CHOLINE NUTRITION AND METABOLISM IN SHEEP

A Thesis

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by

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"Knowledge is proud that he has learned
so much; Wisdom is humble that he
knows no more".

William Cowper (1731-1800) : *The Task*, bk. VI, The Winter Walk at Noon, 1.96.

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SUMMARY

In the literature it has been recently reported that sheep derive relatively little choline from the diet as there is almost complete microbial breakdown of dietary choline to trimethylamine and methane in the rumen. The low amount of dietary choline received by sheep would certainly be, on a body-weight basis, inadequate for preventing severe pathological lesions (e.g. fatty liver, haemorrhagic kidney) and death in several non-ruminant species. Sheep appear to survive on a very limited dietary choline intake, in part, by conserving body choline efficiently. The overall aim of the work in this thesis was to make an important contribution to the knowledge of choline nutrition and metabolism in sheep with particular emphasis on the reasons why sheep have a minimal need for dietary choline.

Dried summer pastures and fresh pasture commonly used to graze sheep in Australia were analysed to compare the concentrations of unesterified choline, glycerophosphocholine, phosphocholine, lipid choline and betaine. In dried wheat, barley and triticale stubbles and dried medic and lucerne hays most of the choline was present as unesterified choline and/or glycerophosphocholine in contrast to fresh grass/clover pasture in which choline mainly existed as lipid choline. There were lower concentrations of total choline and betaine in the dried stubbles and hays compared with the fresh pasture.

A radioenzymic assay of choline using [^{3}H] acetyl-CoA and partially purified choline acetyltransferase was developed for the

analysis of sheep tissues and fluids. The assay procedure was sensitive and specific for choline in the presence of carnitine and carnitine acetyltransferase. The method was used to measure the various forms of choline in digesta, saliva, bile, faeces and urine of sheep fed lucerne hay to assess the flux of choline in the alimentary and urinary tracts. The passage of total choline (mainly in the form of lipid choline) in the rumen, abomasal and duodenal digesta of the sheep was only a few percent of the total choline available in the diet. There was a significant total choline flux in the saliva (largely as unesterified choline) which would enrich the rumen digesta. The total choline flux in the bile was very substantial (mainly as lipid choline) which would supplement the duodenal digesta. Low amounts of choline were excreted in the faeces and urine of the sheep.

The net uptake and output of plasma unesterified choline, glycerophosphocholine, phosphocholine and lipid choline by organs of conscious chronically catheterized sheep were measured. There was significant production of plasma unesterified choline by the alimentary tract, brain, heart, hindlimb muscle and the upper- and lower-body regions drained by the venae cavae and uptake by the liver, lungs and kidneys. The upper- and lower-body regions drained by the venae cavae provided the bulk (about 87%) of the total body venous return of plasma unesterified choline with a major contribution by the muscle mass. Production of plasma unesterified choline by the alimentary tract was approximately balanced by the plasma unesterified choline taken up by the liver, and was almost equal to the amount of choline secreted in the bile. There was a

considerable amount of glycerophosphocholine in the liver and there was production of plasma glycerophosphocholine by the liver and uptake by the lungs and kidneys. The concentration of glycerophosphocholine was higher in the plasma of sheep than in that of cattle and rats. Plasma phosphocholine was produced by the alimentary tract and kidneys. There was production of plasma lipid choline by the upper- and lower-body regions drained by the venae cavae. In rats there was no uptake or output of plasma unesterified choline or lipid choline by the lower-body region drained by the inferior vena cava in contrast to sheep.

The capacity of various sheep and rat tissues to synthesize choline was determined *in vitro* by monitoring the overall methylation of endogenous phosphatidylethanolamine to phosphatidylcholine in isolated microsomal preparations using *S*-adenosyl-L-[*methyl*-^{14}C] methionine as the methyl donor. Phosphatidylcholine was the main phospholipid product and radioactivity was specifically associated with the choline portion of the molecule. In sheep the liver was the major site of choline synthesis, but there was also appreciable production in other tissues, particularly the lungs, kidneys, alimentary tract and skeletal muscle. In rats choline synthesis was of quantitative significance in the liver but not in extrahepatic tissues.

A surgically prepared sheep with portal vein, duodenal and gallbladder catheters and ligated bile duct was infused with radioactively-labelled choline compounds to establish the extent of retention and reutilization of bile choline. The recovery of [*methyl*-^{3}H] choline chloride radioactivity infused *via* the portal vein in bile lipid choline was 48.4% after 24h and 71.2% after

120h of bile collection. The recovery of [^{3}H] lipid choline bile radioactivity infused *via* the duodenum in bile lipid choline was 25.1% and 72.3% after 24h and 120h of bile collection respectively. Radioactivity in the bile lipid choline was almost exclusively associated with the choline moiety of phosphatidylcholine. The amount of choline reincorporated into bile lipid choline for the sheep was several times higher than the levels previously reported for rats and humans.

The main findings of the present investigation were that sheep synthesize considerable amounts of choline in tissues other than the liver and have the capacity for extensive retention and recycling of bile choline. These factors, coupled with a slow turnover of the endogenous choline body pool, explain the insensitivity of sheep to a low dietary choline supply compared to many non-ruminant species.

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the Ph.D. degree.

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BRENTON SCOTT ROBINSON

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Robinson, B.S., Snoswell, A.M., Runciman, W.B. & Upton, R.N. (1985). Extrahepatic synthesis of choline in the sheep. In *Absts. 13th Int. Cong. Biochem., Amsterdam*, p. 563, International Union of Biochemistry.

PREFACE

Abbreviations approved by the Biochemical Journal (1983) for use without definition are used as such throughout this thesis.

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

The recommendations of the Nomenclature Committee of the International Union of Biochemistry (1979, 1980, 1981) on the nomenclature and classification of enzymes have been followed as far as possible. The following enzymes are referred to by name only:

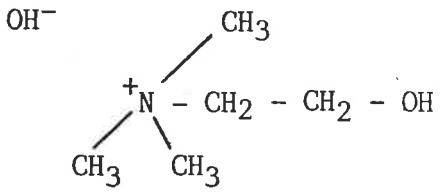
Acetylcholinesterase	EC 3.1.1.7
Acid phosphatase	EC 3.1.3.2
Adenosinetriphosphatase	EC 3.6.1.3
Adenosylhomocysteinase	EC 3.3.1.1
Adenylate cyclase	EC 4.6.1.1
Alkaline phosphatase	EC 3.1.3.1
Betaine-aldehyde dehydrogenase	EC 1.2.1.8
Betaine-homocysteine methyltransferase	EC 2.1.1.5
Carnitine acetyltransferase	EC 2.3.1.7
Ceramide cholinephosphotransferase	EC 2.7.8.3
Choline acetyltransferase	EC 2.3.1.6
Choline dehydrogenase	EC 1.1.99.1
Choline kinase	EC 2.7.1.32
Choline oxidase	EC 1.1.3.17
Cholinephosphate cytidylyltransferase	EC 2.7.7.15
Cholinephosphotransferase	EC 2.7.8.2
Cystathionine β-synthase	EC 4.2.1.22
Cystathionine γ-lyase	EC 4.4.1.1

Glycerophosphocholine phosphodiesterase	EC 3.1.4.2
Guanidinoacetate methyltransferase	EC 2.1.1.2
3α-Hydroxysteroid dehydrogenase	EC 1.1.1.50
Lecithin-cholesterol acyltransferase	EC 2.3.1.43
Lysophospholipase	EC 3.1.1.5
Methionine adenosyltransferase	EC 2.5.1.6
5-Methyltetrahydrofolate-homocysteine methyltransferase	EC 2.1.1.13
NADPH-cytochrome *c* reductase	EC 1.6.2.4
Peroxidase	EC 1.11.1.7
Phosphatidylethanolamine methyltransferase	EC 2.1.1.17
Phospholipase A_1	EC 3.1.1.32
Phospholipase A_2	EC 3.1.1.4
Phospholipase B	EC 3.1.1.5
Phospholipase C	EC 3.1.4.3
Phospholipase D	EC 3.1.4.4
Protein (lysine) methyltransferase	EC 2.1.1.43
Sphingomyelin phosphodiesterase	EC 3.1.4.12

LITERATURE REVIEW

1. Introduction

Choline, (β-hydroxyethyl) trimethylammonium hydroxide, is a quaternary ammonium compound with the following structural formula:



It was first discovered by Strecker in 1862 when it was isolated from hog bile (Gr. *cholĕ*, bile). The correct structure of choline was determined and was chemically synthesized by Baeyer in 1866. Chemically, choline is a strong organic base that exists as a colourless hygroscopic viscous liquid which is soluble in water and alcohol and insoluble in ether. It is usually crystallized as the chloride. Choline is ubiquitously present in animals, plants and micro-organisms in the unesterified form and as a component of phospholipids (phosphatidylcholine, lysophosphatidylcholine, sphingomyelin and choline plasmalogens) and water-soluble esters (mainly glycerophosphocholine, phosphocholine and acetylcholine) (Griffith *et al.*, 1971).

Choline has several important biochemical functions within the animal body:

(1) It is a lipotropic factor which prevents the accumulation of abnormal quantities of fat in the liver. It also prevents haemorrhagic degeneration of the kidneys.

(2) It is a precursor for the biosynthesis of phospholipids which are essential components of cell membranes, lipoproteins and bile.

(3) It is a precursor for the biosynthesis of the neurotransmitter acetylcholine, which is essential for normal brain function.

(4) It is a precursor for the biosynthesis of betaine which is a labile methyl donor for many transmethylation reactions in intermediary metabolism.

The animal body can synthesize choline by a transmethylation process which is dependent on the supply of methionine and betaine as labile methyl donors and vitamin B_{12} (cyanocobalamin) and folic acid as cofactors. In most animal species, especially in the young, choline synthesis is insufficient to meet physiological needs and there is a dietary choline requirement. Choline is therefore an accessory food factor which is often regarded as a vitamin of the B complex necessary for normal growth and metabolism of animals (Lucas & Ridout, 1967; Griffith *et al.*, 1971; Kuksis & Mookerjea, 1978).

The purpose of this review is to highlight aspects in the literature on choline nutrition and metabolism in animals up to the time when the present study was commenced in early 1981. While this review pertains to animals in general, specific reference is made to the situation in the sheep where possible, although in many areas such information is simply not available.

2. The diet as a source of choline

The dietary requirement for choline is influenced by many factors such as the age, sex and strain of the animal, its rate of growth, energy intake and expenditure, intake and type of dietary fat, carbohydrate and protein and the environmental temperature. The presence

of factors in the diet that influence the endogenous biosynthesis of choline such as methionine, betaine, folic acid and vitamin B_{12} also affect the dietary choline requirement. Young animals have a higher dietary choline requirement than mature animals (Griffith *et al.*, 1971; Lucas & Ridout, 1967). The neonatal animal requires a great deal of choline for growth related membrane synthesis and production of lung surfactant (mostly disaturated phosphatidylcholine) and brain acetylcholine (Zeisel *et al.*, 1980*a*).

The choline requirements for non-ruminant species such as rats, mice, guinea pigs, hamsters, rabbits, dogs, cats, pigs and poultry have been found to be 0.1% or more of the total dietary intake of dry matter (Reid, 1955; Lucas & Ridout, 1967; National Research Council, 1974, 1977*a*, *b*, 1978*a*, *b*, 1979). There are no specific choline requirements published for sheep and other ruminant animals (National Research Council, 1975, 1976, 1978*c*; Agricultural Research Council, 1980). Choline is an essential dietary nutrient in the pre-ruminant calf, and it is suggested that an adequate choline intake is 0.26% per unit of milk replacer (National Research Council, 1978*c*). It is thought that all the vitamins of the B complex are synthesized by rumen micro-organisms in amounts adequate for normal metabolism in the ruminant animal and secretion of normal amounts into milk to satisfy the needs of the pre-ruminant (Kon & Porter, 1954; Ling *et al.*, 1961; Clifford *et al.*, 1967). However, ruminal choline synthesis was not studied in any of these experiments, and there are reports that rumen micro-organisms are incapable of net choline synthesis (Broad & Dawson, 1975, 1976; Harfoot, 1978).

Dietary choline deficiency produces severe pathological lesions and death in many non-ruminant animal species. In rats, mice, hamsters, guinea pigs, rabbits, dogs, pigs, monkeys, chickens and ducklings a diet deficient in choline results in fatty infiltration of the liver (Griffith *et al.*, 1971; Lucas & Ridout, 1967) which is probably due to a decrease in phosphatidylcholine synthesis, thus disturbing the synthesis or secretion of plasma lipoproteins involved in the removal of hepatic triglycerides (Mookerjea, 1971; Lombardi, 1971). Haemorrhagic kidney lesions due to choline deficiency have been observed in young rats and pigs (Griffith *et al.*, 1971; Lucas & Ridout, 1967) which is believed to be caused by a concomitant decrease in acetylcholine and deficiency of Factor V involved in blood clotting (Wells, 1971). Cirrhosis, growth failure, bone abnormalities, infertility, muscle defects, haemorrhagic lesions, cardiovascular lesions, neurological lesions, gastric lesions, neoplasms, anaemia, oedema, hypertension and various biochemical alterations have also been reported as consequences of choline - deficient diets in non-ruminant species. There are no reports of dietary choline deficiency in man (Griffith *et al.*, 1971; Lucas & Ridout, 1967; Kuksis & Mookerjea, 1978).

There is very little information in the literature concerning dietary choline deficiency in ruminant animals. Johnson *et al.* (1951) showed that two-day-old dairy calves fed an artificial milk replacer diet devoid of choline developed an acute deficiency syndrome after about seven days. The symptoms included marked weakness and inability to stand, laboured or rapid breathing, anorexia, and in some cases slight fatty liver and renal haemorrhage. Unless the

deficiency had progressed too far, the animals responded to choline supplementation. Later removal of choline from the diet when the calves were a few weeks old did not produce deficiency symptoms. This suggested that body choline synthesis of the calves was adequate once the initial critical period was past. Earlier studies by Waugh *et al.* (1947*a*, *b*) had emphasized the importance of choline in colostrum and milk to the choline nutrition of calves. A choline deficiency syndrome has never been documented for sheep.

Choline, mainly in the form of phosphatidylcholine and unesterified choline, is widely distributed in the foods consumed by monogastric animals. Important dietary sources of choline include egg yolk, meats, cereals and legumes. Fruits and vegetables are generally poor choline sources (Lucas & Ridout, 1967; Griffith *et al.*, 1971; Wurtman, 1979). Some of the free choline ingested by monogastric species is degraded to trimethylamine (De la Huerga & Popper, 1951, 1952; Michel, 1956; Asatoor & Simenhoff, 1965) and betaine (Flower *et al.*, 1972*a*, *b*) by bacteria in the intestine before it can be absorbed.

The main dietary source of choline for mature sheep is phosphatidylcholine and unesterified choline of plant material. Although the choline content of forage is quite low and variable (Glick, 1945; Roughan & Batt, 1969; Storey & Wyn Jones, 1977; Snoswell *et al.*, 1978; Neill *et al.*, 1979; Robinson, 1980), the total amount of choline ingested by sheep may be considerable because of the very large bulk of dietary material consumed. In contrast to choline nutrition of non-ruminant animals, the adult sheep derives very little choline from that present in the diet.

Early studies showed that sheep rumen micro-organisms rapidly hydrolysed a ^{32}P-labelled phosphatidylcholine substrate added in the pure liquid crystalline form (Dawson, 1959). The degradative pathway was primarily through deacylation producing initially glycerophosphocholine which was then decomposed to glycerophosphoric acid and unesterified choline. More recently the rapid decomposition of phosphatidylcholine in plant material fed to sheep without ciliated protozoa in their rumen has been demonstrated (Dawson & Kemp, 1969), as well as the catabolism of phosphatidylcholine in totally-labelled [^{14}C] grass introduced into the normal rumen (Dawson & Hemington, 1974*a*; Broad & Dawson, 1976). It is possible that a certain proportion of dietary plant lipids may be decomposed in the rumen by lipolytic enzymes present in the leaves themselves, rather than exclusively by micro-organisms (Dawson & Hemington, 1974*a*; Faruque *et al.*, 1974). However, the pathway of phosphatidylcholine degradation which was observed to proceed *via* glycerophosphocholine does not conform with the action of the main phospholipid-hydrolysing enzyme found in plant tissue, phospholipase D. In addition, Dawson & Hemington (1974*a*) found little phospholipase activity in the bolus delivered to the rumen and Dawson & Hemington (1974*b*) established that ruminant saliva contains a powerful inhibitor of plant phospholipase D. A bacterium, tentatively identified as a non-cellulolytic strain of *Butyrivibrio fibrisolvens*, has been isolated from the sheep rumen which produces active phospholipases and can completely deacylate phosphatidylcholine (Hazlewood & Dawson, 1975, 1976).

Choline of ingested plant material in the unesterified form and that derived from the hydrolysis of phosphatidylcholine is

rapidly degraded in the rumen by bacteria, and only a negligible amount is absorbed as such and becomes incorporated into tissues. The *N*-methyl groups of choline are almost totally converted into trimethylamine and ultimately methane, which is lost in the gaseous mixture resulting from rumen fermentation (Neill & Dawson, 1977; Neill *et al.*, 1978, 1979). The metabolism of trimethylamine to methane is easily saturated by an excess of substrate, so that trimethylamine accumulates in the rumen of the fed animal. The demethylation of trimethylamine to produce methane is presumably independent of the cleavage of choline and is likely to be carried out by different bacterial species (Neill *et al.*, 1978). Other products of choline metabolism by bacteria in the rumen have been identified as acetate and ethanol (Neill & Dawson, 1977; Neill *et al.*, 1978). The methyl group of methylamine and methionine can also be converted by rumen micro-organisms into methane, but the methyl groups of carnitine can not (Neill *et al.*, 1978). Mitchell *et al.* (1979) showed that rumen micro-organisms rapidly catabolize exogenous betaine *in vitro* and *in vivo*. When [*methyl*-^{14}C] betaine was incubated with rumen contents or added directly to the rumen, radioactivity was recovered in trimethylamine, methane and CO_2. [1-^{14}C] betaine gave rise to labelled acetate and CO_2 and [^{15}N] betaine produced labelled ammonia. Cows fed diets high in betaine excrete trimethylamine-*N*-oxide in the urine (Davies, 1936) and milk (Baldwin, 1953), which reflects the microbial degradation of betaine to trimethylamine in the rumen.

In sheep fed 40g of choline chloride daily for 6 days, there was no accumulation of choline in the liver, kidneys or blood, and

only 0.7 - 2.5% was recovered in the urine on any single day (Luecke & Pearson, 1945). Broad & Dawson (1976) found virtually no free choline in strained rumen contents of fasted sheep and only a transient appearance of unesterified choline at a concentration of less than 1 nmol/ml after feeding 600g of grass. The grass fed contained about 800 - 900 μmol of phosphatidylcholine, which should have yielded approximately 160 nmol of free choline/ml of rumen fluid by microbial hydrolysis. This rapid microbial breakdown of choline in the rumen questions the efficiency of oral choline supplementation to ruminants as discussed by Church (1979*a*) and raises doubts as to the mechanism of the benefit of rumen bacterial growth produced by choline (Swingle & Dyer, 1970). The rumen bacteria are incapable of *de novo* choline synthesis (Broad & Dawson, 1975, 1976), and the bacterial membranes contain negligible amounts of phosphatidylcholine (Harfoot, 1978).

The only dietary choline escaping microbial degradation in the rumen appears to be a very small percentage that is incorporated into the structural membranes of ciliated protozoa as phosphatidylcholine (Broad & Dawson, 1976). The rumen ciliated protozoon *Entodinium caudatum* has phosphatidylcholine as a major component of the phospholipids present in its membranes (Dawson & Kemp, 1967). Broad & Dawson (1976) have shown that this anaerobic protozoon has a nutritional requirement for choline which cannot be replaced by ethanolamine, *N*-methylethanolamine or *N*-dimethylethanolamine. In contrast to many aerobic protozoa, *Entodinium caudatum* cannot synthesize phosphatidylcholine by the methylation of phosphatidylethanolamine (Broad & Dawson, 1975). Unesterified choline is rapidly taken up by the

organism and incorporated into membrane phosphatidylcholine through a phosphorylcholine-CDP-choline pathway but not by base-exchange (Broad & Dawson, 1975; Bygrave & Dawson, 1976). The usual level of free choline available in the rumen would certainly not be able to support growth of *Entodinium caudatum*, even though the organism possesses an extraordinary rapid rate of choline uptake which would help in the accumulation of the base when it appeared transiently during ruminal digestion. Unless any free choline taken up could be stored and used up gradually for necessary membrane synthesis in cell division, the growth of the protozoonin the rumen would be expected to be intermittent. It has been suggested that *Entodinium caudatum* obtains choline for growth mainly from plant membrane phosphatidylcholine which it has ingested, rather than from the free base in the rumen fluid (Broad & Dawson, 1976). The ruminal preservation of dietary phosphatidylcholine is virtually eliminated when the rumen is cleared of ciliated protozoa (Broad & Dawson, 1976; Neill *et al.*, 1979). John & Ulyatt (1979) reported that essentially all dietary phosphatidylcholine is degraded in the rumen of sheep, and that reaching the duodenum is of protozoal origin. There is very limited transfer of phosphatidylcholine present in rumen protozoa to the lower digestive tract due to a selective retention of these micro-organisms in the rumen (Weller & Pilgrim, 1974; Bauchop & Clarke, 1976; Harrison *et al.*, 1979; Neill *et al.*, 1979; Coleman *et al.*, 1980).

Neill *et al.* (1979) calculated that if all the choline present in the abomasal digesta (largely as phosphatidylcholine of ciliated protozoa) was derived from dietary sources, the adult sheep fed a

diet of hay/chaff and oats could receive no more than 20 - 25mg of effective choline per day (0.002 - 0.0025% of dietary total dry-weight intake). This amount of choline is some fifty times less than the minimum required to avoid severe pathological lesions (fatty liver and haemorrhagic kidney) and death in many non-ruminant species (0.1% or more of dietary dry-weight intake). However, Neill *et al.* (1979) also observed that when a sheep was defaunated to remove the phosphatidylcholine - containing ciliated protozoa from its rumen then the concentration of phosphatidylcholine in the abomasal digesta was above that in the ruminal contents. This suggested that some, at least, of the phosphatidylcholine present in the abomasal digesta was derived from non-dietary sources, e.g. from abomasal secretions or regurgitation of bile from the duodenum. Robinson (1980) found that sheep fed on dried cereal stubble receive a sub-optimal choline and betaine intake and much of the choline in the lower digestive tract is of endogenous origin. Sheep whose rumens have been defaunated to remove ciliated protozoa remain completely healthy for long time periods and show no signs of choline deficiency (Neill & Dawson, 1977; Neill *et al.*, 1979). Thus it would appear that the adult sheep is less sensitive to a low dietary choline supply than non-ruminant animals.

3. Metabolism of choline in tissues

3a. Release of unesterified choline from choline-containing esters

Choline-containing phospholipids and water-soluble esters of either dietary or endogenous origin can be degraded in mammalian

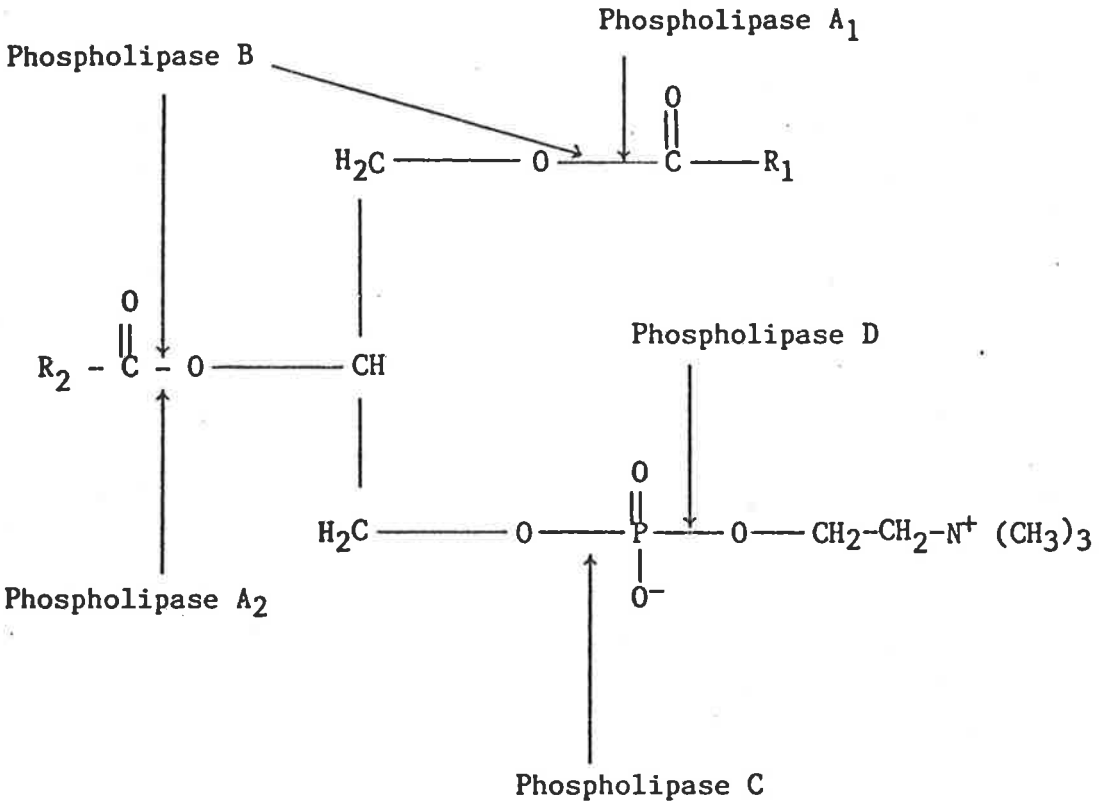
tissues, ultimately releasing unesterified choline.

Phosphatidylcholine is hydrolysed in tissues by specific phospholipases [reviewed by Thompson (1973, 1980), Van Golde & Van den Bergh (1977*a*,*b*), Van den Bosch (1980) and Kanfer (1980)], as indicated in Figure 1. Phospholipase A_1 specifically releases the fatty acid from the 1-position of the phosphatidylcholine glycerol backbone, and phospholipase A_2 releases the fatty acid from the 2-position, yielding 2-acyl and 1-acyl lysophosphatidylcholine respectively. Phospholipase B can bring about successive removal of the two fatty acids of phosphatidylcholine to form glycerophosphocholine. Phospholipase C catalyses the conversion of phosphatidylcholine to phosphocholine and 1,2-diacylglycerol. Phospholipase D hydrolyses phosphatidylcholine to release unesterified choline and phosphatidic acid.

Lecithin-cholesterol acyltransferase mediates the transfer of a fatty acyl group from the 2-position of phosphatidylcholine to the hydroxyl function of free cholesterol, forming cholesterol ester and lysophosphatidylcholine in plasma (Glomset, 1968, 1979) and liver (Akiyama *et al.*, 1967).

In tissues, lysophosphatidylcholine can be converted to glycerophosphocholine by further deacylation, catalysed by lysophospholipase (phospholipase B) (Thompson, 1973; Van Golde & Van den Bergh (1977*a*,*b*); Van den Bosch, 1980). Lysophosphatidylcholine can also be reacylated with acyl-CoA to form phosphatidylcholine again, mediated by an acyltransferase (Lands, 1960; Lands & Merkl, 1963; Hill & Lands, 1970; Van den Bosch *et al.*, 1972).

Figure 1: Sites of attack of phospholipases on phosphatidylcholine


Phospholipase A1
Phospholipase B
H2C
O
C
R1
O
R2 - C
O
CH
Phospholipase D
O
H2C
O
P
O
CH2-CH2-N+ (CH3)3
O−
Phospholipase A2
Phospholipase C

In addition, two lysophosphatidylcholine molecules can undergo a transesterification reaction catalysed by a dismutase to form one molecule of phosphatidylcholine and one molecule of glycerophosphocholine (Marinetti *et al.*, 1958; Erbland & Marinetti, 1965; Van den Bosch *et al.*, 1965).

Glycerophosphocholine is degraded to unesterified choline and glycerophosphate by the enzyme glycerophosphocholine phosphodiesterase (Dawson, 1956; Webster *et al.*, 1957; Wallace & White, 1965; Baldwin & Cornatzer, 1968, 1969; Lloyd-Davies *et al.*, 1972; Mann, 1975). The activity of glycerophosphocholine phosphodiesterase is very low in sheep liver and high in rat liver (Dawson, 1956). This is probably related to the higher concentration of glycerophosphocholine in sheep liver compared with rat liver (Schmidt *et al.*, 1952, 1955; Dawson, 1955*a*). Alkaline and acid phosphatases hydrolyse phosphocholine to free choline and inorganic phosphate (MacFarlane *et al.*, 1934).

Choline plasmalogens (choline phosphoglycerides with a hydrocarbon side-chain attached to position 1 of the glycerol backbone through a vinyl ether linkage) are partially degraded by the action of phospholipases A_2 and C (Thompson, 1973; Dawson, 1973). An enzyme in rat liver microsomes hydrolyses choline lysoplasmalogen to a long-chain aldehyde and glycerophosphocholine. The enzyme does not hydrolyse ethanolamine lysoplasmalogen or intact choline and ethanolamine plasmalogens (Warner & Lands, 1961). Phosphocholine and glycerophosphocholine released from choline plasmalogens can subsequently be degraded to free choline.

Sphingomyelin is degraded to ceramide and phosphocholine by a specific sphingomyelinase (sphingomyelin phosphodiesterase) (Heller & Shapiro, 1966; Barnholz *et al.*, 1966; Stoffel, 1971; Morell & Braun, 1972). Phospholipase C can also act on sphingomyelin, thereby forming phosphocholine (Fujino, 1952; Illingworth & Portman, 1973). As mentioned previously, the phosphocholine produced can be hydrolysed by phosphatases to yield unesterified choline.

Acetylcholine is cleaved by acetylcholinesterase to unesterified choline and acetate (Rosenberry, 1975; Massoulié, 1980).

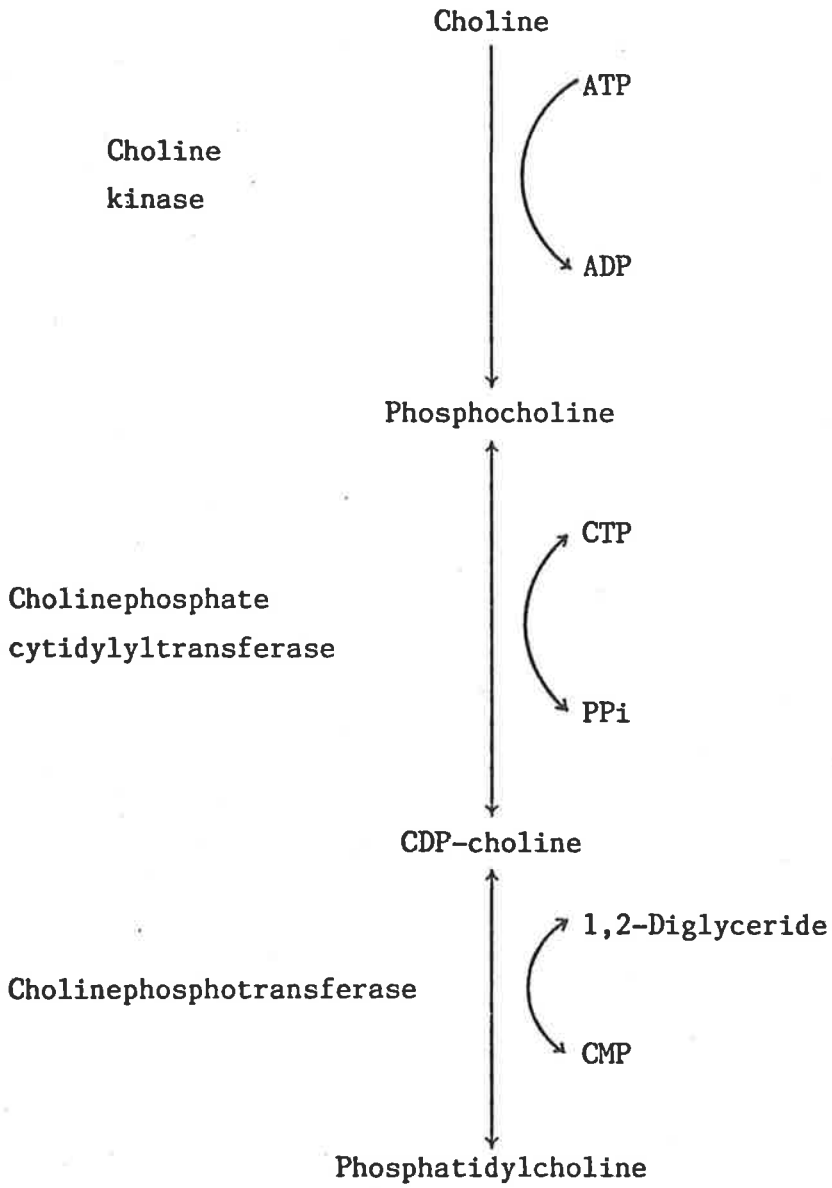
3b. Utilization of unesterified choline

Unesterified choline derived from the diet or from the hydrolysis of dietary and endogenous choline-containing esters can be utilized in several pathways in mammalian tissues.

