METABOLIC INTER-RELATIONSHIPS

BETWEEN CARNITINE, CHOLINE AND CREATINE

IN SHEEP LIVER

A Thesis submitted

by

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PREFACE

The recommendations (1976) of the I.U.P.A.C.-I.U.B. Commission on Biochemical Nomenclature related to the nomenclature of lipids and phosphorus-containing compounds of biological importance have been followed throughout this thesis.

The recommendations [1972 & Supplement 1: Corrections & Additions (1975)] of the I.U.P.A.C.-I.U.B. on enzyme nomenclature have been followed as far as possible. The unit of enzymic activity used was the katal (symbol: kat), and is defined as the amount of activity that converts 1 mol of substrate/s (or 1 mol of measured product formed/s). The following enzymes are referred to by name only:

sn-Glycerol 3-phosphate dehydrogenase (NAD^+)	1.1.1.8
Lactate dehydrogenase	1.1.1.27
Glucose oxidase	1.1.3.4
Choline dehydrogenase	1.1.99.1
Betaine-aldehyde dehydrogenase	1.2.1.8
Peroxidase	1.11.1.7
γ-Butyrobetaine hydroxylase	1.14.11.1
Betaine-homocysteine methyltransferase	2.1.1.5
Tetrahydropteroylglutamate methyltransferase	2.1.1.13
3-*sn*-Phosphatidylethanolamines methyltransferase	2.1.1.17
Glycine methyltransferase	2.1.1.20
Protein(lysine) methyltransferase	2.1.1.43
Lysine methyltransferase	2.1.1.-
Choline acetyltransferase	2.3.1.6

Carnitine acetyltransferase	2.3.1.7
sn-Glycerol-3-*P* acyltransferase	2.3.1.15
Diacylglycerol acyltransferase	2.3.1.20
Carnitine palmitoyltransferase	2.3.1.21
Choline kinase	2.7.1.32
Pyruvate kinase	2.7.1.40
Creatine kinase	2.7.3.2
Ethanolamine phosphotransferase	2.7.8.1
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Cholinesterase	3.1.1.8
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Creatininase	3.5.2.-
Inorganic pyrophosphatase	3.6.1.1
Cystathionine β-synthase	4.2.1.22
Cystathionine γ-lyase	4.4.1.1

Chemical compounds, their sources and degrees of purity are described in the text.

Abbreviations approved by the Biochemical Journal (1976) for use without definition are used as such throughout this thesis. The following abbreviations have been used where appropriate:

PtdEtn	3-*sn*-phosphatidylethanolamines
PtdOEtN$<^{H}_{Me}$	3-*sn*-phosphatidyl *N*-methylethanolamines
PtdOEtN$<^{Me}_{Me}$	3-*sn*-phosphatidyl *N*,*N*-dimethylethanolamines
PtdCho	3-*sn*-phosphatidylcholines
Cer*P*Cho	ceramide-1-phosphocholines

PtdSer	3-*sn*-phosphatidylserines
PtdIns	3-*sn*-phosphatidylinositols
PtdGro	3-*sn*-phosphatidylglycerols
sn-glycerol-3-*P*	*sn*-glycerol 3-phosphate
CDP-Cho	cytidine-5'-diphosphocholine
Gro*P*Cho	*sn*-glycero-3-phosphocholine
P-ethanolamine	phosphoethanolamine
P-choline	phosphocholine
CDP-Etn	cytidine-5'-diphosphoethanolamine
S-Ado-met	*S*-adenosylmethionine
S-Ado-hcys	*S*-adenosylhomocysteine
5-Me-H_4 folate	5-methyltetrahydropteroylglutamate
t.l.c.	thin-layer chromatography
g.l.c.	gas-liquid chromatography
VLDL	very low density lipoproteins
LDL	low density lipoproteins
HDL	high density lipoproteins

Unless otherwise stated, results were analyzed for the statistical significance of differences using Student's t test.

SUMMARY

1. Choline acetyltransferase was partially purified from sheep brain striata and immature human placentae and used in the assay of choline. However, it was not possible to obtain an enzyme preparation free of carnitine acetyltransferase and thus obtain a specific choline assay for tissue extracts.

2. The fatty livers of alloxan-diabetic and pregnant-toxaemic sheep were partially characterized. Normal and alloxan-diabetic rats were studied for comparison. The liver total lipid content increased from 227 mg/g dry fat-free tissue in normal sheep to a maximum of 837 mg/g dry fat-free tissue in alloxan-diabetic uncontrolled responder wethers and 1,215 mg/g dry fat-free tissue in pregnant-toxaemic ewes. Triacylglycerols were the major class of lipid accumulating. The liver total lipid content increased from 232 mg/g dry fat-free tissue in normal rats to 356 mg/g dry fat-free tissue in alloxan-diabetic rats. Alloxan-diabetic insulin stabilized wethers subsequently withdrawn from insulin for 24 h and alloxan-diabetic rats had a significantly higher liver water content than normal animals when the results were expressed per g dry fat-free tissue.

3. A possible relationship between the development of fatty livers and choline metabolism in alloxan-diabetic and pregnant-toxaemic sheep was investigated. 3-*sn*-Phosphatidylcholines (PtdCho) and 3-*sn*-phosphatidylethanolamines (PtdEtn) were the major phospholipids in sheep liver. There was no apparent

relationship between the development of a fatty liver and total hepatic $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratio except that alloxan-diabetic uncontrolled responder wethers had a significantly lower ratio than alloxan-diabetic uncontrolled non-responder wethers. Qualitative argentation t.l.c. of intact total hepatic PtdCho and PtdEtn, and g.l.c. of total hepatic phospholipids suggested decreased levels of PtdCho and PtdEtn containing polyunsaturated fatty acids in alloxan-diabetic uncontrolled responder and pregnant-toxaemic sheep compared to normal wethers.

4. The effect of abomasal infusions of L-methionine in wethers for 32 days on the liver water and lipid contents was investigated. Wethers infused with 0.36 g met/kg body wt.$^{0.75}$/day had a significantly higher liver total lipid content than control wethers. Wethers infused with 0.12 g met/kg body wt.$^{0.75}$/day had a significantly lower total lipid choline content than control wethers.

5. The livers from neonatal lambs were partially characterized. They had a significantly lower total lipid and total $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratio, and a significantly higher water content than the livers from normal adult sheep.

6. There was a significant ($P < 0.001$) negative correlation ($r = -0.94$) and regression between the water and total lipid content (both expressed per g wet tissue wt.) of the livers from adult sheep under various physiological conditions. This relationship was used to estimate the total lipid content of sheep liver.

7. There was a significant ($P < 0.001$) positive correlation ($r = 0.90$) and regression between total hepatic PtdCho and PtdEtn content in sheep under various physiological conditions.

8. Total hepatic carnitine, creatine and PtdCho contents were determined in sheep under various physiological conditions. Normal and alloxan-diabetic rats were studied for comparison. The results were expressed as μequiv. methyl moiety/g dry fat-free tissue and showed:

   (a) alloxan-diabetic wethers and pregnant-toxaemic ewes which developed fatty livers had a significantly higher carnitine content than sheep which did not develop fatty livers. The livers from alloxan-diabetic rats had significantly higher creatine and carnitine contents than livers from normal rats.

   (b) wethers infused with 0.12 g met/kg body wt.$^{0.75}$/day had a significantly lower carnitine and PtdCho content than control wethers. Wethers infused with 0.36 g met/kg body wt.$^{0.75}$/day had a significantly lower creatine content than control wethers.

   (c) neonatal lambs had a significantly lower creatine and PtdCho content than normal adult sheep.

   (d) fatty sheep livers from the abattoirs had a significantly lower creatine content than non-fatty sheep livers from the abattoirs, possibly due to lower guanidinoacetate methyltransferase activity.

9. Hepatic methionine adenosyltransferase specific activity was determined in sheep under various physiological conditions. The activity was similar in pregnant-toxaemic ewes, neonatal lambs and alloxan-diabetic insulin stabilized wethers subsequently withdrawn from insulin for 24 h. However, the activities appeared considerably lower than literature values for normal adult sheep and rat liver.

10. The creatine and carnitine content of skeletal muscle, and the urinary excretion of creatinine and carnitine was determined in normal and alloxan-diabetic sheep. The results were expressed as μequiv. methyl moiety/g dry fat-free tissue or μequiv. methyl moiety excreted/24 h, and showed:

    (a) the body pool of methyl groups in both metabolites was similar in sheep.

    (b) the urinary $\frac{\text{creatinine}}{\text{carnitine}}$ ratio was about 12 for two normal wethers and 61 for a normal ewe. In the alloxan-diabetic state these ratios were about 5 and 2,263 respectively. The large increase in ratio for the diabetic ewe was due mainly to a decreased excretion of carnitine.

11. The total acid-soluble carnitine content of skeletal muscle and liver tissue from neonatal lambs was determined. The carnitine content of skeletal muscle increased rapidly from about 5% of the adult value at birth to about 40% of the adult value at 8 days of age. These results were discussed in relation to the possible role of ewes milk in carnitine metabolism in young lambs.

12. Radioisotopes were used to study the biosynthesis of carnitine and PtdCho, and the metabolism of choline, in isolated hepatocytes prepared from alloxan-diabetic insulin stabilized wethers and alloxan-diabetic insulin stabilized wethers which were subsequently withdrawn from insulin for 24 h. Results were obtained for 2 wethers and indicated:

(a) incorporation of L-[*methyl*-^{3}H]methionine into PtdCho was depressed in an insulin withdrawn wether compared to an insulin stabilized wether, while its incorporation into carnitine was increased in the insulin withdrawn wether compared to the insulin stabilized wether.

(b) incorporation of [1,2-^{14}C]choline into PtdCho was increased in the insulin withdrawn wether compared to the insulin stabilized wether. The incorporation of [1,2-^{14}C] choline and L-[*methyl*-^{3}H]methionine into *sn*-glycero-3-phosphocholine was lower in the insulin withdrawn wether compared to the insulin stabilized wether. The rate of PtdCho synthesis *via* methylation of PtdEtn, expressed as a percentage of the total rate of PtdCho biosynthesis *via* both the methylation and CDP-Cho pathway was about 4.8% for the insulin stabilized wether and 0.6% for the insulin withdrawn wether.

(c) the ratio of [1,2-^{14}C]choline metabolism to betaine and [1,2-^{14}C]choline incorporation into PtdCho after 60 min incubation was about 0.84 for both wethers, compared to a ratio of about 26 calculated from data in the literature for isolated rat hepatocytes.

13. The main findings in this work suggest that under conditions of increased carnitine biosynthesis in sheep liver, choline biosynthesis was depressed. These animals also had fatty livers. The creatine content was depressed in fatty livers, possibly due to lowered activity of guanidinoacetate methyl-transferase. Thus, there may be competition for methyl groups between carnitine, choline and creatine biosynthesis under conditions of metabolic stress in sheep liver which in turn may be related to the development of the fatty state.

STATEMENT

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.

....

Graham Dean Henderson

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LITERATURE REVIEW

1. Introduction

Sheep are particularly prone to the development of fatty livers under field and laboratory conditions. However, the biochemical mechanisms underlying the lipid accumulation are poorly understood.

Carnitine, creatine and choline require methyl groups from L-methionine in the course of their biosynthesis. L-methionine is frequently the first limiting amino acid in sheep. The importance of choline in cell membranes and lipid transport, creatine in methyl group metabolism, and carnitine in fatty acid metabolism suggests a relationship exists between the metabolism of these compounds and the development of fatty livers in sheep.

The purpose of this review is to highlight currently known facts about these metabolites and fatty livers in sheep, to explore interrelationships between these compounds and the fatty state and to point out deficiencies in current knowledge as far as their metabolism in the sheep is concerned. Information from studies on other animals has been included where relevant and where information is lacking for the sheep.

2. The normal sheep liver

2.1. Morphology

The sheep liver is dark-reddish-brown in colour under normal physiological conditions, and weighs about 1%-3% of the live

weight of the animal. However, the weight of the liver is greatly influenced by the food intake (Robinson, 1948). Changes in this percentage of liver weight to body weight may occur under various physiological conditions such as short term starvation (Callaghan & Thompson, 1940; Kirton *et al.*, 1968), pregnancy and lactation (Campbell & Fell, 1970) and after feeding super- or sub-maintenance rations (Robinson, 1948).

Rushton & Murray (1976) have listed a number of references to sheep liver anatomy. The vascular morphology of the sheep liver is not markedly different from other mammalian livers (Rushton & Murray, 1976). An extensive discussion of the morphology of the mammalian liver is provided by Elias & Sherrick (1969) and in Schiff (1975).

According to Rappaport (1975), the hepatic structural, microcirculatory and functional unit is the liver acinus (see Fig. 1), and is defined as follows:

"The simple liver acinus is a small parenchymal mass, irregular in size and shape, and arranged around an axis consisting of a terminal hepatic arteriole, portal venule, bile ductule, lymph vessels and nerves which grow out together from a small triangular portal field. The simple liver acinus lies between two (or more) terminal hepatic venules ("central veins") with which its vascular and biliary axis interdigitates. In a two-dimensional view it occupies sectors only of two adjacent hexagonal fields".

The vascular network of the mammalian liver is regarded as a sinusoidal system that differs from true capillary networks in a number of ways including the absence of a basal lamina and the

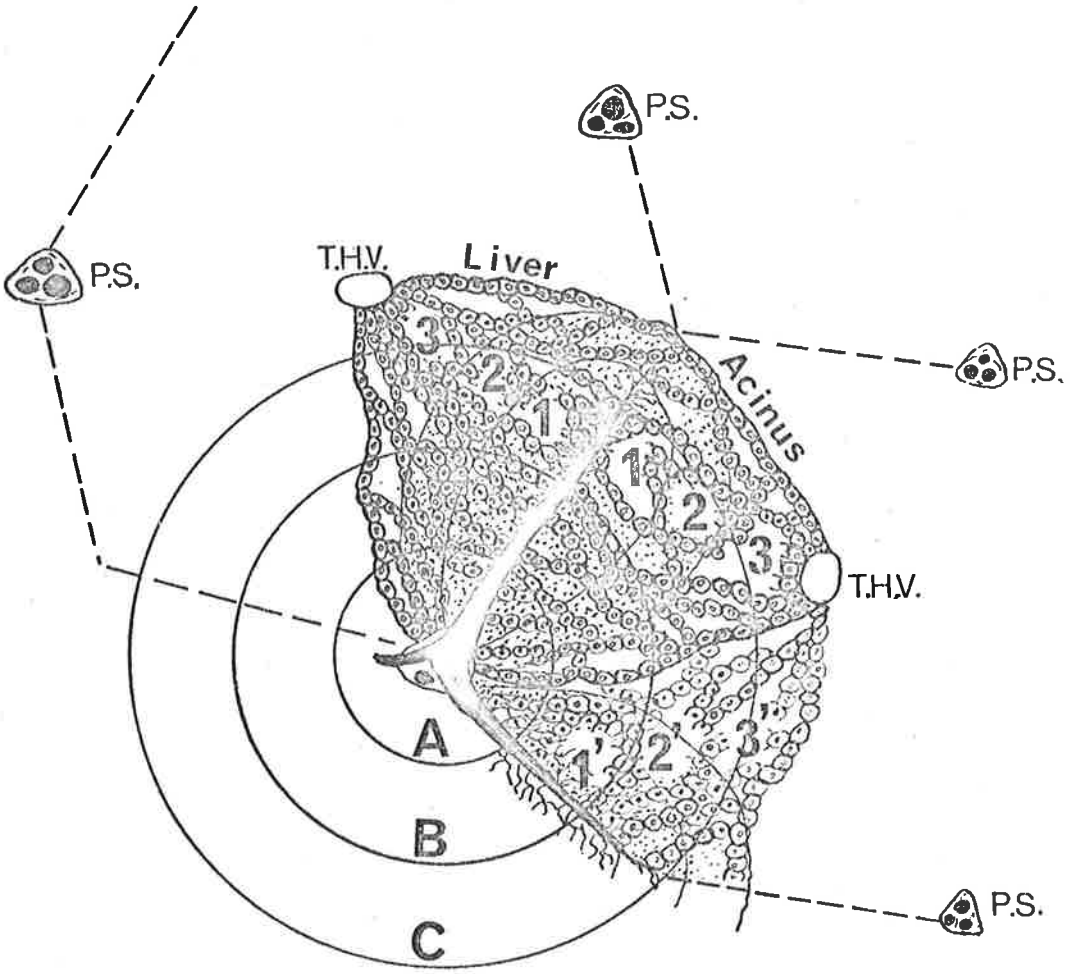
FIGURE 1

Blood supply of the simple liver acinus and the zonal arrangement of cells

[Taken directly from Rappaport (1975)]

The acinus occupies adjacent sectors of neighbouring hexagonal fields. Zones **1**, **2** and **3**, respectively represent areas supplied with blood of first, second, and third quality with regard to oxygen and nutrients. These zones centre about the terminal afferent vascular branches, terminal bile ductules, lymph vessels, and nerves, and extend into the triangular portal field from which these branches crop out.

Zones **1'**, **2'** and **3'** designate corresponding areas in a portion of an adjacent acinar unit. In Zones **1** and **1'** the afferent vascular twigs empty into the sinusoids. Circles **B** and **C** indicate peripheral circulatory areas as commonly described around a "periportal" area, **A**. P.S., portal space; T.H.V., terminal hepatic venules ("central veins").


P.S.
P.S.
P.S.
P.S.
T.H.V.
T.H.V.
Liver
Acinus
3
2
1
1
2
3
A
B
C
1'
2'
3'

presence of large discontinuities in the endothelial lining (Fawcett, 1963). The freely anastomotic sinusoidal system found in sheep is similar to that in other mammals with the exception of the pig. However, in contrast to the livers of a number of mammals, including rats, mice, dogs, rabbits and humans, the sinusoids of goat (Kuhn & Olivier, 1965), sheep (David, 1964; Grubb & Jones, 1971; Gemmell & Heath, 1972) and bovine (Wood, 1963; David, 1964) liver have a distinct basal lamina (see Fig. 2). The hepatic sinusoids of adult sheep are surrounded by a complete basal lamina, and the endothelial lining is discontinuous. The basal lamina in newborn lambs is only partially formed, and the endothelial lining appears more continuous (Gemmell & Heath, 1972).

The function of the basal lamina is not known, but it is possible it plays a part in structural support for the sinusoids (Wood, 1963) and in regulating the movement of large molecules such as lipoproteins between the hepatocytes and the blood (Grubb & Jones, 1971; Gemmell & Heath, 1972). Bergman *et al.* (1971) consider that differences in the metabolism of chylomicrons between sheep and dogs may in part be related to differences in the structure of their hepatic sinusoids.

Largely as a result of histochemical studies it is known that parenchymal cells in different zones within the liver acinus are morphologically and functionally different [Rappaport (1975) has summarized some of these differences]. Manns (1972) has observed this in the sheep liver. These considerations are relevant to understanding the underlying biochemical mechanisms behind histologically detected changes in liver cells under various physiological conditions, such as the accumulation of lipid around the terminal

FIGURE 2

A diagrammatic representation of the ultrastructure of the hepatic sinusoid of adult sheep

The main details of the ultrastructure to be noted include the presence of a continuous basal lamina interposed between the hepatocyte microvilli and the vascular endothelium, the discontinuous endothelial lining, and the numerous hepatocyte microvilli extending into the space of Disse.

Details of the ultrastructure of the hepatic sinusoids of sheep are described by Grubb & Jones (1971) and Gemmell & Heath (1972).

Abbreviations:

B, bile canaliculus with microvilli

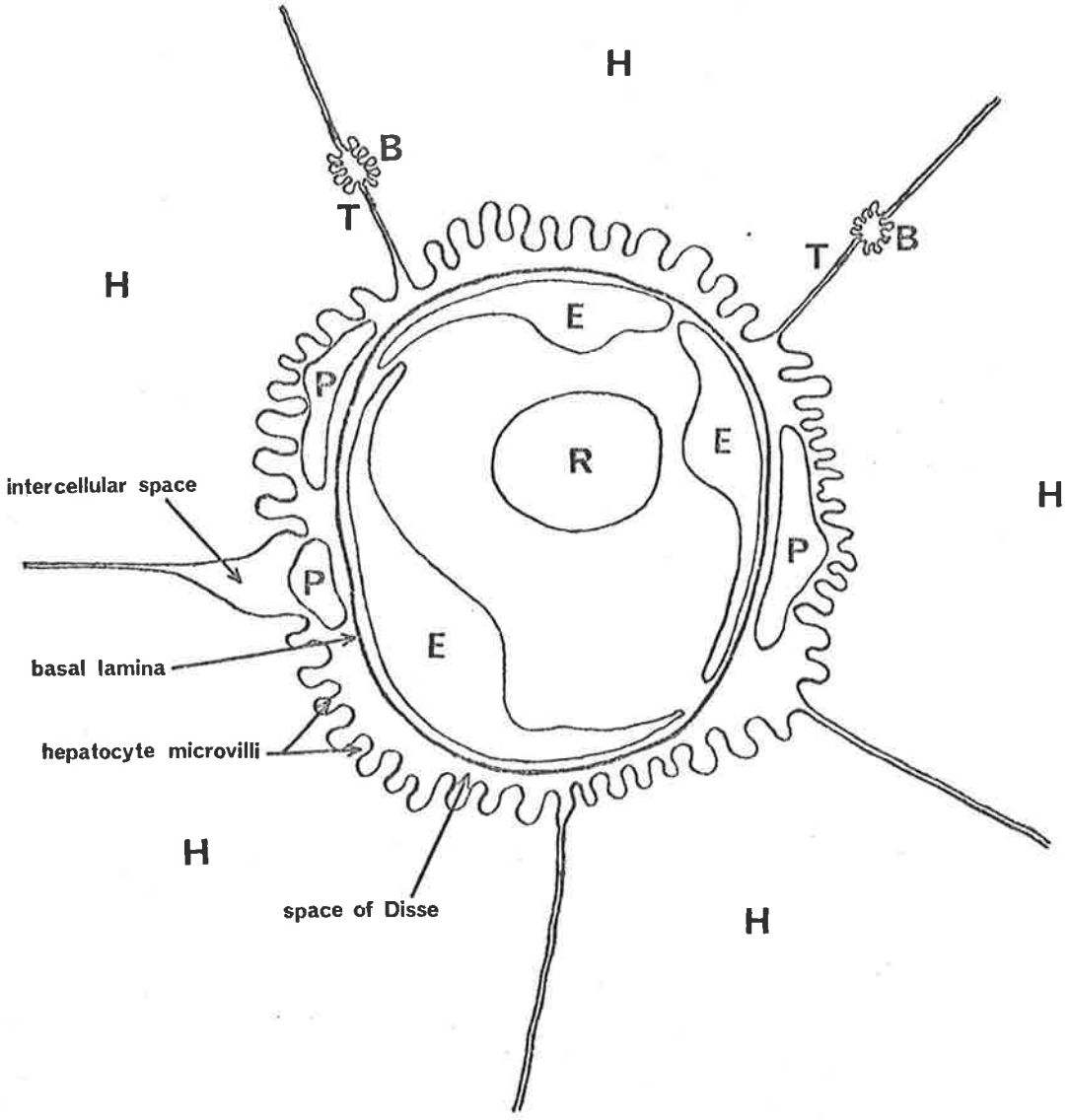
E, endothelial cell

H, hepatocyte

P, perisinusoidal cell

R, red blood cell

T, tight junction


H
B
T
T
B
H
E
P
R
E
intercellular space
H
P
P
basal lamina
E
hepatocyte microvilli
H
space of Disse
H

hepatic venules in choline deficiency (Hartroft, 1950), and around the portal space in protein deficiency (Viviani *et al.*, 1964).

Wanson *et al.* (1975) and Drochmans *et al.* (1975) have physically separated rat hepatocytes from different regions within the liver acinus.

Recent technical developments have enabled the isolation of metabolically viable sheep hepatocytes for *in vitro* biochemical studies (Jarrett *et al.*, 1975; Ash *et al.*, 1975; Clark *et al.*, 1976).

2.2. The lipid composition of sheep liver

Lipid represents about 5% of the fresh weight of sheep liver, of which about 70% is phospholipid and 30% is neutral lipid (Peters & Smith, 1964).

Free cholesterol and triacylglycerols are the major neutral lipids (Peters & Smith, 1964), and 3-*sn*-phosphatidylcholines and 3-*sn*-phosphatidylethanolamines are the major phospholipids (Peters & Smith, 1964; Dawson, 1960; Scott *et al.*, 1967; Getz *et al.*, 1968; Noble *et al.*, 1971*a*).

The major fatty acids in adult sheep liver lipids are palmitic, stearic, oleic and linoleic acids. Arachidonic acid is relatively more important in the phospholipids than the neutral lipids (Horgan & Masters, 1963; Scott *et al.*, 1967). The liver lipids of young suckling lambs are relatively richer in polyunsaturated fatty acids compared to an adult ewe (Noble *et al.*, 1970, 1971*b*).

3. The fatty sheep liver

Sheep appear prone to the development of fatty livers as

evidenced by their high incidence at the local abattoirs and numerous reports of their incidence in the literature. However, relatively few studies have investigated the cause or nature of these fatty livers.

Fatty livers are usually larger and paler than normal livers, and the increase in weight is due mainly to an accumulation of lipid and water (Lucas & Ridout, 1967). Fatty livers are associated with a large number of physiological conditions, such as protein deficiency, choline deficiency, diabetes, pregnancy and fasting. Species differ in their susceptibility to fatty livers of various aetiology. For instance, rats are less susceptible than sheep to fatty livers associated with fasting (Harrison, 1953; Manns, 1972), and guinea pigs are less susceptible than rats to fatty livers associated with choline deficiency (Lucas & Ridout, 1967).

Triacylglycerols are usually the main lipids which accumulate in the fatty livers of various aetiology (Lombardi, 1966).

3.1. Causes of fatty livers

When the formation of triacylglycerol exceeds its utilization or removal, a fatty liver results.

Generally, hepatic triacylglycerols arise either from plasma fatty acids, or fatty acids synthesized within the liver. The liver does not synthesize significant amounts of fatty acids in sheep (Ingle *et al.*, 1972*a*,*b*), and consequently the plasma is the principal source of fatty acids. The plasma fatty acid concentration is elevated in fasted, pregnant-toxaemic and alloxan-diabetic sheep

(Lindsay & Leat, 1975; Patterson, 1966; Jarrett *et al.*, 1974). This results in an increased flux of fatty acids through the oxidation and esterification pathways as fatty acids are taken up in proportion to their concentration in the blood (Katz & Bergman, 1969). The mechanisms controlling the balance between oxidation and esterification are not well understood (Mayes, 1976), but some factors involved are discussed in section 4 of this review. Enhanced triacylglycerol synthesis may be the principal factor in the fatty livers associated with noradrenaline or cortisone administration (Coleman, 1973).

The liver is the major site of synthesis of VLDL, a lipoprotein class rich in triacylglycerols (Coleman, 1973). Hepatic triacylglycerols are the immediate precursors of triacylglycerols present in VLDL (Mayes, 1976). Consequently, the formation and secretion of these lipoproteins represents a major route of utilization of hepatic triacylglycerols. Fatty livers associated with dietary protein and choline deficiency are due to impaired synthesis and/or secretion of lipoproteins (Coleman, 1973). The levels of chylomicrons and VLDL are low in normal sheep plasma compared with some animals (Mills & Taylaur, 1971; Nelson, 1973; Kubasek *et al.*, 1974; Leat *et al.*, 1976). These low levels are probably associated with the low intake of dietary lipid under normal husbandry conditions (Leat *et al.*, 1976). A major cause of fatty livers in sheep may be an inherent incapacity to synthesize significant quantities of VLDL. The physiological significance of the unique structure of the sheep liver sinusoids with regard to lipoprotein secretion is not known but may also be an important factor limiting the release of triacylglycerols from the liver.

3.2. The incidence of fatty livers in sheep

There are numerous reports of fatty livers in sheep under a variety of physiological conditions, such as, acute and chronic starvation and undernutrition (Roderick *et al.*, 1937; Fraser *et al.*, 1938; Snook, 1939; Ferguson, 1954; Parry & Shelley, 1958; Ford, 1962; Jackson *et al.*, 1964; Patterson, 1966; Taylor & Jackson, 1968; Manns, 1972), pregnancy toxaemia (Roderick *et al.*, 1937; Dryerre & Robertson, 1941; Ferguson, 1954; Patterson, 1966; Pethick, 1975), severe diabetes (Jarrett, 1946; Jarrett *et al.*, 1956), normal pregnancy (Dryerre & Robertson, 1941; Smith & Walsh 1975), lactation (Smith & Walsh, 1975), facial eczema (Peters & Smith, 1964) and cobalt deficiency (Smith & Osborne-White, 1973). Fatty infiltration of the liver is one of the most characteristic features of pregnancy toxaemia in ewes (Reid, 1968).

A number of other domesticated ruminants have been shown to develop fatty livers. Spontaneous bovine ketosis, a condition metabolically similar to pregnancy toxaemia in sheep (Baird, 1977) is characterized by fatty infiltration of the liver (Baird *et al.*, 1968; Schultz, 1968, 1971; Bergman, 1971). Starved lactating and non-lactating cows develop fatty livers (Brumby *et al.*, 1975; Reid *et al.*, 1976; Reid *et al.*, 1977*a*,*b*), and alloxan-diabetic goats have fatty livers (Schwalm & Schultz, 1976).

3.3. Characterization of the fatty livers in sheep

3.3.1. Histology of fat accumulation

Manns (1972) reported that in fresh-frozen liver sections from sheep starved for 1 to 4 days, lipid was distributed evenly

throughout the liver acini. Snook (1939), Dryerre & Robertson (1941) and Patterson *et al.* (1964) observed that in the fatty livers of pregnant and pregnant-toxaemic ewes, lipid tended to accumulate around the portal space of the liver acini (see Fig. 1). The pattern of lipid accumulation in the liver acini may give some indication of the cause of the fatty liver, as reviewed in section 2.

3.3.2. The nature of the fat accumulating

Few studies have characterized the lipid components of the fatty livers from sheep. Dryerre & Robertson (1941) reported that neutral fat was the main component of the increased liver lipid in pregnant ewes, pregnant-toxaemic ewes, and abattoirs wethers. Total phospholipid content was relatively unchanged in these animals. Smith & Walsh (1975) identified triacylglycerol and cholesteryl ester as the principal elevated liver lipid classes in pregnant and lactating ewes.

Triacylglycerol is the major lipid class contributing to the elevated lipid content of the livers of alloxan-diabetic goats (Schwalm & Schultz, 1976) and fasted non-lactating and lactating cows (Brumby *et al.*, 1975; Reid *et al.*, 1976, 1977*b*).

3.3.3. Ultrastructural changes in the liver

Liver mitochondria are enlarged and more fragile in starved and alloxan-diabetic sheep compared to those of normal sheep (Taylor *et al.*, 1971). Liver mitochondrial enlargement is also observed in starved (Herdson *et al.*, 1964) and alloxan-diabetic rats (Harano *et al.*, 1972; Reaven *et al.*, 1973), and in a number of other physiological conditions [Harano *et al.* (1972) list a number of conditions and appropriate references].

Reaven *et al.* (1973) report that in alloxan-diabetic rats, there is a reduction and disorganisation of the rough endoplasmic reticulum, and ribosomal protein synthesis is depressed. The abnormalities were reversed within 24 h of insulin treatment, indicating they were not the result of a toxic effect due to alloxan.

An increase in the size, and a decrease in the number of mitochondria per hepatocyte is characteristic of fasted lactating and non-lactating dairy cows (Reid, 1973; Reid *et al.*, 1977*a*,*b*). These changes may be associated with impaired hepatic respiration in fasted cows (Baird *et al.*, 1972).

The volume of the rough endoplasmic reticulum is reduced in the hepatocytes of fasted cows (Reid, 1973; Reid *et al* , 1977*b*). Seawright (1965) observed some abnormalities in the appearance of the endoplasmic reticulum in the hepatocytes of starved wethers compared to a normal wether.

The significance of these membrane changes in relation to the metabolism of their component lipids is unknown in ruminants. Brumby *et al.* (1975) reported no significant differences in the total hepatic phospholipids, 3-*sn*-phosphatidylcholines and 3-*sn*-phosphatidylethanolamines between fed and 6 day fasted lactating cows. Getz *et al.* (1968) report that 3-*sn*-phosphatidylcholines and 3-*sn*-phosphatidylethanolamines are major phospholipid components of the sheep liver membranes. These compounds are metabolically heterogeneous (van den Bosch, 1974), and the possibility exists that changes in molecular classes of these compounds are associated with the observed membrane changes.

4. The regulation of liver lipid metabolism

Long chain fatty acids entering liver cells undergo metabolism *via* two major routes, oxidation to CO_2 and ketone bodies, and esterification to form triacylglycerols, phospholipids and other fatty acid esters (Scow & Chernick, 1970). Recent reviews have considered some aspects of the regulation of liver lipid metabolism (Mayes, 1976; Bremer, 1977). The major pathways, and some key enzymes involved in these pathways, are shown in Fig. 3.

A possible site of regulation is the partition of fatty acyl-CoA between acylation of carnitine, catalyzed by carnitine palmitoyltransferase (enzyme 1 in Fig. 3), and acylation of *sn*-glycerol-3-*P*, catalyzed by *sn*-glycerol-3-*P* acyltransferase (enzyme 2 in Fig. 3). In rat liver, the activities of these enzymes vary in opposite directions, depending on the physiological state of the animal. In fasted, fat-fed and alloxan-diabetic rats, the activity of carnitine palmitoyltransferase increases, and in fasted and fat-fed rats, the activity of *sn*-glycerol-3-*P* acyltransferase decreases (Norum, 1965; Aas & Daae, 1971; van Tol, 1974).

Fasting or alloxan-diabetes increase the content of carnitine in the livers of rats previously fed high carbohydrate diets (McGarry *et al.*, 1975). Fasting or alloxan-diabetes also increase the content of carnitine in the livers of sheep, but to a considerably greater extent (Snoswell & Henderson, 1970; Snoswell & Koundakjian, 1972). Addition of carnitine to the perfusion fluid in perfusion studies using the livers from rats fed a high carbohydrate diet accelerates ketogenesis (McGarry *et al.*, 1975). The

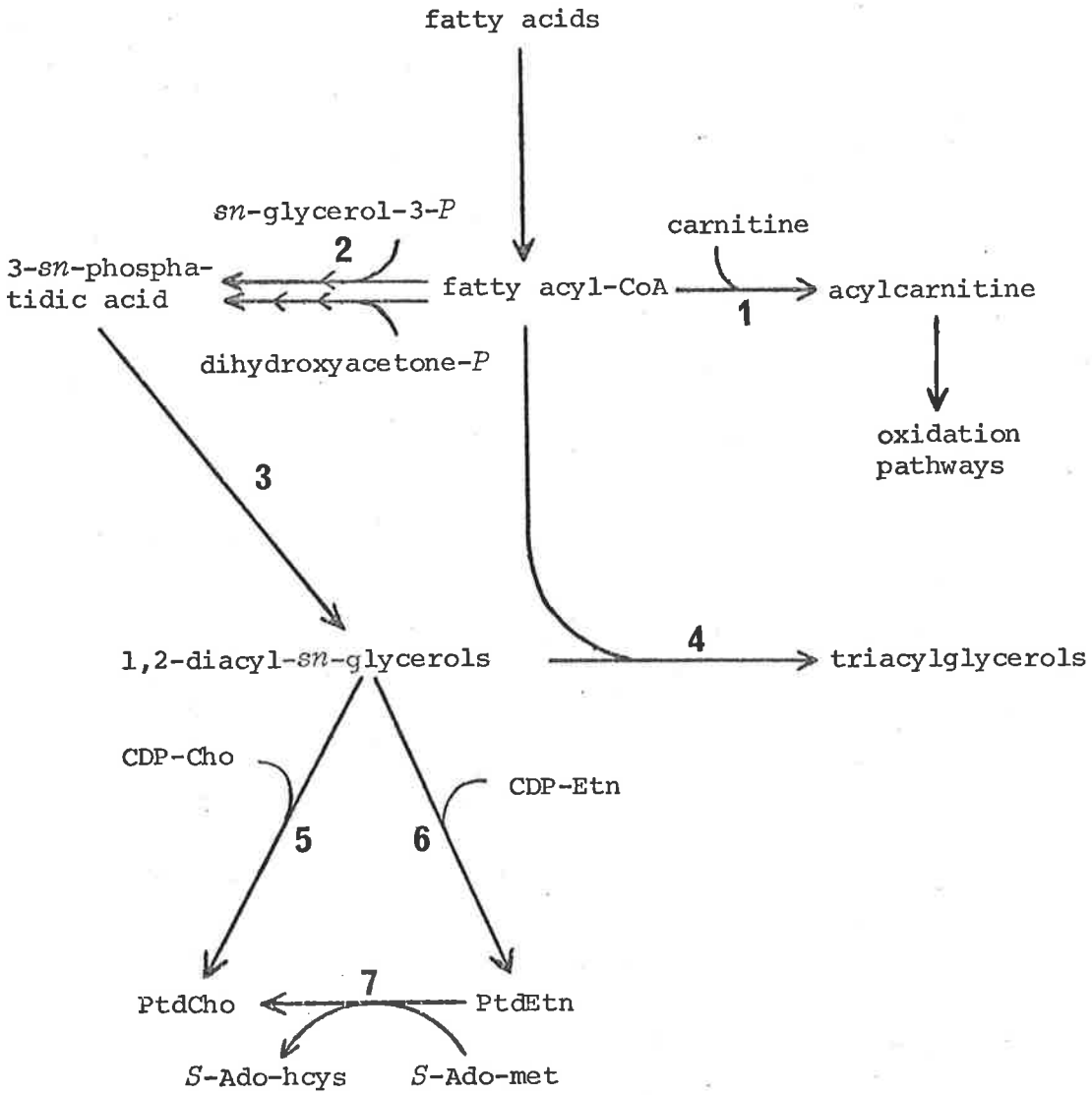
FIGURE 3

Several pathways of hepatic lipid metabolism

A summary of the key pathways of lipid metabolism is presented. Details such as reversibility of reactions, subcellular compartmentation of enzymes and substrates, and details of many reactions are omitted for clarity.

Key to the numbered enzymes:

1. Carnitine palmitoyltransferase
2. *sn*-Glycerol-3-*P* acyltransferase
3. Phosphatidate phosphatase
4. Diacylglycerol acyltransferase
5. Choline phosphotransferase
6. Ethanolamine phosphotransferase
7. 3-*sn*-Phosphatidylethanolamines methyltransferase


fatty acids
sn-glycerol-3-P
2
3-sn-phospha-
tidic acid
fatty acyl-CoA
carnitine
1
acylcarnitine
dihydroxyacetone-P
oxidation
pathways
3
4
1,2-diacyl-sn-glycerols
triacylglycerols
CDP-Cho
CDP-Etn
5
6
7
PtdCho
PtdEtn
S-Ado-hcys
S-Ado-met

rate of fatty acid oxidation and ketogenesis increased, and the rate of esterification decreased when isolated hepatocytes prepared from normal and fasted rats were incubated with carnitine (Christiansen *et al.*, 1976). The role of the tissue content of *sn*-glycerol-3-*P* in the regulation of esterification is uncertain (van Tol, 1974; van den Bosch, 1974; Christiansen *et al.*, 1976).

These observations suggest that both the relative activities of enzymes 1 and 2 (see Fig. 3) and changes in the concentration of carnitine may regulate the partition of fatty acids in the liver. Additional regulation may also occur by esterification *via* the dihydroxyacetone phosphate pathway (see Fig. 3) (Hajra, 1977).

Phosphatidate phosphatase (enzyme 3 in Fig. 3) may also be important in the regulation of triacylglycerol formation *in vivo* (Fallon *et al.*, 1977).

1,2-Diacyl-*sn*-glycerols occupy a branchpoint in the esterification pathways, leading to the synthesis of triacylglycerols, 3-*sn*-phosphatidylcholines and 3-*sn*-phosphatidylethanolamines, as shown in Fig. 3.

In vitro experiments with isolated hepatocytes indicate that under various experimental conditions, the biosynthesis of triacylglycerols are more markedly influenced than the biosynthesis of phospholipids (Ontko, 1972; Sundler *et al.*, 1974; Christiansen *et al.*, 1976; Groener & van Golde, 1977). These metabolic studies are consistent with the frequent observation that the content of triacylglycerols in liver is more variable than that of phospholipids under various physiological conditions [e.g. fasted, choline-deficient and alloxan-diabetic rats (Groener & van Golde, 1977; Lucas &

Ridout, 1967; van Harken *et al.*, 1967), fasted cows (Brumby *et al.*, 1975; Reid *et al.*, 1977*b*), alloxan-diabetic goats (Schwalm & Schultz, 1976) and pregnant and lactating ewes (Smith & Walsh, 1975)], and indicate the existence of metabolic control at the 1,2-diacyl-*sn*-glycerol branchpoint.

Factors that may be involved in metabolic regulation at this point are reviewed by van den Bosch (1974) and Åkesson & Sundler (1977). The fatty acid composition of triacylglycerols differs from that of the phospholipids in the same mammalian tissue (Hubscher, 1970). This has been shown in sheep liver (Horgan & Masters, 1963; Scott *et al.*, 1967; Noble *et al.*, 1970, 1971*b*; Read, 1976). An explanation for this could be substrate selectivity of enzymes 4, 5, and 6 in Fig. 3 for different molecular species of 1,2-diacyl-*sn*-glycerols. *In vitro* enzyme studies with rat liver indicate that diacylglycerol acyltransferase (enzyme 4 in Fig. 3) and choline phosphotransferase (enzyme 5 in Fig. 3) do not exhibit a high selectivity for molecular classes of 1,2-diacyl-*sn*-glycerols (Holub & Piekarski, 1977; Kanoh & Ohno, 1975), but that ethanolamine phosphotransferase (enzyme 6 in Fig. 3) has a high selectivity for hexaenoic 1,2-diacyl-*sn*-glycerols (Kanoh & Ohno, 1975). Recent studies suggest that choline phosphotransferase may exhibit some selectivity for molecular species of 1,2-diacyl-*sn*-glycerols which is a function of the 1,2-diacyl-*sn*-glycerol concentration and may be mediated by substrates for enzymes which compete for the common 1,2-diacyl-*sn*-glycerol precursors (Holub, 1977). The physiological significance of the *in vitro* inhibition of rat liver choline phosphotransferase and ethanolamine phosphotransferase by ATP and pantothenic acid derivatives is unknown (Sribney *et al.*, 1976; Liteplo & Sribney,

1977). *In vitro* investigations into metabolic regulation at this point are complicated owing to the insolubility of 1,2-diacyl-*sn*-glycerols in water and their molecular heterogeneity.

Åkesson & Sundler (1977) conclude that the need for 1,2-diacyl-*sn*-glycerols in phospholipid synthesis is met first, and that additional 1,2-diacyl-*sn*-glycerols are converted into triacylglycerols.

3-*sn*-Phosphatidylcholines and 3-*sn*-phosphatidylethanolamines are the major membrane phospholipids in animal tissues (McMurray, 1973). They are not homogeneous compounds but consist of molecular species differing from each other, principally by the unsaturated fatty acid esterified at the 2 position (Kuksis, 1972).

The two major pathways of 3-*sn*-phosphatidylcholines biosynthesis are the CDP-Cho-pathway (enzyme 5 in Fig. 3 is the last enzyme in this pathway) and the methylation pathway [enzyme(s) 7 in Fig. 3 catalyzes this pathway]. Evidence indicates that in rat liver the monoenoic and dienoic molecular classes of 3-*sn*-phosphatidylcholines are synthesized largely *via* the CDP-Cho-pathway, tetraenoic classes are formed mainly by acyltransfer reactions, and that the methylation pathway is involved mainly in the synthesis of tetraenoic and polyenoic (> 4 double bonds) classes of 3-*sn*-phosphatidylcholines [reviewed by van den Bosch (1974) and MacDonald & Thompson (1975)].

Evidence from *in vivo* studies with male rats suggests that the rate of 3-*sn*-phosphatidylcholines synthesis *via* the methylation pathway, expressed as a percentage of the total rate of 3-*sn*-phosphatidylcholines synthesis *via* both the methylation and CDP-Cho-pathways, is

about 3.4%-10% (Wise & Elwyn, 1965; Bjørnstad & Bremer, 1966; Trewhella & Collins, 1973). *In vitro* studies with isolated hepatocytes prepared from male rats suggest the percentage may be considerably higher (about 30%) (Sundler & Åkesson, 1975).

The physiological significance of the methylation pathway may be a role in the synthesis of specific 3-*sn*-phosphatidylcholines for a specific function such as structural lipids (Coleman, 1973). However, as this pathway represents the only known pathway for choline biosynthesis in animals (see section 7), its major physiological function may be the synthesis of this compound.

The regulation of this pathway is discussed in section 7 of this review. 3-*sn*-Phosphatidylethanolamines are successively transmethylated to form 3-*sn*-phosphatidylcholines, using methyl groups from L-methionine. Therefore it is pertinent to now consider methionine metabolism and transmethylation.

5. Methionine metabolism

Methionine is an essential amino acid in animals, and its major metabolic functions include protein synthesis, synthesis of *S*-adenosylmethionine, the primary biological methyl group donor (see section 6 of this review), and a source of cysteine, cystine and other derivatives of cysteine.

Methionine is usually regarded as the first limiting amino acid for sheep (Chalupa, 1972). As wool proteins are rich in cystine (Crewther *et al.*, 1965) the conversion of methionine to cyst(e)ine may represent a major metabolic route for methionine in sheep. Abomasal or parenteral administration of sulphur-containing

amino acids to sheep has a significant influence on the rate of wool growth. Reis *et al.* (1973*a*,*b*) observed that abomasal infusion of small amounts of methionine (up to 2.5 g/day) resulted in large increases in wool growth but relatively small changes in the pattern of plasma free amino acids.

Elwyn (1970) suggested that the liver may be the key organ in the regulation of amino acid supply to the tissues. The quantitative importance of the sheep liver in amino acid metabolism, including methionine metabolism, has been demonstrated both *in vivo* (Wolff *et al.*, 1972) and *in vitro* using the perfused liver (Lindsay *et al.*, 1975).

The principal pathways involved in methionine metabolism in mammalian liver are shown in Fig. 4. Reactions 1 and 2 in Fig. 4 are discussed in relation to transmethylation in section 6 of this review. Reactions 9 and 10 are discussed in section 7 in relation to choline metabolism.

Fig. 4 shows that hepatic methionine metabolism is a cycle, with a single major outlet to homocysteine. The regulation of degradation *versus* conservation of methionine occurs at this stage, where four known enzymes compete for the common substrate homocysteine (enzymes 3, 4, 5 & 6 in Fig. 4).

Betaine-homocysteine methyltransferase (enzyme 4) and tetrahydropteroylglutamate methyltransferase (enzyme 5) remethylate homocysteine and complete the methionine conservation cycle. Adenosylhomocysteinase (enzyme 3) catalyzes the reversible formation of *S*-adenosylhomocysteine, a powerful inhibitor of most trans-methylation reactions (Duerre & Walker, 1977). Cystathionine

FIGURE 4

Known pathways of methionine metabolism in animals

[After Finkelstein *et al.* (1974)]

The key enzymes of these pathways are as follows:

1 Methionine adenosyltransferase

2 Enzyme Nomenclature [1972 & Supplement 1: Corrections & Additions (1975)] lists 46 methyltransferases that utilize *S*-adenosylmethionine as the methyl donor.
Three such methyltransferases are:
Guanidinoacetate methyltransferase, the terminal enzyme in creatine biosynthesis.
3-*sn*-Phosphatidylethanolamines methyltransferase, the enzyme(s) associated with choline biosynthesis.
Protein(lysine) methyltransferase, a postulated enzyme in carnitine biosynthesis.

3 Adenosylhomocysteinase

4 Betaine-homocysteine methyltransferase

5 Tetrahydropteroylglutamate methyltransferase

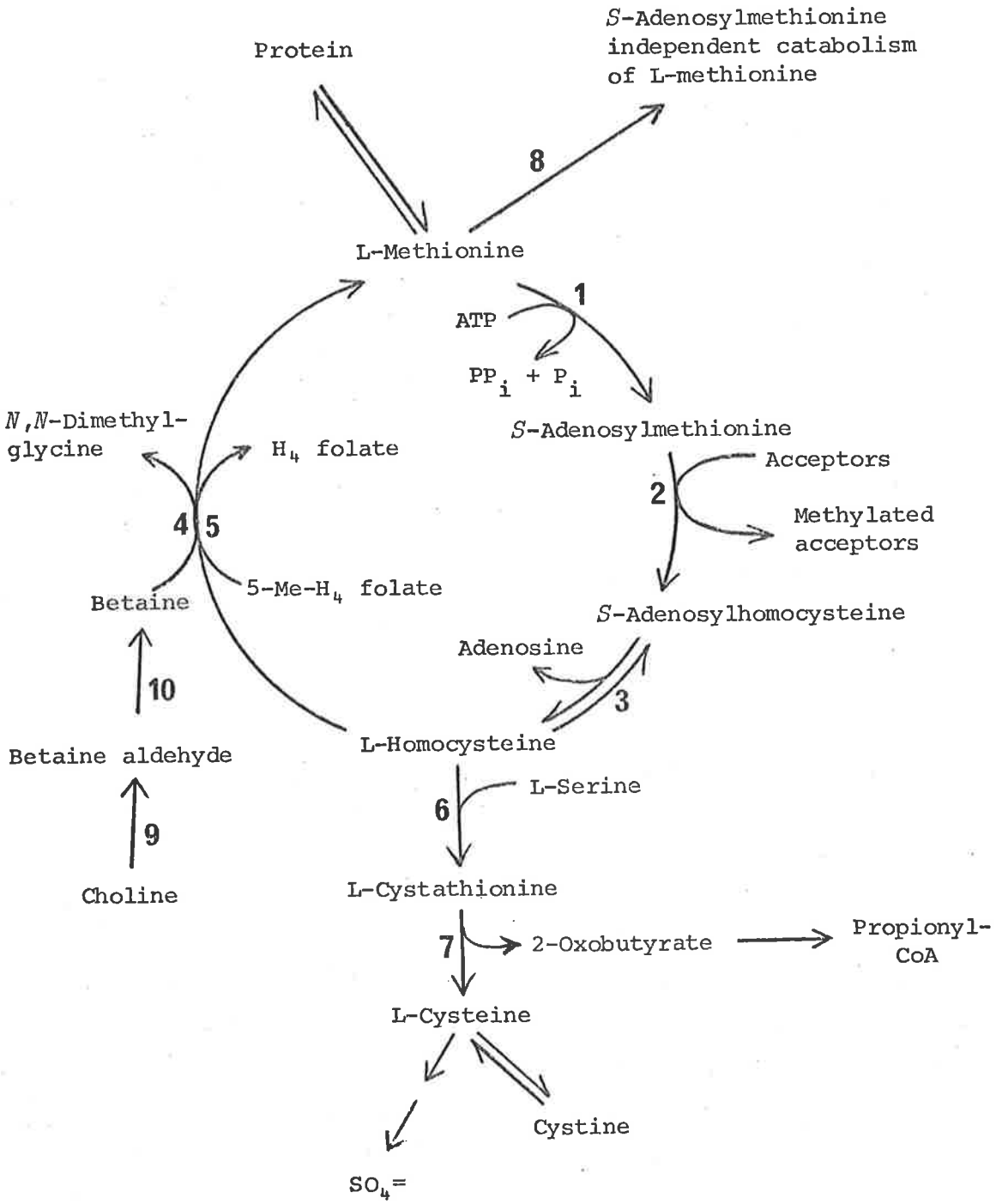
6 Cystathionine β-synthase

7 Cystathionine γ-lyase

8 Unknown enzymes in *S*-Ado-met independent catabolism of L-methionine

9 Choline dehydrogenase

10 Betaine-aldehyde dehydrogenase


Protein
S-Adenosylmethionine independent catabolism of L-methionine
8
L-Methionine
1
ATP
PPi + Pi
S-Adenosylmethionine
N,N-Dimethyl-glycine
H4 folate
Acceptors
2
Methylated acceptors
4
5
5-Me-H4 folate
Betaine
S-Adenosylhomocysteine
Adenosine
3
10
L-Homocysteine
Betaine aldehyde
L-Serine
6
9
L-Cystathionine
Choline
7
2-Oxobutyrate
Propionyl-CoA
L-Cysteine
Cystine
SO4=

β-synthase (enzyme 6) catalyzes the formation of cystathionine, the first reaction in the trans-sulphuration pathway, which is the major route for methionine degradation in mammals (Finkelstein & Mudd, 1967; Finkelstein, 1974). The distribution of homocysteine between these competing reactions is determined by the kinetic properties and tissue content of these enzymes (Finkelstein, 1974).

In vitro studies of the rat liver enzymes 3, 4, 5 & 6 (see Fig. 4) (Finkelstein, 1967; Finkelstein *et al.*, 1971; Finkelstein & Harris, 1973), the perfused rat liver (Finkelstein, 1974) and isolated rat hepatocytes (Krebs & Hems, 1976; Krebs *et al.*, 1976), and *in vivo* studies with humans (Mudd & Poole, 1975), indicate that folate and methionine metabolism are intimately related, and reveal the importance of tetrahydropteroylglutamate methyltransferase (enzyme 5 in Fig. 4) in the regulation of methionine metabolism. The specific activity of this enzyme appears similar in rat and sheep liver (Finkelstein *et al.*, 1971; Gawthorne & Smith, 1974). Regulation is mediated, at least in part, by specific effects on a number of enzymes by *S*-adenosylmethionine (Kutzbach & Stokstad, 1967; Finkelstein *et al.*, 1975; Buchanan *et al.*, 1964) and *S*-adenosylhomocysteine (Finkelstein *et al.*, 1974). In relation to these regulatory concepts, it is interesting that Eloranta (1977) observed transient increases in the liver content of *S*-adenosylmethionine and *S*-adenosylhomocysteine after intraperitoneal administration of methionine to rats.

