine and 4-aminobutyric acid by sheep hepatocyte suspensions

L-[U-^{14}C]-lysine and [U-^{14}C]-4-aminobutyric acid were incubated in tandem with sheep hepatocytes to allow direct comparison of rates of carnitine synthesis from the two compounds.

The rate of synthesis of carntine from lysine was approximately three-fold greater than the comparative rate from 4-aminobutyric acid in insulin-stabilized sheep hepatocytes. In alloxan-diabetic sheep hepatocytes, the rate of carnitine synthesis from lysine was approximately 4½-fold greater than the comparative rate from 4-aminobutyric acid (Figure 39).

The total acid-soluble carnitine concentration of insulin-stabilized sheep liver was 120 nmol/g wet weight while that of 24 hour withdrawn sheep liver was 140 nmol/g wet weight (Mr. G.D. Henderson, Ph.D. thesis). The slight increase in carnitine concentration in 24 hour withdrawn sheep liver was not significant. There was no detectable concentrations of 4-aminobutyric acid in any blood samples from normal or 5-day starved sheep.

4.2.5 The incorporation of ^{14}C-label from L-[methyl-^{14}C]-methionine into carnitine by sheep hepatocyte suspensions

^{14}C-label from L-[methyl-^{14}C]-methionine was incorporated into carnitine at a rate of 3.2 pmols of carnitine synthesized/mg protein after 60 minutes incubation by 24 hour withdrawn sheep hepatocyte suspensions (Figure 40). L-lysine and 4-aminobutyric acid were

FIGURE 39

Comparison of rates of synthesis of carnitine from 4-aminobutyric acid and L-lysine in hepatocytes isolated from alloxan-diabetic, 24 h withdrawn from insulin sheep

The values shown were obtained from Figures 37 and 38.

GABA: 4-aminobutyric acid

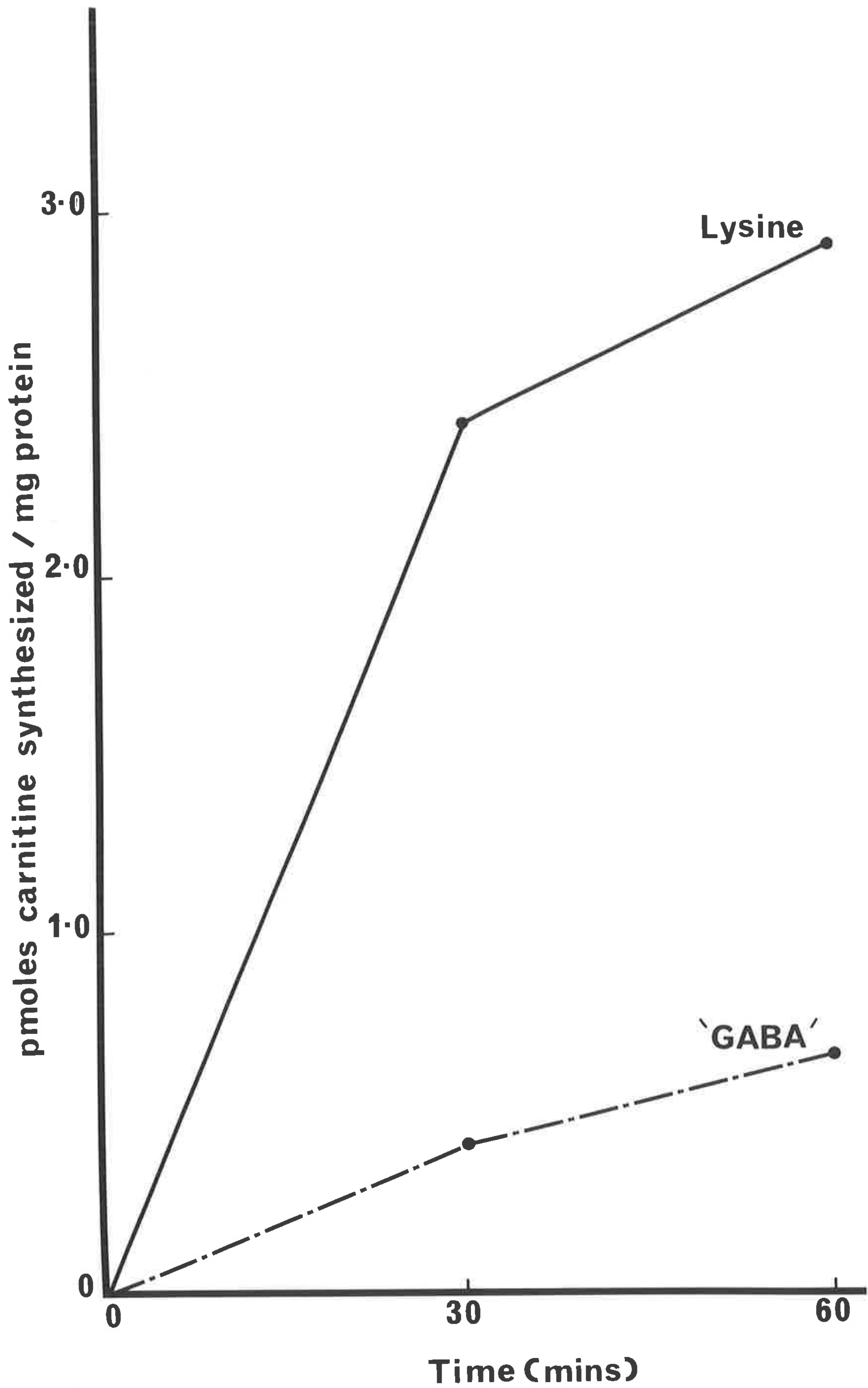


Figure 39

FIGURE 40

The rate of incorporation of methionine into carnitine by hepatocytes isolated from alloxan-diabetic, 24 h withdrawn from insulin sheep