3b.1 Biosynthesis of phosphatidylcholine *via* the CDP-choline pathway

In mammalian tissues unesterified choline is converted to phosphatidylcholine *via* the CDP-choline pathway (Kennedy & Weiss, 1956; Kennedy, 1962; Thompson, 1973; Van Golde & Van den Bergh (1977*a*,*b*) as shown in Figure 2. Choline is phosphorylated to phosphocholine utilizing ATP by the cytosolic enzyme choline kinase. Phosphocholine reacts with CTP to form CDP-choline in a reversible reaction catalysed by cholinephosphate cytidylyltransferase which is distributed in the endoplasmic reticulum and cytoplasm. The CDP-choline is subsequently combined with 1,2-diacylglycerol (derived principally from the dephosphorylation of phosphatidic acid)

Figure 2: Conversion of unesterified choline to phosphatidylcholine *via* the CDP-choline pathway


Choline
ATP
Choline
kinase
ADP
Phosphocholine
CTP
Cholinephosphate
cytidylyltransferase
PPi
CDP-choline
1,2-Diglyceride
Cholinephosphotransferase
CMP
Phosphatidylcholine

to form phosphatidylcholine in a reversible reaction catalysed by the microsomal enzyme cholinephosphotransferase. The presence of an ether-linkage in the diacylglycerol precursor can lead to the synthesis of a choline plasmalogen [reviewed by Wykle & Snyder (1976)]. The CDP-choline pathway is the major route for the synthesis of phosphatidylcholine in tissues, which is the main phospholipid of cellular membranes (McMurray, 1973; White, 1973). In the lung the CDP-choline pathway is important in the synthesis of disaturated phosphatidylcholine, the principal component of surfactant (Frosolono, 1977). Phosphatidylcholine formed *via* the CDP-choline pathway is a major constituent of plasma lipoproteins and bile secreted by the liver (Coleman, 1973).

The rat liver can synthesize phosphatidylcholine *via* CDP-choline at a rate between 1.7 - 3.1 mmol/day per kg body wt. (Sundler & Åkesson, 1975*a*). In rat liver the monoenoic and dienoic molecular classes of phosphatidylcholines are synthesized largely *via* the CDP-choline pathway (Van den Bosch, 1974; MacDonald & Thompson, 1975). In the rat the activity of cholinephosphotransferase is much higher in the liver compared with extrahepatic tissues (Skurdal & Cornatzer, 1975). Skurdal & Cornatzer (1975) found the greatest activity of cholinephosphotransferase in the liver microsomes of the chicken followed by mouse, human, rat, rabbit, turtle, pigeon, guinea pig, beef and dog.

In recent years significant advances have been made on how the synthesis of phosphatidylcholine *via* the CDP-choline

pathway is controlled in tissues (Porcellati, 1972; Infante, 1977; Vance & Choy, 1979; Rooney, 1979). Most of the evidence suggests that the enzyme cholinephosphate cytidylyltransferase catalyses the rate-limiting reaction for phosphatidylcholine biosynthesis from free choline (Vance & Choy, 1979). In rat liver, the cytidylyltransferase is activated by several phospholipids, of which the most important appears to be lysophosphatidylethanolamine (Choy *et al.*, 1977; Choy & Vance, 1978). The enzyme from rat lung is also activated by phospholipid and in this case phosphatidylglycerol appears to be the most significant (Feldman *et al.*, 1978). Another type of regulation of this enzyme has been described in He La cells, where the concentration of CTP in the cytoplasm correlated with the rate of the reaction catalysed by the cytidylyltransferase and the rate of phosphatidylcholine synthesis (Vance *et al.*, 1980; Choy *et al.*, 1980). On the other hand, theoretical and some experimental work have implicated a regulatory and rate-limiting role for choline kinase (Infante, 1977; Infante & Kinsella, 1978).

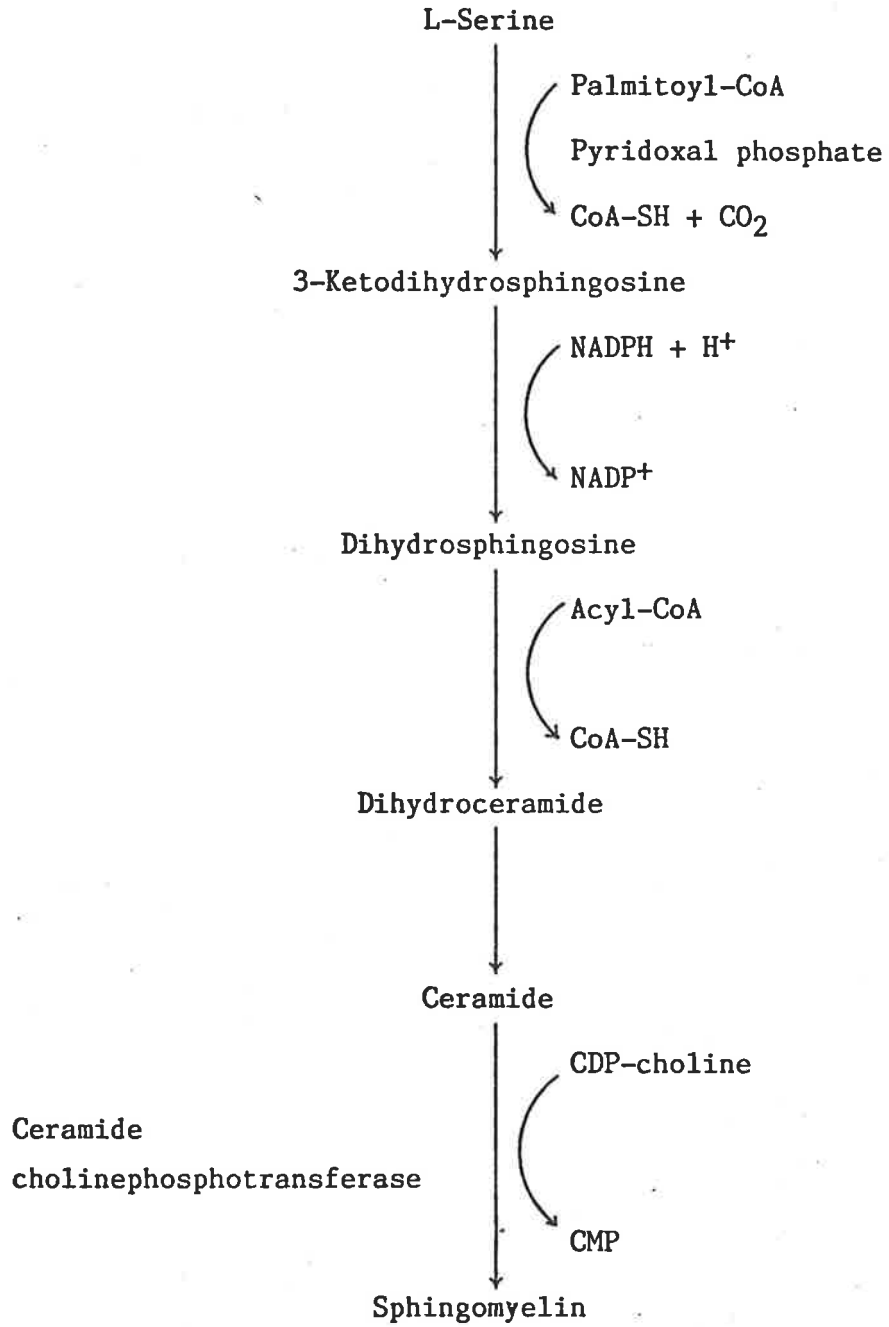
Schneider & Vance (1978) found that the activity of cholinephosphate cytidylyltransferase is depressed in the choline-deficient rat liver. The activity of cholinephosphotransferase is decreased in the liver of rats fed a diet free of methionine and choline (Hoffman *et al.*, 1980*b*). Ethanol administration stimulates the activity of cholinephosphotransferase in rat liver (Uthus *et al.*, 1976). The activities of choline kinase, cholinephosphate cytidylyltransferase and cholinephosphotransferase in rat liver, lung and brain are

maximal immediately before or shortly after birth (Artom, 1968; Weinhold *et al.*, 1973; Farrell *et al.*, 1974). Noble *et al.* (1971) showed that the total hepatic phosphatidylcholine to phosphatidylethanolamine ratio of young lambs was markedly lower than that of adult sheep and that this ratio increased rapidly during the first week of life. The rapid increase in phosphatidylcholine content of the liver was attributed to increased biosynthesis *via* the CDP-choline pathway on the basis of the fatty acid composition of the ewe's milk and the lamb tissues. Ewe's colostrum is rich in preformed choline (Tsielens, 1954) and this would presumably favour the CDP-choline pathway for phosphatidylcholine biosynthesis in lamb liver.

3b.2 Biosynthesis of sphingomyelin

Although there are still some unsolved problems with regard to the biosynthesis of sphingomyelin in mammalian tissues, the essential reactions have been largely elucidated [reviewed by Van Golde & Van den Bergh (1977[a]) and Thompson (1980)] as shown in Figure 3. Pyridoxal phosphate-bound serine reacts with palmitoyl-CoA, producing 3-ketodihydrosphingosine, which is reduced in the presence of NADPH to yield dihydrosphingosine. Dihydrosphingosine is converted into dihydroceramide, which is then desaturated to ceramide. Subsequently, ceramide cholinephosphotransferase, an enzyme found in many mammalian tissues, catalyses the formation of sphingomyelin from ceramide and CDP-choline (derived from free choline *via* phosphocholine, as described in Section 3b.1.

Figure 3: Synthesis of sphingomyelin


L-Serine
Palmitoyl-CoA
Pyridoxal phosphate
CoA-SH + CO_2
3-Ketodihydrosphingosine
NADPH + H^+
$NADP^+$
Dihydrosphingosine
Acyl-CoA
CoA-SH
Dihydroceramide
Ceramide
CDP-choline
Ceramide
cholinephosphotransferase
CMP
Sphingomyelin

An alternative pathway for sphingomyelin synthesis has been observed in homogenates of immature rat brain. This involves the transfer of phosphocholine from CDP-choline to free sphingosine. The sphingosylphosphocholine is then acylated with fatty acyl-CoA to yield sphingomyelin (Brady *et al.*, 1965). Diringer *et al.* (1972) and Marggraf & Anderer (1974) have raised the possibility of yet another mechanism for sphingomyelin biosynthesis. In cultured virus-transformed mouse fibroblasts, sphingomyelin appears to arise through the transfer of phosphocholine from phosphatidylcholine to ceramide.

It is pertinent to mention that the erythrocytes of sheep and other ruminants are characterized by high levels of sphingomyelin and negligible amounts of phosphatidylcholine. In contrast, in the erythrocytes of monogastric animals, phosphatidylcholine replaces sphingomyelin as the major single phospholipid constituent (Dawson *et al.*, 1960; Christie, 1978). The erythrocyte membrane of the sheep, unlike those of non-ruminants, contains an active phospholipase A that preferentially hydrolyses phosphatidylcholine and it has been suggested that this enzyme might play a role in maintaining the low levels of phosphatidylcholine in sheep erythrocytes (Kramer *et al.*, 1974; Zwaal *et al.*, 1974).

3b.3 Biosynthesis of phosphatidylcholine by base-exchange

Free choline can exchange with serine, ethanolamine or inositol present within phospholipids to form phosphatidyl-

choline in several mammalian tissues including liver, brain, heart and lung [reviewed by Van Golde & Van den Bergh (1977*a*,*b*), Thompson (1973, 1980) and Kanfer (1980)] as depicted in Figure 4. The base-exchange reaction is reversible and requires the presence of calcium ions, but is not energy dependent. It is catalysed by an enzyme that shows highest activity in the microsomal fraction. The incorporation of choline into phosphatidylcholine by the base-exchange process is of minor quantitative importance *in vivo* (Stein & Stein, 1969; Sundler *et al.*, 1972; Bjerve, 1973; Salerno & Beeler, 1973). Sundler *et al.* (1972) calculated that at least 20 times more choline is incorporated into phosphatidylcholine *via* CDP-choline than *via* base-exchange in rat liver. Choline is preferentially incorporated into monoenoic molecular classes of phosphatidylcholines by base-exchange in rat liver *in vivo* (Balint *et al.*, 1967; Rytter *et al.*, 1968; Spitzer *et al.*, 1969).

3b.4 Biosynthesis of acetylcholine

Choline reacts with acetyl-CoA to form acetylcholine in a reversible reaction catalysed by the enzyme choline acetyltransferase (Nachmansohn & Machado, 1943) as shown in Figure 5. Choline acetyltransferase is highly concentrated in cholinergic nerve terminals (Fonnum, 1973; Wajda *et al.*, 1973), although it has been localized in some non-nervous tissues such as placenta (Sastry *et al.*, 1976). Although only a small fraction of unesterified choline is acetylated in the body (Cohen & Wurtman, 1975; Haubrich *et al.*, 1975*a*, *b*), this pathway is very

Figure 4: Conversion of unesterified choline to phosphatidylcholine by base-exchange

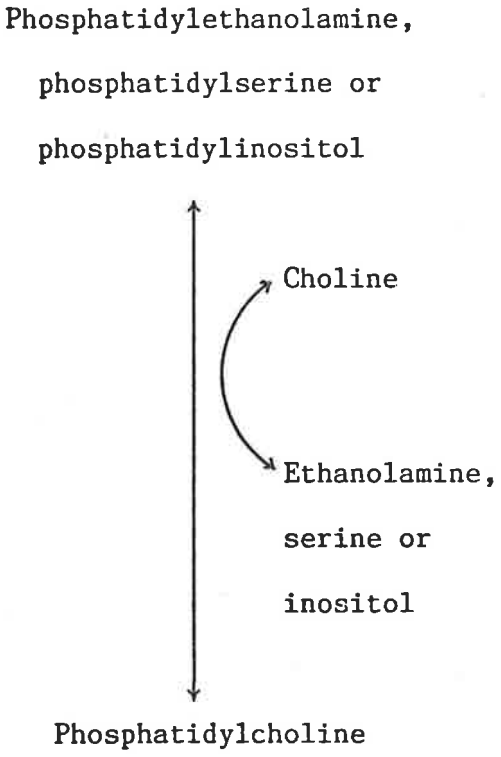
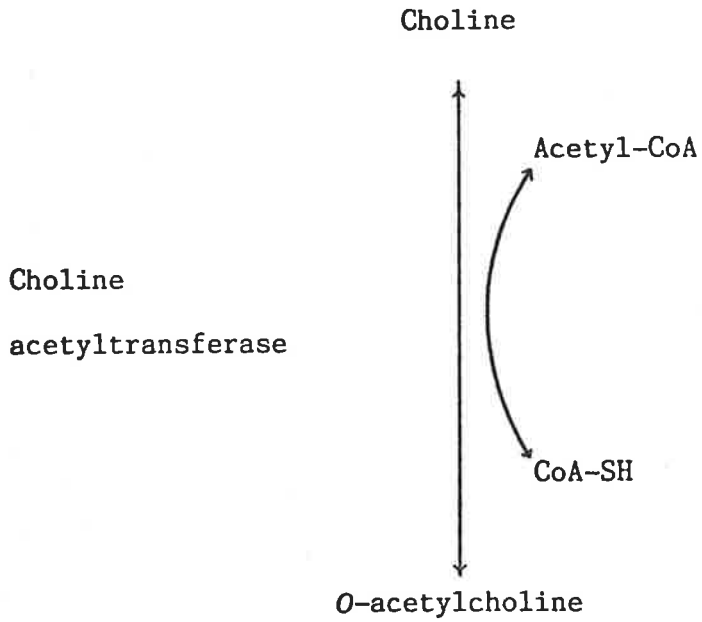

Phosphatidylethanolamine,
phosphatidylserine or
phosphatidylinositol
Choline
Ethanolamine,
serine or
inositol
Phosphatidylcholine

Figure 5: Biosynthesis of acetylcholine from unesterified choline

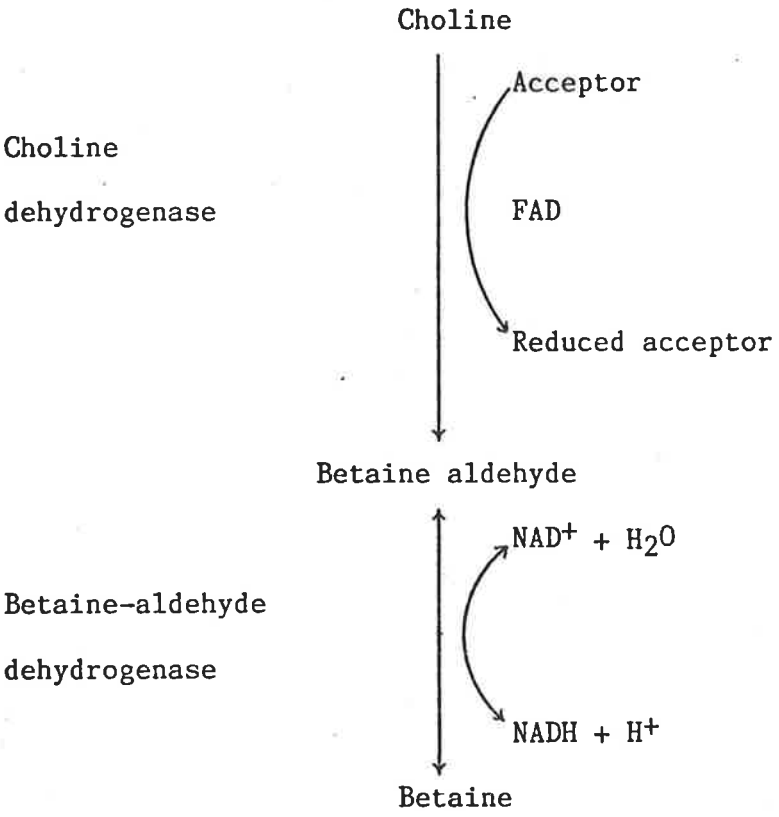

Choline
Acetyl-CoA
Choline
acetyltransferase
CoA-SH
O-acetylcholine

important because of the role of acetylcholine as a neurotransmitter.

3b.5 Oxidation to betaine and other losses

Unesterified choline is oxidized to betaine aldehyde, which is then converted to betaine by the enzyme system choline oxidase (choline dehydrogenase and betaine-aldehyde dehydrogenase) (Hatefi & Stiggall, 1976) as shown in Figure 6. This is the only known mechanism for irretrievable degradation of choline in higher animals. Betaine cannot be reduced to form choline; it can, however, donate one of its methyl groups to homocysteine, producing methionine and dimethylglycine, in a reaction catalysed by betaine-homocysteine methyltransferase (McGilvery, 1970; Hatefi & Stiggall, 1976; Finkelstein, 1974, 1978). Thus, although the choline oxidase pathway acts to remove unesterified choline from the body, it is able to scavenge valuable methyl groups. Choline oxidase activity is present in several mammalian tissues, including liver (where it is most active) and kidney. It is not present in the brain, muscle or blood of the rat (Bernheim & Bernheim, 1933, 1938; Mann & Quastel, 1937; Hatefi & Stiggall, 1976). Choline oxidase in the liver and kidney is important in maintaining physiological levels of free choline in the blood (Haubrich *et al.*, 1975*a*; Zeisel *et al.*, 1980*b*). Choline dehydrogenase is mainly a mitochondrial enzyme (Williams, 1960; Tyler *et al.*, 1966; De Ridder & Van Dam, 1975), whereas betaine-aldehyde dehydrogenase is cytosolic (Williams, 1952; Yue *et al.*, 1966; Hatefi & Stiggall, 1976). The two enzymes have been partially

Figure 6: Oxidation of choline to betaine


Choline
Acceptor
Choline
dehydrogenase
FAD
Reduced acceptor
Betaine aldehyde
NAD+ + H2O
Betaine-aldehyde
dehydrogenase
NADH + H+
Betaine

purified and kinetic properties established (Rothschild & Barron, 1954; Rendina & Singer, 1959; Hatefi & Stiggall, 1976).

The choline oxidase activity of hepatic tissue of different monogastric species varies considerably (Bernheim & Bernheim, 1933; Dubnoff, 1949; Dinning *et al.*, 1949; Kensler & Langemann, 1954; Sidransky & Farber, 1960; Guha & Wegmann, 1963). Sidransky & Farber (1960) found the highest liver choline oxidase activity in the rat followed by chick, mouse, dog, hamster, rabbit, monkey, guinea pig and human. An apparent correlation between hepatic choline oxidase activity of a species and the tendency to develop choline-deficient fatty liver has been suggested (Handler, 1949; Handler & Bernheim, 1949). Rat hepatic mitochondrial choline dehydrogenase activity has been shown to increase markedly postnatally (Weinhold & Sanders, 1973; Streumer-Svobodová & Drahota, 1977) and choline oxidase activity in preparations of rat liver slices is lower in foetal (19 days post-conception) and neonatal (7 days old) than in adult rats (Weinhold & Sanders, 1973). Liver choline oxidase activity decreases in the choline-deficient rat (Wong & Thompson, 1972; Schneider & Vance, 1978). Acute ethanol administration increases choline oxidase activity in the rat liver (Tuma *et al.*, 1973; Thompson & Reitz, 1976).

The choline oxidase activity of calf liver is very much less than that of rat liver (Hopper & Johnson, 1956). Neill *et al.* (1979) found that the intact sheep oxidizes an injected dose of [1,2-^{14}C] choline to $^{14}CO_2$ over a 5h period at a rate that is one-third of that in the rat. The real difference in

the rate of oxidation of choline between the two species may be even greater than that observed, because the measurements were made (from economic necessity) before the injected choline had equilibrated with the total choline body pool. Henderson (1978) found that, in sheep hepatocyte preparations, the ratio of incorporation of label from [1,2-^{14}C] choline into betaine as compared with phosphatidylcholine for a 1h incubation was 0.84:1. A similar ratio for rat hepatocytes calculated from the data of Sundler & Åkesson (1975*b*) is 26:1. If these values can be directly compared, they indicate that sheep liver cells can use available choline more effectively for membrane maintenance than can cells of rat liver. These results help to explain the insensitivity of sheep to a low dietary choline intake.

Mammals excrete only small amounts of choline in the urine which is entirely in the free form. The urinary excretion of choline for the sheep, dog and human is about 20, 30 and 70 µmol per day respectively (Luecke & Pearson, 1944, 1945; Johnson *et al.*, 1945). It has been demonstrated in the chicken that transport of choline across the kidney tubule is an active process (Acara & Rennick, 1972, 1976; Acara, 1975; Acara *et al.*, 1975; Rennick *et al.*, 1977), with the rate determined by choline concentration (Acara & Rennick, 1972). Hemicholinium-3 at low concentrations enhances renal excretion of choline, whereas at high concentrations it markedly inhibits choline output (Acara *et al.*, 1975). Quinine, cyanine 863, and tetraethylammonium also inhibit transport

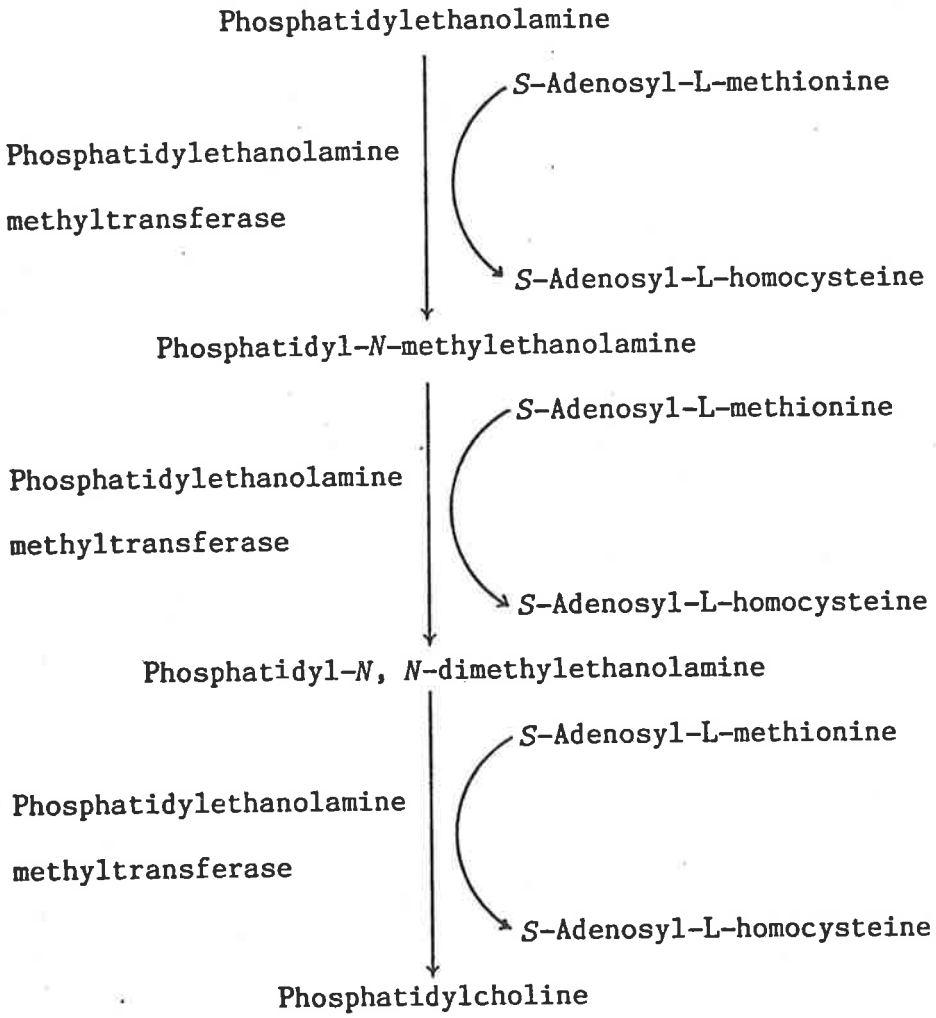
(Acara & Rennick, 1972; Rennick *et al.*, 1977), whereas betaine and carnitine have no effect (Sung & Johnstone, 1969). Much of the choline taken up by the kidney is oxidized to form betaine (Sung & Johnstone, 1969; Acara & Rennick, 1972). Net active choline excretion does not occur until sufficient choline is presented to the kidney to saturate choline oxidase. Betaine is passively transported by the kidney and is a major metabolite of choline excreted in the urine (Haubrich *et al.*, 1975*a*).

Negligible amounts of free choline are lost from the body in sweat and faeces (Johnson *et al.*, 1945). During pregnancy considerable quantities of free choline are transported from maternal blood to the foetus *via* the placenta (Biezenski *et al.*, 1971; Jorswieck, 1974; Welsch, 1978). Significant amounts of unesterified and lipid choline are transferred from mother to neonate during the lactation period (Johnson *et al.*, 1951; Tsielens, 1954; Nayman *et al.*, 1979).

3c. Biosynthesis of choline by the methylation pathway in tissues

The only known pathway for the *de novo* synthesis of choline in mammalian tissues is by the stepwise methylation of phosphatidylethanolamine to phosphatidylcholine (Figure 7). The synthesis of phosphatidylcholine by the transmethylation of the amino moiety of phosphatidylethanolamine proceeds with the intermediate formation of phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*, *N*-dimethylethanolamine. *S*-Adenosyl-L-methionine derived from

Figure 7: Biosynthesis of choline by the methylation of phosphatidylethanolamine to phosphatidylcholine


Phosphatidylethanolamine
S-Adenosyl-L-methionine
Phosphatidylethanolamine
methyltransferase
S-Adenosyl-L-homocysteine
Phosphatidyl-N-methylethanolamine
S-Adenosyl-L-methionine
Phosphatidylethanolamine
methyltransferase
S-Adenosyl-L-homocysteine
Phosphatidyl-N, N-dimethylethanolamine
S-Adenosyl-L-methionine
Phosphatidylethanolamine
methyltransferase
S-Adenosyl-L-homocysteine
Phosphatidylcholine

L-methionine serves as the immediate methyl donor for this biological process that modifies the polar head groups leading to the interconversion of phospholipid classes, and *S*-adenosyl-L-homocysteine is a second product which is a potent competitive inhibitor. The conversion of phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine is the rate-limiting step (Bremer & Greenberg, 1961; Gibson *et al.*, 1961; Schneider & Vance, 1979).

It is not certain whether the three successive methyl group transfer reactions involved in the conversion of phosphatidylethanolamine to phosphatidylcholine are catalysed by more than one phosphatidylethanolamine methyltransferase enzyme in mammalian tissues. In studies using solubilized methyltransferase from rat liver microsomes the results indicate that a single enzyme catalyses the methylation of phosphatidylethanolamine to phosphatidylcholine (Rehbinder & Greenberg, 1965; Tanaka *et al.*, 1979; Schneider & Vance, 1979). There is genetic evidence for the existence of two distinct enzymes for the phospholipid methylation process in the micro-organism *Neurospora crassa* (Scarborough & Nyc, 1967*a*, *b*). There are reports of two separate enzymes involved in phospholipid methylation in rat erythrocyte membranes (Hirata & Axelrod, 1978), bovine adrenal medulla microsomes (Hirata *et al.*, 1978) and rat brain synaptosomes (Crews *et al.*, 1980*a*), distinguished with respect to pH optimum, magnesium requirement and affinity for *S*-adenosyl-L-methionine. The first enzyme (methyltransferase I) catalyses the methylation of phosphatidylethanolamine to form phosphatidyl-*N*-monomethylethanolamine, and the second enzyme (methyltransferase II) catalyses the incorporation of two methyl groups into phosphatidyl-*N*-

monomethylethanolamine to form phosphatidylcholine *via* phosphatidyl-*N*, *N*-dimethylethanolamine. Hirata & Axelrod (1978) reported that these two enzymes, like phospholipids (Rothman & Lenard, 1977; Chap *et al.*, 1977), are asymmetrically distributed in the plasma membrane. Methyltransferase I and its substrate phosphatidylethanolamine are localized on the cytoplasmic surface of the membrane, whereas methyltransferase II and its product phosphatidylcholine are on the outer surface. Phosphatidyl-*N*-monomethylethanolamine, the product of the first enzyme and the substrate for the second enzyme appears to be embedded in the membrane. The phospholipid asymmetry may, therefore, be maintained in the plasma membrane as a result of their pathway of synthesis and the methyltransferase asymmetry.

The methylation pathway has been detected in a variety of tissues of the rat, but is only quantitatively important in the liver (Bremer & Greenberg, 1961; Bjørnstad & Bremer, 1966; Skurdal & Cornatzer, 1975; Vance & De Kruijff, 1980). The principal intracellular site of *N*-methylation is the endoplasmic reticulum (Bremer & Greenberg, 1960; Gibson *et al.*, 1961; Skurdal & Cornatzer, 1975; Jelsema & Morré, 1978; Hirata *et al.*, 1978). The methylation pathway quantitatively synthesizes about 15-40% of the total phosphatidylcholine in the rat liver, with most of the rest derived from the CDP-choline pathway (Artom, 1965; Bremer & Greenberg, 1961; Sundler & Åkesson, 1975*b*). In the extrahepatic tissues of rats the contribution of the methylation pathway to the total synthesis of phosphatidylcholine is less than 5% (Artom, 1965). In rat liver, phosphatidylethanolamine that is used as the substrate for the methylation pathway can be synthesized from free ethanolamine by a

CDP-ethanolamine pathway or by base-exchange or made *de novo* by the decarboxylation of phosphatidylserine (Thompson, 1973; Van Golde & Van den Bergh, 1977*b*). The methylation of phosphatidylethanolamine is mainly involved in the synthesis of tetraenoic and polyenoic (>4 double bonds) classes of phosphatidylcholines in rat liver (Van den Bosch, 1974; MacDonald & Thompson, 1975). Le Kim *et al.* (1973) found that the methylation of phosphatidyl-*N*, *N*-dimethylethanolamine proceeds more rapidly as the degree of unsaturation rises. Apparently the structure of the phosphatidyl residue is very important for the methyltransferase since the related ceramide phosphoryl-*N*, *N*-dimethylethanolamine is not methylated.