When laboratory animals are fed diets containing more than about 2% methionine, a number of physiological changes occur such as, increased urinary excretion of creatinine, loss of body fat, increased liver fat and atrophy of the liver [an extensive list of

physiological effects and appropriate references is given by Strecker *et al.* (1970)]. Infusion of large amounts of methionine (about 2.5-10 g/day) into the abomasum of sheep results in a dose dependent reduction of the wool growth response obtained when smaller amounts (up to 2.5 g/day) are infused, and substantial changes in the pattern of plasma free amino acids, in particular, large increases in the plasma concentration of methionine (Reis *et al.*, 1973*a*,*b*).

Alternative catabolic pathways not involving the synthesis of *S*-adenosylmethionine may become important at these unphysiological levels of methionine (Benevenga, 1974). These pathways are shown as number 8 in Fig. 4. The initial step may involve transamination of methionine to α-keto-γ-methiol-butyrate (Mitchell & Benevenga, 1975). Radcliffe & Egan (1976) report that transamination may be important in situations of methionine loading in the sheep. However, relatively little is known about these pathways.

6. Transmethylation

The concept of biological transmethylation, that is, the transfer of a complete methyl group, and the important role of methionine in transmethylation reactions was established by du Vigneaud and colleagues in the 1940's (reviewed by du Vigneaud & Rachele, 1965).

Subsequent studies demonstrated that methionine must be activated before it can act as a methyl donor (Cantoni, 1951), and the active intermediate was identified as *S*-adenosylmethionine (Cantoni, 1952, 1953).

The dominant mechanism of transmethylation from methionine is attack by nucleophilic sites (e.g. N, O, S) within methyl acceptor molecules upon the positively charged methyl group attached to the sulphonium pole of *S*-adenosylmethionine (Mudd & Cantoni, 1964; Mudd, 1973*a*). The key pathways of transmethylation are shown in Fig. 4.

6.1. The biosynthesis of *S*-adenosylmethionine

S-Adenosylmethionine is synthesized in animal tissues by the direct enzymatic adenosylation of L-methionine at the expense of ATP, by methionine adenosyltransferase (enzyme 1 in Fig. 4). The properties of this enzyme have been reviewed by Mudd (1973) and Chou *et al.* (1977).

The enzyme is most active in the liver of various mammalian species (Mudd *et al.*, 1965; Sturman *et al.*, 1970; Eloranta, 1977) where it occurs in the cytosol fraction (Allsop & Watts, 1975).

There are conflicting reports in the literature on the effect of age on the liver enzyme. The foetal livers of rats, mice, rabbits and humans have a lower activity than that of adult animals (Hancock, 1966; Sheid, 1968; Gaull *et al.*, 1972). The enzyme activity of rat liver has been reported to increase (Artom, 1969), decrease (Finkelstein, 1967) or change relatively little (Eloranta, 1977) with increasing age. Eloranta *et al.* (1976) report that the liver enzyme activity decreases with age in male chicks. Presumably this confusion results from differences in the method of expression of the enzyme activity, and difficulties associated with measuring its activity (Chou *et al.*, 1977).

The enzyme activity increased in the livers of alloxan-diabetic rats and rabbits (Finkelstein, 1967; Mudd *et al.*, 1965), and decreased in starved rats (Pan & Tarver, 1967). A specific deficiency of methionine adenosyltransferase has been demonstrated in the liver of a human child with hypermethionemia (Gaull, 1977).

The significance of the observed changes in the enzyme activity under various physiological conditions is obscure, as it is generally considered that the rate-limiting factor in *S*-adenosyl-methionine synthesis is the tissue concentration of methionine (Lombardini & Talalay, 1971; Eloranta, 1977).

6.2. The *S*-adenosylmethionine content of liver

The *S*-adenosylmethionine content of rat tissues is low, with the liver having the highest content [about 50-80 nmol/g fresh tissue (Lombardini & Talalay, 1971; Salvatore *et al.*, 1971; Eloranta, 1977)]. In the adult sheep and calf liver, the content is about 17 and 55 nmol/g fresh tissue respectively (Gawthorne & Smith, 1974; Salvatore *et al.*, 1971). The *S*-adenosylmethionine content of rat liver does not change markedly with age (Eloranta, 1977), except that it may be lower in aged rats (Stramentinoli *et al.*, 1977).

S-Adenosylmethionine has a very rapid turnover in tissues (Lombardini & Talalay, 1971). The tissue concentration appears to be controlled by rates of synthesis rather than rates of utilization (Chou *et al.*, 1977).

6.3. The utilization of *S*-adenosylmethionine in transmethylation reactions

S-Adenosylmethionine functions in a number of biochemical reactions (Lombardini & Talalay, 1971; Cantoni, 1977), the most significant of which is its methyl donor function in hundreds of transmethylation reactions [these are reviewed by Mudd & Cantoni (1964), Cantoni (1975), and in Salvatore *et al.* (1977)].

S-Adenosylmethionine is the sole methyl donor in all methyl transfer reactions except those resulting in the biosynthesis of methionine (reactions 4 & 5 in Fig. 4) (Cantoni, 1975; Fuller, 1976). The methyl acceptors have not been fully characterized in many instances, particularly in the case of macromolecules such as proteins. 46 methyltransferases have been identified that utilize the common substrate *S*-adenosylmethionine (Enzyme Nomenclature, 1972, 1975), but relatively few have been highly characterized.

The relative quantitative importance of the transmethylation reactions, and the factors involved in their regulation are largely unknown (Cantoni, 1977). The methylation of guanidinoacetic acid is quantitatively the most important methyl transfer reaction in mammals (Mudd & Poole, 1975). However, relatively little is known concerning the regulation of the methyl transferase involved in this reaction, guanidinoacetate methyltransferase. The enzyme probably occurs in the cytosol of the liver (Cantoni & Vignos, 1954). The activity of the sheep liver enzyme appears several fold lower than that of the rat (Salvatore & Schlenk, 1962). The activity of the rat liver enzyme has been investigated under a variety of physiological conditions, and the activity was elevated in the livers

of rats fed a guanidinoacetate supplemented diet (van Pilsum & Carlson, 1970; Carlson & van Pilsum, 1973).

A preliminary investigation of the kinetic parameters of various transmethylases indicates that differences in the K_m for the different methyl-acceptor molecules, the tissue content of the enzymes and their K_i for *S*-adenosylhomocysteine may form a basis for regulation of *S*-adenosylmethionine utilization (Cantoni, 1977).

S-Adenosylhomocysteine, one of the products of all *S*-adenosylmethionine utilizing enzymes, is a potent inhibitor of most methyltransferases (Duerre & Walker, 1977). Tissue levels of *S*-adenosylhomocysteine in the rat are lower than that of *S*-adenosylmethionine, and the liver has the highest content (Eloranta, 1977). The activity of adenosylhomocysteinase (enzyme 3 in Fig. 4) is about 10-fold higher than that of methionine adenosyltransferase (enzyme 1 in Fig. 4) in rat liver (Eloranta, 1977). The activity of the rat liver enzyme has been investigated under various physiological conditions (Finkelstein & Harris, 1973).

Regulation of the tRNA methyltransferases may be mediated in part through the action of competing methyltransferase systems in animal tissues (Swiatek *et al.*, 1973; Kerr & Heady, 1974). A competing methyltransferase system has also been reported in embryos of *Musca domestica* (Law *et al.*, 1976). Glycine methyltransferase may play a regulatory role in the utilization of *S*-adenosylmethionine in the liver (Kerr, 1972; Eloranta, 1977).

In addition to the possible regulatory factors mentioned above, metabolic compartmentation of *S*-adenosylmethionine (Judes & Jacob, 1972), and an *S*-adenosylmethionine binding protein (Smith,

1976) may play a regulatory role in *S*-adenosylmethionine utilization in animal tissues.

7. Choline metabolism

Choline (β-hydroxyethyltrimethyl ammonium hydroxide) is present in a wide variety of biological materials (Griffith & Nyc, 1954). A large number of animal species appear to require choline in their diet. Deprivation of dietary choline results in a large number of metabolic changes, the principal manifestation of which is the accumulation of large amounts of triacylglycerols in the liver (Lucas & Ridout, 1967). The biochemical basis of the lipotropic action of choline is believed to be associated with the secretion or synthesis of plasma lipoproteins by the liver (Mookerjea, 1971). Dietary choline deficiency has been implicated as a cause of fatty livers in calves (Johnson *et al.*, 1951).

The principal known metabolic pathways for choline in the animal liver are its conversion to phosphocholine and subsequent incorporation into phospholipids, and its oxidation to betaine, which plays a role as a methyl donor in the methionine cycle described in section 5.

7.1. The choline content of liver

There are relatively few accurate estimates of the tissue content of free choline and some of its compounds owing to difficulties in their assay (Schuberth *et al.*, 1970). Nevertheless, it is apparent the level of free choline in the rat liver is relatively low compared to the level of some of its compounds (Ansell & Spanner, 1971; Sundler *et al.*, 1972; Wong & Thompson,

1972; White, 1973).

3-*sn*-Phosphatidylcholines represent the major form of choline in the rat liver, as shown in Table 1. In the sheep liver, in addition to 3-*sn*-phosphatidylcholines, *sn*-glycero-3-phosphocholine is a major form of choline (see Table 1). *sn*-Glycero-3-phosphocholine is an intermediate in the catabolism of 3-*sn*-phosphatidylcholines (Dawson, 1955), and its high level in sheep liver is probably due to the absence of *sn*-glycero-3-phosphocholine phosphodiesterase in this tissue (Dawson, 1956).

Metabolic interrelationships between the various forms of choline in the liver are summarized by Thompson (1973) and Coleman (1973). Recent concepts concerning the regulation of some of the pathways associated with the choline phospholipids are discussed by van den Bosch (1974) and Åkesson & Sundler (1977), and highlight the complexity of the interrelationships.

7.2. The biosynthesis of choline, and in particular its methyl groups

The early known details of choline biosynthesis are summarized by Mudd & Cantoni (1964). Some relevant observations from this early work, and more recent concepts of choline biosynthesis are included in this review.

7.2.1. The site of biosynthesis

The principal organ involved in the biosynthesis of choline appears to be the liver (Bremer & Greenberg, 1961*a*; Bjørnstad & Bremer, 1966), and the principal intracellular site of *N*-methylation is the microsomes (Bremer & Greenberg, 1960).

TABLE 1

The hepatic content of choline and some of its compounds

Values have been taken from the literature, as indicated.

Form of tissue choline	Hepatic content µmol/g fresh tissue	
	Rat	Sheep
Free choline	0.24*	1.65§
P-choline	1.45*	n.d.
CDP-choline	0.009*	n.d.
PtdCho	13.47*	19.35Π
Cer*P*Cho	1.72†	2.44Π
Gro*P*Cho	0.24**	7.44¶
Total choline	22.53‡	28.59¶

n.d. not determined

* Sundler *et al.* (1972)

† White (1973)

‡ Strength *et al.* (1965)

§ Luecke & Pearson (1945)

Π Scott *et al.* (1967)

¶ Schmidt *et al.* (1952, 1955)

** Dawson (1955)

7.2.2. The origin of the methyl groups

The origin of the 3 methyl groups of choline is methionine, and *S*-adenosylmethionine is the immediate methyl donor, as reviewed by Mudd & Cantoni (1964).

7.2.3. The nature of the methyl acceptor and methyltransferase(s)

Evidence indicates that methylation of ethanolamine occurs at the phosphatide level (Bremer & Greenberg, 1959; Bremer *et al.*, 1960; Gibson *et al.*, 1961). The phosphatidyl structure is important for at least the last methylation step in rat liver (Le Kim *et al.*, 1973). The methylation pathway for 3-*sn*-phosphatidylcholines biosynthesis in rat liver [enzyme(s) 7 in Fig. 3] is involved mainly in the synthesis of tetraenoic and polyenoic (> 4 double bonds) species (see section 4 of this review). *In vitro* evidence indicates that for the last methylation step, substrates with a higher degree of unsaturation are methylated more rapidly (Le Kim, 1973).

In vitro, choline formation is stimulated by exogenous mono- and di-methylated derivatives of 3-*sn*-phosphatidylethanolamines, but not by exogenous 3-*sn*-phosphatidylethanolamines. These observations have been attributed to the presence of saturating levels of endogenous 3-*sn*-phosphatidylethanolamines in the enzyme systems studied (Bremer, 1969). However, Rehbinder & Greenberg (1965) prepared the rat liver enzyme system free of endogenous phospholipid substrates, but still failed to obtain methylation of exogenous 3-*sn*-phosphatidylethanolamines.

The mammalian liver enzyme, 3-*sn*-phosphatidylethanolamines methyltransferase has not been purified to any extent, and it is not known if a single enzyme catalyzes all three methylations (Bremer, 1969). Studies with microorganisms suggest two separate enzymes are involved, one catalyzing the first methylation, and a second catalyzing the remaining two methylations (Bremer, 1969; Waechter & Lester, 1973; Matysiak *et al.*, 1974).

Introduction of the first methyl group is rate limiting in rat and guinea pig liver (Bremer & Greenberg, 1960, 1961 ; Kobayashi, 1970). The levels of PtdOEtN$\langle^{H}_{Me}$ and PtdOEtN$\langle^{Me}_{Me}$ are very low in rat liver (Lester & White, 1967). Exogenous choline did not inhibit the transmethylation pathway in isolated rat hepatocytes (Sundler & Åkesson, 1975). The choline-synthesizing enzyme system has been found in the liver of a number of animal species including the sheep, where its activity appears about 64% of that of the rat liver (Bremer & Greenberg, 1961*a*). The rat liver microsomal enzyme system is inhibited by *S*-adenosylhomocysteine (Gibson *et al.*, 1961), and nonphysiological sulphydryl compounds due to a competing methyltransferase (Bremer & Greenberg, 1961*a*,*b*).

The specific activity of the rat liver microsomal methyltransferase decreases on fasting (Bremer & Greenberg, 1961*a*) and increases in phenobarbital treated rats (Young *et al.*, 1971). The liver enzyme activity increases with age in the rat (Artom, 1969). The liver enzyme activity is unchanged in choline-deficient guinea pigs (Kobayashi, 1970). The enzyme(s) was assayed using endogenous substrates in each of these studies, and consequently the activities probably reflect the first methylation step.

A partially purified, soluble, methyltransferase has been prepared from dog lung (Morgan, 1969).

7.3. Turnover of choline

Choline is rapidly oxidized to betaine in normal rat liver *in vivo* (Wong & Thompson, 1972; Sundler *et al.*, 1972) and *in vitro* (Weinhold & Sanders, 1973; Sundler & Åkesson, 1975). Choline oxidation may be depressed in choline-deficient rat liver (Wong & Thompson, 1972). Tyler (1977) estimates that a normal rat liver has the capacity to synthesize about 450 µmol betaine/100 g body wt. per day. This is in considerable excess of the estimated daily synthesis of choline of about 70 µmol/100 g body wt. (Wise & Elwyn, 1965). Compared to a number of different species, the rat liver choline oxidase activity is high (Sidransky & Farber, 1960), and this may partly explain the high dietary requirement for choline by this species.

Two enzymes are involved in the oxidation of choline to betaine, choline dehydrogenase (enzyme 9 in Fig. 4), and betaine-aldehyde dehydrogenase (enzyme 10 in Fig. 4). Finkelstein *et al.* (1971) consider that betainehomocysteine methyltransferase (enzyme 4 in Fig. 4) may play an important role in the catabolism of choline in the rat.

The urinary excretion of choline in normal sheep is low (Luecke & Pearson, 1945). Luecke & Pearson (1945) considered that failure to detect an increase in either free or total choline in the liver, kidney and blood of sheep after ingesting large doses of choline may have been due to an active choline oxidase in sheep tissues. However, Neill & Dawson (1977) have demonstrated that choline is rapidly metabolized by the microorganisms in the sheep rumen.

8. Carnitine metabolism

Carnitine (3-hydroxy-4-*N*-trimethylaminobutyrate) plays a regulatory role in the transport of activated long-chain fatty acyl groups from sites of activation to sites of β-oxidation within the mitochondria (Bremer, 1977).

8.1. The carnitine content of tissues

Carnitine is found in a large number of animal tissues (Brooks & McIntosh, 1975; Snoswell & Linzell, 1975). Skeletal muscle generally contains the highest content; however there are pronounced species differences in the absolute levels. For example, in rat, human and sheep skeletal muscle, the carnitine content is about 1, 3.5 and 13 μmol/g fresh wt. respectively (Brooks & McIntosh, 1975; Cederblad *et al.*, 1974; Snoswell & Koundakjian, 1972). Species differences in the carnitine content of liver appear less marked. For example, in rat and sheep liver, the carnitine content is about 0.27 and 0.13 μmol/g fresh wt. respectively (Brooks & McIntosh, 1975; Snoswell & Koundakjian, 1972).

Tissue carnitine contents can be significantly influenced by diet, physiological status and environmental temperature (Strength *et al.*, 1965; Therriault & Mehlman, 1965; Mehlman *et al.*, 1969, 1971; Pearson & Tubbs, 1967; Snoswell & Henderson, 1970; Snoswell & Koundakjian, 1972; Cox & Hoppel, 1973*a*; McGarry *et al.*, 1975; Borum & Broquist, 1977*a*).

8.2. The biosynthesis of carnitine and in particular its methyl groups

8.2.1. The site of biosynthesis

The hydroxylation of γ-butyrobetaine (4-*N*-trimethylamino-butyrate) to carnitine by the enzyme γ-butyrobetaine hydroxylase is the only established enzyme reaction in the biosynthetic pathway of carnitine in animals. Lindstedt (1967) reported that in the rat the enzyme was only found in the liver, and Bøhmer (1974) reported that *in vivo* the liver was the only site of hydroxylation. However, a species difference in the tissue distribution of this enzyme exists as Erfle (1975) reported the presence of the hydroxylase in sheep liver, kidney and skeletal muscle, and Costa (1977) reported the presence of the hydroxylase in sheep and goat liver, kidney, heart and skeletal muscle, and in the liver of 1-day-old lambs. The hydroxylase from *Pseudomonas* sp. AK 1 has been purified to homogeneity (Lindstedt *et al.*, 1977).

In vivo radioisotope studies with rats, using radiolabelled precursors, L-[*methyl*-^{14}C]methionine, 6-*N*-[*methyl*-^{3}H]trimethyl-L-lysine, and 4-*N*-[*methyl*-^{3}H]trimethylaminobutyrate, implied that the liver was the principal site of carnitine biosynthesis, and that carnitine was subsequently transported to other tissues (Strength *et al.*, 1965; Broquist & Tanphaichitr, 1973; Bøhmer, 1974).

Subsequent *in vitro* studies using rat liver slices (Cox & Hoppel, 1974*a*) and perfused rat liver (La Badie *et al.*, 1976), and *in vivo* studies with sheep (Snoswell & McIntosh, 1974) have established that the liver synthesizes carnitine. *In vitro* studies using tissue slices have established that the liver is the primary

site of carnitine biosynthesis from the precursor 6-*N*-trimethyl-L-lysine (Cox & Hoppel, 1974*b*; Haigler & Broquist, 1974).

However, as Cox & Hoppel (1974*a*) and Haigler & Broquist (1974) failed to detect any conversion of L-lysine to carnitine in rat liver slices, and Cox & Hoppel (1974*a*) failed to detect any incorporation of L-[*methyl*-^{3}H]methionine and *S*-adenosyl-L-[*methyl*-^{14}C]methionine into carnitine in rat liver slices, the site of methylation of the carnitine precursor, L-lysine has not been established.

The combined observations in rats and sheep prevent the conclusion that synthesis of the carnitine precursor 6-*N*-trimethyl-L-lysine occurs in the sheep liver, although this is likely because of the important role of this organ in methionine metabolism (refer to sections 5 & 6).

8.2.2. Origin of the methyl groups

In vivo studies with rats injected with L-[*methyl*-^{14}C]- or L-[*methyl*-^{3}H]methionine, and subsequently analyzed for the presence of radioactivity in the 4-*N*-methyl groups of carnitine have established that the origin of the methyl groups of carnitine is methionine (Wolf & Berger, 1961; Bremer, 1961; Strength & Yu, 1962; Cox & Hoppel, 1973*a*).

Corredor *et al*. (1967) reported that rat liver homogenates incubated for 30 min incorporated a small amount of *S*-adenosyl-L-[*methyl*-^{14}C]methionine into carnitine. However, Cox & Hoppel (1974*a*) failed to detect any incorporation of L-[*methyl*-^{3}H]methionine and *S*-adenosyl-L-[*methyl*-^{14}C]methionine into the carnitine of rat liver slices incubated for 2 h.

Subsequent studies with *Neurospora crassa* have shown that *S*-adenosylmethionine is a methyl donor in carnitine biosynthesis (Rebouche & Broquist, 1976; Borum & Broquist, 1977*b*).

8.2.3. Nature of the methyl acceptor and methyltransferase

The free amino acid, 6-*N*-trimethyl-lysine is a precursor of carnitine in *Neurospora crassa* (Horne & Broquist, 1973; Kaufman & Broquist, 1977) and the rat both *in vitro* (Cox & Hoppel, 1974*a*,*b*; Haigler & Broquist, 1974; Hoppel *et al.*, 1976; La Badie *et al.*, 1976) and *in vivo* (Tanphaichitr & Broquist, 1973; Cox & Hoppel, 1973 ; Hoppel *et al.*, 1976; La Badie *et al.*, 1976).

Free L-lysine is methylated sequentially to 6-*N*-methyl-lysine, 6-*N*-dimethyl-lysine and 6-*N*-trimethyl-lysine by a single enzyme in *Neurospora crassa* (Rebouche & Broquist, 1976; Borum & Broquist, 1977*b*). *S*-Adenosylmethionine is a methyl donor, and the first methylation reaction is rate limiting. The enzyme has been purified to near homogeneity, and is a soluble, monomeric protein of molecular weight 22,000.

The metabolic state of L-lysine at the time of methylation, and consequently the origin of the carnitine precursor, 6-*N*-trimethyl-lysine is uncertain. Apparently, mammalian tissues cannot methylate free L-lysine (Paik & Kim, 1975). The purified lysine methyltransferase from *Neurospora crassa* cannot methylate free L-lysine in rat liver (Borum & Broquist, 1977*b*).

6-*N*-Methylated-lysines have been identified in many animal proteins, and isolated free from the urine and plasma [an extensive list of proteins and relevant references is given by Paik & Kim

(1975)]. Cox & Hoppel (1973*b*), and Tanphaichitr & Broquist (1973) postulated that protein-bound 6-*N*-trimethyl-lysine is an intermediate in carnitine biosynthesis, and that its release as free 6-*N*-trimethyl-lysine by proteolysis is a prerequisite for carnitine biosynthesis.

La Badie *et al.* (1976) obtained *in vivo* and *in vitro* evidence that protein bound 6-*N*-trimethyl-lysine is a precursor of carnitine in the rat. Administered 6-*N*-[*methyl*-^{14}C]trimethyl-lysine-labelled asialo-fetuin was rapidly hydrolyzed within the hepatocyte lysosomes, releasing 6-*N*-[*methyl*-^{14}C]trimethyl-lysine which was rapidly metabolized to tissue carnitine. Administered 6-*N*-[*methyl*-^{14}C]monomethyl- and -dimethyl-lysine-labelled asialo-fetuin were similarly rapidly hydrolyzed, but the released 6-*N*-[*methyl*-^{14}C]monomethyl-lysine and 6-*N*-[*methyl*-^{14}C]dimethyl-lysine amino acids were not metabolized to carnitine. This data supports the concept that free partially methylated lysines are not converted into free 6-*N*-trimethyl-lysine in the rat. Relatively high concentrations of 6-*N*-monomethyl-lysine are found in sheep plasma (Weatherall & Haden, 1969; Bergen & Potter, 1971; Wolff *et al.*, 1972). Furthermore, Wolff *et al.* (1972) report that the net hepatic metabolism of 6-*N*-monomethyl-lysine in sheep is small. The significance of these observations in sheep is unknown, but it is possible they indicate a lack of enzyme(s) in sheep liver capable of further methylating free partially methylated lysines.

Paik *et al.* (1977) postulate that protein (lysine) methyltransferase is involved in carnitine biosynthesis in animal tissues. The enzyme is found in various rat tissues, including the liver, and its subcellular location is the cell nucleus. The enzyme has been

partially purified, and it is considered that it may represent more than one enzyme (Paik & Kim, 1975). The methyltransferase involved in carnitine biosynthesis in *Neurospora crassa* does not exhibit protein (lysine) methyltransferase activity (Borum & Broquist, 1977*b*).

8.3. Turnover of carnitine

Carnitine is metabolized slowly in normal animals (Yue & Fritz, 1962; Lindstedt & Lindstedt, 1961*a*,*b*; Wolf & Berger, 1961; Brooks & McIntosh, 1975; Cederblad & Lindstedt, 1976). The 4-*N*-methyl groups of carnitine are not labile [Fraenkel & Friedman (1957) discuss this aspect briefly], and are not significantly oxidized to respiratory CO_2 in rats (Lindstedt & Lindstedt, 1961*a*; Khairallah & Wolf, 1967).

Isotope dilution experiments indicate the daily rate of synthesis of carnitine in the rat is about 2 μmol/100 g body weight. However, when the daily rate of synthesis is estimated by the enzymatic measurement of urine carnitine, values of 1-1.5 μmol/100 g body weight are obtained (Cederblad & Lindstedt, 1976). The reason for the discrepancy in these estimations is unknown, but it may indicate other routes of carnitine excretion besides the urine, or errors in the isotope dilution techniques.

Cederblad & Lindstedt (1976) consider there are two body pools of carnitine in the rat, a smaller pool of about 9 μmol/100 g body wt. and a larger one of about 25-30 μmol/100 g body wt. Tsai *et al.* (1975) estimate the two carnitine pools have turnover times of about 7 and 40 days respectively. Brooks & McIntosh (1975) report that the rat liver has 2 pools of carnitine, a large pool

(about 90% of the liver carnitine content) with a rapid turnover, and a small pool (about 10% of the total carnitine content) with a slow turnover. The skeletal muscle carnitine appears to exist mainly in a large pool (80% of the total carnitine) of slow turnover, with a smaller pool (about 20% of the total carnitine) of rapid turnover.

Significant decarboxylation of carnitine with extensive urinary excretion of β-methylcholine can occur in rats under various physiological conditions such as choline-deficiency, pregnancy, starvation and alloxan-diabetes (Khairallah & Wolf, 1967; Mehlman *et al.*, 1969; Mehlman *et al.*, 1971). Isotope dilution experiments suggest the turnover time and body pool of carnitine decreases under these conditions (Khairallah & Mehlman, 1965; Mehlman *et al.*, 1969, 1971). Enzymatic estimations of total body carnitine have shown that the total body carnitine content of choline-deficient and lysine-deficient rats is lower than that of control rats (Cox & Hoppel, 1973*a*; Tsai *et al.*, 1975).

9. Interrelationships between choline and carnitine biosynthesis

From the foregoing discussion it is evident that, in the rat, choline biosynthesis has a higher requirement for methionine methyl groups than carnitine biosynthesis under most physiological conditions.

Rats fed a choline-deficient diet have a lower tissue carnitine content than control rats (Strength & Yu, 1962; Strength *et al.*, 1965; Corredor *et al.*, 1967; Mehlman *et al.*, 1971; Cox

& Hoppel, 1973*a*; Tsai *et al.*, 1974, 1975). Mehlman *et al.* (1971) report that the turnover time of carnitine is decreased in choline-deficient rats, while Tsai *et al.* (1975) report that it is increased in choline-deficient rats. The reason for this discrepancy is unknown.

Corredor *et al.* (1967) argue that in choline-deficient rats, choline biosynthesis is increased, and that methionine methyl groups are used preferentially for choline biosynthesis at the expense of carnitine biosynthesis.

However, there is no unequivocal evidence that choline biosynthesis is increased in choline deficiency [Lyman *et al.* (1973), and Wong & Thompson (1972) discuss this aspect and list a number of relevant references], the *S*-adenosylmethionine content of rat liver is not depressed in choline-deficiency (Wong & Thompson, 1972), and *in vitro* enzyme assays indicate that rat liver 3-*sn*-phosphatidyl-ethanolamines methyltransferase(s) has a low ability to compete for available *S*-adenosylmethionine in the presence of other methyltransferases (Bremer & Greenberg, 1961*a*,*b*). Therefore, the evidence for a competing methyltransferase system between choline and carnitine biosynthesis in choline-deficient rats is inconclusive.

Rebouche & Broquist (1976) observed a sparing effect of choline on the methyl groups of methionine for carnitine biosynthesis in a *Neurospora crassa* lysine auxotroph grown in the presence of exogenous choline. However, the significance of this finding in relation to mammalian metabolism is unknown.

There is virtually no information available on a possible competition of choline *versus* carnitine biosynthesis for methyl groups in the sheep.

GENERAL INTRODUCTION

Conditions of metabolic stress such as starvation, alloxan-diabetes and pregnancy toxaemia result in a net release of fatty acids into the blood stream from adipose tissue triacylglycerols for transport to other target organs for their energy requirements. This is generally indicated by an increase in the blood fatty acid concentration (Reid, 1968; Scow & Chernick, 1970). Fatty acids are taken up by the sheep liver as a constant proportion of their transport to the liver (Katz & Bergman, 1969) and therefore the net uptake of fatty acids under these conditions of metabolic stress can be considerable. The fatty acids are activated to their CoA derivatives within the liver cells and enter various metabolic pathways including oxidation to ketones or CO_2, and esterification to triacylglycerols and phospholipids.

The metabolic role of carnitine in facilitating the entry of long chain fatty acids into the liver mitochondria for oxidation is established, and its role in the regulation of the oxidative pathways has become evident (Bremer, 1977). The importance of carnitine in the oxidation of fatty acids in sheep liver mitochondria has been established (Koundakjian & Snoswell, 1970).

Associated with these conditions of metabolic stress in sheep are varying degrees of lipid accumulation in the liver (Roderick *et al.*, 1937; Dryerre & Robertson, 1941; Jarrett, 1946; Ferguson, 1954), presumably as a consequence of the increased liver uptake of fatty acids (Patterson, 1966). The lipid components in the livers of such animals have not been fully characterized and

therefore it seemed necessary to investigate these aspects.

The liver carnitine content is greatly increased under conditions of starvation (Snoswell & Henderson, 1970), alloxan-diabetes (Snoswell & Koundakjian, 1972) and pregnancy toxaemia (Pethick, 1975) in sheep. This increase is due to increased biosynthesis in alloxan-diabetic sheep (Snoswell & McIntosh, 1974). The liver mitochondria appear enlarged and fragile in starved and alloxan-diabetic sheep (Taylor *et al.*, 1971). Phospholipids are essential components of subcellular organelles and membranes (McMurray, 1973), and 3-*sn*-phosphatidylcholines form a major fraction of the sheep liver mitochondrial phospholipids (Getz *et al.*, 1968). Strength *et al.* (1965) reported depressed tissue levels of carnitine in choline-deficient rats, and Corredor *et al.* (1967) postulated that under these conditions, the methyl groups of methionine are used preferentially for choline biosynthesis at the expense of carnitine biosynthesis. Carnitine and choline biosynthesis both involve methylation from L-methionine (Bremer *et al.*, 1960; Gibson *et al.*, 1961; Wolf & Berger, 1961; Bremer, 1961). Competition for available labile methyl groups during their biosynthesis seemed possible in sheep liver under these conditions of metabolic stress, particularly in view of the implication of L-methionine as the first limiting amino acid in sheep (Chalupa, 1972), and that carnitine biosynthesis may occur at the expense of choline biosynthesis under these conditions. Consequently, it was necessary to investigate the liver phospholipid content under these conditions, and undertake *in vitro* radioisotope studies to obtain more direct evidence of a possible competition for methyl groups. The use of a specific enzymatic assay for choline was

considered desirable in the course of these investigations.

The quantitative importance of creatine metabolism in labile methyl group metabolism (Mudd & Poole, 1975) made it necessary to investigate aspects of creatine metabolism in sheep under those metabolic conditions where a possible competition for methyl groups was investigated.

An opportunity arose for obtaining liver samples in the course of experiments being conducted in another department on the effects of abomasal infusions of L-methionine in sheep, and some of the aforementioned studies were extended to this metabolic condition.

CHAPTER 1. THE ENZYMATIC ESTIMATION OF CHOLINE

1.1. Introduction

The measurement of free choline and choline compounds in tissue extracts from sheep under various physiological conditions was considered necessary as a result of work reported in the Literature Review and General Introduction. There are numerous methods for the quantitative determination of choline in biological materials, but many are nonspecific, insensitive, costly or tedious (Engel *et al.*, 1954; Griffith & Nyc, 1954; Schuberth *et al.*, 1970). The advantages associated with the enzymatic assay of tissue metabolites (Bergmeyer, 1974), including specificity, sensitivity and convenience, favoured the use of an enzymatic method for the quantitative determination of choline, and the radiochemical assay using choline acetyltransferase (Schuberth *et al.*, 1970; Potter, 1971) was selected for this purpose. This enzyme was not available commercially at the time, and the aim of the work reported in this chapter was to purify the enzyme from a convenient source such as mammalian brain (Potter & Glover, 1971) and immature human placenta (Morris, 1966*a*).

1.2. Methods and Materials

1.2.1. Source of crude enzymes

Sheep brains and a bovine brain were collected from the Adelaide abattoirs (Gepps Cross) and transported to the laboratory in a bucket of ice. The striata were dissected from the brains as described by Potter & Glover (1971). About 3.5 g of tissue was obtained from each sheep brain.

Brains were taken from male hooded Wistar rats (200-300 g) fed a pelleted rat diet (Charlicks, Adelaide, S. Australia).

Immature human placentae of 14-20 weeks gestation, weighing 25-150 g, and produced by clinical termination of pregnancy, were obtained as fresh as possible from the Queen Victoria Hospital (Rose Park).

1.2.2. Enzyme assays

Choline acetyltransferase was assayed using a slight modification of the method of Mannervick & Sörbo (1970). The following components were incubated for 20 min at 30°C in glass tubes (5 cm x 0.5 cm, internal dimensions) in a final volume of 200 µl.

> 100 mmol/l-Potassium phosphate buffer (pH 7.0), 300 mmol/l-NaCl, 0.25 mmol/l-physostigmine sulphate, 2.5 ml/l-n-butanol, 0.15 mmol/l-[1-^{14}C]acetyl-CoA (820 c.p.m./nmol), 5 mmol/l-choline chloride, and sufficient enzyme solution to metabolize up to 15% of the acetyl-CoA (a maximum of about 3,700 c.p.m.).

The reaction was started by addition of enzyme solution, and terminated by transfer (with a Pasteur pipette) of the contents in the reaction tube to a disposable ion-exchange column prepared in a truncate Pasteur pipette plugged with a small wad of glass wool, and containing Dowex AG1 (X2; 200-400 mesh; Cl^- form) of bed volume about 0.8 ml. The column was washed with 1.5 ml (3 x 0.5 ml additions) of distilled water to elute the [1-^{14}C]acetylcholine from the column, and the 1.7 ml of effluent was collected directly

into a glass scintillation vial, previously background counted, containing 15 ml of scintillation fluid [toluene containing 7 g of 2,5-diphenyloxazole and 0.3 g of 1,4-di[2-(4-methyl-5-phenyl-oxazolyl)]-benzene per litre, mixed 2:1 (v/v) with Triton X-100]. The vials were mixed thoroughly and counted in a Packard Tri-Carb scintillation counter Model 3375 operating at ambient temperature. Control incubations containing no added enzyme were processed in the same manner as the test incubations. Channels ratios showed there were no significant differences in counting efficiency between the vials, and c.p.m. were not corrected to d.p.m.

Carnitine acetyltransferase was assayed as described for choline acetyltransferase except that 5 mmol/l-L-carnitine hydrochloride replaced choline chloride in the incubation mixture.

The specific activity of both enzymes was calculated using the following equation:

$$\text{specific activity } (\mu\text{kat/kg protein}) = \frac{(\text{sample c.p.m.} - \text{control c.p.m.})}{\text{c.p.m./}\mu\text{mol acetyl-CoA/incubation}} \times \frac{1}{20 \times 60} \times \frac{10^6}{\text{mg protein/incubation}}$$

The differential assay for choline acetyltransferase using cholinesterase to correct for the presence of carnitine acetyltransferase in the enzyme preparations was used in some instances (Hamprecht & Amano, 1974).

1.2.3. Preparation of choline acetyltransferase

1.2.3.1. Sheep brain striata

Preliminary investigations showed the specific activity of

choline acetyltransferase was similar in bovine and sheep brain striate nuclei, and that the striate nuclei were a relatively rich source of enzyme compared to other areas of the sheep brain and the whole rat brain. Sheep brains were more convenient to collect than bovine brains and therefore sheep brain striata were used as the source of enzyme.

Choline acetyltransferase was prepared as described by Potter & Glover (1971). Two preparations were attempted, each using about 80 g of striata (from about 22 brains). The final enzyme preparation was concentrated by precipitation with ammonium sulphate.

1.2.3.2. Immature human placentae

The placentae were washed with cold 154 mmol/l-NaCl, the required pulpy tissue removed from the adjacent membranes, chopped into small pieces and homogenized in 154 mmol/l-NaCl containing neutralized thioglycollic acid (50 mmol/l) to form a freely flowing slurry that was freeze-dried (Morris, 1966*a*).

The following purification procedure was based on a personal communication from C. Hebb & S.P. Mann.

5 g freeze-dried powder was homogenized in 50 ml of 500 mmol/l-NaCl, centrifuged for 15 min at 12,000 *g* and 4°C, and the pellet reextracted with 15 ml of 500 mmol/l-NaCl. The supernatants were pooled and diluted to 20 mg protein/ml. $(NH_4)_2SO_4$ was added to a final concentration of 16% (w/v) and the solution left to stand for several h at 4°C. The precipitated protein was discarded, the pH of the supernatant adjusted to 5.5 with acetic

acid (500 mmol/l), and $(NH_4)_2SO_4$ added to a final concentration of 32% (w/v). This solution was left overnight at 4°C and then centrifuged at 10,000 *g* for 10 min. The pellet was taken up in 5 ml of 100 mmol/l-potassium phosphate buffer (pH 6.5) and $(NH_4)_2SO_4$ removed by Sephadex G-25 column (30 cm x 1.3 cm) chromatography. The enzyme preparation was concentrated (3.0 ml) by ultra-filtration and applied to a DEAE-Sephadex column (20 cm x 1.3 cm) equilibrated with 100 mmol/l-potassium phosphate buffer (pH 6.5). The enzyme was eluted with 100 mmol/l-potassium phosphate buffer (pH 6.5) containing a linear gradient of 0-800 mmol/l-NaCl. Fractions 9-20 were pooled to yield 18 ml of enzyme solution (26 mg protein).

This procedure was repeated on a larger scale using 30 g of freeze-dried powder. Attempts to purify the enzyme further by CM-Sephadex and hydroxylapatite column chromatography were unsuccessful owing to the instability of the partially purified enzyme.

1.2.4. Choline assay

The partially purified enzyme preparations were used to assay free choline in sheep tissue extracts prepared as described by Potter (1971). The choline assay incubations were the choline acetyltransferase assay incubations minus added choline chloride. Choline standards from 0-400 nmol (0-2 mmol/l) were used to construct a standard curve.

1.2.5. Preparation of acetyl-CoA and [1-^{14}C]acetyl-CoA

Acetyl-CoA was prepared from free CoA and redistilled acetic anhydride by the method of Stadtman (1957), and assayed as described by Decker (1974). [1-^{14}C]acetyl-CoA was prepared in

the same manner except that acetylation of the CoA was carried out in the borosilicate glass ampoule (P1) containing the [1-^{14}C]acetic anhydride (about 5 μmol). The ampoule was immersed in liquid N_2 before breaking the seal with a metal rod to minimize the loss of [1-^{14}C]acetic anhydride. [1-^{14}C]acetic acid remaining after acetylation was removed by 15 successive extractions with ether at pH 1.0, and the [1-^{14}C]acetyl-CoA preparation purified by DEAE-cellulose and Sephadex G-10 column chromatography as described by Morris *et al.* (1971). The specific activity of the [1-^{14}C]acetyl-CoA preparation was 8,900 c.p.m./nmol.

1.2.6. Protein

This was measured by the Biuret method or by ultraviolet absorption as described by Layne (1957). Bovine albumin was used as the reference standard.

1.2.7. Chemicals

L-Carnitine hydrochloride was generously supplied by Otsuka Pharmaceuticals, Osaka, Japan. CoA was obtained from P.L. Biochemicals Inc., Milwaukee, Wisconsin, U.S.A., enzymes were from C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany and bromoacetyl-L-carnitine was prepared as described by Costa & Snoswell (1975). Other chemicals were obtained as follows: bovine albumin (essentially fatty acid-free), cholinesterase (type IV), choline chloride, and 1,4-di[2-(4-methyl-5-phenyloxazolyl)]-benzene from Sigma Chemical Co., St. Louis, Mo., U.S.A.; 2,5-diphenyloxazole from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; Dowex AG 1 (X2; 200-400 mesh; Cl^- form) and Hydroxylapatite from Bio-Rad Laboratories, Richmond, Calif., U.S.A.; Whatman DE 32-cellulose

from W. & R. Balston (Modified Cellulose) Ltd., Kent, U.K. and other column chromatography materials from Pharmacia, Uppsala, Sweden; [1-^{14}C]acetic anhydride (sp. radioactivity 122 mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K.

Other materials were reagent-grade chemicals or the best commercially available grade.

1.3. Results

The results in Table 1.1. show that choline acetyltransferase from sheep brain striata was purified about 60 fold at step 3, but lost activity in the Sephadex G-200 step. This loss in activity was restored slightly by an ammonium sulphate precipitation. Comparison to the data of Potter & Glover (1971) shows the present purification from sheep brains was considerably less successful than that from bovine brains.

The results in Table 1.2. show that choline acetyltransferase from immature human placentae was purified about 9 fold, to a specific activity similar to the best value obtained for the sheep brain enzyme. A larger scale purification than that shown in Table 1.2. was relatively unsuccessful owing to the instability of the enzyme. Attempts to further purify this enzyme by CM-Sephadex and hydroxylapatite column chromatography were similarly unsuccessful owing to the instability of the enzyme.

The partially purified choline acetyltransferase preparations from both sources were used to assay free choline levels in sheep tissue extracts. However, very high values were obtained. This was shown to be due to the presence of carnitine acetyltransferase

TABLE 1.1.

Purification of choline acetyltransferase from sheep brain striata

Sheep brain striata were collected as described in section 1.2.1., and used in the purification procedure as described in section 1.2.3.1. Choline acetyltransferase was assayed as described in section 1.2.2.

Step	Sheep striata (80 g)			Bovine striata*
	Volume ml	Protein mg	Specific activity µkat/kg protein	Specific activity µkat/kg protein
Homogenate	850	11,200	6.3	4.7
1. Extract	720	510	50.0	43.3
2. CM-Sephadex	55	94	301.7	450.0
3. Ammonium sulphate	5	23	376.7	1,463.3
4. Sephadex G-200	26	18	73.3	5,250.0
5. Ammonium sulphate	2	3	120.0	

* Taken from Potter & Glover (1971) for comparison.

TABLE 1.2.

Purification of choline acetyltransferase from immature human placentae

Immature human placentae were collected as described in section 1.2.1. 5 g of freeze-dried powder was used in a pilot purification procedure as described in section 1.2.3.2. Choline acetyltransferase was assayed as described in section 1.2.2.

Step	Volume ml	Protein mg	Specific activity µkat/kg protein
1. Extract	66.5	1,330	41.7
2. Ammonium sulphate	3.0	318	76.7
3. DEAE-Sephadex	18.0	26	367.7

in the partially purified enzyme preparations. Fig. 1.1. shows the presence of carnitine acetyltransferase in the sheep brain preparation. Similar results were observed for the placental enzyme preparation. The ratio of specific activities of carnitine acetyltransferase and choline acetyltransferase in the initial extracts from both sources varied from 2-6. This ratio narrowed in the partially purified preparations, although the instability of choline acetyltransferase sometimes caused the ratio to increase in the more advanced steps of the purification. Studies with rat brain extracts (where the ratio of specific activities was about 12) indicated that it was difficult to separate the two enzymes.

Inclusion of bromoacetyl-L-carnitine in the enzyme preparations and choline assay incubations to inhibit carnitine acetyltransferase were unsuccessful.

1.4. Discussion

Eade *et al.* (1973) compared three methods for the assay of free choline in rat brain extracts; bioassay, radiometric assay by choline acetyltransferase, and radiometric assay by choline kinase. The radiometric assay by choline acetyltransferase compared favourably with the other two methods.

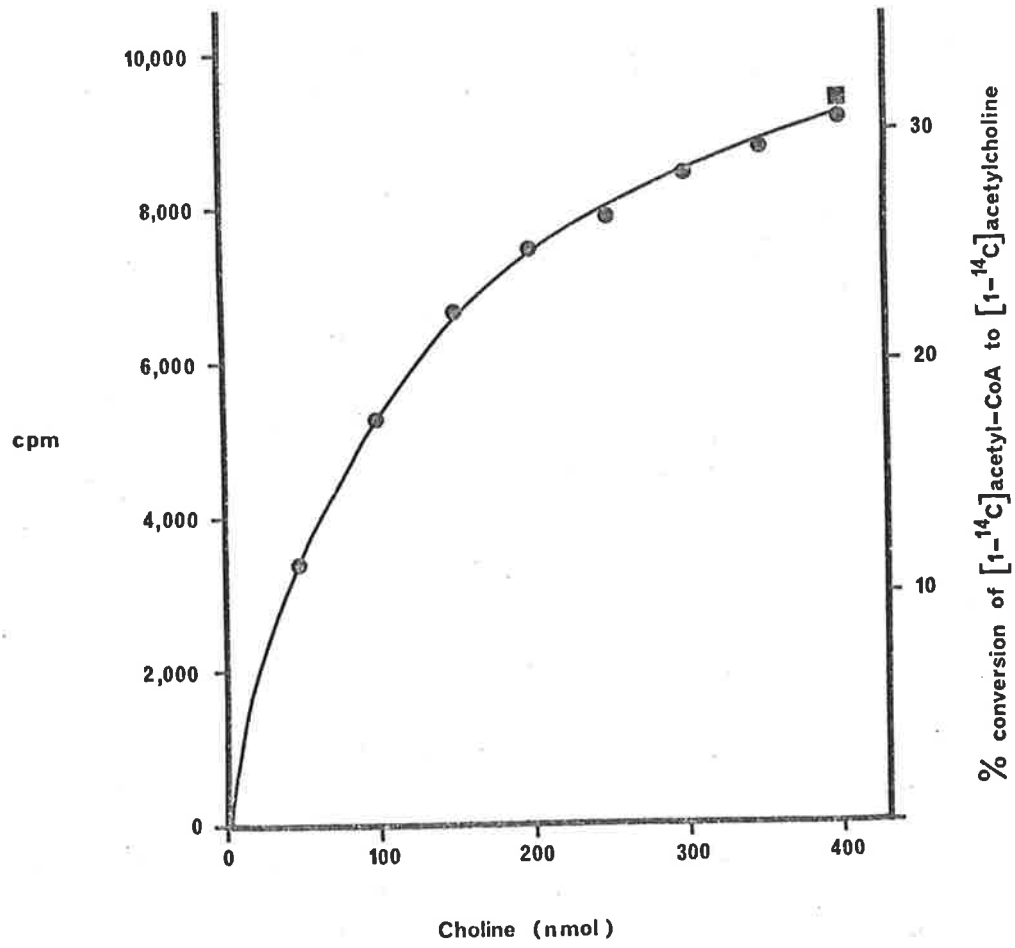
The purpose of the work reported in this chapter was to partially purify choline acetyltransferase for use in a sensitive, specific, accurate and convenient assay for choline in sheep tissue extracts. The specific activity of choline acetyltransferase in animal tissues is low. Rossier (1976) purified rat

FIGURE 1.1.

A choline standard curve constructed using partially purified choline acetyltransferase from sheep brain striata

The incubation conditions are described in section 1.2.4. Each incubation contained 30 µl (5.4 pkat of choline acetyltransferase) of enzyme fraction 5 (see Table 1.1.). Each point represents a single incubation.

■ an incubation where choline was replaced with 400 nmol L-carnitine.


10,000
8,000
6,000
4,000
2,000
0
cpm
0
100
200
300
400
Choline (nmol)
30
20
10
% conversion of [1-14C]acetyl-CoA to [1-14C]acetylcholine

brain choline acetyltransferase 30,000-fold to a specific activity of about 333 mkat/kg protein, and this preparation was only 20% pure. Ryan & McClure (1976) purified bovine caudate nuclei choline acetyltransferase 6,500-fold to a specific activity of about 267 mkat/kg protein, and this preparation was 80-90% pure.

The procedure of Potter & Glover (1971) was used to prepare choline acetyltransferase from sheep brain striata as the initial specific activity was similar to that of bovine striata, and the sheep brains were more convenient to obtain. Glover & Potter (1971) indicated that the partially purified bovine enzyme was stable, but contaminated with carnitine acetyltransferase. The present work showed that the partially purified enzyme from sheep brains was also contaminated with carnitine acetyltransferase. Preliminary studies revealed that it was difficult to separate the two enzymes. This precluded the use of the sheep brain enzyme preparation in a specific assay for choline in sheep tissue extracts, as sheep tissues generally have high levels of carnitine and carnitine compounds (Snoswell & Koundakjian, 1972), and consequently acetylcarnitine contaminated the acetylcholine separated from the radioactive substrate by anion exchange chromatography in the choline assays. The 30,000-fold purified rat brain choline acetyltransferase was contaminated with carnitine acetyltransferase (about 3 mkat/kg protein), and Rossier (1976) concluded that the mammalian brain is not a practical source for the purification of choline acetyltransferase. Squid head ganglia choline acetyltransferase has been purified 325-fold to homogeneity, with a specific activity of about 1,111 mkat/kg protein (Husain & Mautner, 1973); however this was an expensive and inconvenient source of the enzyme for the present work.

The opportunity to obtain immature human placentae led to attempts to purify choline acetyltransferase from this source, as it was known that the enzyme is present in high levels in this tissue (Bull *et al.*, 1961; Welsch, 1974). The purification attempts were largely unsuccessful, owing partly to the instability of the enzyme as it was purified [as similarly reported by Morris (1966*a*)], and its contamination with carnitine acetyltransferase. Attempts to improve the specificity of the partially purified enzyme by inhibiting carnitine acetyltransferase with bromoacetyl-L-carnitine (Chase & Tubbs, 1966) were similarly unsuccessful, owing in part to the difficulty of preparation and instability of this compound.

Recent improvements to the assay for choline using partially purified choline acetyltransferase and the convenient anion exchange chromatographic separation of radioactive substrate and products include the use of [1-^{14}C]acetyl-dephospho-CoA as substrate and the use of cholinesterase control incubations to minimize and correct for the contaminating activity of carnitine acetyltransferase in the partially purified choline acetyltransferase preparations (Snoswell & Mann, 1978). Inclusion of EDTA in the enzyme preparation and incubation mixtures improved the accuracy of the assays, as EDTA inhibits the activity of *sn*-glycero-3-phosphocholine phosphodiesterase, a contaminating enzyme in the partially purified choline acetyltransferase preparations. This is probably important in the assay of choline in sheep liver extracts as the levels of *sn*-glycero-3-phosphocholine are high in this tissue (Schmidt *et al.*, 1952, 1955).

CHAPTER 2. A PARTIAL CHARACTERIZATION OF THE LIVER LIPIDS OF SHEEP UNDER SEVERAL PHYSIOLOGICAL CONDITIONS

2.1. Introduction

Arising out of the work reported in the Literature Review and General Introduction, it was necessary to partially characterize the fatty livers associated with pregnancy toxaemia and alloxan-diabetes in sheep, and this involved analyses of the liver water, total lipids, phospholipids and triacylglycerols.

The relationship between the development of these fatty livers and a possible choline-deficient state induced by increased carnitine biosynthesis in sheep was investigated. Thus the work involved some studies of sheep liver morphology, and measurements of total hepatic PtdCho, PtdEtn and $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratios as characteristic changes in these parameters have been observed in choline-deficient rats (Hartroft, 1950; Blumenstein, 1968; Chalvardjian, 1970). These studies were extended to qualitative analyses of molecular subclasses of PtdCho and PtdEtn, as the methylation pathway of PtdCho biosynthesis involves selectively the synthesis of tetraenoic and polyenoic classes (> 4 double bonds) of PtdCho (van den Bosch, 1974; MacDonald & Thompson, 1975) and changes in these subclasses may occur in choline-deficient rats (Lyman *et al.*, 1973, 1975).

Similar analyses were undertaken with normal and alloxan-diabetic rats for comparison. Some of the above studies were extended to the livers of neonatal lambs, as Noble *et al.* (1971*a*) reported age dependent changes in the liver phospholipids of young lambs, and to the livers of sheep infused abomasally with L-methionine, as it has been reported that the liver lipids of rats fed excessive

quantities of methionine are elevated (Roth *et al.*, 1950; Klain *et al.*, 1963).