Hepatocytes were prepared as described in Part III, 4.1.6., and incubated with L-[methyl - ^{14}C]-methionine (2.5 μCi) as described in Part III, 4.1.8. The values shown were means of duplicate samples obtained from one alloxan-diabetic, 24 h withdrawn from insulin sheep.

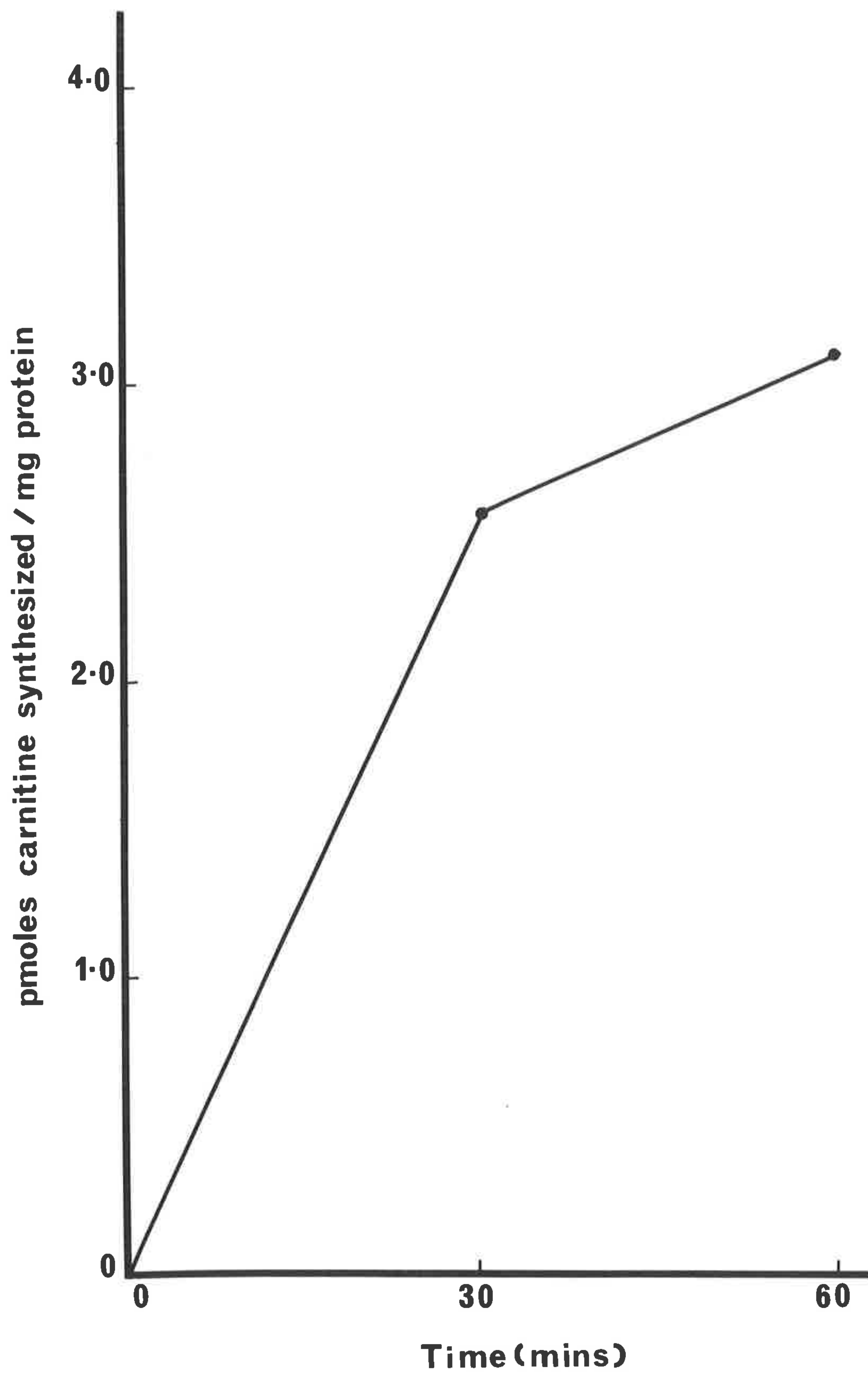


Figure 40

incubated with L-[methyl-^{14}C]-methionine resulting in this rate of carnitine synthesis while the combined carnitine synthesis rate from 4-aminobutyric acid and lysine (Figure 39) was 3.55 pmols of carnitine synthesized/mg protein after 60 minutes.