The methylation pathway has been reported to be regulated by the intracellular concentration ratio of *S*-adenosyl-L-methionine to *S*-adenosyl-L-homocysteine (Chiang & Cantoni, 1979; Hoffman *et al.*, 1980*a*) and phosphatidylethanolamine to phosphatidylcholine (Åkesson, 1978) and many signals acting on the cell surface, including hormones (Geelen *et al.*, 1979; Hirata *et al.*, 1979*a*; Castaño *et al.*, 1980), immunoglobulins (Ishizaka *et al.*, 1979, 1980; Crews *et al.*, 1980*b*) and attractants (Pike *et al.*, 1979; Hirata *et al.*, 1979*b*; Alemany *et al.*, 1980). The methylation of phosphatidylethanolamine to phosphatidylcholine plays an important role in various biological processes related to membrane structure and function including modulation of membrane fluidity, the number of β-adrenergic binding sites and the coupling between the β-adrenergic receptor and the enzyme adenylate cyclase, Ca^{2+}-ATPase enzyme activity, mast cell histamine release, leucocyte chemotaxis and lymphocyte mitogenesis. It has been proposed that enzymatic methylation of phospholipids is

linked to transduction of receptor-mediated signals through the membranes of a variety of cells [reviewed by Hirata & Axelrod (1980) and Borchardt (1980)]. Since the contribution of the methylation pathway to total phosphatidylcholine synthesis is relatively low compared with the CDP-choline pathway in most cells and tissues, Vance & De Kruijff (1980) have questioned the reports of significant physiological changes attributed to phospholipid methylation in these systems. Phosphatidylcholine synthesized by the methylation pathway in the liver contributes to the maintenance of structural membranes and secretion of plasma lipoproteins. The methylation pathway does not appear to be involved in hepatic bile phosphatidylcholine synthesis (Coleman, 1973).

Skurdal & Cornatzer (1975) found the greatest phosphatidylethanolamine methyltransferase activity in the liver microsomes of the mouse followed by chicken, rat, dog, pigeon, human, beef, rabbit, turtle and guinea pig which is similar to the pattern of choline oxidase activity in the liver of various species reported by Sidransky & Farber (1960). Wise & Elwyn (1965) estimated from *in vivo* experiments with [3-^{14}C] serine that the male rat liver can synthesize 0.7 mmol of choline/day per kg body wt. by the methylation of phosphatidylethanolamine to phosphatidylcholine, which is nearly equivalent to the normal dietary choline intake per day for the rat. *In vivo* experiments with [1,2-^{14}C] ethanolamine showed that the male rat liver can synthesize about 1.0 mmol of choline/day per kg body wt., and the value is about 25% higher for the female rat liver (Bjørnstad & Bremer, 1966). Neill *et al.* (1979) found that the sheep liver can synthesize approximately 0.1 mmol of choline/day per

kg body wt. from [2-^{14}C] ethanolamine both *in vivo* and *in vitro*, which is many times less than that reported for rat liver. This confirmed the earlier report of Neill & Dawson (1977) that the methylation pathway did not appear more significant in sheep liver than rat liver. Henderson (1978) found that the incorporation of isotope from methyl-labelled methionine into phosphatidylcholine in sheep isolated hepatocytes was about 10% of that for rat isolated hepatocytes reported by Sundler & Åkesson (1975*b*). Bremer & Greenberg (1961) demonstrated that isolated microsomal fractions from sheep liver were less effective at synthesizing choline from *S*-[*methyl*-^{14}C] adenosyl-L-methionine when compared with those from species known to be susceptible to choline deficiency, e.g. rat, mouse and chicken. Thus, these observations in no way help to explain the apparent minimal requirement of sheep for dietary choline.

Rat liver phosphatidylethanolamine methyltransferase activity is minimal in the foetus but increases rapidly after birth during the suckling period, and then declines moderately to the adult value (Artom, 1969; Hoffman *et al.*, 1979). It is not known if similar developmental changes occur for sheep liver methyltransferase activity. There is a discrepancy in the literature as to whether the methylation of phosphatidylethanolamine to phosphatidylcholine in rat liver increases (Corredor *et al.*,1967; Fallon *et al.*, 1969; Lombardi *et al.*, 1969; Thompson *et al.*, 1969; Glenn& Austin, 1971; Schneider & Vance, 1978; Hoffman *et al.*, 1980*b*) or decreases (Blumenstein, 1964; Haines, 1966; Yamamoto *et al.*, 1969; Lyman *et al.*, 1973; Skurdal & Cornatzer, 1974; Åkesson *et al.*, 1978, 1979) during dietary deficiency of choline and other lipotropic

compounds, which may be due to an inherent problem with analysis (Åkesson, 1978). Exposure to ethanol increases the activity of the methylation pathway in rat liver (Fallon *et al.*, 1969; Uthus *et al.*, 1976).

Under conditions of metabolic stress such as starvation, alloxan-diabetes and pregnancy toxaemia sheep are particularly prone to the development of fatty livers as a result of marked accumulation of triacylglycerols (Read, 1976; Henderson, 1978). Henderson (1978) suggested that the accumulation of triacylglycerols in the livers of stressed sheep is due to the apparent low rate of very low density lipoprotein biosynthesis in sheep liver (Leat *et al.*, 1976) and the presence of a continuous basal lamina in the hepatic sinusoids of sheep (Grubb & Jones, 1971; Gemmell & Heath, 1972) which may impede the movement of large molecules such as very low density lipoproteins from the parenchymal cells across the space of Disse to the sinusoids. Henderson (1978) provided evidence that the synthesis of choline by the methylation of phosphatidylethanolamine to phosphatidylcholine is depressed in the fatty livers of sheep suffering from alloxan-diabetes and pregnancy toxaemia. The decreased biosynthesis of phosphatidylcholine may well be responsible for some of the degenerative changes in hepatic membrane and subcellular organelle structure of alloxan-diabetic and pregnancy toxaemic sheep (Taylor *et al.*, 1971) and, in turn, on total liver function.

3d. Role of methionine in transmethylation reactions in tissues

Methionine is an essential amino acid in mammals. The metabolic

functions of methionine include: (1) utilization for protein synthesis, (2) precursor of polyamines, (3) conversion to cystathionine, cysteine, cystine and other derivatives of cysteine by the transsulphuration pathway, and (4) methyl group donor in many transmethylation reactions (Finkelstein, 1978). There is evidence that methionine can also be degraded by a transamination pathway (Case & Benevenga, 1976; Mitchell & Benevenga, 1978; Steele & Benevenga, 1978; Benevenga & Haas, 1979). Figure 8 is a general representation of the metabolic pathways of methionine in mammalian tissues.

The concept of biological transmethylation, that is the transfer of a complete methyl group to an acceptor molecule, 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), Griffith *et al.* (1954) and Jukes (1980)]. L-Methionine must be activated before it can act as a methyl donor (Cantoni, 1951), and the active intermediate is *S*-adenosyl-L-methionine (Cantoni, 1952, 1953). *S*-Adenosyl-L-methionine is synthesized in animal tissues by direct enzymatic adenosylation of L-methionine at the expense of ATP, by methionine adenosyltransferase (Figure 8, enzyme 1) (Mudd, 1973; Chou *et al.*, 1977). *S*-Adenosyl-L-methionine serves as the immediate methyl donor in numerous transmethylation reactions [reviewed by Mudd & Cantoni (1964), Cantoni (1975) and Salvatore *et al.* (1977)]. Examples include the methylation of phosphatidylethanolamine to phosphatidylcholine (described in Section 3c., of this review), guanidoacetate to creatine, L-lysine to 6-*N*-trimethyl-L-lysine which is an intermediate in carnitine synthesis. The methyl acceptors have not been fully characterized in many instances,

Figure 8: Major pathways of methionine metabolism in mammalian tissues

[modified from Finkelstein (1974, 1978)]

The key enzymes of these pathways are as follows:

1). Methionine adenosyltransferase

2). Nomenclature Committee of the International Union of Biochemistry (1979, 1980, 1981) lists 49 methyltransferases that utilize *S*-adenosyl-methionine as the methyl donor. Three such methyltransferases are:

   i) Guanidinoacetate methyltransferase, the terminal enzyme in creatine biosynthesis;

   ii) Phosphatidylethanolamine methyltransferase, the enzyme(s) associated with choline biosynthesis;

   iii) Protein (lysine) methyltransferase, a postulated enzyme in carnitine biosynthesis.

3). Adenosylhomocysteinase

4). Betaine-homocysteine methyltransferase

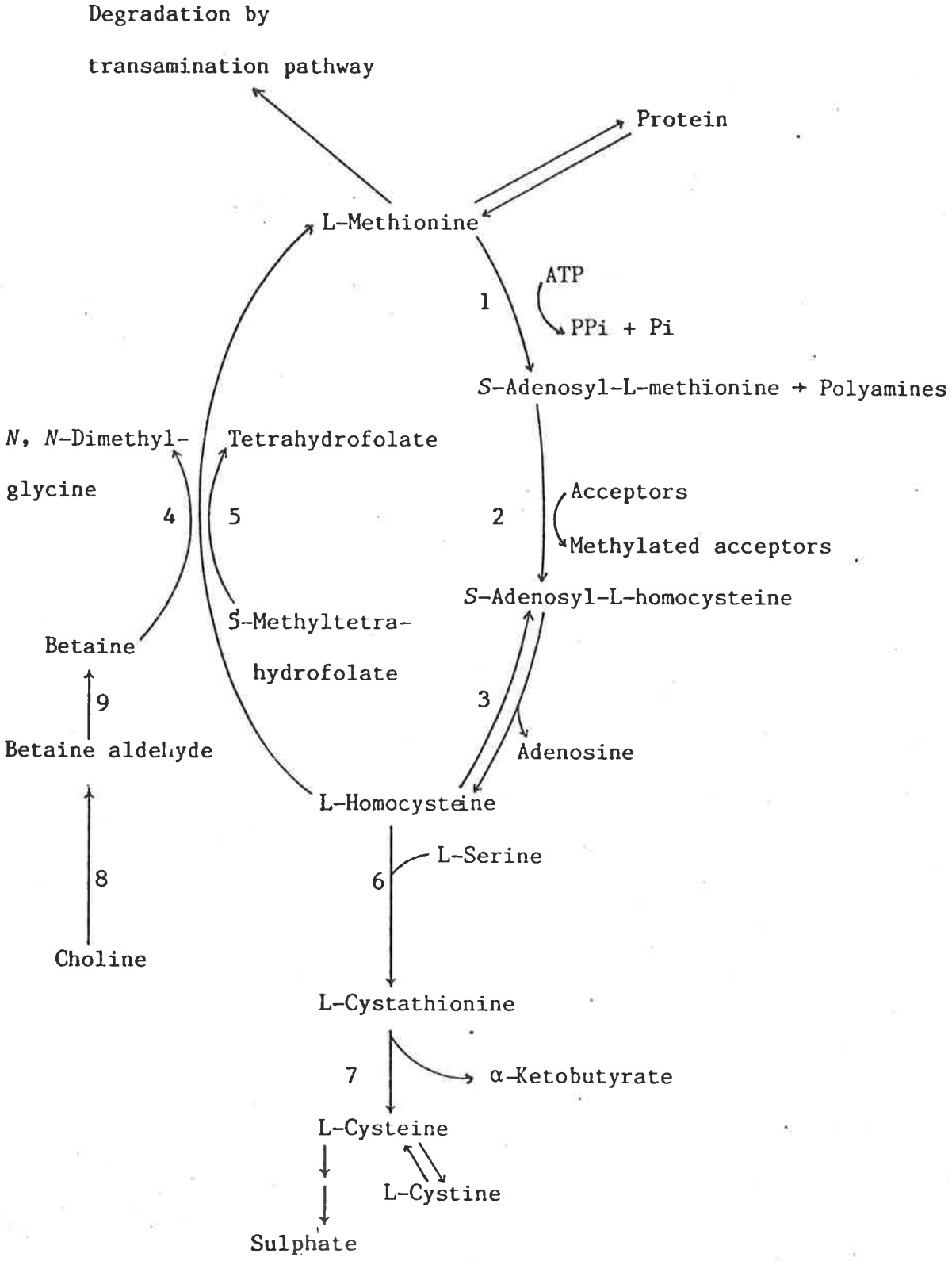
5). 5-methyltetrahydrofolate-homocysteine methyltransferase

6). Cystathionine β-synthase

7). Cystathionine γ-lyase

8). Choline dehydrogenase

9). Betaine-aldehyde dehydrogenase


Degradation by
transamination pathway
Protein
L-Methionine
ATP
1
PPi + Pi
S-Adenosyl-L-methionine → Polyamines
N, N-Dimethyl-
glycine
Tetrahydrofolate
4
5
2
Acceptors
Methylated acceptors
S-Adenosyl-L-homocysteine
5-Methyltetra-
hydrofolate
Betaine
9
3
Adenosine
Betaine aldehyde
L-Homocysteine
8
6
L-Serine
Choline
L-Cystathionine
7
α-Ketobutyrate
L-Cysteine
L-Cystine
Sulphate

particularly in the case of macromolecules such as proteins. Forty-nine methyltransferases (Figure 8, enzyme 2) have been identified that utilize the common substrate *S*-adenosyl-L-methionine (Nomenclature Committee of the International Union of Biochemistry, 1979, 1980, 1981), but few have been characterized. The relative quantitative importance of the transmethylation reactions and the factors involved in their regulation are largely unknown (Cantoni, 1977), except that methylation of guanidoacetate to creatine is quantitatively the major route of methyl group utilization in mammals (Mudd & Poole, 1975). *S*-Adenosyl-L-homocysteine, one of the products of all transmethylation reactions involving *S*-adenosyl-L-methionine, acts as a potent competitive inhibitor of most of these reactions (Duerre & Walker, 1977; Hoffman *et al.*, 1979). Many of the methyltransferases have a higher affinity for *S*-adenosyl-L-homocysteine than *S*-adenosyl-L-methionine (Hoffman *et al.*, 1979). A specific hydrolase, adenosylhomocysteinase (Figure 8, enzyme 3), catalyses the reversible hydrolysis of *S*-adenosyl-L-homocysteine to yield adenosine and L-homocysteine (De la Haba & Cantoni, 1959). The equilibrium of the reaction favours condensation; however, the compound will readily undergo hydrolysis if the products of the reaction are removed enzymatically.

The utilization of L-methionine for transmethylation and other reactions in body tissues is balanced either by dietary intake or by the formation of L-methionine by the methylation of L-homocysteine (Mudd & Poole, 1975; Mudd *et al.*, 1980). The reconversion of L-homocysteine back to L-methionine completes a cycle in which the methyl group of L-methionine has been transferred to an acceptor

molecule, but the L-homocysteine moiety has been conserved. L-Methionine can be regenerated by the transfer of a methyl group from betaine (derived from the oxidation of choline, as discussed in Section 3b.5 of this review) to homocysteine by betaine-homocysteine methyltransferase (Figure 8, enzyme 4). The other reaction product, *N*, *N*-dimethyl glycine, is converted to sarcosine and glycine and the methyl groups enter the one-carbon pool *via* formaldehyde. In the absence of dietary choline, the betaine-homocysteine methyltransferase reaction would result in depletion of the methionine methyl group, since three methionine molecules are utilized to synthesize a single choline (or betaine) molecule by the methylation of phosphatidylethanolamine.

L-Methionine can also be replenished by the transfer of a methyl group from 5-methyltetrahydrofolate to L-homocysteine by 5-methyltetrahydrofolate-homocysteine methyltransferase (Figure 8, enzyme 5) which requires methyl B_{12} (methylcobalamin) as a coenzyme and trace amounts of *S*-adenosyl-L-methionine as a cofactor. The methyl group of 5-methyltetrahydrofolate is synthesized *de novo* from one-carbon units attached to tetrahydrofolate which correspond to the formate and formaldehyde levels of oxidation. The one-carbon units can be obtained from formate, formaldehyde, L-serine, glycine and L-histidine. The 5-methyltetrahydrofolate-homocysteine methyltransferase reaction is the site of interdigitation of folate, vitamin B_{12}, one-carbon fragment and methionine metabolism. Choline and betaine are dispensable in the diet as sources of methyl groups for methionine synthesis so long as an adequate supply of folate, vitamin B_{12} and one-carbon precursors is present (McGilvery, 1970; Finkelstein, 1974, 1978).

In rats (Kutzbach *et al.*, 1967) and sheep (Smith *et al.*, 1974; Gawthorne & Smith, 1974), a deficiency of vitamin B_{12} has been shown to decrease hepatic 5-methyltetrahydrofolate-homocysteine methyltransferase activity resulting in impaired folate metabolism and reduced methionine synthesis.

Although some L-homocysteine can be recovered as L-methionine by methylation reactions, part is constantly lost *via* the transsulphuration pathway. In this process, L-homocysteine reacts with L-serine to form L-cystathionine in an irreversible reaction catalysed by cystathionine β-synthase (Figure 8, enzyme 6) which requires pyridoxal phosphate (active vitamin B_6) as cofactor. L-Cystathionine is cleaved to L-cysteine and α-ketobutyrate by cystathionine γ-lyase (Figure 8, enzyme 7) which also requires pyridoxal phosphate. Further metabolism of L-cysteine converts what once was the sulphur atom of L-methionine to L-cystine and ultimately inorganic sulphate. L-Cysteine and L-cystine are incorporated into proteins, particularly α-keratin. The transsulphuration pathway is the major route for L-methionine degradation in mammals (Finkelstein & Mudd, 1967; Finkelstein, 1974).

Finkelstein (1978) reported that the activities of the enzymes of methionine metabolism in various rat tissues are not constant. Indeed the scheme depicted in Figure 8 is incomplete in all rat tissues except the liver. In rats the liver is the only tissue in which there is a substantial amount of betaine-homocysteine methyltransferase. In all other tissues, 5-methyltetrahydrofolate-homocysteine methyltransferase appears to be the major means of L-homocysteine remethylation. Heart, testes, lung and adrenal gland

are deficient in cystathionine β-synthase and cystathionine γ-lyase, while the small intestinal mucosa lacks both homocysteine methyltransferases. Methionine adenosyltransferase and adenosylhomocysteinase are present in all tissues. All rat tissues have the capacity to synthesize *S*-adenosyl-L-methionine and contain at least one enzyme capable of metabolizing L-homocysteine. The activities of the enzymes involved with methionine conservation in rat liver decrease with age. Conversely, the activities of transsulphuration enzymes in rat liver increase with maturation. Radcliffe & Egan (1974) determined the activities of methionine adenosyltransferase and cystathionine γ-lyase in various tissues of the neonatal lamb, pre-ruminant lamb and calf, and adult sheep, cattle and goat. Methionine adenosyltransferase was widely distributed in the tissues of all ruminant species. Greatest activities were found in liver, kidney, spleen and duodenal wall in adult ruminants, and also in skeletal muscle in pre-ruminant lambs and calves. Hepatic methionine adenosyltransferase activity declined with age in sheep and cattle. Cystathionine γ-lyase activity was not detectable in skeletal muscle or heart of any ruminant species. Greatest activities were found in liver, kidney and pancreas of all species. Neonatal lamb tissues had the highest activities. Cystathionine γ-lyase activity in sheep liver, pancreas, kidney cortex and spleen declined with age.

The partitioning of homocysteine either to cystathionine or to methionine is the branch point which determines the balance between transsulphuration and methionine conservation in tissues. Mudd & Poole (1975) and Mudd *et al.* (1980) have determined the rate of *de novo* methyl formation versus utilization of preformed methyl groups

and the average number of times a homocysteinyl moiety passes through the conservation cycle before it is converted to cystathionine in humans on various dietary regimens. Diets restricted in methionine and choline causes methylneogenesis to increase to compensate for the decreased dietary intake of methyl moieties. Studies with rat tissues suggested that conservation of methionine, in response to deprivation of this essential amino acid, is achieved by an increase in 5-methyltetrahydrofolate-homocysteine methyltransferase activity relative to cystathionine β-synthase activity which results in an increase in the remethylation of homocysteine. Catabolism of excess methionine depends on degradation of homocysteine to cystathionine as a consequence of a relative increase in cystathionine β-synthase activity. Fundamental to this regulatory mechanism are both the inherent kinetic properties of the constituent enzymes and the ability of hormones and diet to alter the tissue levels of the enzymes. The role of betaine-homocysteine methyltransferase in the maintenance of hepatic levels of methionine has not been fully elucidated. It is thought that betaine-homocysteine methyltransferase may be of major importance in the catabolism of excessive amounts of choline and homocysteine in the liver (Finkelstein *et al.*, 1971; Finkelstein, 1974, 1978).

Both direct and indirect evidence indicate that sulphur-containing amino acids are of critical importance in the nutrition of ruminants, and more specifically that methionine is the first limiting amino acid for sheep (Chalupa, 1972; Barry *et al.*, 1973; Schelling *et al.*, 1973). As wool proteins are rich in cystine (Crewther *et al.*, 1965), the conversion of methionine to cysteine

and cystine may represent a major metabolic route for methionine in sheep. Wool has a nitrogen : sulphur ratio of 4-6 compared with body protein and rumen bacterial protein which have a nitrogen : sulphur ratio of about 15 and 13 respectively (Radcliffe & Egan, 1974). Reis *et al.* (1973*a*) showed that there is a large increase in wool growth after small amounts of methionine (up to 2.5g/day) are infused into the abomasum of sheep. In sheep subjected to methionine loading (about 2.5 - 10g/day) there is a dose dependent reduction of wool growth and large increases in plasma concentration of methionine (Reis *et al.*, 1973*a*, *b*). This appears to be due to reduced entry of methionine into the transsulphuration pathway because of a depression of methionine adenosyltransferase activity and an increase in betaine-homocysteine methyltransferase activity (Radcliffe & Egan, 1978).

In sheep the liver carnitine content is greatly increased under conditions of starvation (Snoswell & Henderson, 1970), alloxan-diabetes (Snoswell & Koundakjian, 1972; Snoswell & McIntosh, 1974) and in pregnancy toxaemia (Pethick, 1975) which corresponds to the increase in liver triacylglycerol content (Snoswell & Henderson, 1980). The increase in liver carnitine content has been shown to be due to increased biosynthesis in alloxan-diabetic sheep. The blood carnitine concentration rises up to 10-fold and the daily loss of carnitine in the urine increases markedly in sheep in the terminal diabetic state (Snoswell & McIntosh, 1974). Carnitine is a vital cofactor involved in fatty acid oxidation (Bremer, 1977), and the unique dramatic response of liver carnitine levels to liver lipid levels in sheep under conditions of metabolic stress is presumably

to increase the flux of fatty acids *via* the oxidative pathway thus reducing the extent of esterification to triacylglycerols which cannot be readily transported out of the liver (Henderson, 1978; Snoswell & Henderson, 1980). Carnitine has three methyl groups which are derived from methionine (*via* *S*-adenosyl-L-methionine) and when large amounts of carnitine are synthesized in the liver of sheep, as in the severe diabetic state, and then passes into the blood and urine, this represents a major drain on the methyl pool and hence methionine. This is particularly critical in the sheep where methionine is the first limiting amino acid. There is evidence that in the diabetic state increased hepatic carnitine biosynthesis in sheep can proceed to a large extent because utilization of the methionine methyl group for choline and creatine biosynthesis is substantially reduced (Henderson, 1978). In rats injected with azo-adenosine to block transmethylation reactions, the liver carnitine content increases while the liver creatine content decreases, suggesting that under these circumstances carnitine synthesis takes preference for available methyl groups (Silipramdi, 1980). 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 group of methionine is used preferentially for choline biosynthesis at the expense of carnitine biosynthesis.

4. Aims of the work in this thesis

The information presented in the literature review makes it clear that there are some unique aspects of choline nutrition and metabolism in the sheep which have only been recognized recently.

Much remains to be elucidated, particularly the reasons why the sheep survives on an apparently minimal intake of dietary choline. This is the main focus of the work in this thesis. The specific aims are as follows:

(1) To examine the choline and betaine content of a range of dried and fresh pastures which would normally be grazed by sheep in Australia, with particular emphasis on dry summer pastures.

(2) To develop a sensitive and specific assay for choline in the presence of rather high amounts of interfering metabolites (e.g. carnitine) in order to analyse sheep tissues and fluids.

(3) To determine the flux of choline in the alimentary and urinary tracts of the sheep.

(4) To investigate the uptake and output of choline by organs of sheep.

(5) To study the synthesis of choline in sheep tissues by the methylation of phosphatidylethanolamine to phosphatidylcholine.

(6) To examine the retention and recycling of choline in the sheep body.

CHAPTER 1

EXAMINATION OF THE CHOLINE AND BETAINE CONTENT OF PASTURES

1.1 Introduction

In Australia sheep are extensively grazed on open pastures in contrast to European countries where sheep are under more intensive management and given supplements of high calorific value. During the hot dry summers, when fresh pastures have dried off, it is common practice to allow sheep to graze cereal stubble paddocks and residual grain left on the ground after harvesting. Dried hays are often used as supplementary feedstuffs to sustain the nutritional requirements of grazing animals under these harsh conditions (Wheeler & Hutchinson, 1973; Allden, 1984).

Preliminary work by Snoswell *et al.* (1978) showed that in dried wheaten hay most of the choline is in the unesterified form rather than as phosphatidylcholine.(Similar results were obtained by Neill *et al.*, 1979). This is in direct contrast to fresh pasture plants in which the concentration of phosphatidylcholine is significantly higher than unesterified choline (Roughan & Batt, 1969; Storey & Wyn Jones, 1975, 1977). Robinson (1980) found that dried wheat, barley and oat stubbles and dried medic plants contain more free choline and glycerophosphocholine than phosphatidylcholine, in marked contrast to the equivalent fresh plant material. In addition, dried stubbles and medic were found to contain lower concentrations of all forms of choline compared with the fresh material. Since the choline content of dried pastures is low and in a form more susceptible to breakdown in the rumen by micro-organisms (Neill *et*

al., 1978, 1979), this raises the question of the adequacy of choline intake for sheep on such pastures. The closely related compound, betaine, accumulates in water-and salt-stressed growing plants (Storey & Wyn Jones, 1977; Wyn Jones & Storey, 1978; Hanson & Nelsen, 1978; Hanson & Scott, 1980; Hitz & Hanson, 1980). However, Robinson (1980) observed a much lower concentration of betaine in dried stubbles and medic than in the equivalent fresh plants. This implies that sheep grazing dried summer pastures would receive minimal amounts of dietary betaine to act as a methyl donor for choline synthesis in tissues.

The aim of the work in this Chapter was to confirm and extend the choline and betaine analyses of dried and fresh pastures which would normally be consumed by sheep in Australia, with specific emphasis on dry summer pastures. This work seemed particularly vital in view of Australian summer conditions, where sheep grazing dried pastures may become choline deficient which would be further aggravated in times of drought and restricted feed intake.

1.2 Methods and Materials

1.2.1 Collection of pasture material

Wheat (*Triticum aestivum* L., cultivar Warigal), barley (*Hordeum vulgare* L., cultivar Galleon) and triticale (X *Triticosecale* Wittmack, cultivar Coorong) stubbles and barrel medic hay (*Medicago truncatula* Gaertn., cultivar Jemalong) were obtained in the late summer of 1980/1981 from The Charlick Experimental Research Station, The University of Adelaide, Strathalbyn, S. Aust., Australia. The cereal stubbles were from crops which had been harvested about two months previously.

The stubbles were standing in the paddock approximately 40cm high, with threshings, residual grain and weed seedlings on the ground. The respective stubbles were cut 2-4cm from ground level with hedge shears over an area of about 20 m^2 and all material on the ground raked up and collected into bags. The barrel medic hay had been cut and baled about three months previously and stored under cover on the property. The three types of cereal stubble and medic hay were chaffed to a uniform consistency using an Agserv Hy-Put hammer-mill fitted with a 2.54cm cutting sieve (Agserv Industries Pty. Ltd., Adelaide, S.Aust. Australia).

Chaffed lucerne hay (*Medicago sativa* L., cultivar Hunter River) was obtained from The Waite Agricultural Research Institute, The University of Adelaide. It is routinely used as a feed for housed sheep, cattle and goats.

Fresh pasture consisting mainly of perennial rye grass (*Lolium perenne* L., cultivar Medea), annual rye grass (*Lolium rigidum* Gaudin, cultivar Wimmera), barley grass (*Hordeum leporinum* Link) and sub-terranean clover (*Trifolium subterraneum* L., cultivar Mount Barker) was cut just above ground level with scissors from several areas of a paddock at The Waite Agricultural Research Institute in the mid-winter of 1981. The stems and leaves were chopped into small pieces and thoroughly mixed. The fresh plant material was collected immediately before extraction to minimize water-stress.

1.2.2 Extraction of pasture material

Quadruplicate samples were taken from each type of bulk pasture material and were extracted by a procedure based on that of

Toyosawa & Nishimoto (1967) and Storey & Wyn Jones (1977). All solvents contained the antioxidant 2,6-di-*tert*-butyl-4-methylphenol (50mg/l) to minimize autoxidation of polyunsaturated fatty acids (Wren & Szczepanowska, 1964; Holman, 1967). Chopped pasture material (10g fresh wt.) was homogenized in *iso*-propan-2-ol (100ml) with a Sorvall omni-mixer homogenizer (Ivan Sorvall Inc., Norwalk, Connecticut, U.S.A.) on setting 7 for 5 min and filtered under vacuum through filter paper (previously boiled in chloroform). The residue was homogenized with a further volume of *iso*-propan-2-ol (75ml) and filtered. The residue was then blended with two volumes of methanol (50ml) using a Polytron Type PT 10 20350D tissue homogenizer and sonicator fitted with a PCU-2 speed control on setting 7 (Kinematica, G.m.b.H., Luzern, Switzerland) for 2 min and filtered. Finally, the residue was boiled in methanol (25ml) for 10 min and filtered. The 5 combined extracted volumes were taken to dryness *in vacuo* at 30-40°C. The flask containing the concentrate was washed successively with chloroform (7.50ml); methanol (3.75ml); water (2.25ml); chloroform (5.00ml); methanol (2.50ml) and water (1.50ml). The total extract was placed in a stoppered 50ml glass measuring cylinder and shaken vigorously for 5 min. After centrifugation at 1,000g for 10 min, the upper water-soluble phase was removed as completely as possible with a pasteur pipette and microlitre syringe. The tube-wall and interphase of the lower lipid phase were rinsed 3 times with 4ml of the upper phase of a pure solvent mixture of chloroform/methanol/water (10:5:3, by vol.). The water-soluble phase and the 3 washings were evaporated to dryness *in vacuo* at 30-40°C and the residue redissolved in 3ml of water. This water-soluble fraction was shaken well with diethylether (3x 1ml) to

remove lipid contamination, and the ether phases were combined into the lipid phase. The lipid phase was taken to dryness *in vacuo* at 30-40°C and the residue redissolved in 10ml of chloroform. The water-soluble and lipid fractions were stored at -15°C until required for analysis.

1.2.3 Determination of various forms of choline and betaine

A 5ml portion of the lipid fraction of extracted pasture samples containing lipid choline (choline phospholipids) was evaporated to dryness *in vacuo* at 30-40°C and hydrolysed to unesterified choline with 3ml of 6M-HCl at 110°C for 24h in a sealed test tube (Collins & Shotlander, 1961). The fraction was taken to dryness *in vacuo* at 40°C and the residue redissolved in 2.0ml of distilled water. This was then centrifuged at 8,000g for 4 min in 1ml stoppered plastic centrifuge tubes in an Eppendorf 3200 microcentrifuge (Eppendorf Gerätebau, Netheler and Hinz G.m.b.H., Hamburg, Germany) and assayed for unesterified choline as described below.