2.2. Methods and Materials

2.2.1. Animals

2.2.1.1. Normal animals

Merino wethers and rams, 1½-4 year-old, weighing 30-40 kg, were fed 800-1000 g of lucerne-hay chaff each morning.

Virgin female hooded Wistar rats, weighing 200 ± 5 g were fed a pelleted rat diet (Charlicks, Adelaide, S. Australia) *ad libitum*.

All animals had ready access to water.

2.2.1.2. Alloxan-diabetic animals

A solution of alloxan (200 g/l) in sterile saline (9 g/l, NaCl) was prepared immediately before injection into the animals because of the short half life of alloxan in solution (Patterson *et al.*, 1949).

Merino wethers 2-3 year-old, weighing 39 ± 2 kg, and a male 1-year-old crossbred goat (predominantly Chamois), weighing 30 kg, were made diabetic by injecting the alloxan solution (60 mg alloxan/kg body wt.) into the jugular vein.

Virgin female hooded Wistar rats, weighing 226 ± 4 g were made diabetic by the intraperitoneal injection of alloxan solution (230 mg alloxan/kg body wt.). The procedure was facilitated by administration of light diethyl ether anaesthesia to the rats.

Animals were considered diabetic if they exhibited glucosuria

and hyperglycemia when tested 24 h later using Dextrostix and Keto-Diastix reagent strips (Miles Laboratories Inc.).

2.2.1.2.1. Uncontrolled diabetes

Wethers which spontaneously reduced their food intake to 250 g or less per day were slaughtered 5-7 days after alloxan administration, and are referred to as "responders". These animals were extremely ill at the time of slaughter.

Three wethers did not spontaneously reduce their food intake, and were slaughtered 10, 28 and 34 days after alloxan administration. These wethers were diabetic, but otherwise healthy at the time of slaughter, and are referred to as "non-responders". The variable response of sheep to alloxan treatment is discussed by Jarrett (1946).

The rats were all "responders", and were slaughtered 46-58 h after alloxan administration. At the time of slaughter, the rats were extremely ill, and urine analysis indicated moderate ketones (30-40 mg % acetoacetate concentration) and high glucose (2%) concentration. Rats that died overnight were not used in the experimental analysis.

2.2.1.2.2. Controlled diabetes

A group of 7 diabetic wethers which spontaneously reduced their food intake to 250 g or less per day, 4-10 days after alloxan administration, were given daily, a single subcutaneous injection (in the neck) of 15-20 Units of Lente Novo Insulin (Novo Industri A/S, Copenhagen, Denmark). Except for one animal (wether 3-29), all wethers returned to full intake of food (800-1000 g of lucerne-hay chaff) 2-5 days after commencement of insulin treatment.

Two wethers were given daily injections of insulin until their slaughter, 27 and 30 days after the alloxan injection. These animals are referred to as "insulin treated responders".

Five wethers were withdrawn from insulin treatment 24 h before their slaughter, 10-44 days after alloxan administration. These animals are referred to as "insulin withdrawn responders".

Details of the insulin therapy are reported more fully in Chapter 4. Some metabolic effects of insulin treatment and insulin withdrawal in alloxan-diabetic sheep are described by Jarrett *et al.* (1974).

2.2.1.3. Pregnant-toxaemic ewes

In late May 1975, 800 Merino crossbred ewes, in advanced stages of pregnancy, were delivered to the Adelaide abattoirs (Gepps Cross) from the mid-north (Peterborough) of South Australia. The ewes refused to eat at the abattoirs and many would not have eaten for at least 7 days. They were diagnosed as suffering from pregnancy toxaemia, syndrome 3 (Pethick, 1975). Large numbers of ewes were shot in the yards, and removed for disposal. Ewes in more reasonable condition were walked to the slaughtering line and processed as usual. Many of these ewes had twin foetuses, and their livers appeared extremely fatty. Liver tissue was collected from 5 such ewes, and these are referred to as "untreated pregnant-toxaemic ewes".

Twelve animals were taken from this flock and used in a pregnancy toxaemia treatment trial (Pethick, 1975). Liver tissue was collected at the time of slaughter from 4 of the ewes that

survived the treatment trial. These ewes were slaughtered at the Waite Institute 11 days after their treatment and 8 days later than the 5 ewes slaughtered at the abattoirs. These ewes are referred to as the "treated pregnant-toxaemic ewes". A clinical assessment of the treatment trial with respect to these 4 ewes is presented in Table 2.1.

Liver tissue was collected at the time of slaughter, from a Corriedale ewe (ewe 11) suffering from severe pregnancy toxaemia, on a property near Mount Pleasant, South Australia. The ewe had twin foetuses and a very fatty liver. Details of the condition of the ewe, and a sample of liver tissue were provided by courtesy of Mr. D. Pethick.

2.2.1.4. Neonatal lambs

At the time of slaughter of ewes 3, 6, 8 and 10 (see section 2.2.1.3. and Table 2.1.), liver tissue was collected from the foetal lamb (sex not recorded) of ewe 3 and the twin foetal lambs (a male and female) of ewe 6. Liver tissue was collected from the lamb (a male) of ewe 8, 6 days after its delivery by caesarean section.

2.2.1.5. Wethers infused abomasally with L-methionine

Twelve Merino x Dorset wethers, each weighing about 25 kg, were divided into 3 treatment groups, and received abomasal infusions of 0, 0.12 or 0.36 g of L-methionine/kg body weight $^{0.75}$/day for 32 days. They were held in metabolism crates and fed a poor quality hay-chaff diet during the experimental period. At the end of the infusion period, the wethers were slaughtered and liver tissue was

TABLE 2.1.

Clinical assessment of the ewes treated for pregnancy toxaemia

The results of the pregnancy toxaemia treatment trial for the 4 ewes have been taken from Pethick (1975).

T.A., Trienbolone acetate. *p.m.*, *post mortem*.

Ewe number	Clinical condition	Treatment	Recovery
3	Standing. Sight almost normal. Dull.	Glycerol†	Recovered* slowly. *p.m.* 1 lamb. Liver slightly fatty.
6	Standing. Sight normal. Dull.	T.A.‡	Recovered slowly. *p.m.* 2 lambs. Liver slightly fatty.
8	Standing. Sight normal. Alert, clinically the least affected.	Glycerol	Recovered slowly. *p.m.* 1 lamb. Liver normal.
10	Standing. Sight normal. Nervous disorders.	T.A.	Recovered slowly. *p.m.* 1 lamb. Very fatty liver.

* Considered to be an increase in alertness and gradual regaining of appetite.

† Applied as a drench.

‡ Administered intramuscularly.

collected. Permission to collect tissue was kindly provided by Dr. B. Radcliffe and Dr. A. Egan (Agronomy Department, Waite Institute).

2.2.1.6. Abattoirs sheep

Liver tissue was collected from the slaughtering line at the Adelaide abattoirs (Gepps Cross). The livers were from sheep of unknown breed, sex and age.

2.2.1.7. Slaughter of animals

Sheep were killed by severing the neck. Rats were killed by cervical dislocation and exsanguination.

2.2.1.8. Housing of animals

Rats were housed in wire cages in the laboratory.

Sheep were housed in the animal sheds in steel pens or metabolism crates. All sheep were unshorn.

2.2.2. Tissue collection and storage

Tissues from animals slaughtered at the Waite Institute and C.S.I.R.O. (Hackney, Adelaide) were placed in liquid N_2 within 1 min of slaughter.

Liver tissue from the 6-day-old lamb (see section 2.2.1.4.) was placed in liquid N_2 1 h after its death.

Liver tissue collected at the abattoirs was placed in liquid N_2 within 30 min of slaughter.

A stainless steel mortar and heavy pestle, both precooled in liquid N_2, were used to powder the frozen tissue samples. The powdered tissues were stored in 30 ml plastic sample tubes (Camelec Ltd., Camden, Adelaide) immersed in liquid N_2 until processed. A small hole was punched in their lids to prevent excessive pressure increase when the tubes were withdrawn from the liquid N_2.

2.2.3. Extraction of tissue lipids

The method of Folch *et al.* (1957) was mainly followed. The general recommendations of Entenman (1961), Rouser *et al.* (1966*a*), Radin (1969) and Johnson (1971) were followed to minimize lipid contamination, autoxidation and extraction losses as described below.

Freshly distilled chloroform and methanol containing 4-methyl-2,6-di-*tert.*-butylphenol (50 mg/l) antioxidant was used (Wren & Szczepanowska, 1964), and all operations were performed at laboratory temperature and where practicable, under N_2 gas. The following procedure was used:

2.0 g of powdered tissue was weighed into a 50 ml glass extraction tube (MF 24/3; Quickfit and Quartz Ltd., Staffordshire, England) precooled in liquid N_2. 12 ml of methanol was added and the tissue homogenized (about 60 s) using a Polytron PT 10 (Kinematica, G.m.b.H.). 24 ml of chloroform was added and the homogenization repeated. During homogenization, the glass tube was immersed in ice-cold water, and N_2 gas directed into it from a fine tube. The extraction tube was flushed with N_2, stoppered, and left to stand for about 1 h. The contents were then filtered through a fluted filter paper (previously boiled in chloroform) into a graduated glass tube (MF 24/3). The extraction tube and filter

paper were rinsed with chloroform/methanol (2:1, v/v) until a filtrate of 40.0 ml was obtained. Filtration was performed in a N_2 chamber.

8 ml of KCl solution in water (8.8 g/l) was added to the filtrate, the stoppered tube shaken thoroughly for 10 min, and centrifuged (Heraeus Christ bench centrifuge) for about 45 min. The upper phase was removed by aspiration, and the sides of the tube and surface of the lower phase rinsed 3 times with 2 ml of chloroform/methanol/KCl solution in water (7.4 g/l) (3:48:47, by vol.).

The lower phase (about 30 ml) was concentrated by rotary evaporation to 1-2 ml, and residual water and methanol removed by repeated chloroform addition (about 4 ml each time) and rotary evaporation to about one ml. After removal of water and methanol (judged by absence of cloudiness when the chloroform was added to the concentrated lipid extract), the lipid extract was filtered through a glass wool column (a truncated Pasteur pipette containing a plug of glass wool), to remove any suspended matter, into a graduated 7 ml capacity glass bottle. The tube and column were rinsed with chloroform to ensure quantitative transfer of lipid, and the filtration step was carried out in a N_2 chamber. The glass bottle was gently flushed with N_2 gas, the volume adjusted to 5.0 ml with further chloroform, and the bottle sealed with a Teflon lined metal cap.

The lipid extracts (20-80 g lipid/l) were stored in the dark at -14°C until required for analysis. The weights of the various fractions obtained in the lipid extraction procedure are

shown in Table 2.2. With this scale of operation, about 100-450 mg of lipid was obtained per extraction. This was considered sufficient for the analyses performed or contemplated (e.g. g.l.c.).

A convenient apparatus for the extraction of lipids from small samples of tissue has been reported which may be superior (Walker, 1974).

2.2.4. Estimation of total lipid and moisture content of tissues

Duplicate samples of frozen powdered tissue were used to determine the water content, by oven drying 0.5-1.5 g samples in tared glass weighing bottles at 80°C for 48 h.

The total lipid content of tissues was determined by oven drying an aliquot (1.0 ml) of the total lipid extract in tared glass weighing bottles at 60°C for 2 h. The lipid residue was discarded after weighing. As this procedure is wasteful, the method of Rouser & Fleischer (1967) may be superior.

2.2.5. Thin-layer chromatography of total hepatic lipids

2.2.5.1. General techniques

The general techniques used were based on those described by Skipski & Barclay (1969) and Shenstone (1971). In particular, all operations were conducted under N_2 gas, and the antioxidant, 4-methyl-2,6-di-*tert.*-butylphenol, was included in the samples, standards and developing solvents to minimize the risk of autoxidation of polyunsaturated fatty acids (Wren & Szczepanowska, 1964; Neudoerffer & Lea, 1966; Holman, 1967).

TABLE 2.2.

Fractions obtained in the extraction of sheep liver lipids

Sheep livers were classed as normal or fatty by visual assessment. Lipids were extracted as described in section 2.2.3., and moisture and lipid content estimated as described in section 2.2.4. The weight of residue remaining on the filter paper was determined by oven drying at 80°C for 48 h. The weight of solids in the F.U.P. (less the weight of KCl) was determined by oven drying at 80°C for 7 days. Values represent mean ± S.E.M. of the livers extracted.

F.L.P., Folch Lower Phase; F.U.P., Folch Upper Phase.

Type of sheep liver	Number of livers extracted	Wet wt. of liver tissue extracted mg	Wt. of solids extracted into F.L.P. mg	Wt. of solids extracted into F.U.P. mg	Wt. of oven dry residue left on filter paper mg	Water content of tissue mg	Recovery of starting weight %
normal	10	2,000	124 ± 7	24 ± 5	434 ± 16	1,427 ± 4	101 ± 1
fatty	8	2,000	378 ± 35	20 ± 2	353 ± 11	1,227 ± 40	99 ± 1

Silica gel with calcium sulphate binder [silica gel G (type 60)] was used for the separation of triacylglycerols. Silica gel without calcium sulphate binder [silica gel H (type 60)] was used for the separation of phospholipids, and was washed before use as described by Parker & Peterson (1965). Siliconized silica gel H was prepared from unwashed silica gel H (type 60) and magnesium silicate by the method of Rouser & Fleischer (1967). Thin-layers, 0.25, 0.30, 0.5, 0.75 and 1.0 mm thick were prepared, using an adjustable applicator (Desaga, G.m.b.H., Heidelberg, West Germany), on 50 x 200, 100 x 200 and 200 x 200 mm glass plates (Camag Co., Muttenz, Switzerland). Silica gel G was slurried in 2 volumes (w/v) of distilled water, and 50 g was sufficient to prepare five, 200 x 200 x 0.5 mm thin-layers. Silica gel H was slurried in 2.3 volumes (w/v) of distilled water, and 60 g was sufficient to prepare five 200 x 200 x 0.5 mm thin-layers. Freshly poured thin-layers were air dried for at least 1 h before activating for 1 h at 110°C. The silica gel G thin-layers were developed with diethyl ether before activating and spotting, to remove possible contaminants to the top, and a narrow band of gel removed (Mahadevan, 1967).

Thin-layers were developed in standard glass chromatography tanks lined on all sides with filter paper saturated with the developing solvent. The solvent mixtures were prepared and thoroughly mixed in 250 ml glass stoppered graduated cylinders. The tanks were placed in a draught free position, and the chromatographic separations were performed at laboratory temperature.

2.2.5.2. Identification of the sample lipids

Identification of the sample lipids separated on chromatograms was based on:

(a) calibration standards chromatographed side by side with the samples on the same plate. Apparent R_F values were not reproducible because the t.l.c. conditions were not rigorously controlled (Jänchen, 1977), but the pattern of the separated classes appeared reproducible. The standard compounds used to identify the sample lipids were palmitic acid, stearic acid, 1,2-dipalmitoyl-*sn*-glycerol, cholesterol, cholesteryl palmitate, tripalmitoylglycerol, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phospho *N*,*N*-dimethylethanolamine, 1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine, asolectin (purified soya phosphatides, Associated Concentrates, Inc., Woodside, N.Y.), and t.l.c. standard PL-3 (The Hormel Institute, Austin, Minn., U.S.A.) containing PtdCho, PtdSer, PtdEtn, and Cer*P*Cho.

(b) specific spray reagents were used for lipids containing phosphorus (Vaskovsky & Kostetsky, 1968), choline (Wagner *et al.*, 1961; Vaskovsky & Suppes, 1971), alk-1'-enyl substituents (Reitsema, 1954), free amino groups, cholesterol and cholesteryl esters, and were prepared as described in the original reference or by Skipski & Barclay (1969).

Several chromatographic systems were used to verify identifications, as suggested by Skipski & Barclay (1969).

2.2.5.3. Thin-layer chromatography of triacylglycerols

2.2.5.3.1. Quantitative analysis of triacylglycerols

Aliquots of total lipid extract, estimated to contain up to 1 μmol of triacylglycerols (about 100 μl for a non-fatty sheep

liver, and 10-20 µl for a fatty sheep liver) were spotted on 200 mm x 200 mm x 0.5 mm thin-layers and developed in light petroleum (b.p. 40-60°C)/diethyl ether/acetic acid (90:10:1, by vol.) (Malins & Mangold, 1960) to 10 cm above the origin. Six samples were spotted per plate, together with 2 lanes of calibration standards. The R_F values of cholesteryl esters, triacylglycerols and cholesterol were about 0.90, 0.40 and 0.06 respectively (see Fig. 2.1.). The marker lanes were sprayed with 2'7'-dichlorofluorescein solution [0.5 g/l in methanol/water (50:50, v/v)], viewed under u.v. light, and the sample spots corresponding to triacylglycerols scraped from the plate and extracted twice with 4 ml of chloroform/methanol (2:1 v/v). The solutions of triacylglycerols, freed of silica gel by filtration, were evaporated to dryness at 65-80°C, and saponified according to Denton & Randle (1967). Glycerol was assayed according to the method of Pinter *et al.* (1967). Instrumentation is described in Chapter 3.

2.2.5.3.2. Preparation of triacylglycerols

400-1000 µl of the total lipid extracts were spotted as bands 10-15 cm long, on 200 mm x 200 mm x 0.5 mm thin-layers, developed and identified as described in section 2.2.5.3.1. The triacylglycerol bands were scraped from the plates, and extracted twice with 10 ml of chloroform/methanol (2:1, v/v). The solutions of triacylglycerols, freed of silica gel by filtration, were reduced in volume by rotary evaporation, and stored, in a total volume of 1 ml, in calibrated glass tubes (5 cm long x 0.5 cm internal diameter) sealed with several layers of 0.06 mm Teflon film, and stored in the dark at -14°C under N_2, until required. The purity of the triacylglycerol preparations was verified by t.l.c. as

FIGURE 2.1.

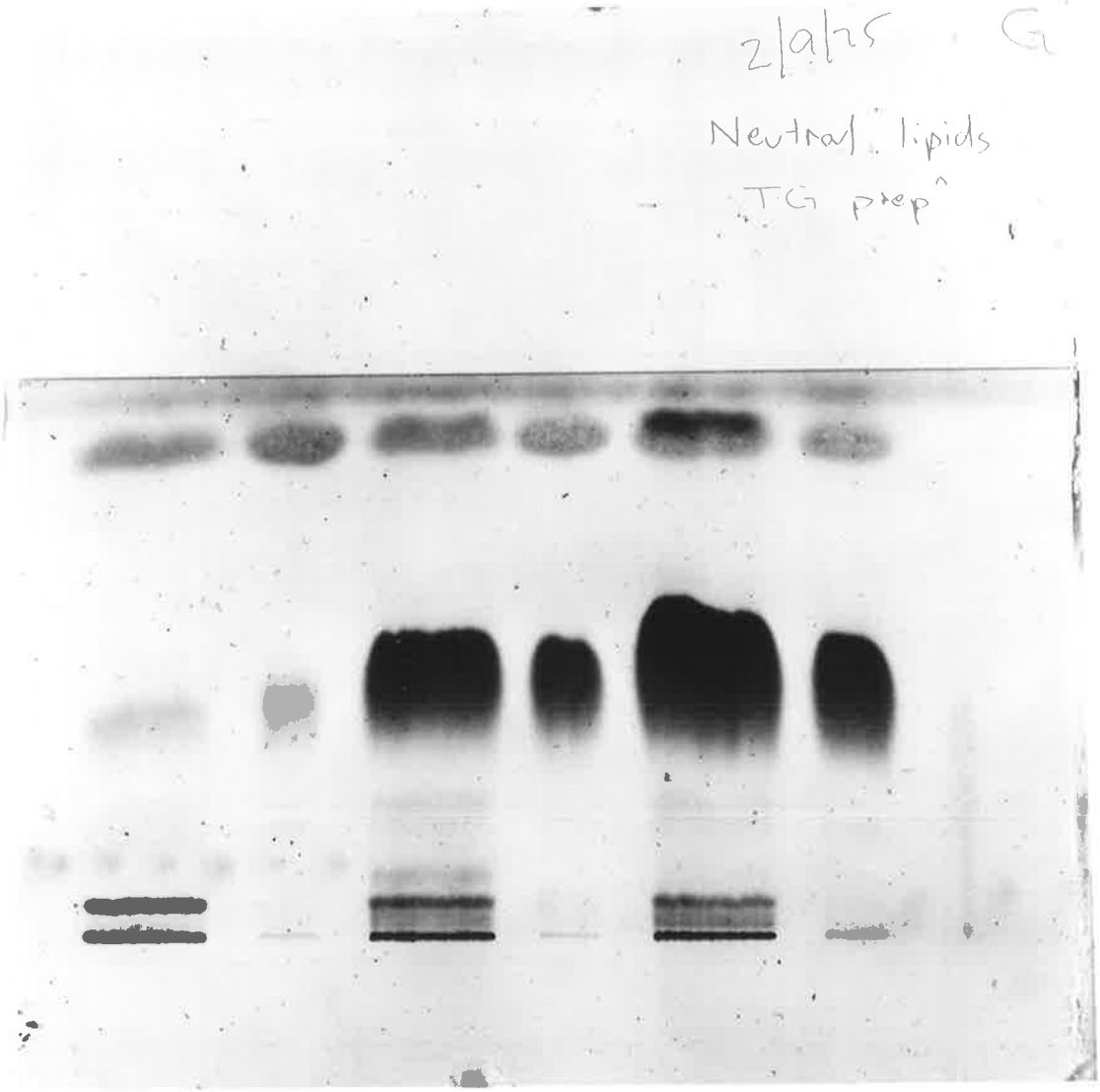
A thin-layer chromatogram of total hepatic lipids and triacylglycerol preparations

The triacylglycerols were prepared as described in section 2.2.5.3.2. The thin-layer was prepared and developed as described in sections 2.2.5.1. and 2.2.5.3.1., and visualized by spraying with a saturated solution of $K_2Cr_2O_7$ in aqueous sulphuric acid (12.5 mol/l) and heating in an oven at 180°C for 1 h. The spot at R_F about 0.88 in each lane was due to the presence of antioxidant in the samples, and in the total lipid extracts the antioxidant migrated immediately behind cholesteryl esters (R_F about 0.91).

The number in parentheses represents the weight (µg) of total lipid extract (equivalent to 4 mg wet wt. of liver for each sheep) and amount (µmol) of triacylglycerol preparation spotted. 10 µl of solution was spotted in each lane.

From left to right, the lanes were spotted with:

1. Total lipid extract from a normal wether (210)
2. The triacylglycerol preparation from 1. (0.03)
3. Total lipid extract from an uncontrolled alloxan-diabetic responder wether (620)
4. The triacylglycerol preparation from 3. (0.22)
5. Total lipid extract from a pregnant-toxaemic ewe (1,030)
6. The triacylglycerol preparation from 5. (0.37)


2/9/15
Neutral lipids
TG prep^

shown in Fig. 2.1.

2.2.5.3.3. Argentation t.l.c. of triacylglycerols

Qualitative argentation t.l.c. was performed using 200 mm x 100 mm x 0.25 mm layers of silica gel G (type 60) impregnated with $AgNO_3$ [$AgNO_3$/silica gel (5/100, w/w) as recommended by Morris (1966)]. The thin-layers were activated at 110°C for 1 h, and 14-190 nmol of sheep liver triacylglycerols were spotted over 5 mm and developed to 10 cm above the origin. Solvent systems of various polarities were investigated, and included benzene/diethyl ether (100:0 to 0:100, v/v) and chloroform/ethyl alcohol (100:0 to 97:3, v/v) as suggested by Litchfield (1972), and light petroleum (b.p. 40-60°C)/diethyl ether/acetic acid (85:15:1, by vol.) (Schmid *et al.*, 1966). Ethanol-free chloroform was used in these investigations. Triacylglycerols were detected by spraying the thin-layers with aqueous orthophosphoric acid (7.5 mol/l) (Barrett *et al.*, 1963) and heating in an oven at 180°C for 1 h.

The solvent systems containing chloroform/ethyl alcohol generally gave the best resolution of bands. The system chloroform/ethyl alcohol (99:1, v/v) gave 7 bands above the origin, ranging in R_F from 0.002-0.82, presumably representing groups of triacylglycerols containing from 0 (near the solvent front) to about 6 (near the origin) double bonds per molecule, assuming that separation had been on the basis of degree of unsaturation (Morris, 1966; Litchfield, 1972). Subsequent work by Read (1976) showed that the separations were based on the degree of unsaturation in the sheep liver triacylglycerol molecules. The more polar solvent systems [e.g. chloroform/ethyl alcohol (97:3, v/v)] suggested that

a significant proportion of the sheep liver triacylglycerols contained more than 6 double bonds per molecule (results not shown). An argentation chromatogram is shown in Fig. 2.2.

The two dimensional t.l.c. procedures of Cubero & Mangold (1965) and Schmid *et al.* (1966) were investigated, and the procedure of Schmid *et al.* (1966) showed promise as a rapid technique for the qualitative assessment of sheep liver triacylglycerols subfractionated on the basis of the degree of unsaturation of their fatty acids. The major advantage of this method was that the total lipid extracts could be used directly, without the need to prepare purified triacylglycerols. This method may be useful for quantitative analysis using the improved charring technique of Chobanov *et al.* (1976).

2.2.5.4. Thin-layer chromatography of phospholipids

2.2.5.4.1. Quantitation of total hepatic PtdCho and PtdEtn by phosphorus measurement after t.l.c. separation

The measurement of PtdCho and PtdEtn was based on the non-specific assay of phosphorus, and it was necessary to use a separation system that was accurate, reproducible and convenient for routine use.

2.2.5.4.1.1. Specificity of the separations

2.2.5.4.1.1.1. Chromatographic separation of PtdCho and PtdEtn

A number of one dimensional t.l.c. solvent systems were investigated (Wagner *et al.*, 1961; Skipski *et al.*, 1964; Nichols, 1964; Lepage, 1967; Roozemond, 1967; Chalvardjian,

FIGURE 2.2.

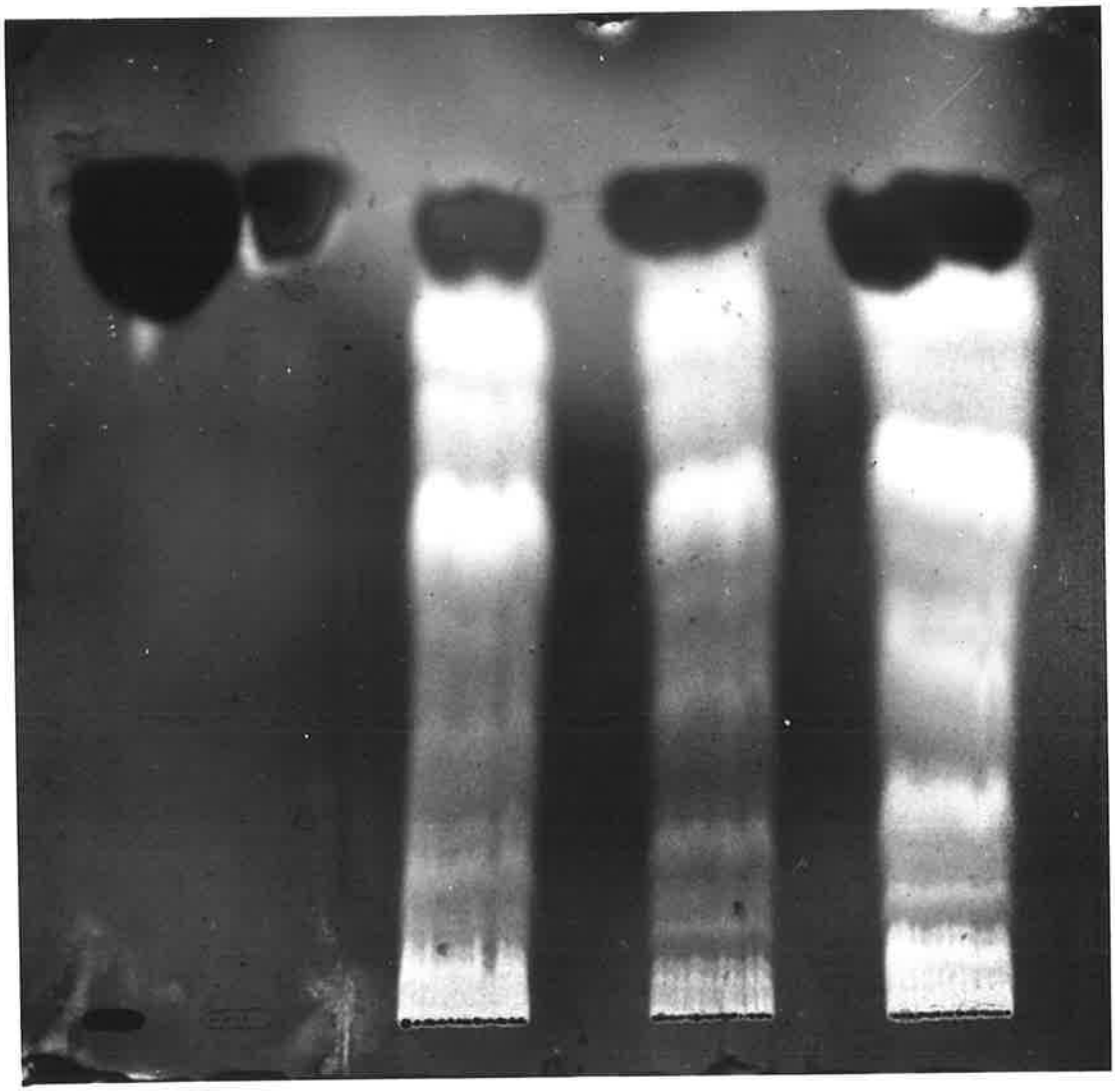
An argentation thin-layer chromatogram of total hepatic triacylglycerols from sheep

(Courtesy of L.C. Read, Department of Agricultural Biochemistry, University of Adelaide).

The triacylglycerols were prepared essentially as described in section 2.2.5.3.2. The thin-layer was prepared as described in section 2.2.5.3.3., and developed in chloroform/ethyl alcohol (99:1, v/v). The bands were visualized by spraying with 2',7'-dichlorofluorescein solution [0.5 g/l in methanol/water (50:50, v/v)] and viewing under u.v. light. The light bands are triacylglycerols, and the dark areas near the apparent solvent front are due to a reaction between the antioxidant in the triacylglycerol preparations and $AgNO_3$ in the thin-layer. About 2.45 µmol of triacylglycerols was spotted for each sheep.

From left to right, the lanes were spotted with:

1. antioxidant plus a trace of tripalmitoylglycerol
2. tripalmitoylglycerol plus antioxidant
3. normal sheep liver triacylglycerols
4. uncontrolled alloxan-diabetic responder liver triacylglycerols
5. pregnant-toxaemic ewe liver triacylglycerols



1969). Chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) (Skipski *et al.*, 1964) gave the best separation of PtdCho and PtdEtn from the other major phospholipid classes (identified as described in section 2.2.5.2.) found in sheep liver lipid extracts (Dawson, 1960; Peters & Smith, 1964; Scott *et al.*, 1967; Getz *et al.*, 1968). Noble *et al.* (1971*a*) used this solvent system to analyze sheep liver phospholipids.

2.2.5.4.1.1.2. Purity of the separated PtdEtn

The solvent system chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) does not separate PtdEtn from PtdGro and some glycosphingolipids that occur in animal tissues (Skipski & Barclay, 1969).

In man, the liver contains only minute amounts of glycosphingolipids (Mårtensson, 1969). This may be so in sheep too, as Noble *et al.* (1971*a*) obtained the same estimate for the relative concentration of PtdEtn in sheep liver lipid extracts by phosphorus analysis (glycosphingolipid contamination of the PtdEtn spots would not interfere) and by g.l.c. analysis (glycosphingolipid fatty acids would contaminate the PtdEtn fatty acids). Peters & Smith (1964) report that ceramide, a constituent of all glycosphingolipids, represents about 2.4% (w/w) of the total lipids of sheep liver. In the work reported in this thesis, glycosphingolipid contamination of the PtdEtn spots would not interfere with the quantitation by phosphorus estimation, but may influence the qualitative assessment of the density of charring of the PtdEtn spots separated by argentation t.l.c.

Scott *et al.* (1967) report that PtdGro may account for 5% of the total phospholipid phosphorus of sheep liver. One dimensional t.l.c. with chloroform/acetone/methanol/acetic acid/water (5:2:1:1:0.5, by vol.) (Lepage, 1967) and two dimensional t.l.c. (on siliconized silica gel H) with chloroform/methanol/water (65:25:4, by vol.) followed by butan-1-ol/acetic acid/water (3:1:1, by vol.) (Rouser *et al.*, 1967) did not detect PtdGro in sheep liver lipid extract when 9-18 µg total phospholipid phosphorus (400-700 µg total lipid) was chromatographed and visualized by charring. Two dimensional t.l.c. (as above) of 30 µg total phospholipid phosphorus (1,200 µg total lipid) detected two spots in sheep liver lipid extract in positions that may indicate the presence of PtdGro and ceramide dihexoside (Rouser *et al.*, 1967). Assuming the sensitivity of charring to be about 1 µg of lipid (Mangold, 1961), PtdGro probably accounts for less than 1% of the total phospholipid phosphorus in these sheep. Noble *et al.* (1971*a*) do not report any contamination of their PtdEtn spots.

One dimensional t.l.c. of sheep liver lipid extract (30 µg of phosphorus) with chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) gave one positive spot when sprayed with ninhydrin reagent, corresponding in position to PtdEtn. Two dimensional t.l.c. (30 µg of phosphorus) as described above, gave two positive spots when sprayed with ninhydrin reagent, corresponding in position to PtdEtn and PtdSer. Calibration standards indicate that the PtdSer and PtdIns of sheep liver comigrate (see Fig. 2.3.), as reported by Arvidson (1968) for rat liver lipids using chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). Skipski *et al.* (1964), using "basic" silica gel H plates, and Parker & Peterson (1965),

FIGURE 2.3.

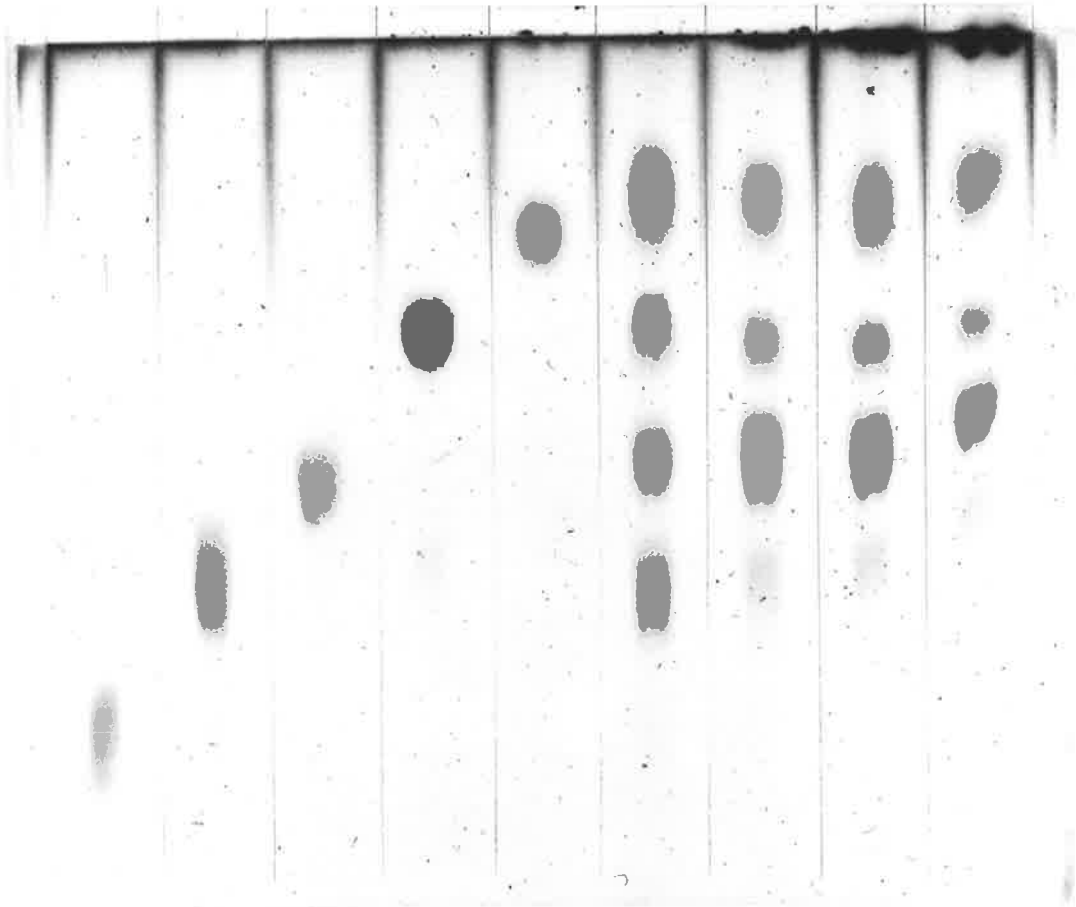
One dimensional thin-layer chromatogram of phospholipid standards and total hepatic phospholipids of sheep

The thin-layer was prepared and developed as described in sections 2.2.5.1. and 2.2.5.4.1.1.1., and visualized as described in Fig. 2.1. The number in parentheses represents µg of phosphorus spotted.

Key to figure:

1. 1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine (1 µg)
2. Cer*P*Cho (from bovine brain) (1 µg)
3. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (1 µg)
4. 1,2-dipalmitoyl-*sn*-glycero-3-phospho *N*,*N*-dimethylethanolamine (2 µg)
5. 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (1 µg)
6. standard PL-3 (Hormel Institute) consisting of from bottom to top: Cer*P*Cho (1 µg), PtdCho (1 µg), PtdSer (1 µg), PtdEtn (1 µg)
7. total lipid extract from a normal wether (5 µg)
8. total lipid extract from an uncontrolled alloxan-diabetic responder wether (5 µg)
9. total lipid extract from a pregnant-toxaemic ewe (5 µg)

TOTAL PHOSPHOLIPID SEPARATION



1 2 3 4 5 6 7 8 9

using "neutral" plates developed in a "saturation chamber", obtained clear separations of rat liver PtdEtn, PtdIns and PtdSer. The reason for the failure to detect PtdSer by ninhydrin spray on the one dimensional plates is unknown if PtdEtn and PtdSer are not comigrating. The reported difficulty of separating natural PtdEtn from PtdSer [Strickland (1973) lists a number of references related to this problem], and the fact that considerable emphasis has been placed on chromatographic methods for the identification of unknown lipids, suggests that independent techniques (such as infrared spectrometry) could have been used for unambiguous identification of the lipids studied in this work.

The results in Fig. 2.12. (see section 2.3.7.1.2.) suggest that PtdEtn separated by the one dimensional t.l.c. system used in this work are contaminated with other compounds (such as PtdGro, PtdSer and glycosphingolipids), as minor bands appear above the 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine calibration standard on the argentation chromatogram, visualized by charring. However, the contamination appeared to be minor because there were no significant differences in the phosphorus content of the PtdEtn spots separated by one dimensional t.l.c. [chloroform/methanol/ acetic acid/water (25:15:4:2, by vol.) and chloroform/methanol/ water (13:6:1, by vol.)] and two dimensional t.l.c. (as described above) for liver lipid extracts from 8 sheep.

2.2.5.4.1.1.3. Alk-1'-enyl derivatives of PtdCho and PtdEtn

The reported presence of low levels of alk-1'-enyl derivatives of PtdCho and PtdEtn in sheep liver lipid extracts (Dawson, 1960; Scott *et al.*, 1967; Getz *et al.*, 1968) was verified

using a specific spray reagent on developed chromatograms (Reitsema, 1954) and by t.l.c. of liver lipid extracts (2 sheep only) according to the method of Owens (1966) but using an increased loading of lipid material as suggested by Skipski & Barclay (1969). Accordingly, the terms PtdEtn and PtdCho used throughout this thesis include possibly occurring alk-1'-enyl derivatives. Alkyl derivatives may also be present in the PtdEtn and PtdCho classes separated in this work.

2.2.5.4.1.2. Quantitation of PtdCho and PtdEtn by one dimensional t.l.c. without elution

The procedure of Parker & Peterson (1965) was mainly followed.

2.2.5.4.1.2.1. Chromatographic procedures

200 mm x 200 mm x 0.25 mm silica gel H thin-layers were divided into 9 2 cm lanes by etching with a fine needle prior to activation.

20 µl of total lipid extract, containing 9-15 µg of total phosphorus, was applied in quadruplicate, as a narrow band about 1 cm long in each lane. Two samples were spotted per plate, and the remaining lane was used as the blank lane for phosphorus analysis.

The spotted plates were developed first with acetone/light petroleum (b.p. 40-60^{o}C) (1:3, v/v) (Skipski & Barclay, 1969) to remove non-polar lipids to the top. A line was then etched across the plates, 10-15 cm above the origin, and the plates developed with chloroform/methanol/acetic acid/water (25:15:4:2,

by vol.). The proportion of water was varied slightly under some laboratory conditions to improve resolution of the spots. The first development took about 30 min, and the second about 60 min to complete.

2.2.5.4.1.2.2. Detection and removal of spots from the plates

The PtdCho and PtdEtn spots were detected either by iodine vapours in a closed tank (Sims & Larose, 1962), or by spraying with aqueous sulphuric acid (9 mol/l) and heating in an oven at 180°C for 1 h (Owens, 1966). A typical charred chromatogram used for quantitative analysis is shown in Fig. 2.4. The spots were outlined with a fine needle.

When detected by iodine vapours, the iodine was evaporated from the plates in an oven at 60°C for 15 min, and each outlined spot aspirated into 20 ml tubes (Corning Pyrex, MA 9820) using a slight modification of the device of Rouser *et al.* (1966*b*). Precautions were taken to minimize contamination and loss of gel, as described by Rouser *et al.* (1966*b*).

When detected by charring, the outlined spots were scraped into the 20 ml tubes using a sharpened, stainless steel spatula. Provided the plates were slightly damp with acid, this procedure was quicker than the aspiration technique. To minimize the risk of phosphorus contamination by diffusion of excess acid through the thin-layer, it may be necessary to scrape the plates as soon as possible after charring (Robinson & Phillips, 1963).

Areas of silica gel, corresponding in size and position to the areas containing PtdCho and PtdEtn, were scraped (or aspirated)

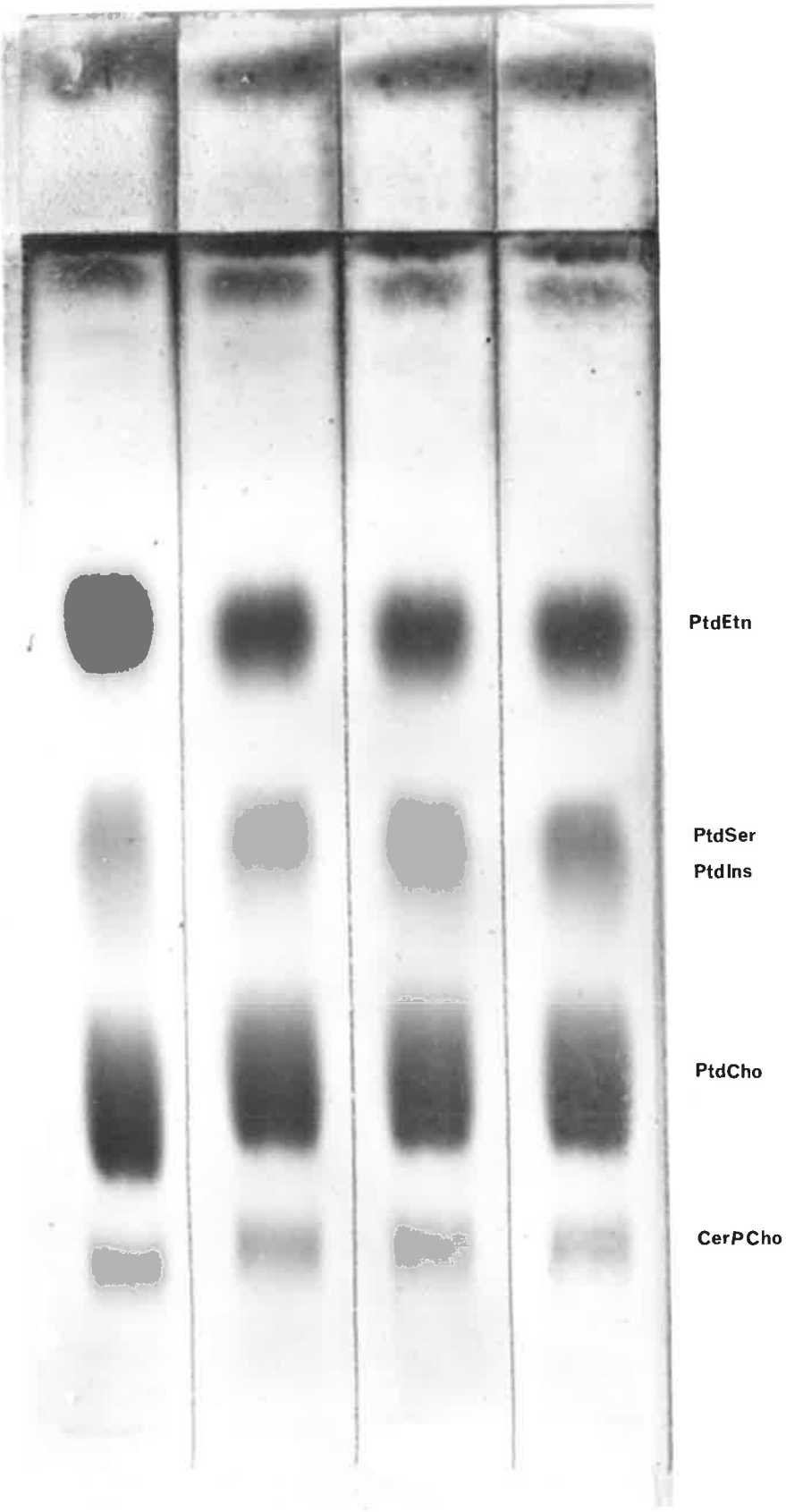
FIGURE 2.4.

A typical one dimensional thin-layer chromatogram used for quantitative analysis of PtdEtn and PtdCho without elution

The plate was prepared, developed and visualized as described in sections 2.2.5.1., 2.2.5.4.1.2.1. and 2.2.5.4.1.2.2.

20 µl of total lipid extract from a normal sheep liver was spotted per lane. equivalent to about 9 µg of phosphorus.

The identity of the spots was determined as described in section 2.2.5.2.


PtdEtn
PtdSer
PtdIns
PtdCho
CerPCho

from the blank lanes into 20 ml tubes.

2.2.5.4.1.2.3. Phosphorus determination

0.5 ml of conc. sulphuric acid (18 mol/l) was added to each tube and digestion carried out, according to Parker & Peterson (1965), in an electrically heated, aluminium block (manufactured by Southcott Pty. Ltd., Adelaide) with holes for 30 tubes. The upper two-thirds of each tube extended outside of the block.

A set (11) of standards containing 0-10 µg of phosphorus was prepared (in duplicate) in 20 ml digestion tubes, from a standard solution of potassium phosphate (monobasic) KH_2PO_4 (10 mg of phosphorus/l). Water was removed from the standards in an oven at 80°C before the addition of 0.5 ml of sulphuric acid. Before preparation of the standard phosphorus solution, the KH_2PO_4 (Univar, assay 99.0-101.0%, Ajax Chemicals Ltd., Sydney) was dried at 110°C for 1-2 h. A set of standards containing washed silica gel H was prepared, as recommended by Skipski & Barclay (1969). The standard and blank tubes were processed in the same way as the sample tubes.

Phosphorus was measured in the digests as described by Parker & Peterson (1965). The 1-amino-2-naphthol-4-sulphonic acid (BDH Chemicals Ltd., Poole, England) was recrystallized as described by Skipski & Barclay (1969), but remained slightly pink, and was used in this condition. The reducing reagent was prepared fresh each week, and stored in a dark bottle.

The standard curves were linear over the range 0-10 µg of phosphorus, and 1 µg of phosphorus had an OD_{820nm} of 0.091 ± 0.002

(mean ± S.D. of 5 curves). A new standard curve was made for each experiment. The presence of silica gel did not influence colour development during the phosphorus determination under these conditions. The results are expressed as µmol of PtdCho and PtdEtn on the basis that 1 µmol of these compounds contain 30.975 µg of phosphorus.

2.2.5.4.1.2.4. Recovery of phosphorus from the plates

Phosphorus determinations were performed (see section 2.2.5.4.1.2.3.) on identical aliquots (10 µl), of total liver lipid extract from 7 sheep, placed directly in digestion tubes, and on a thin-layer plate and scraped into digestion tubes after developing the plate with acetone/light petroleum (b.p. 40-60^{o}C) (1:3, v/v) and charring (see section 2.2.5.4.1.2.2.). Each determination was performed in triplicate.

The recovery of phosphorus was 95 ± 3% (mean ± S.D.). Parker & Peterson (1965) obtained a recovery of 96 ± 2% (mean ± S.D. of 9 determinations) for chromatographed total lipid phosphorus of 2 pooled rat livers. As the recovery of standard PtdCho and PtdEtn phosphorus appeared similar (96%) to that of the total lipid phosphorus in rats (Parker & Peterson, 1965), it was assumed that the recovery of total hepatic PtdCho and PtdEtn phosphorus of sheep was 95%.

The results reported in this thesis have not been corrected for this recovery.

2.2.5.4.1.2.5. Repeatability of the phosphorus determinations

An indication of the repeatability of the phospholipid determinations is shown in Table 2.3. Repeatability, estimated by the standard deviation (S.D.) of the mean of the quadruplicate measurements, varied between t.l.c. plates. The extremes of this variation are shown in Table 2.3.

The problem of phosphorus contamination of the glassware, solvents and reagents, of the accurate pipetting of small volumes of volatile solvent, and of high t.l.c. adsorbent blank phosphorus values have contributed to this variation.

Improvements to the procedure would include: the use of fewer (and wider) lanes (6 or 7) per plate, to minimize phosphorus contamination associated with the lateral movement of solvents during development; scraping the plates, visualized by charring, as soon as possible after charring to avoid the possible problem of acid diffusion through the thin-layer of gel (Robinson & Phillips, 1963); lowering the phosphorus content of the silica gel adsorbent and ensuring that the same weight of gel is scraped into each digestion tube.

Parker & Peterson (1965) reduced the phosphorus content of their silica gel H from 0.003 µg/mg gel to 0.0006 µg/mg gel by a washing procedure. The same washing procedure reduced the phosphorus content of the silica gel H used in this work from 0.008 µg/mg gel to 0.004 µg/mg gel.

The phosphorus content of the silica gel H blanks varied between plates and with position on the same plate. The

TABLE 2.3.

Repeatability of the quantitative analysis of PtdCho and PtdEtn by t.l.c. and phosphorus determination without elution

The basis of the classification "best" and "worst" t.l.c. plate was the coefficient of variation of the determination. The number in parentheses is the number of replicate determinations used in calculating the mean. The second value [mean ± S.D.(3)] for the two t.l.c. plates was obtained when the worst replicate of the four was rejected. Data from 2 references is included for comparison.

Reference	Rouser *et al.* (1970)		Parker & Peterson (1965)		This work			
					Best t.l.c. plate		Worst t.l.c. plate	
Phospholipid separated	%*	µg phosphorus/spot mean ± S.D.	%	µg phosphorus/spot mean ± S.D.	%	µg phosphorus/spot mean ± S.D.	%	µg phosphorus/spot mean ± S.D.
PtdCho	1	3.50 ± 0.06(4)	1	3.58 ± 0.09(9)	6	3.76 ± 0.07(4) 3.79 ± 0.02(3)	3	4.70 ± 0.69(4) 4.35 ± 0.05(3)
PtdEtn	1	3.75 ± 0.06(4)	2	1.63 ± 0.03(9)	4	2.01 ± 0.07(4) 2.04 ± 0.01(3)	10	1.67 ± 0.63(4) 1.95 ± 0.32(3)

* Adsorbent blank phosphorus as a % of the total phosphorus in the separated spot.

phosphorus content of the PtdCho and PtdEtn silica gel blanks was about 0.15 µg (0.01-0.50). As shown in Table 2.3., Rouser *et al.* (1970) and Parker & Peterson (1965) had much lower blank values, and this probably contributed greatly to their better repeatability.

To avoid the problem of unrealistic phosphorus values, the worst replicate (the one that deviated the most from the other 3) was rejected, and the mean of the 3 remaining values used to calculate the results. The effect of this procedure on the results is shown for several samples in Table 2.3.

2.2.5.4.2. Qualitative argentation t.l.c. of intact PtdCho and PtdEtn

The procedures of Arvidson (1965, 1968) were mainly followed.

2.2.5.4.2.1. Isolation of PtdCho and PtdEtn

The PtdCho and PtdEtn fractions were isolated from the total lipid extracts by preparative t.l.c. using 200 mm x 200 mm x 0.5 mm thin-layers. Precautions were taken to minimize autoxidation and contamination (see section 2.2.5.1.). 1 ml of extract (containing 500-600 µg of phosphorus) was spotted as a band 15 cm long. The plates were developed as described in section 2.2.5.4.1.2.1., and the separated bands (5 major phospholipid bands for sheep and rats, identified as described in section 2.2.5.2.) were visualized, scraped and eluted according to Arvidson (1968). Residual methanol, acetic acid and water were removed by solvent replacement with chloroform (see section 2.2.3.), and the preparations (containing 3-5 µmol PtdEtn and 3-9 µmol PtdCho, 6-18 mmol/l) were placed in

graduated glass tubes (5 cm long x 0.4 cm internal diameter) and sealed with several layers of 0.06 mm Teflon film. The tubes were stored in the dark at -14°C under N_2 until required. The recovery of PtdCho and PtdEtn was 85 ± 14% (mean ± S.D. of 10 preparations).