The free methionine concentration of insulin-stabilized sheep liver was 255 nmol/g wet weight while that of 24 hour withdrawn sheep liver was 326 nmol/g wet weight (Mr. G.D. Henderson, Ph.D. thesis). The increase in free methionine concentration in 24 hour withdrawn as compared to insulin-stabilized sheep liver was not significant.

5. Discussion

The results presented in Part III, 2.2.1 clearly indicate that, in rat tissues, the liver was the only site of 4-trimethylaminobutyric acid hydroxylase activity. This distribution of the enzyme in rat tissues is in agreement with the findings of Lindstedt (1967). It is interesting to note that there was no significant change in 4-trimethylaminobutyric acid hydroxylase activity in liver homogenates from alloxan-diabetic as compared to normal rats, although the turnover time of carnitine in alloxan-diabetic rats is four-fold faster than in normal rats (Mehlman *et al.*, 1969). The activity of the 4-trimethylaminobutyric acid hydroxylase enzyme in liver homogenates from normal rats has sufficient capacity to easily account for the average total carnitine excretion rate of 10.4 μmol/day in normal rat tissues, under steady-state conditions, reported by Brooks and McIntosh (1975).

4-Dimethylaminobutyric acid and 4-monomethylaminobutyric acid were also hydroxylated by rat liver homogenates indicating that 4-trimethylaminobutyric acid hydroxylase is not specific for 4-trimethylaminobutyric

acid. The stimulation of 4-trimethylaminobutyric acid hydroxylase by s-adenosylmethionine is an interesting result that may have significance in the control of carnitine biosynthesis.

Haigler and Broquist (1974) and Bohmer (1974), on the basis of the conversion of 4-trimethylaminobutyric acid to carnitine occurring solely in rat liver, suggested that the liver contained the full complement of enzymes for the biosynthesis of carnitine. Although this would be ideal from the point of view of controlling the biosynthesis of carnitine in the rat, more evidence than the distribution of 4-trimethyl-aminobutyric acid hydroxylase (the final step in carnitine biosynthesis) is required as a basis for these suggestions.

4-Trimethylaminobutyric acid hydroxylase activity was more widely distributed in the two ruminant species, sheep and goats. The specific activity of the hydroxylase enzyme in sheep and goat tissues was lower in all cases than that found in rat liver. These results confirm the findings of Erfle (1975) in sheep (with the exception that he did not detect 4-trimethylaminobutyric acid hydroxylase activity in homogenates of sheep heart) and extends them to encompass goat tissues. It would appear that the wider distribution of the hydroxylase enzyme may be a feature of the ruminant animal as compared to the monogastric animal. On the basis of specific activity coupled with mass, skeletal muscle of sheep and goats would be quantitatively the most important site of hydroxylation of 4-trimethylaminobutyric acid, in agreement with the suggestion of Erfle (1975). However, Snoswell and McIntosh (1974) suggested that the liver was the only site of carnitine biosynthesis in sheep that were severely alloxan-diabetic, but concentration of carnitine in skeletal muscle increased from 12900 nmol/g wet weight to 17100 nmol/g wet weight in normal as compared to alloxan-diabetic sheep respectively. There were no

significant changes in 4-trimethylaminobutyric acid hydroxylase activity in tissues from alloxan-diabetic as compared to normal sheep, although under these physiological conditions Snoswell and Koundakjian (1972) and Snoswell and McIntosh (1974) reported significant increases (up to twenty-fold increases in severely alloxan-diabetic) in liver total acid-soluble carnitine concentration. The 4-trimethylaminobutyric acid hydroxylase activity in homogenates of 1-day-old lambs was almost the same as that detected in 2-year-old sheep. A point of interest being, the carnitine concentration of skeletal muscle of lambs doubles each day after birth for approximately a week proceeded subsequently by a slower increase to adult concentrations (A.M. Snoswell, unpublished results).

Since the liver in rats was the sole site of 4-trimethylaminobutyric acid hydroxylase activity, and in sheep contained the highest specific activity of hydroxylase enzyme, hepatocytes isolated from these two species were used to establish whether the liver did contain the full complement of enzymes for the biosynthesis of carnitine from 4-aminobutyric acid, L-lysine and methionine. Results from only those hepatocyte suspension whose morphological and metabolic integrity had been established by the criteria described in the text were presented.

The results presented in Part III, 3.2.2 and 4.2.2 indicate that there are no permeability barriers to the uptake of 4-aminobutyric acid present in hepatocytes prepared from rats and sheep. This finding is in direct contrast to the suggestions of Lindstedt and Lindstedt (1965) that permeability barriers to 4-aminobutyric acid may exist outside the blood-brain barrier in mice. Indeed, from the substantial inhibition of carbon dioxide production from 4-aminobutyric acid in the presence of iso-nicotinylhydrazine, it would appear that rat liver contains the enzymes, 4-aminobutyric acid glutamate transaminase and

succinic semialdehyde dehydrogenase, required for the oxidation of 4-aminobutyric acid to carbon dioxide via the TCA cycle. In this regard Haber *et al.* (1970) have detected 4-aminobutyric acid transaminase activity in rabbit liver (88% of brain activity) but could not detect any 4-aminobutyric acid in rabbit liver.