A 1ml portion of the lipid fraction was evaporated to dryness under N_2 in a 1ml plastic centrifuge tube and the residue redissolved in 200µl of chloroform. A portion of the chloroform extract (20µl) and 10µg of choline-containing phospholipid standards (phosphatidylcholine, lysophosphatidylcholine and sphingomyelin) were applied to a 0.2mm pre-coated silica gel 60 thin-layer chromatography plate (E. Merck, Darmstadt, Germany) that had been prewashed chromatographically with diethyl ether. The plate was first developed in the solvent chloroform/methanol (9:1, v/v) to move pigments and non-

polar lipids to the top. After drying, the plate was again developed with chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) solvent in the same direction to separate the phospholipids. The plate was dried and the phospholipids visualized with I_2 vapour. Comparison with the standards showed which choline-containing phospholipids were present in the lipid fraction of respective pasture samples.

A 0.5ml portion of the water-soluble fraction of extracted pasture samples (containing glycerophosphocholine, phosphocholine, unesterified choline and betaine) was centrifuged at 8,000g for 2 min in a 1ml stoppered plastic centrifuge tube in an Eppendorf 3200 microcentrifuge and the pH adjusted to 8-9 with NH_3 vapour. The solution was applied to a column (0.8cm x 10cm) of Dowex AG 50W (X8; 100-200 mesh; H^+ form) cation-exchange resin equilibrated with distilled water (pH 5-6) and successively washed with 25ml of water, 30ml of 0.4M-HCl and 25ml of 3M-HCl to elute glycerophosphocholine, phosphocholine, and unesterified choline plus betaine respectively (Webster & Cooper, 1968; Illingworth & Portman, 1972). The glycerophosphocholine and phosphocholine fractions were taken to dryness *in vacuo* at 30-40°C. The glycerophosphocholine residue was hydrolysed to unesterified choline in 5ml of 1M-HCl at 100°C for 30 min. The phosphocholine residue was hydrolysed to unesterified choline in 5ml of 3M-HCl at 123°C for 24h in a sealed test tube (Dawson, 1955*b*). The glycerophosphocholine and phosphocholine hydrolysates were evaporated to dryness *in vacuo* at 30-40°C and redissolved in 500µl and 250µl of distilled water respectively and the unesterified choline determined. The fraction containing unesterified choline and betaine was taken to dryness *in vacuo* at 30-40°C and the

residue redissolved in 500µl of distilled water which was then assayed for both choline and betaine. The recoveries of mixed or individual standards of glycerophosphocholine, phosphocholine, unesterified choline and betaine (2 µmol of each/0.5ml of distilled water) from the column and subsequent processing were at least 93%.

The specificity of the column cation-exchange chromatography technique was investigated for all pasture samples by quantitative thin-layer chromatography. Another 0.5ml portion of the water-soluble fraction of extracted pasture samples was fractionated on the Dowex column as before. The glycerophosphocholine, phosphocholine, and unesterified choline plus betaine column fractions were taken to dryness *in vacuo* and redissolved in 250µl of distilled water respectively. A portion (50µl) of each concentrated column fraction along with 20-50µg of corresponding standards were applied to 0.2mm pre-coated silica gel 60 thin-layer chromatography plates and developed in the solvent systems methanol/0.6% NaCl/NH_3 (sp. gr. 0.88) (10:10:1, by vol.), methanol/acetone/11M-HCl (45:5:2, by vol.) and chloroform/methanol/NH_3 (sp. gr. 0.88) (6:3:1, by vol.). After the plates were dried, the glycerophosphocholine, phosphocholine, unesterified choline and betaine spots were located with I_2 vapour,scraped off and eluted with 3ml of methanol. A portion (2ml) of the glycerophosphocholine and phosphocholine extracts was taken to dryness under N_2 and the residue hydrolysed and redissolved in 200µl of distilled water which was assayed for unesterified choline. A portion (2ml) of the choline and betaine extracts was taken to dryness under N_2 and the residue assayed for choline or betaine after being redissolved in 200 µl of distilled water.

There is a tendency for small amounts of lysophosphatidylcholine to distribute in the upper phase of a chloroform/methanol/

water extraction (Bjerve *et al.*, 1974). The washing of the water-soluble phase of extracted pasture samples with diethyl ether should have removed most of this phospholipid contamination if present. Any lysophosphatidylcholine still remaining in the water-soluble fraction loaded on the Dowex column would not have been eluted under the conditions used (Blusztajn *et al.*, 1979), and therefore would not have interfered with the glycerophosphocholine, phosphocholine and unesterified choline plus betaine fractions.

Unesterified choline and betaine in pasture samples were both determined essentially by the colorimetric periodide assay for determination of quaternary ammonium compounds described by Storey (1976) and Storey & Wyn Jones (1977). It involves the precipitation of the periodide ion (the ennaiodide, I_8I^- or I_6I^-, Wall *et al.*, 1960) of the quaternary ammonium compound in water, which is redissolved in ethylene dichloride and quantitatively determined from absorption measurements at 365nm. While all quaternary ammonium compounds are precipitated by KI_3 in acidic solutions, only choline is also reactive in the alkaline pH range (Wall *et al.*, 1960). This selective precipitation at an alkaline pH (7-11) has been used as a specific assay for choline. It has also been shown for some, but not all plant species, that quaternary ammonium compounds other than choline and betaine are negligible so that the difference in periodide ion precipitation of total quaternary ammonium compounds (pH 2) and choline (pH 8) gives a satisfactory assay of the betaine content (Storey, 1976; Storey & Wyn Jones, 1977). This procedure was used to determine betaine in the unesterified choline plus betaine column fraction of the pasture samples

investigated in this work. Its validity was checked in each case by separating the quaternary ammonium compounds by thin-layer chromatography and quantifying choline and betaine independently by the periodide assay.

The alkaline potassium triiodide reagent (for choline) was prepared by dissolving 7.5g of I_2 and 10g of KI in 100ml of 0.4M-KH_2PO_4/NaOH buffer (pH 8.0) which was stirred for 30min and filtered. The acid potassium triiodide reagent (for total quaternary ammonium compounds) was prepared by dissolving 7.5g of I_2 and 10g of KI in 100ml of 1M-HCl. The solution was stirred for 30min and filtered. To a 100µl aqueous plant sample (containing between 0-0.3 µmol of quaternary ammonium compounds) in a 15ml tapered glass centrifuge tube, 40µl of the alkaline or acid potassium triiodide reagent was added accurately. Equivalent choline chloride and betaine hydrochloride standards were treated with 40µl of the alkaline and acid potassium triiodide reagent respectively. The mixture was shaken and left for 90min in a reciprocating ice-water bath to cause periodide ion precipitation. Cold distilled water (400µl) was then added rapidly to the mixture and shaken, followed immediately with 1,2-dichloroethane (4ml) at -10°C. The two layers were mixed by a constant stream of N_2 gas for 5min, while the temperature was maintained at 4°C. A portion of the lower organic layer was pipetted into a 1ml semi-micro cuvette (avoiding contamination from the upper aqueous layer) and the absorbance measured at 365nm in a Zeiss PMQII spectrophotometer (Carl Zeiss, Oberkocken, Germany) against an identical water blank. The colour intensity was stable for at least 1h. The choline chloride and betaine hydrochloride standard

curves were linear over the 0-0.3 µmol range, and 0.1 µmol of choline chloride and betaine hydrochloride had an absorbance at 365nm of 0.649 ± 0.015 and 0.415 ± 0.025 units (mean ± S.E.M. of 12 curves) respectively. The absorbance was similar when choline chloride standards were assayed with the alkaline or acid potassium triiodide reagent. When betaine hydrochloride standards were assayed with the alkaline potassium triiodide reagent the absorbance was zero. This demonstrated that choline was exclusively precipitated by alkaline potassium triiodide. All pasture samples and standards were assayed in duplicate.

1.2.4 Determination of dry weights of pasture material

Quadruplicate chopped samples (0.5 - 1.5g fresh wt.) of each type of bulk pasture material were placed in tared glass petri dishes and oven-dried at 100°C for 48h to a constant weight. The petri dishes were cooled to room temperature in an evacuated desiccator for at least 1h prior to weighing.

1.2.5 Chemicals

Choline chloride (3 x crystallized), betaine hydrochloride, L-α-glycerophosphocholine (grade I, from egg yolk, cadmium chloride complex), phosphocholine chloride (calcium salt), L-α-phosphatidylcholine (type V-E, from egg yolk), L-α-lysophosphatidylcholine (type I, from egg yolk) and sphingomyelin (from bovine brain) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. The salts of glycerophosphocholine and phosphocholine were converted to their free forms for the Dowex column cation-exchange chromatography step

by being treated with a mixture of analytical grade Amberlite IRC-50(H) cation-exchange resin and Amberlite IR-4B(OH) anion-exchange resin (Baer & Robinson, 1967) obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Dowex AG 50W (X8; 100-200 mesh; H^+ form) cation-exchange resin was from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and 2,6-di-*tert*-butyl-4-methylphenol from Calbiochem, Los Angeles, Calif., U.S.A. Reagent grade chloroform, methanol, *iso*-propan-2-ol and 1,2-dichloroethane were glass-distilled. The re-distilled 1,2-dichloroethane was water-saturated as follows: 200ml of 1,2-dichloroethane and 200ml of distilled water were added to a 1 litre separation funnel. The funnel was shaken for 2min and then allowed to stand for 5min before the 1,2-dichloroethane was removed. Other chemicals were reagent-grade or the best commercially available grade. Glass double-distilled water was used throughout.

1.3 Results

1.3.1 Concentrations of various forms of choline in pasture material

The concentrations of unesterified choline, lipid choline, glycerophosphocholine, phosphocholine and total choline in wheat, barley and triticale stubbles, medic and lucerne hays, and fresh grass/clover pasture are shown in Table 1.1. In the wheat, barley and triticale stubbles most of the choline was present as unesterified choline and glycerophosphocholine, with quite small amounts of lipid choline and phosphocholine. In the medic and lucerne hays the concentration of unesterified choline was substantially higher

Table 1.1: Concentrations of various forms of choline in pasture material

The different types of pasture material were collected and cut to uniform consistency as described in Section 1.2.1. Representative samples of each type of bulk pasture material were extracted and unesterified choline, lipid choline, glycerophosphocholine and phosphocholine fractionated and determined in duplicate as described in Sections 1.2.2 and 1.2.3. The dry weights of pasture samples were determined as described in Section 1.2.4. The values shown are means ± S.E.M. for four samples of each type of pasture material. Abbreviation used: N.D. = not detectable.

Pasture material	Concentration (μmol/g dry wt.)				
	Unesterified choline	Lipid choline	Glycerophospho-choline	Phosphocholine	Total choline
Wheat stubble	0.57 ± 0.02	0.30 ± 0.02	3.32 ± 0.21	0.07 ± 0.02	4.26
Barley stubble	0.48 ± 0.02	0.28 ± 0.02	2.59 ± 0.19	0.04 ± 0.01	3.39
Triticale stubble	0.64 ± 0.02	0.34 ± 0.02	4.17 ± 0.20	0.13 ± 0.01	5.28
Medic hay	3.18 ± 0.18	0.63 ± 0.11	1.42 ± 0.32	0.10 ± 0.02	5.33
Lucerne hay	6.32 ± 0.07[a]	1.36 ± 0.21[b]	N.D.	N.D.	7.68[c]
Fresh grass/ clover pasture	2.90 ± 0.27	6.38 ± 0.47	2.77 ± 0.17	0.52 ± 0.04	12.57

[a] Equivalent to 5.64 μmol/g fresh wt. of lucerne hay.
[b] Equivalent to 1.21 μmol/g fresh wt. of lucerne hay.
[c] Equivalent to 6.85 μmol/g fresh wt. of lucerne hay.

than the other forms of choline. It is interesting to note that the lucerne hay contained no glycerophosphocholine or phosphocholine. The major form of choline in the fresh grass/clover pasture was lipid choline, which is in direct contrast to the dried pasture material. The concentrations of lipid choline, phosphocholine and total choline were considerably higher in the fresh pasture than in the dried stubbles and hays. Thin-layer chromatography revealed that the lipid choline in all of the pasture material analysed was almost exclusively phosphatidylcholine. There were only trace amounts of lysophosphatidylcholine and no sphingomyelin or choline plasmalogens present. This was also observed by Roughan & Batt (1969) and Robinson (1980). The choline values presented in Table 1.1 are comparable with those previously reported for plant material in general (Toyosawa & Nishimoto, 1967; Roughan & Batt, 1969; Storey & Wyn Jones, 1975, 1977; Snoswell *et al.*, 1978; Neill *et al.*, 1979; Robinson, 1980).

1.3.2 Concentration of betaine in pasture material

Table 1.2 shows the concentration of betaine in wheat, barley and triticale stubbles, medic and lucerne hays, and fresh grass/clover pasture. The betaine content of the fresh pasture was much higher than that of the dry stubbles and hays. In the dry stubbles and hays the concentration of betaine was less than that of total choline. This was in contrast to the fresh pasture in which the concentration of betaine was higher than that of total choline. In this study the betaine content of the pasture material was determined by the expression: total quaternary ammonium compounds (pH 2) -

Table 1.2: Concentration of betaine in pasture material

The different types of pasture material were collected and cut to uniform consistency as described in Section 1.2.1. Representative samples of each type of bulk pasture material were extracted and betaine fractionated and determined in duplicate as described in Sections 1.2.2 and 1.2.3. The dry weights of pasture samples were determined as described in Section 1.2.4. The values shown are means ± S.E.M. for four samples of each type of pasture material.

Pasture material	Betaine concentration (µmol/g dry wt.)
Wheat stubble	3.42 ± 0.31[a]
Barley stubble	1.69 ± 0.32[a]
Triticale stubble	3.97 ± 0.14[a]
Medic hay	0.35 ± 0.02[b]
Lucerne hay	0.24 ± 0.02[b]
Fresh grass/clover pasture	22.81 ± 1.31[a]

[a] Betaine determined by the expression: total quaternary ammonium compounds (pH 2)-choline (pH 8) = betaine and also by quantitative thin-layer chromatography using the colorimetric periodide assay.

[b] Betaine determined by quantitative thin-layer chromatography using the colorimetric periodide assay.

choline (pH 8) = betaine and also by quantitative thin-layer chromatography using the colorimetric periodide assay. Both methods gave essentially identical betaine values for the three types of stubble and the fresh pasture because they contained negligible amounts of other quaternary ammonium compounds in addition to choline and betaine. The agreement between the two methods was unsatisfactory for the betaine content of the lucerne and medic hays because they contained substantial amounts of other quaternary ammonium compounds besides choline and betaine which caused the expression: total quaternary ammonium compounds (pH 2) - choline (pH 8) = betaine to be overestimated. There are reports that lucerne contains high amounts of the quaternary ammonium compound prolinebetaine (stachydrine) (Sethi & Carew, 1974; Wyn Jones & Storey, 1981). The betaine values shown in Table 1.2 are consistent with those previously published for plant material in general by other workers (Cromwell & Rennie, 1953; Storey & Wyn Jones, 1975, 1977; Storey *et al.*, 1977; Hitz & Hanson, 1980; Hanson & Scott, 1980; Wyn Jones & Storey, 1981).

1.4 Discussion

The work in this Chapter showed that there is a pronounced difference in the choline and betaine content of dried summer pastures and fresh green pastures commonly used for grazing sheep in Australia. The results support the preliminary findings of Snoswell *et al.* (1978), Neill *et al.* (1979) and Robinson (1980).

The reason for the higher ratio of unesterified choline and/or glycerophosphocholine to phosphatidylcholine in dried stubbles and

hays compared with fresh pasture is presumably due to the activation of plant phospholipases during the processes of senescence and drying. Activation phospholipase D in senescent and drying plants would cause the direct conversion of phosphatidylcholine to unesterified choline. The combined activation of phospholipases A_1 or A_2 and lysophospholipase in such plant material would form glycerophosphocholine from phosphatidylcholine. It is also feasible that some of the phosphatidylcholine in dried plant material is degraded to free choline and glycerophosphocholine by the action of aerobic bacteria. The lower level of total choline in dried stubbles and hays compared to fresh pasture is probably due to aerobic bacterial degradation and/or the action of degradative enzymes during aging and drying. It is possible that the choline present in the stem and leaves of mature cereal crops is transported to the harvested grain which is reported to have a high choline content (Glick, 1945; Chittenden *et al.*, 1978).

Betaine occurs in turgid leaves of certain cereal crops and grasses at a concentration of about 20 µmol/g dry wt. (Storey *et al.*, 1977; Hanson & Scott, 1980; Wyn Jones & Storey, 1981). The betaine concentration increases several fold in most of these plants upon moderate water stress (Hanson & Nelsen, 1978; Wyn Jones & Storey, 1978; Hanson & Scott, 1980; Hitz & Hanson, 1980) and upon salination (Storey & Wyn Jones, 1977; Wyn Jones & Storey, 1978). This accumulation of betaine is very likely to be of adaptive significance, perhaps as a nontoxic cytoplasmic osmoticum and/or as a protectant against enzyme inactivation (Wyn Jones & Storey, 1981; Paleg *et al.*, 1981; Nash *et al.*, 1982). Betaine accumulation in

leaves of stressed plants is due to enhanced *de novo* synthesis from serine and ethanolamine rather than by the oxidation of pre-existing choline pools. The betaine amassed during stress is not metabolized upon stress-relief, but can be translocated in the phloem from mature leaves to shoot growing points (Hanson & Nelsen, 1978; Hanson & Scott, 1980; Ladyman *et al.*, 1980; Hitz *et al.*, 1981, 1982; Hanson & Wyse, 1982; Coughlan & Wyn Jones, 1982; Hanson & Rhodes, 1983). The present work showed that the betaine content of dried stubbles and hays is much less than fresh pasture plants. This implies that the betaine accumulated during the growth period of plant material undergoes extensive loss or breakdown during senescence and drying, probably by the same mechanisms proposed above for choline.

Since the choline in dried summer pastures mainly exists as free choline and glycerophosphocholine instead of phosphatidylcholine as in fresh pasture, it is unlikely to make a significant contribution to the choline requirements of sheep as it will be highly vulnerable to microbial degradation in the rumen. Unesterified choline and glycerophosphocholine will be converted through the intermediary of trimethylamine into methane, which is lost in the gaseous mixture resulting from rumen fermentation (Dawson, 1959; Neill *et al.*, 1978, 1979). The only choline in dried summer pastures escaping such degradation will be a negligible amount of phosphatidylcholine that is directly incorporated into the membranes of ciliated protozoa (Neill *et al.*, 1979). Not only is the choline in dried summer pastures in a form susceptible to rumen microbial breakdown, there is also a lower total amount than in fresh pasture.

Dried summer pastures are very fibrous and therefore have low digestibility. Sheep could not physically ingest enough dried plant material to compensate for the low choline content. Dietary betaine is also degraded in the rumen by micro-organisms (Mitchell *et al.*, 1979), and sheep grazing dry summer pastures containing low amounts of betaine would receive virtually none of this compound to act as a methyl donor for choline biosynthesis in tissues.

In view of the results presented here it would seem pertinent to suggest that sheep fed on dried pastures under Australian summer conditions would receive a suboptimal choline and betaine intake, and thus may become marginally choline deficient with an associated loss in production. However, soon after the present investigation was completed Dawson *et al.* (1981) reported that injection of [*methyl*-^{14}C] choline into sheep indicated that the small amount of phosphatidylcholine present in abomasal digesta was largely (69%) of non-dietary or ruminal origin. Long-term feeding of [*methyl*-^{3}H] choline to sheep produced insignificant labelling of plasma phosphatidylcholine, indicating that more than 99% of the choline body pool was of non-dietary origin. In contrast, when rats were fed with [*methyl*-^{3}H] choline for similar time periods, 18-54% of the tissue phosphatidylcholine was derived from dietary origin. The loss of [^{14}C] choline and ^{32}P from the plasma phosphatidylcholine after a single injection of these isotopes indicated a markedly slower turnover of choline in the sheep compared with the rat. This subsequent information provides good evidence that sheep are not dependent on dietary choline for maintaining the choline body pool. Thus it is unlikely, as first thought, that sheep would suffer from dietary choline deficiency even when grazing on Australian summer pastures which contain extremely low levels of choline and betaine.

CHAPTER 2

THE RADIOENZYMIC ASSAY OF CHOLINE AND ITS APPLICATION TO THE STUDY OF CHOLINE PASSAGE IN THE ALIMENTARY AND URINARY TRACTS OF SHEEP

2.1 Introduction

In order to investigate the nutrition and metabolism of choline in sheep it was essential to be able to accurately measure choline in body tissues and fluids. The colorimetric periodide method (Storey, 1976; Storey & Wyn Jones, 1977) was suitable for measuring choline in plant samples in Chapter 1 because there were generally high amounts of choline present and it also enabled betaine to be determined simultaneously. However, preliminary work revealed that the periodide method lacked sensitivity and specificity for the quantitative determination of choline in some animal samples where the concentrations of this metabolite are extremely low. The advantages associated with enzymic radiochemical assays of choline (Hanin, 1974), including sensitivity, specificity and reasonable convenience, favoured their use for the animal studies in this thesis. The radioenzymicassay of choline involving the reaction: choline + [^{32}P] ATP $\longrightarrow$ [^{32}P] phosphocholine + ADP catalysed by the enzyme choline kinase (Reid *et al.*, 1971; Goldberg & McCaman, 1973) was rejected because the labelled substrate is expensive to purchase, is a safety hazard due to the emission of high energy radiation, and is inconvenient to use due to a relatively short half-life. The radioenzymic assay of choline involving the reaction: choline + [^{3}H] acetyl-CoA $\longrightarrow$ [^{3}H] acetylcholine + CoA catalysed by the enzyme choline acetyltransferase (Shea & Aprison, 1973; Hebb *et*

al., 1975) was considered more appropriate since the labelled substrate can be synthesized from CoA and [^{3}H] acetic anhydride for a reasonable cost, emits low energy radiation, and has a long half-life. The work in this Chapter was devoted to the development of this radioenzymic choline assay. This method was subsequently applied to examine the passage of choline in the alimentary and urinary tracts of sheep fed chaffed lucerne hay (the standard diet of sheep used in subsequent investigations in this thesis). This involved measuring the various forms of choline in digesta, saliva, bile, faeces and urine of these animals.

2.2 Methods and Materials

2.2.1 Partial purification of choline acetyltransferase from sheep brain caudate nuclei

When this study was initiated partially purified choline acetyltransferase from bovine brains was commercially available from Sigma Chemical Co., St. Louis, Mo., U.S.A. but was not economically practicable for routine use in the radioenzymic assay of choline because the preparation had a very low specific activity. Consequently, choline acetyltransferase was partially purified essentially by the method of Ryan & McClure (1979) from sheep brain caudate nuclei, which were a relatively rich and convenient source of the enzyme.

2.2.1.1 General techniques

Sheep brains were collected as soon as possible after slaughter from the local abbatoirs (South Australian Meat

Corporation, Gepps Cross, S.Aust., Australia) and the caudate nuclei immediately dissected and placed in liquid N_2. The frozen caudate nuclei were powdered using a stainless steel mortar and heavy pestle which had been precooled in liquid N_2. The powdered brain tissue was stored under liquid N_2 until processed. About 140g of caudate nucleus tissue was obtained from 35 sheep brains. All steps in the purification of choline acetyltransferase were performed at 0-4°C. Centrifugations were carried out at 20,000g for 20min unless stated otherwise. Dialysis tubing was purified by boiling in 0.1 mM-EDTA for 60min and soaking in glass double-distilled water for two periods of 12h each. Buffers containing citrate were made up by dissolving the stated concentrations of the basic form of the buffer salt, and any added components, and titrating to the desired pH with citric acid. All other buffers were made up by dissolving the stated concentrations of the acidic form of the buffer salt, and any added components, and titrating to the desired pH with NaOH.

2.2.1.2 Initial solubilization

Powdered caudate nucleus tissue (125g) was homogenized in 25g lots in 100ml of 25 mM-sodium phosphate buffer (pH 7.4) using a Teflon pestle in a smooth glass mortar (Kenco Scientific, Adelaide, S.Aust., Australia) driven by a Dynamax laboratory stirrer SM 650 (Betts & Co. Pty. Ltd., Milperra, N.S.W., Australia) at full speed for 20 strokes. A further 100ml of buffer was added and the mixture blended using a Polytron Type PT 10 2035 OD tissue homogenizer and

sonicator fitted with a PCU-2 speed control on setting 10 (Kinematica, G.m.b.H., Luzern, Switzerland) for 30s. The homogenate was centrifuged for 1h and the supernatant decanted and retained.

2.2.1.3 Acid precipitation

The supernatant was adjusted to pH 4.5 with 50% acetic acid (5-7ml required for a 125g preparation) added slowly with rapid stirring over a 45min period. After gentle stirring for another 50min, the solution was centrifuged and the supernatant collected.

2.2.1.4 Ammonium sulphate precipitation

The supernatant was titrated to pH 6.0 with 1M-NaOH. The solution was stirred and solid ammonium sulphate was added slowly to a saturation of 40% without adjusting the pH. After the ammonium sulphate had completely dissolved, the solution was stirred for a further 30min. The suspension was centrifuged and the pellets were discarded. The supernatant was then made 70% saturated with ammonium sulphate, again without adjusting the pH, and stirred for another 30min. Following centrifugation, the pellets were resuspended in 10 mM-citrate-sodium phosphate buffer (pH 7.2) containing 0.1 mM-EDTA and 0.1 mM-dithiothreitol (CPED buffer, 0.5ml/g of brain tissue). The resulting solution was dialysed overnight against 150 volumes of CPED buffer.

2.2.1.5 CM-Sephadex chromatography

The dialysed enzyme solution was loaded onto a

column (2.5cm x 16cm) of carboxymethyl-Sephadex C-50 (particle size 40-120μm, Na^+ form) cation-exchange resin which had been pre-equilibrated with CPED buffer. After being washed with 30-35ml of CPED buffer, the column was eluted with a linear gradient from 0 to 0.4M-NaCl in the same buffer, using a gradient volume of 700ml pumped at 20ml/h. Column fractions (130 x 5ml) were collected while monitoring protein and those containing a significant amount of enzyme were pooled and concentrated by pressure dialysis (N_2, 300 kPa) using an Amicon ultrafiltration stirred cell model 52 (65ml maximum process volume) and Diaflo PM 10 membrane (nominal molecular weight cut-off of 10,000) (Amicon Corp., Lexington, Ma., U.S.A.). After the solution was reduced in volume to about 15ml, 30ml of CPED buffer was added and the volume then taken to 25ml. During this process, most of the NaCl was removed. The resulting enzyme solution was divided into 83 x 300 μl aliquots and stored in 50mm x 7mm plastic tubes (Disposable Products Pty. Ltd., Adelaide, S.Aust., Australia) at -80°C, where it remained stable for at least 30 months.

2.2.2 Determination of choline acetyltransferase activity

Choline acetyltransferase activity was assayed by modification of the methods described by Mannervik & Sörbo (1970), Hebb *et al.* (1975) and Ryan & McClure (1979). The following components were incubated for 15min at 37°C in 7mm x 50mm plastic tubes (Disposable Products Pty. Ltd., Adelaide, S.Aust., Australia) in a final volume of 50μl : 50 mM-sodium phosphate buffer (pH 7.4); 0.2 mM-eserine

(physostigmine) salicylate salt; 1 mM-EDTA (pH 7.4); 0.5mg of bovine serum albumin/ml; 300 mM-NaCl; 10 mM-choline chloride; 0.4 mM-[^{3}H] acetyl-CoA (specific radioactivity 100,000 d.p.m./nmol) and enzyme solution. The incubation mixture consisted of 20µl of reaction mixture, 5µl of [^{3}H] acetyl-CoA and 0-25µl of enzyme solution (the volume adjusted with water). The reaction was initiated by the addition of enzyme solution and terminated by the addition of 0.25cm^3 of Amberlite IRA 410 (30-72 mesh; Cl^- form) anion-exchange resin and 350µl of water. The tubes were stoppered, shaken for 10min, and centrifuged at 1,000g for 10min. Two hundred µl of the aqueous supernatant containing the product [^{3}H] acetylcholine was added to 3.5ml of scintillation fluid [7g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(4-methyl-5-phenyl-2-oxazolyl) benzene per litre of toluene and Triton X-100 (2:1, v/v)] in a polyethylene scintillation vial. Radioactivity was determined by counting for 10min in a Packard TRI-CARB 460 CD liquid scintillation system (Packard Instrument Co. Inc., Downers Grove, Il., U.S.A.) which automatically corrected for quenching by the sample channels ratio technique. All enzyme samples were assayed in duplicate.

Carnitine acetyltransferase activity was assayed as described for choline acetyltransferase except that 10 mM-L-carnitine hydrochloride replaced choline chloride in the incubation mixture and the radioactivity of the [^{3}H] acetylcarnitine product measured.

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

2.2.3 Synthesis and purification of acetyl-CoA and [^{3}H] acetyl-CoA

Acetyl-CoA was synthesized and purified by the following procedure which was based on personal communication with Dr. D.B. Keech, Department of Biochemistry, The University of Adelaide, and the method of Stadtman (1957).

CoA (10mg) was dissolved in 0.5ml of water in a 10ml stoppered glass extraction tube (Quickfit & Quartz Ltd., Stone, Staffordshire, England). The pH was adjusted to 7.0 - 7.4 with 1M-$KHCO_3$ and approximately 1mg of sodium borohydride added. The solution was left on ice for 5min. About 1-2 drops of 5M-HCl was added and after the frothing had subsided the pH was readjusted to 7.4 with $KHCO_3$. Acetic anhydride was added in 1µl aliquots. The solution was stirred after each addition, the pH adjusted to 7.4, and a 10µl portion added to 1ml of 100 mM-sodium phosphate buffer (pH 7.2) plus 10µl of 10 mM-5,5'-dithiobis-(2-nitrobenzoic acid) in ethanol to test for unreacted CoA. A yellow colour due to the formation of thio-nitro-benzoate anion indicated the presence of free sulphydryl groups. When the test was positive, further 1µl aliquots of acetic anhydride were added. Approximately 2-3µl of acetic anhydride was usually sufficient. Finally, when the sulphydryl test was negative, unreacted acetic anhydride was removed by adjusting the pH to 2.0 with 1-2 drops of H_2SO_4 and extracting the solution three times with an equal volume of ether. (It was essential to remove excess acetic anhydride in case it reacted a second time with the CoA molecule to produce a diacetylated derivative). After the final ether extraction, the aqueous solution was blown with N_2 to

remove traces of ether and the pH readjusted to 6.0 with $KHCO_3$.

The crude acetyl-CoA solution was applied as a strip (approximately 1mg/2.5cm) along with 250µg of CoA and acetyl-CoA standards on a sheet of 3MM chromatography paper (Whatman Ltd., Maidstone, Kent, England) that had previously been washed with 7% acetic acid. The paper was developed in the ascending direction in the solvent system *n*-butanol/glacial acetic acid/water (5:2:3, by vol.). After the chromatogram was air-dried, the acetyl-CoA band was located under ultra-violet light, cut into pieces, and eluted with 5 x 0.5ml of 0.1 mM-EDTA (pH 6.0). The combined washings were adjusted to pH 6.0 if necessary and evaporated to near dryness *in vacuo* at 25-30°C. The sample was taken up in less than 1ml of 0.1 mM-EDTA (pH 6.0) and assayed as described by Decker (1974). The purified acetyl-CoA preparation usually had a concentration of 15-25 mM and was stored at -15°C where it was stable for at least 3 months. It was important that the stored product be at pH 6.0 to maintain stability.

[^{3}H] acetyl-CoA was prepared in a similar manner except that acetylation of the CoA was carried out in a borosilicate glass ampoule containing the [^{3}H] acetic anhydride (about 50 µmol) and then transferred to a 10ml stoppered glass extraction tube for extractions with ether. The purified [^{3}H] acetyl-CoA preparation normally had a concentration of 3-5 mM with a specific activity of 2-5 x 10^5 dpm/nmol.