The purity of the preparations was checked by t.l.c. using the solvent system of Skipski *et al.* (1964), and only those preparations migrating as a single spot, corresponding in position to authentic PtdCho and PtdEtn, were used for argentation t.l.c. This was possibly a poor check of purity, and other tests could have been made (see section 2.2.5.4.1.1.2.).

2.2.5.4.2.2. Preparation, storage and activation of $AgNO_3$-impregnated thin layers

Argentation t.l.c. was performed using 200 mm x 100 mm x 0.30 mm thin-layers of silica gel H (type 60) impregnated with $AgNO_3$ [$AgNO_3$/silica gel (3:10, w/w)]. The silica gel was slurried in 2.5 volumes (w/v) of aqueous $AgNO_3$ solution (0.71 mol/l), and 35 g of gel was sufficient to prepare 10 thin-layers. The glass plates were not roughened on the face to be coated, as recommended by Barrett *et al.* (1963). The adjustable applicator was washed immediately after thin-layer preparation to minimize corrosion. Washed silica gel H (see section 2.2.5.1.) could not be used for argentation t.l.c. as the thin-layers turned black very rapidly.

The plates were dried on the bench for 2 h, placed in a carrying rack, enclosed in aluminium foil to exclude light, and stored in a small room for 24 h away from wooden surfaces (Morris, 1964).

The thin-layers were activated for 5 h at 175°C or 3 h at 195°C, cooled in a cabinet at 60-80°C to minimize cracking of the glass plates, and stored in a large glass bell jar over P_2O_5 in the dark. Thin-layers remained active for at least 2 weeks when stored under these conditions.

2.2.5.4.2.3. Chromatographic procedures

Samples containing 1-5 µg of phosphorus were applied to the thin-layers as spots or 5-10 mm bands. A dark area formed at the point of application of the samples and migrated near the solvent front during development. The dark area was apparent on charred chromatograms (see Fig. 2.12.) and chromatograms viewed under u.v. light (see Fig. 2.2.), and was the result of a reaction between the antioxidant in the samples and $AgNO_3$. The antioxidant appeared to be concentrated in the samples during the solvent replacement technique (see section 2.2.3.) used in their preparation, as the reference standards, containing dilute antioxidant (0.23 mmol/l) did not produce the same large dark areas near the solvent front (see Fig. 2.12.).

Thin-layers activated at 195°C for 3 h, and spotted with PtdEtn, were developed in chloroform/methanol/water (55:35:7, by vol.). Thin-layers activated at 195°C for 3 h or 175°C for 5 h, and spotted with PtdCho, were developed in chloroform/methanol/water (60:30:5, by vol.), or chloroform/methanol/water (65:25:4, by vol.). The developing chambers were closed glass tanks (internal dimensions 14 cm x 11 cm x 21 cm) lined on all sides with solvent saturated filter paper. The chambers were equilibrated with solvent for 30 min and the thin-layers were developed until the

apparent solvent front had reached 10-15 cm above the origin.

2.2.5.4.2.4. Detection of the bands

The thin-layers were very fragile after development, and consequently the plates were kept horizontal at all times, and spray reagents were applied gently. The bands were poorly visualized when the thin-layers were sprayed with a solution of 2',7'-dichlorofluorescein in 95% ethanol (2 g/l) and viewed under u.v. light, but were readily detected when sprayed with aqueous ammonium bisulphate solution (1.74 mol/l) and charred at 175°C or 195°C for 1 h (Ziminski & Borowski, 1966; Renkonen, 1966). The saturated PtdCho and PtdEtn standards did not char as well as the samples (see Fig. 2.12.).

A qualitative estimate of the PtdCho and PtdEtn subfractions was obtained by visual assessment of the density of charring when equivalent amounts (2 or 5 µg of phosphorus) of total hepatic PtdCho and PtdEtn, prepared from animals under a variety of metabolic conditions, were spotted, developed and charred on the same plates. Interpretation of the results is difficult because of the problems of differential charring of saturated and unsaturated fatty acids, the influence of R_F and spot shape on charring, the anomalous charring by PtdEtn, the effect on charring of variations in gel thickness, uneven spraying of the plates, damage to the gel surface during spraying, the negative effect of $AgNO_3$ and the tendency of the gel surface to bubble during charring (Barrett *et al.*, 1963; Blank *et al.*, 1964; Jones *et al.*, 1966). Some of these problems may be overcome using the procedure of Chobanov *et al.* (1976), by the addition of bromine to the double

bonds in the separated bands prior to charring in the presence of sulphuryl chloride.

Despite the difficulties of interpreting the results of charred chromatograms, it was considered that comparisons between the same subfractions obtained for different animals on the same plate were useful. The separations were repeated on a number of plates (3 plates for PtdEtn and 6 for PtdCho) to reduce the effect of some of the difficulties.

2.2.6. Estimation of the total phospholipid content of liver tissue

This was determined by taking an aliquot (usually 10 µl) of the total lipid extract (5 ml) and analyzing for total phosphorus, as described in section 2.2.5.4.1.2.3. Assays were performed in triplicate.

2.2.7. Expression of the analytical results

The work reported in this thesis often involved comparisons of metabolite contents between sheep with fatty and non-fatty livers. In rats, fatty livers are often larger and heavier than non-fatty livers, and the increase in weight is due mainly to an increase in the lipid and water content (Lucas & Ridout, 1967). This may be true for the fatty livers associated with vitamin B_{12} deficiency in sheep (Smith & Osborne-White, 1973; Smith *et al.*, 1974).

On the assumption that this is generally true for fatty livers in sheep, the analytical data reported in this thesis has been expressed using dry fat-free tissue weight as the base of

reference, as this removes the two major weight variables, and may represent a more accurate index of the liver cell count than the fresh weight. As shown in Table 2.2., the dry fat-free tissue wt./unit fresh liver wt. was lower in the fatty livers than in the normal livers.

2.2.8. Chemicals

Reagent-grade chloroform and methanol were glass distilled. Ethanol-free chloroform was prepared as described by Vogel (1957), and stabilized with 4-methyl-2,6-di-*tert.*-butylphenol (Wren & Szczepanowska, 1964). Except where specified, distilled chloroform containing ethanol stabilizer was used. Distilled chloroform was stored in dark bottles in the cold to minimize decomposition. Aqueous ammonia (14.8 mol/l) was prepared as described by Rouser & Fleischer (1967). Glass distilled water was used throughout. All other solvents were reagent-grade and were not distilled before use.

Chemicals were obtained as follows: silica gel G (type 60) and silica gel H (type 60) from E. Merck AG, Darmstadt, Germany; magnesium silicate from Sigma Chemical Co., St. Louis, Mo., U.S.A.; alloxan from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; 4-methyl-2,6-di-*tert.*-butylphenol from Calbiochem, Los Angeles, Calif., U.S.A.; lipid standards (see section 2.2.5.2.) from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., Sigma Chemical Co., St. Louis, Mo., U.S.A., and the Hormel Institute standard PL-3 by courtesy of Dr. A. Marker, Department of Plant Physiology, Waite Agricultural Research Institute.

Other materials were reagent-grade chemicals or the best commercially available grade.

2.3. Results

2.3.1. The morphology of sheep liver

2.3.1.1. The macroscopic appearance

Fig. 2.5. shows a healthy liver from a normal Merino wether, and a fatty liver from a pregnant-toxaemic Merino ewe.

2.3.1.2. Histological studies

2.3.1.2.1. Light microscopy

Fig. 2.6a. shows liver sections from two abattoirs sheep stained with a general stain, haematoxylin and eosin. Fat, which does not stain, was evident in the fatty liver as white globules that occupied a large fraction of the cells. Fig. 2.6b. shows liver sections from the same sheep stained with a fat specific stain, Oil Red O, and the fat, which stains red, was evident throughout the acini of both livers although the staining appeared more intense in the fatty liver. No obvious pattern of fat deposition in the acini was evident. The histological method of fat detection is very sensitive, and stainable fat may be observed before there is any significant increase in the amount of chemically extracted fat (Lucas & Ridout, 1967). Sheep are slaughtered at the abattoirs after a 24-48 h fast. Thus it was difficult to obtain a non-fatty liver (at least from the viewpoint of the histologist) from the abattoirs, as histologically detectable fat accumulation in sheep liver can occur within 24 h of fasting (Manns, 1972). The muralium simplex typical of the liver of higher vertebrates (Elias & Sherrick, 1969), and the extensive network of sinusoids can be seen in Figs. 2.6a. and 2.6b. The centre of each

FIGURE 2.5.

The macroscopic appearance of sheep liver

(The photographs were provided by courtesy of Dr. A.M. Snoswell)

Top: A healthy liver from a normal wether.

Bottom: A fatty liver from a pregnant-toxaemic ewe.

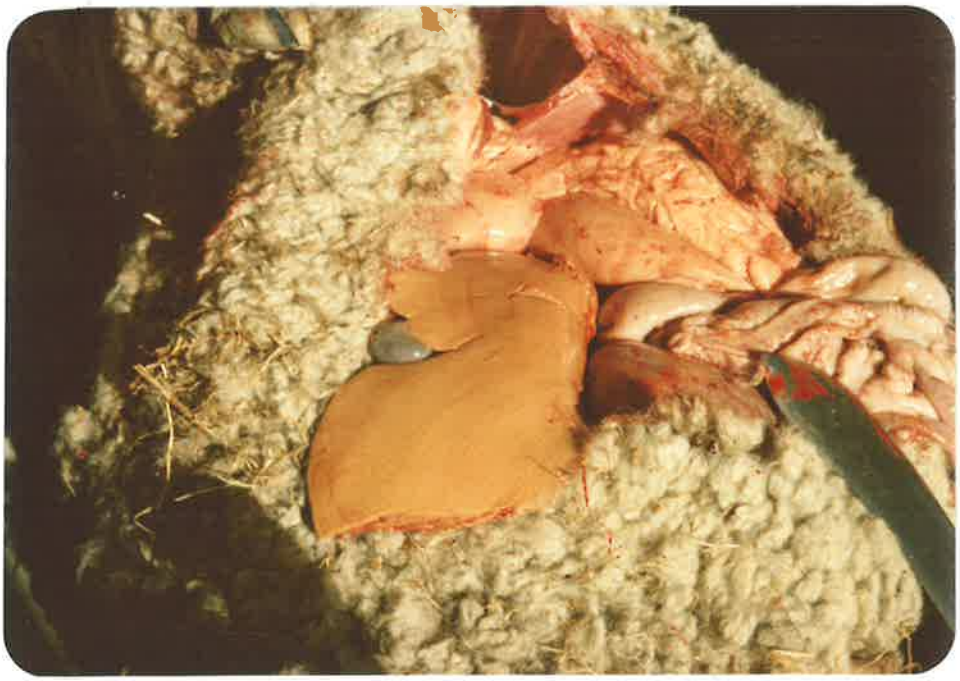


FIGURE 2.6a.

Low-power photomicrographs of liver sections from sheep

(Courtesy of the
Institute of Medical and Veterinary Science, Adelaide)

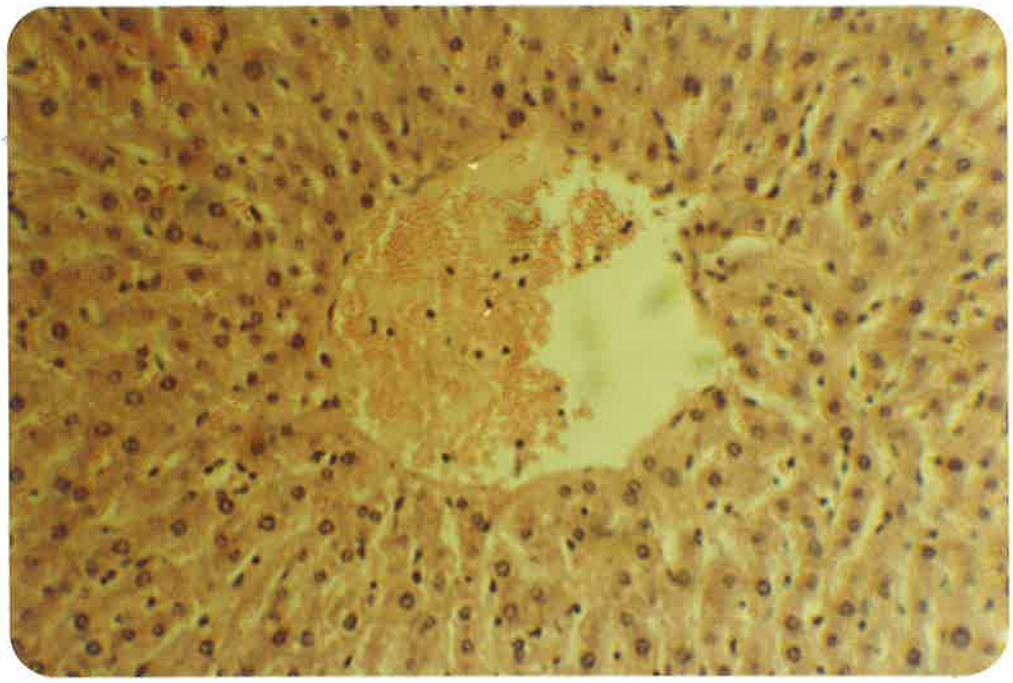
The sections were stained with haematoxylin and eosin.

Magnification x 100.

The samples of non-fatty and fatty sheep liver, classified as such by visual assessment, were collected into formalin [formaldehyde/ aqueous NaCl (9 g/l), 40 g/l)] at the Adelaide abattoirs (Gepps Cross).

Top: A non-fatty sheep liver

Bottom: A fatty sheep liver



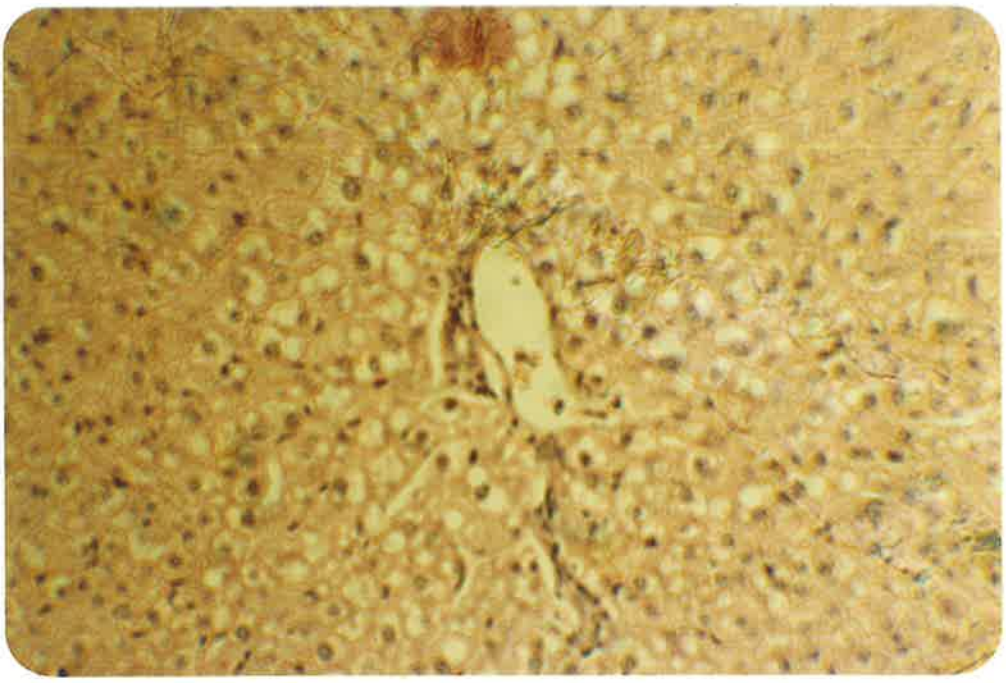


FIGURE 2.6b.

Low-power photomicrographs of liver sections from sheep

(Courtesy of the
Institute of Medical and Veterinary Science, Adelaide)

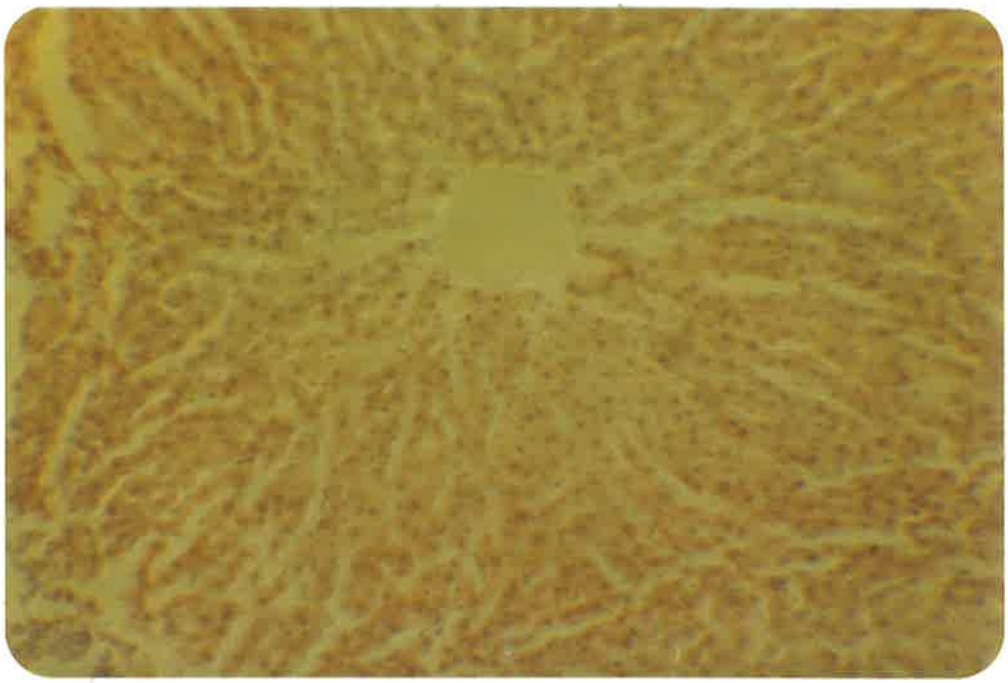
The sections were stained with Oil Red O.

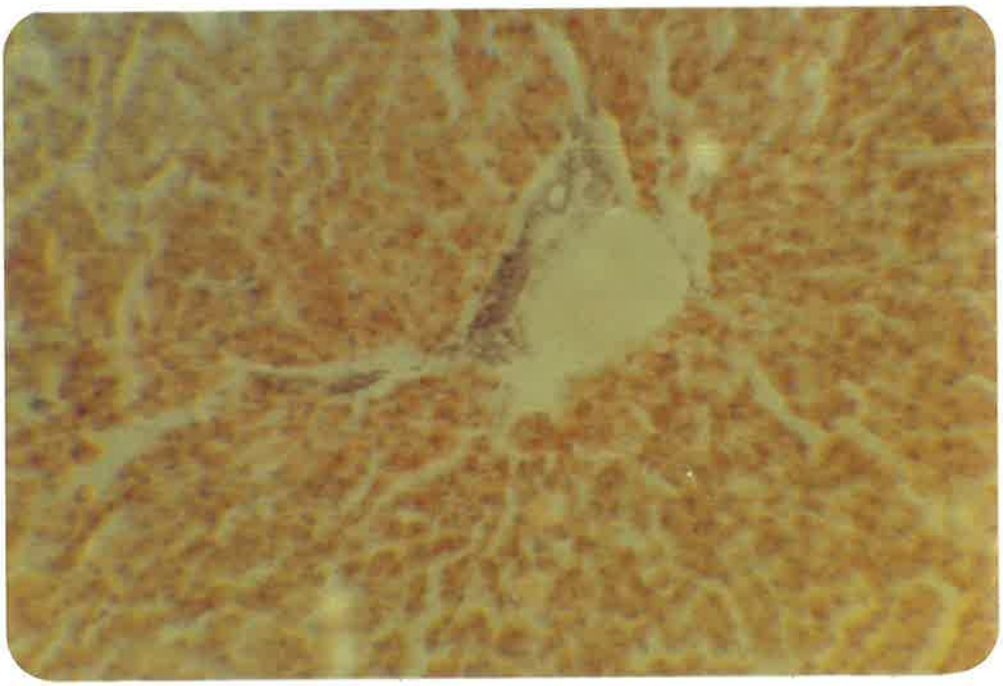
Magnification x 30.

Tissues were collected as described in Fig. 2.6a.

Top: A non-fatty sheep liver.

Bottom: A fatty sheep liver.





section in Figs. 2.6a. and 2.6b. is occupied by a terminal hepatic venule or portal space (Rappaport, 1975).

2.3.1.2.2. Electron microscopy

Fig. 2.7. shows a comparison of sections taken from the liver of a normal and an alloxan-diabetic sheep. Numerous large lipid droplets and enlarged and distorted mitochondria are evident in the hepatocytes from the alloxan-diabetic sheep in contrast to the hepatocytes from the normal sheep. A distinct basal lamina was not evident in these sections of sheep liver. Subcellular fractionation studies using marker enzymes revealed that liver mitochondria from diabetic sheep were more fragile than those from normal sheep liver, as reported by Taylor *et al.* (1971). This may indicate some abnormality of membrane structure under these conditions.

2.3.2. The total lipid and water content of several tissues of normal and alloxan-diabetic animals

The results in Tables 2.4. and 2.5. were not analyzed statistically because of the small number of animals involved. The data in Table 2.4. suggests that, of the four tissues studied, liver and to a lesser extent kidney cortex were the main tissues associated with an increased lipid content in alloxan-diabetic responder sheep. Data from a severely alloxan-diabetic responder goat was included for comparison, as severely alloxan-diabetic responder sheep were not used in this work, and indicates the high lipid levels that can occur in liver and kidney cortex under these physiological conditions. The total lipid content of skeletal muscle shown in Table 2.4. was similar to that reported by Pryor &

FIGURE 2.7.

Electron micrographs of liver sections from sheep

(Courtesy of Dr. J.C. Wallace and Dr. D.B. Keech,
Department of Biochemistry, University of Adelaide)

The sections were prepared as described by Taylor *et al.* (1971).

Magnification x 8000.

Top: A normal Merino ewe.

Bottom: An alloxan-diabetic responder Merino ewe (3 days after alloxan administration).

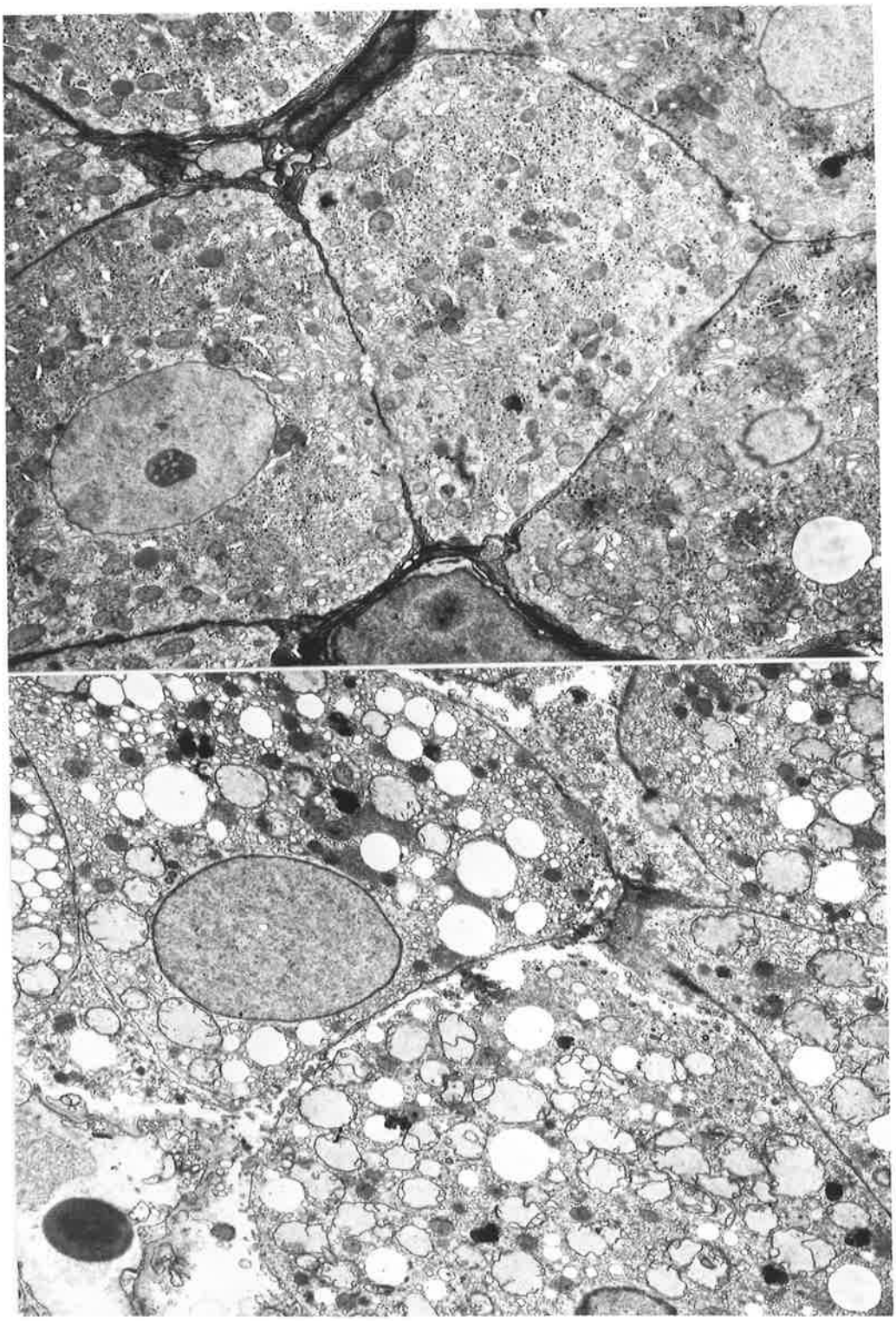


TABLE 2.4.

Total lipid content of several tissues of sheep and a goat

Experimental treatment of the animals is described in sections 2.2.1.1. and 2.2.1.2.1. Tissues were collected, lipid extracted, and their total lipid content determined as described in sections 2.2.2., 2.2.3. and 2.2.4. Values are means, with individual figures in parentheses. n.d., not determined.

Metabolic condition of the animals	Total lipid content of the tissue mg/g dry fat-free tissue			
	Liver	Kidney cortex	Heart	Skeletal muscle M. *biceps femoris*
Normal fed sheep*	229 (216, 242)	261 (262, 259)	184 (180, 188)	178 (164, 191)
Alloxan-diabetic wethers				
Uncontrolled non-responders	292 (243, 341)	190 (211, 169)	192 (236, 148)	148 (148,n.d.)
Uncontrolled responders	651 (465, 837)	371 (415, 327)	258 (187, 328)	147 (118, 176)
Alloxan-diabetic goat				
Uncontrolled responder	2,156	1,157	n.d.	n.d.

* One animal was a ram, and the other a wether.

TABLE 2.5.

Total water content of several tissues of sheep and a goat

The moisture contents of the tissues were determined as described in section 2.2.4. Other details are as described in Table 2.4.

Metabolic condition of the animals	Total water content of the tissue mg/g dry fat-free tissue			
	Liver	Kidney cortex	Heart	Skeletal muscle M. *biceps femoris*
Normal fed sheep	3092 (3021, 3163)	5795 (6675, 4914)	4573 (4808, 4337)	4133 (4301, 3964)
Alloxan-diabetic wethers				
Uncontrolled non-responders	3257 (3332, 3181)	4631 (4813, 4449)	4872 (4975, 4769)	4317 (4317, n.d.)
Uncontrolled responders	3371 (3143, 3598)	5005 (5164, 4846)	4112 (3995, 4228)	3735 (3621, 3849)
Alloxan-diabetic goat				
Uncontrolled responder	6,018	5,306	n.d.	n.d.

Warren (1973) for a variety of sheep. Using measured organ weights and the data in Table 2.4. it was possible to estimate that the net accumulation of lipid in the livers of the alloxan-diabetic responder sheep was about 40 g above the normal levels of about 30 g/liver in sheep of that size. Table 2.5. indicates that liver tissue had the lowest water content of the four tissues examined, and that this may increase in alloxan-diabetic sheep while it may decrease in the other tissues under these conditions.

2.3.3. The total lipid and water content of the livers of rats and sheep

2.3.3.1. Normal and alloxan-diabetic sheep and rats

The results are shown in Table 2.6. The alloxan-diabetic uncontrolled non-responders, uncontrolled responders and insulin withdrawn responders had a significantly ($P < 0.02$, $P < 0.01$ and $P < 0.05$ respectively) higher liver total lipid content than the group of normal sheep. The alloxan-diabetic insulin withdrawn responders had a significantly higher ($P < 0.05$) liver water content than the normal sheep. There were no significant differences between the various groups of alloxan-diabetic sheep.

The alloxan-diabetic rats had a significantly higher liver total lipid content ($P < 0.02$) and liver water content ($P < 0.01$) than the normal rats.

The liver total lipid content of the alloxan-diabetic uncontrolled responder wethers was about 2.9-fold higher than that of the normal sheep, and the corresponding figure for the rats was 1.5-fold.

TABLE 2.6.

The total lipid and water content of the livers of normal and alloxan-diabetic rats and sheep

Experimental treatment of the animals is described in sections 2.2.1.1. and 2.2.1.2. Tissues were collected, lipid extracted and analyzed as described in sections 2.2.2., 2.2.3. and 2.2.4. Results are means ± S.E.M. for the number of animals in parentheses.

Experimental group of animals	Water content mg/g dry	Lipid content fat-free tissue
Merino sheep		
Normal sheep* (5)	3,063 ± 37	227 ± 7
Alloxan-diabetic wethers		
Uncontrolled non-responders (3)	3,200 ± 71	307 ± 32
Uncontrolled responders (2)	3,371 ± 228	651 ± 186
Insulin treated responders (2)	3,319 ± 170	267 ± 30
Insulin withdrawn responders (5)	3,561 ± 182	418 ± 73
Hooded Wistar rats		
Normal rats (4)	2,801 ± 63	232 ± 16
Alloxan-diabetic rats (2)	3,583 ± 9	356 ± 28

* 2 rams and 3 wethers.

2.3.3.2. Wethers infused abomasally with L-methionine

Analysis of the results shown in Table 2.7. revealed no significant differences in liver water contents between the three groups of wethers. The wethers infused with 0.36 g met/kg body wt.$^{0.75}$/day had a significantly ($P < 0.05$) higher liver total lipid content than the control group of wethers. There were no other significant differences in liver total lipid contents between the three groups of wethers.

2.3.3.3. Pregnant-toxaemic ewes and neonatal lambs

There were no significant differences in the liver total lipid and water contents between the treated and untreated pregnant-toxaemic ewes shown in Table 2.7. Included in the group of treated pregnant-toxaemic ewes was ewe 10 which had a fatty liver in contrast to the other ewes in that group (refer to Appendix 1 for details of the individual animals). When the data for ewe 10 was excluded from the statistical analysis, or included with the group of untreated ewes, the group of untreated ewes had a significantly ($P < 0.001$) higher liver total lipid content than the treated ewes. The data for the single Corriedale ewe (Table 2.7.) was not included in the statistical analyses.

The neonatal lambs shown in Table 2.7. had a significantly ($P < 0.05$) higher liver water content and a significantly ($P < 0.001$) lower liver total lipid content than the group of untreated pregnant-toxaemic ewes. When the data for ewe 10 was excluded from the statistical analysis, the group of treated pregnant-toxaemic ewes (representing the dams of the neonatal lambs) had a significantly ($P < 0.01$) higher liver total lipid content than the

TABLE 2.7.

The total lipid and water content of the livers of methionine treated wethers, pregnant-toxaemic ewes, and neonatal lambs

Experimental treatment of the animals is described in sections 2.2.1.3., 2.2.1.4. and 2.2.1.5. Other details are as described in Table 2.6.

Experimental group of animals	Water content mg/g dry fat-free tissue	Lipid content mg/g dry fat-free tissue
Wethers infused abomasally with L-methionine		
0 g met/kg body wt.$^{0.75}$/day (4)	2,872 ± 23	186 ± 7
0.12 g met/kg body wt.$^{0.75}$/day (4)	2,900 ± 40	180 ± 12
0.36 g met/kg body wt.$^{0.75}$/day (4)	2,962 ± 116	211 ± 6
Pregnant-toxaemic ewes		
Untreated ewes, slaughtered at abattoirs (5)	2,979 ± 280	1,040 ± 61
Treated ewes, slaughtered at Waite (4)	3,393 ± 102	569 ± 215
A single Corriedale ewe	3,523	1,159
Neonatal lambs (4)	4,185 ± 351	116 ± 24

neonatal lambs. The liver water contents of the neonatal lambs and the treated ewes (with the data for ewe 10 excluded) did not differ significantly.

2.3.3.4. The relationship between water content and total lipid content of adult sheep liver

Fig. 2.8. shows the relationship between the water content (expressed as % of wet tissue wt.) and total lipid content (expressed per g wet tissue wt.) of the livers from a number of adult sheep under a variety of physiological conditions. There was a significant ($P < 0.001$) negative correlation and regression between the two measurements. The correlation coefficient (r) was -0.94, and details of the regression line are given in Fig. 2.8. The values for water contents expressed in this form do not indicate the true change in the water content of the livers under these physiological conditions (the true changes are given in sections 2.3.3.1., 2.3.3.2., and 2.3.3.3.), but the significance of this relationship was that it enabled an estimate of the liver total lipid content from the more readily measured water content.

2.3.3.5. Abattoirs sheep

The total lipid contents of fourteen sheep livers, obtained from the abattoirs and visually classified as fatty or non-fatty, were estimated from their measured water contents using the relationship obtained in section 2.3.3.4. The non-fatty livers were estimated to have had a total lipid content of 49 ± 8(7) mg/g wet tissue wt., and the fatty livers 146 ± 11(7) mg/g wet tissue wt. (means ± S.E.M. for the number of livers in parentheses). Two non-fatty livers (numbers 6 and 7 as shown in Appendix 4) had

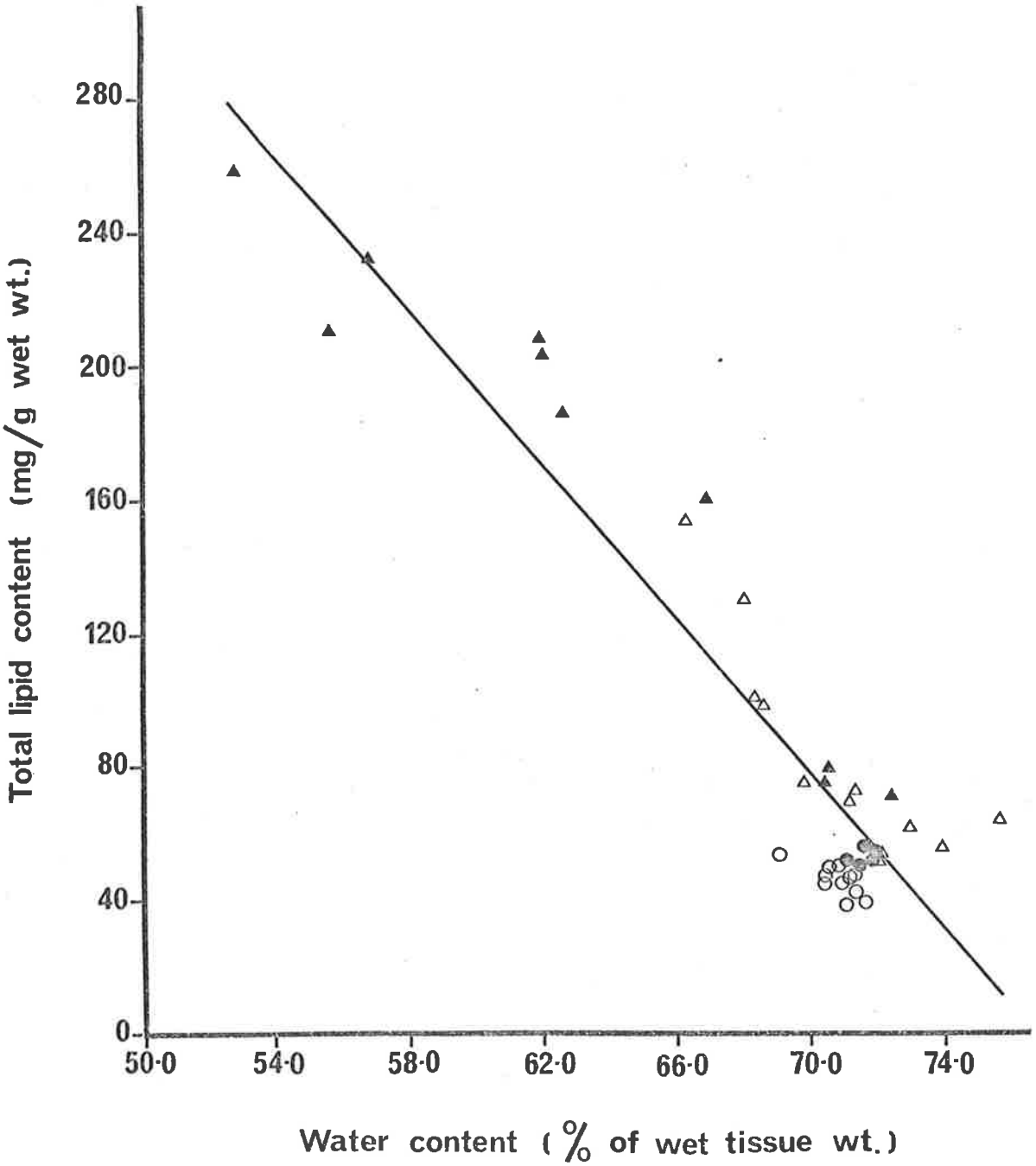
FIGURE 2.8.

The relationship between moisture content and total lipid content of adult sheep liver

The data was obtained from 39 adult sheep under a variety of physiological conditions. The total lipid and moisture contents were determined as described in section 2.2.4. The regression line, which exhibits the linear regression of total lipid content on moisture content was $y = 887.80 - 11.57x$. The coefficient of determination (r^2) was 0.87, and the regression coefficient (± standard error) of total lipid content on moisture content was -11.57 ± 0.73. The number in parentheses represents the number of animals in each group.

Key to figure:

- ▲ pregnant-toxaemic ewes (10)
- △ alloxan-diabetic wethers (12)
- ● normal wethers and rams (5)
- ○ wethers infused abomasally with L-methionine (12)


Total lipid content (mg/g wet wt.)
280
240
200
160
120
80
40
0
50·0
54·0
58·0
62·0
66·0
70·0
74·0
Water content (% of wet tissue wt.)

unrealistic total lipid contents when estimated in this manner. These results (except for livers 6 and 7) were used in Chapter 3 to express the analytical data obtained for these livers per g dry fat-free tissue.

2.3.4. The nature of the lipid accumulating in the livers of stressed sheep

Fig. 2.9. and Table 2.8. show that triacylglycerol was the major class of lipid accumulating in the fatty livers investigated in this thesis. These results are similar to those obtained by Read (1976) for a larger number of similarly stressed sheep. The total hepatic PtdCho and PtdEtn did not change markedly under these conditions. PtdCho and PtdEtn are the major phospholipids of sheep liver. Thus it appeared that the total phospholipids did not change markedly under these conditions, as similarly reported by Dryerre & Robertson (1941) for pregnant-toxaemic ewes, and Read (1976) for alloxan-diabetic wethers and pregnant-toxaemic ewes.

2.3.5. The total hepatic phospholipids of rats and sheep under a variety of physiological conditions

2.3.5.1. The total phospholipid content of rat and sheep liver

The results are shown in Table 2.9. There was no significant difference in total phospholipid content between the alloxan-diabetic insulin treated and insulin withdrawn responders. One wether (3-40, as shown in Appendix 1) had a markedly lower total phospholipid content than the other four wethers in the group of alloxan-diabetic insulin withdrawn responders. When the data for wether 3-40 was omitted from the statistical analysis,

FIGURE 2.9.

A thin-layer chromatogram of lipid standards and total hepatic lipids of sheep

The thin-layer was prepared, developed and visualized as described in Fig. 2.1. The wt. of lipid (µg) spotted is given in parentheses. The wt. of total lipid extract spotted for each sheep was equivalent to the lipid contained in 4 mg wet wt. of liver.

Key to the lanes spotted, from left to right:

1. 1,2-dipalmitoyl-*sn*-glycerol (20)
2. cholesterol (50)
3. stearic and palmitic acid mixture (50)
4. tripalmitoylglycerol (50)
5. methyl stearate (50)
6. cholesteryl palmitate (50)
7. mixture of 1 to 6 (200)
8. total lipid extract from a normal wether (210)
9. total lipid extract from an uncontrolled alloxan-diabetic responder wether (620)
10. total lipid extract from a pregnant-toxaemic ewe (1,030)

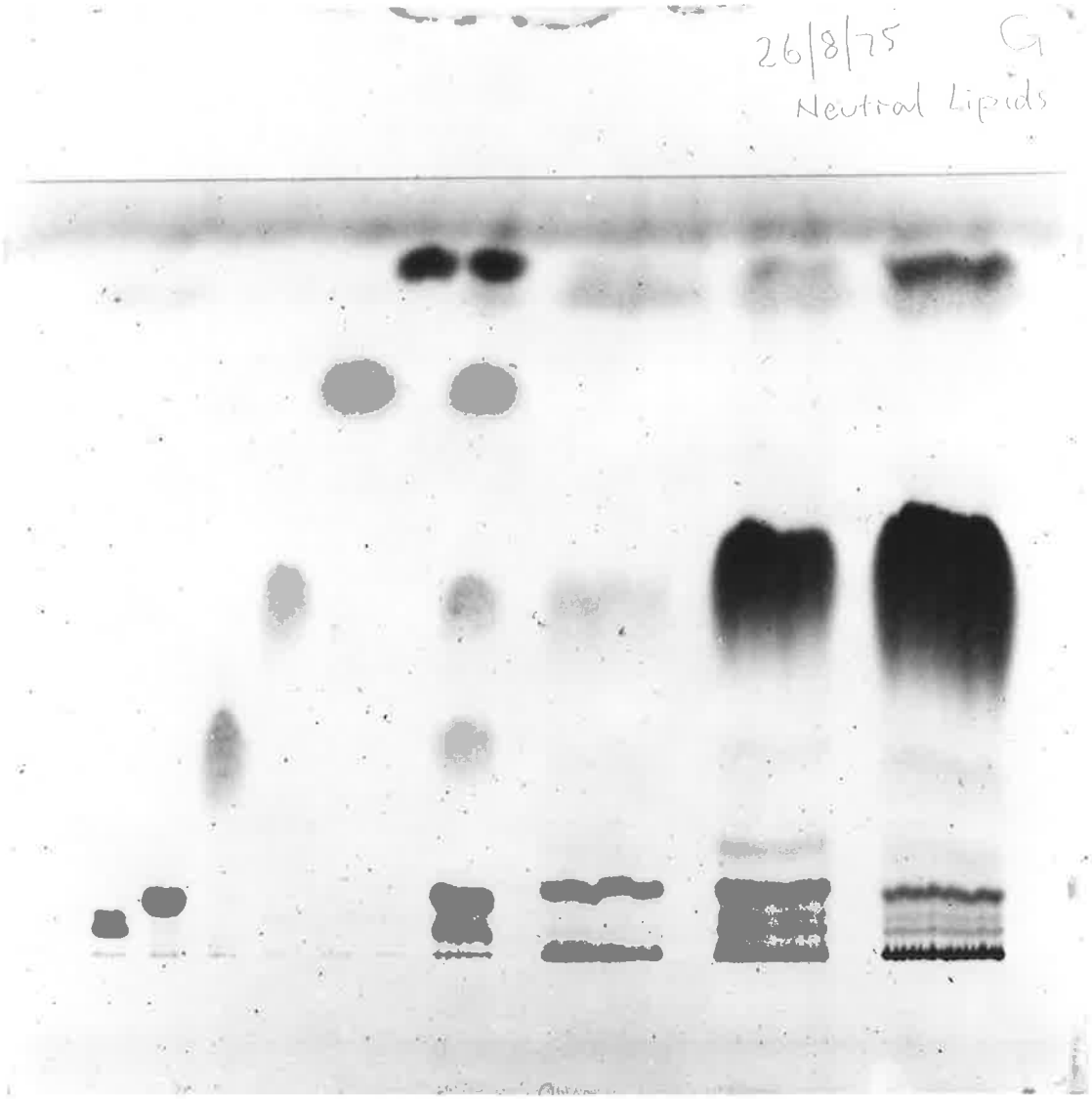

26/8/75
Neutral Lipids

TABLE 2.8.

The nature of the lipid accumulating in the livers of stressed sheep

The total hepatic lipid, triacylglycerols, PtdCho and PtdEtn contents were determined as described in sections 2.2.4., 2.2.5.3.1. and 2.2.5.4.1.2. The data represents a single animal for each physiological condition.

Physiological condition of the animal	Total lipid content		Triacylglycerols content*			ΣPtdCho, PtdEtn content†	
	mg/g dry fat-free tissue	fold increase	mg/g dry fat-free tissue	% of the total lipid	fold increase	mg/g dry fat-free tissue	% of the total lipid
A normal wether	218	1	25	11.5	1	126	58
An uncontrolled alloxan-diabetic responder wether	837	3.8	536	64	21.2	148	18
An untreated pregnant-toxaemic ewe	1,206	5.5	965	80	38.6	94	8

* mol. wt. assumed to be 885.

† mol. wt. of PtdCho and PtdEtn assumed to be 770 and 745 respectively.

TABLE 2.9.

The total phospholipid content of the livers of rats and sheep under a variety of physiological conditions

The total phospholipid contents were determined as described in section 2.2.6. Results are means ± S.E.M. for the number of animals in parentheses.

Experimental group of animals	Total phospholipid content µg phosphorus/g dry fat-free tissue
Alloxan-diabetic wethers	
Uncontrolled non-responder (1)	6,686
Insulin treated responders (2)	6,935 ± 27
Insulin withdrawn responders (5)	7,195 ± 350
Hooded Wistar rats	
Normal rats (3)	5,312 ± 206
Alloxan-diabetic rats (2)	6,181 ± 54
Wethers infused abomasally with L-methionine	
0 g met/kg body wt.$^{0.75}$/day (4)	5,256 ± 143
0.12 g met/kg body wt.$^{0.75}$/day (4)	4,810 ± 251
0.36 g met/kg body wt.$^{0.75}$/day (4)	5,050 ± 351

the liver total phospholipid content of the group of insulin withdrawn responders was significantly ($P < 0.02$) higher than that of the group of insulin treated responders. The single alloxan-diabetic uncontrolled non-responder (Table 2.9.) was not included in the statistical analysis.

The group of alloxan-diabetic rats (Table 2.9.) had a significantly ($P < 0.05$) higher liver total phospholipid content than the group of normal rats.

There were no significant differences in the liver total phospholipid contents between the three groups of wethers infused abomasally with various levels of L-methionine shown in Table 2.9.

2.3.5.2. The fatty acid composition of the total phospholipids of sheep liver

Table 2.10. shows that the percentage of oleic acid [18:1(9)] was higher, and the percentage of polyunsaturated fatty acids (> 18:3) lower, in the liver total phospholipids of the group of alloxan-diabetic uncontrolled responder wethers and untreated pregnant-toxaemic ewes compared to the group of normal wethers. The percentage of stearic acid (18:0) was higher in the liver total phospholipids of the group of untreated pregnant-toxaemic ewes compared to the group of normal wethers. The fatty acid differences may reflect differences in the fatty acid composition of PtdEtn and PtdCho, as these are the major phospholipids of sheep liver.

TABLE 2.10.

Fatty acid composition of the total phospholipids of sheep liver

(Courtesy of Ms. L.C. Read, Department of Agricultural Biochemistry, Waite Agricultural Research Institute)

Experimental treatment of the animals is described in sections 2.2.1.1., 2.2.1.2.1. and 2.2.1.3. Details of the analytical techniques are described by Read (1976). The fatty acids were grouped as shown for statistical analysis, which involved variance ratio tests. The diabetic non-responder was excluded from the analysis. Values represent means ± S.E.M. for the number of animals in parentheses.

Experimental group of animals	Fatty acid (area percentage)					
	< 16:0	16:0	18:0	18:1 (9)	Σ18:2, 18:3	> 18:3
Normal wethers (2)	1.08 ± 1.08	8.68 ± 0.94	19.55 ± 1.90	12.36 ± 0.67	11.25 ± 0.17	47.10 ± 1.50
Alloxan-diabetic wethers						
Uncontrolled non-responder (1)	3.00	5.47	16.78	17.42	13.13	43.91
Uncontrolled responders (2)	2.23 ± 0.16	9.36 ± 0.21	20.14 ± 1.43	20.76 ± 0.35 *	10.74 ± 1.24	34.98 ± 0.96 *
Untreated pregnant-toxaemic ewes (4)	1.59 ± 0.27	7.42 ± 0.98	23.78 ± 0.57 *	18.05 ± 3.65 **	12.37 ± 0.70	36.37 ± 4.11 **

* Significantly different from normal ($P < 0.1$) ** Significantly different from normal ($P < 0.05$)

2.3.5.3. The effect of abomasal infusion of L-methionine on several sheep liver phospholipids

The data for the individual animals is presented in Fig. 2.10. and Appendix 2. Three wethers, 53, 54 and 61 had a higher lyso-PtdCho plus Cer*P*Cho content than all the other wethers, and a lower PtdCho and PtdEtn content than the other wethers in their respective treatment groups. The results for these wethers were considered artifactual as each wether was in a different treatment group and consequently the results were unlikely to be due to treatment effects, and the total hepatic lyso-PtdCho plus Cer*P*Cho content in normal adult sheep is less than 10% of the total hepatic phospholipid content (Peters *et al.*, 1964; Noble *et al.*, 1971*a*). The artifactual results may have been due to acid hydrolysis of PtdCho and PtdEtn during the lipid extraction procedure owing to the possible presence of HCl in the extraction solvents (Schmid *et al.*, 1973).

When the data for wethers 53, 54 and 61 was omitted from the statistical analysis, the group of wethers infused with 0.12 g met/kg body wt.$^{0.75}$/day had a significantly ($P < 0.05$) lower PtdCho and total lipid choline content than the control group of wethers. There were no other significant differences between the three groups of wethers.

2.3.6. Total hepatic PtdCho and PtdEtn contents, and $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratios of sheep and rats

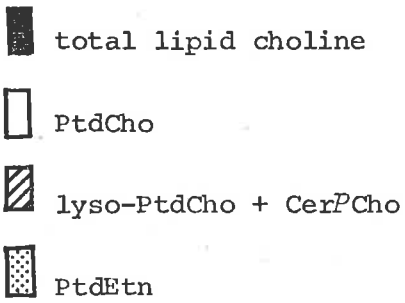
2.3.6.1. Preliminary observations with sheep

The data for the wether Al-567 shown in Table 2.11. was

FIGURE 2.10.

Liver phospholipid contents in methionine treated sheep

Experimental treatment of the wethers is described in section 2.2.1.5. The phospholipid classes were separated, identified, and quantitated as described in sections 2.2.5.2. and 2.2.5.4.1.2. Data for the individual animals is presented, and total lipid choline represents the sum of PtdCho, lyso-PtdCho and Cer*P*Cho.