In contrast to the observations *in vivo* of Bremer (1962*b*) and Lindstedt and Lindstedt (1965), 4-aminobutyric acid was converted to carnitine by hepatocyte preparations from rats and sheep. Thus the pathway of carnitine biosynthesis from 4-aminobutyric acid appears to proceed via successive 4-amino methylation to form 4-trimethylamino-butyric acid which is subsequently rapidly converted to carnitine. Bremer (1962*b*) and Lindstedt and Lindstedt (1965) may have failed to detect conversion of 4-aminobutyric acid to carnitine due to the less specific technique of intraperitoneal injections of [1-^{14}C]-4-aminobutyric acid which was subsequently rapidly metabolized to carbon dioxide. Also, in this study, very high concentrations (1 mM) of high specific radioactivity of 4-aminobutyric acid were used.

It is important to note that in hepatocytes prepared from normal rats and insulin-stabilized, alloxan-diabetic sheep 4-aminobutyric acid was more readily converted to 4-dimethylaminobutyric acid than the subsequent conversion of 4-dimethylaminobutyric acid to carnitine. The hydroxylation of 4-dimethylaminobutyric acid may proceed at a faster rate that the N-methylation to 4-trimethylaminobutyric acid, thus rationalizing the slow rate of conversion of 4-dimethylaminobutyric acid to carnitine. This concept is consistent with the observations of Bremer (1962*b*) and Lindstedt and Lindstedt (1965), where there was a very small conversion (0.1% in mice) of 4-dimethylaminobutyric acid to carnitine.

The rate of synthesis of carnitine from 4-aminobutyric acid was

almost six-fold faster in hepatocytes isolated from alloxan-diabetic as compared to normal rats. This result is consistent with the four-fold decrease in turnover time of carnitine in alloxan-diabetic rats compared to normal rats (Mehlman *et al.*, 1969). The concentrations of 4-aminobutyric acid and L-methionine were the same in incubations with alloxan-diabetic and normal rat hepatocytes and there was no increase in 4-trimethylaminobutyric acid hydroxylase activity under these conditions. Thus a possible explanation of the increased rate of carnitine biosynthesis observed may be an increased activity of the 4-aminobutyric acid N-methylases in the alloxan-diabetic state. Indeed in alloxan-diabetic rats (with one exception, see Figure 26) there was a rapid conversion of 4-dimethylaminobutyric acid to carnitine (c.f. normal rats). These results also indicate that hepatocyte suspensions reflect the metabolic changes in the animals from which they are prepared. The change in the rate of carnitine synthesis from 4-aminobutyric acid in hepatocytes prepared from alloxan-diabetic sheep that had been stabilized on insulin as compared to similar sheep that had been withdrawn from insulin was much smaller in magnitude (1½-fold increase in withdrawn animals) than the changes observed in alloxan-diabetic as compared to normal rats. A possible explanation is that the rate of carnitine biosynthesis may not have altered greatly after 24 hour insulin withdrawal from the insulin-stabilized, alloxan-diabetic sheep. If the rates of carnitine biosynthesis from 4-aminobutyric acid are compared after 60 minutes in hepatocytes prepared from normal rats and insulin-stabilized sheep, it can be seen that these rates (0.42 pmol/mg protein in stabilized sheep and 0.59 pmol/mg in normal rats) are reasonably similar, suggesting that turnover time of carnitine in these two species may possibly be similar (14 days in normal rats; Mehlman *et al.*, 1969).

Since 4-aminobutyric acid was not detected in the portal blood of sheep, 4-aminobutyric acid must be synthesized within the liver of sheep (and possibly rats) to be available as a precursor of the carbon skeleton of carnitine *in vivo*. 4-Aminobutyric acid could be synthesized from glutamate via glutamate decarboxylase or from the oxidation of putrescine. Even if these enzymes were present in rat and sheep liver, the results presented here and those of Bremer (1962*b*) in rats and Lindstedt and Lindstedt (1965) indicate that the greatest proportion of the 4-aminobutyric acid possibly synthesized in sheep and rat liver would be oxidized via the TCA cycle and very little would be converted to carnitine. Although 4-aminobutyric acid (present in high concentrations) is converted to carnitine *in vitro* by hepatocyte suspensions prepared from both rats and sheep, its very low availability *in vivo* in rat and sheep liver may preclude 4-aminobutyric acid as a significant precursor of the carbon skeleton of carnitine.

The results in hepatocytes isolated from rats and sheep showing that radioactivity from L-[U-^{14}C]-lysine but not DL-[1-^{14}C]-lysine was incorporated into carnitine are in agreement with the *in vivo* results in rats reported by Tanphaichitr and Broquist (1973) and Cox and Hoppel (1973*a*) and are also consistent with their suggestions that only carbons 3 to 6 of lysine are incorporated into the carbon skeleton of carnitine. The incorporation of radioactivity from lysine into carnitine in rat and sheep hepatocyte preparations contrasts with the lack of incorporation in rat liver slices reported by Cox and Hoppel (1974) and Haigler and Broquist (1974).