2.2.4 Determination of protein

The protein content of samples produced during the purification

of sheep brain caudate nuclei choline acetyltransferase was measured by a modification of the micro-biuret method of Itzhaki & Gill (1964). Modified biuret reagent was prepared by dissolving 300g of NaOH and 2.1g of $CuSO_4.5H_2O$ in 1*l* of water and stored at 4°C. 0.2% sodium deoxycholate (10µl) was added to 10µl of sample (containing 0-1mg of protein) in 10ml glass centrifuge tubes, thoroughly mixed, and left for a few minutes to solubilize the protein. Water (980µl) and 2ml of modified biuret reagent were then added and the solution mixed and incubated at room temperature for 60min. The solution was immediately transferred to a 1ml semi-micro cuvette and the absorbance read at 310nm in a Zeiss PM QII spectrophotometer (Carl Zeiss, Oberkocken, Germany) against an identical water blank. A protein standard curve was constructed by adding 2ml of modified biuret reagent to 1ml of water containing 0-1mg of bovine serum albumin and reading the absorbance after incubation at room temperature for 60min as before. The protein standard curve was linear over the 0-1mg range, and 0.5mg of bovine serum albumin had an absorbance at 310nm of 0.410 ± 0.006 units (mean ± S.E.M. of 6 series of standards). All samples and standards were assayed in duplicate.

2.2.5 The radioenzymic assay of choline using [^{3}H] acetyl-CoA and partially purified choline acetyltransferase

Unesterified choline was assayed by modification of the radioenzymic method involving the reaction: choline + [^{3}H] acetyl-CoA $\longrightarrow$ [^{3}H] acetylcholine + CoA catalysed by the enzyme choline acetyltransferase as described by Shea & Aprison (1973) and Hebb *et al.* (1975). The techniques of Fonnum (1969*a*, *b*) and Tučcek *et al.* (1978) were adopted for specifically assaying choline in the presence of carnitine

acetyltransferase and carnitine. The following components were incubated for 15min at 37°C in 1ml plastic centrifuge tubes (Eppendorf Gerätebau, Netheler and Hinz G.m.b.H., Hamburg, Germany) in a final volume of 50µl : 50 mM-sodium phosphate buffer (pH 7.8); 0.2 mM-eserine (physostigmine) salicylate salt; 1 mM-EDTA (pH 7.8); 0.5mg of bovine serum albumin/ml; 0.04 mM-sample or standard choline chloride; 0.4 mM-[^{3}H] acetyl-CoA (specific radioactivity 100,000 d.p.m./nmol) and partially purified choline acetyltransferase enzyme solution (pH 7.2, total activity 0.4 nmol acetylcholine formed/min). The incubation mixture consisted of 20µl of reaction mixture, 20µl of sample or standard, 5µl of [^{3}H] acetyl-CoA and 5µl of enzyme solution. The reaction was started by the addition of enzyme solution and stopped with 550µl of 100 mM-sodium phosphate buffer (pH 7.8), followed immediately with 300µl of heptan-2-one containing sodium tetraphenylboron (25mg/ml). The tubes were stoppered, shaken for 10min, and centrifuged for 8min at 8,000g in an Eppendorf microcentrifuge 3200 (Eppendorf Gerätebau, Netheler and Hinz G.m.b.H., Hamburg, Germany). One hundred and fifty µl of the heptan-2-one layer containing essentially only [^{3}H] acetylcholine was added to 3.5ml of scintillation fluid in a polyethylene scintillation vial and the radioactivity measured as described in Section 2.2.2. All samples and standards were determined in duplicate. Some modifications of this standard assay procedure were used and are described in the legends to figures in the results section.

2.2.6 Animals

Merino ewes (*Ovis aries*) obtained from the flock of The Waite Agricultural Research Institute, The University of Adelaide, aged 2-3

years and weighing 35-40kg, were housed indoors in pens. They were given water *ad libitum* and fed once daily (at 09.00h) a ration of 1.1kg of chaffed lucerne hay.

2.2.7 Surgical preparation and post-operative treatment of sheep

Sheep were surgically prepared with standard cannulae in rumen, abomasal and duodenal fistulae by Dr. A.R. Egan, Mr. B.J. Hosking and Dr. P.I. Hynd in The Department of Animal Sciences, Waite Agricultural Research Institute, The University of Adelaide, as outlined by Hynd (1982). The duodenal cannula was inserted about 4-5cm caudal to the pylorus of the abomasum. Sheep were allowed to stabilize on full feed intake for 30 days after surgery before samples were taken.

2.2.8 Collection of digesta, saliva, bile, faeces and urine samples

Samples were collected from sheep 6h after feeding (at 15.00h) the daily ration of chaffed lucerne hay which was totally consumed within 2h. All samples were placed in ice-cold plastic containers. Digesta was collected through the rumen cannulae by gentle suction through a wide-bore rubber tube. Digesta was collected from the abomasal and duodenal cannulae by gravity drainage. Fresh faeces and urine were obtained from sheep spontaneously or in response to external stimuli. Saliva was aspirated from the mouth with a plastic syringe and tube. Finally, bile was aspirated from the gallbladder with needle and plastic syringe after slaughtering the sheep by severing the neck.

2.2.9 Extraction of digesta, saliva, bile, faeces and urine samples and determination of the various forms of choline

Digesta and faeces samples were extracted by the method described by Neill *et al.* (1979). All solvents contained the antioxidant 2,6-di-*tert*-butyl-4-methylphenol (50mg/l) (Wren & Szczepanowska, 1964; Holman, 1967). Digesta (20ml) or faeces (20g fresh wt.) were homogenized with 50ml of methanol for 1min and then blended with 25ml of chloroform for 2min using a Sorvall omnimixer homogenizer on setting 7 (Ivan Sorvall Inc., Norwalk, Connecticut, U.S.A.). A further 25ml of chloroform and 25ml of water were then added and the mixture reblended for 2min. The upper water-soluble phase was removed, taken to dryness *in vacuo* at 30-40°C, and the residue redissolved in 2ml of water. This water-soluble fraction was shaken with diethyl ether (3 x 1ml) to remove lipid contaminants and the ether washes were added to the lower lipid phase. The lipid phase was filtered through a small glasswool pad and the filtrate evaporated to dryness *in vacuo* at 30-40°C. The residue was taken up in 5ml of chloroform. The water-soluble and lipid fractions were stored at -15°C until they were analysed.

Urine, saliva and bile samples were extracted essentially by the method of Folch *et al.* (1957) but with the modifications described by Henderson (1978). 1.5 ml portions of bile (pH 6.5 - 7.0) were initially acidified (pH 4.0 - 4.5) with about 10 µl of 5M-HCl. Urine, saliva and acidic bile (1ml in each case) were mixed with 18ml of ice-cold chloroform/methanol (2:1, v/v) containing the antioxidant 2,6-di-*tert*-butyl-4-methylphenol (50mg/l) in a 50ml glass

vial. The extract was filtered through fluted filter paper (previously boiled in chloroform) into a 25ml glass measuring cylinder. The extraction vial and filter paper were rinsed with chloroform/methanol (2:1, v/v) until a filtrate of 20ml was obtained. Water (4ml) was added to the filtrate and the stoppered measuring cylinder shaken thoroughly for 5min. After centrifugation at 1,000g for 10min, the upper water-soluble phase was removed by aspiration, and the tube-wall and surface of the lower lipid phase washed 3 times with 2ml of the upper phase of a pure solvent mixture of chloroform/methanol/water (8:4:3, by vol.). The water-soluble phase and the 3 washings were taken to dryness *in vacuo* at 30-40°C and the residue redissolved in 2ml of water. This water-soluble fraction was shaken with diethyl ether (3 x 1ml), and the ether phases combined with the lipid phase. The lipid phase was taken to dryness *in vacuo* at 30-40°C and the residue redissolved in 10ml of chloroform. The water-soluble and lipid fractions were kept at -15°C until analysis.

A 2ml portion of the lipid fraction of extracted digesta, faeces, urine, saliva and bile samples containing lipid choline (choline phospholipids) was evaporated to dryness under N_2 and hydrolysed to unesterified choline with 3ml of 6M-HCl at 110°C for 24h in a sealed test tube. The hydrolysate was taken to dryness *in vacuo* at 40°C and stored in a vacuum desiccator containing KOH pellets for at least 12h to remove traces of acid. The residue was redissolved in 250µl-50ml of 5 mM-sodium phosphate buffer (pH 7.8) and the unesterified choline measured in duplicate by the radioenzymic assay described in Section 2.2.5.

The water-soluble fraction (2ml) of extracted digesta, faeces, urine, saliva and bile samples (containing glycerophosphocholine, phosphocholine and unesterified choline) was centrifuged at 8,000g for 2min in 1ml stoppered plastic centrifuge tubes in an Eppendorf 3200 microcentrifuge (Eppendorf Gerätebau, Netheler and Hinz G.m.b.H., Hamburg, Germany) and the pH adjusted to 8-9 with NH_3 vapour. Glycerophosphocholine, phosphocholine and unesterified choline were fractionated by Dowex AG 50W (X 8; 100-200 mesh; H^+ form) cation-exchange column chromatography (Webster & Cooper, 1968; Illingworth & Portman, 1972) as outlined in Section 1.2.3 of Chapter 1. The glycerophosphocholine and phosphocholine column fractions were taken to dryness *in vacuo* at 40°C. The glycerophosphocholine and phosphocholine residues were hydrolysed to unesterified choline in 5ml of 1M-HCl at 100°C for 30min and 3M-HCl at 123°C for 24h respectively. All fractions were taken to dryness *in vacuo* at 30-40°C and stored for at least 12h in a desiccator containing KOH pellets. The glycerophosphocholine, phosphocholine and unesterified choline residues were redissolved in 500µl, 250µl and 500µl of 5 mM-sodium phosphate buffer (pH 7.8) respectively and the unesterified choline determined in duplicate by the radioenzymic assay (Section 2.2.5). The authenticity of the various forms of water-soluble choline in the samples was ascertained by subjecting a concentrated portion of each column fraction to quantitative thin-layer chromatography as explained in Chapter 1 (Section 1.2.3).

2.2.10 Determination of dry weights of digesta and faeces samples

Duplicate portions of digesta samples (2ml) and faeces samples

(2g fresh wt.) were placed in tared glass vials and oven-dried at 80°C for 96h to a constant weight. The vials were cooled to room temperature in an evacuated desiccator for at least 1h prior to weighing.

2.2.11 Chemicals

[^{3}H] Acetic anhydride (specific radioactivity 500mCi/mmol) was purchased from Amersham Australia Pty. Ltd., Sydney, N.S.W., Australia. CoA (lithium salt) was obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. L-Carnitine hydrochloride was generously supplied by Otsuka Pharmaceuticals, Osaka, Japan. Other chemicals were obtained as follows: choline chloride (3 x crystallized), eserine (physostigmine) salicylate salt, 5,5'-dithiobis-(2-nitrobenzoic acid), 2,5-diphenyloxazole, 1,4-bis-(4-methyl-5-phenyl-2-oxazolyl) benzene, acetyl-CoA (lithium salt) from Sigma Chemical Co., St. Louis, Mo., U.S.A; 2,6-di-*tert*-butyl-4-methylphenol from Calbiochem, Los Angeles, Calif., U.S.A.; sodium tetraphenylboron from BDH Chemicals Australia Pty. Ltd., Pt. Fairy, Vic., Australia; carboxymethyl-Sephadex C-50 (particle size 40-120μm, Na^{+} form) cation-exchange resin from Pharmacia Fine Chemicals AB, Uppsala, Sweden; Amberlite IRA 410 (30-72 mesh, Cl^{-} form) anion-exchange resin from BDH Chemicals Ltd., Poole, Dorset, U.K.; and Dowex AG 50W (X 8; 100-200 mesh; H^{+} form) cation-exchange resin from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Reagent grade chloroform, methanol and acetic anhydride were glass redistilled. Other chemicals were reagent-grade or the best commercially available grade. Glass double-distilled water was used.

2.3 Results

2.3.1 Partial purification of choline acetyltransferase from sheep brain caudate nuclei

The results in Table 2.1 show that choline acetyltransferase from the caudate nuclei of sheep brains was purified approximately 13 fold and had a specific activity of 0.02 µmol acetylcholine formed/min/mg protein. This purification of choline acetyltransferase from sheep brain caudate nuclei was somewhat less successful at the CM-Sephadex chromatography step than that reported by Ryan & McClure (1979) from rat brain cerebra and bovine brain caudate nuclei. However the present enzyme preparation had about twice the specific activity of that produced by the Sigma Chemical Co. from bovine brains and provided a considerable quantity for use in the radioenzymic assay of choline.

The enzyme preparation lost activity with repeated freezing and thawing. Hence the preparation was dispensed into small plastic tubes in 300µl aliquots and stored at -80°C. A fresh tube was used for each batch of choline assays and was only thawed once.

The enzyme preparation was contaminated with carnitine acetyltransferase but any interference in the radioenzymic choline assay due to this enzyme was overcome by the assay procedure adopted (see Section 2.2.5).

2.3.2 The radioenzymic assay of choline using [^{3}H] acetyl-CoA and partially purified choline acetyltransferase

Figure 2.1 shows a typical standard curve for the radioenzymic assay of choline using [^{3}H] acetyl-CoA and partially purified choline

Table 2.1: Partial purification of choline acetyltransferase from sheep brain caudate nuclei

Choline acetyltransferase was partially purified from 125g of sheep brain caudate nuclei as described in Section 2.2.1. The enzyme activity and protein content of the fractions were determined in duplicate as described in Sections 2.2.2 and 2.2.4 respectively.

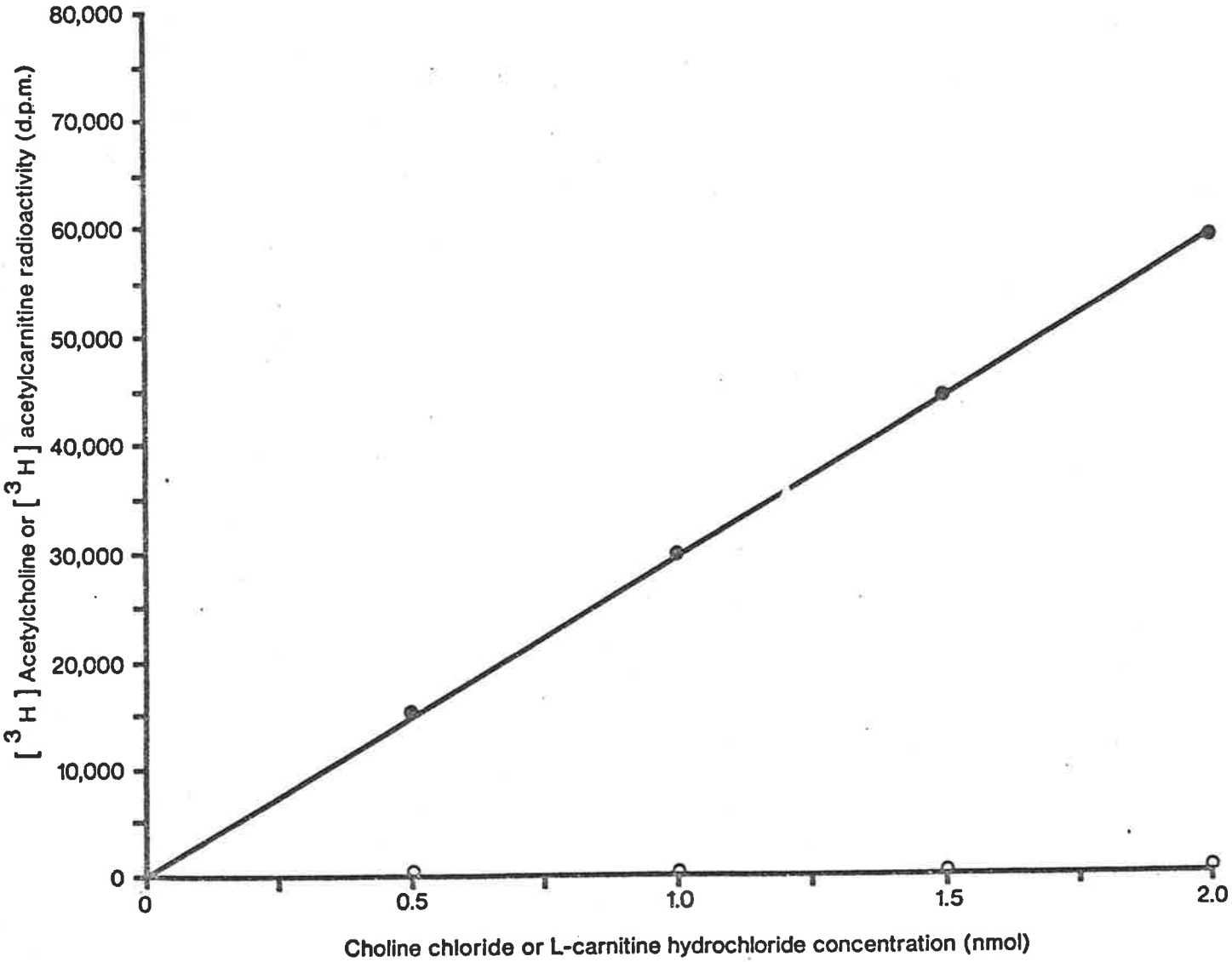
Step	Volume (ml)	Total protein (mg)	Total activity (µmol acetylcholine formed/min)	Specific activity (µmol acetylcholine formed/min/mg protein)	Yield (%)	Purification (x-fold)
Crude supernatant	900	4797	7.38	0.0015	100	1
pH 4.5 supernatant	865	1713	2.94	0.0017	40	1.1
Dialysed 40-70% $(NH_4)_2\ SO_4$ fraction	48	400	2.48	0.0062	34	4.1
Concentrated CM^- Sephadex pool	25	102	2.04	0.02	28	13.3

Figure 2.1 A standard curve for the radioenzymic assay of choline using [^{3}H] acetyl-CoA and partially purified choline acetyltransferase

Choline chloride standards (0-2 nmol) were assayed as described in Section 2.2.5.

Each data point represents a duplicate incubation.

O = choline chloride standards replaced with L-carnitine hydrochloride standards.


80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
[3 H] Acetylcholine or [3 H] acetylcarnitine radioactivity (d.p.m.)
0
0.5
1.0
1.5
2.0
Choline chloride or L-carnitine hydrochloride concentration (nmol)

acetyltransferase in the reaction : choline + [^{3}H] acetyl-CoA $\xrightarrow{\text{choline acetyltransferase}}$ [^{3}H] acetylcholine + CoA. The choline chloride standard curve was linear over the 0-2 nmol range, and 1 nmol of choline chloride produced [^{3}H] acetylcholine with radioactivity of 30,000 d.p.m. The radioactivity of blanks containing no added choline chloride was about 2,000 d.p.m. Since the partially purified choline acetyltransferase preparation used in the radioenzymic choline assay was contaminated with carnitine acetyltransferase a considerable amount of [^{3}H] acetylcarnitine may be formed by the reaction:

carnitine + [^{3}H] acetyl-CoA $\xrightarrow{\text{carnitine acetyltransferase}}$ [^{3}H] acetylcarnitine + CoA

if carnitine is present. When L-carnitine hydrochloride standards (0-2 nmol) replaced choline chloride in the assay system virtually no radioactivity was detected as shown in Figure 2.1. This indicates that any [^{3}H] acetylcarnitine formed was not extracted from the incubation mixture by heptan-2-one containing sodium tetraphenylboron at alkaline pH, in contrast to [^{3}H] acetylcholine. This agrees with the observations of Fonnum (1969*a*, *b*) and Tuček *et al.* (1978). Thus the radioenzymic assay was specific for choline in the presence of carnitine acetyltransferase and carnitine.

The amount of [^{3}H] acetylcholine formed from choline chloride standard (1 nmol) in the radioenzymic assay increased as the pH of the incubation mixture became more alkaline over the range 7.0 - 8.0 as shown in Figure 2.2. An incubation pH of 7.8 was used in the routine assay of choline to promote sensitivity.

Figure 2.3 shows the formation of [^{3}H] acetylcholine from

Figure 2.2: Effect of incubation pH on [^{3}H] acetylcholine production in the radioenzymic assay of choline using [^{3}H] acetyl-CoA and partially purified choline acetyltransferase

Choline chloride standard (1 nmol) was assayed as described in Section 2.2.5 using 50 mM-sodium phosphate buffer at pH 7.0 - 8.0 in the incubation mixture. Each data point represents a duplicate incubation.

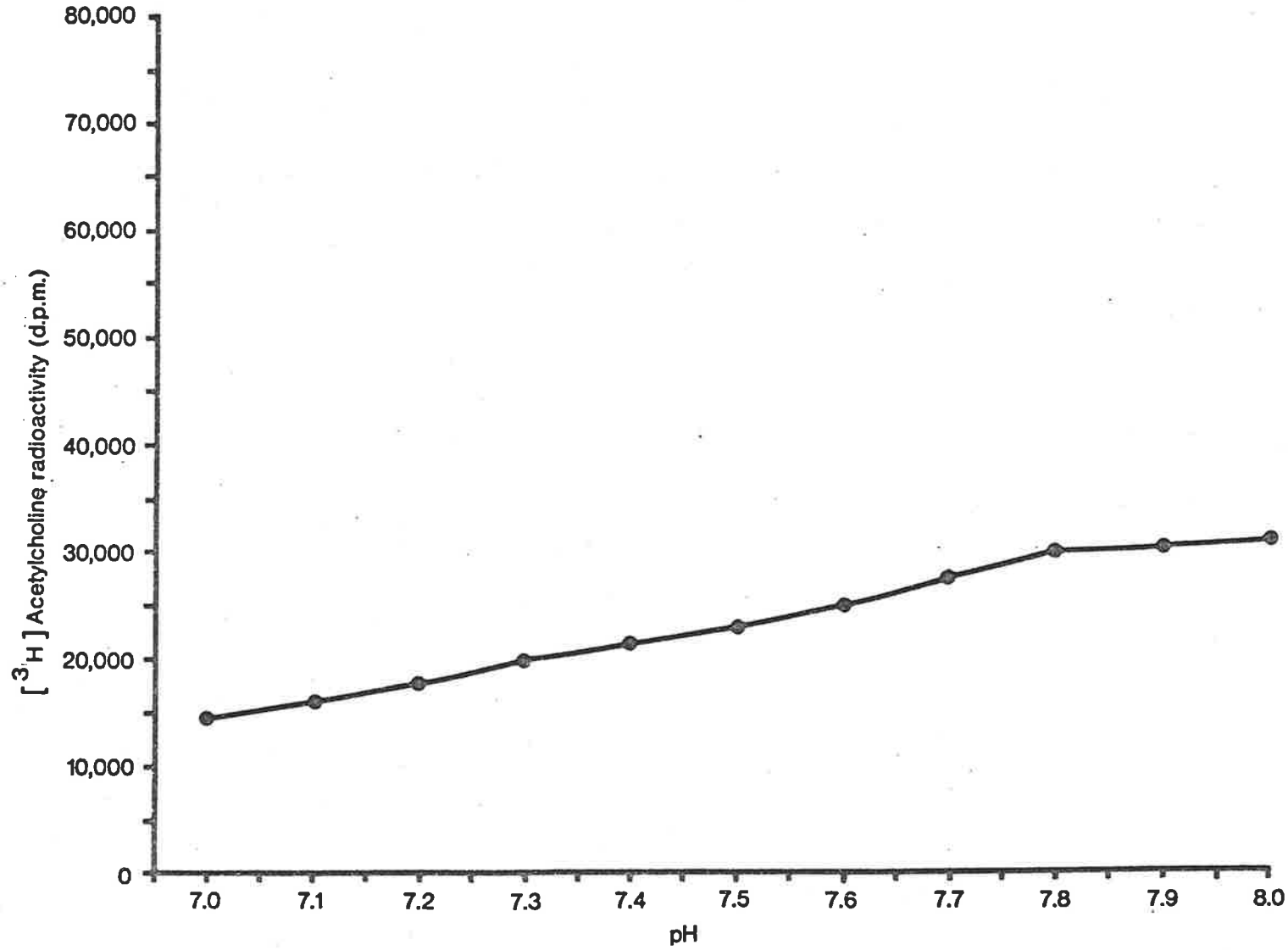
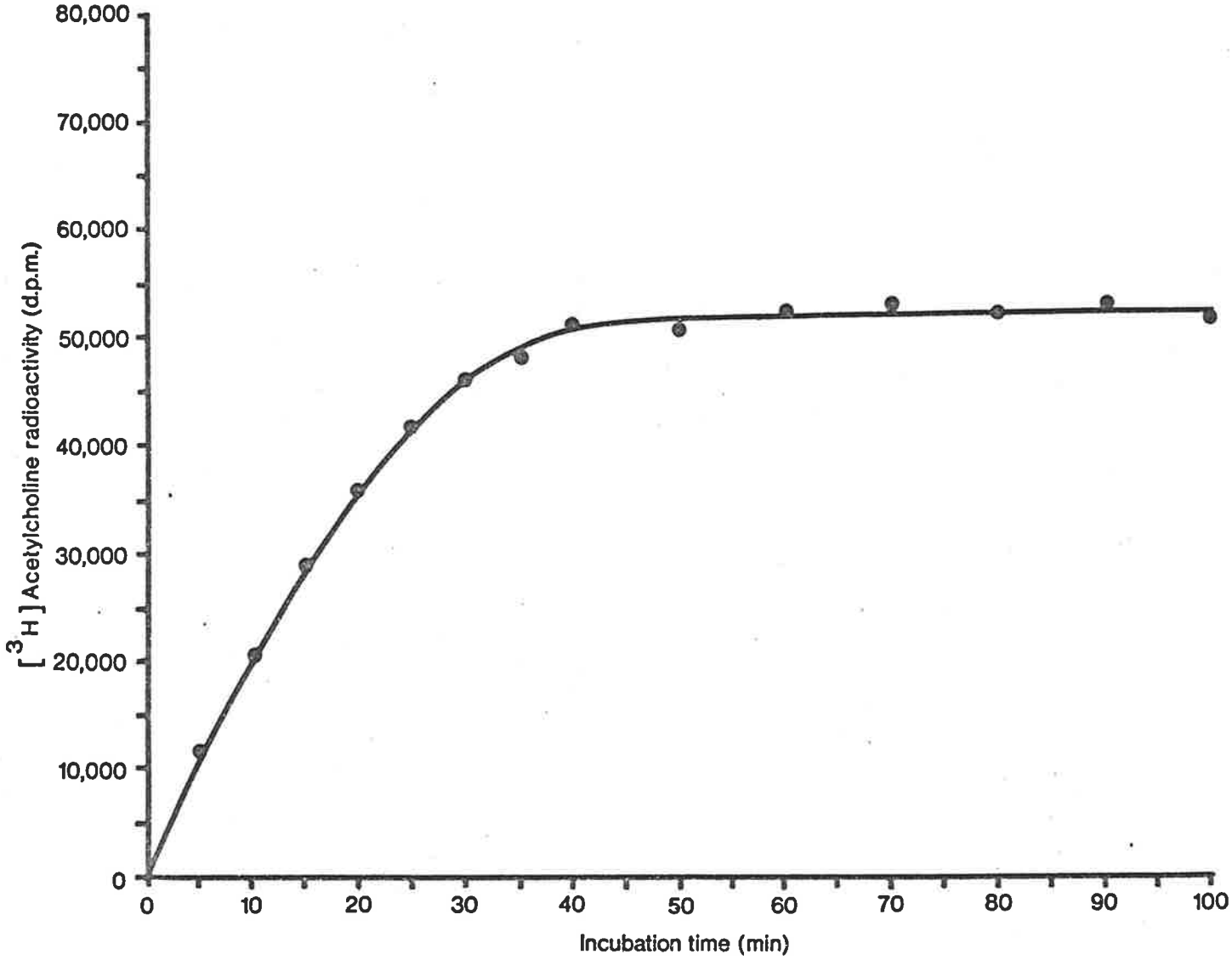

80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
[3H] Acetylcholine radioactivity (d.p.m.)
7.0
7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8
7.9
8.0
pH

Figure 2.3: Effect of incubation time on [^{3}H] acetylcholine production in the radioenzymic assay of choline using [^{3}H] acetyl-CoA and partially purified choline acetyltransferase.

Choline chloride standard (1 nmol) was assayed as described in Section 2.2.5 using incubation times of 5-100min. Each data point represents a duplicate incubation.


80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
[3H] Acetylcholine radioactivity (d.p.m.)
0
10
20
30
40
50
60
70
80
90
100
Incubation time (min)

choline chloride standard (1 nmol) in the radioenzymic assay as a function of incubation time. There was an increase in the production of [^{3}H] acetylcholine with incubation time up to approximately 40min after which it levelled off. An incubation time of 15min was used in the routine assay of choline because it gave a highly reproducible standard curve and was convenient.

2.3.3 Concentrations of various forms of choline in digesta and faeces of sheep

The concentrations of the various forms of choline in digesta and faeces of sheep fed chaffed lucerne hay are shown in Table 2.2. Most of the choline in the sheep digesta was present as lipid choline. The concentration of lipid choline was significantly higher in the rumen and duodenal digesta than in the abomasal digesta ($P<0.05$, Student's t test). The digesta lipid choline values in Table 2.2 are in the range previously reported for sheep by Neill *et al.* (1979) and Robinson (1980). The concentration of unesterified choline was significantly higher in the rumen digesta than in the abomasal and duodenal digesta ($P<0.05$, Student's t test). The digesta unesterified choline levels in Table 2.2 are comparable with those previously documented for sheep (Neill *et al.*, 1979; Robinson, 1980). The concentrations of glycerophosphocholine and phosphocholine were significantly higher in the abomasal and duodenal digesta than in the rumen digesta ($P<0.05$, Student's t test). The total choline content of the duodenal digesta was several fold higher than that of the rumen and abomasal digesta because of the greater amount of lipid choline. No forms of choline were detectable in the sheep faeces (Table 2.2).

Table 2.2: Concentrations of various forms of choline in digesta and faeces of sheep

Digesta and faeces samples were collected from Merino ewes fed 1.1kg of chaffed lucerne hay per day as described in Section 2.2.8. The samples were extracted and the various forms of choline fractionated and determined in duplicate by the radioenzymic assay as described in Section 2.2.9. The dry weights of the samples were determined as described in Section 2.2.10. The values shown are means ± S.E.M. for four sheep. Abbreviation used: N.D. = not detectable.

Sample	Concentration (nmol/g dry wt.)				
	Unesterified Choline	Lipid choline	Glycerophospho-choline	Phosphocholine	Total choline
Rumen digesta	54.9 ± 15.2	201 ± 34.1	1.9 ± 0.4	3.8 ± 1.9	262[a]
Abomasal digesta	13.4 ± 5.4	156 ± 56.3	29.5 ± 8.0	85.8 ± 29.5	285[b]
Duodenal digesta	19.2 ± 3.3	869 ± 201	33.5 ± 8.4	20.9 ± 6.3	943[c]
Faeces	N.D.	N.D.	N.D.	N.D.	N.D.

[a] Equivalent to 13.8 nmol/ml of digesta.
[b] Equivalent to 10.6 nmol/ml of digesta.
[c] Equivalent to 45.0 nmol/ml of digesta.

2.3.4 Concentrations of various forms of choline in saliva, bile and urine of sheep

Table 2.3 shows the concentrations of the various forms of choline in saliva, bile and urine of sheep fed chaffed lucerne hay. In the sheep urine and saliva choline was mainly present in the unesterified form, with only small amounts of lipid choline, glycerophosphocholine and phosphocholine. The urine unesterified choline and lipid choline levels in Table 2.3 are consistent with those reported for humans by Luecke & Pearson (1944). The saliva unesterified choline value in Table 2.3 is similar to that found for cattle by Broad & Dawson (1976). In the sheep bile there was a very high concentration of lipid choline and relatively small amounts of unesterified choline, glycerophosphocholine and phosphocholine. The bile lipid choline value in Table 2.3 is comparable with those published for sheep by other workers (Leat & Harrison, 1975; Christie, 1978). The total choline content of the bile was much higher than that of the urine and saliva due to the greater amount of lipid choline (Table 2.3).