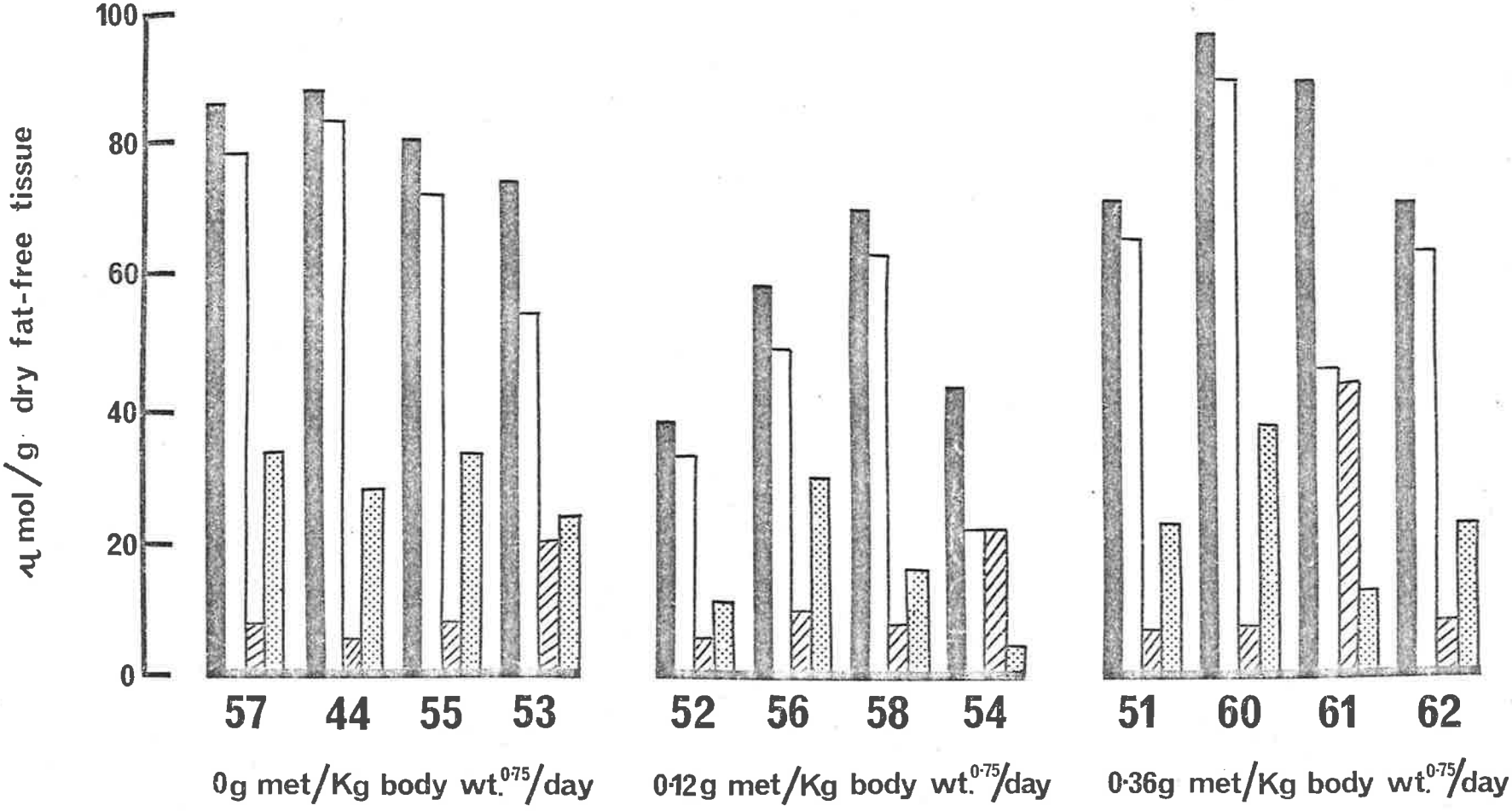

μ mol/g dry fat-free tissue
100
80
60
40
20
0
57 44 55 53
52 56 58 54
51 60 61 62
0g met/Kg body wt.0·75/day
0·12g met/Kg body wt.0·75/day
0·36g met/Kg body wt.0·75/day

TABLE 2.11.

Preliminary estimates of total hepatic PtdCho and PtdEtn contents and $\frac{PtdCho}{PtdEtn}$ *of sheep*

The experimental treatment and metabolic condition of Merino wether A1-567 is described by Snoswell & McIntosh (1974). The liver biopsy sample (before alloxan administration) was taken during surgery, following a 48 h fast. Details of the abattoirs sheep are given in section 2.2.1.6. Total hepatic PtdCho, PtdEtn, and phospholipid contents were determined as described in sections 2.2.5.4.1.2. and 2.2.6. Values for the abattoirs sheep are means, with the individual figures in parentheses.

Animal	Total phospholipid* content µg phosphorus/g wet wt.	PtdCho* µmol/g wet wt.	PtdEtn* µmol/g wet wt.	PtdCho / PtdEtn
Merino wether A1-567				
Before alloxan administration	1,750	27.7	13.7	2.02
10 days after alloxan administration	1,470	20.5	14.0	1.46
Sheep livers from abattoirs	1,445 (1,730, 1,160)	24.4 (25.1, 23.6)	11.7 (12.9, 10.5)	2.10 (1.95, 2.25)

* Results are expressed per g wet wt. as moisture and total lipid contents were not determined.

significant because it enabled a comparison between the normal and alloxan-diabetic condition using the same animal, thus avoiding between animal variation. Furthermore, this animal was the only severely alloxan-diabetic uncontrolled responder sheep studied in this work. Table 2.11. shows the $\frac{\text{PtdCho}}{\text{PtdEtn}}$ was lower in the alloxan-diabetic condition than the normal condition. This change in ratio may have been due to a decrease in PtdCho content, an increase in PtdEtn content, or both, as the liver was fatty 10 days after alloxan administration and the data was expressed per g wet tissue wt. An approximate estimate of the PtdCho and PtdEtn contents using dry fat-free tissue wt. as the base of reference suggested that the decrease in the ratio was due mainly to an increase in the PtdEtn content.

The data for the two abattoirs sheep shown in Table 2.11. demonstrates the variability in the sheep data and the desirability of using as uniform animals as possible in this work.

2.3.6.2. Normal and alloxan-diabetic sheep and rats

There were no significant differences in total hepatic PtdCho and PtdEtn contents between the normal sheep and the various groups of alloxan-diabetic wethers shown in Table 2.12. The $\frac{\text{PtdCho}}{\text{PtdEtn}}$ was significantly ($P < 0.01$) lower in the alloxan-diabetic uncontrolled responders compared to the alloxan-diabetic uncontrolled non-responders, probably due to the fall (though not significant) in PtdCho content. There were no other significant differences between the various groups of sheep. When the alloxan-diabetic sheep were grouped into those with a fatty liver (wethers 3-29, 3-70 and 3-59 shown in Appendix 1.) and those with

TABLE 2.12

Total hepatic PtdCho and PtdEtn contents and $\frac{PtdCho}{PtdEtn}$ of normal and alloxan-diabetic sheep and rats

PtdCho and PtdEtn were determined as described in section 2.2.5.4.1.2. Values represent means ± S.E.M. for the number of animals in parentheses.

Experimental group of animals	PtdCho	PtdEtn	$\frac{PtdCho}{PtdEtn}$
	µmol/g dry fat-free tissue		
Merino sheep			
Normal sheep* (3)	96.38 ± 9.07	52.63 ± 2.79	1.88 ± 0.10
Alloxan-diabetic wethers			
Uncontrolled non-responders (3)	121.97 ± 7.70	58.92 ± 2.33	2.07 ± 0.05
Uncontrolled responders (2)	97.74 ± 25.30	59.08 ± 12.04	1.58 ± 0.08
Insulin treated responders (2)	108.29 ± 5.44	58.84 ± 2.64	1.84 ± 0.01
Insulin withdrawn responders (5)	100.39 ± 10.09	58.48 ± 7.69	1.75 ± 0.12
Hooded Wistar rats			
Normal rats (3)	65.08 ± 5.07	30.69 ± 4.54	2.18 ± 0.22
Alloxan-diabetic rats (2)	80.99 ± 0.12	34.27 ± 9.27	2.50 ± 0.40

* two wethers and one ram.

a non-fatty liver (the remainder of the alloxan-diabetic wethers shown in Appendix 1.), there were no significant differences in PtdCho and PtdEtn contents, and $\frac{\text{PtdCho}}{\text{PtdEtn}}$ between the two groups of wethers.

There were no significant differences between the normal and alloxan-diabetic rats shown in Table 2.12.

2.3.6.3. Wethers infused abomasally with L-methionine

The results are shown in Table 2.13. The data for wethers 53, 54 and 61 (see Appendix 2.) was excluded from the statistical analysis for the reasons described in section 2.3.5.3. The wethers infused with 0.12 g met/kg body wt.$^{0.75}$/day had a significantly ($P < 0.05$) lower PtdCho content than the control group of wethers. There were no other significant differences between the three groups of wethers.

2.3.6.4. Pregnant-toxaemic ewes and neonatal lambs

There were no significant differences between the treated and untreated groups of pregnant-toxaemic ewes shown in Table 2.13. The data for the single Corriedale ewe (Table 2.13.) was excluded from the statistical analyses. When the pregnant-toxaemic ewes were grouped into those with a fatty liver (the untreated ewes plus ewe 10 shown in Appendix 1.) and those with a non-fatty liver (ewe 3 and ewe 8 in Appendix 1.), there were no significant differences in PtdCho and PtdEtn contents, and $\frac{\text{PtdCho}}{\text{PtdEtn}}$ between the two groups of ewes.

The group of neonatal lambs shown in Table 2.13. had a significantly ($P < 0.001$) lower PtdCho and PtdEtn content than the

TABLE 2.13.

Total hepatic PtdCho and PtdEtn contents and $\frac{PtdCho}{PtdEtn}$ *of methionine treated wethers, pregnant-toxaemic ewes, and neonatal lambs*

PtdCho and PtdEtn were determined as described in section 2.2.5.4.1.2. Values represent means ± S.E.M. for the number of animals in parentheses.

Experimental group of animals	PtdCho	PtdEtn	$\frac{PtdCho}{PtdEtn}$
	µmol/g dry fat-free tissue		
Wethers infused abomasally with L-methionine			
0 g met/kg body wt.$^{0.75}$/day (3)	78.02 ± 2.95	31.79 ± 1.81	2.48 ± 0.24
0.12 g met/kg body wt.$^{0.75}$/day (3)	48.49 ± 8.76	18.71 ± 5.62	2.92 ± 0.70
0.36 g met/kg body wt.$^{0.75}$/day (3)	72.77 ± 8.50	27.49 ± 4.84	2.70 ± 0.14
Pregnant-toxaemic ewes			
Untreated ewes, slaughtered at abattoirs (5)	85.06 ± 7.40	55.78 ± 3.08	1.51 ± 0.06
Treated ewes, slaughtered at Waite (4)	76.40 ± 8.94	55.62 ± 5.28	1.37 ± 0.05
A single Corriedale ewe	72.90	53.86	1.35
Neonatal lambs (4)	32.54 ± 6.22	22.59 ± 3.29	1.42 ± 0.08

group of untreated pregnant-toxaemic ewes, and a significantly ($P < 0.01$) lower PtdCho and PtdEtn content than the group of treated pregnant-toxaemic ewes. The $\frac{\text{PtdCho}}{\text{PtdEtn}}$ of the group of neonatal lambs did not differ significantly from either group of pregnant-toxaemic ewes, but was significantly ($P < 0.02$) lower than the ratio of the normal sheep shown in Table 2.12.

2.3.6.5. The apparent relationship between the total hepatic PtdCho and PtdEtn contents of adult sheep

Analysis of the PtdCho and PtdEtn data for the normal and alloxan-diabetic sheep, the pregnant-toxaemic ewes, and the wethers treated with L-methionine revealed a significant positive correlation and regression between the two measurements for each of the three groups of sheep. The data for the normal and alloxan-diabetic sheep and the methionine treated wethers has been pooled in Fig. 2.11. as there were no significant differences between the correlation and regression coefficients for these two groups of sheep. There was a significant ($P < 0.001$) positive correlation and regression between total hepatic PtdCho and PtdEtn. The correlation coefficient (r) was 0.90, and details of the regression line are given in Fig. 2.11.

2.3.6.6. Variability in the total hepatic $\frac{\text{PtdCho}}{\text{PtdEtn}}$ of sheep

The data in Table 2.14. suggests that breed, age and sex differences may influence the $\frac{\text{PtdCho}}{\text{PtdEtn}}$ of sheep liver, and reveals the need to select uniform animals when comparing ratios and the influence of physiological perturbations.

FIGURE 2.11.

The relationship between total hepatic PtdCho and PtdEtn content of adult sheep

The data was obtained from 24 adult sheep under a variety of physiological conditions. PtdCho and PtdEtn were determined as described in section 2.2.5.4.1.2. The regression line, which exhibits the linear regression of PtdCho content on PtdEtn content was $y = 31.515 + 1.286x$. The coefficient of determination (r^2) was 0.80, and the regression coefficient (± standard error) of PtdCho on PtdEtn was 1.286 ± 0.135. The number in parentheses represents the number of animals in each group.

Key to figure:

- ● normal wethers and ram (3)
- Δ alloxan-diabetic wethers (12)
- o wethers infused abomasally with L-methionine (9)

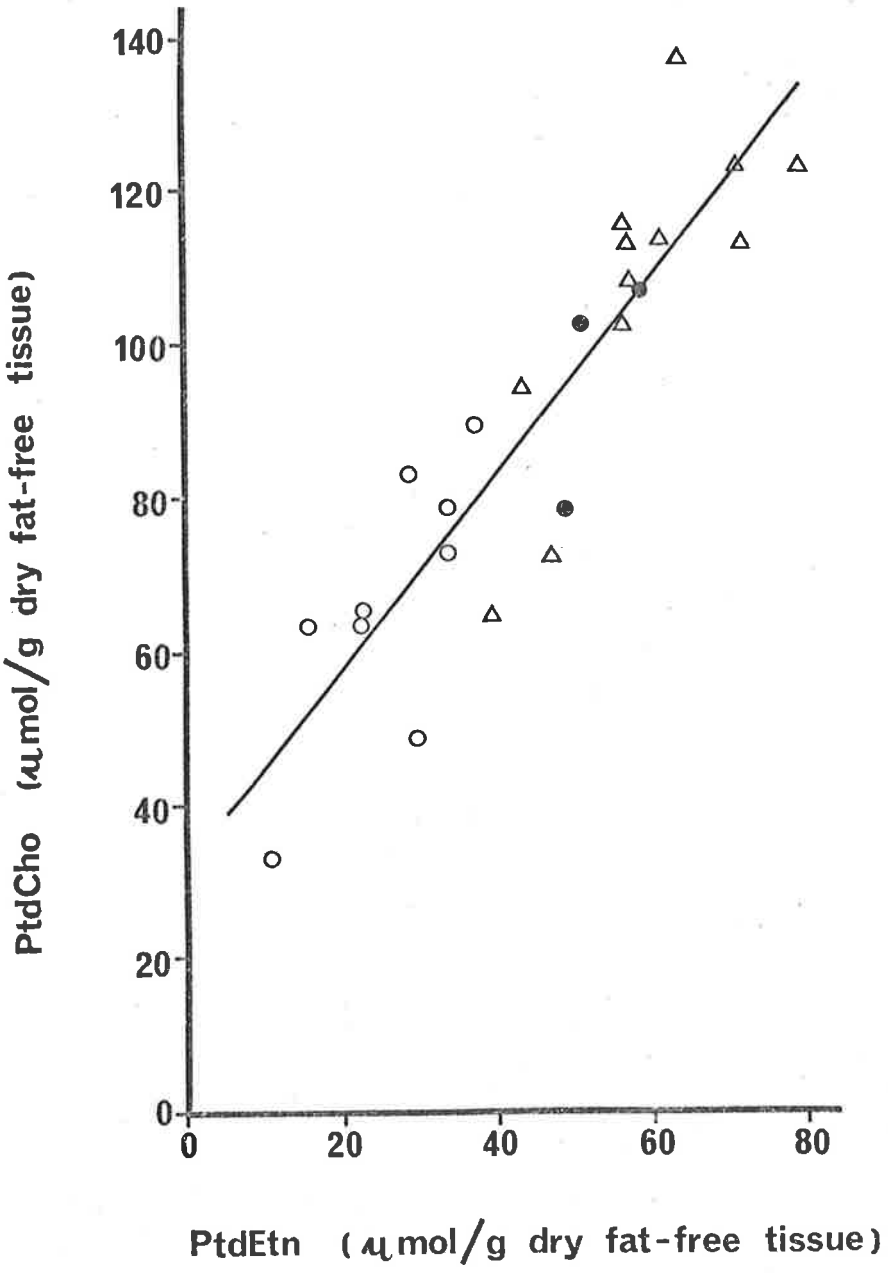

140
120
100
80
60
40
20
0
0
20
40
60
80
PtdCho (μmol/g dry fat-free tissue)
PtdEtn (μmol/g dry fat-free tissue)

TABLE 2.14.

Total hepatic $\frac{PtdCho}{PtdEtn}$ of sheep

$\frac{PtdCho}{PtdEtn}$ for several groups of sheep studied in this thesis have been summarized. Values are means ± S.E.M. for the number of animals in parentheses. Ratios calculated from data reported in the literature have been included for comparison.

Experimental group of animals	$\frac{PtdCho}{PtdEtn}$	Reference
Normal Merino ram (1)	2.01	This work
Normal Merino wethers* (3)	1.83 ± 0.12	This work
Normal Merino x Dorset wethers (3)	2.48 ± 0.24	This work
Normal Cheviot wethers (4)	2.10	Noble *et al.* (1971*a*)
Normal Romney and Romney x Southdown wethers and ewes (4)	1.64	Peters *et al.* (1964)
Abattoirs sheep (3)	3.61	Getz *et al.* (1968)
Abattoirs sheep (2)	2.10	This work
Pregnant-toxaemic, Merino crossbred ewes (9)	1.45 ± 0.04	This work
A 50 kg ewe (1)	2.07	Dawson (1960)
A pregnant, near term, Border Leicester x Merino ewe (1)	1.29	Scott *et al.* (1967)
Neonatal, Merino crossbred lambs (4)	1.42 ± 0.08	This work
Neonatal (0-8 days old) Cheviot lambs (13)	1.06	Noble *et al.* (1971*a*)
Foetal lambs, about 17 days prepartum (2)	2.12 (1.64, 2.60)	Scott *et al.* (1967)

* Includes the data for wethers 1 & 2 (shown in Appendix 1) and wether Al-567 before alloxan administration (see Table 2.11.).

The higher (though not significantly) ratio for the normal Merino x Dorset wethers compared to the normal Merino wethers appeared to be due to their significantly ($P < 0.01$) lower PtdEtn contents. The pregnant-toxaemic ewes shown in Table 2.14. had a significantly ($P < 0.01$) lower ratio than the normal Merino wethers, possibly due to a lower (though not significantly) PtdCho content.

The data for two apparently normal ewes reported in the literature are included in Table 2.14. for comparison to the pregnant-toxaemic ewes. The high ratio calculated from the data of Dawson (1960) compared to that calculated from the data of Scott *et al.* (1967) was due to the high hepatic PtdCho content reported by Dawson (1960). The pregnant-toxaemic ewes had lower PtdCho and PtdEtn contents than the ewes of Scott *et al.* (1967) and Dawson (1960).

The high ratio reported by Getz *et al.* (1968) was due to a low total hepatic PtdEtn content in their abattoirs sheep.

The neonatal lambs shown in Table 2.14. had lower ratios than the adult sheep, although the data of Scott *et al.* (1967) for two foetal lambs was at variance owing mainly to their relatively high PtdCho values compared to the neonatal lambs studied in this work.

2.3.7. Argentation t.l.c. of intact total hepatic PtdCho and PtdEtn of sheep

2.3.7.1. Separations obtained

2.3.7.1.1. PtdCho

An argentation thin-layer chromatogram of sheep liver

PtdCho is shown in Fig. 2.12. Sheep liver PtdCho separated into 5 or 6 subfractions visible by charring, under conditions in which rat liver PtdCho, chromatographed on the same thin-layer, separated into 3 subfractions. Arvidson (1965) resolved bovine liver PtdCho into 5 subfractions under conditions in which rat liver PtdCho separated into 3 subfractions.

2.3.7.1.2. PtdEtn

An argentation thin-layer chromatogram of sheep liver PtdEtn is shown in Fig. 2.12. Sheep liver PtdEtn separated into 5 or 6 subfractions visible by charring, under conditions in which rat liver PtdEtn, chromatographed on the same thin-layer, separated into 3 subfractions. A better resolution of intact PtdEtn subfractions may possibly be obtained by converting the PtdEtn into a non-polar form by dinitrophenylation and methylation before argentation t.l.c. (Renkonen, 1967). The presence of minor bands above the least unsaturated PtdEtn subfractions (those with R_F values similar to the calibration standard, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine) in Fig. 2.12. indicates impurities in the total hepatic PtdEtn preparations. Such impurities could be PtdSer, PtdGro, glycosphingolipids (see section 2.2.5.4.1.1.2.), or autoxidation products of PtdEtn (Lea, 1956). 4-Methyl-2,6-di-*tert*.-butylphenol was included in all PtdEtn preparations, but may not have prevented autoxidation as reported by Hopkins *et al.* (1968).

2.3.7.1.3. Tentative identification of the PtdCho and PtdEtn subfractions

A tentative identification of the sheep liver PtdCho

FIGURE 2.12.

Argentation thin-layer chromatograms of intact total hepatic PtdCho and PtdEtn of sheep

The thin-layer on the left of Fig. 2.12. resolved PtdCho subfractions, and that on the right, PtdEtn subfractions. Total hepatic PtdCho and PtdEtn were prepared from 3 sheep as described in section 2.2.5.4.2.1. The thin-layers were prepared as described in section 2.2.5.4.2.2., and activated at 195°C for 3 h. The total sample load per lane was 5 µg phosphorus. The PtdCho preparations were developed in chloroform/methanol/water (60:30:5, by vol.) and the PtdEtn preparations in chloroform/methanol/water (55:35:7, by vol.), and the subfractions visualized as described in section 2.2.5.4.2.4. The large dark areas near the apparent solvent fronts are not char spots, but the result of a reaction between the antioxidant in the samples and $AgNO_3$ (see section 2.2.5.4.2.4.).

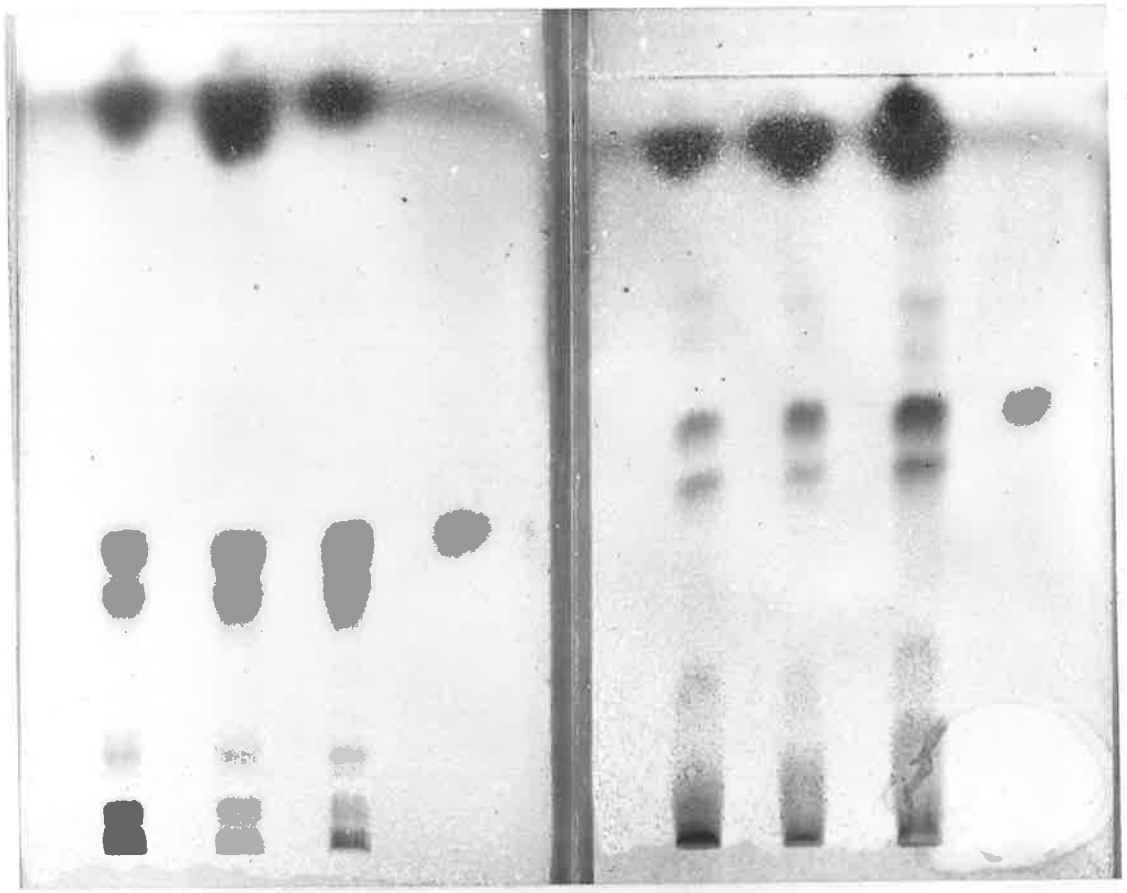
Key to the figure:

N a normal wether

D an uncontrolled alloxan-diabetic responder wether

P a pregnant-toxaemic ewe

S calibration standards, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine


N D P S
N D P S

and PtdEtn subfractions, based on their R_F values, R_F values of rat liver PtdCho and PtdEtn subfractions chromatographed on the same thin-layers, and comparison to R_F values calculated from the data of Arvidson (1965, 1968) is shown in Table 2.15. Positive identification would require g.l.c. analysis of the various subfractions.

2.3.7.2. Qualitative assessment of the PtdCho and PtdEtn subfractions of sheep under various physiological conditions

Visual assessment of the density of charring of the argentation thin-layer chromatograms (see Fig. 2.12. as an example) suggested that alloxan-diabetic uncontrolled responder wethers and untreated pregnant-toxaemic ewes had a lower proportion of the more unsaturated PtdCho and PtdEtn subfractions (those nearest the origin), and a higher proportion of the less unsaturated PtdCho and PtdEtn subfractions (those with R_F values similar to the saturated PtdCho and PtdEtn calibration standards shown in Fig. 2.12.) than normal wethers. A better comparison to the pregnant-toxaemic ewes would have been PtdCho and PtdEtn subfractions from normal pregnant or non-pregnant ewes.

2.4. Discussion

2.4.1. Partial characterization of the liver lipids of sheep

Fatty livers generally are larger and heavier than non-fatty livers. Lucas & Ridout (1967) showed that water and lipid accumulation accounted for the major component of weight increase in the fatty livers of choline-deficient rats. Calculations from

TABLE 2.15.

Argentation t.l.c. of intact total hepatic PtdCho and PtdEtn of sheep and rats: tentative identification of subfractions

Argentation t.l.c. of PtdCho and PtdEtn from rats and sheep was performed as described in section 2.2.5.4.2. R_F values for the subfractions separated are compared to R_F values calculated for g.l.c. identified subfractions from the data of Arvidson (1965, 1968). R_F values represent the means for 2 rats and 3 sheep developed on the same thin-layer.

S, 1,2-dipalmitoyl-*sn*-glycero-3-(phosphocholine or phosphoethanolamine); N.D., not detected.

Phospholipid		PtdCho								PtdEtn	
t.l.c. conditions		175°C/5h chloroform/methanol/water (65:25:4, by vol.)		195°C/3h chloroform/methanol/water (60:30:5, by vol.)						195°C/3h chloroform/methanol/water (55:35:7, by vol.)	
Reference		Arvidson (1965)	This work			Arvidson (1968)	This work			Arvidson (1968)	This work
Animal		rat	bovine	rat	sheep	rat	rat		sheep	rat	rat	sheep
Spot number	Subfraction	R_F										
Solvent front												
	S			0.49	0.49		0.61	0.61		0.76	0.76
1	*monoenoic*	0.53 *	0.55 *	0.46 †	0.48 †	0.57	0.60 †	0.60	0.67	0.77 †	0.74 †
2	*dienoic*					0.51		0.56	0.62		
3	*trienoic*	N.D.	0.47			N.D.		0.35	N.D.		0.55
4	*tetraenoic*	0.33	0.33	0.29	0.32	0.21	0.30	0.25	0.22	0.39	0.39
5	*pentaenoic*	N.D.	0.21		0.22	N.D.		0.13	N.D.		0.29
6	*hexaenoic*	0.13	0.14	0.10	0.14	0.08	0.05	0.05	0.11	0.16	0.17
origin											

* The *monoenoic* and *dienoic* subfractions did not separate.

† Speculating that the observed single spot consisted of two subfractions that did not separate.

the data of Smith *et al.* (1974) indicate that this also applies to the fatty livers associated with cobalt deficiency in sheep. The present work revealed a negative relationship between the water content and total lipid content (both expressed per unit wet tissue wt.) of livers from adult sheep under various physiological conditions which gave the impression that lipid replaced water in the fatty livers (see Fig. 2.8.). Ferguson (1954) indicated this to be so in pregnant ewes subjected to various dietary treatments. However, Dryerre & Robertson (1941) observed a similar negative relationship ($r = -0.82$) between the water and total lipid content (both expressed per unit wet tissue wt.) in pregnant and non-pregnant ewes and considered there was an increase in liver weight without an increase in water content and hence the water content appeared to decline. The results reported in this thesis suggest the base of reference used to calculate tissue metabolite contents may be especially important when fatty and non-fatty livers are compared. Reid (1973) observed a significant increase in the volume of hepatocytes in 6-day-fasted lactating cows with fatty livers compared to normal cows, but subsequently Reid *et al.* (1977*a*,*b*) reported a significant decrease in the volume of hepatocytes in 6-day-fasted lactating and non-lactating cows with fatty livers compared to normal cows. Assuming that hypertrophy rather than hyperplasia of hepatocytes was associated with the probable increase in weight of the fatty sheep livers investigated in the present work, each unit weight of fatty liver would contain fewer hepatocytes than each unit weight of non-fatty liver. DNA or total phospholipid content would probably provide the best base of reference for comparing analytical data from fatty and non-fatty livers. These were not measured however, and therefore dry

fat-free tissue weight was considered a better reference base than wet tissue weight as this removed the two major components of the weight change in the fatty livers. The dry fat-free tissue consists mainly of protein [about 150 mg/g wet tissue wt. for non-fatty sheep liver (Smith *et al.*, 1974)] and to a lesser extent, glycogen [about 20 mg/g wet tissue wt. for normal sheep liver (Ballard & Oliver, 1965)] and nucleic acids [about 10 mg/g wet tissue wt. for normal sheep liver (Masters, 1963)]. The sheep liver appears more resistant than the rat liver to glycogen depletion upon fasting (Filsell *et al.*, 1969), and consequently dry fat-free tissue wt. per sheep liver may not vary markedly in fatty and non-fatty sheep livers and therefore provide a reliable base of reference for analytical data.

The present work indicated that the liver and to a lesser extent the kidney cortex were the main tissues of the four studied (liver, kidney cortex, heart and skeletal muscle as shown in Table 2.4.) which had elevated total lipid contents in alloxan-diabetic uncontrolled responder sheep compared to normal and alloxan-diabetic uncontrolled non-responder sheep. Dryerre & Robertson (1941) reported relatively small changes in the total lipid content of whole kidneys from pregnant and pregnant-toxaemic ewes compared to non-pregnant ewes. Histological studies showed that if fat did occur, it appeared mainly in the cells of the kidney cortex. The uptake of plasma fatty acids by these four tissues is a function of the fatty acid concentration in the blood, the rate of blood flow through the tissue and the avidity of the tissue for fatty acids (Scow & Chernick, 1970). Consequently, the uptake of fatty acids by these four tissues probably increased in

the alloxan-diabetic uncontrolled responder wethers compared to the other sheep. Apparently, the liver and kidney cortex had less capacity to oxidize the increased uptake of fatty acids than the muscle tissues under these conditions, and hence increased esterification with concomitant elevated lipid levels resulted.

The lipid content in the liver, heart and skeletal muscle (M. *gastrocnemius*) of alloxan-diabetic rats is elevated compared to normal rats (Denton & Randle, 1967; van Harken *et al.*, 1967), due mainly to increased levels of triacylglycerols. The increase in total lipid content of the livers from alloxan-diabetic rats was relatively less than that for the alloxan-diabetic uncontrolled responder wethers in the present work (see Table 2.6.). Triacylglycerols were the major lipid class accumulating in the livers of alloxan-diabetic responder and pregnant-toxaemic sheep. The relatively larger accumulation of total lipid in the alloxan-diabetic responder sheep compared to the alloxan-diabetic rats may be associated with the unique structure of the hepatic sinusoid of sheep (David, 1964; Grubb & Jones, 1971; Gemmell & Heath, 1972) (see Fig. 2 in the Literature Review) and the apparent inability of sheep liver to synthesize significant quantities of VLDL as evidenced by the relatively low level of this lipoprotein in sheep plasma (Mills & Taylaur, 1971; Nelson, 1973; Kubasek *et al.*, 1974; Leat *et al.*, 1976). Jarrett *et al.* (1974) reported that blood triacylglycerols concentration increased nearly 4-fold in alloxan-diabetic responder sheep, which may indicate increased VLDL synthesis by the liver under these conditions or alternatively their decreased removal from the blood by extrahepatic tissues.

The results in Table 2.9. suggested the total hepatic phospholipids may have increased in alloxan-diabetic rats compared to normal rats and in alloxan-diabetic insulin stabilized responder wethers which were subsequently withdrawn from insulin for 24 h compared to the insulin stabilized wethers. These results conflict with the data of van Harken *et al.* (1967) and Meier *et al.* (1972) for alloxan-diabetic rats and with the data of Read (1976) for the alloxan-diabetic wethers. Generally, total hepatic phospholipids are prone to relatively little change in content under a variety of physiological conditions such as fasting in cows (Brumby *et al.*, 1975; Reid *et al.*, 1977), pregnancy and lactation in ewes (Smith & Walsh, 1975), alloxan-diabetes in goats (Schwalm & Schultz, 1976), and pregnancy toxaemia in ewes (Dryerre & Robertson, 1941). Almost invariably, these results use wet tissue wt. as the base of reference for the analytical data, and it would be instructive to recalculate the data using dry fat-free tissue wt. as the base of reference, especially where very fatty livers were investigated. However, regardless of the base of reference used, it is likely that total hepatic phospholipid contents do not change markedly under many different physiological conditions.

The wethers infused abomasally with 0.36 g met/kg body wt.$^{0.75}$/day had a slightly elevated liver total lipid content compared to the control group of wethers (see Table 2.7.), and therefore appear similar to rats fed excessive quantities of dietary methionine where small increases in the liver total lipid occur (Roth *et al.*, 1950; Klain *et al.*, 1963) due mainly to increased levels of neutral lipids (Klain *et al.*, 1963). The physiological significance of this lipid accumulation is unknown,

but may represent a toxic effect of the methionine with subsequent increased mobilization of adipose tissue triacylglycerols. Total hepatic phospholipids did not change in these wethers, as similarly reported for methionine fed rats (Klain *et al.*, 1963). The significance of the depressed total hepatic PtdCho and total lipid choline content of the group of wethers infused with 0.12 g met/kg body wt.$^{0.75}$/day (refer to Fig. 2.10.) compared to the control group of wethers is obscure, particularly in view of the lack of effect in the livers of wethers infused with the higher level of methionine.

2.4.2. The possible relationship between the fatty livers of sheep and choline metabolism

The limited histological studies in the present work confirmed the concept that sheep are particularly prone to the development of fatty livers, but gave no indication of the possible cause for the lipid accumulation in the liver acini in the sense that no specific pattern of fat accumulation was evident. Manns (1972) reported neutral fat in ordinary frozen liver sections from 24 h fasted sheep was mainly restricted to hepatocytes near the terminal hepatic venules (refer to Fig. 1 in the Literature Review for the structure of the simple liver acinus), but that in fresh-frozen sections, fat was evenly distributed throughout the acini of 24 h fasted sheep. Hartroft (1950) described lipid accumulation in the liver acini of rats fed a choline-deficient diet for 24 h as restricted mainly to those hepatocytes near the terminal hepatic venules. The possibility exists therefore that the initial accumulation of lipid in the liver acini of 24 h fasted sheep is related to a deficiency of choline. The sheep derives little

choline from the diet (Neill & Dawson, 1977), and fasting may reduce this even further. The accumulation of lipid in the hepatocytes in the vicinity of the terminal hepatic venules in choline deficiency may be related to the fact that these cells receive blood after those cells nearer the portal space (refer to Fig. 1 in the Literature Review for the blood flow pattern in the simple liver acinus). Fasted rats (which therefore receive no dietary choline) generally do not develop fatty livers (Harrison, 1953) in sharp contrast to sheep. Since a 4-day fast for sheep is similar to a 12-14 h post absorptive fast for rats and would only result in moderate stress, the lipid accumulation in the fasted sheep may have a basic mechanism similar to that for choline-deficient rats. The accumulation of lipid around the portal space of the liver acini in the fatty livers of pregnant and pregnant-toxaemic ewes (Snook, 1939; Dryerre & Robertson, 1941; Patterson *et al.*, 1964) may indicate that an amino acid or protein deficiency is the dominant mechanism underlying the lipid accumulation in very fatty livers in sheep. This may be related to an inability to synthesize specific apoproteins for VLDL synthesis in sheep.

The derangement of membrane structures apparent in the alloxan-diabetic responder ewe liver section (see Fig. 2.7.) and in alloxan-diabetic rat livers (Harano *et al.*, 1972; Reaven *et al.*, 1973) does not appear to be associated with any major change in the total hepatic phospholipids under these conditions (see Table 2.9.). The limited data in the present work suggests that the total hepatic phospholipids may increase to some extent in alloxan-diabetic responder sheep and rats. The possibility exists that changes in molecular subclasses of PtdCho and PtdEtn

had occurred under these conditions. A characteristic finding in choline-deficient rats is a lowered total hepatic $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratio due to a lowered PtdCho content and an increased PtdEtn content (Blumenstein, 1968; Chalvardjian, 1970; Lyman *et al.*, 1973, 1975). This change in ratio has been interpreted as evidence of impaired methylation of PtdEtn to PtdCho, the only known pathway for choline biosynthesis in the rat. The present work showed total hepatic $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratios decreased in alloxan-diabetic uncontrolled responder wethers (see Table 2.12.) compared to alloxan-diabetic uncontrolled non-responder wethers; however the limited data and considerable between animal variation made it difficult to determine if the observed decrease was due to an elevated PtdEtn content or a decreased PtdCho content or both. The significant positive relationship between total hepatic PtdCho and PtdEtn contents for adult sheep under a variety of physiological conditions (see Fig. 2.11.) may indicate that the metabolic pathways of PtdCho and PtdEtn are under a common control mechanism, perhaps *via* 1,2-diacyl-*sn*-glycerols in the CDP-Cho and CDP-Etn pathways. The rate of PtdCho biosynthesis *via* the methylation pathway, expressed as a percentage of the rate of PtdCho biosynthesis *via* both the methylation and CDP-Cho pathways is in the range 3.4%-30% in male rats (Wise & Elwyn, 1965; Bjørnstad & Bremer, 1966; Trewhella & Collins, 1972; Sundler & Åkesson, 1975). Consequently, changes in the methylation pathway may not be readily detected by measuring total hepatic $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratios. Furthermore, the data in Table 2.14. indicates that these ratios are extremely variable in sheep and caution must be exercised when making comparisons between different groups of animals.

The limited data obtained in the present work on argentation t.l.c. of intact total hepatic PtdCho and PtdEtn suggested the relative proportions of the polyenoic classes (> 4 double bonds) of PtdCho and PtdEtn were depressed in alloxan-diabetic uncontrolled responder wethers and untreated pregnant-toxaemic ewes compared to normal wethers (see Fig. 2.12.). These observations correlate with the g.l.c. data on the total hepatic phospholipids where the relative proportions of polyunsaturated fatty acids (> 18:3) were lower in these stressed sheep compared to normal wethers (see Table 2.10.). The concomitant apparent increase in the relative proportions of the more saturated classes of PtdCho and PtdEtn (see Fig. 2.12.) may help to explain the relatively few significant changes observed in total hepatic PtdCho and PtdEtn contents between the various experimental groups of sheep. These qualitative observations also reinforce the concept (shown in Fig. 2.11.) that parallel changes occur in total hepatic PtdCho and PtdEtn of sheep under a variety of physiological conditions.

The results presented here are somewhat preliminary, and definitive g.l.c. studies are required to identify the molecular subclasses of PtdCho and PtdEtn and to quantitate their relative proportions. Lyman *et al.* (1973) observed elevated levels of hexaenoic PtdEtn subclasses in choline-deficient rat livers and attributed this to impaired methylation of PtdEtn to PtdCho. The parallel changes in total hepatic PtdCho and PtdEtn in sheep under the physiological conditions reported in the present work tend to suggest that the mechanism of the observed changes may be different from that in choline-deficient rats.

CHAPTER 3. A PARTIAL CHARACTERIZATION OF METHYLATED METABOLITES IN THE LIVERS OF SHEEP UNDER SEVERAL PHYSIOLOGICAL CONDITIONS

3.1. Introduction

The pathways of choline and carnitine biosynthesis in sheep liver may compete for the common substrate L-methionine under conditions of metabolic stress, as indicated in the Literature Review and General Introduction. This possibility was investigated in the work reported in this chapter and involved the measurement of total hepatic carnitine and PtdCho contents in sheep under various physiological conditions. An attempt was made to estimate the *sn*-glycero-3-phosphocholine content of sheep liver in view of the relatively high liver content of this metabolite in lambs (Schmidt *et al.*, 1952, 1955).

The quantitative importance of creatine in mammalian methyl group metabolism (Mudd & Poole, 1975) required that some aspects of creatine metabolism be investigated concurrently. This involved measurements of tissue creatine contents, urinary creatinine and creatine excretion, and some studies of guanidinoacetate methyltransferase activity in liver tissue.

The important role of methionine adenosyltransferase in the synthesis of *S*-adenosylmethionine, the immediate methyl donor for choline, carnitine and creatine biosynthesis, required that some studies were made on the liver activity of this enzyme in sheep under various physiological conditions.

Several of the aforementioned studies were extended to the tissues of rats for comparative purposes, and to the tissues of

neonatal lambs to investigate the effect of age on the tissue levels of these metabolites.

3.2. Methods and Materials

3.2.1. Animals

Lambs born to normal, pasture grazed Merino ewes were slaughtered at the Mortlock Experimental Station, South Australia, and tissue samples were collected. The ages of the lambs were < 12 h (1 lamb), 24 h (2 lambs), and 72 h (1 lamb). The abomasums of the lambs were full of milk.

Except where specified, all other animals were as described in Chapter 2.

3.2.2. Tissue collection and storage

Tissues were collected and stored as described in Chapter 2. Sheep urine was collected daily into plastic bottles containing 500 ml (100 ml would have been sufficient) of perchloric acid (50 g/l) as preservative. Blood was collected from the jugular vein of sheep, and 2 ml added to 2 ml of perchloric acid (150 g/l).

Liver tissue used for *in vitro* enzyme assays was collected into ice-cold solutions of the homogenizing media, or placed in crushed ice until the enzyme extracts were prepared.

3.2.3. Tissue extractions

3.2.3.1. Perchloric acid and trichloroacetic acid extraction

About 1 g of powdered tissue was weighed into a centrifuge tube precooled in liquid N_2, and homogenized in 3 ml of either acid solution (100 g/l) using a Polytron PT 10 homogenizer.

The probe was rinsed with a further 1 ml of acid, the homogenate mixed on a vortex mixer and centrifuged for 30 min at 4°C and 12,000 *g*. The supernatant was decanted, its volume recorded, and stored at -14°C until analyzed.

The trichloroacetic acid was subsequently removed by five successive extractions with an equal volume of diethyl ether. Perchloric acid was removed by neutralization with potassium hydroxide, cooling, and centrifugation at 4°C.

3.2.3.2. Alkali extraction of liver tissue

About 0.5 g of powdered tissue was weighed into a centrifuge tube precooled in liquid N_2, and homogenized in 4 ml of potassium hydroxide (0.1 mol/l) containing EDTA (1 mmol/l) using a Polytron PT 10 homogenizer. The probe was rinsed with a further 1 ml of potassium hydroxide solution and the homogenate mixed on a vortex mixer. The homogenate was incubated at 50°C for 4 h, cooled to 0°C, acidified with 70 µl of perchloric acid (500 g/l), and centrifuged as described in section 3.2.3.1. The supernatant was decanted, the volume recorded, and stored at -14°C until analyzed.

No loss of carnitine occurs during this procedure (Brooks & McIntosh, 1975).

3.2.4. Metabolite determinations

All assays involved the use of an Eppendorf Spectrum line photometer, fitted with an automatic cell changer and recorder (Eppendorf Gerätebau, Netheler und Hinz G.m.b.H., Hamburg, Germany) or a Zeiss PMQII spectrophotometer fitted with an automatic sample changer and TE converter (Carl Zeiss, Oberkocken, Germany) connected

to a Rikadenki model B-16 electronic recorder (Rikadenki Kogyo Co., Ltd., Tokyo, Japan). Both instruments were fitted with a temperature controlled cell holder.

3.2.4.1. L-carnitine

L-carnitine was determined using the enzymatic assay of Marquis & Fritz (1964). When 4,4'-dithiodipyridine (4-PDS) was used instead of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), the pH was 7.2, the wavelength 324 nm, and the concentration of L-carnitine calculated assuming an extinction coefficient of 1.98×10^4 cm^2/mol for 4-thiopyridone (4-TP) (Grassetti & Murray, 1967). 4-PDS is superior to DTNB because it does not inactivate carnitine acetyltransferase and is more sensitive (Ramsay & Tubbs, 1975).

Total acid-soluble carnitine fractions were prepared for L-carnitine assay by the method of Tubbs *et al.* (1965). Perchloric acid extracts of whole blood, and the liver total carnitine extracts were prepared for L-carnitine assay by neutralization.

3.2.4.2. Creatinine

In acid solutions, the formation of creatinine from creatine and phosphocreatine is complete (Cannan & Shore, 1928; Cantoni & Vignos, 1954). As the acid extracts of the tissues and urine had been stored at $-14^{o}C$ for up to 12 months, the formation of creatinine would have been complete. It has been assumed that the creatinine was stable during the storage period, as in the frozen state, creatinine in untreated serum, plasma and urine is stable indefinitely (Wahlefeld *et al.*, 1974).

The procedure of Wahlefeld *et al.* (1974) was mainly followed for the determination of creatinine. The final assay volume was 1 ml, and 150 nkat of creatine kinase and 30 nkat of creatininase were added to each cuvette simultaneously, and the decrease in optical density at 340 nm followed for 40-60 min until the optical density changes became linear with respect to time. The pyruvate kinase and lactate dehydrogenase used were crystalline suspensions in ammonium sulphate solution, and consequently the glycine/phosphate buffer could not be used in the assay, with the result that the equilibrium 'creep' in optical density at 30°C was extensive (0.022 optical density units/10 min).

Under these conditions, the recovery of creatinine over the range of cuvette concentrations 10-60 µmol/l was 86 ± 1% (mean ± S.E.M. for 15 assays), due to the low levels of creatine kinase and creatininase used (Wahlefeld *et al.*, 1974). The results reported in this thesis have been corrected for this low recovery.

The reaction between creatine kinase and guanidinoacetic acid (see section 3.2.4.3.) does not affect the assay because of the very low levels of guanidinoacetic acid in mammalian liver (Rosenberg, 1959).

3.2.4.3. Creatine

During the investigation of guanidinoacetate methyltransferase activities in liver tissue from rats and sheep (see section 3.2.5.2.), it was necessary to measure creatine in the presence of high levels of guanidinoacetic acid. Creatine was measured in the neutralized acid extracts of the enzyme incubations using the assay system for creatinine (see section 3.2.4.2.) except

that creatininase was omitted from the assay.

The accuracy of the enzymatic assay for creatine depends on the substrate specificity of creatine kinase. This enzyme reacts with guanidinoacetic acid, but at substrate concentrations of 2.4 mmol/l, the rate of the reaction is 240 times greater with creatine than guanidinoacetic acid (Tanzer & Gilvarg, 1959). Tanzer & Gilvarg (1959) found under their conditions of assay, a molar ratio of guanidinoacetic acid/creatine of 1:1 and 10:1 resulted in an overestimation of 2% and 17% respectively for creatine.

The molar ratios of guanidinoacetic acid/creatine in this work varied between 50:1 and 100:1, and consequently the spectrophotometric assays for creatine had an extensive equilibrium "creep" in optical density, as shown in Fig. 3.1. Minus-tissue controls for the guanidinoacetate methyltransferase incubations (see section 3.2.5.2.) were processed and assayed for creatine in the same manner as the test and zero-time incubations. Changes in optical density due to the presence of guanidinoacetic acid were corrected for as shown in Fig. 3.1.

The validity of this procedure was checked using an internal creatine standard. The recovery of creatine by this assay procedure was 86%, as found for creatinine (see section 3.2.4.2.), and the results have been corrected for this low recovery.

3.2.4.4. *sn*-Glycero-3-phosphocholine

The Gro*P*Cho content of sheep liver was estimated by measuring *sn*-glycerol-3-*P* in the liver acid extracts (prepared as

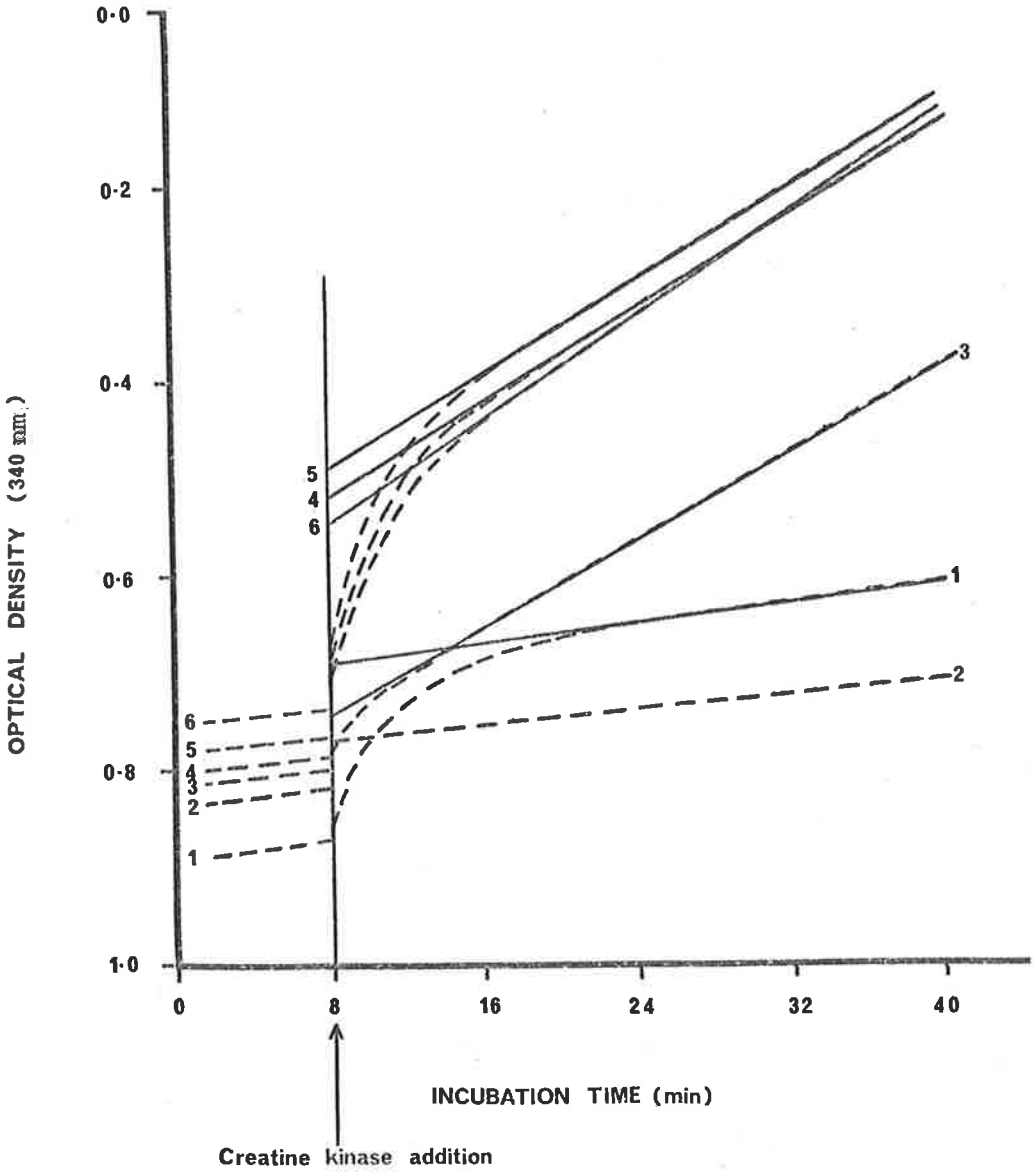
FIGURE 3.1.

The enzymatic estimation of creatine in the presence of high levels of guanidinoacetic acid

The assays were performed as described in section 3.2.4.3. Each dashed line represents the change in optical density for a single cuvette. The chart speed was 0.5 cm/min, and each cuvette was monitored for 8 s/cycle.

Key to the figure:

1. Cuvette containing creatine (20 µmol/l).
2. Cuvette containing water.
3. Cuvette containing guanidinoacetic acid (1,400 µmol/l).
4. & 5. Cuvettes containing creatine (about 26 µmol/l) and guanidinoacetic acid (about 1,374 µmol/l), representing duplicate assays for creatine in aliquots (160 µl) of the neutralized acid extracts of guanidinoacetate methyltransferase incubations for a normal sheep liver (see section 3.2.5.2.).
6. Cuvette containing creatine (about 19 µmol/l) and guanidinoacetic acid (1,400 µmol/l), representing the zero-time control for the normal sheep liver guanidinoacetate methyltransferase incubations.


0·0
0·2
0·4
0·6
0·8
1·0
OPTICAL DENSITY (340 nm)
0
8
16
24
32
40
INCUBATION TIME (min)
Creatine kinase addition
1
2
3
4
5
6

described in section 3.2.3.1.) after acid hydrolysis (1 mol/l-HCl) at 100°C for 20 min, as esters of *sn*-glycerol-3-*P* are quantitatively hydrolyzed under these conditions (Schmidt *et al.*, 1952). Acid hydrolysis of phospholipids may have released some *sn*-glycerol-3-*P* esters during preparation of the liver extracts (Ansell & Norman, 1953; Dawson, 1955). *sn*-Glycerol-3-*P* was determined in the neutralized hydrolyzates as described by Michal & Lang (1974).

The recovery of *sn*-glycerol-3-*P* from Gro*P*Cho under the conditions of hydrolysis was 42%, probably due to the formation of *sn*-glycerol-2-*P* and *sn*-glycerol-1-*P* (Schmidt *et al.*, 1955), as *sn*-glycerol 3-phosphate dehydrogenase (NAD^+) is specific for *sn*-glycerol-3-*P* (Michal & Lang, 1974).