The four-fold increase in the rate of carnitine biosynthesis from lysine in hepatocytes prepared from alloxan-diabetic as compared to normal rats is again consonant with the four-fold decrease in the turnover time

of carnitine under these conditions (Mehlman *et al.*, 1969). The greater than two-fold increase in the rate of carnitine biosynthesis from lysine in hepatocytes prepared from 24 hour withdrawn from insulin as compared to insulin-stabilized, alloxan-diabetic sheep indicates that, in spite of the fact that total acid-soluble carnitine concentrations in the liver did not change significantly, the turnover time of carnitine may have decreased under these conditions in sheep. The rates of synthesis of carnitine from lysine in hepatocytes prepared from normal rats and insulin-stabilized alloxan-diabetic sheep were identical after 60 minutes (1.30 pmol carnitine synthesized/mg protein), suggesting again that the carnitine turnover time in these two species may be similar.

Since the rates of carnitine synthesis from 4-aminobutyric acid and L-lysine were investigated concurrently in each hepatocytes preparation it can be seen that the rate of carnitine synthesis from lysine was always greater than from 4-aminobutyric acid in both rats and sheep under the physiological conditions prevailing. These results alone suggest that L-lysine is the more significant precursor of the carbon skeleton of carnitine.

Radioactivity from methyl-labelled methionine was incorporated into carnitine in hepatocytes prepared from alloxan-diabetic rats and alloxan-diabetic sheep 24 hours after insulin withdrawal, in agreement with the *in vivo* results in rats reported by Wolf and Berger (1961), Bremer (1961), Tanphaichitr and Broquist (1973) and Cox and Hoppel (1973*a*). The incorporation of methionine label into carnitine in hepatocyte preparations described here contrasts again with the lack of incorporation in rat liver slices reported by Cox and Hoppel (1974). The amount of carnitine synthesized (per mg hepatocyte protein) from methionine incubated with

both lysine and 4-aminobutyric acid was only slightly greater (approximately 20%) than the amount of carnitine synthesized when methionine was incubated with lysine in hepatocytes isolated from alloxan-diabetic rats and insulin-withdrawn, alloxan-diabetic sheep. This evidence strongly suggests that lysine rather than 4-aminobutyric acid is the significant precursor of the carbon skeleton of carnitine.

Thus, the results using rat and sheep hepatocytes presented in Part III, 3.2 and 4.2 respectively represent the first *in vitro* demonstration of biosynthesis of carnitine from 4-aminobutyric acid, lysine and methionine. The additional significant advantage of using hepatocyte preparationsin the study of carnitine biosynthesis was the fact that 4-aminobutyric acid and lysine could be compared as precursors of the carbon skeleton of carnitine simultaneously within the same animal preparation. This advantage is particularly valuable in the study of carnitine biosynthesis in the sheep where between animal variation can be substantial (P. Fennessey, Ph.D. thesis) and the use of radioactive compounds in *vivo* quite expensive.

It is interesting to speculate on a possible biosynthetic sequence whereby lysine and methionine are converted to carnitine and the controls that may influence carnitine biosynthesis.

Lysine and methionine are both essential amino acids and as such the dietary supply and uptake by the liver will directly influence their availability for carnitine biosynthesis. Substantial proportions of methionine (>90%) and lysine (>80%) are removed from the portal system by the liver of sheep (Wolff *et al.*, 1972).

The lysine in liver may be methylated as free lysine by an analogous enzymic process to that present in *Neurospora crassa* (Rebouche and Broquist, 1976) or only lysine incorporation into histone proteins may be methylated via protein methylase III (Paik and Kim, 1975). The

methylation of lysine may be the significant control point in carnitine biosynthesis, in that this is the stage at which the two precursors, namely lysine and methionine, come together.

The results of Aronson *et al.* (1976) suggest that in rat liver lysine is methylated only in the protein-bound form. The results reported by Tanphaichitr and Broquist (1973) and Cox and Hoppel (1973*b*) in lysine-deficient rats, showing that free 6-trimethyl-lysine was more readily incorporated into carnitine than free lysine, are also consistent with protein-bound lysine as the methyl acceptor. By this argument, the rate and extent of histone protein synthesis would initially determine the lysine available for ultimate conversion to carnitine.

The factors which control the methylation of the lysine residues in the histone proteins would be important controls of carnitine biosynthesis. The liver concentration of methionine in alloxan-diabetic sheep 24 hours after insulin withdrawal was not significantly different from the concentration in insulin-stabilized, alloxan-diabetic sheep, suggesting at first glance that the concentration of free methionine in liver may not be of prime importance in determining protein methylation. Prior to methyl transfer, methionine must be activated by methionine activating enzyme to form s-adenosylmethionine which is the actual methyl-donor compound involved in enzymatic transmethylation reactions (Cantoni, 1953; Mudd and Cantoni, 1964). The activity of methionine activating enzyme, which in the species surveyed this far is highest in the liver (Sturman *et al.*, 1970), would determine the rate of production of s-adenosymethionine. Alloxan administration results in a three-fold increase in activity of this enzyme in liver but not in kidney, pancreas or brain of rats (Finkelstein, 1967). The activities of the competing methyltransferases and their affinities for s-adenosylmethionine have been suggested as determinants of priority of methylation (Kerr, 1972). The activity of