2.4 Discussion

In this Chapter a radioenzymic assay of choline, using partially purified choline acetyltransferase and [^{3}H] acetyl-CoA, was developed for the routine analysis of sheep tissues and fluids. The assay procedure adopted was sensitive and specific for choline in the presence of carnitine acetyltransferase and carnitine and more convenient than the technique described by Snoswell & Mann (1978). Since sheep tissues and fluids generally contain high levels of

Table 2.3: Concentrations of various forms of choline in saliva, bile and urine of sheep

Urine, saliva and bile samples were collected from Merino ewes fed 1.1kg of chaffed lucerne hay per day as described in Section 2.2.8. The samples were extracted and the various forms of choline fractionated and determined in duplicate by the radioenzymic assay as described in Section 2.2.9. The values shown are means ± S.E.M. for four sheep.

Sample	Concentration (nmol/ml)				
	Unesterified Choline	Lipid choline	Glycerophospho-choline	Phosphocholine	Total choline
Saliva	6.5 ± 0.8	0.1 ± 0.1	0.8 ± 0.2	0.2 ± 0.1	7.6
Bile	3.1 ± 0.4	16,900 ± 430	0.1 ± 0.0	0.3 ± 0.2	16,904
Urine	8.4 ± 0.5	0.3 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	9.5

carnitine and carnitine compounds (Snoswell & Koundakjian, 1972), it was particularly important that carnitine interference in the choline radioenzymic assay was eliminated. The method was successfully used to measure the various choline forms in digesta, saliva, bile, faeces and urine of sheep fed lucerne hay to assess the flux of choline in the alimentary and urinary tracts.

In the present study sheep ingested 1.1kg of lucerne hay which contained 5.64 µmol of unesterified choline/g fresh wt. and 1.21 µmol of lipid choline/g fresh wt. (from Table 1.1 in Chapter 1). Calculation from these values shows that the sheep had a total dietary choline intake of 7.53 mmol/day (6.20 mmol of dietary unesterified choline and 1.33 mmol of dietary lipid choline/day). The passage of total choline (mainly in the form of lipid choline) in the rumen, abomasal and duodenal digesta of the sheep was 0.08 - 0.30, 0.07 - 0.17 and 0.32 - 1.08 mmol/day respectively (as indicated in Table 2.4) which is only a few per cent of the total choline available in the diet. The flux of choline in the abomasal digesta of the sheep represents approximately 0.001 - 0.002% of the total dietary dry-weight intake. This is about 70 times less than the minimum required to avoid pathological lesions and death in many non-ruminant species. Similar values have been reported for sheep by Neill *et al.* (1979) and Dawson *et al.*(1981). The minimal passage of choline to the lower digestive tract of the sheep is due to the extensive microbial destruction of dietary choline in the rumen and omasal digesta with its N-methyl groups being converted to trimethylamine and eventually methane (Neill *et al.*, 1978, 1979; Dawson *et al.*, 1981). The only dietary choline escaping such degradation

Table 2.4: Total choline flux in digesta, saliva, bile, faeces and urine of sheep

Sheep ingested 1.1kg of lucerne hay per day containing 1.64 umol of unesterified choline/g fresh wt. and 1.21 umol of lipid choline/g fresh wt. (from Table 1.1 in Chapter 1) and thus received a total of 7.53 mmol of dietary choline per day. The total choline flux in digesta, saliva, bile, faeces and urine of the sheep was calculated by the product of the total choline concentration (from Tables 2.2 and 2.3) and the documented flow rate of each sample. The main form of choline in each sample is indicated. Abbreviation used: N.D. = not detectable.

Sample	Total choline concentration (nmol/ml)	Main form of choline	Flow rate (litre/day)	Total flux of choline (mmol/day)
Rumen digesta	13.8	Lipid choline	6-22[a]	0.08 - 0.30
Abomasal digesta	10.6	Lipid choline	7-16[b]	0.07 - 0.17
Duodenal digesta	45.0	Lipid choline	7-24[c]	0.32 - 1.08
Saliva	7.6	Unesterified choline	6-16[d]	0.05 - 0.12
Bile	16,904	Lipid choline	0.7-1[e]	11.83 -16.90
Faeces	N.D.	N.D.	0.5-1*[f]	N.D.
Urine	9.5	Unesterified choline	1-2[g]	0.01 - 0.02

* kg/day.
a Faichney & Weston (1971), Faichney (1972), Church (1979*b*).
b Faichney & Weston (1971), Faichney (1972), Church (1979*b*).
c Church (1979*b*), Smith (1979).
d Phillis (1976), Church (1979*c*).
e Harrison (1962), Adams & Heath (1963), Heath *et al*. (1970), Phillis (1976), Noble (1978).
f Church (1979*b*), A.R. Egan, B.J. Hosking & P.I. Hynd (personal communication).
g Church (1979*d*).

appears to be a very small amount that is incorporated into the membranes of ciliated protozoa as phosphatidylcholine (Broad & Dawson, 1976) and passes on to the abomasum. Most protozoa are selectively retained in the rumen (Weller & Pilgrim, 1974; Bauchop & Clarke, 1976; Harrison *et al.*, 1979; Neill *et al.*, 1979; Coleman *et al.*, 1980).

Some of the choline present in the sheep digesta would have been derived from non-dietary sources. Table 2.4 shows that the total choline flux in the sheep saliva (largely as unesterified choline) was 0.05 - 0.12 mmol/day which would enrich the rumen digesta. The most outstanding figure shown in Table 2.4 is that for the total choline flux in the sheep bile (mainly as lipid choline) which was 11.83 - 16.90 mmol/day, and is consistent with values reported by others (Adams & Heath, 1963; Noble, 1978). The large amount of choline flowing in bile would enter the duodenal digesta *via* the common bile duct. It is feasible that there is some regurgitation of bile choline into the upper duodenum and even the abomasum of sheep. There is evidence that even the minimal concentration of choline in the abomasal digesta of sheep is substantially of endogenous origin, e.g. from abomasal secretions or sloughed-off abomasum epithelial cells (Neill *et al.*, 1979; Robinson, 1980; Dawson *et al.* 1981).

No choline could be detected in the faeces of the sheep (Table 2.4). Indeed, by the time digesta reaches the ileum it contains a negligible amount of phosphatidylcholine (Leat & Harrison, 1975; Noble, 1978), indicating appreciable choline absorption in the duodenum and jejunum of the sheep small intestine. The total

choline excreted in the sheep urine was only 0.01 - 0.02 mmol/day (predominately unesterified choline) as shown in Table 2.4. Luecke & Pearson (1945) also reported a low daily urinary excretion of choline in sheep, indicating efficient conservation of a vital nutrient.

The most prominent observation arising from the work in this Chapter was the enormous amount of choline secreted in the bile of sheep per day. Considering that sheep receive a very low amount of dietary choline per day, almost entire retention and reutilization of bile choline would be necessary to avoid choline deficiency in this species. This important feature of choline nutrition and metabolism in sheep thus warranted thorough investigation and is the subject of the work reported in Chapters 3 and 5.

CHAPTER 3

UPTAKE AND OUTPUT OF VARIOUS FORMS OF CHOLINE BY ORGANS OF THE CONSCIOUS CHRONICALLY CATHETERIZED SHEEP

3.1 Introduction

It has been shown that sheep receive an insignificant amount of choline from the diet per day, as there is almost complete microbial destruction of dietary choline in the alimentary tract before absorption (Neill *et al.*, 1978, 1979; Dawson *et al.*, 1981; results in Chapter 2). In sheep nearly all of the choline body pool is of endogenous origin, and this is in contrast with rats, in which a sizable proportion of the body choline is of dietary origin (Dawson *et al.*, 1981). Sheep liver is less effective at synthesizing choline through the methylation of phosphatidylethanolamine to phosphatidylcholine than is rat liver (Bremer & Greenberg, 1961; Henderson, 1978; Neill *et al.*, 1979). Sheep appear to survive successfully on a low dietary choline intake owing to a slow turnover of the choline body pool (Dawson *et al.*, 1981, results in Chapter 2).

It can be calculated from the data of Neill *et al.* (1979) and Dawson *et al.* (1981) that hepatic synthesis and dietary supply of choline only account for 24% of the daily choline requirement of the adult sheep. This suggests that extrahepatic tissues of sheep may be capable of substantial choline production in order to maintain the balance of the endogenous body pool. This would be in contrast with rats, in which choline synthesis by extrahepatic tissues is of minor importance (Bremer & Greenberg, 1961; Bjørnstad & Bremer, 1966).

Sheep also secrete considerable amounts of choline mainly as

phosphatidylcholine into the intestinal lumen through bile which is produced by the liver (Adams & Heath, 1963; Noble, 1978; results in Chapter 2). Since sheep derive limited amounts of choline from the diet and hepatic synthesis, there must be almost complete re-absorption of bile choline from the alimentary tract and subsequently extensive reutilization of that choline in the liver for new bile phosphatidylcholine synthesis.

Insight into the sites and rates of formation and elimination of endogenous metabolites in the whole animal may be gained if the blood flow rates through, and blood metabolite gradients across, various organs can be measured. A sheep preparation with chronic intravascular catheters has been developed which has facilitated direct and simultaneous measurement of drug and oxygen blood concentration gradients across organs and organ blood flow in conscious unrestrained animals under steady state conditions (Mather *et al.*, 1982; Runciman, 1982; Runciman *et al.*, 1984). In the present study this preparation was adopted to measure the net uptake and output of various forms of endogenous choline by organs of the sheep. The amounts of choline compounds in sheep tissues and in blood of cattle and rats were also determined. This experimental approach allowed the origin of the bulk of the endogenous choline body pool and the potential for the retention and recycling of bile choline in sheep to be examined.

3.2 Methods and Materials

3.2.1 Animals

All animals were obtained from holdings of The Waite Agricultural

Research Institute, The University of Adelaide.

Specially selected Merino wether sheep (*Ovis aries*) of blood haemoglobin type A or B (determined by the method of Moore *et al.*, 1966), aged 1-2 years and weighing 30-45kg, were acclimatised indoors in metabolism crates for at least one month and used for conscious chronically catheterized sheep preparations. They were given chaffed lucerne hay and water *ad libitum*. Prior to surgery the animals were fasted for 24h. Merino wether sheep, approximately 2 years old and weighing 35-45kg, were maintained indoors in pens and used for obtaining tissue samples. They were provided with water *ad libitum* and a daily ration (09.00h) of 1kg of chaffed lucerne hay.

Intact Hereford bulls (*Bos taurus*), approximately 3 years old and weighing 600-700kg were used for obtaining blood samples. The cattle were grazed on irrigated perennial pasture consisting mainly of subterranean clover and rye grass and supplemented *ad libitum* with chaffed lucerne hay.

Adult male Hooded Wistar rats (*Rattus norvegicus*), weighing 250-300g, were housed in wire cages indoors and used for obtaining blood samples. They were given water and a pelleted rat diet (Charlicks, Adelaide, S. Aust., Australia) *ad libitum*.

3.2.2 Surgical preparation and post-operative treatment of conscious chronically catheterized sheep

Sheep were surgically prepared with chronic catheters in various veins and arteries of the blood circulation system (Figure 3.1) and maintained by Dr. W.B. Runciman and members of his research group

Figure 3.1: Diagrammatic representation of the adult sheep blood circulation system

[modified from Runciman (1982)]

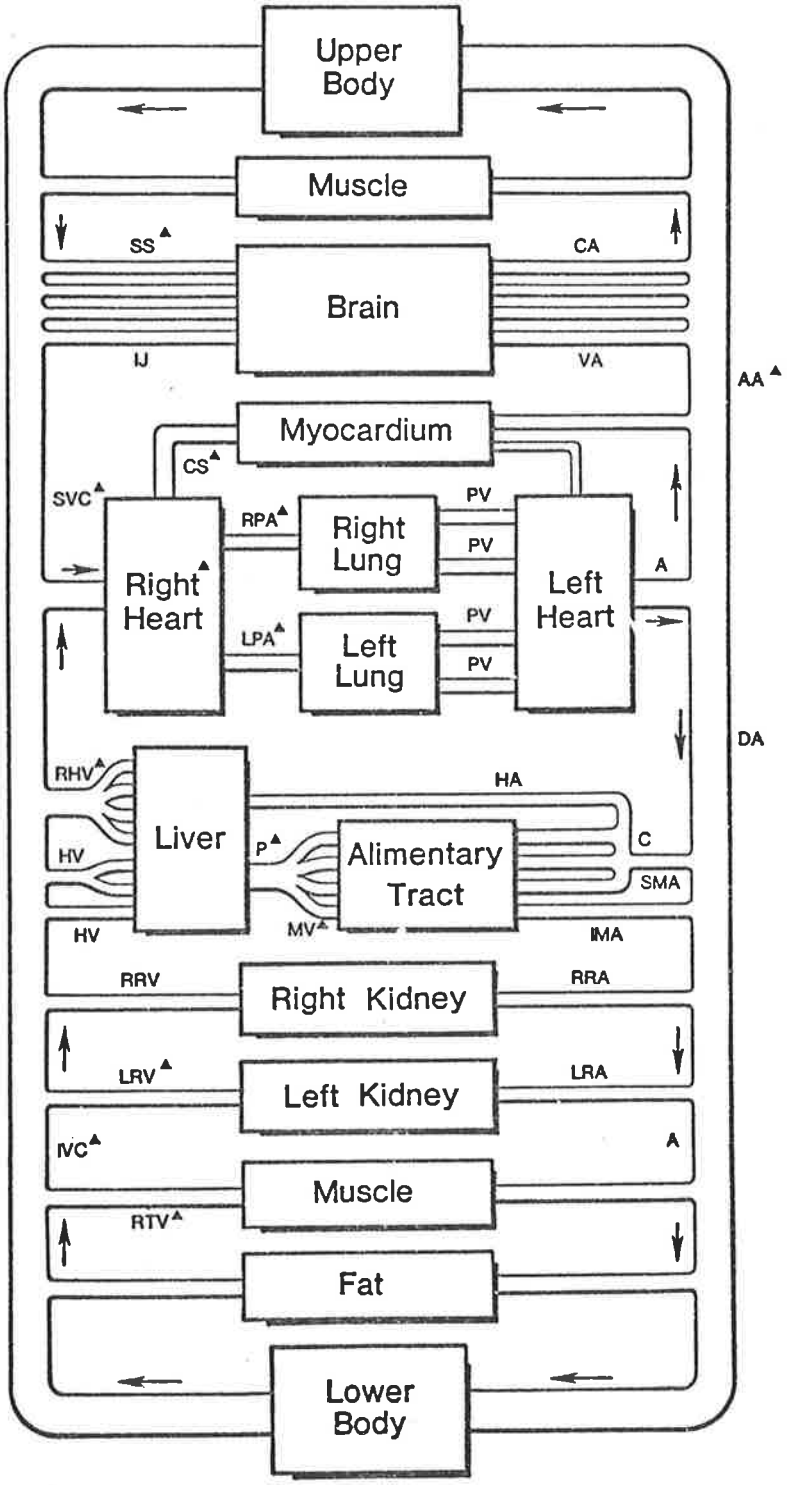
Oxygenated blood is pumped from the left-side of the heart to the body tissues *via* arteries and deoxygenated blood is returned to the right-side of the heart *via* veins (systemic circulation). Deoxygenated blood is pumped from the right-side of the heart through the pulmonary arteries to the lungs and oxygenated blood passes to the left-side of the heart in the pulmonary veins (pulmonary circulation).

Key to the figure:

SS = superior sagittal sinus.
IJ = internal jugular vein.
CA = carotid artery.
VA = vertebral artery.
SVC = superior vena cava.
IVC = inferior vena cava.
CS = coronary sinus.
RPA = right pulmonary artery.
LPA = left pulmonary artery.
PV = pulmonary vein.
A = aorta.
AA = ascending aorta.
DA = descending aorta.
RHV = right hepatic vein.
HV = hepatic vein.
P = portal vein.
MV = mesenteric vein.
HA = hepatic artery.
C = coeliac artery.
SMA = superior mesenteric artery.
IMA = inferior mesenteric artery.
RRV = right renal vein.
LRV = left renal vein.
RRA = right renal artery.
LRA = left renal artery.
RTV = recurrent tarsal vein.

⟶ = direction of blood flow.

▲ = blood vessel catheterized in this study.


Upper Body
Muscle
SS
CA
Brain
IJ
VA
AA
Myocardium
CS
SVC
RPA
Right Lung
PV
PV
Right Heart
Left Heart
A
LPA
Left Lung
PV
PV
DA
RHV
HA
Liver
HV
P
Alimentary Tract
C
SMA
HV
MV
IMA
RRV
Right Kidney
RRA
LRV
Left Kidney
LRA
IVC
A
Muscle
RTV
Fat
Lower Body

in the Department of Anaesthesia and Intensive Care at Flinders Medical Centre, The Flinders University of South Australia as follows:

After induction with intravenous sodium thiopental, sheep were anaesthetized with halothane using a closed circuit and artificial ventilation. Under image intensifier control, the right or left pulmonary artery, left renal vein, right hepatic vein, coronary sinus, superior vena cava (proximal to the tricuspid valve in the right atrium of the heart), inferior vena cava (caudal to the renal veins), three right atrial and three ascending aorta catheters were inserted *via* the right carotid artery and jugular vein using a Seldinger technique as described by Runciman (1982) and Runciman *et al*. (1984).

The recurrent tarsal vein of the right hind limb was catheterized after incising the skin and freeing the vein from adherent connective tissue. The catheter was inserted about 20cm into the recurrent tarsal vein from the junction of its dorsal and plantar rami and tied in place. This allowed the sampling of venous blood draining approximately 900g of muscle and 40g of intermuscular fat, with very little contribution from skin or subcutaneous tissue (Domanski *et al*., 1974; Pethick, 1980).

A catheter was implanted into the superior sagittal sinus through a trephine hole near the point of intersection of the lines joining each eye with the opposite ear using a Seldinger technique. The tip of the catheter was advanced caudally about 4cm until it lay near the confluence of the sinuses. The catheter was led cranially through a small hole drilled through the skull at the edge

of the trephine hole, through the skin and then turned and threaded back under the skin to the neck. This arrangement enabled venous blood samples draining the brain to be obtained without disturbing the animal (Lindsay & Setchell, 1972; Gardiner, 1980).

Portal and mesenteric vein catheters were inserted directly *via* an abdominal incision and secured using a purse string suture (Runciman, 1982; Runciman *et al.*, 1984). The mesenteric vein catheter was used for the infusion of indicator to determine hepatic blood flow, and the other catheters were used for obtaining blood samples, blood pressure measurements and for the infusion of indicators to determine cardiac output and renal blood flow.

On recovery, the sheep was returned to the metabolism crate and each catheter attached to an extension line (Cat. No. 19080, Surgimed Pty. Ltd., Frankston, Vic., Australia), a pressure infusion system (Intraflo CFS-03F, Sorrensen Research Company, Salt Lake City, Utah, U.S.A.), and one limb of a triple connector (Deseret multi-flow adaptor, Cat. No. 1270, The Deseret Company, Sandy, Utah, U.S.A.), the input side of which was attached *via* a drip set (Type A-FCP-32-000, Tuta Laboratories, Lane Cove, N.S.W., Australia), and an in-line burette (Travenol Laboratories, Sydney, N.S.W., Australia) to a one-litre bag of 0.9% (w/v) sodium chloride injection B.P. containing 5,000 units heparin/l (heparin sodium injection B.P. [mucous] 5,000 units/ml). Each one-litre bag was placed in a pressure infusion pump (BD-10, 4R4403-Fenwal Laboratories, Morton Grove, Il., U.S.A.) which was kept at 300mm Hg *via* a reducing valve (TR63, The Commonwealth Industrial Gases Ltd., Sydney, N.S.W., Australia) attached to a compressed air cylinder (Runciman, 1982; Runciman *et*

al., 1984). Each conscious chronically catheterized sheep was self-contained in its mobile metabolism crate having its own catheter flushing system and containers for feed, water, urine and faeces as shown in Figure 3.2. These techniques for securing the catheters and maintaining their patency have allowed data collection for up to 18 weeks. The positions of the catheters were checked by post-mortem examination and in all cases were found to be correct. Each sheep was allowed to stabilize on full feed intake for 5-7 days post-operatively before taking blood samples and measuring blood flow rates.

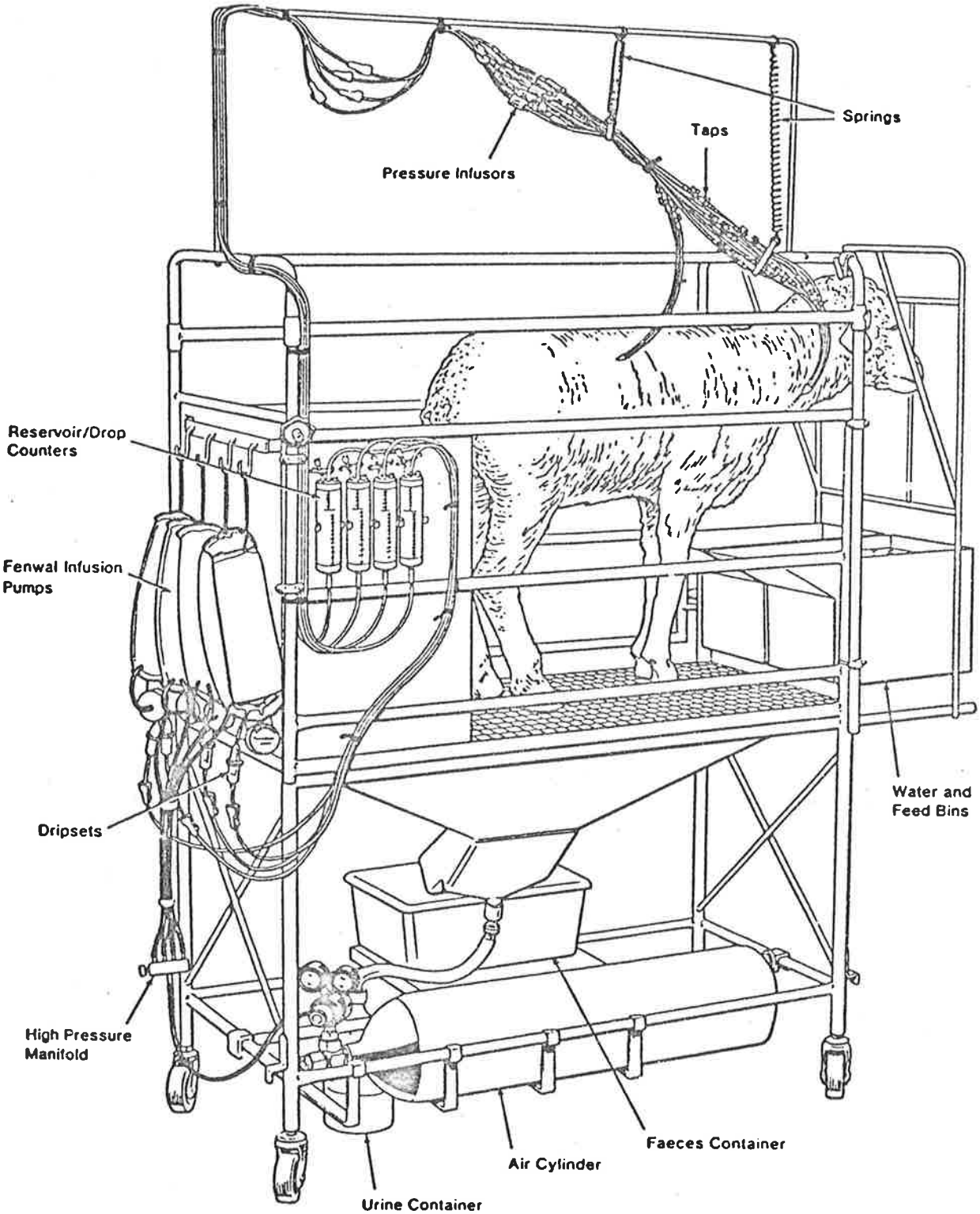
3.2.3 Determination of blood flow rates in conscious chronically catheterized sheep

Blood flow rate measurements were made in each chronically catheterized sheep on the same day blood samples were collected for choline analysis (see below) by Dr. W.B. Runciman and members of his research group (Department of Anaesthesia and Intensive Care, Flinders Medical Centre, The Flinders University of South Australia). Cardiac output was routinely determined by a thermodilution method using cold (0°C) 5% (w/v) dextrose injection B.P. as the indicator and the calculated value corrected for thermal losses as calibrated by dye dilution measurements (Runciman, 1982; Runciman *et al.*, 1984). Kidney blood flow was measured by the Fick method using sodium [^{125}I] iodohippurate as the indicator and assayed by a gamma scintillation counting method (Runciman, 1982; Runciman *et al.*, 1984). Liver blood flow was measured by the Fick method using sulphobromophthalein as the indicator and assayed by a high-pressure liquid-chromatographic technique (Runciman, 1982; Runciman *et al.*,

Figure 3.2: The conscious chronically catheterized sheep preparation

(Diagram provided by courtesy of Dr. W.B. Runciman, Department of Anaesthesia and Intensive Care, Flinders Medical Centre, The Flinders University of South Australia).

Each sheep was surgically prepared with a combination of chronic intravascular catheters and maintained in a mobile metabolism crate equipped with a catheter-flushing system, feed and water containers, and urine and faeces collectors as described in Section 3.2.2.


Springs
Taps
Pressure Infusors
Reservoir/Drop Counters
Fenwal Infusion Pumps
Dripsets
Water and Feed Bins
High Pressure Manifold
Faeces Container
Air Cylinder
Urine Container

1984). Portal blood flow was determined by an indicator dilution method with sodium [^{125}I] iodohippurate (Runciman, 1982; Runciman *et al.*, 1984). Blood flow increases with decreases in blood packed cell volume (haematocrit) so that oxygen consumption and venous pO_2 remain unchanged (Messmer *et al.*, 1972; Peter *et al.*, 1975). For all blood flow rate measurements, the packed cell volumes of blood samples remained approximately 30% over the periods of analysis, which indicated that no excessive removal of blood from sheep had occurred and that blood flows were normal.

3.2.4 Collection of blood samples from conscious chronically catheterized sheep and determination of various forms of choline

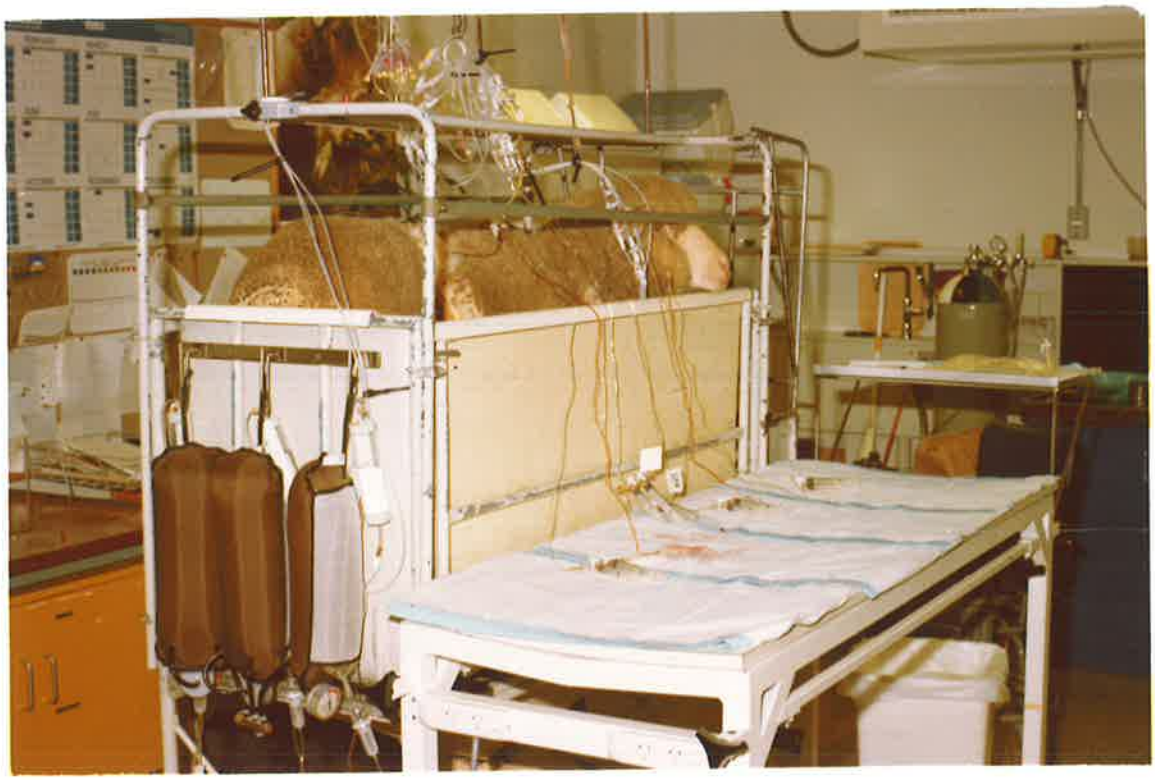
Blood samples (6ml) were simultaneously collected with 10ml plastic syringes (Terumo Pty. Ltd., Melbourne, Vic., Australia) from respective catheters of conscious chronically prepared sheep at hourly intervals for at least 4h. Catheter "dead-space" blood samples (5ml) were simultaneously aspirated with different syringes directly beforehand. The blood sampling procedure is shown in Figure 3.3. Not more than 120ml of blood was removed from each sheep per experiment (i.e. less than 5% of the total blood volume).

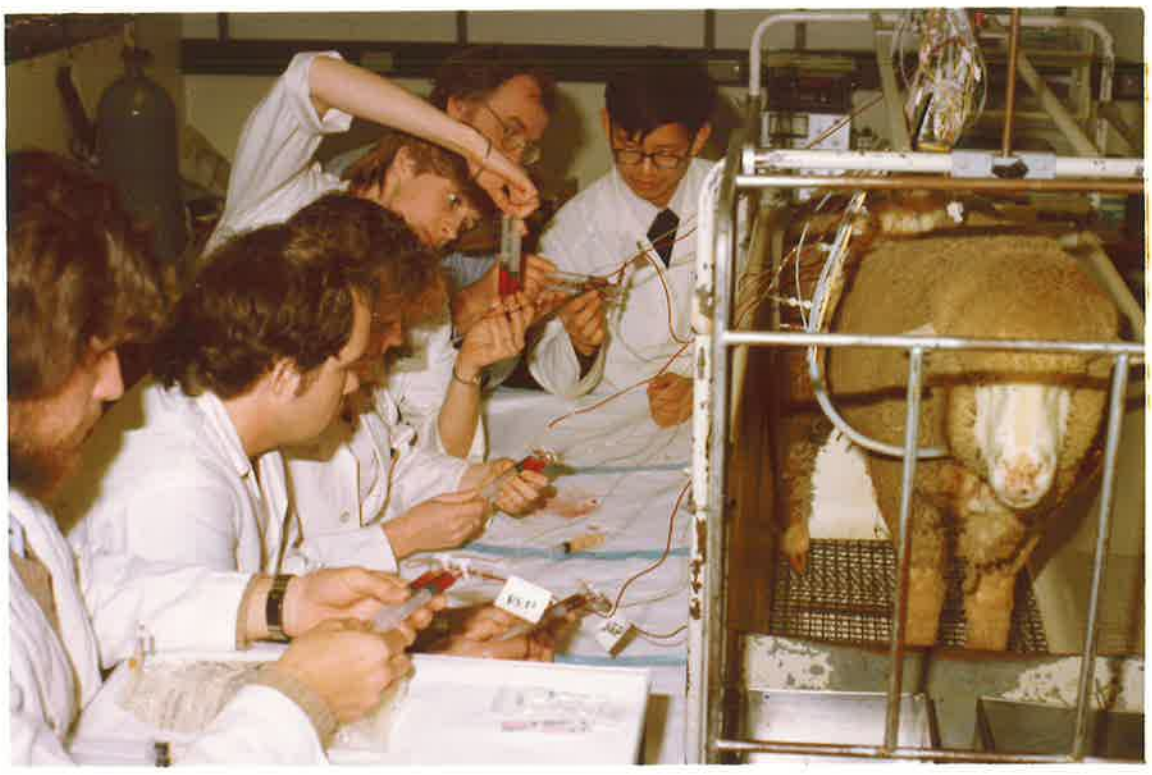
The blood samples were immediately transferred to ice-cold glass centrifuge tubes containing approximately 100 units of heparin (heparin sodium injection B.P. [mucous] 5,000 units/ml) and centrifuged at 1,200g for 10min at 4°C. The plasma was removed and, in some cases, the cells were resuspended to the original blood volume with ice-cold 0.9% (w/v) sodium chloride solution. The packed cell volumes of blood samples were all about 30%. It was important

Figure 3.3: Collection of blood samples from conscious chronically catheterized sheep for choline analysis

Top: A double tap joined each catheter to a pressure infusor of the continuous flushing system. Two blood sampling extension lines were connected *via* this double tap to a further remote double tap fitted with two syringes. This enabled blood to be taken from each catheter *via* this loop without disturbing the animal, and each catheter to be flushed continuously between sampling.