The *sn*-glycerol-3-*P* content of sheep liver is about 0.14 µmol/g wet tissue wt. (Herriman *et al.*, 1976), and this may show marked change in ischaemia (Michal & Lang, 1974). The *sn*-glycerol-3-*P* content of the liver extracts was not determined before the acid hydrolysis procedure as the tissues were not freeze-clamped at the time of slaughter and the acid extracts had been stored at -14°C for up to 12 months.

The estimations assume the *sn*-glycerol-3-*P* released on acid hydrolysis originated largely from *sn*-glycerol-3-*P* esters, that Gro*P*Cho accounts for 51% of the total *sn*-glycerol-3-*P* esters of sheep liver (Schmidt *et al.*, 1955) and that recovery of the esters as *sn*-glycerol-3-*P* was 42%.

When this procedure was applied to liver acid extracts (prepared as described in section 3.2.3.1.) from 5 normal rats,

12.1 ± 0.03 mg of *sn*-glycerol-3-*P* phosphorus/100 g wet tissue wt. was obtained, assuming the recovery of *sn*-glycerol-3-*P* from *sn*-glycerol-3-*P* esters was 42% under these conditions. Schmidt *et al.* (1955) obtained 8.8 mg of total glycerophosphate ester phosphorus/100 g wet tissue wt. for rat liver.

3.2.4.5. Protein

This was determined as described in Chapter 1.

3.2.4.6. 3-*sn*-phosphatidylcholines

These were determined as described in Chapter 2.

3.2.5. *In vitro* enzyme assays

3.2.5.1. ATP:L-methionine *S*-adenosyltransferase EC 2.5.1.6. (Methionine adenosyltransferase).

3.2.5.1.1. Preparation of liver enzyme extracts

Homogenates (20% w/v) of sheep liver were prepared in ice-cold 30 mmol/l potassium phosphate buffer (pH 6.9) using a Polytron PT 10 homogenizer, and centrifuged for 30 min at 4°C and 12,000 *g*. The supernatants were used for enzyme assay.

3.2.5.1.2. Assay conditions

There are numerous published assays for methionine adenosyltransferase, based on spectrophotometric, radioisotopic or amino acid analyzer techniques (Cantoni & Durell, 1957; Greene, 1969; Chou & Lombardini, 1972; McKenzie & Gholson, 1973; Tallan & Cohen, 1976; Eloranta *et al.*, 1976). For assay of the sheep

liver enzyme, a modification of the methods of Mudd *et al.* (1965), Gaull *et al.* (1969) and Lombardini *et al.* (1970) was used.

The following components, in a total volume of 0.5 ml, were incubated for 30 min at 37°C in a reciprocating water bath:

> 0.12 mol/l-Tris/HCl buffer (pH 7.2), 0.24 mol/l-$MgAc_2$, 0.16 mol/l-KCl, 4 mmol/l-dithiothreitol, 24 mmol/l-ATP, 5 mmol/l-L-[*methyl*-^{14}C]methionine (0.1 μCi/μmol), inorganic pyrophosphatase (82 nkat) and 1.5-3.0 mg of crude enzyme protein.

The incubations were performed in 20 ml glass tubes in duplicate, and were initiated by the addition of enzyme extract. Blanks were performed similarly except that ATP or enzyme extract was omitted from the incubation mixture. The reaction was stopped by adding 10 ml of ice-cold water.

The diluted incubation mixture was applied to a column (1 cm x 24 cm) containing 3 ml of Dowex AG50W (X 2; 100-200 mesh; NH_4^+ form), and 200 ml of water was used to wash unreacted L-[*methyl*-^{14}C]methionine through the column. *S*-Adenosylmethionine was eluted from the column with 10 ml of aqueous ammonia (14.8 mol/l) into glass scintillation vials, previously background counted, and evaporated to dryness at 80°C.

The residue was dissolved in 1 ml of water, 10 ml of scintillation fluid [toluene containing 7 g of 2,5-diphenyloxazole and 0.3 g of 1,4-di[2-(4-methyl-5-phenyloxazolyl)]-benzene per litre, mixed 2:1 (v/v) with Triton X-100] added, the vials shaken vigorously, left to equilibrate for about 1 h, and the radioactivity

determined in a Packard Tri-Carb scintillation counter Model 3375 operating at ambient temperature.

Vials were counted for 10 min (2 x 5 min) or until 10,000 counts had accumulated in the counting channel. Vials from the control incubations contained less than 0.15% of the total radioactivity added, and vials from the test incubations contained 2-5 times the radioactivity of the control vials. Channels ratios showed there were no significant differences in counting efficiency between the vials, and c.p.m. were not corrected to d.p.m.

The specific activity of methionine adenosyltransferase was calculated using the following equation:

$$\text{specific activity (}\mu\text{kat/kg protein)} = \frac{(\text{Sample c.p.m.} - \text{control c.p.m.})}{\text{c.p.m./}\mu\text{mol L-methionine/incubation}} \times \frac{1}{30 \times 60} \times \frac{10^6}{\text{mg protein/incubation}}$$

As the recovery of *S*-adenosylmethionine in this procedure was not determined, and in view of the interfering reactions that either degrade or further utilize *S*-adenosylmethionine in most crude tissue extracts, the values obtained for the specific activity of the enzyme in this work are probably underestimates of their absolute values (Chou *et al.*, 1977). The dual isotope procedure of Matthysse *et al.* (1972) evaluates and compensates for these interfering reactions.

3.2.5.1.3. Attempted purification

Preliminary attempts were made to purify the enzyme

from *Escherichia coli* W and baker's yeast by the procedures of Tabor & Tabor (1971) and Lombardini *et al.* (1970), for use in the enzymatic estimation of L-methionine (Lombardini *et al.*, 1971).

The requirement for a sensitive and rapid assay for the enzyme during purification led to the use of a modification of the methods of Lombardini *et al.* (1970) and Tabor & Tabor (1971). The following concentrations of components were incubated for 30 min at 37°C in a total volume of 1 ml:

> 0.1 mol/l-Tris/Ac (pH 8.2), 0.1 mol/l-$MgAc_2$, 0.16 mol/l-KCl, 2 mmol/l-dithiothreitol, 10 mmol/l-L-methionine, 5.2 mmol/l-[8-^{14}C]adenosine 5'-triphosphate (0.03 µCi/µmol) and crude enzyme protein.

The incubations were performed in 1.5 ml capped plastic tubes. After 30 min, 0.1 ml of the incubation mixture was diluted with 1 ml of water and 0.2 ml of a suspension (500 g/l) of Dowex AG1 (X 10; 200-400 mesh; Cl^- form). The tubes were mixed thoroughly and centrifuged. 1 ml of supernatant was added to 10 ml of scintillation fluid, the vials shaken vigorously, and counted, as described in section 3.2.5.1.2. Blanks were made similarly except that crude enzyme was not included in the incubation mixture.

This assay procedure was not specific for methionine adenosyltransferase, as the incubations had the same activity in the presence or absence of L-methionine. This was not realized at first because the incubation mixture was prepared in batches, as recommended by Tabor & Tabor (1971), and suitable controls without L-methionine were not included.

Dialysis and Sephadex G-25 chromatography of the crude enzyme extracts did not influence the L-methionine blanks, and subcellular fractionation of a rat liver homogenate revealed that the enzyme activity was mainly associated with the mitochondrial fraction. As the enzyme only occurs in the cytosol fraction of rat liver homogenates (Allsop & Watts, 1975), this assay procedure was rejected, and the attempted enzyme purification discontinued. Lombardini *et al.* (1970) do not refer to any lack of specificity in their [8-^{14}C]adenosine 5'-triphosphate assay procedure.

As ATP is reported to act as a substrate for over 120 enzymes (Yount, 1975), it is not surprising that this assay procedure is not specific for methionine adenosyltransferase. The identity of the radioactive products was not determined, but Cohn & Carter (1950) report that most adenine derivatives apart from adenosine and *S*-adenosylmethionine are adsorbed on Dowex 1 anion exchange resin at neutral pH. Cantoni & Durell (1957) reported that adenosine was not formed enzymatically from ATP under their conditions of assay.

3.2.5.2. *S*-Adenosyl-L-methionine:guanidinoacetate *N*-methyltransferase EC 2.1.1.2. (Guanidinoacetate methyltransferase)

3.2.5.2.1. Preparation of liver enzyme extracts

Homogenates (20% w/v) of rat liver were prepared in ice-cold 0.1 mol/l-Tris-HCl buffer (pH 7.4) and 0.25 mol/l-sucrose using a Polytron PT 10 homogenizer, and centrifuged for 30 min at 4^{o}C and 12,000 *g*.

Sheep liver homogenates were prepared in ice-cold 0.25 mol/l-sucrose as described above, 3-4 h after slaughter of the animals at the abattoirs.

The supernatants were used for enzyme assay.

3.2.5.2.2. Assay conditions

To determine the optimum conditions for assay of guanidinoacetate methyltransferase, the effect of varying substrate concentration, incubation time, and level of crude enzyme protein on the reaction was investigated using the liver from a normal rat. The incubation conditions are based on those of Cantoni & Vignos (1954).

Incubations, of final volume 500 µl, were performed in capped plastic microcentrifuge tubes in a reciprocating water bath at 37°C and 150 oscillations/min. Except for the variable investigated, the following conditions were used:

0.1 mol/l-Tris-HCl buffer (pH 7.4), 0.2 mmol/l-dithiothreitol, 5 mmol/l-guanidinoacetic acid, 2 mmol/l-*S*-adenosylmethionine and 4.78 mg of crude enzyme protein.

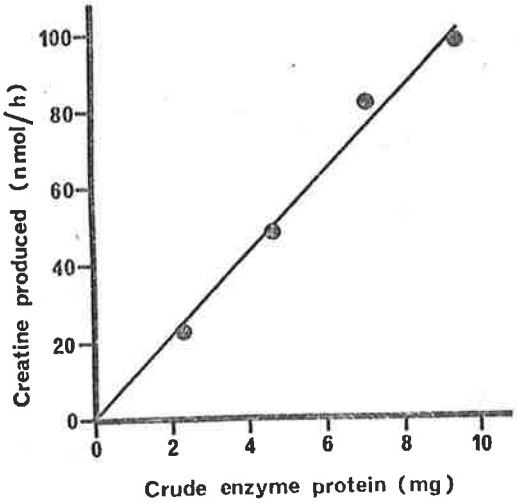
The incubation tubes were preincubated at 37°C for 5 min, and the reaction initiated by the addition of enzyme extract. The reaction was terminated after 1 h with 30 µl of perchloric acid (500 g/l). The acid extracts were centrifuged, and an aliquot of supernatant taken for neutralization and creatine estimation as described in section 3.2.4.3. Incubations and creatine determinations were not replicated.

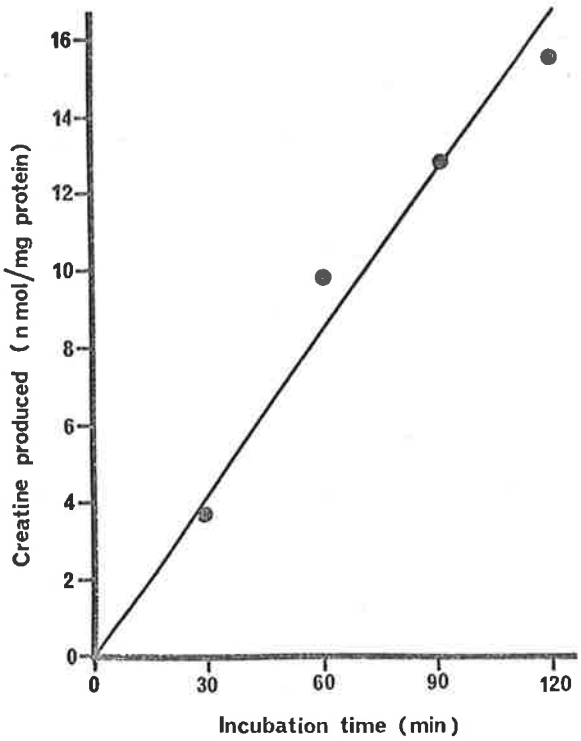
As shown in Fig. 3.2., under these conditions the reaction appears linear with time and protein up to about 100 min and 9 mg of protein. The significance of the lower reaction rate at the highest concentrations of guanidinoacetic acid and *S*-adenosylmethionine

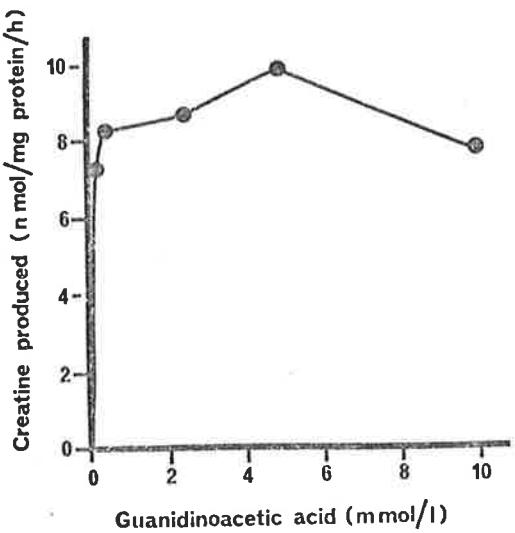
FIGURE 3.2.

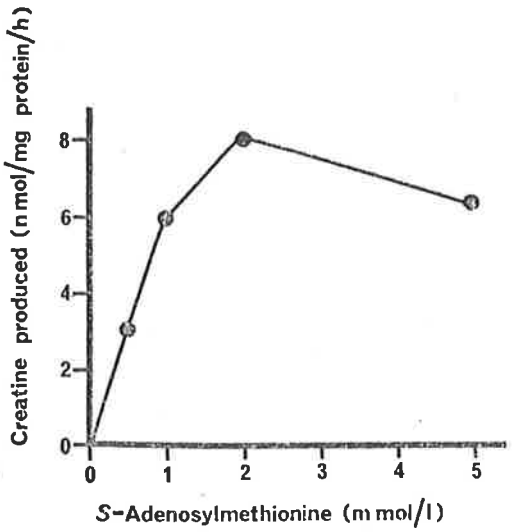
Factors affecting the synthesis of creatine by a crude extract of rat liver

The liver enzyme extract was prepared in 0.1 mol/l-Tris-HCl buffer (pH 7.4) as described in section 3.2.5.2.1., immediately after slaughter of a normal rat (172 g). Incubations were performed as described in section 3.2.5.2.2.


Creatine produced (n mol/h)
100
80
60
40
20
0
0
2
4
6
8
10
Crude enzyme protein (mg)


Creatine produced (n mol/mg protein)
16
14
12
10
8
6
4
2
0
0
30
60
90
120
Incubation time (min)


Creatine produced (n mol/mg protein/h)
10
8
6
4
2
0
0
2
4
6
8
10
Guanidinoacetic acid (m mol/l)


Creatine produced (n mol/mg protein/h)
8
6
4
2
0
0
1
2
3
4
5
S-Adenosylmethionine (m mol/l)

is uncertain because of the smaller number of experimental points, the lack of replication, and the difficulty of estimating creatine in the presence of large amounts of guanidinoacetic acid (see section 3.2.4.3.). The apparent K_m for guanidinoacetic acid at 2 mmol/l-*S*-adenosylmethionine is low, probably less than 0.1 mmol/l, and the apparent K_m for *S*-adenosylmethionine at 5 mmol/l-guanidinoacetic acid is in the range 0.5-1.0 mmol/l.

On the basis of these observations, the following conditions were used for the sheep liver incubations:

> 0.1 mol/l-Tris-HCl buffer (pH 7.4), 0.2 mmol/l-dithiothreitol, 2 mmol/l-*S*-adenosylmethionine, 10 mmol/l-guanidinoacetic acid, and 2-6 mg of crude enzyme protein.

Incubations were for 1 h at 37°C, and were performed in duplicate. Zero time controls were run with each incubation.

3.2.5.2.3. Stability of the rat liver enzyme

Initially it was considered the sheep liver extracts had no significant enzyme activity. In estimating activity, the creatine determinations had an extensive "creep" in optical density, and there appeared little difference between the test incubations and the zero time control (see Fig. 3.1.).

Preliminary assays with rat liver involved the use of 0.1 mol/l-Tris-HCl buffer (pH 7.4) extraction medium, and were performed immediately after slaughter. In contrast, sheep livers from the abattoirs were extracted in 0.25 mol/l-sucrose, and assays were performed 3-4 h after slaughter.

TABLE 3.1a.

The stability of rat liver guanidinoacetate methyltransferase

The enzyme extract was prepared as described in section 3.2.5.2.1., immediately and 3.5 h after slaughter of a normal rat. Incubations were performed as described in section 3.2.5.2.2. except that the substrate concentration of guanidinoacetic acid was 10 mmol/l, and 2.50-5.42 mg of crude enzyme protein was added per incubation. Student's paired '*t*' test was used to determine the significance of the difference between the two extraction media. n.s., not significant.

Timing details of liver extraction			Enzyme activity μkat/kg protein	
Time after slaughter that liver extract was prepared‡ h	Time after extract preparation that assay was started h	Time after slaughter that the assay was started h	0.25 mol/l-sucrose extraction medium	0.1 mol/l-Tris-HCl buffer (pH 7.4) extraction medium
0	0	1*	3.75	2.61
0	1	2*	2.22	2.22
0	2	3†	3.19	2.86
3.5	0	4.5†	3.11	2.64
Significance of difference				n.s.

* Duplicate incubations † Single incubations ‡ Liver kept on ice until extract preparation

To determine the effect of extraction medium and *post mortem* changes on enzyme activity, an experiment was performed with a rat, and is summarized in Table 3.1a. During this experiment the effect of high levels of guanidinoacetic acid on the creatine determinations was realized, and re-examination of the creatine assays for the sheep liver incubations revealed significant enzyme activity.

As shown in Table 3.1a, the nature of the extraction medium had little effect on enzyme activity, and the enzyme was stable for at least 4.5 h. It is likely therefore, that the sheep liver enzyme had not lost significant activity during the 3-4 h between slaughter and assay.

3.2.6. Expression of the analytical results

The tissue contents of metabolites are expressed as equivalents of methyl moiety per g dry fat-free tissue, and were obtained by multiplying the molar content of each metabolite by the number of *N*-methyl groups it contains.

No distinction has been made between the creatine and creatinine content of the tissues, and both metabolites are included in the term creatinine.

3.2.7. Chemicals

L-[*methyl*-^{14}C]Methionine (sp. radioactivity 56 mCi/mmol), L-[*methyl*-^{3}H]methionine (sp. radioactivity 6.0 Ci/mmol), and [8-^{14}C]adenosine 5'-triphosphate, ammonium salt (sp. radioactivity 58 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. *S*-Adenosylmethionine, and enzymes for metabolite

assays were obtained from C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Creatine, creatinine, *sn*-glycerol 3-phosphate, guanidinoacetic acid, *sn*-glycero-3-phosphocholine (cadmium chloride complex), 5,5'-dithio-bis(2-nitrobenzoic acid) and dithiothreitol were from the Sigma Chemical Co., St. Louis, Mo., U.S.A. Other chemicals were obtained as follows: 4,4'-dithiodipyridine from the Aldrich Chemical Co., Milwaukee, Wis., U.S.A.; L-methionine from BDH Chemicals Ltd., Poole, Dorset, U.K.; Dowex AG50W (X 2; 100-200 mesh; H^+ form) from Bio-Rad Laboratories, Richmond, Calif., U.S.A. This was converted to the NH_4^+ form before use. Other materials used were prepared, or were obtained from sources as described in Chapters 1 and 2. Glass-distilled water was used throughout this work.

3.3. Results

3.3.1. Total hepatic carnitine, creatinine and PtdCho contents of sheep and rats under a variety of physiological conditions

3.3.1.1. Normal and alloxan-diabetic sheep and rats

Analysis of the results shown in Table 3.1. revealed no significant differences in total hepatic PtdCho contents between the normal Merino sheep and the various groups of alloxan-diabetic Merino wethers. There were no significant differences in total hepatic PtdCho contents between the various groups of alloxan-diabetic Merino wethers.

Total hepatic carnitine and creatinine contents were not determined for the normal Merinos shown in Table 3.1. (wethers 1, 2 and ram 3, shown in Appendix 1). Measurements of two other normal

TABLE 3.1.

Total hepatic carnitine, creatinine, and PtdCho contents of normal and alloxan-diabetic sheep and rats

Experimental treatment of the animals is described in Chapter 2. Tissues were extracted and analyzed for carnitine, creatinine and PtdCho as described in sections 3.2.3., 3.2.4. and Chapter 2. Values are means ± S.E.M. for the number of animals in parentheses. n.d., not determined.

Experimental group of animals	Hepatic content µequiv. Me moiety/g dry fat-free tissue			PtdCho / carnitine
	carnitine*	creatinine	PtdCho	
Adult Merino x Dorset sheep				
Normal wethers (4)†	1.07 ± 0.07	7.02 ± 0.49	234.06 ± 8.86	219
Adult Merino sheep				
Normal sheep (3)‡	n.d.	n.d.	289.14 ± 27.22	
Alloxan-diabetic wethers				
Uncontrolled non-responders (3)	3.80 ± 0.86	10.38 ± 1.95	365.92 ± 23.09	96
Uncontrolled responders (2)	45.25 ± 8.23	8.68 ± 2.17	293.23 ± 75.90	6
Insulin treated responders (2)	3.81 ± 1.19	7.43 ± 0.20	324.87 ± 16.32	85
Insulin withdrawn responders (5)	9.00 ± 6.38	7.06 ± 0.61	301.17 ± 30.27	33
Hooded Wistar rats				
Normal rats (3)	2.54 ± 0.44§	2.66 ± 0.94	195.24 ± 15.22	77
Alloxan-diabetic rats (2)	6.59 ± 0.75	7.96 ± 0.90	242.96 ± 0.31	37

* this was total tissue or total acid-soluble carnitine as indicated for the individual animals in Appendices 1 & 2.
† the control group from the methionine infusion experiment shown in Table 3.2.
‡ two wethers and one ram.
§ mean ± S.E.M. for four rats.

Merino sheep gave unusually high total hepatic creatinine contents and unusually low total hepatic carnitine contents and consequently the data for these sheep was omitted from Table 3.1. but included in Appendix 1 (wether 4278 and ram 4127). The reason for these unusual values is unknown. The data for the control group of Merino x Dorset wethers from the L-methionine infusion experiment (see Table 3.2.) has been included in Table 3.1. for comparison, but not included in the statistical analyses. Table 3.1. shows the total hepatic creatinine contents for these normal Merino x Dorset wethers was not markedly different from the various groups of alloxan-diabetic Merino wethers. There were no significant differences in total hepatic creatinine contents between the various groups of alloxan-diabetic wethers. The total hepatic $\frac{\text{PtdCho}}{\text{creatinine}}$ ratios did not differ markedly between the various groups of sheep shown in Table 3.1. and varied from 31-44.

Table 3.1. shows the normal Merino x Dorset wethers had a lower total hepatic carnitine content than the various groups of alloxan-diabetic Merino wethers, in particular, the alloxan-diabetic uncontrolled responders. Calculations using the liver carnitine data for normal Merino wethers reported by Snoswell & Koundakjian (1972) and liver lipid and water data for the normal Merino wethers listed in Appendix 1 show the total hepatic carnitine content of normal adult Merino wethers to be about 1.70 μequiv. methyl moiety/g dry fat-free tissue, which is lower than that shown for the various groups of alloxan-diabetic Merino wethers shown in Table 3.1. The alloxan-diabetic uncontrolled responder wethers had a significantly ($P < 0.05$ or $P < 0.01$) higher total hepatic carnitine content than the other groups of alloxan-diabetic wethers.

There were no significant differences in total hepatic carnitine contents between the other groups of alloxan-diabetic wethers. The total hepatic $\frac{\text{PtdCho}}{\text{carnitine}}$ ratio was about 170 for normal adult Merino wethers [using the carnitine data from Snoswell & Koundakjian (1972) and the PtdCho data from Table 3.1.]. The total hepatic $\frac{\text{PtdCho}}{\text{carnitine}}$ ratio for the alloxan-diabetic uncontrolled responder group of wethers was markedly lower (see Table 3.1.) than the other groups of sheep, due to the significantly higher total hepatic carnitine content of this group of wethers.

When the alloxan-diabetic sheep were grouped into those with a fatty liver (wethers 3-29, 3-59 and 3-70, shown in Appendix 1) and those with a non-fatty liver (the nine remaining alloxan-diabetic wethers shown in Appendix 1), there were no significant differences in total hepatic creatinine and PtdCho contents between the two groups, but the group with fatty livers had a significantly ($P < 0.001$) higher total hepatic carnitine content than the sheep with non-fatty livers. The $\frac{\text{PtdCho}}{\text{carnitine}}$ ratio was 101 for the non-fatty group and 7 for the fatty group. The $\frac{\text{PtdCho}}{\text{creatinine}}$ ratio was 40 and 35 respectively for these two groups of sheep.

The alloxan-diabetic rats shown in Table 3.1. had a significantly ($P < 0.05$) higher total hepatic creatinine content and a significantly ($P < 0.01$) higher total hepatic carnitine content than the normal rats shown in Table 3.1., but the total hepatic PtdCho contents of the two groups of rats did not differ significantly. Table 3.1. shows the $\frac{\text{PtdCho}}{\text{carnitine}}$ ratio for the alloxan-diabetic rats did not decrease as markedly as that for the alloxan-diabetic uncontrolled responder wethers compared to their groups of normal animals. The total hepatic $\frac{\text{PtdCho}}{\text{creatinine}}$ ratio was

73 for the normal rats and 31 for the alloxan-diabetic rats.

3.3.1.2. Wethers infused abomasally with L-methionine

The results are shown in Table 3.2. The Merino x Dorset wethers infused with 0.12 g met/kg body wt.$^{0.75}$/day had a significantly ($P < 0.02$) lower total hepatic carnitine content and a significantly ($P < 0.05$) lower total hepatic PtdCho content than the control group of wethers. The wethers infused with 0.36 g met/kg body wt.$^{0.75}$/day had a significantly ($P < 0.05$) lower total hepatic creatinine content than the control group of wethers. There were no other significant differences between the three groups of wethers. The $\frac{\text{PtdCho}}{\text{carnitine}}$ ratios for these groups of wethers (see Table 3.2.) are markedly higher than the ratios shown for the alloxan-diabetic wethers in Table 3.1. The $\frac{\text{PtdCho}}{\text{creatinine}}$ ratios for the wethers shown in Table 3.2. varied from 26-41.

3.3.1.3. Pregnant-toxaemic ewes

There were no significant differences between the treated and untreated groups of pregnant-toxaemic ewes shown in Table 3.2. The data for the single Corriedale ewe (Table 3.2.) were omitted from the statistical analyses. When the pregnant-toxaemic ewes were grouped into those with fatty livers (the untreated ewes plus ewe 10, shown in Appendix 1) and those with non-fatty livers (ewes 3, 6 and 8, shown in Appendix 1), the group with fatty livers had a significantly ($P < 0.001$) higher total hepatic carnitine content than the group with non-fatty livers. The total hepatic PtdCho contents of the two groups did not differ significantly, and the total hepatic creatinine contents were not statistically analyzed because of the small number of ewes (only ewe 10, shown in Appendix

TABLE 3.2.

Total hepatic carnitine, creatinine, and PtdCho contents of methionine treated wethers, pregnant-toxaemic ewes, and abattoirs sheep

Experimental treatment of the animals is described in Chapter 2. Tissues were extracted and analyzed for carnitine, creatinine and PtdCho as described in sections 3.2.3., 3.2.4. and Chapter 2. Values are means ± S.E.M. for the number of animals in parentheses. n.d., not determined.

Experimental group of animals	Hepatic content µequiv. Me moiety/g dry fat-free tissue			PtdCho / carnitine
	carnitine*	creatinine	PtdCho	
Wethers infused abomasally with L-methionine				
0 g met/kg body wt.$^{0.75}$/day (4)	1.07 ± 0.07	7.02 ± 0.49	234.06 ± 8.86†	219
0.12 g met/kg body wt.$^{0.75}$/day (4)	0.59 ± 0.12	5.64 ± 0.35	145.48 ± 26.29†	247
0.36 g met/kg body wt.$^{0.75}$ /day (4)	1.09 ± 0.27	5.39 ± 0.41	218.31 ± 25.51†	200
Pregnant-toxaemic ewes				
Untreated ewes, slaughtered at abattoirs (5)	24.61 ± 4.74	n.d.	255.18 ± 22.21	10
Treated ewes, slaughtered at Waite (4)	8.05 ± 6.36	8.95 ± 0.65	229.19 ± 26.81‡	28
A single Corriedale ewe	230.01	n.d.	218.70	1
Abattoirs sheep				
Non-fatty livers (5)§	5.26 ± 0.71	7.69 ± 1.41	n.d.	
Fatty livers (7)	5.65 ± 0.36	3.36 ± 0.30	n.d.	

* this was total tissue or total acid-soluble carnitine as indicated for the individual animals in Appendices 1,2 & 4

† mean ± S.E.M. for three of the four wethers in each group, for the reasons described in section 2.3.5.3. in Chapter 2.

‡ mean ± S.E.M. for three of the four ewes in this group.

§ the data for livers 6 & 7 (shown in Appendix 4) was omitted from the analysis.

1) in the group with fatty livers in which creatinine estimations were made.

The total hepatic $\frac{\text{PtdCho}}{\text{carnitine}}$ ratio was 121 for the group of ewes with non-fatty livers, and 10 for the group with fatty livers. This large difference in ratio between the two groups was due to the significantly higher total hepatic carnitine content of the group of ewes with fatty livers. The total hepatic $\frac{\text{PtdCho}}{\text{creatinine}}$ ratio was 22 for two ewes with non-fatty livers (ewes 3 and 8, shown in Appendix 1) and 39 for ewe 10 which had a fatty liver (see Appendix 1).

The single Corriedale ewe shown in Table 3.2. had a total hepatic $\frac{\text{PtdCho}}{\text{carnitine}}$ ratio of 1, due to a very high carnitine content. This is the highest total hepatic carnitine content recorded for any species, and was higher than that obtained for sheep skeletal muscle (see Table 3.6.).

3.3.1.4. Abattoirs sheep

Analysis of the data in Table 3.2. for the sheep livers collected from the abattoirs indicated the fatty livers had a significantly ($P < 0.01$) lower total hepatic creatinine content than the non-fatty livers. The total hepatic carnitine contents of the two groups of livers did not differ significantly. The data for these livers was expressed per g dry fat-free tissue using total hepatic lipid contents estimated as described in section 2.3.3.5. in Chapter 2.

3.3.2. Attempted estimation of the *sn*-glycero-3-phosphocholine content of sheep liver

The results shown in Table 3.3. indicate the approximate values obtained for the Gro*P*Cho content of sheep liver in the present work are similar to those reported by Schmidt *et al.* (1952, 1955) for lamb liver using a more specific assay procedure. The values obtained in the present work probably overestimate the Gro*P*Cho content, for the reasons described in section 3.2.4.4., but indicate that this metabolite probably represents a significant fraction of the total choline in the sheep livers investigated.

3.3.3. Hepatic methionine adenosyltransferase activity of animals under a variety of physiological conditions

The results are shown in Table 3.4. There were no significant differences in specific activity between the treated and untreated pregnant-toxaemic ewes. This also applied when the ewes were grouped into two on the basis of a fatty or non-fatty liver. The specific activity of the neonatal lambs did not differ significantly from either group of pregnant-toxaemic ewes. The alloxan-diabetic wethers shown in Table 3.4. had a specific activity similar to the neonatal lambs and pregnant-toxaemic ewes. Values obtained by Radcliffe & Egan (personal communication) for normal adult Merino x Dorset wethers are markedly higher (see Table 3.4.) than those obtained for the various groups of sheep in the present work. This difference may be due to the physiological condition of the sheep studied in the present work, or due to differences in the method of enzyme assay.

TABLE 3.3.

Hepatic sn-glycero-3-phosphocholine content of sheep under various physiological conditions

Experimental treatment of the animals is described in Chapter 2. Tissues were extracted with perchloric acid as described in section 3.2.3.1. Gro*P*Cho was estimated as described in section 3.2.4.4. Values are means ± S.E.M. for the number of animals in parentheses. The results are expressed in two forms for comparison with two literature reports.

Experimental group of animals	Hepatic Gro*P*Cho µequiv. Me moiety per g wet tissue wt.	Hepatic Gro*P*Cho µequiv. Me moiety per g dry fat-free tissue
Adult Merino sheep		
Normal sheep (2)*	41.31 ± 5.91	173.88 ± 24.51
Alloxan-diabetic wethers		
Uncontrolled non-responder (1)	21.24	93.99
Insulin treated responders (2)	26.03 ± 5.33	118.26 ± 19.23
Insulin withdrawn responders (4)	25.60 ± 3.40	128.02 ± 13.57
Pregnant-toxaemic ewes		
Treated ewes, slaughtered at Waite (4)	26.06 ± 2.30	130.76 ± 18.05
Neonatal lambs (4)	17.45 ± 1.80	93.14 ± 14.00
Literature values for lambs		
Schmidt *et al.* (1952) (3)	26.74 ± 4.09	n.d.
Schmidt *et al.* (1955) (3)	17.89 ± 1.51	n.d.

* one wether and one ram.

n.d., not determined

TABLE 3.4.

Methionine adenosyltransferase activity of bovine, neonatal lamb, sheep and rat liver under various physiological conditions

Experimental treatment of the animals is described in Chapter 2. Tissues were collected and assayed as described in sections 3.2.2. and 3.2.5.1. Values are means ± S.E.M. for the number of animals in parentheses. Literature values for rat and bovine liver are included for comparison.

Experimental group of animals	Enzyme specific activity μkat/kg protein
Normal adult Merino x Dorset wethers (12)*	19.46 ± 3.01
Merino crossbred, pregnant-toxaemic ewes: Untreated ewes, slaughtered at abattoirs (5)	2.65 ± 0.46
Treated ewes, slaughtered at Waite (4)	3.24 ± 0.32
Merino crossbred, neonatal lambs (3)	3.37 ± 0.69
Alloxan-diabetic, Merino wethers: Insulin withdrawn responders (2)	2.00 ± 0.39
A single non-lactating cow†	2.83
Literature values	
bovine liver (Mudd *et al.*, 1965)	2.33
normal male adult rat liver (Mudd *et al.*, 1965)	9.44
(Sturman *et al.*, 1970; Lombardini & Talalay, 1973; Eloranta, 1977)	40-128

* a personal communication from Dr. B.C. Radcliffe and Dr. A.R. Egan.

† the tissue was a biopsy sample from a 3-year-old, predominantly Jersey cow, provided by courtesy of Dr. J. McLean.

The literature values shown in Table 3.4. indicate that the specific activity of the rat liver enzyme reported by Mudd *et al.* (1965) is considerably lower than that reported by other workers. This is probably due to the substrate limiting levels of L-methionine used by Mudd *et al.* (1965) to obtain high sensitivity in their assay procedure. The specific activity obtained for a non-lactating cow in the present work (see Table 3.4.) was similar to that reported by Mudd *et al.* (1965) for a bovine liver (also shown in Table 3.4.). Therefore, it is possible the specific activities obtained in the present work for sheep liver are underestimates of the methionine adenosyltransferase activity of this tissue. The reason for these low activities is unknown as the incubation conditions used for the enzyme assays were considered optimal (see section 3.2.5.1.2.).

The data in Table 3.4. indicates that the rat liver enzyme is considerably more active than the sheep liver enzyme.

3.3.4. Hepatic guanidinoacetate methyltransferase activity of rats and sheep

The results in Table 3.5. indicate the specific activity of guanidinoacetate methyltransferase was similar in two normal rat livers and a non-fatty sheep liver. The specific activity of the sheep enzyme appeared lower in two fatty livers compared to the non-fatty liver.

The specific activities obtained in the present work for rat liver correspond to values reported for rat liver by van Pilsum & Carlson (1970) and Carlson & van Pilsum (1973), but are 4.5-fold higher than that reported by Salvatore & Schlenk (1962) (see Table 3.5). The value obtained for the non-fatty sheep liver in the

TABLE 3.5.

Hepatic guanidinoacetate methyltransferase activity of rats and sheep

Experimental treatment of the animals is described in Chapter 2. Tissues were collected and assayed as described in sections 3.2.2. and 3.2.5.2. Values represent a single animal or the mean of two animals, with the individual figures in parentheses. Results are expressed in two forms for comparison with literature values. n.d., not determined.

Experimental group of animals		Enzyme specific activity μkat/ kg protein	Enzyme specific activity nkat/ g wet tissue wt.
Abattoirs sheep			
Non-fatty liver		2.96	0.20
Fatty livers		1.77	0.13
		(1.58, 1.97)	(0.12, 0.14)
Hooded Wistar rats			
Normal rats		2.79	0.28
		(2.75, 2.83)	(0.31, 0.25)
Literature values:			
Normal rats	van Pilsum & Carlson (1970); Carlson & van Pilsum (1973)*	n.d.	0.17-0.31
	Salvatore & Schlenk (1962)†	0.62	n.d.
Normal pig	Cantoni & Vignos (1954)*	4.72	n.d.
	Salvatore & Schlenk (1962)†	0.24	n.d.
Normal sheep	Salvatore & Schlenk (1962)†	0.19	n.d.

* enzyme assay based on colourimetric estimation of creatine
† enzyme assay based on radioisotopic estimation of creatine

present work was 16-fold higher than that reported by Salvatore & Schlenk (1962). Cantoni & Vignos (1954) obtained a value for the pig liver enzyme 20-fold higher than that reported by Salvatore & Schlenk (1962). The possibility therefore exists that the radioisotope assay procedure of Salvatore & Schlenk (1962) underestimated the sheep liver enzyme activity. However, it is also possible the present work had considerably overestimated the enzyme activity in view of the difficulties associated with its assay (see section 3.2.4.3.).

3.3.5. The total carnitine and creatinine content of skeletal muscle of normal and alloxan-diabetic sheep

The limited data in Table 3.6. suggests that the body pool of methyl groups in carnitine and creatinine (creatine) was similar in these sheep as skeletal muscle makes up about 45% of the body weight in sheep and this tissue has a high carnitine content (Snoswell & Koundakjian, 1972) and a high creatinine (creatine) content [measurements of a single Merino ewe gave the following tissue contents: liver, 0.9; kidney cortex, 2.49; heart, 15.87; skeletal muscle (M. *biceps femoris*), 21.80; each expressed as μequiv. methyl moiety/g wet tissue wt.] relative to other tissues.

The effect of alloxan-diabetes on the muscle creatinine (creatine) content in sheep (see Table 3.6.) was unclear because of the small number of sheep studied and the variability of the data. The carnitine data in Table 3.6. was also very variable. However, Snoswell & Koundakjian (1972) report that the total carnitine content of skeletal muscle does not change with alloxan-diabetes in sheep.

TABLE 3.6.

Carnitine and creatinine content of skeletal muscle of normal and alloxan-diabetic sheep

Experimental treatment of the sheep is described in Chapter 2. Tissues were extracted with perchloric acid and assayed for total acid-soluble carnitine and creatinine as described in sections 3.2.3.1., 3.2.4.1. and 3.2.4.2. Values are means with the individual figures in parentheses.

Experimental group of animals	Skeletal muscle content (M. *biceps femoris*) µequiv. Me moiety per g dry fat-free tissue	
	creatinine	carnitine
Adult Merino sheep		
Normal sheep*	146.94 (123.17, 170.71)	104.68 (125.38, 83.97)
Alloxan-diabetic wethers		
Uncontrolled non-responders	106.39 (93.74, 119.03)	139.33 (184.08, 94.57)
Uncontrolled responders	138.68 (129.69, 147.66)	195.44 (183.98, 206.89)

* one wether and one ram.

3.3.6. Urinary excretion of carnitine and creatinine in normal and alloxan-diabetic sheep

The limited data in Table 3.7. indicates that creatinine was quantitatively more significant than carnitine in the excretion of methyl groups in the two normal wethers and the single normal ewe. The urinary $\frac{\text{creatinine}}{\text{carnitine}}$ ratio was higher in the normal ewe than the normal wethers. A decrease in carnitine excretion increased this ratio markedly in the ewe when it spontaneously reduced its food intake to about 250 g of lucerne-hay chaff per day 5 days after alloxan treatment. The wethers did not spontaneously reduce their food intake following alloxan administration. However the ratio appeared to fall in these animals after alloxan treatment. Snoswell & McIntosh (1974) report the urinary excretion of carnitine can increase up to 20-fold in severely alloxan-diabetic responder sheep, so it is conceivable that the $\frac{\text{creatinine}}{\text{carnitine}}$ ratio would decrease in these animals.

3.3.7. Tissue metabolite contents in neonatal lambs

3.3.7.1. Total hepatic carnitine, creatinine and PtdCho contents

The near term foetal lambs shown in Table 3.8. had a significantly ($P < 0.01$) lower hepatic creatinine and PtdCho content, and a significantly ($P < 0.001$) higher hepatic carnitine content than the normal adult Merino x Dorset wethers. The 6-day-old ram lamb had a higher hepatic carnitine content than both the foetal lambs and adult wethers. The hepatic PtdCho and creatinine contents of the 6-day-old lamb were about 61% of the adult values shown in Table 3.8. but 1.2 to 1.7-fold higher than that of the foetal lambs.

TABLE 3.7.

Urinary excretion of carnitine and creatinine in sheep

Experimental treatment of the sheep is described in Chapter 2. Urine was collected and assayed for total acid-soluble carnitine and creatinine as described in sections 3.2.2., 3.2.4.1. and 3.2.4.2. Values represent a single 24 h collection, or the mean of two consecutive 24 h collections, with the individual figures in parentheses. Two literature reports are included for comparison. n.d., not determined.

Experimental group of sheep		Urinary excretion µequiv. Me moiety/24 h creatinine	carnitine	creatinine / carnitine
Adult Merino sheep				
Normal wethers	3-188 (body wt. 30.5 kg)	9,421 (10,667, 8,174)	897 (1,029, 765)	11
	3-66 (body wt. 32.5 kg)	21,615 (28,070, 15,159)	1,515 (1,671, 1,359)	14
A normal ewe	3-683* (body wt. 33.8 kg)	12,677	208	61
Alloxan-diabetic sheep				
Uncontrolled non-responder wethers, 18 & 19 days after alloxan				
	3-188 (body wt. 30.9 kg)	6,560 (6,070, 7,050)	1,098 (1,017, 1,179)	6
	3-66 (body wt. 25.1 kg)	14,998 (16,001, 13,995)	5,540 (5,598, 5,481)	3
Uncontrolled responder ewe, 5 days after alloxan				
	3-683* (n.d.)	15,845	7	2264
Literature values:				
Masters & Horgan (1962)	Normal Merino ewes [body wt. 30 ± 2 kg (4)]	11,402	n.d.	
Blanch & Setchell (1960)	Normal aged Corriedale ewes	17,140	n.d.	
	Aged Corriedale ewes, fasted 6 days	16,272	n.d.	

* ewe 3-683 was housed, fed and made diabetic as described in Chapter 2 for the wethers, and a catheter used to collect the urine.

TABLE 3.8.

Total hepatic carnitine, creatinine, and PtdCho contents of neonatal lambs

Experimental treatment of the neonatal lambs is described in Chapter 2. Tissues were extracted and assayed for total acid-soluble carnitine, creatinine and PtdCho as described in sections 3.2.3.1., 3.2.4.1., 3.2.4.2., and Chapter 2. Data for the control group from the methionine infusion experiment (see Tables 2.13. & 3.2.) has been included for comparison. Values are means ± S.E.M. for the number of animals in parentheses.

Experimental group of animals	Hepatic content μequiv. Me moiety/g dry fat-free tissue			PtdCho / carnitine	PtdCho / PtdEtn
	carnitine	creatinine	PtdCho		
Merino crossbred sheep					
Neonatal lambs					
Near term foetal lambs (3)	1.97 ± 0.10	3.56 ± 0.36	82.67 ± 15.79	42	1.36
A 6-day-old ram	3.70	4.30	142.44	38	1.59
Merino x Dorset sheep					
Normal adult wethers (4)	1.07 ± 0.07	7.02 ± 0.49	234.06 ± 8.86	219	2.48

The total hepatic $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratio for the group of near term foetal lambs was significantly ($P < 0.02$) lower than that for the group of adult wethers and appeared lower than the ratio for the 6-day-old lamb. The low $\frac{\text{PtdCho}}{\text{carnitine}}$ ratios for the neonatal lambs compared to the adult wethers was due mainly to the relatively lower hepatic PtdCho contents in the lambs.

3.3.7.2. Total acid-soluble carnitine content of the liver and skeletal muscle of young lambs

The results in Fig. 3.3. show that the total acid-soluble carnitine content of the skeletal muscle of the young lambs increased rapidly from about 5% of the adult value at birth to about 40% of the adult value at 8 days of age. The liver carnitine content in contrast showed relatively little change with age, except a slight increase at about 1 day of age. The age of the near term foetal lambs shown in Fig. 3.3. was estimated only.

3.3.8. A possible relationship between the total hepatic carnitine and lipid content of adult sheep

The results in Fig. 3.4. suggest that a positive relationship exists between the total lipid and carnitine content of adult sheep liver. The data was variable, probably due to the diverse nature of the physiological condition of the sheep, and no attempt was made to establish a mathematical relationship between the two parameters.

The line drawn in Fig. 3.4. was fitted visually to indicate that the lipid content may increase linearly with increasing carnitine to a carnitine content of about 10 μequiv. methyl moiety/g dry fat-

FIGURE 3.3.

Changes with age in the carnitine content of skeletal muscle and liver of young lambs

The data was obtained from 14 Merino and Merino crossbred neonatal lambs. Experimental treatment of the lambs is described in sections 2.2.1.4., 3.2.1. and by Snoswell & Koundakjian (1972). Results for 6 of the lambs were provided by courtesy of Dr. A.M. Snoswell. Tissues were extracted with perchloric acid and assayed for total acid-soluble carnitine as described in sections 3.2.3.1. and 3.2.4.1. The results are expressed per g wet tissue wt. as total lipid and water contents were not determined for all the lambs. Corresponding values for normal adult Merino wethers have been included for comparison, and were taken from Snoswell & Koundakjian (1972).

Key to figure:

● skeletal muscle (M. *biceps femoris)*

■ liver

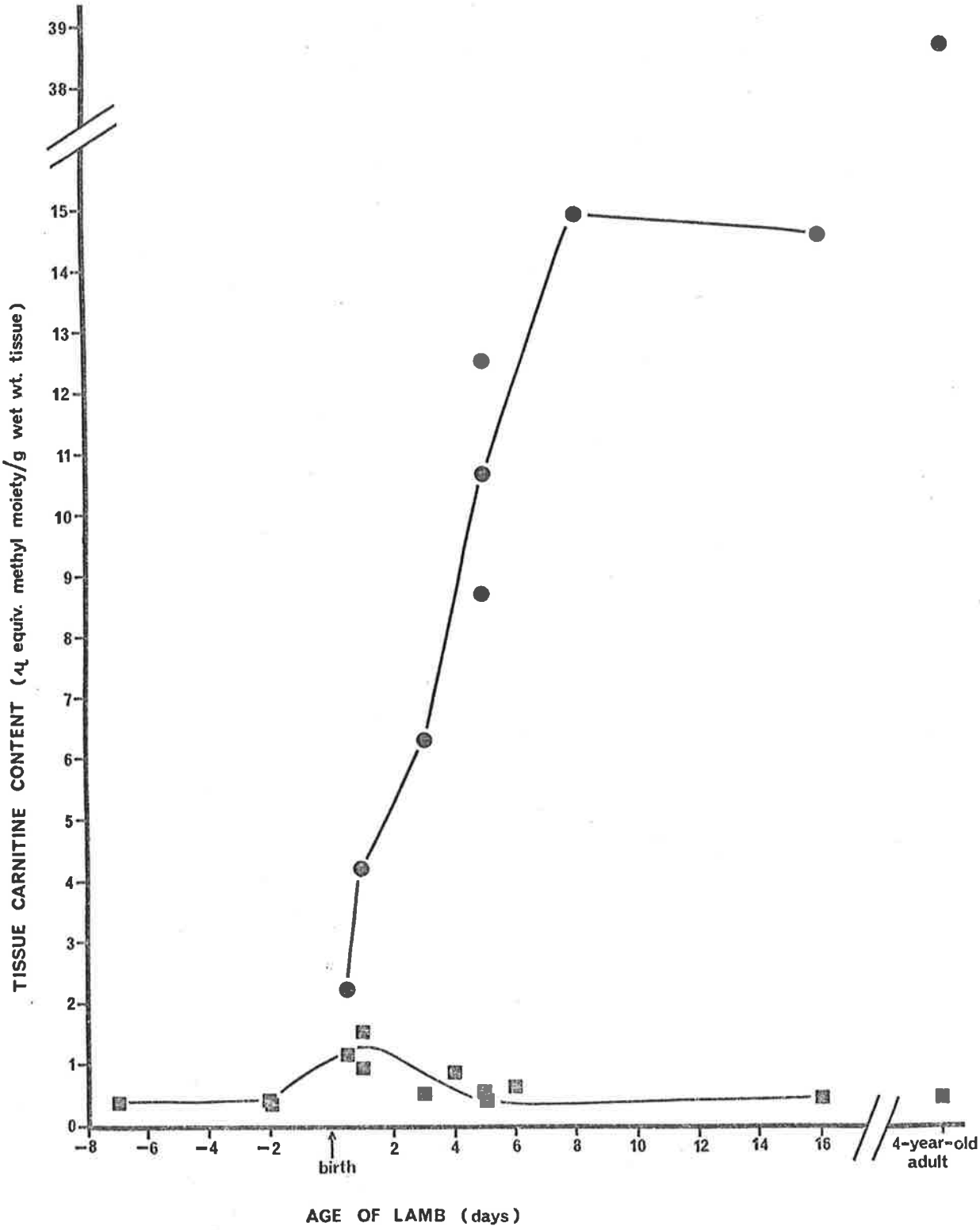

TISSUE CARNITINE CONTENT (μ equiv. methyl moiety/g wet wt. tissue)
39
38
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
−8
−6
−4
−2
birth
2
4
6
8
10
12
14
16
4-year-old adult
AGE OF LAMB (days)

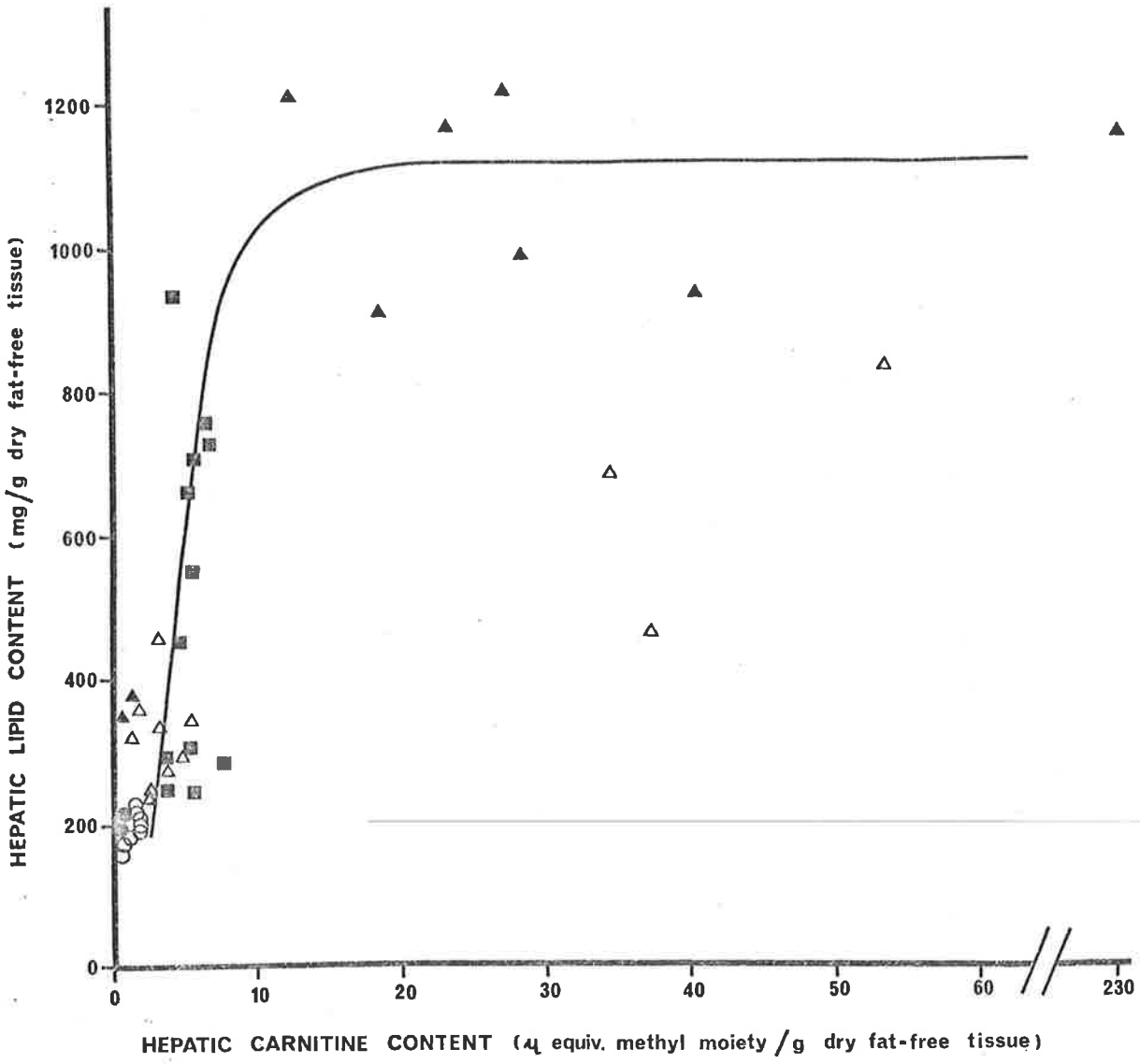
FIGURE 3.4.