protein methylase III in liver is very low (1.07 pmol/min per mg protein) compared to the activities of tRNA methylase and glycine methyltransferse (Paik and Kim, 1975; Kerr, 1972). In addition, protein methylase III is chromatin-bound and only methylates the 6-N-amino groups of lysine residues of histone proteins during the period between the entry of histone molecules into the nucleus and its conjugation in the chromatin complex (Paik and Kim, 1975). The subcellular localization of the s-adenosylmethionine pools in liver would be of prime importance, but at this stage little is known of their localization. The increased rates of synthesis of carnitine in hepatocytes isolated from alloxan-diabetic rats and sheep indicate that lysine is being methylated at a faster rate. This result may be rationalized on the basis of increased methionine activating enzyme activity and protein methylase III activity. The results presented in sheep hepatocytes indicate that the increased methylation activity occurs within 24 hours of insulin withdrawal from insulin-stabilization. Protein-bound trimethyl-lysine formed from methylation of protein-bound lysine must be released from peptide linkage by proteolytic enzymes. The nature of these enzymes and their subcellular localization remains to be elucidated.

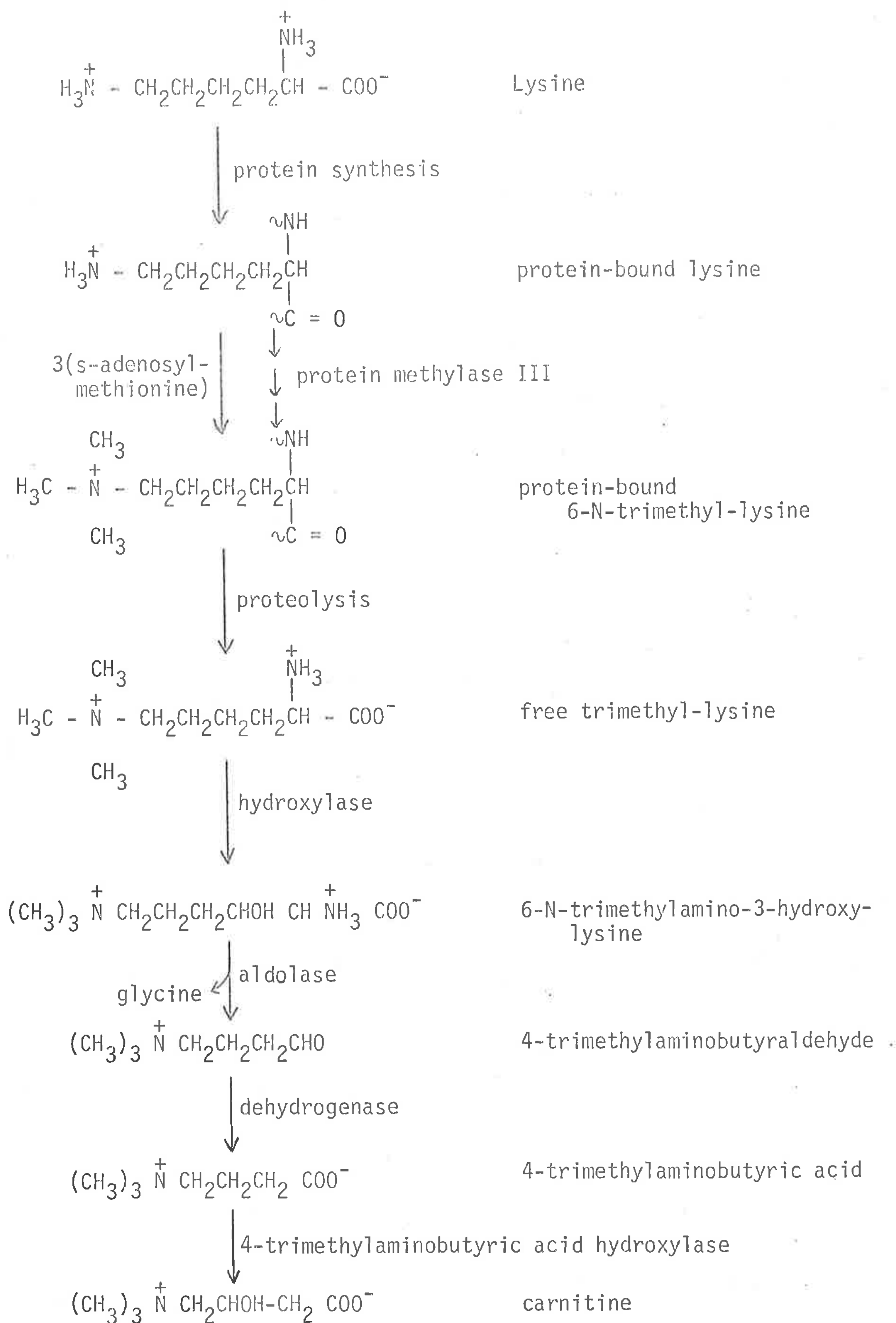
The results in Part III, 3.2 and 4.2 where incorporation of radioactivity from L-[U-^{14}C]-lysine into carnitine was detected in hepatocytes preparations from rats and sheep after only 60 minutes incubation suggests that lysine may be methylated as free lysine (without the necessity of incorporation into peptides) in agreement with the findings of *Neurospora crassa* by Rebouche and Broquist (1976) and Borum (1976). Methylation of free lysine may be controlled by the activity of s-adenosylmethionine:L-lysine 6-N-methyltransferase in rat and sheep liver and the affinity of the enzyme for s-adenosylmethionine in relation to other methyltransferases, a concept consistent with the proposals of Kerr (1972). The first