Bottom: Blood samples were taken simultaneously from respective catheters with two 10ml syringes at hourly intervals for at least 4h. An initial 5ml blood sample was withdrawn from each catheter using the left syringe representing catheter "dead-space", followed by a 6ml blood sample with the right syringe for choline analysis. The 6ml blood samples for choline determination were immediately transferred to heparinized glass centrifuge tubes on ice and treated as described in Section 3.2.4. The 5ml "dead-space" blood samples were returned to the animal and the sampling extension lines and catheters flushed with a 0.9% sodium chloride solution containing 5,000 units heparin/litre.





to maintain and centrifuge blood at 4°C after sampling, as there was a rapid rise in unesterified choline in plasma and cells if the blood remained at room temperature. In whole blood, plasma and cells maintained at 4°C, no increase in the amount of unesterified choline could be detected in a time interval of 3h. Spanner *et al.* (1976) and Eckernäs & Aquilonius (1977) reported that a significant increase in unesterified choline occurs in the blood of non-ruminant animals at room temperature which is attributed to phospholipases liberating unesterified choline from choline-containing phospholipids. A reduction in temperature was found to be adequate for completely controlling artifactual increases in blood unesterified choline of these species.

Plasma samples (approximately 3ml) were immediately transferred to membrane cones of selective permeability (Centriflo CF 25; Amicon Corp., Lexington, Ma., U.S.A.; mol. wt. >25,000 excluded) and centrifuged at 1,000g for 20min at 4°C (Eckernäs & Aquilonius, 1977). The membrane cones were soaked in water overnight prior to blood sampling and the absorbed water was removed by centrifuging the empty cones (1,000g for 10min) before plasma samples were added. The clear ultrafiltrates of plasma (pH 7.5; approximately 0.5ml) were stored in 3ml plastic containers (Disposable Products Pty. Ltd., Adelaide, S. Aust., Australia) at -80°C until assayed in duplicate for unesterified choline by the radioenzymic method using partially purified choline acetyltransferase and labelled acetyl-CoA described in Section 2.2.5 of Chapter 2. The plasma ultrafiltrates were stable at -80°C for at least 3 months and stable at 22°C for at least 24h. This indicates that no plasma phospholipases were present to degrade choline esters which agrees with the

findings of Eckernäs & Aquilonius (1977).

Plasma samples (1ml) were immediately extracted with ice-cold chloroform/methanol (2:1, v/v) (containing the antioxidant 2,6-di-*tert*-butyl-4-methylphenol, 50mg/l) and water by the method of Folch *et al.* (1957) but with modifications used by Henderson (1978), as previously described for saliva, bile and urine samples in Section 2.2.9 of Chapter 2. Resuspended blood cell samples (1ml) were immediately extracted by the same procedure, but initially blended with 6ml of ice-cold methanol for 1min using a Polytron Type PT10 20350D tissue homogenizer and sonicator fitted with a PCU-2 speed control on setting 6 (Kinematica, G.m.b.H., Luzern, Switzerland) and then reblended for 1min after adding 12ml of ice-cold chloroform. If resuspended blood cells were directly treated with a mixture of chloroform/methanol (2:1, v/v) they formed into a coagulated mass which resulted in poor lipid extraction. This phenomenon was previously observed by Dawson *et al.* (1960).

A 2ml portion of the lipid fraction (10ml) of extracted plasma and blood cell samples was evaporated to dryness under N_2 and the lipid choline hydrolysed to unesterified choline with 3ml of 6M-HCl at 110°C for 24h in a sealed test tube. The fraction was taken to dryness *in vacuo* at 30-40°C and stored in a vacuum desiccator containing KOH pellets for at least 12h. The residue was redissolved in 2ml of 5 mM-sodium phosphate buffer (pH 7.8) and the unesterified choline determined in duplicate by the radioenzymic assay.

The water-soluble fraction (2ml) of extracted plasma and blood cell samples (previously washed three times with 1ml of diethyl ether to remove lipid contaminants) was adjusted to pH 8-9

with NH_3 vapour after being centrifuged at 8,000g for 2min in 1ml stoppered plastic centrifuge tubes in an Eppendorf 3200 micro-centrifuge (Eppendorf Gerätebau, Netheler and Hinz G.m.b.H., Hamburg, Germany). Glycerophosphocholine, phosphocholine and unesterified choline in the sample were fractionated by the column cation-exchange chromatography technique of Webster & Cooper (1968) and Illingworth & Portman (1972) described in Chapter 1 (Section 1.2.3). The glycerophosphocholine and phosphocholine column fractions were taken to dryness *in vacuo* at 30-40°C and hydrolysed to free choline in 5ml of 1M-HCl at 100°C for 30min and 3M-HCl at 123°C for 24h respectively. All fractions were evaporated to dryness *in vacuo* at 30-40°C and placed in a desiccator containing KOH pellets for at least 12h. The glycerophosphocholine, phosphocholine and unesterified choline residues were redissolved in 500µl, 250µl and 500µl of 5 mM-sodium phosphate buffer (pH 7.8) respectively and assayed for unesterified choline in duplicate by the radioenzymic method. The specificity of this technique was checked by quantitative thin-layer chromatography as mentioned in Section 1.2.3 of Chapter 1.

3.2.5 Collection of blood samples from rats and cattle and determination of various forms of choline

Blood samples (5ml maximum) were simultaneously withdrawn by needle (19 gauge) and syringe from the descending aorta and inferior vena cava of rats (anaesthetized with diethyl ether) after abdominal incision. Blood samples (6ml) were taken by needle (18 gauge) and syringe directly from a jugular vein of cattle. Blood was treated and the various forms of choline determined in plasma and cells as described in Section 3.2.4.

3.2.6 Collection of tissues from sheep and determination of various forms of choline

Sheep were slaughtered by severing the neck, and samples of liver, kidney cortex, heart, skeletal muscle (M. biceps femoris) and small intestine (jejunum) were rapidly excised and freeze-clamped with aluminium-faced tongs previously cooled in liquid N_2 (Zeisel & Wurtman, 1981). The entire procedure took less than 1min. Immediate freezing of tissues after slaughter was necessary to prevent rapid post-mortem increases in unesterified choline due to the degradation of choline-containing phospholipids by phospholipases (Dross & Kewitz, 1972; Haubrich *et al.*, 1981). 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 plastic sample containers (Camelec Ltd., Adelaide, S. Aust., Australia) immersed in liquid N_2 until processed. Powdered tissues (1g) were extracted and the various forms of choline fractionated and hydrolysed to unesterified choline, as described for blood cell samples in Section 3.2.4. The lipid choline, glycerophosphocholine, phosphocholine and unesterified choline fractions were redissolved in 5-50ml, 2-30ml, 1-15ml and 2-15ml of 5 mM-sodium phosphate buffer (pH 7.8) respectively and the choline measured in duplicate by the radioenzymic assay outlined in Section 2.2.5 of Chapter 2.

3.2.7 Chemicals

Sodium [^{125}I] iodohippurate (specific radioactivity 35μCi/mg) was purchased from Amersham Australia Pty. Ltd., Sydney, N.S.W., Australia. Sulphobromophthalein (sodium salt) was obtained from

Sigma Chemical Co., St. Louis, Mo., U.S.A. Other chemicals were obtained as follows: 0.9% (w/v) sodium chloride injection B.P. and 5% (w/v) dextrose injection B.P. from Travenol Laboratories Pty. Ltd., Sydney, N.S.W., Australia; heparin sodium injection B.P. [mucous] 5,000 units/ml from David Bull Laboratories Pty. Ltd., Mulgrave, Vic., Australia; halothane B.P. (Fluothane) from I.C.I. Australia Operations Pty. Ltd., Villawood, N.S.W., Australia; sodium thiopentone from May & Baker Australia Pty. Ltd., West Footscray, Vic., Australia; 2,6-di-*tert*-butyl-4-methylphenol from Calbiochem, Los Angeles, Calif., U.S.A. Reagent grade chloroform and methanol were glass redistilled. Other chemicals were reagent-grade or the best commercially available grade. Glass double-distilled water was used throughout this work.

3.3 Results

3.3.1 Unesterified choline concentrations in blood plasma from various blood vessels of conscious chronically catheterized sheep

The results in Table 3.1 show the blood plasma unesterified choline concentrations in ten different blood vessels of ten conscious chronically catheterized sheep. Sheep with blood haemoglobin type A showed significantly lower plasma unesterified choline concentrations than sheep with blood haemoglobin type B in corresponding vessels (Student's *t* test). The values reported in the present work are lower than those reported for non-ruminant species (Wang & Haubrich, 1975; Zeisel & Wurtman, 1981; Zeisel, 1981).

Differences in plasma unesterified choline concentrations

Table 3.1: Unesterified choline concentrations in blood plasma from various blood vessels of conscious chronically catheterized sheep

At hourly intervals blood samples were collected simultaneously from different vessels of conscious chronically catheterized Merino wethers. Unesterified choline was determined in plasma ultrafiltrates in duplicate as described in Section 3.2.4. The values shown are means ± S.E.M. with the numbers of blood samples taken from sheep given in parentheses. Significant mean plasma concentration differences between blood haemoglobin type A and B sheep, as determined by Student's *t* test, are indicated.

Sheep	Blood haemoglobin type	Unesterified choline concentration in blood plasma (nmol/ml)									
		Portal vein	Ascending aorta	Hepatic vein	Pulmonary artery	Renal vein	Superior vena cava	Inferior vena cava	Coronary sinus	Sagittal sinus	Tarsal vein
1	B	10.4±1.9(7)	6.3±1.3(7)								
2	B	8.9±1.3(4)	4.6±0.2(4)	3.7±0.2(4)	6.0±0.3(4)						
3	B	12.8±0.9(5)	3.7±0.2(5)	2.8±0.2(5)		2.7±0.2(5)					
Mean		10.7	4.9	3.3	6.0	2.7					
4	A		3.0±0.4(4)	1.6±0.3(4)	3.4±0.4(4)	2.9±0.8(4)	4.2±0.6(4)	4.2±0.7(4)			
5	A		2.4±0.2(4)	1.2±0.4(4)	2.7±0.3(4)	2.1±0.3(4)	3.4±0.3(4)	3.7±0.3(4)			
6	A	13.4±2.2(4)	4.2±0.5(4)	1.1±0.3(4)	3.7±0.5(4)			5.3±0.5(4)	5.6±0.4(4)		
7	A		4.1±0.1(8)							5.2±0.3(8)	
8	A		2.5±0.1(4)				3.8±0.3(4)	3.4±0.2(4)	2.7±0.2(4)	3.6±0.2(4)	
9	A		3.1±0.1(4)					4.6±0.1(4)			4.0±0.2(4)
10	A		3.3±0.0(4)					4.8±0.1(4)			4.1±0.1(4)
Mean		13.4	3.2*	1.3**	3.3*	2.5	3.8	4.3	4.2	4.4	4.1

*$P<0.05$ and **$P<0.02$ compared with blood haemoglobin type B sheep.

between blood vessels can give a measure of the net uptake and output of choline by various organs of the body. The values shown in Table 3.2 indicate a significant production of unesterified choline by the alimentary tract, brain, heart, hindlimb muscle and the upper- and lower-body regions drained by the venae cavae and uptake by the liver, lungs and kidneys of sheep (Student's paired *t* test). The production of unesterified choline by the alimentary tract was substantial and was particularly pronounced in sheep 3 and 6. Unesterified choline production by the alimentary tract was approximately balanced by hepatic uptake if a correction is made for the input into the liver *via* the hepatic artery (approximately 20% of the total liver blood flow) in addition to the main input *via* the portal vein. Brain, heart and hindlimb muscle clearly contributed to the unesterified choline production by the venae cavae-drained upper- and lower-body regions. There was almost equal production of unesterified choline by the two body regions. The uptake of unesterified choline by the lungs and kidneys was relatively low in sheep with blood haemoglobin type B.

3.3.2 Lipid choline concentrations in blood plasma from various blood vessels of conscious chronically catheterized sheep

The blood plasma concentrations of lipid choline in different blood vessels of eight conscious chronically catheterized sheep are presented in Table 3.3. Lipid choline constitutes the major form of choline in plasma and the values shown are in the range reported for sheep by Lindsay & Leat (1975). Plasma lipid choline concentrations were similar in blood haemoglobin type A and B sheep. There was significant production of lipid choline by the venae

Table 3.2: Differences in blood plasma unesterified choline concentration across various organs of conscious chronically catheterized sheep

The values shown are means ± S.E.M. for the numbers of blood samples taken from sheep given in parentheses. + indicates net output and − net uptake by an organ. The significance of the differences across organs, as determined by Student's pared *t* test, is indicated. Abbreviations used: N.S. = not significant; PV = portal vein; A = Ascending aorta; HV = hepatic vein; PA = pulmonary artery; RV = renal vein; SVC = superior vena cava; IVC = inferior vena cava; CS = coronary sinus; SS = sagittal sinus; TV = tarsal vein. See Table 3.1 for absolute plasma unesterified choline concentrations in various vessels.

		Differences in blood plasma unesterified choline conentration (nmol/ml)									
Sheep	Blood haemo-globin type	Alimentary tract (PV-A)	Liver (HV-PV)	Liver (HV-A)	Lung (A-PA)	Kidney (RV-A)	Upper-body region (SVC-A)	Lower-body region (IVC-A)	Heart (CS-A)	Brain (SS-A)	Hindlimb muscle (TV-A)
1	B	+4.0±1.4(7) *P*<0.05									
2	B	+4.3±1.2(4) *P*<0.05	−5.2±1.4(4) *P*<0.05	−0.9±0.3(4) N.S.	−1.4±0.4(4) *P*<0.05						
3	B	+9.1±0.9(5) *P*<0.001	−10.0±0.8(5) *P*<0.001	−0.9±0.3(5) *P*<0.05		−1.0±0.3(5) *P*<0.05					
Mean		+5.8	−7.6	−0.9	−1.4	−1.0					
4	A			−1.4±0.4(4) *P*<0.05	−0.5±0.2(4) N.S.	−0.1±0.4(4) N.S.	+1.3±0.3(4) *P*<0.02	+1.2±0.4(4) *P*<0.05			
5	A			−1.1±0.3(4) *P*<0.05	−0.4±0.1(4) *P*<0.05	−0.3±0.1(4) *P*<0.05	+1.1±0.1(4) *P*<0.01	+1.4±0.1(4) *P*<0.01			
6	A	+9.2±1.7(4) *P*<0.02	−12.4±2.0(4) *P*<0.01	−3.2±0.3(4) *P*<0.01	+0.5±0.2(4) N.S.			+1.1±0.2(4) *P*<0.02	+1.4±0.2(4) *P*<0.01		
7	A									+1.1±0.3(8) *P*<0.02	
8	A						+1.3±0.3(4) *P*<0.02	+0.9±0.1(4) *P*<0.01	+0.2±0.0(4) *P*<0.02	+1.1±0.3(4) *P*<0.05	
9	A							+1.5±0.1(4) *P*<0.001			+0.9±0.2(4) *P*<0.05
10	A							+1.5±0.1(4) *P*<0.001			+0.8±0.1(4) *P*<0.02
Mean		+9.2	−12.4	−1.9	−0.1	−0.2	+1.2	+1.3	+0.8	+1.1	+0.9

Table 3.3: Lipid choline concentrations in blood plasma from various blood vessels of conscious chronically catheterized sheep

Blood samples were collected as described in the legend to Table 3.1. Plasma samples were extracted with chloroform/methanol and lipid choline separated from other forms of choline and determined in duplicate, as described in Section 3.2.4. The values shown are means ± S.E.M. for the numbers of blood samples taken from sheep given in parentheses. Significant plasma concentration differences across organs, as determined by Student's paired *t* test, are also indicated. Other abbreviations are defined in the legend to Table 3.2.

		Lipid choline concentration in blood plasma (nmol/ml)									
Sheep	Blood haemo-globin type	Portal vein (PV)	Ascending aorta (A)	Hepatic vein (HV)	Pulmonary artery (PA)	Renal vein (RV)	Superior vena cava (SVC)	Inferior vena cava (IVC)	Coronary sinus (CS)	Sagittal sinus (SS)	Tarsal vein (TV)
2	B	410±48(4)	391±44(4)	376±20(4)	389±18(4)						
3	B	452± 8(5)	451± 7(5)	455±12(5)		451±17(5)					
Mean		431	421	416	389	451					
5	A		387±17(4)	392± 6(4)	402±14(4)	369±20(4)	435±15(4)[a]	415±20(4)[b]			
6	A	436±34(4)	404±41(4)	413±11(4)	372±14(4)			441±30(4)	439±26(4)		
7	A		325±11(8)							300±11(8)	
8	A		547±12(4)				556±13(4)	542± 8(4)	534± 9(4)	539± 4(4)	
9	A		555±14(4)					574±11(4)			541±13(4)
10	A		548±22(4)					553±25(4)			550±22(4)
Mean		436	461	403	387	369	496	505	487	420	546

[a] Net output by upper-body : (SVC-A), $P<0.05$.
[b] Net output by lower-body : (IVC-A), $P<0.05$.

cavae-drained upper- and lower-body regions of sheep 5 (Student's paired *t* test). No uptake or output of lipid choline was detected across any other organs of the eight animals.

3.3.3 Glycerophosphocholine and phosphocholine concentrations in blood plasma from various blood vessels of conscious chronically catheterized sheep

Table 3.4 shows the blood plasma concentrations of glycerophosphocholine and phosphocholine in different blood vessels of three conscious chronically catheterized sheep. The data presented appear to be the first accurate values for glycerophosphocholine and phosphocholine concentrations in plasma from the use of a highly sensitive radioenzymic choline assay. Hinton & Setchell (1980) measured glycerophosphocholine and phosphocholine in rat plasma using a relatively insensitive chemical phosphorus assay and could merely suggest that the levels were less than 100 nmol/ml. Plasma glycerophosphocholine and phosphocholine concentrations were comparable in blood haemoglobin type A and B sheep. Table 3.4 indicates significant production of glycerophosphocholine by the liver of sheep 2 and 3 and uptake by the lungs and kidneys of sheep 2 and 3 respectively. There was significant production of phosphocholine by the alimentary tract and kidneys of sheep 3. No uptake or output of glycerophosphocholine and phosphocholine was observed across the upper- and lower-body regions drained by the venae cavae of sheep 5.

3.3.4 Blood flow rates measured in conscious chronically catheterized sheep

The results of cardiac output, liver blood flow and kidney

Table 3.4: Glycerophosphocholine and phosphocholine concentrations in blood plasma from various blood vessels of conscious chronically catheterized sheep

Blood samples were collected as described in the legend to Table 3.1. Plasma samples were extracted with chloroform/methanol and glycerophosphocholine (Gro-*P*-Cho) and phosphocholine (*P*-Cho) fractionated and determined in duplicate, as described in Section 3.2.4. The values shown are means ± S.E.M. for the numbers of blood samples taken from sheep given in parentheses. Significant plasma concentration differences across organs, as determined by Student's paired *t* test, are also indicated. Other abbreviations are defined in the legend to Table 3.2.

Sheep	Blood haemo-globin type	Choline ester	Concentration in blood plasma (nmol/ml)						
			Portal vein (PV)	Ascending aorta (A)	Hepatic vein (HV)	Pulmonary artery (PA)	Renal vein (RV)	Superior vena cava (SVC)	Inferior vena cava (IVC)
2	B	Gro-*P*-Cho	2.4±0.3(4)	2.1±0.3(4)	4.3±0.7(4)[a]	2.8±0.6(4)[b]			
		P-Cho	1.7±0.1(4)	1.5±0.2(4)	2.4±0.4(4)	1.2±0.1(4)			
3	B	Gro-*P*-Cho	3.3±0.5(5)	3.0±0.2(5)	3.5±0.3(5)[c]		2.4±0.1(5)[d]		
		P-Cho	0.3±0.0(5)[e]	0.1±0.0(5)	0.3±0.1(5)		0.4±0.1(5)[f]		
Mean		Gro-*P*-Cho	2.9	2.6	3.9	2.8	2.4		
		P-Cho	1.0	0.8	1.4	1.2	0.4		
5	A	Gro-*P*-Cho		2.5±0.2(4)				2.3±0.3(4)	3.0±0.3(4)
		P-Cho		0.4±0.1(4)				0.3±0.1(4)	0.2±0.1(4)

a Net output by liver : (HV-PV), $P<0.05$; (HV-A), $P<0.02$.
b Net uptake by lung : (A-PA), $P<0.05$.
c Net output by liver : (HV-A), $P<0.01$.
d Net uptake by kidney: (RV-A), $P<0.02$.
e Net output by alimentary tract : (PV-A), $P<0.001$.
f Net output by kidney : (RV-A), $P<0.01$.

blood flow measurements in ten conscious chronically catheterized sheep are presented in Table 3.5. The values are consistent with those previously obtained for sheep by others (Katz & Bergman, 1969; Hales, 1973; Runciman, 1982), with the mean liver and kidney blood flows being 0.27 - 0.44 and 0.18 of the mean cardiac output respectively. The mean cardiac output of blood haemoglobin type A sheep was significantly higher than that of blood haemoglobin type B sheep ($P<0.05$, Student's t test). This phenomenon is almost certainly related to the lower oxygen-carrying capacity of haemoglobin type A compared with haemoglobin type B in sheep blood (Blunt & Huisman, 1975). The higher cardiac output in the haemoglobin type A sheep may be responsible for the lower plasma unesterified choline concentrations in the various blood vessels of these animals, as shown in Table 3.1.

It was not possible to obtain reliable, individual measurements of portal blood flow using the sodium [^{125}I] iodohippurate indicator dilution method, as streaming consistently occurred in the portal vein. Unequivocal evidence of streaming was provided by the fact that measured portal blood flow was sometimes apparently greater than measured hepatic blood flow. This is not surprising on examination of the anatomy; the splenic vein enters the portal vein very close to the porta hepatis (Runciman, 1982). Examination of results obtained by others also provides evidence of streaming with the apparent relative contribution of the portal vein to hepatic flow being subject to wide variation (Katz & Bergman, 1969). However, pooling weighted-mean sodium [^{125}I] iodohippurate values from 140 measurements in six sheep yielded an average portal vein fraction of 0.80 of the total liver blood flow (Runciman, 1982).

Table 3.5: Blood flow rates measured in conscious chronically catheterized sheep

Blood flow rates were determined as described in Section 3.2.3. The values shown are means ± S.E.M. for the numbers of measurements in sheep given in parentheses. Abbreviation used: N.D. = not determined. Significant mean blood flow rate differences between blood haemoglobin type A and B sheep, as determined by Student's *t* test, are indicated.

Sheep	Blood haemoglobin type	Blood flow rate (litre/min)		
		Cardiac output	Liver blood flow	Kidney blood flow
1	B	N.D.	2.16(1)	N.D.
2	B	3.91±0.11(6)	1.36±0.03(6)	0.74±0.01(6)
3	B	3.75±0.15(6)	1.50±0.16(6)	0.62±0.03(6)
Mean		3.83	1.67	0.68
4	A	6.26±0.20(6)	1.80±0.08(6)	N.D.
5	A	6.35±0.17(6)	2.14±0.05(6)	1.41±0.10(3)
6	A	4.70±0.12(4)	1.30±0.13(4)	N.D.
7	A	6.27±0.10(8)	N.D.	N.D.
8	A	4.26±0.11(4)	1.03±0.03(4)	0.49±0.03(4)
9	A	4.64±0.19(4)	1.15±0.08(4)	N.D.
10	A	5.39±0.20(4)	1.35±0.09(4)	N.D.
Mean		5.41*	1.46	0.95

*$P<0.05$ compared with blood haemoglobin type B sheep.

Thismeans that the hepatic artery fraction of the total liver blood flow is 0.20, obtained by difference. The value of 0.80 for the portal vein fraction is similar to that reported for several species (Richardson & Withrington, 1981) and is the value used by others working with sheep (Katz & Bergman, 1969; Thompson *et al.*, 1978).

3.3.5 Unesterified choline and lipid choline concentrations in blood plasma from the inferior vena cava and descending aorta of rats

The concentrations of unesterified choline and lipid choline in blood plasma from the inferior vena cava and descending aorta of rats are presented in Table 3.6. The values are similar to those previously reported for rat blood plasma (Ansell & Spanner, 1971; Wang & Haubrich, 1975; Zeisel & Wurtman, 1981; Jope, 1982; Hicks *et al.*, 1982). There were no significant differences in plasma unesterified choline and lipid choline concentrations from the inferior vena cava and descending aorta of the animals (Table 3.6). This supports the data of Dross & Kewitz (1972) which implied that there was no significant difference in whole blood unesterified choline concentration from the femoral vein and artery of rats. Collectively the results suggest there is no uptake or output of choline by the lower-body region of this species.

3.3.6 Concentrations of various forms of choline in plasma and cells of sheep, cattle and rat blood

Table 3.7 shows that the concentrations of the various forms of choline are higher in the cells than the plasma of sheep, cattle and rat blood, which is consistent with the findings of Spanner *et al.* (1976). In Table 3.7 lipid choline constitutes the major form

Table 3.6: Unesterified choline and lipid choline concentrations in blood plasma from the inferior vena cava and descending aorta of rats

Blood samples were collected simultaneously from the inferior vena cava and descending aorta of anaesthetized rats. Plasma samples were extracted with chloroform/methanol and lipid choline separated from other forms of choline and determined in duplicate. Plasma unesterified choline was determined in ultrafiltrates in duplicate as described in Section 3.2.4. The values shown are means ± S.E.M. for the numbers of rats given in parentheses. The significance of the difference between inferior vena cava and descending aorta plasma concentrations, as determined by Student's paired *t* test, is also indicated. Abbreviation used: N.S. = not significant.

	Choline concentration in blood plasma (nmol/ml)			
Form of choline	Inferior vena cava (IVC)	Descending aorta (A)	(IVC-A)	Significance
Unesterified choline	9.3± 0.9(17)	9.5± 0.7(17)	-0.2± 0.9(17)	N.S.
Lipid Choline	847.7±40.2(10)	818.8±37.5(10)	+28.9±24.5(10)	N.S.

Table 3.7: Concentrations of various forms of choline in plasma and cells of sheep, cattle and rat blood

Blood samples were collected from the inferior vena cava of sheep and rats and from the jugular vein of cattle. Blood plasma and cells were extracted with chloroform/methanol and the various forms of choline fractionated and determined in duplicate as described in Section 3.2.4. Plasma unesterified choline was determined in ultrafiltrates. The values shown are means ± S.E.M. for the numbers of animals of each species given in parentheses.

Species	Blood fraction	Concentration (nmol/ml) Glycerophosphocholine	Phosphocholine	Unesterified choline	Lipid choline
Sheep	Plasma	4.3±0.1(2)	0.4±0.1(2)	8.4±0.1(2)	452.7±45.2(2)
	Cells	12.0±0.7(2)	6.5±0.7(2)	8.8±0.2(2)	2751.8±95.0(2)
Cattle	Plasma	1.4±0.3(4)	0.8±0.3(4)	10.1±2.4(4)	653.5±74.7(4)
	Cells	4.9±0.6(4)	3.7±0.7(4)	25.6±4.1(4)	1680.8±89.4(4)
Rats	Plasma	1.4±0.1(2)	0.5±0.1(2)	9.6±0.7(2)	1045.1±73.9(2)
	Cells	17.6±1.8(2)	11.2±1.8(2)	12.2±0.5(2)	3516.8±54.9(2)

of choline in blood plasma and cells of the three species examined. The concentration of lipid choline was lower in plasma and higher in cells of sheep with respect to cattle. The lipid choline concentrations in blood plasma and cells were significantly lower in sheep and cattle compared to rats ($P<0.05$, Student's t test). In addition to the difference in absolute amounts of lipid choline observed here, a striking difference between the composition of choline-containing phospholipids in blood cells of ruminants and those of non-ruminants has been reported. Blood cells of ruminants contain high amounts of sphingomyelin and negligible amounts of phosphatidylcholine, which is the complete opposite to the blood cell composition of non-ruminant species (Dawson *et al.*, 1960; Christie, 1978). The species variations seen in the choline phospholipids of blood cells are in no way reflected in the composition of blood plasma (Dawson *et al.*, 1960).

In Table 3.7 the ratio of unesterified choline concentration in the cells compared with that in plasma is between 1 and 3 for the three species examined, which is comparable with the range reported for humans (Hanin *et al.*, 1979; Jope *et al.*, 1980; Barclay *et al.*, 1982). The concentration of unesterified choline in plasma and cells was essentially the same between species. The plasma concentration of glycerophosphocholine was approximately 3 times greater in sheep than in cattle and rats ($P<0.01$, Student's t test). The concentration of glycerophosphocholine was higher in the blood cells of rats compared to the ruminant species ($P<0.05$, Student's t test). Phosphocholine was the minor form of choline in the blood components of the animals. The phosphocholine concen-

tration was higher in the cells of rats compared to sheep and cattle ($P<0.05$, Student's t test).

3.3.7 Concentrations of various forms of choline in sheep tissues

The concentrations of unesterified choline and choline esters in adult sheep tissues are presented in Table 3.8. Lipid choline constitutes the major form of choline in all tissues examined, with the highest concentration being in the liver. The lipid choline values are comparable with those of other species reported by White (1973). The concentration of glycerophosphocholine is high in sheep liver (Table 3.8) and is about 11 times greater than the level reported in rat liver (Dawson, 1955*a*). Other workers have also observed a high concentration of glycerophosphocholine in sheep and cattle liver (Schmidt *et al.*, 1952, 1955; R.M.C. Dawson, personal communication). The other sheep tissues examined did not show the same high level of this choline derivative as the liver. The results in Table 3.8 indicate that the concentration of phosphocholine is relatively low in most tissues except the liver and kidney cortex. The phosphocholine values for sheep liver and small intestine in Table 3.8 are lower than those determined for the rat by Dawson (1955*b*). Conversely, the level of phosphocholine observed in sheep kidney appears to be higher than that of rat kidney (Dawson, 1955*b*). The results obtained for unesterified choline in sheep tissues are higher than those reported in corresponding guinea-pig and rat tissues (Haubrich *et al.*, 1975*a*, 1976). The highest levels of unesterified choline were found in the small intestine and skeletal muscle of sheep (Table 3.8).

Table 3.8: Concentrations of various forms of choline in sheep tissues

Merino wethers were slaughtered and tissue samples immediately frozen with aluminium-faced tongs previously cooled in liquid N_2. The frozen tissue powders were extracted with chloroform/methanol and the various forms of choline fractionated and determined in duplicate as described in Section 3.2.5. The values shown are means ± S.E.M. for three adult sheep.

	Concentration (µmol/g wet wt.)			
Tissue	Glycerophosphocholine	Phosphocholine	Unesterified choline	Lipid choline
Liver	2.65±0.15	1.48±0.02	0.48±0.18	21.06±0.96
Kidney (cortex)	0.59±0.14	0.86±0.09	0.15±0.05	5.50±0.10
Skeletal muscle (M.biceps femoris)	0.16±0.02	0.10±0.01	0.56±0.12	2.45±0.15
Heart	0.28±0.02	0.15±0.01	0.49±0.01	7.45±0.15
Small intestine (jejunum)	0.61±0.03	0.38±0.04	1.25±0.01	4.03±0.63

3.4 Discussion

Glycerophosphocholine is an intermediate in the catabolism of phosphatidylcholine to unesterified choline (Dawson, 1955*a*), and the concentration in sheep liver reported here is several times higher than that in rat liver (Dawson, 1955*a*; R.M.C. Dawson, personal communication). The activity of glycerophosphocholine phosphodiesterase, which liberates unesterified choline from glycerophosphocholine, is negligible in sheep liver and high in rat liver (Dawson, 1956; R.M.C. Dawson, personal communication). These observations in sheep liver suggest a mechanism for conserving the choline moiety of phosphatidylcholine by preventing the ready release of unesterified choline as a substrate for oxidation to betaine and CO_2. The present work also shows that in sheep a portion of the liver glycerophosphocholine is transported to the lungs and kidneys *via* the blood plasma, which would help to supply the choline pool of these tissues. There appears to be production of phosphocholine by the alimentary tract and kidneys of the sheep. The latter observation fits in with the relatively high concentration of phosphocholine found in sheep kidney, and may be related to the low amounts of choline secreted in the urine (Luecke & Pearson, 1945; results in Chapter 2).