A possible relationship between total hepatic lipid and carnitine content of adult sheep

The data was obtained from 48 adult sheep under a variety of physiological conditions. Total lipid and carnitine contents were determined as described in sections 2.2.4., 2.3.3.5. and 3.2.4.1. The number in parentheses represents the number of animals in each group.

Key to the figure:

- ▲ pregnant-toxaemic ewes (10)
- △ alloxan-diabetic wethers (12)
- ● normal wether and ram (2)
- ○ wethers infused abomasally with L-methionine (12)
- □ abattoirs sheep (12)


HEPATIC LIPID CONTENT (mg/g dry fat-free tissue)
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
230
HEPATIC CARNITINE CONTENT (μ equiv. methyl moiety / g dry fat-free tissue)

free tissue. Thereafter, the total lipid content may level at about 1,100 mg/g dry fat-free tissue as the carnitine content continues to rise.

3.4. Discussion

The present work indicated that the total hepatic $\frac{\text{PtdCho}}{\text{carnitine}}$ ratios for normal wethers were in the range 170-220. Alloxan-diabetic and pregnant-toxaemic sheep which did not develop fatty livers had ratios of 101 and 121 respectively. These ratios decreased to 7 and 10 respectively in those alloxan-diabetic and pregnant-toxaemic sheep which developed fatty livers. One severely pregnant-toxaemic ewe with a fatty liver had a total hepatic $\frac{\text{PtdCho}}{\text{carnitine}}$ ratio of 1 (see Table 3.2.). The data in Tables 3.1. and 3.2. show that these changes in $\frac{\text{PtdCho}}{\text{carnitine}}$ ratios were due to increases in the total hepatic carnitine contents and that the total hepatic PtdCho contents did not change markedly. The increase in total hepatic carnitine content in the alloxan-diabetic responder sheep was due to increased biosynthesis of carnitine (Snoswell & McIntosh, 1974). The $\frac{\text{PtdCho}}{\text{carnitine}}$ ratio was 77 for normal rats and this decreased to 37 for the alloxan-diabetic rats with fatty livers, thus indicating the change in ratio for these rats was less than that for the similarly stressed sheep.

The total hepatic $\frac{\text{PtdCho}}{\text{creatine}}$ ratios for normal wethers were about 33. Alloxan-diabetic and pregnant-toxaemic sheep which did not develop fatty livers had ratios of 40 and 22 respectively. The effect of the development of a fatty liver on the $\frac{\text{PtdCho}}{\text{creatine}}$ ratios for these sheep was uncertain owing to the limited and variable data obtained. However, studies with sheep livers

obtained from the abattoirs suggested the creatine content decreased in fatty livers compared to non-fatty livers (see Table 3.2.) possibly due to a lower guanidinoacetate methyltransferase activity in the fatty livers (see Table 3.5.). Thus it could be expected the total hepatic $\frac{\text{PtdCho}}{\text{creatine}}$ ratios would increase in fatty compared to normal sheep livers. The total hepatic $\frac{\text{PtdCho}}{\text{creatine}}$ ratios for normal rats were 73, and this decreased to 31 in alloxan-diabetic rats, due to an increase in the creatine content of the alloxan-diabetic rat livers (see Table 3.1.).

The approximate values obtained for the Gro*P*Cho contents of sheep livers (see Table 3.3.) are similar to those reported by Schmidt *et al.* (1952, 1955) for lamb livers and suggest that this compound represented a major form of choline in the sheep livers investigated in the present work. This metabolite is involved in the catabolism of PtdCho (Dawson, 1955) and its high level in sheep liver is probably associated with the absence of *sn*-glycero-3-phosphocholine phosphodiesterase in this tissue (Dawson, 1956). The significance of these observations in sheep is unknown, but it may represent a mechanism for conserving choline if free choline is rapidly metabolized in sheep as it is in other animals (Lucas & Ridout, 1967). Neill & Dawson (1977) report that sheep derive little choline from the diet. Consequently, sheep must depend heavily on tissue choline biosynthesis for their choline requirements. However, Neill & Dawson (1977) mention that the methylation pathway of PtdCho biosynthesis was similar in sheep and rat liver, and Bremer & Greenberg (1961*a*) showed the specific activity of sheep liver microsomal 3-*sn*-phosphatidylethanolamines methyltransferase was about 64% of that of rat liver. These observations imply that

choline catabolism is less in sheep tissues than rat tissues because rats are readily susceptible to dietary choline deficiency, possibly due to the rapid choline catabolism in these animals (Lucas & Ridout, 1967).

The body pool of methyl groups in carnitine and creatine appeared similar in normal sheep (see Table 3.6.). Creatinine was more significant than carnitine in the excretion of methyl groups (see Table 3.7.), as similarly reported by Mudd & Poole (1975) for humans. Thus the requirement for methyl groups in the biosynthesis of creatine must greatly exceed that for carnitine biosynthesis as these compounds are not metabolized to any significant extent in normal animals (Bloch *et al.*, 1941; Brooks & McIntosh, 1975; Cederblad & Lindstedt, 1976). The limited data in Table 3.7. suggested that urinary excretion of carnitine in the normal ewe was lower than that in the normal wethers. This may be a real effect as human females have a lower urinary excretion of carnitine than human males (Cederblad & Lindstedt, 1971; Maebashi *et al.*, 1976). The response to metabolic stress may be different in ewes than wethers. Whereas urinary excretion of carnitine increased in alloxan-diabetic wethers [see Table 3.7., Koundakjian (1974) and Snoswell & McIntosh (1974)] it appeared to decrease in the alloxan-diabetic ewe (see Table 3.7.). Interestingly, urinary excretion of carnitine increases in fasted human males (Maebashi *et al.*, 1976). However similar studies were not conducted with human females, so at the moment it is uncertain whether there are fundamental differences in the metabolism of carnitine in male and female animals subjected to metabolic stress.

Table 3.9. shows that creatine metabolism is similar in rats, humans and sheep. The similar specific activities obtained for rat and sheep liver guanidinoacetate methyltransferase (see Table 3.5.) correlate with the observations for these animals shown in Table 3.9. However, marked differences in carnitine metabolism are evident between the three species shown in Table 3.9. Relative to the rat, the sheep and human conserve body carnitine as evidenced by their small percentage excretion of this metabolite. The relative rate of carnitine biosynthesis must be similar in the rat and sheep as evidenced by the similar relative levels of carnitine excretion in these species, and is about 10-20 μmol/kg body wt./day. The sheep has a much larger body pool of carnitine than the rat and therefore, either the biosynthesis of carnitine was more rapid at some stage in the growth of sheep or else sheep acquire considerable quantities of carnitine in the diet. This is unlikely in view of the low levels of carnitine in plants.

The rapid increase in muscle carnitine content of the young lambs shown in Fig. 3.3. may have been due to a rapid rate of carnitine biosynthesis in this tissue, or alternatively, due to uptake from the ewe's milk which has high levels of carnitine (Snoswell & Linzell, 1975). The data of Snoswell & Linzell (1975) shows that a suckling lamb could receive about 30-40 g of carnitine *via* the milk during the course of a 10-week lactation and thus it would not be necessary to postulate a period of rapid carnitine biosynthesis in sheep tissues during development. Furthermore, an accelerated rate of carnitine biosynthesis under the anabolic conditions of rapid growth in young lambs may be unlikely if protein catabolism is required to release the carnitine precursor 6-*N*-trimethyl-lysine (Paik *et al.*, 1977). The concept that ewe's milk

TABLE 3.9.

Carnitine and creatine metabolism in three mammalian species

The values for sheep have been taken from Tables 3.6. and 3.7. in this thesis, and the values for the human and rat taken from the literature as indicated. Creatine represents Σcreatine, creatinine.

Parameter	carnitine			creatine		
	Sheep	Human	Rat‡	Sheep	Human§	Rat¶
Total body content µequiv. Me moiety/kg body wt.	18,000	5,000*	1,000	18,000	14,000	14,000
Urinary excretion µequiv. Me moiety/kg body wt.	35	16†	45	300	200	300
Urinary excretion as % body content	0.2	0.3	4	1.7	1.4	2

* Cederblad *et al.* (1974) † Maebashi *et al.* (1976) § Crim *et al.* (1976)

‡ Cederblad & Lindstedt (1976) ¶ Bloch *et al.* (1941)

may provide a significant proportion of the body pool of carnitine in young lambs is strengthened by the observations of Robles-Valdes *et al.* (1976) that the carnitine content of liver and heart tissue in young suckling rats increased during the suckling period due to the uptake of carnitine from the nursing mother's milk. The high body content of creatine in sheep (see Table 3.9.) may suggest that significant quantities of this metabolite are also contributed to lambs *via* the milk, but no data is available on this point. The limited data in Table 3.8. showed the total hepatic PtdCho content of neonatal lambs was lower than that of normal adult sheep, and that the total hepatic $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratio may increase with age due to a relatively rapid increase in PtdCho content. Noble *et al.* (1971*a*) showed the total hepatic $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratios of young lambs were markedly lower than the ratios of adult sheep and that this ratio increased rapidly during the first week of life due to a rapid increase in PtdCho content. These workers attributed this rapid increase in PtdCho content to increased biosynthesis *via* the CDP-Cho-pathway on the basis of the fatty acid composition of the ewe's milk and the lamb tissues. Ewe's colostrum is reportedly rich in preformed choline (Tsielens, 1954) and this would presumably favour the CDP-Cho-pathway for PtdCho biosynthesis in lamb liver. Based on the observations of Artom (1969) for neonatal rats, it is possible the capacity of lamb liver to synthesize choline *via* the methylation pathway of PtdCho biosynthesis is limited at birth but that this capacity increases during the suckling period. The observations of Waugh *et al.* (1947*a*,*b*) suggest that cow's colostrum and milk choline may contribute significantly to choline metabolism in calves. The methionine content of ewe's colostrum and milk proteins is relatively low compared to other essential amino acids

(Kuiken & Pearson, 1949), and consequently the intake of methionine by the lamb may be rather restricted. Methionine is often the first limiting amino acid in sheep (Chalupa, 1972), and the data in Table 3.4. shows that the specific activity of neonatal lamb liver methionine adenosyltransferase was similar to or lower than adult values for this enzyme. These combined observations may reflect a limited capacity of the neonatal lamb liver to synthesize *S*-adenosylmethionine, the immediate methyl group donor for carnitine, creatine and choline biosynthesis. The rapid growth of young lambs compared to some other species (White *et al.*, 1973) in conjunction with the aforementioned observations may require that the ewe provides a substantial proportion of preformed methylated compounds to the lambs *via* the milk.

The specific activity of methionine adenosyltransferase in sheep liver was markedly lower than that reported for rat liver (see Table 3.4.). There was no apparent relationship between the development of a fatty liver in pregnant-toxaemic ewes and the specific activity of this enzyme. The lower content of *S*-adenosylmethionine in sheep liver (Gawthorne & Smith, 1974) compared to rat liver (Eloranta, 1977) may be related to the lower specific activity of methionine adenosyltransferase in sheep liver as Chou *et al.* (1977) consider the tissue content of *S*-adenosylmethionine is controlled by rates of synthesis rather than rates of utilization. However, Lombardini & Talalay (1971) and Eloranta (1977) consider the rate-limiting factor in *S*-adenosylmethionine synthesis is the tissue content of L-methionine. The levels of free-methionine in sheep liver are not accurately known. The different response of rats and sheep to alloxan-diabetes, as shown in Table 3.1., may be

related to the greater capacity of rat liver to generate *S*-adenosylmethionine.

The biochemical explanation underlying the depressed total hepatic carnitine and PtdCho contents in the sheep infused abomasally with 0.12 g met/kg body wt.$^{0.75}$/day for 32 days (see Table 3.2.) was obscure, particularly in view of the lack of effect on these metabolites of the higher level of L-methionine infusion. The specific activity of hepatic methionine adenosyltransferase appeared lower in the 0.12 g met group compared to the control group (B.C. Radcliffe & A.R. Egan, personal communication). This may have conceivably resulted in a lower hepatic *S*-adenosylmethionine content in these sheep. A possible consequence of this may have been competition for methyl groups between choline and carnitine biosynthesis, and an active sheep liver methyltransferase that transmethylates non-physiological sulphydryl compounds including methylmercaptan (Bremer & Greenberg, 1961*b*), a postulated product (or intermediate) in the *S*-adenosylmethionine independent catabolism of methionine (Benevenga, 1974). The significance of the depressed hepatic creatine levels in the wethers infused at the highest level of L-methionine (see Table 3.2.) was unknown.

The possible relationship between total hepatic lipid (triacylglycerols) and total hepatic carnitine content in sheep under various physiological conditions (see Fig. 3.4.) may indicate a role for carnitine in regulating the flux of fatty acids into the oxidative pathways of metabolism (McGarry *et al.*, 1975). The susceptibility of sheep to the development of fatty livers, presumably due to their inability to synthesize and/or secrete VLDL, may require an active role for carnitine in redirecting as large a

proportion as possible of the incoming fatty acids *via* the carnitine palmitoyltransferase reaction to the oxidative pathways to relieve the increased accumulation of triacylglycerols in the hepatocytes. However, as this probably involved increased carnitine biosynthesis (Snoswell & McIntosh, 1974), choline biosynthesis may have been impaired as suggested by the observations reported in Chapter 2 of this thesis. This in turn may have exacerbated the lipid accumulation in the hepatocytes. Fig. 3.4. shows the lipid content did not exceed about 1,200 mg total lipid/g dry fat-free tissue. A meaningful relationship between carnitine and total lipid content may not exist at this extreme level in view of the extensive membrane aberrations (see Fig. 2.7. in Chapter 2) and pathological condition of the hepatocytes with a resultant breakdown in metabolic control.

CHAPTER 4. RADIOISOTOPE STUDIES ON THE BIOSYNTHESIS OF CARNITINE AND 3-*sn*-PHOSPHATIDYLCHOLINES, AND CHOLINE METABOLISM, USING ISOLATED HEPATOCYTES FROM SHEEP

4.1. Introduction

The principal purpose of the work reported in this chapter was to obtain more direct evidence of a possible competition for L-methionine methyl groups between choline and carnitine biosynthesis in sheep liver under physiological conditions in which the rate of carnitine biosynthesis may be accelerated.

Snoswell & Koundakjian (1972) observed a marked rise in the liver carnitine content of an alloxan-diabetic insulin stabilized sheep which was subsequently withdrawn from insulin for 24 h. Assuming this rapid rise in carnitine content was due to increased biosynthesis (Snoswell & McIntosh, 1974), the methyl group requirement for carnitine biosynthesis would be maximal and any likely competition for methyl groups between choline and carnitine biosynthesis would become evident. Consequently, radioisotope studies were conducted using isolated hepatocytes prepared from alloxan-diabetic insulin stabilized and 24 h insulin withdrawn sheep.

The relative significance of the two pathways for *de novo* biosynthesis of 3-*sn*-phosphatidylcholines (Bremer *et al.*, 1960; Wilson *et al.*, 1960; Kennedy & Weiss, 1956) in sheep liver is unknown. Concurrent with the studies on methyl group competition, an attempt was made to evaluate the relative significance of these pathways and obtain information relating to the oxidation of choline to betaine.

4.2. Methods and Materials

4.2.1. Animals

Eight 2-3 year-old Merino wethers were brought into the animal sheds directly from the Waite Institute pastures at intervals of 4-9 days, weighed, placed in metabolism crates and immediately made alloxan-diabetic as described in section 2.2.1.2. of Chapter 2. Abrupt treatment of the wethers in this way appeared to reduce the variable response of the animals to alloxan as seven of the eight wethers were subsequently responders (see section 2.2.1.2.1. in Chapter 2 for the definition of this term). The wethers were administered insulin subcutaneously as described in section 2.2.1.2.2. of Chapter 2. Details of the synchronization of insulin treatment and feeding are shown in Fig. 4.1. These animals were used to prepare isolated hepatocytes as described in section 4.2.2. Five wethers were insulin withdrawn responders and two were insulin treated responders (see section 2.2.1.2.2. in Chapter 2 for the definition of these terms). The blood glucose concentration of the insulin treated responders was about 45-130 mg/100 ml (Dextrostix) at 2000 h, and all animals had a blood glucose concentration > 250 mg/100 ml (Dextrostix) at the time of slaughter (0930 h - 1000 h). A summary of the experimental treatment of the wethers is given in Table 4.1.

4.2.2. The preparation of isolated sheep hepatocytes

The method of Ash *et al.* (1975) was mainly followed.

4.2.2.1. Preparation of buffers

Three slightly modified and one unmodified Krebs-Henseleit

FIGURE 4.1.

Synchronization of insulin treatment
and feeding of the alloxan-diabetic responder wethers

The alloxan-diabetic responder wethers were injected with insulin (as described in section 2.2.1.2.2. of Chapter 2) at 0930 h, and fed 800-1000 g lucerne-hay chaff at 1500 h. The synchronization of insulin treatment and feeding was designed to correspond to the normal pattern of plasma insulin concentration under this feeding regimen. The wethers were receiving no insulin for 2 h/day (1000 h - 1200 h) under this schedule. A wether was considered stabilized on insulin if it consumed 800-1000 g of feed/day. The daily pattern of plasma insulin concentration for normal adult wethers fed at 1500 h was taken from Basset (1974). The action spectrum of the Lente Novo insulin was taken from the manufacturer's specifications for the product.

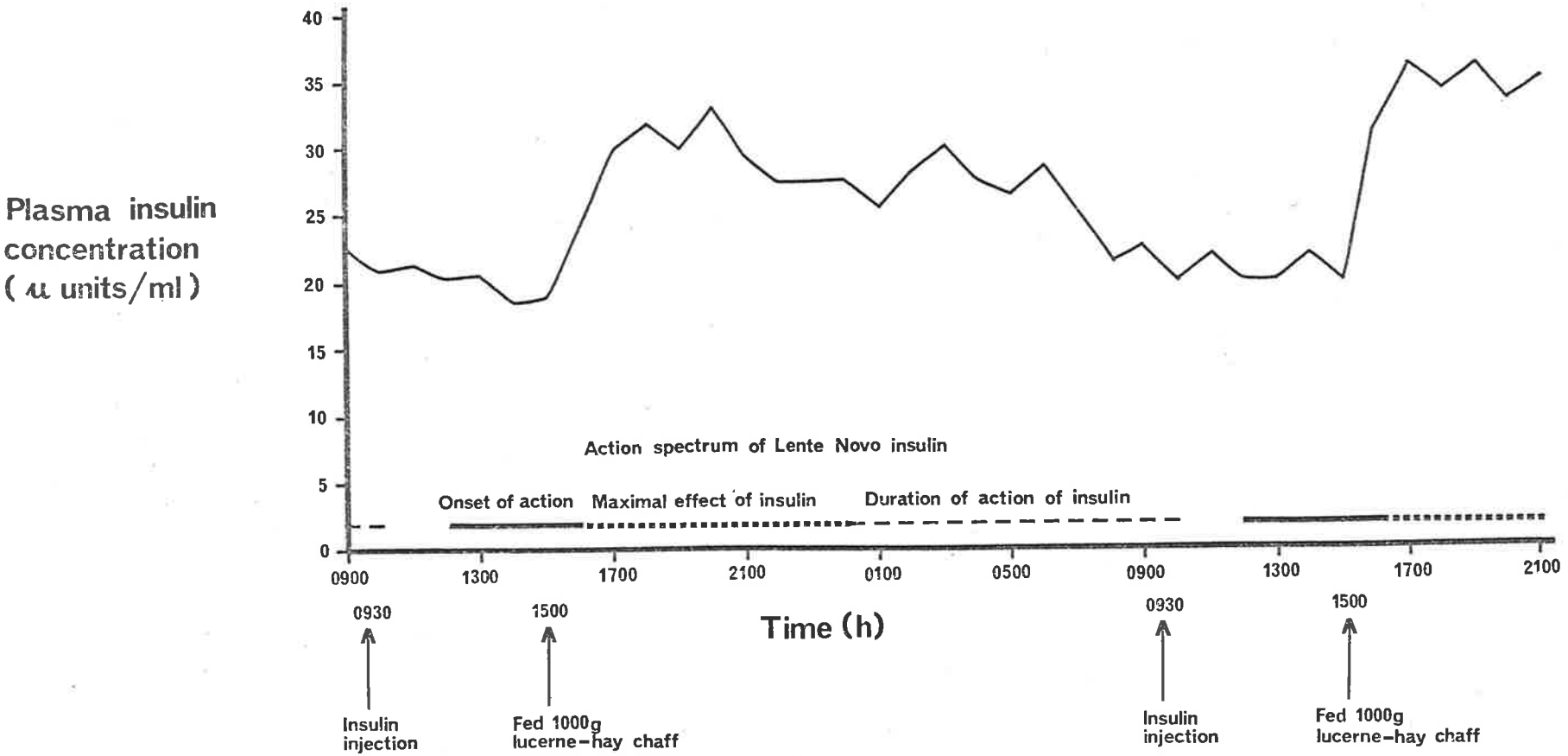

Plasma insulin concentration (μ units/ml)
40
35
30
25
20
15
10
5
0
Action spectrum of Lente Novo insulin
Onset of action
Maximal effect of insulin
Duration of action of insulin
0900
1300
1700
2100
0100
0500
0900
1300
1700
2100
Time (h)
0930
1500
0930
1500
Insulin injection
Fed 1000g lucerne-hay chaff
Insulin injection
Fed 1000g lucerne-hay chaff

TABLE 4.1.

Experimental treatment of the Merino wethers

Wether	Initial body wt. kg	Insulin treatment: Days after alloxan that treatment started	Insulin treatment: Food intake at the start g/24 h	Insulin treatment: Days taken to stabilize	Time of slaughter: Days after alloxan	Time of slaughter: Insulin status	Time of slaughter: Appearance of liver	Blood carnitine nmol/ml	Isolated cell preparation
3-29	40.0	4	140	*	10	withdrawn	fatty	91	failed
3-40	40.0	7	260	3	16	withdrawn	normal	27	satisfactory
3-53	44.1	11	280	3	24	withdrawn	normal	n.d.†	failed
3-115	39.3	7	140	3	27	stabilized	normal	22	satisfactory
3-43	38.5	4	0	2	28	withdrawn	normal	20	satisfactory
3-161	50.3	9	40	2	30	stabilized	normal	24	satisfactory
3-131	45.0	4	200	2	44	withdrawn	normal	31	failed

* this wether did not fully stabilize, and was scouring at time of slaughter

† not determined

original Ringer bicarbonate solutions were freshly prepared before each isolated cell experiment. The unmodified Krebs-Henseleit bicarbonate solution was prepared as described by Dawson *et al.* (1969), and the three modified solutions altered as described below.

5 litres of Ca^{2+}-free Krebs-Henseleit bicarbonate was prepared for *in situ* liver perfusion.

100 ml of Ca^{2+}-free Krebs-Henseleit bicarbonate containing bovine albumin (essentially fatty acid-free, 20 g/l) and 10 mmol/l-sodium propionate was prepared for the enzymatic digestion of the liver slices.

200 ml of Krebs-Henseleit bicarbonate was prepared for washing the isolated cells.

50 ml of Krebs-Henseleit bicarbonate containing bovine albumin (essentially fatty acid-free, 20 g/l) was prepared for the final resuspension of the isolated hepatocytes.

Each solution was gassed with O_2 + CO_2 (95:5) for about 10-15 min before use.

4.2.2.2. Preparation of the cell suspension

The wethers were killed by severing the neck or shooting in the head, and the liver quickly exposed by cutting open the chest. A plastic tube was immediately inserted into the portal vein, the hepatic artery severed, and the liver perfused (2 l/min) with about 2-3 litres of cold (4°C) Ca^{2+}-free Krebs-Henseleit bicarbonate solution.

Several pieces of liver (from various lobes) were taken, the capsule removed, and the liver pieces bathed in oxygenated perfusion solution. Tissue slices about 1 cm square by 1 mm thick were cut by hand using siliconized scalpels, and these slices subsequently cut into 0.2 mm thick slices using a McIlwain-Buddle tissue slicer (The Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.). A more satisfactory method for preparing the tissue slices involved the use of siliconized skin graft knife blades (Costa, 1977).

About 3 g of slices were placed in 10 ml of oxygenated digestion solution (see section 4.2.2.1.) contained in 100 ml plastic bottles. About 6 bottles were prepared for each sheep. Collagenase (8-10 mg) and hyaluronidase (10 mg) were added to each bottle, the bottles flushed with O_2 + CO_2 (95:5), capped, fixed in a reciprocating water bath (Model RW1812, Paton Industries Pty. Ltd., Stepney, Adelaide) at 37°C and incubated at 150 oscillations/min. After 20 min incubation, $CaCl_2$ was added (2.8 mg, to full strength Krebs-Henseleit bicarbonate solution) to each bottle and the incubation continued for a further 20 min.

The incubation mixtures were then pooled and eased through 1 mm mesh Terylene cloth using a Teflon pestle, and subsequently filtered through nylon mesh (0.1 mm diam.). These procedures were facilitated by rinsing with Krebs-Henseleit bicarbonate solution. The cells were separated from debris by centrifuging at about 40 *g* (Heraeus Christ bench centrifuge) for 2.5 min. The supernatant was discarded and the cells were washed twice (centrifuging each time at about 40 g for 2.5 min) with Krebs-Henseleit bicarbonate solution (about 30 ml each time), and finally

resuspended in 10-15 ml (depending on the yield of cells) of Krebs-Henseleit bicarbonate solution containing bovine albumin (essentially fatty acid-free, 20 g/l). The liver-cell suspension was used immediately for the metabolism studies (see section 4.2.3.). All operations were performed at laboratory temperature and as quickly as possible to minimize the risk of anoxia. Generally, about 1.5-2 h elapsed between the slaughter of the wethers and commencement of the metabolic studies using the cell suspensions.

4.2.2.3. Cell viability tests

4.2.2.3.1. Light microscopy

The ability of the hepatocytes to exclude the dye Trypan Blue was used as a routine test of viability. A drop of aqueous Trypan Blue (0.02 g/l) was placed on an aliquot of cell suspension and exclusion of the dye from the cells assessed under the microscope. Exclusion was generally high, but this may have overestimated the viability, as the cell suspension medium contained bovine albumin which may have interfered with the dye uptake by the cells (Pappenheimer, 1917). The appearance of the cells is shown in Fig. 4.2a. and 4.2b. Several non-viable cells (those with blebs) are arrowed in Fig. 4.2b.

4.2.2.3.2. Glucose production from propionate

The ability of the cells to produce glucose from sodium propionate was used as a test of metabolic activity. The following components, in a total volume of 500 µl, were incubated at 37°C in a reciprocating water bath at 150 oscillations/min:

FIGURE 4.2a.

Low-power photomicrograph of isolated hepatocytes from an alloxan-diabetic wether

(Courtesy of Dr. J.F. Jackson,
Department of Agricultural Biochemistry, University of Adelaide)

The hepatocytes were prepared as described in section 4.2.2. from the alloxan-diabetic insulin stabilized wether 3-43 which was subsequently withdrawn from insulin for 24 h. The hepatocytes were stained with Trypan Blue.

Magnification X 100.

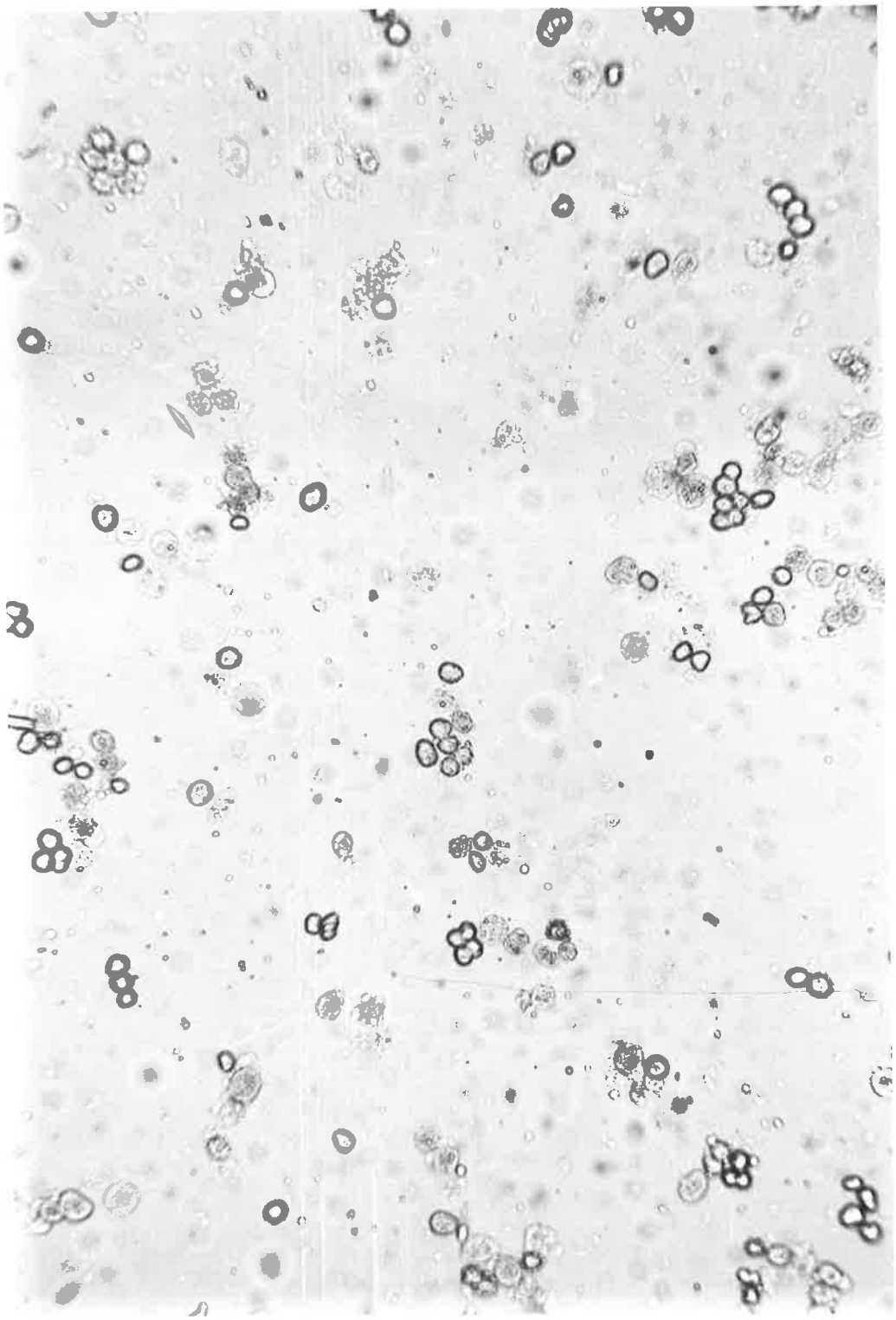
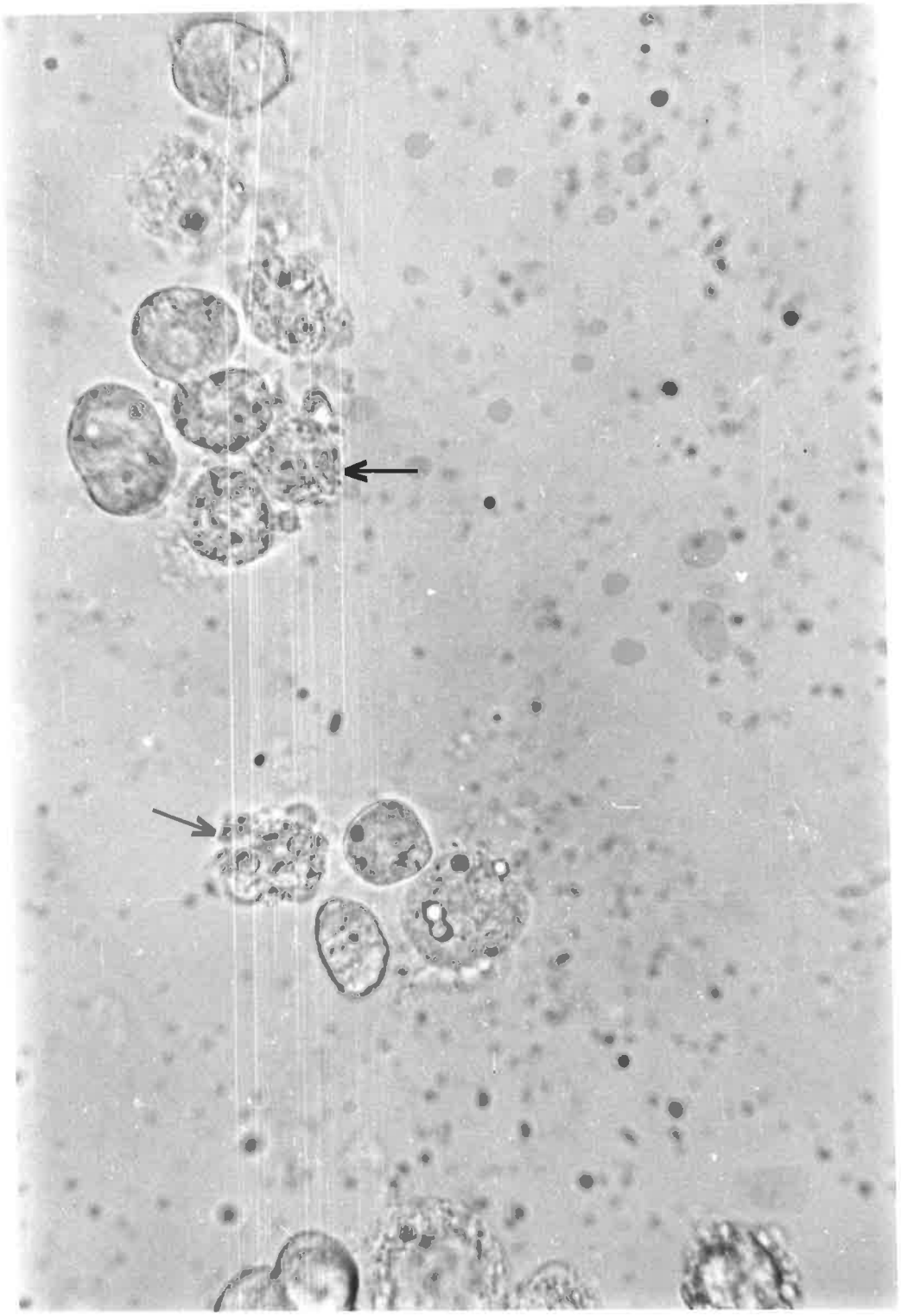


FIGURE 4.2b.

Low-power photomicrograph of isolated hepatocytes from an alloxan-diabetic wether.

Details as for Fig. 4.2a.

Magnification X 400.



200 µl liver-cell suspension (1.9-2.3 mg dry wt. cells), 300 µl of Krebs-Henseleit bicarbonate containing bovine albumin (essentially fatty acid-free, 20 g/l) and 10 mmol/l-Na propionate.

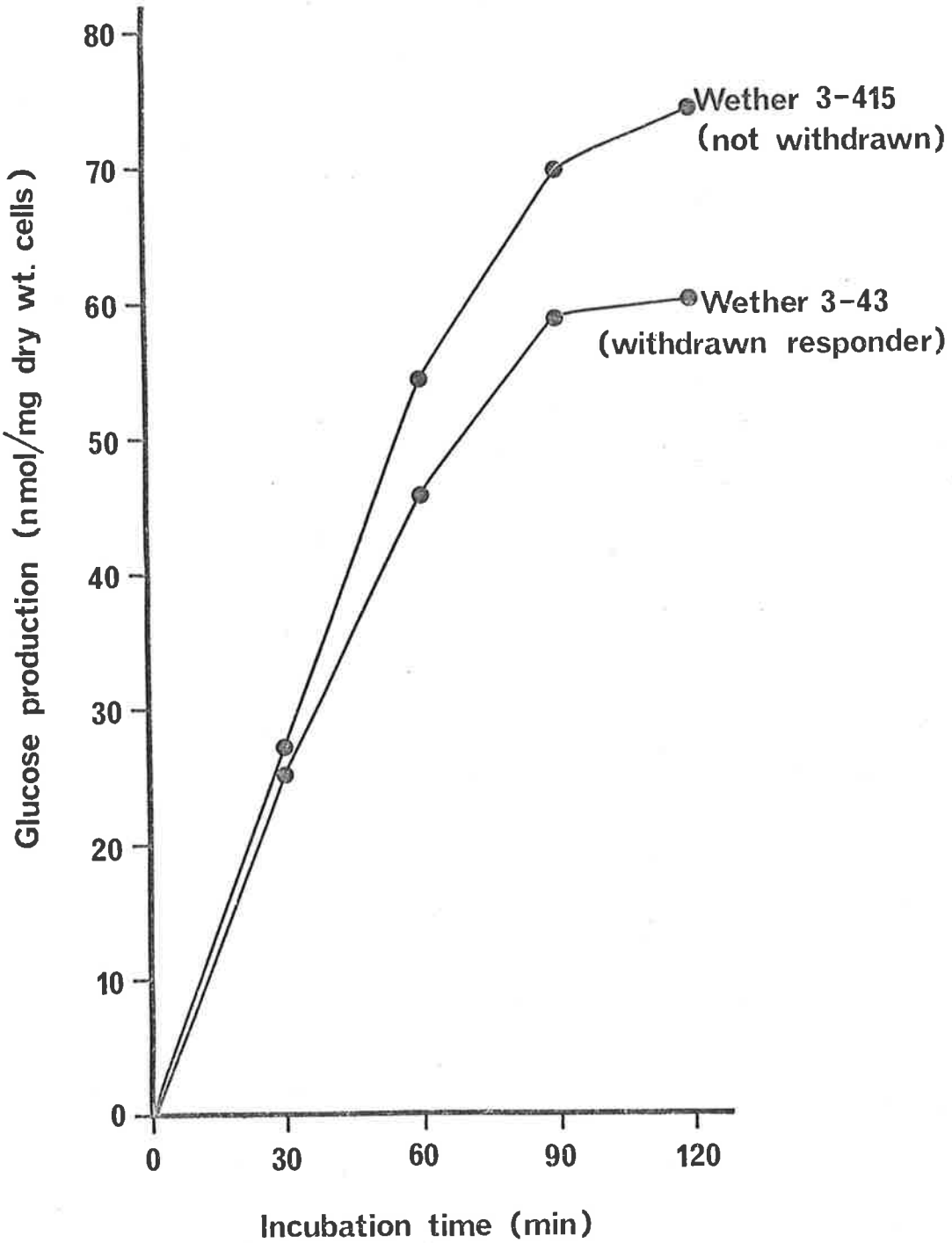
The incubations were performed in 20 ml plastic scintillation vials and were initiated by the addition of the liver-cell suspension. The vials were flushed with O_2 + CO_2 (95:5) before adding the caps. Incubation times were 0, 30, 60, 90 and 120 min, and each incubation was performed singly. The reactions were stopped with 1 ml of perchloric acid (1.8 g/l), and glucose estimated in the acid extracts as described in section 4.2.7.1.

Owing to the low yield of cells obtained, minus propionate controls were not routinely performed. The glucose production curves for the two successful cell preparations reported in this Chapter are shown in Fig. 4.3. Values for glucose production by these liver-cell suspensions were very low. The values were only 28% of those reported by Ash *et al.* (1975), despite the fact that gluconeogenesis is accelerated in alloxan-diabetic sheep (Filsell *et al.*, 1969). Glucose production rates by liver-cell suspensions prepared from alloxan-diabetic insulin stabilized rats which were subsequently withdrawn from insulin did not reach a peak until 72 h after the last injection of insulin (Wagle & Ingebretsen, 1975). Thus a residual effect of insulin may have depressed glucose production rates in the sheep liver-cell suspensions shown in Fig. 4.3. Clark *et al.* (1976) obtained glucose production rates from 10 mmol/l-propionate in considerable excess of those reported by Ash *et al.* (1975) and the present work for sheep liver-cell suspensions.

FIGURE 4.3.

Glucose production by isolated sheep hepatocytes in the presence of propionate

The isolated hepatocytes were incubated in the presence of 10 mmol/l -sodium propionate as described in section 4.2.2.3.2. Glucose was determined as described in section 4.2.7.1.


Glucose production (nmol/mg dry wt. cells)
80
70
60
50
40
30
20
10
0
0
30
60
90
120
Incubation time (min)
Wether 3-415
(not withdrawn)
Wether 3-43
(withdrawn responder)

4.2.2.4. Yield of cells

The yield of cells obtained by this procedure was low. Recovery of the original liver tissue dry weight in the cell suspensions was about 3%.

4.2.2.5. Expression of the results

The dry weight of the liver-cell suspension was used as the base of reference for the analytical data, and this was determined from the difference in dry weight between 1 ml of cell suspension and 1 ml of suspension buffer, each dried at 70°C for 48 h.

The wide disparity in the absolute rates of glucose production (from 10 mmol/l-propionate) by sheep liver-cell suspensions reported by Ash *et al.* (1975), Clark *et al.* (1976) and the present work (see section 4.2.2.3.2.) may in part be due to difficulties in standardizing the dry weights of the cell suspensions used as the base of reference for the data. A better, more standardized, base of reference for the data may be perchloric-acid-insoluble, glycogen-free, defatted dry weight, which is probably 95% protein (Katz *et al.*, 1975).

4.2.3. Radioisotope studies using the isolated hepatocytes

4.2.3.1. Experimental design

The basic design of the radioisotope experiments is shown in Fig. 4.4.

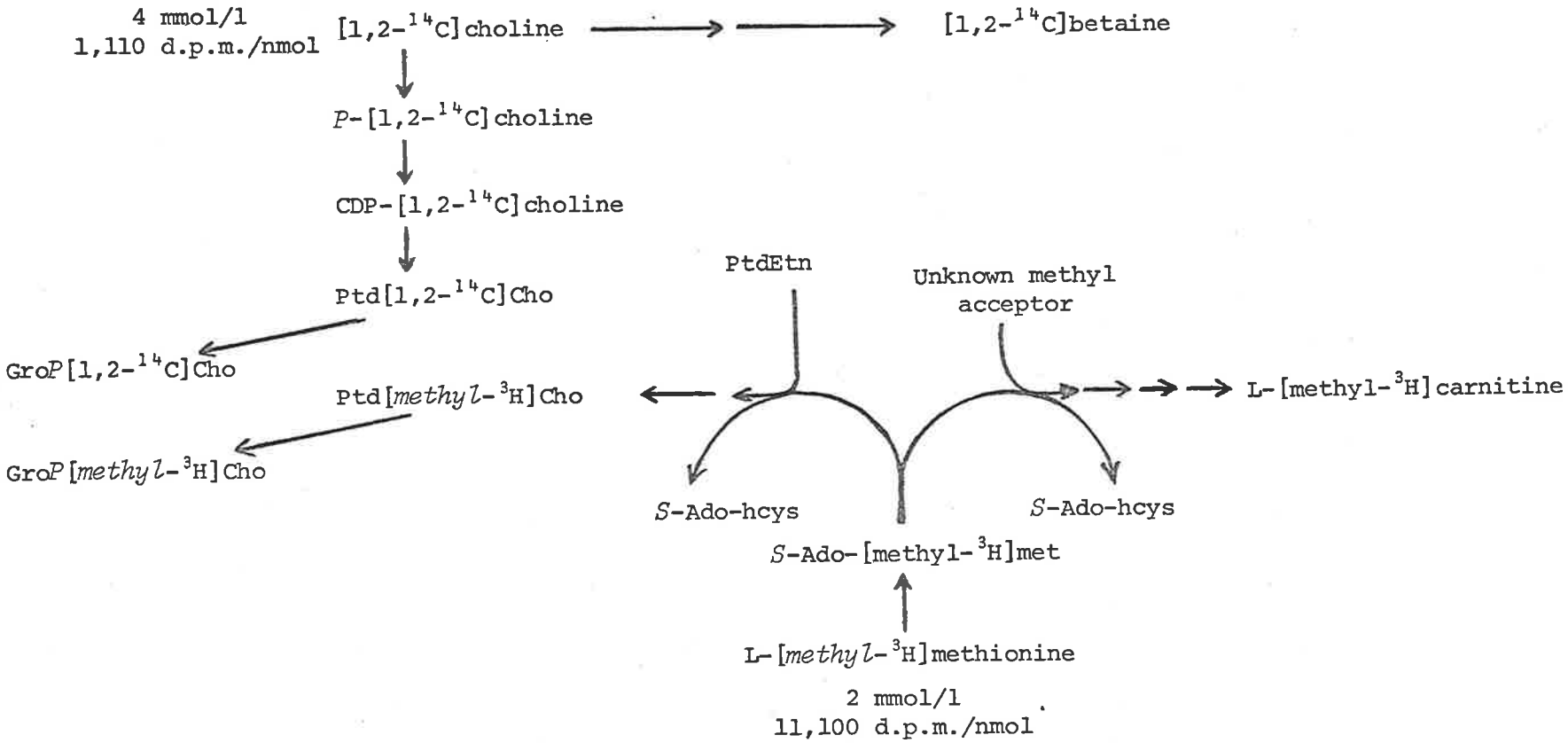


FIGURE 4.4.

Experimental design of the radioisotope studies using isolated hepatocytes

4.2.3.2. Incubation conditions

The following components, in a total volume of 500 µl, were incubated at 37°C in a reciprocating water bath at 150 oscillations/min:

100 µl Krebs-Henseleit bicarbonate solution, 10 mmol/l-Na propionate, 10 mmol/l-Na glutamate, 10 mmol/l-Na fumarate, 10 mmol/l-Na pyruvate, 4 mmol/l-lysine monochloride, 1 mmol/l-aminobutyrate, 2 mmol/l-L-[*methyl*-^{3}H]methionine (5 µCi/µmol), 4 mmol/l-[1,2-^{14}C]choline chloride (0.5 µCi/µmol), and 400 µl of liver-cell suspension (3.8-4.6 mg dry wt. cells).

The incubations were performed in 20 ml plastic scintillation vials and were initiated by addition of the liver-cell suspension. The vials were flushed with O_2 + CO_2 (95:5) before adding the caps. Incubation times were 0, 30, 60, 90 and 120 min, and each incubation was performed singly. The reaction was stopped by transferring the incubation mixture (using a Pasteur pipette) to 4 ml of chloroform/methanol (1:1, v/v) contained in a glass tube. The tube was then flushed with N_2 and stoppered. The chloroform/methanol (1:1, v/v) contained 4-methyl-2,6-di-*tert*.-butylphenol (50 mg/l) antioxidant.

4.2.3.3. Extraction of labelled metabolites

The method of Sundler *et al.* (1974) was mainly followed. Precautions were taken to minimize autoxidation, lipid contamination and extraction losses as described in section 2.2.3. in Chapter 2. All lipid solvents contained 4-methyl-2,6-di-*tert*.-butylphenol (50 mg/l) antioxidant, and all operations were performed under N_2

gas where practicable.

The glass tubes containing the terminated incubations were left at laboratory temperature for 1 h. 1.5 ml of aqueous NaCl (10 g/l) was added, the tubes vigorously mixed, and centrifuged (Heraeus Christ bench centrifuge) for about 20 min. The upper phase was removed by aspiration, freeze-dried, resuspended in 300 µl of distilled water and stored at -14°C until required. The lower phase was rinsed 3 times with aqueous NaCl (10 g/l)/methanol/ chloroform (47:48:3, by vol.), filtered through a fluted filter paper (previously boiled in chloroform) and rotary evaporated to about 1 ml. Residual water and methanol was removed by solvent replacement with chloroform (as described in section 2.2.3. in Chapter 2) and the lipid extracts placed in graduated glass tubes (5 cm long x 0.4 cm internal diam.), adjusted to 100 µl with chloroform, sealed with several layers of 0.06 mm Teflon film and stored in the dark at -14°C under N_2 until required.

4.2.3.4. Separation of labelled metabolites

Radioactive PtdCho was separated from the other lipid classes by thin-layer chromatography as described in section 2.2.5.4.1.2.1. in Chapter 2. 10 µl (containing 4-7 µg phosphorus) of liver total lipid extract (prepared from each liver as described in section 2.2.3. in Chapter 2) was used as carrier lipid in the t.l.c. separation. The spots were detected with iodine vapours, and the PtdCho (identified as described in section 2.2.5.2.) spots scraped into glass tubes using a sharpened, stainless steel spatula. PtdCho was eluted from the gel with 4 ml of chloroform/methanol/ acetic acid/water (50:39:1:10, by vol.) (Arvidson, 1968) for 1 h at

laboratory temperature, and then filtered through fluted filter paper into previously background counted glass scintillation vials, rinsed with a further 5 ml of chloroform, and evaporated to dryness in a cabinet at 60°C.

The radioactive water-soluble metabolites were separated by thin-layer chromatography on 200 mm x 200 mm x 0.5 mm thin-layers of silica gel H (type 60) prepared as described in section 2.2.5.1. in Chapter 2. The developing solvent was methanol/aqueous NaCl (5 g/l)/ammonia (14.8 mol/l) (50:50:5, by vol.) (Sundler & Åkesson, 1975). A mixture of standard compounds was dissolved in isopropanol/water (1:10, v/v) (Eneroth & Lindstedt, 1961) and spotted as carriers. The mixture (50 mmol/l) spotted as carrier contained 200 nmol of each of the following compounds: L-methionine, choline chloride, *P*-choline, β-methyl Cho, L-carnitine HCl, Gro*P*Cho (cadmium chloride complex), betaine HCl and CDP-Cho. 10-20 µl of the radioactive water-soluble extract was spotted. A developed thin-layer chromatogram is shown in Fig. 4.5. Carnitine, *P*-choline and Gro*P*Cho comigrated in this system. The developed thin-layers were visualized with I_2 vapours, and the spots corresponding in position to betaine, CDP-Cho and "carnitine" were circled with a fine needle, and scraped into glass tubes. Gro*P*Cho was separated from carnitine and *P*-choline by eluting these compounds from the silica gel with 1.5 ml of water (pH 8.0) and applying the eluate to an ion-exchange column prepared in a truncate Pasteur pipette plugged with a small wad of glass wool, and containing Dowex AG50W (X 8; 200-400 mesh; H^+ form) of bed volume about 1 ml. The column was washed with 5 ml of water, and the eluant containing Gro*P*Cho (Ansell & Spanner, 1971) was collected in a previously

FIGURE 4.5.

Thin-layer chromatograms of water soluble metabolites

The thin-layers were 200 mm x 200 mm x 0.5 mm silica gel H (type 60) prepared and activated as described in section 2.2.5.1. in Chapter 2. The thin-layers were developed in methanol/aqueous NaCl (5 g/l)/ NH_3 (14.8 mol/l) (50:50:5, by vol.) and visualized by I_2 vapours.

The thin-layer on the left resolved standard compounds (100 nmol) which were from left to right: choline, β-methyl Cho, *P*-choline, carnitine, Gro*P*Cho, betaine, CDP-Cho, methionine, and mixtures of all standards.

The thin-layer on the right resolved radiolabelled metabolites from isolated cell incubations. Carrier standard compounds were also applied as described in section 4.2.3.4.

The following compounds appeared to migrate with higher R_F values than carnitine in this solvent system: glycine, serine, sarcosine, *N*,*N*-dimethylglycine, creatine and creatinine.

3-EB
120p
120
90
60
30
0

A
MA
PA
C
GPA
B
CPA
m

background counted glass scintillation vial and evaporated to dryness at 80°C. The separated Gro*P*Cho was subsequently redissolved in 1 ml of distilled water in preparation for counting. Betaine, CDP-Cho and the mixture of carnitine, *P*-Cho and Gro*P*Cho, were eluted from the scraped silica gel with 1.5 ml of water. 1 ml of the eluate was taken for counting in each case.

4.2.4. Liquid Scintillation Counting

The general recommendations of Bush (1964), Kobayashi & Maudsley (1970) and Bransome (1975) for double label counting, and Kobayashi & Maudsley (1974) for counting in Triton X-100/toluene systems were followed. Radioactivity was determined in a Packard Tri-Carb scintillation counter Model 3375 operating at ambient temperature.

The radioactivity of PtdCho in the background counted scintillation vials was determined after the addition of 10 ml of scintillation fluid [toluene containing 7 g of 2,5-diphenyloxazole and 0.3 g of 1,4-di[2-(4-methyl-5-phenyloxazolyl)]-benzene per litre]. Discriminator settings were selected in such a way that the counting efficiency of ^{14}C was 60.8% and that of ^{3}H was 29.4%. The spillover of ^{14}C into the ^{3}H channel under these conditions was 10.6% whereas that of ^{3}H into the ^{14}C channel was 0.77%. Counting efficiency was determined by internal standardization with n-[1,2(n)-^{3}H]hexadecane and [*methyl*-^{14}C]toluene.