methylation of lysine to 6-N-monomethyl-lysine may be the rate limiting step in rat and sheep liver, as was the case in *Neurospora crassa* (Rebouche and Broquist, 1976). Continuing the comparison with *Neurospora crassa* findings of Rebouche and Broquist (1976), the second methylation to 6-N-dimethyl-lysine and third methylation to 6-N-trimethyl-lysine would be successively more rapid steps. The sensitivity of different methyltransferase to inhibition by s-adenosylhomocysteine (Paik and Kim, 1975; Kerr, 1972) may also be an important control fracture of the methylation of lysine. Thus the methylation of free lysine represents a much simpler model for the formation of free 6-trimethyl-lysine.

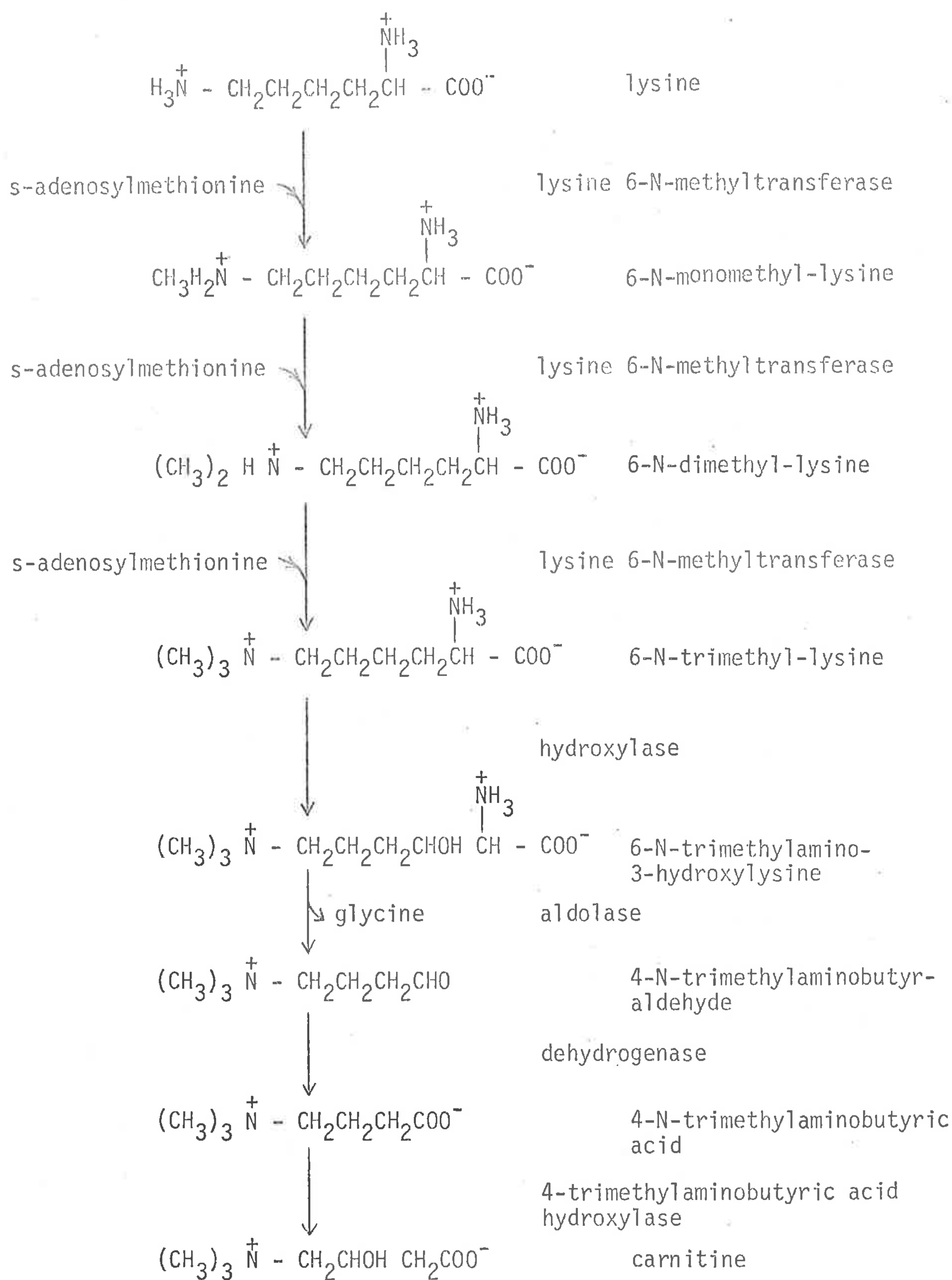
Hochalter and Henderson (1976) propose that free 6-trimethyl-lysine is hydroxylated to form 6-trimethylamino-3-hydroxy-lysine in order that carbons 1 and 2 can be removed as glycine, by a aldolase reaction analogous to threonine aldolase (Braunshtein and Vilenkina, 1949), resulting in the formation of 4-trimethylaminobutyraldehyde. The final reactions would involve oxidation of 4-trimethylbutyraldehyde to 4-trimethylaminobutyrate which could be hydroxylated to carnitine by 4-trimethylaminobutyric acid hydroxylase present in the cytoplasm of rat and sheep liver.