1 ml of the eluates containing the various water-soluble metabolites were added to previously background counted glass scintillation vials containing 10 ml of scintillation fluid [toluene containing 7 g of 2,5-diphenyloxazole and 0.3 g of

1,4-di[2-(4-methyl-5-phenyloxazolyl)]-benzene per litre, mixed 2:1 (v/v) with Triton X-100]. Discriminator settings were selected in such a way that the counting efficiency of ^{14}C was 60.0% and that of ^{3}H was 19.1%. The spillover of ^{14}C into the ^{3}H channel under these conditions was 7.8% whereas that of ^{3}H into the ^{14}C channel was 0.49%. Counting efficiency was determined by internal standardization with a standard aqueous solution of [1,2-^{14}C]choline chloride and tritiated water. The [1,2-^{14}C]choline chloride solution was standardized using benzoic acid-^{14}C secondary standard (sp. radioactivity 40,000 d.p.m./mg).

Corrections for both the spillover of the ^{14}C into the tritium channel and for the ^{3}H spillover into the ^{14}C channel were made by solving the following simultaneous equations:

$$^{3}H^{\text{corrected}}_{\text{c.p.m.}} = {}^{3}H_{\text{c.p.m.}} - \frac{\%\ ^{14}C\ \text{spill}}{100}\left({}^{14}C_{\text{c.p.m.}} - \frac{\%\ ^{3}H\ \text{spill}}{100} \times {}^{3}H^{\text{corrected}}_{\text{c.p.m.}}\right)$$

$$^{14}C^{\text{corrected}}_{\text{c.p.m.}} = {}^{14}C_{\text{c.p.m.}} - \frac{\%\ ^{3}H\ \text{spill}}{100}\left({}^{3}H_{\text{c.p.m.}} - \frac{\%\ ^{14}C\ \text{spill}}{100} \times {}^{14}C^{\text{corrected}}_{\text{c.p.m.}}\right)$$

The radioactivity due to carnitine was determined by calculating the corrected ^{3}Hc.p.m. for the eluted spot that contained carnitine, *P*-Cho and Gro*P*Cho, and subtracting from this the ^{3}Hc.p.m. due to Gro*P*Cho which were obtained after ion-exchange chromatography.

4.2.5. Tissue collection and storage

Liver tissue was collected at the time of slaughter of the wethers and stored as described in Section 2.2.2. in Chapter 2.

4.2.6. Tissue extractions

Liver tissue was extracted into trichloroacetic acid (50 g/l) containing thiodiglycol (4 g/l) as described in section 3.2.3.1. in Chapter 3.

4.2.7. Metabolite assays

Instrumentation was as described in section 3.2.4. in Chapter 3.

4.2.7.1. Glucose

Glucose was determined in the acid extracts of the gluconeogenesis incubations according to the method of Huggett & Nixon (1957). A glucose standard curve (0-400 nmol) was prepared for each isolated cell experiment.

4.2.7.2. Free methionine

Free methionine was estimated in the trichloroacetic acid extracts prepared as described in section 4.2.6. after ether extraction, using a Beckman Model 119 amino acid analyzer.

4.2.8. Chemicals

[*methyl*-^{14}C]Toluene (sp. radioactivity 1.12 x 10^6 d.p.m./g), n-[1,2(n)-^{3}H]hexadecane (sp. radioactivity 5.04 x 10^6 d.p.m./g) and tritiated water (sp. radioactivity 1.93 μCi/ml) were obtained from

The Radiochemical Centre, Amersham, Bucks., U.K. [1,2-^{14}C]Choline chloride (sp. radioactivity 6.35 µCi/µmol) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Sodium propionate, sodium pyruvate, sodium fumarate, sodium glutamate, 4-aminobutyrate, lysine monochloride, glucose oxidase (type II), peroxidase (type I), O-dianisidine dihydrochloride, sarcosine, *N*,*N*-dimethylglycine hydrochloride, collagenase (Sigma, Type I, Lot 94C-0019), and hyaluronidase (Sigma, Type I, Lot 24C-3290) were from the Sigma Chemical Co., St. Louis, Mo., U.S.A. Other materials were obtained from sources as described in Chapters 1, 2 and 3. Glass-distilled water was used throughout this work.

4.3. Results

4.3.1. Incorporation of L-[*methyl*-^{3}H]methionine and [1,2-^{14}C]choline into PtdCho of the isolated hepatocytes

The results in Fig. 4.6. show that the incorporation of methyl label into PtdCho was markedly less in the hepatocytes from the insulin withdrawn responder wether compared to those from the insulin stabilized responder wether. The initial lag in incorporation of the methyl label into PtdCho (i.e. the 30 min incubation) may have been due to an initial deficiency of intermediary metabolites that were leached from the hepatocytes during their isolation. ATP was needed for the methionine adenosyltransferase catalyzed biosynthesis of *S*-adenosylmethionine, and a shortage of this metabolite may have contributed towards the observed lag. A similar lag has been observed in freshly prepared isolated rat hepatocytes involving gluconeogenesis from lactate (Cornell *et al.*, 1974).

FIGURE 4.6.

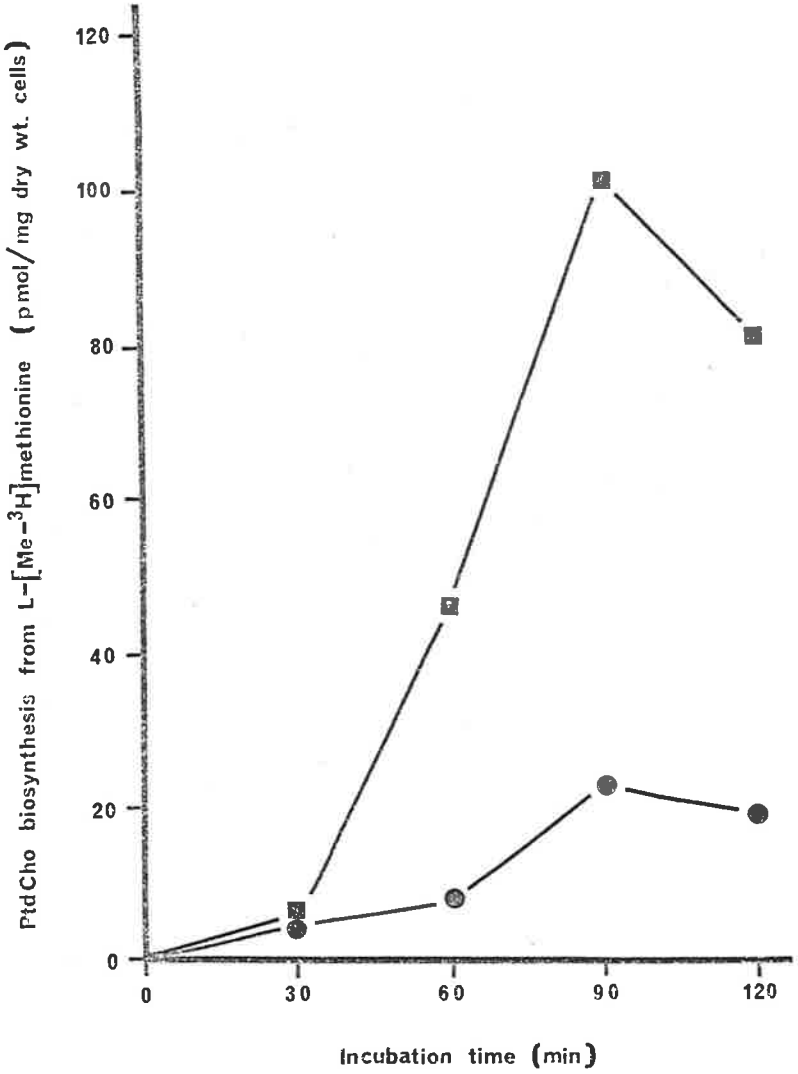
PtdCho biosynthesis from L-[methyl-^{3}H]methionine and [1,2-^{14}C]choline in isolated hepatocytes from alloxan-diabetic sheep*

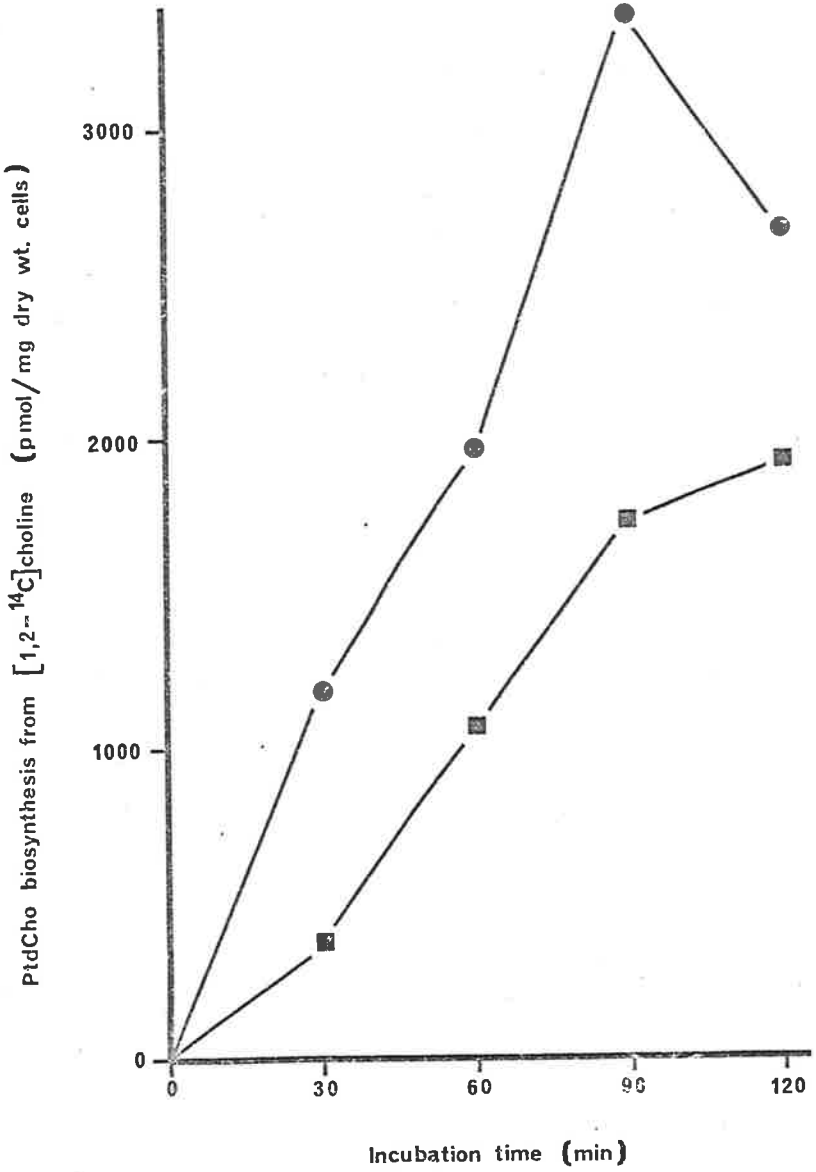
The hepatocytes were prepared as described in section 4.2.2. The incubation conditions are described in section 4.2.3.2. The PtdCho were extracted and separated as described in sections 4.2.3.3. and 4.2.3.4., and analyzed for radioactivity as described in section 4.2.4. Each point represents a single incubation containing 400 µl of cell suspension (3.8 mg and 4.6 mg dry wt. cells for wethers 3-43 and 3-115 respectively).

Key to the Figure:

- ● hepatocytes prepared from the insulin withdrawn wether 3-43
- ■ hepatocytes prepared from the insulin stabilized wether 3-115

* picomoles of L-[*methyl*-^{3}H]methionine incorporated x $^{1}/_{3}$


PtdCho biosynthesis from L-[Me-^{3}H]methionine (p mol/mg dry wt. cells)
120
100
80
60
40
20
0
0
30
60
90
120
Incubation time (min)


PtdCho biosynthesis from [1,2-^{14}C]choline (p mol/mg dry wt. cells)
3000
2000
1000
0
0
30
60
90
120
Incubation time (min)

The results in Fig. 4.6. show that in contrast to the methyl label, incorporation of [1,2-^{14}C]choline into PtdCho was markedly higher in the hepatocytes from the insulin withdrawn responder wether compared to those from the insulin stabilized responder wether. No lag period in label incorporation was evident for the CDP-Cho-pathway of PtdCho biosynthesis, perhaps owing to a stronger competition by this pathway for available endogenous substrates.

The decrease in incorporation of label for both pathways of PtdCho biosynthesis at 120 min was probably related to a breakdown in the metabolic integrity of the hepatocytes as possibly shown by the sharp fall in glucose production by these hepatocytes (see Fig. 4.3.) after 90 min of incubation. Clark *et al.* (1976) reported that lamb liver cells deteriorated rapidly at 37°C even under the best conditions of storage.

4.3.2. The metabolism of PtdCho in the isolated hepatocytes

The results in Table 4.2. show that the rate of PtdCho biosynthesis *via* methylation of PtdEtn, expressed as a percentage of the total rate of PtdCho biosynthesis *via* both the methylation and CDP-Cho-pathways was about 4.8% for the insulin stabilized wether and about 0.6% for the insulin withdrawn wether. This assumes that the rate of PtdCho biosynthesis *via* direct exchange of choline was negligible compared to the CDP-Cho-pathway. The incorporation of L-[*methyl*-^{3}H]methionine and [1,2-^{14}C]choline into Gro*P*Cho was lower in the hepatocytes from the insulin withdrawn wether and suggested that PtdCho catabolism was less in these hepatocytes compared to those from the insulin stabilized wether.

TABLE 4.2.

The metabolism of 3-sn-phosphatidylcholines in isolated sheep hepatocytes

The hepatocytes were prepared as described in section 4.2.2., incubated as described in section 4.2.3.2. and analyzed as described in sections 4.2.3.3., 4.2.3.4., and 4.2.4. n.d., not determined

Incubation time (min)	Metabolism of PtdCho							
	Insulin stabilized wether 3-115				24 h insulin withdrawn wether 3-43			
	Biosynthesis pmol/mg dry wt. cells			Degradation*	Biosynthesis pmol/mg dry wt. cells			Degradation*
	Total	% of Total			Total	% of Total		
		CDP-Cho-pathway	Methylation pathway			CDP-Cho-pathway	Methylation pathway	
0	0			0	0			0
30	383	98.4	1.6	n.d.	1,195	99.6	0.4	0
60	1,116	95.9	4.1	75	1,974	99.6	0.4	0
90	1,837	94.5	5.5	263	3,395	99.3	0.7	153
120	2,004	96.0	4.0	440	2,697	99.3	0.7	248

* estimated by the incorporation of [1,2-^{14}C]choline and L-[*methyl*-^{3}H]methionine into Gro*P*Cho

4.3.3. Incorporation of L-[*methyl*-^{3}H]methionine into carnitine of the isolated hepatocytes

The results in Fig. 4.7. show that the incorporation of methyl label into carnitine of the hepatocytes from the insulin withdrawn wether was slightly higher than that into the hepatocytes from the insulin stabilized wether. The initial lag in methyl label incorporation and the depressed incorporation at 120 min may be explained as described in section 4.3.1.

4.3.4. The metabolism of [1,2-^{14}C]choline in the isolated hepatocytes

The results in Fig. 4.8. show that betaine was an important metabolite of choline in the isolated cell preparations, particularly at later stages in the incubation. The ratio of [1,2-^{14}C] choline metabolism to betaine and [1,2-^{14}C]choline incorporation into PtdCho was 0.87, 2.04 and 4.02 for the insulin stabilized wether 3-115 at 60, 90 and 120 min incubation respectively. The corresponding values for wether 3-43 were 0.80, 1.85 and 2.22.

4.3.5. Total hepatic metabolite contents of the two Merino wethers used for the radioisotope studies with the isolated hepatocytes

The results in Table 4.3. have been taken from Chapters 2 and 3, and included in this Chapter to attempt to correlate the radioisotope data with tissue metabolite levels. The carnitine level appeared slightly higher in the insulin withdrawn wether, whereas the total hepatic $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratio appeared lower in this animal. The free methionine level appeared higher in the

FIGURE 4.7.

Carnitine biosynthesis from L-[methyl-^{3}H]methionine in isolated hepatocytes from alloxan-diabetic sheep*

Carnitine was extracted and separated as described in sections 4.2.3.3. and 4.2.3.4. Carnitine was not determined in the 30 min incubation for wether 3-115 due to experimental error. Other details were as described in Fig. 4.6.

Key to the Figure:

- ● hepatocytes prepared from the insulin withdrawn wether 3-43
- ■ hepatocytes prepared from the insulin stabilized wether 3-115

* picomoles of L-[*methyl*-^{3}H]methionine incorporated x $\frac{1}{3}$

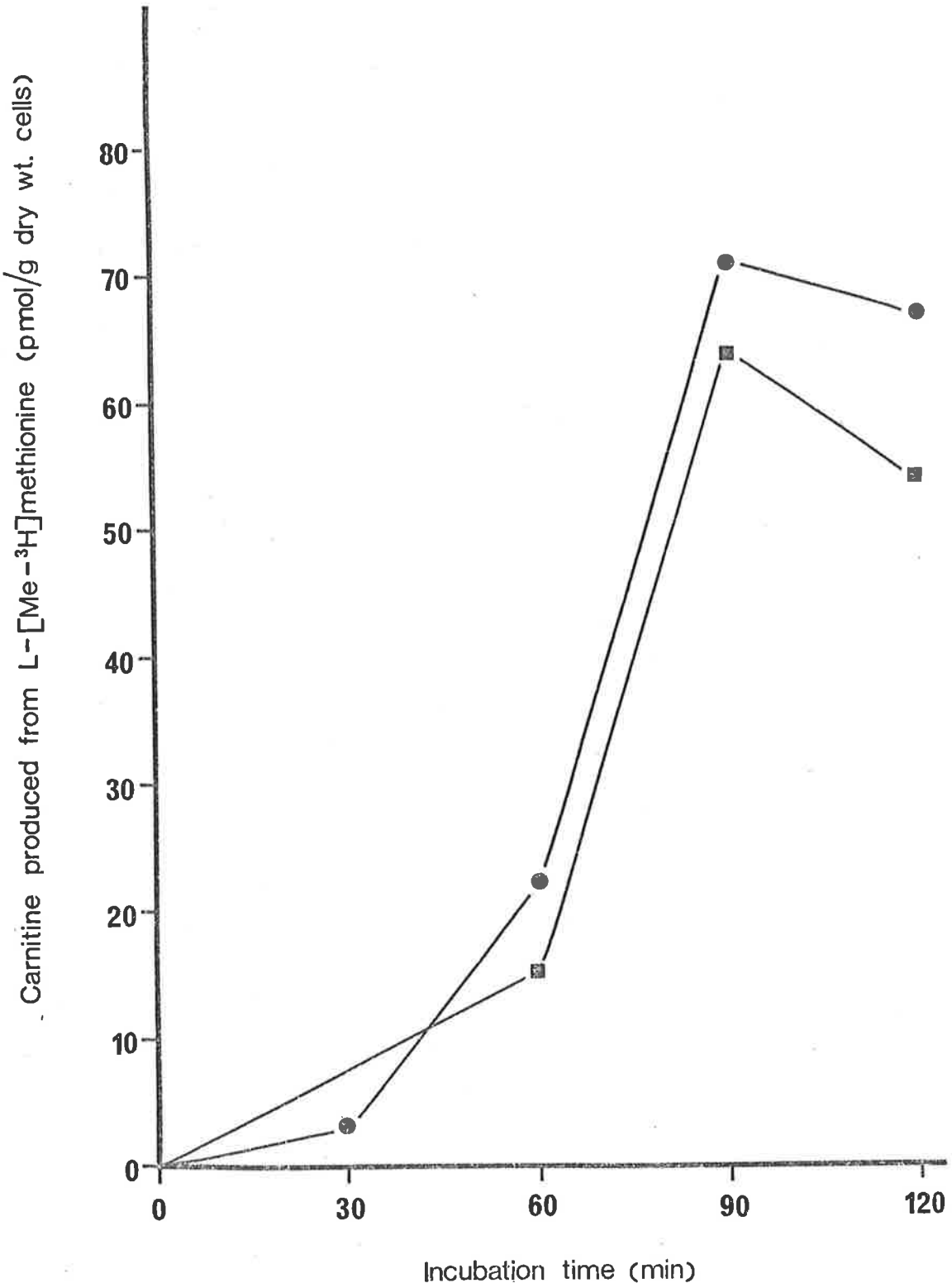

Carnitine produced from L-[Me-³H]methionine (pmol/g dry wt. cells)
80
70
60
50
40
30
20
10
0
0
30
60
90
120
Incubation time (min)

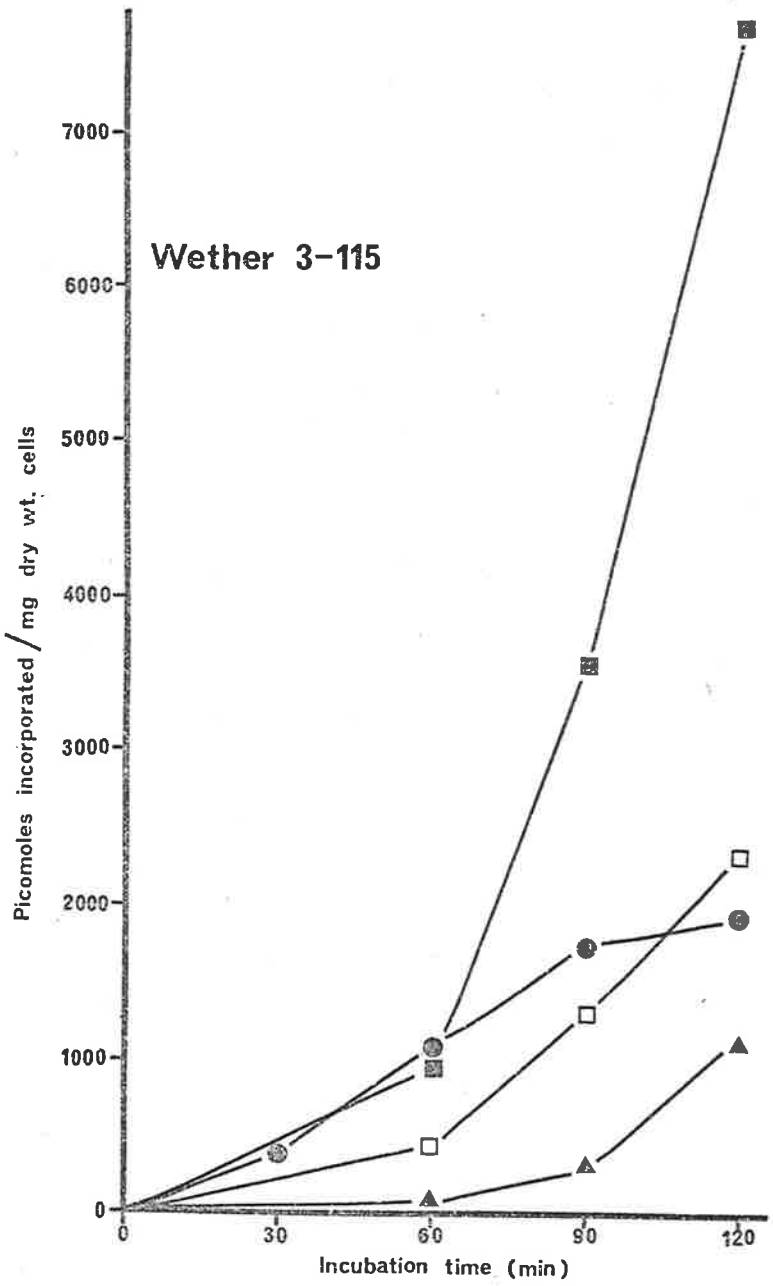
FIGURE 4.8.

[1,2-^{14}C]Choline metabolism in isolated hepatocytes from alloxan-diabetic sheep

The metabolites were extracted and separated as described in sections 4.2.3.3. and 4.2.3.4. The water-soluble metabolites in the 30 min incubation for wether 3-115 were not determined due to experimental error. Other details were as described in Fig. 4.6.

Key to the figure:

- ● PtdCho
- □ CDP-Cho
- ▲ *P*-choline
- ■ Betaine


Wether 3-115
Picomoles incorporated / mg dry wt. cells
7000
6000
5000
4000
3000
2000
1000
0
0
30
60
90
120
Incubation time (min)

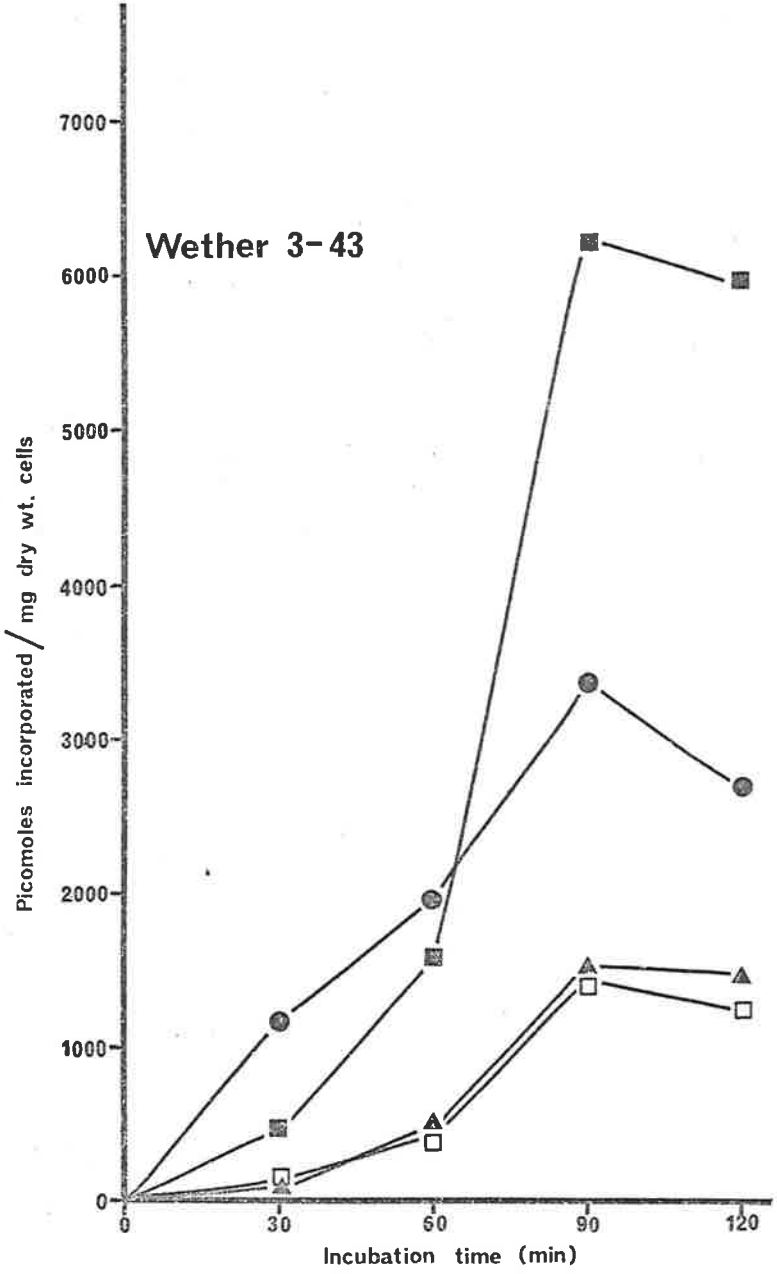

Wether 3-43
Picomoles incorporated / mg dry wt. cells
7000
6000
5000
4000
3000
2000
1000
0
0
30
60
90
120
Incubation time (min)

TABLE 4.3.

Hepatic metabolite contents in the two Merino wethers used for the radioisotope studies with isolated hepatocytes

Experimental treatment of the wethers is described in section 4.2.1. The tissues were collected, stored and extracted as described in sections 4.2.5. and 4.2.6. Metabolites were determined as described in Chapters 2 and 3 and section 4.2.7.

Metabolic parameter determined	Insulin stabilized wether 3-115	24 h insulin withdrawn wether 3-43
Hepatic metabolite content, expressed per g dry fat-free tissue		
creatinine (μequiv. Me moiety)	7.23	8.81
carnitine (μequiv. Me moiety)	2.61	3.99
PtdCho (μequiv. Me moiety)	308.55	338.34
Gro*P*Cho (μequiv. Me moiety)	137.49	151.77
free methionine (μequiv. Me moiety)	1.16	2.28
total lipid (mg)	237	273
total water (mg)	3,149	3,605
total phospholipid (μg phosphorus)	6,908	7,527
PtdEtn (μmol)	56.20	71.83
Total hepatic $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratio	1.83	1.57
Qualitative estimations		
Total hepatic triacylglycerols*	slightly higher in wether 3-43	
Total hepatic PtdCho molecular† subfractions	no differences apparent	

* estimated by visual assessment of the density of charring of thin-layer chromatograms processed as described in Chapter 2.

† estimated by visual assessment of the density of charring of the 6 molecular subfractions separated and processed as described in Chapter 2.

withdrawn animal, perhaps due to increased mobilization from muscle tissue. In general, these metabolite levels correlate with the radioisotope data.

4.4. Discussion

The present study demonstrated that the rate of PtdCho biosynthesis *via* methylation of PtdEtn, expressed as a percentage of the total rate of PtdCho biosynthesis *via* both the methylation and CDP-Cho-pathways was about 4.8% for the insulin treated responder wether and about 0.6% for the insulin treated responder wether which was subsequently withdrawn from insulin for 24 h. This value for the insulin treated responder was similar to values reported for adult male rats from *in vivo* studies (Wise & Elwyn, 1965; Bjørnstad & Bremer, 1966; Trewhella & Collins, 1972), but lower than that reported by Sundler & Åkesson (1975) using isolated hepatocytes from male rats. These workers used saturating concentrations of L-methionine (0.4 mmol/l) in their incubations, and obtained a value for the rate of PtdCho biosynthesis *via* methylation of PtdEtn, expressed as a percentage of the total rate of PtdCho biosynthesis *via* both pathways of about 30%. The conditions used in the present study were saturating with respect to L-methionine (2 mmol/l) and choline (4 mmol/l), as the concentration of these metabolites in sheep plasma are about 0.025 mmol/l (Reis *et al.*, 1973*b*) and 0.007 mmol/l (Snoswell & Mann, 1978) respectively.

The absolute rate of PtdEtn methylation in the present study was about 10% of that reported for rats by Sundler & Åkesson (1975) when their data was converted to a dry weight of cells basis by using for this conversion the data of Seglen (1973).

This rate was reduced about ten-fold in the insulin withdrawn responder, and strongly indicates that choline biosynthesis may be depressed in such animals. It is pertinent to note that the total hepatic $\frac{\text{PtdCho}}{\text{PtdEtn}}$ ratio was lower for the insulin withdrawn wether compared to the insulin stabilized wether (see Table 4.3.), as this ratio was used in Chapter 2 as a possible indicator of choline biosynthesis. Bremer & Greenberg (1961*a*) showed the specific activity of rat liver 3-*sn*-phosphatidylethanolamines methyltransferase was about 1.6-fold higher than that of sheep liver. Neill & Dawson (1977) reported that the methylation pathway did not appear more significant in sheep liver than rat liver. Thus the results obtained in the present study may be a true reflection of a relatively low rate of choline biosynthesis in sheep compared to rats. The incubations were performed in the presence of choline and methionine and consequently the effect of the saturating level of choline on the methylation pathway is unknown, although Sundler & Åkesson (1975) reported that choline did not inhibit PtdEtn methylation in their system.

The total rate of PtdCho biosynthesis appeared higher in the insulin withdrawn responder wether (see Table 4.2.) than the insulin treated responder. This correlates with the higher total phospholipid and total hepatic PtdCho contents measured for this wether compared to the insulin treated responder wether (see Table 4.3.). The higher rate was due to the higher rate of PtdCho biosynthesis *via* the CDP-Cho-pathway in the insulin withdrawn responder. This data may correlate with the qualitative argentation t.l.c. data (see section 2.3.7.2. and Fig. 2.12. in Chapter 2) and the fatty acid composition data for the liver total

phospholipids (see section 2.3.5.2. and Table 2.10. in Chapter 2), as the CDP-Cho-pathway appears more active *via* the formation of monoenoic and dienoic classes of PtdCho and the methylation pathway more active for the tetraenoic and polyenoic classes (> 4 double bonds) (van den Bosch, 1974; MacDonald & Thompson, 1975). The total rate of PtdCho biosynthesis *via* both pathways was about 50% in the insulin treated responder and about the same in the insulin withdrawn wether when compared to the data of Sundler & Åkesson (1975) for rat hepatocytes incubated in the presence of saturating levels of choline and methionine.

The rate of degradation of PtdCho, as estimated by the incorporation of [1,2-^{14}C]choline and L-[*methyl*-^{3}H]methionine into GroPCho appeared less in the insulin withdrawn responder wether (see Table 4.2.). This may partly explain the higher level of total hepatic PtdCho in the insulin withdrawn responder compared to the insulin treated responder (see Table 4.3.).

The initial observation of Snoswell & Koundakjian (1972) concerning the rapid increase in the liver carnitine content of an alloxan-diabetic insulin stabilized sheep that was subsequently withdrawn from insulin for 24 h was not repeated in this work, as shown in Table 4.3., probably due to technical differences in the method of insulin stabilization and withdrawal. Withdrawal of intravenous insulin was probably more sudden than the more gradual withdrawal obtained using sheep treated subcutaneously with insulin as performed in the present work. However, the data in Fig. 4.7. shows that the incorporation of L-[*methyl*-^{3}H]methionine into carnitine of the isolated hepatocytes from the insulin withdrawn responder was higher than that for the insulin treated responder,

in contrast to the data for PtdCho shown in Fig. 4.6. where incorporation of methyl label was decreased. The higher incorporation of L-[*methyl*-^{3}H]methionine into carnitine by the insulin withdrawn responder compared to the insulin stabilized responder was only about 18% of the observed decrease in choline biosynthesis between the two wethers at 60 min incubation (see Fig. 4.6.) and this reduced further to a value of about 9% after 90 min incubation. This was not convincing evidence of a direct competition for methyl groups between choline and carnitine biosynthesis under these experimental conditions, as a closer reciprocal change could be expected if a direct competition for substrate was involved. However, it is possible that a significant increase in methyl label had not entered carnitine itself at this stage and that the methyl label was still protein-bound, as protein-bound lysine is trans-methylated to protein-bound 6-*N*-trimethyl-lysine before proteolytic release of the 6-*N*-trimethyl-lysine from the proteins and its subsequent incorporation into carnitine (Cox & Hoppel, 1973*b*; Tanphaichitr & Broquist, 1973; La Badie *et al.*, 1976) presumably *via* the pathway recently elaborated in *Neurospora crassa* (Kaufman & Broquist, 1977). The total hepatic carnitine content was higher in the insulin withdrawn responder wether compared to the insulin stabilized responder wether (see Table 4.3.), thus the radioisotope data (see Fig. 4.7.) may reflect a very sensitive initiation of accelerated carnitine biosynthesis that had not yet been reflected in an elevated liver carnitine content.

Cox & Hoppel (1974*a*) failed to detect any incorporation of L-[*methyl*-^{14}C]methionine and *S*-adenosyl-L-[*methyl*-^{14}C]methionine into the carnitine of rat liver slices incubated for 2 h, and

ascribed this to the complex series of reactions necessary for their incorporation into carnitine. Corredor *et al.* (1967), in contrast, obtained a very small incorporation of *S*-adenosyl-L-[*methyl*-^{14}C]methionine into carnitine in rat liver homogenates incubated for 30 min. However, the chromatographic and identification procedures used by these workers were not as rigorous as those of Cox & Hoppel (1974*a*).

Hepatic triacylglycerols were apparently starting to accumulate in the insulin withdrawn wether compared to the insulin stabilized wether (qualitative estimation shown in Table 4.3. that was verified to some extent by the quantitative data for the total lipid contents of the livers). This correlates with the possible increased rate of carnitine biosynthesis in the insulin withdrawn wether and the apparent relationship between the total hepatic carnitine content and total lipid content of sheep liver shown in Fig. 3.4. in Chapter 3.

The ratio of [1,2-^{14}C]choline metabolism to betaine and [1,2-^{14}C]choline incorporation into PtdCho after 60 min of incubation was 0.84 (mean of similar values for the two sheep) under the present conditions (see Fig. 4.8.). This contrasts with the corresponding ratio of about 26 calculated from the data of Sundler & Åkesson (1975) for rat hepatocytes incubated in the presence of 4 mmol/l [*methyl*-^{3}H]choline. These results suggest that choline oxidation may be relatively less in sheep liver than rat liver as intimated by Neill & Dawson (1977). Finkelstein *et al.* (1971) postulated that betaine-homocysteine methyltransferase (enzyme 4 in Fig. 4 of the Literature Review) may be of major importance in

the catabolism of choline in rat liver. Consequently, it is interesting that the specific activity of this enzyme appears to be about 14-fold higher in rat liver (Finkelstein *et al.*, 1971) than sheep liver (Radcliffe & Egan, personal communication).

GENERAL DISCUSSION

The work presented in this thesis confirmed and extended the widely reported observation that sheep are prone to develop fatty livers under field and laboratory conditions. Triacylglycerols are the major form of lipid accumulating in the livers of stressed sheep, as similarly found for other animal species under various physiological conditions.

The apparent inability of sheep liver to synthesize VLDL which function to transport triacylglycerols to extrahepatic tissues (Coleman, 1973) is probably the prime cause of the accumulation of triacylglycerols in the livers of stressed sheep. This inherent inability to synthesize VLDL may be associated with the low rate of fatty acid synthesis in sheep liver (Ingle *et al.*, 1972*a*,*b*) as Windmueller & Spaeth (1967) showed that in perfused livers from fed rats, release of triacylglycerol was highly correlated with the rate of fatty acid synthesis. The basal lamina present in the hepatic sinusoids of sheep (see section 2.1. of the Literature Review) may impede the ready movement of large molecules such as VLDL between the hepatocytes and the blood (Karnovsky, 1968). The relatively high levels of LDL and in particular HDL in sheep blood (Leat *et al.*, 1976) may be related to the unique structure of the sinusoid as these lipoproteins are smaller than VLDL (Coleman, 1973) and can presumably traverse the basal lamina readily. Sheep in contrast to rats have not apparently evolved a mechanism for transporting significant quantities of triacylglycerols from the liver to extrahepatic tissues. Under normal physiological conditions this is unnecessary

as the liver does not synthesize significant quantities of fatty acids, the precursors of triacylglycerols.

Dible (1951) obtained a positive correlation between the total lipid content of liver and the total body fat content in rats and rabbits subjected to metabolic stress. Scow & Chernick (1960) and Mayes (1962) found the accumulation of hepatic triacylglycerols less in stressed rats which had depleted fat stores. These observations suggest the susceptibility of sheep to fatty liver development may be related to the total body stores of fat in these animals.

The hundreds of breeds of domesticated sheep, *Ovis aries* (Mason, 1969) probably originate from the wild sheep the mouflon (*Ovis musimon*, *Ovis orientalis*) (Short, 1976). The Asiatic mouflon (*Ovis orientalis*) has a body fat content of about 5% of the carcass weight (Ahmedov, 1968), as similarly reported for other wild ruminants (Rowett Research Institute and Hill Farming Research Organisation, 1974; Crawford, 1975*a*,*b*). Modern breeds of sheep have been selected for rapid growth and this has resulted in an increase in body fat in these animals (Mitchell, 1962) which lies in the range about 15%-40% of the carcass weight (Body & Shorland, 1964; Body *et al.*, 1966; Russel *et al.*, 1971; McClelland & Russel, 1972; Warren, 1976). This body fat is principally in the form of triacylglycerols (Body *et al.*, 1966). There are breed differences in the body fat % (Zubairov, 1966; Ahmedov, 1968; Searle & Griffiths, 1976; McClelland *et al.*, 1976). McClelland *et al.* (1976) showed that Soay sheep, perhaps the most primitive breed of domestic sheep found nowadays, had a significantly lower body fat % than similarly fed modern breeds of sheep. These

observations suggest that domesticated sheep may be prone to develop fatty livers because of the high body fat levels acquired over about 13,000 years of selection. During this time the ability to synthesize VLDL in the liver and the development of a more open liver sinusoid structure by loss or reduction of the basal lamina has not occurred.

The large increases in the liver carnitine content of sheep under conditions of metabolic stress (Snoswell & Henderson, 1970; Snoswell & Koundakjian, 1972; Pethick, 1975) which appear positively related to the total lipid content of the liver (see Fig. 3.4. in Chapter 3) may represent a metabolic adaptation whereby the fatty acids, whose liver uptake is increased under these conditions, are diverted to a greater extent to the oxidative pathways of metabolism *via* the carnitine palmitoyltransferase reaction (reaction 1 in Fig. 3. of the Literature Review) thus reducing the extent of esterification to triacylglycerols which cannot be readily transported out of the liver.

This increase in liver carnitine content is due to increased biosynthesis in alloxan-diabetic sheep (Snoswell & McIntosh, 1974) and would thus involve an increased utilization of L-methionine methyl groups *via* *S*-adenosylmethionine, the immediate methyl donor. *S*-adenosylmethionine is a substrate for many other methylation reactions besides those involved in carnitine biosynthesis (Salvatore *et al.*, 1977). The capacity of the sheep liver to synthesize *S*-adenosylmethionine *via* the methionine adenosyltransferase catalyzed reaction appears to be considerably less than that of some other animals including the rat liver (see Table 3.4.

in Chapter 3 and Sturman *et al.*, 1970). This is reflected in the lower liver content of *S*-adenosylmethionine in sheep (Gawthorne & Smith, 1974) compared to rats (Eloranta, 1977).

The relative quantitative importance of the numerous transmethylation reactions and the factors involved in their regulation are largely unknown (Cantoni, 1977), except that creatine metabolism is quantitatively the major route of methyl group utilization in mammals (Mudd & Poole, 1975). The factors that regulate the activity of guanidinoacetate methyltransferase are unknown. The ratio of hepatic specific activities $\frac{\text{methionine adenosyltransferase}}{\text{guanidinoacetate methyltransferase}}$ are in the range 1-6 for sheep and 14-46 for rats (see Tables 3.4 and 3.5 in Chapter 3), thus it is evident that the sheep liver may be more prone to the development of a deficiency of active methyl groups than the rat.

Early studies with rats (Mackenzie *et al.*, 1949) suggested that choline biosynthesis is a major factor in methionine methyl group utilization as well as creatine; however, 3-*sn*-phosphatidylethanolamines methyltransferase is a poor competitor for available methyl groups under certain conditions (Bremer & Greenberg, 1961*a*, *b*). Evidence reported in this thesis showed total hepatic $\frac{\text{PtdCho}}{\text{carnitine}}$ ratios fell dramatically in stressed sheep that developed fatty livers, implying that the relative importance of carnitine metabolism in methyl group utilization was significantly increased. McCarthy *et al.* (1968) reported depressed levels of choline phospholipids in the VLDL and LDL of ketotic cows compared to non-ketotic cows. This is a typical finding in choline-deficient rats (Mookerjea, 1971). A similar effect is likely to occur in pregnant-toxaemic ewes as this condition is metabolically similar to bovine

ketotis (Baird, 1977). These observations, together with the observations reported in this thesis, suggest that choline biosynthesis may be impaired in sheep liver under those conditions where the liver carnitine content is significantly elevated. Preliminary observations in Chapter 4 of this thesis provide more direct evidence that choline biosynthesis is depressed in alloxan-diabetic sheep, and further, that carnitine biosynthesis may be increased. The decreased biosynthesis of choline under these conditions could be expected to exacerbate the fatty liver, as choline is required in the synthesis and secretion of lipoproteins (Mookerjea, 1971).

The significance of the depressed creatine levels in the livers of the abattoirs sheep (see Table 3.2. in Chapter 3) is not entirely clear. However, as creatine biosynthesis represents the major drain on methyl groups in the sheep which in turn has a limited capacity to synthesize these groups, a decrease in creatine biosynthesis may represent an adaptation to conserve methyl groups. This may be necessary to help sustain the biosynthesis of choline under metabolic conditions in which carnitine biosynthesis competes for an increasing proportion of the methyl pool. The important role of carnitine in the oxidation of fatty acids in sheep liver mitochondria (Koundakjian & Snoswell, 1970) and its wider role in the regulation of fatty acid metabolism (Bremer, 1977) may result in its biosynthesis taking preference over that of other methylated metabolites under those physiological conditions that lead to an increased liver uptake of fatty acids. These metabolic changes may all stem from the high body fat content of sheep which renders these animals prone to the development of fatty livers under conditions of metabolic stress due to

the inability to transport triacylglycerols from the liver.

Important questions remain to be clarified in this general area of metabolism in sheep. The metabolism of choline is poorly understood, due in no small way to the lack of a specific, sensitive assay for this metabolite in the past. Estimates of the total choline content of sheep liver vary from 28-52 μmol/g wet tissue wt. (Luecke & Pearson, 1945; Schmidt *et al.*, 1952; Smith *et al.*, 1974). Thus, an important area for future studies is the measurement of free choline and choline compounds in sheep tissues and the effect upon these levels of conditions of metabolic stress. Investigations into the significance of the relatively high levels of GroPCho in sheep liver in relation to choline metabolism are needed. Studies on choline metabolism are needed in view of the low dietary intake of choline in sheep (Neill & Dawson, 1977). The significance of choline catabolism in relation to the overall choline statis of sheep needs clarification. Virtually nothing is known concerning the regulation of creatine metabolism in sheep. This is an important area in view of the fact that this is the major methylated metabolite in sheep, as in other mammals, and L-methionine is frequently the first limiting amino acid (Chalupa, 1972). Technical advances in relation to the preparation of metabolically active isolated sheep hepatocytes (Ash *et al.*, 1975; Clark *et al.*, 1976) opens the way to answer many of these questions by enabling the use of radioisotopes to follow fluxes of metabolites and pathways of metabolism.

APPENDICES

The analytical data reported in this thesis has been expressed using dry fat-free tissue weight as the reference base, for reasons outlined in section 2.2.7. of Chapter 2. Most of the analytical data reported in the literature is expressed using wet tissue weight as the reference base. Thus it was considered desirable to include the data for the individual animals studied in this work in the form of several Appendices, to enable accurate conversion of the results into the more common form, and hence enable more direct comparisons with values reported in the literature. A further reason for including data for individual animals was due to the difficulty in grouping animals for statistical analysis. This was particularly true in the alloxan-diabetic and pregnant-toxaemic animals where differing individual responses and severity of the disease were observed. Thus by including the data for individual animals it was possible to prepare alternative groupings and analyses based on selected metabolic responses.

APPENDIX 1.

Hepatic metabolite contents in normal and alloxan-diabetic sheep and rats, pregnant-toxaemic ewes and neonatal lambs

Experimental group of animals	Animal	Total water mg/g wet tissue	Total lipid wt.	PtdCho µmol per g dry fat-free tissue	PtdEtn µmol per g dry fat-free tissue	Total phospholipid µg phosphorus per g dry fat-free tissue	Creatinine µ equiv. Me moiety per g dry fat-free tissue	Carnitine µ equiv. Me moiety per g dry fat-free tissue
Normal, adult Merino sheep	wether 1	713	50	107.97	58.05	n.d.+	n.d.	n.d.
	wether 2	710	52	78.49	48.75	n.d.	n.d.	n.d.
	wether 4278	713	51	n.d.	n.d.	n.d.	20.63	0.13*
	ram 4127	718	55	n.d.	n.d.	n.d.	13.38	0.85*
	ram 3	716	56	102.68	51.08	n.d.	n.d.	n.d.
Alloxan-diabetic Merino wethers								
Uncontrolled non-responders	3-188	719	55	113.01	57.08	n.d.	7.62	2.79*
	3-66	713	73	137.29	63.56	n.d.	14.14	5.52*
	3-41	698	76	115.62	56.13	6,686	9.37	3.09
Uncontrolled responders	3-59	682	101	72.44	47.04	n.d.	6.51	37.02*
	3-70	662	154	123.04	71.12	n.d.	10.84	53.48*
Insulin treated responders	3-115	718	54	102.85	56.20	6,908	7.23	2.62
	3-161	729	62	113.73	61.48	6,962	7.63	5.00
Insulin withdrawn responders	3-131	756	64	108.06	58.41	7,572	5.23	1.95
	3-53	711	70	122.56	79.07	7,434	6.28	1.48
	3-40	685	99	64.44	39.53	5,801	7.60	3.13
	3-29	680	130	94.11	43.57	7,237	7.37	34.45
	3-43	739	56	112.78	71.83	7,527	8.81	3.98
Hooded Wistar rats								
Normal rats	5	698	53	n.d.	n.d.	n.d.	1.87	1.49
	6	692	63	74.32	36.24	5,359	4.52	3.20
	7	688	51	64.07	34.14	4,935	1.58	3.33
	8	700	62	56.84	21.70	5,643	n.d.	2.13
Alloxan-diabetic rats	9	722	77	81.11	43.53	6,234	7.06	7.33
	10	729	67	80.87	25.00	6,127	8.85	5.84
Pregnant-toxaemic ewes								
Untreated Merino crossbred	1a	667	161	101.59	59.83	n.d.	n.d.	40.41*
ewes slaughtered at abattoirs	2a	556	211	68.76	50.75	n.d.	n.d.	18.57*
	3a	626	186	104.11	65.90	n.d.	n.d.	28.35*
	4a	528	258	73.36	49.78	n.d.	n.d.	12.51*
	5a	567	233	77.48	52.66	n.d.	n.d.	23.19*
Treated Merino crossbred	3	723	72	61.02	47.85	n.d.	9.72	0.89*
ewes slaughtered at Waite	6	703	75	n.d.	n.d.	n.d.	9.97	2.72*
	8	705	80	76.19	53.30	n.d.	9.00	1.48*
	10	619	209	91.98	65.70	n.d.	7.09	27.09*
A single Corriedale ewe	11	620	204	72.90	53.86	5,028	n.d.	230.01
Neonatal, Merino crossbred lambs	3	767	15	18.94	14.13	n.d.	2.85	1.80*
	ram 6	781	17	26.63	21.65	n.d.	4.06	1.99*
	ewe 6	782	32	37.10	24.73	n.d.	3.76	2.13*
	ram 8	819	22	47.48	29.86	n.d.	4.30	3.70*

+ n.d., not determined * Total acid-soluble carnitine; Other values were total hepatic carnitine

APPENDIX 2.

Hepatic metabolite contents in wethers infused abomasally with L-methionine

Experimental group of animals	Animal	Total water	Total lipid	Total phospholipid	PtdCho	PtdEtn	Σlyso-PtdCho, CerPCho	Total lipid choline	Creatinine	Carnitine*
		mg/g wet tissue wt.		µg phosphorus per g dry fat-free tissue	µmol per g dry fat-free tissue				µequiv. Me moiety per g dry fat-free tissue	
0 g met/kg body wt.$^{0\cdot75}$/day	53	704	45	4,881	54.18	24.12	20.26	74.44	7.23	1.11
	55	713	42	5,480	72.80	33.60	8.40	81.20	6.47	0.88
	44	705	50	5,480	83.02	28.17	5.27	88.29	8.30	1.22
	57	709	46	5,184	78.24	33.60	7.58	85.82	6.06	1.08
0.12 g met/kg body wt.$^{0\cdot75}$/day	54	710	39	4,442	21.54	4.02	21.54	43.08	5.10	0.47
	52	716	40	4,754	33.08	10.92	5.46	38.54	5.43	0.69
	56	704	48	4,506	48.98	29.62	9.93	58.91	5.37	0.32
	58	713	49	5,536	63.42	15.60	7.12	70.54	6.65	0.89
0.36 g met/kg body wt.$^{0\cdot75}$/day	51	690	54	4,395	65.10	22.86	6.31	71.41	4.21	0.36
	62	712	47	4,502	63.46	22.44	7.87	71.33	5.60	0.98
	61	727	50	5,762	45.60	12.31	43.97	89.57	6.14	1.53
	60	708	51	5,539	89.75	37.17	7.03	96.78	5.59	1.49

* Total hepatic carnitine

APPENDIX 3.

The total lipid and water contents of several tissues of normal and alloxan-diabetic sheep

Experimental group of animals	Animal	Total water mg/g wet tissue wt.			Total lipid mg/g wet tissue wt.		
		Kidney cortex	Heart	Skeletal muscle (M. *biceps femoris*)	Kidney cortex	Heart	Skeletal muscle (M. *biceps femoris*)
Normal, adult Merino sheep	Ram 4127	841	803	787	33	30	30
	Wether 4278	796	785	769	42	34	37
Alloxan-diabetic Merino wethers							
Uncontrolled non-responders	3-66	799	801	790	35	38	27
	3-188	792	806	796	30	25	n.d.*
Uncontrolled responders	3-70	785	771	764	63	36	25
	3-59	785	761	766	53	59	35

* n.d., not determined

APPENDIX 4.

Hepatic metabolite contents in abattoirs sheep

Visual classification of the liver	Liver number	Total water mg/g wet tissue wt.	Total lipid* mg/g wet tissue wt.	Creatinine µequiv. Me moiety per g dry fat-free tissue	Carnitine† µequiv. Me moiety per g dry fat-free tissue
Non-fatty	1	707	68	9.93	5.11
"	2	717	56	10.92	5.64
"	3	710	64	8.47	3.86
"	4	717	56	5.97	3.97
"	5	710	64	3.14	7.74
"	6	758	9‡	0.74§	1.81§
"	7	741	28‡	0.86§	0.70§
Fatty	8	643	142	2.92	6.04
"	9	636	150	3.36	5.87
"	10	679	100	2.06	4.59
"	11	633	154	3.91	6.90
"	12	598	194	3.91	4.18
"	13	627	161	2.98	6.20
"	14	664	118	4.41	5.77

* estimated using the relationship in section 2.3.3.4.
† total acid-soluble carnitine
‡ values considered unrealistic
§ metabolites expressed per g wet tissue wt.

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