The sequence of reactions suggested here for the biosynthesis of carnitine from lysine and methionine are complex both enzymically and in regard to the compartmentation of the metabolites and enzymes in the liver cell. The suggested pathways are presented in Schemes 1 and 2 differing in methylation of protein-bound (Scheme 1) or free lysine (Scheme 2).

SCHEME 1



SCHEME 2



General Discussion

The results presented in this thesis clearly modify the hypothesis that carnitine and carnitine acetyltransferase buffer 'acetyl pressure' to form acetylcarnitine. The demonstration of an alternate enzymic utilization of acetylcarnitine (other than via carnitine acetyltransferase) invalidates the concept that acetylcarnitine is a metabolic sink, acting as a reservoir of acetyl groups (Pearson and Tubbs, 1967). The presence of acetylcarnitine hydrolase enables the transfer of acetyl groups of matrix acetyl-CoA, initially catalyzed by carnitine acetyltransferase, to result in the production of acetate in the liver. Also the presence of acetylcarnitine hydrolase in the liver enables carnitine to be 'recycled' through the carnitine acetyltransferase - acetylcarnitine hydrolase couple.

The consequences of this enzyme couple in rat liver remain to be quantitated, although Walter and Söling (1976) suggest that in rat liver the acetyl-CoA-hydrolase activity of this enzyme couple could account for up to 20% of the transfer of acetyl groups from matrix acetyl-CoA (80% transferred via the citrate cleavage pathway) resulting in acetate formation.

The importance of the carnitine acetyltransferase - acetylcarnitine hydrolase couple in ruminant liver has been demonstrated in this thesis. The quantitative capacity of this enzyme couple to account for endogenous acetate production from the liver in lactating ewes has been demonstrated also. However, it is interesting to note the quantitative difference of liver carnitine concentrations in sheep and dairy cows under conditions of 'acetyl pressure'. In response to increases in 'acetyl pressure' in the liver of sheep the concentrations of acetylcarnitine and total acid-soluble carnitine increase markedly (Snoswell and Henderson, 1970;

Snoswell and Koundakjian, 1972; Snoswell and McIntosh, 1974). In contrast, the total acid-soluble carnitine concentrations in the liver of dairy cows did not increase (in fact they decreased) under conditions of increase 'acetyl pressure'.

There must be a fundamental difference in the metabolic responses of carnitine in the liver of sheep as compared to both the rat and the cow. Alloxan administration increased the concentration of total acid-soluble carnitine up to 24-fold in sheep liver (Snoswell and McIntosh, 1974) but only two-fold in rat liver (McGarry *et al.*, 1975). The effects of alloxan administration in the dairy cow have yet to be investigated. The increased carnitine concentrations in liver from severely alloxan-diabetic sheep may be precipitated by the decrease in carnitine recycling via acetylcarnitine hydrolase resulting in a compensating synthesis of carnitine to maintain adequate concentrations of free carnitine for the important roles in fatty acid oxidation and relief of 'acetyl pressure'. This compensating response suggested here may not be needed in liver from starved rats or lactating dairy cows because of the increase in carnitine recycling via increased acetylcarnitine hydrolase activity.

The use of hepatocyte preparations from rats and sheep for *in vitro* investigations of carnitine biosynthesis represent a significant advance when compared to the limited success of the use of liver slices for this purpose (Cox and Hoppel, 1974; Haigler and Broquist, 1974). The simultaneous comparisons of 4-aminobutyric acid and lysine as precursors of the carbon skeleton of carnitine in rat and sheep hepatocyte suspensions provided convincing evidence that lysine is the more significant precursor of the carbon skeleton of carnitine. It was interesting to note the similarities in the rate of carnitine biosynthesis in rat and sheep hepatocyte suspensions. The enzymic mechanism whereby lysine is methylated to

form 6-trimethyl-lysine in animal tissues remains an important area for future investigations. Establishing whether lysine is methylated in the free or protein-bound form will provide an important insight into the control of carnitine biosynthesis in animal tissues. Each of the two possibilities have their advantages and disadvantages. Methylation of protein-bound lysine has the advantage that only the 6-amino position is methylated, and not the 2-amino position involved in the peptide-bond, intrinsically ensuring specificity of methylation. Also the methylation enzyme, protein methylase III, has been detected in animal tissues (Paik and Kim, 1975). However, the methylation of free lysine provides a much simpler reaction sequence (Scheme 2) enzymically and particularly in regard to cellular compartmentation of the enzymes and substrates. In ruminant species the possibility that carnitine may be synthesized in extrahepatic tissues (inferred by the presence of 4-trimethylaminobutyric acid hydroxylase) also remains to be investigated. If carnitine were to be synthesized in muscle tissue from ruminant species this may in part help to explain the high concentrations of carnitine found in these tissues.

The aspect of carnitine metabolism possibly least understood at this stage is the transport systems for carnitine that are present in animal tissues. The transport system for carnitine present in the liver of rats, sheep and dairy cows may have different characteristics which may result in the different quantitative responses of carnitine observed in these species. Hepatocyte preparations may prove to be an important aid in the investigation of the transport of carnitine from the liver cell.

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