Plasma unesterified choline is taken up by the liver, lungs and kidneys of sheep, as summarized in Tables 3.9 and 3.10, and is likely to be used efficiently for lipid choline synthesis *via* the CDP-choline pathway. Uptake of unesterified choline by the lungs may be used in the synthesis of (dipalmitoyl) phosphatidylcholine, a principal pulmonary surfactant (Frosolono, 1977; Zeisel, 1981).

Table 3.9: Summary of uptake and output of unesterified choline by organs of conscious chronically catheterized sheep with blood haemoglobin type A

The mean uptake or output of plasma unesterified choline by an organ of the sheep was calculated by the product of the mean venous-arterial plasma unesterified choline concentration difference across the organ (from Table 3.2) and the mean organ blood flow rate. The measured mean cardiac output and liver and kidney blood flow rates are from Table 3.5. Mean organ blood flow rates that have been derived are indicated.

Organ or tissue	Mean venous-arterial plasma unesterified choline concentration difference (nmole/ml)	Mean blood flow rate (litre/min)	Mean uptake(-) or output(+) of plasma unesterified choline (mmole/day)
Alimentary tract	+ 9.2	1.17[a]	+15.5
Liver (portal vein input)	-12.4	1.17[a] 1.46	-20.9) -21.7[¶]
(hepatic artery input)	- 1.9	0.29[b]	- 0.8)
Lungs	- 0.1	5.41[c]	- 0.8
Kidneys	- 0.2	0.95	- 0.3
Upper-body region	+ 1.2	1.50[d]	+ 2.6
Lower-body region	+ 1.3	1.50[d]	+ 2.8
Heart muscle	+ 0.8	0.17[e]	+ 0.2
Brain	+ 1.1	0.13[f]	+ 0.2
Hindlimb muscle	+ 0.9	0.228	+ 0.3
Whole body muscle	+ 0.9[§]	2.40[h]	+ 3.1

[a] Mean portal vein blood flow assumed to be 0.80 of mean total liver blood flow (Runciman, 1982).
[b] Mean hepatic artery blood flow assumed to be 0.20 of mean total liver blood flow (Runciman, 1982).
[c] Mean blood flow through lungs assumed to be equal to mean cardiac output.
[d] Mean total blood flow through venae cavae-drained upper- and lower-body regions assumed to be equal to the mean cardiac output minus mean liver and kidney blood flows (see Figure 3.1). Thus mean blood flow through each region assumed to be half of mean total blood flow.
[e] Mean coronary blood flow calculated using rate of 1.09ml/min/g sheep heart (Lindsay & Setchell, 1974) and sheep heart wt. of 0.4% of sheep body wt., i.e. 160g for a 40kg sheep (May, 1970).
[f] Mean brain blood flow calculated using rate of 1.00ml/min/g sheep brain (Pappenheimer & Setchell, 1972) and sheep brain wt. of 130g (May, 1970).
[g] Mean hindlimb muscle blood flow calculated using rate of 0.24ml/min/g sheep hindlimb muscle (Domanski *et al.*, 1974; Pethick *et al.*, 1981) and sheep hindlimb muscle wt. of 900g (Domanski *et al.*, 1974).
[h] Mean whole body muscle blood flow calculated using rate of 0.24ml/min/g sheep hindlimb muscle (Domanski *et al.*, 1974; Pethick *et al.*, 1981) and sheep total muscle mass equal to 25% of sheep body wt., i.e. 10kg for a 40kg sheep (Pethick *et al.*, 1981).
[§] Assumed to be same as hindlimb muscle studied.
[¶] Total liver uptake derived using the equation of Bergman & Wolff (1971).

Table 3.10: Summary of uptake and output of unesterified choline by organs of conscious chronically catheterized sheep with blood haemoglobin type B

The mean uptake or output of plasma unesterified choline by an organ of the sheep was calculated by the product of the mean venous-arterial plasma unesterified choline concentration difference across the organ (from Table 3.2) and the mean organ blood flow rate. The measured mean cardiac output and liver and kidney blood flow rates are from Table 3.5. Mean organ blood flow rates that have been derived are indicated.

Organ or tissue	Mean venous-arterial plasma unesterified choline concentration difference (nmole/ml)	Mean blood flow rate (litre/min)	Mean uptake(-) or output(+) of plasma unesterified choline (mmole/day)
Alimentary tract	+5.8	1.34[a]	+11.2
Liver (portal vein input)	-7.6	1.34[a])	-14.7)
(hepatic artery input)	-0.9	0.33[b])[1.67]	-0.4)[-15.1¶]
Lungs	-1.4	3.83[c]	-7.7
Kidneys	-1.0	0.68	-1.0

[a] Mean portal vein blood flow assumed to be 0.80 of mean total liver blood flow (Runciman, 1982).
[b] Mean hepatic artery blood flow assumed to be 0.20 of mean total liver blood flow (Runciman, 1982).
[c] Mean blood flow through lungs assumed to be equal to mean cardiac output.
¶ Total liver uptake derived using the equation of Bergman & Wolff (1971).

Dawson *et al.* (1981) calculated that adult sheep require approximately 17.3 mmol of choline per day in order to maintain the choline body pool, which is almost entirely of endogenous origin. Sheep derive about 0.1 mmol of choline from the diet per day, as there is almost complete microbial destruction of dietary choline in the alimentary tract (Neill *et al.*, 1978, 1979; Dawson *et al.*, 1981; results in Chapter 2). Neill *et al.* (1979) showed that the sheep liver can synthesize a maximum of 0.1 mmol of choline per day per kg body wt. by the methylation of phosphatidylethanolamine to phosphatidylcholine (i.e. 4.0 mmol of choline per day for a 40kg sheep). Thus these two sources of choline provide only 24% of the daily choline requirement, which implies that the bulk of the endogenous choline body pool is maintained by substantial choline production in extrahepatic tissues of sheep. The data for conscious chronically catheterized sheep with blood haemoglobin type A support this conclusion. The mean total body venous return of plasma unesterified choline for these sheep is 14.2 μmol/min, on the basis of the mean hepatic venous plasma unesterified choline return of 1.9 μmol/min, and the mean superior and inferior venae cavae plasma unesterified choline return of 12.3 μmol/min, representing the rest of the body (taking the mean systemic venous blood flow as approximately the mean cardiac output minus mean liver and kidney blood flows). The mean total body venous return of plasma unesterified choline is almost equal to the mean total body arterial output of 16.6 μmol/min, if corrections are made for uptake of plasma unesterified choline by the liver *via* the hepatic artery (approximately 20% of the total mean liver blood flow) and uptake by the lungs and kidneys. Thus this whole-body

calculation indicates that only 13% of the total body plasma unesterified choline return is provided by the liver, and the major contribution (87%) is by the venae cavae draining the upper- and lower-body regions of the sheep.

The upper- and lower-body regions of sheep 5 produced substantial amounts of lipid choline in addition to unesterified choline. Since there was no significant uptake of lipid choline and no uptake or output of glycerophosphocholine and phosphocholine in the plasma across the upper- and lower-body of any of the sheep, it is unlikely that unesterified choline production is derived from breakdown of choline esters that are delivered to these regions in the blood. Presumably the major source of the unesterified choline is from net synthesis of phosphatidylcholine by the methylation of phosphatidylethanolamine in upper- and lower-body tissues of sheep that is subsequently hydrolysed by phospholipases or undergoes base-exchange. Table 3.9 shows that the upper- and lower-body regions drained by the venae cavae of blood haemoglobin type A sheep produced almost equal amounts of plasma unesterified choline, with a total mean value of 5.4 mmol per day. Sheep brain and heart muscle only contributed about 4% to this whole body unesterified choline production. Hind-limb muscle alone made a relatively minor contribution, however approximately 57% of the unesterified choline production by the upper- and lower-body of sheep can be accounted for by the total muscle mass (Table 3.9). The remainder of the unesterified choline production is probably supplied by other upper- and lower-body tissues of the sheep, e.g. skin, endocrine glands, lymph nodes, endothelium of veins, bone marrow. Choline may also originate from bile and net synthesis in the mucosa of the intestine, reaching the

venae cavae in lymph *via* the thoracic duct. The present work showed that there was no uptake or output of lipid choline or unesterified choline by the lower-body region of rats which is in direct contrast to sheep. In rats (Dross & Kewitz, 1972; Choi *et al.*, 1975; Spanner *et al.*, 1976) and humans (Aquilonius *et al.*, 1975) there is an efflux of plasma unesterified choline across the brain but this only makes a minor contribution to the whole body as observed here for sheep. In rats choline synthesis is of quantitative significance in the liver but not in extrahepatic tissues (Bremer & Greenberg, 1961; Bjørnstad & Bremer, 1966), and a substantial part of the choline body pool is of dietary origin (Dawson *et al.*, 1981).

Sheep secrete 11-17 mmol of phosphatidylcholine into the intestinal lumen through bile per day (Adams & Heath, 1963; Noble, 1978; results in Chapter 2). Balint *et al.* (1967) and Treble *et al.* (1970) have demonstrated in rat liver that bile phosphatidylcholine is preferentially synthesized from unesterified choline *via* the CDP-choline and base-exchange pathways, rather than by the methylation pathway. Since sheep receive only limited amounts of dietary choline, there must be efficient reabsorption and reutilization of the bile choline from the intestine in order to maintain the balance of the endogenous choline pool and the daily rate of secretion in bile. The mean net plasma unesterified choline production by the alimentary tract of conscious chronically catheterized sheep with blood haemoglobin type A and B are 15.5 and 11.2 mmol per day respectively, as indicated in Tables 3.9 and 3.10. It is improbable that this unesterified choline arises from the hydrolysis

of choline esters as the blood flows through the alimentary tract, since there was production of glycerophosphocholine and phosphocholine and no uptake or output of lipid choline in the plasma across this region. The amount of choline produced is much higher than that received from the diet (Neill *et al.*, 1979; Dawson *et al.*, 1981; results in Chapter 2) and is probably mainly derived from the phosphatidylcholine delivered in bile. The total mean uptake of plasma unesterified choline by the liver of blood haemoglobin type A and B chronically catheterized sheep are 21.7 and 15.1 mmol per day respectively [calculated from the equation of Bergman & Wolff (1971) as shown in Tables 3.9 and 3.10] and are almost equal to the mean productions of unesterified choline by the alimentary tract of the animals. Thus there appears to be considerable reabsorption and enterohepatic recirculation of the unesterified choline moiety of bile phosphatidylcholine from the intestine of the sheep. Presumably the unesterified choline taken up by the sheep liver is mainly reutilized for bile phosphatidylcholine synthesis by the CDP-choline and base-exchange pathways. In non-ruminant species less than 10% of the choline moiety of bile lipid choline that enters the enterohepatic circulation is reutilized for bile phosphatidylcholine synthesis in the liver (Saunders, 1970; Robins, 1975). This explains the dependence of bile phosphatidylcholine synthesis and secretion in rat liver on dietary choline (Robins, 1974; Robins & Armstrong, 1976).

It may be calculated from the data of Harrison & Leat (1972), Leat & Harrison (1974) and Christie (1978) that in sheep 3-7 mmol of phosphatidylcholine is transported in thoracic duct lymph per day,

most of which is derived from bile delivered to the intestinal mucosa (Leat & Harrison, 1974, 1977, 1984; Noble, 1978). The total choline output by the sheep intestine into the lymph and enterohepatic circulation adequately accounts for the choline from bile and dietary sources. It is possible that some of the choline in the lymph and enterohepatic circulation is synthesized by the methylation pathway in the intestine of sheep. Administration of [^{14}C] ethanolamine to sheep clearly indicated that the small intestine is an active site for the synthesis of phosphatidylcholine (R.M.C. Dawson, personal communication). In contrast, the synthesis of choline by the methylation pathway is insignificant in the rat small intestine (Bremer & Greenberg, 1961; Wise & Elwyn, 1965; Bjørnstad & Bremer, 1966).

The use of conscious chronically catheterized sheep has provided good evidence that the sheep synthesizes substantial amounts of choline in other tissues besides the liver and has the potential for the efficient retention and recycling of bile choline in contrast to non-ruminant species. These aspects were subsequently examined in more detail as set out in Chapters 4 and 5.

CHAPTER 4

CHOLINE SYNTHESIS BY THE METHYLATION OF PHOSPHATIDYLETHANOLAMINE TO PHOSPHATIDYLCHOLINE IN SHEEP AND RAT TISSUES

4.1 Introduction

Phosphatidylcholine is the major phospholipid in the membranes of mammalian tissues (McMurray, 1973; White, 1973). It is important not only as a structural component of membranes, but also as a potential source of choline molecules. Of the enzymic pathways known to exist in tissues that synthesize phosphatidylcholine, only the methylation of phosphatidylethanolamine to phosphatidylcholine actually generates new choline molecules (Bremer & Greenberg, 1961; Bjørnstad & Bremer, 1966). As described in the literature review, the stepwise methylation of phosphatidylethanolamine to phosphatidylcholine proceeds with the intermediate formation of phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*, *N*-dimethylethanolamine. *S*-adenosyl-L-methionine serves as the immediate methyl donor for the reaction and *S*-adenosyl-L-homocysteine is a second product which is a potent competitive inhibitor. Whether the three successive methyl transfer reactions are catalysed by more than one phosphatidylethanolamine methyltransferase enzyme in mammalian tissues is a controversial issue, but it is generally accepted that the first methylation step is rate-limiting (Schneider & Vance, 1979; Hirata & Axelrod, 1980; Vance *et al.*, 1982; Mato & Alemany, 1983; Audubert & Vance, 1983; Pajares *et al.*, 1984; Mato *et al.*, 1984). The entire methylation pathway in tissues can be assayed *in vitro* using a high pH and a high concentration of radioactive *S*-adenosyl-

L-methionine (Bremer & Greenberg, 1961; Hirata *et al.*, 1978; Hoffman & Cornatzer, 1981; Sastry *et al.*, 1981).

The use of a conscious chronically catheterized sheep preparation in Chapter 3 showed that in this species there is a substantial production of plasma unesterified choline by the alimentary tract and the upper- and lower-body regions which is predominantly supplied by skeletal muscle and supplemented by the brain and heart. Conversely, in rats there is insignificant plasma unesterified choline production by the lower-body (results in Chapter 3). The sheep liver is less efficient at synthesizing choline by the methylation pathway than is rat liver (Bremer & Greenberg, 1961; Henderson, 1978; Neill *et al.*, 1979), and calculation in Chapter 3 using the data of Neill *et al.* (1979) and Dawson *et al.* (1981) revealed that hepatic synthesis alone is insufficient to maintain the endogenous choline body pool of sheep. Collectively, these findings strongly implied that the extrahepatic tissues of the sheep are capable of appreciable choline synthesis by the methylation pathway in contrast to the rat in which synthesis is confined primarily to the liver and is localized in the microsomal fraction (Bremer & Greenberg, 1961; Bjørnstad & Bremer, 1966; Skurdal & Cornatzer, 1975). The aim of the work in this Chapter was to investigate whether corresponding sheep and rat tissues do indeed have different capacities to synthesize choline. This involved measuring the overall methylation of endogenous phosphatidylethanolamine to phosphatidylcholine in tissue fractions of the two species using *S*-adenosyl-L-[*methyl*-^{14}C] methionine as the methyl donor.

4.2 Methods and Materials

4.2.1 Animals

Animals were obtained from The Waite Agricultural Research Institute, The University of Adelaide.

The sheep used were 1 year old Merino ewes (*Ovis aries*), weighing 35-40kg. They were kept indoors in pens and given water *ad libitum* and a daily ration (09.00h) of 1kg of chaffed lucerne hay.

The rats used were adult Hooded Wistar females (*Rattus norvegicus*), weighing 200-250g. They were housed in wire cages indoors and given water and a pelleted rat diet (Charlicks, Adelaide, S.Aust., Australia) *ad libitum*.

4.2.2 Collection of tissue samples

Sheep were slaughtered by severing their necks and rats were killed by cervical dislocation and exsanguination. Tissue samples were rapidly excised and placed in ice-cold 0.25 M-sucrose/10 mM-Tris-HCl buffer/1.0 mM - $MgCl_2$ (pH 7.4). Various segments of the alimentary tract were initially irrigated with the ice-cold medium to remove digesta contamination. In some instances the alimentary tract tissue was cut longitudinally and placed on an ice-cold glass plate and the inner epithelium collected into the medium by gentle scraping with a wooden spatula (Hübscher *et al.*, 1965). Brain and bone marrow samples were removed from surrounding bone using a cold chisel and hammer.

4.2.3 Preparation of tissue homogenates and subcellular fractions

Tissue homogenates were prepared and the subcellular fractions separated by differential centrifugation by modification of the methods of Hoffman *et al*. (1981*a*) and Sastry *et al*. (1981).

Tissue samples (6g) were minced with scissors in 30ml of ice-cold 0.25M-sucrose/10 mM-Tris-HCl buffer/1.0 mM-$MgCl_2$ (pH 7.4) and then homogenized for 1min using a Polytron Type PT 10 20350D tissue homogenizer and sonicator fitted with a PCU-2 speed control on setting 8 (Kinematica, G.m.b.H., Luzern, Switzerland). The homogenate was filtered through 4 layers of gauze to remove cellular debris and then diluted with 18ml of ice-cold homogenizing medium.

The nuclear fraction was sedimented by centrifugation of the total homogenate at 1,000g for 10min. The postnuclear supernatant was centrifuged at 12,000g for 20min to sediment the mitochondrial fraction. The microsomal and supernatant (cytosolic) fractions were prepared by centrifugation of the postmitochondrial supernatant at 95,000g for 70min. All centrifugations were performed at 4°C. The nuclear, mitochondrial and microsomal pellets were resuspended in 2-5ml of ice-cold 0.25M-sucrose/10 mM-Tris-HCl buffer/1.0 mM-$MgCl_2$ (pH 7.4) using a glass-Teflon hand homogenizer (size A) (Arthur H. Thomas Co., Philadelphia, U.S.A.). The tissue homogenates and subcellular fractions were stored at -15°C. Phospholipid methylation activity remained stable for several weeks under such storage conditions. However, upon repeated freezing and thawing about one-third of the activity was lost.

Most of the studies on the methylation of phosphatidylethanola-

mine to phosphatidylcholine were carried out using the microsomal fraction of tissues. The integrity of the microsomal fraction was established by determining the activity of the microsomal marker enzyme NADPH-cytochrome *C* reductase (rotenone-insensitive) in the subcellular fractions. This was measured essentially by the spectrophotometric assay described by Sottocasa *et al*. (1967). The reaction mixture consisted of 50 mM-sodium phosphate buffer (pH 7.4) containing 0.25% Triton X-100, 0.1 mM-cytochrome C, 0.3 mM-KCN, 2µM-rotenone, 20-100µl of the subcellular fraction (0.2 - 2.0mg protein) and 0.1 mM-NADPH in a final volume of 1ml. The reaction was started by the addition of NADPH and the rate of increase of absorbance at 550nm monitored in a semi-micro cuvette using a Zeiss PMQII spectrophotometer fitted with an automatic sample changer and transmittance extinction converter (TE) (Carl Zeiss, Oberkocken, Germany) connected to a Rikadenki model B16 chart recorder (Rikadenki Kogyo Co. Ltd., Tokyo, Japan). The instrument was fitted with a temperature-controlled cuvette-holder and the temperature was maintained at 30°C. In all cases the marker enzyme activity was greater in the microsomal fraction compared to the nuclear, mitochondrial and cytosolic fractions.

4.2.4 Determination of protein

The protein concentration of tissue homogenates and subcellular fractions was determined in duplicate as described in Chapter 2 (2.2.4).

4.2.5 Determination of phospholipid methylation

The overall methylation of endogenous phosphatidylethanolamine to phosphatidylcholine in tissue homogenates and subcellular

fractions was assayed by measuring the incorporation of the [^{14}C] methyl group from *S*-adenosyl-L-[*methyl*-^{14}C] methionine into *N*-methylated phospholipid derivatives of phosphatidylethanolamine. The procedure adopted was a modification of the methods described by Hoffman & Cornatzer (1981) and Sastry *et al.* (1981) using a high pH and a high concentration of radioactive *S*-adenosyl-L-methionine. Some of the general recommendations of Audubert & Vance (1983) were followed.

The incubation medium, in a 10ml stoppered plastic centrifuge tube (Disposable Products Pty. Ltd., Adelaide, S.Aust., Australia), contained 125 mM-Tris/HCl buffer (pH 9.3), 10 mM-$MgCl_2$, 0.1 mM-EDTA, 0.2 mM-*S*-adenosyl-L-[*methyl*-^{14}C] methionine (0.9mCi/mmol) and tissue extract (1-4mg of protein) in a total volume of 1.4ml. The components of the incubation mixture were added as 1ml of Tris/HCl buffer containing $MgCl_2$ and EDTA, 300µl of tissue extract and 100µl of *S*-adenosyl-L-[*methyl*-^{14}C] methionine. No exogenous phosphatidylethanolamine was included in the assay medium because there are saturating levels of this substrate in tissue fractions (Bremer, 1969; White, 1973). In addition there is the possible complication of metabolic heterogeneity among molecular species of phosphatidylethanolamine as substrate for phospholipid methylation (Sundler & Åkesson, 1975*b*; Åkesson, 1983). The reaction was initiated by the addition of radioactive *S*-adenosyl-L-methionine and the mixture incubated at 37°C for 30min. The reaction was stopped by adding 0.15ml of HCl (11M). The radioactive phospholipid products were extracted by the addition of 4.25ml of methanol/chloroform (2.5:1, v/v)

followed by 1.5ml of water and 1.5ml of chloroform with intermediate mixing. The chloroform and methanol solvents contained the antioxidant 2,6-di-*tert*-butyl-4-methylphenol (50mg/l). The biphasic extract was shaken vigorously for 10min and then centrifuged at 1,000g for 15min. After the upper aqueous phase was aspirated and discarded, a 2ml portion of the lower chloroform phase was transferred to a polyethylene scintillation vial (Packard Instrument Co. Inc., Downers Grove, Il., U.S.A.) and evaporated to dryness in a water bath at 80°C for 1h. The residue was redissolved in 3.5ml of scintillation fluid [7g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(4-methyl-5-phenyl-2-oxazolyl) benzene per litre of toluene and Triton X-100 (2:1, v/v)], and the radioactivity determined by counting for 10min in a Packard TRI-CARB 460 CD liquid scintillation system (Packard Instrument Co. Inc., Downers Grove, Il., U.S.A.) which automatically corrected for quenching by the sample channels ratio technique. Heated tissue extract (100°C for 10min) was used as a blank which was run in parallel with the sample tissue extract. All tissue extracts were assayed in duplicate. Some modifications to this standard procedure were used and are described in the legends to tables and figures in the results section. Total phospholipid methylation in tissue extracts are expressed as pmol of [^{14}C] methyl groups incorporated into phospholipids/mg protein/min.

4.2.6 Identification of the products of phospholipid methylation

The [^{14}C] methylated phospholipid reaction products were identified by quantitative thin-layer chromatography. A portion (2ml) of the chloroform phase was evaporated to dryness under N_2 in

a 1ml plastic centrifuge tube (Eppendorf, Hamburg, Germany). Unlabelled carrier standards (50µg/5µl of chloroform) of phosphatidylethanolamine, phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*, *N*-dimethylethanolamine, phosphatidylcholine, lysophosphatidylcholine and sphingomyelin were also added to the tube and taken to dryness under N_2. The residue was redissolved in 50µl of chloroform and quantitatively applied on a 0.2mm x 20cm x 20cm pre-coated silica gel 60 thin-layer chromatography plate (E. Merck, Darmstadt, Germany) that had previously been chromatographically washed with diethyl ether. The plastic sample tube was washed twice with 25µl of chloroform and each washing was again applied to the plate. The chromatogram was developed in the solvent system chloroform/propionic acid/*n*-propyl alcohol/water (3:2:6:1, by vol.). Phospholipid standards (50µg) were run concomitantly. After the plate was air-dried, the phospholipid strips were located with I_2 vapour and scraped into 10ml stoppered plastic centrifuge tubes. The phospholipids were eluted from the silica gel by adding 4ml of chloroform/methanol (1:1, v/v) and thoroughly shaking the tubes periodically over the course of 3h. The tubes were then centrifuged at 1,000g for 15min and 2ml portions of the supernatants were evaporated to dryness in scintillation vials in a water bath at 80°C for 1h. The residues were redissolved in 3.5ml of scintillation fluid and the radioactivity determined as above.

To establish whether the radioactivity found in the phosphatidylcholine extract was actually present as [^{14}C] choline, a 1-2ml portion was dried under N_2 and the residue hydrolysed with 3ml of 6M-HCl at 110°C for 24h in a sealed test tube. The hydrolsate was taken to dryness *in vacuo* at 40°C, redissolved in 2ml of water, and then extracted 3 times with 1ml of diethyl ether to remove free fatty acid

contamination. The extract was concentrated and applied on a silica gel 60 thin-layer chromatography plate along with 20µg of standard choline chloride. The plate was developed in the solvent system methanol/acetone/11M-HCl (45:5:2, by vol.). After the plate was air-dried, the choline spot was located with I_2 vapour, scraped off, and eluted from the silica gel with 3ml of methanol. A 2ml portion was evaporated in a scintillation vial in a water-bath at 80°C for 1h and the radioactivity measured after the addition of 3.5ml of scintillation fluid.

4.2.7 Chemicals

S-adenosyl-L-[*methyl*-^{14}C] methionine (specific radioactivity 59mCi/mmol and radiochemical purity 98%) was purchased from Amersham Australia Pty. Ltd., Sydney, N.S.W., Australia. *S*-Adenosyl-L-methionine (chloride salt, grade II) used to dilute *S*-adenosyl-L-[*methyl*-^{14}C] methionine, along with *S*-adenosyl-L-homocysteine, L-α-phosphatidylethanolamine (type IV, from soybean), L-α-phosphatidylcholine (type V-E, from egg yolk), sphingomyelin (from bovine brain), L-α-lysophosphatidylcholine (type I, from egg yolk), choline chloride (3 x crystallized), bovine albumin (fraction V, powder), cytochrome C (type II-A, from horse heart), β-NADPH (tetrasodium salt, type I) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. L-α-phosphatidyl-*N*, *N*-dimethylethanolamine and 2,5-diphenyloxazole were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England. Other chemicals were obtained as follows: rotenone from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.; 1,4-bis-(4-methyl-5-phenyl-2-oxazolyl) benzene from Packard Instrument Co. Inc., Downers Grove, Il., U.S.A.; 2,6-di-*tert*-butyl-4-methylphenol from Calbiochem, Los Angeles,

Calif., U.S.A.; and L-α-phosphatidyl-*N*-monomethylethanolamine dipalmitoyl from Calbiochem-Behring Corp., La Jolla, Calif., U.S.A.

All other materials were reagent-grade chemicals or the best commercially available grade. Glass double-distilled water was used throughout.

4.3 Results

4.3.1 Products of phospholipid methylation in sheep and rat tissue fractions

The [^{14}C] methylated phospholipids formed by the methylation of endogenous phosphatidylethanolamine in sheep and rat tissue fractions with *S*-adenosyl-L-[*methyl*-^{14}C] methionine were identified by quantitative thin-layer chromatography of the assay chloroform phase. The [^{14}C] methylated phospholipid products accounted for approximately 90% of the total radioactivity in the assay chloroform phase, of which about 88%, 10% and 2% was associated with phosphatidylcholine, phosphatidyldimethylethanolamine and phosphatidylmonomethylethanolamine respectively. The radioactivity in phosphatidylcholine was specifically associated with the choline portion of the molecule. There was negligible radioactivity present in phosphatidylethanolamine, lysophosphatidylcholine or sphingomyelin. Phosphatidylcholine was clearly the major product of endogenous phosphatidylethanolamine methylation in tissue extracts using a high pH (9.3) and a high concentration of radioactive *S*-adenosyl-L-methionine (200μM) in the incubation medium and is consistent with the findings of others (Hirata *et al.*, 1978; Sastry *et al.*, 1981; Hoffman & Cornatzer, 1981). Considerable amounts of phosphatidylmonomethylethanolamine

and phosphatidyldimethylethanolamine are formed as reaction products when tissue fractions are incubated at an intermediate pH with a low concentration of radioactive *S*-adenosyl-L-methionine (Hirata *et al.*, 1978; Sastry *et al.*, 1981). In tissues there are negligible amounts of endogenous phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine (Bremer & Greenberg, 1961; Lester & White, 1967; Katyal & Lombardi, 1976) and are formed as intermediates during the course of the reaction.

The overall methylation of phosphatidylethanolamine to phosphatidylcholine measured in tissue extracts did not account for possible breakdown of newly synthesized phosphatidylcholine to water-soluble forms of choline (Blusztajn & Wurtman, 1981) or for minor interference due to the methylation of lipids besides phosphatidylethanolamine using *S*-adenosyl-L-methionine as the methyl donor (Mogelson & Sobel, 1981; Zatz *et al.*, 1981, 1982; Alemany *et al.*, 1982).

4.3.2 Distribution of phospholipid methylation in sheep and rat tissue fractions

The distribution of phosphatidylethanolamine methylation to phosphatidylcholine in liver and kidney fractions of sheep and rats is shown in Table 4.1. Phospholipid methylation was highest in the microsomal fraction of the sheep and rat tissues, although substantial amounts were also present in the mitochondrial and nuclear fractions. Phospholipid methylation was not detectable in the cytosolic fraction of any of the tissues examined. This pattern of phospholipid methylation is in accordance with that previously reported by others for tissues (Bremer & Greenberg, 1960; Skurdal & Cornatzer, 1975; Hirata *et al.*, 1978; Sastry *et al.*, 1981). The

Table 4.1: Distribution of phospholipid methylation in liver and kidney fractions of sheep and rats

Tissues were collected and homogenates and subcellular fractions prepared as described in Sections 4.2.2 and 4.2.3. Phospholipid methylation in tissue fractions was assayed in duplicate as described in Section 4.2.5. Tissue fraction protein was determined in duplicate as described in Section 4.2.4. The values shown are means ± S.E.M. for two animals of each species. Abbreviation used: N.D. = not detectable.

Tissue fraction	Phospholipid methylation (pmol [^{14}C] methyl groups incorporated/mg protein/min)			
	Sheep		Rat	
	Liver	Kidney	Liver	Kidney
Homogenate	6.5±0.1	1.3±0.1	30.0±0.7	0.8±0.1
Nuclear	2.9±0.4	1.8±0.4	32.8±0.4	0.6±0.1
Mitochondrial	7.0±2.5	2.2±0.1	44.7±0.3	1.0±0.1
Microsomal	19.3±4.4	3.8±0.2	64.1±0.1	1.5±0.1
Cytosolic	N.D.	N.D.	N.D.	N.D.

microsomal fraction of sheep and rat tissues was used in subsequent investigations because of its high capacity for phospholipid methylation.

4.3.3 Effect of microsomal protein on phospholipid methylation in sheep and rat tissue microsomal fractions

Figure 4.1 shows that the methylation of phosphatidylethanolamine to phosphatidylcholine in sheep and rat liver microsomal fractions was linear with up to 5mg of microsomal protein using pH 9.3 and 200μM-*S*-adenosyl-L-methionine in the incubation medium. A microsomal protein content of 1-4mg was used in the subsequent assay of phospholipid methylation in the microsomal fraction of various sheep and rat tissues to ensure linearity.

4.3.4 Time course of phospholipid methylation in sheep and rat tissue microsomal fractions

The methylation of phosphatidylethanolamine to phosphatidylcholine in sheep and rat liver microsomal fractions was linear with time up to about 35min in the presence of 200μM-*S*-adenosyl-L-methionine at pH 9.3 (Figure 4.2). A 30min incubation time was used in the assay of phospholipid methylation in the microsomal fraction of various sheep and rat tissues to ensure linearity.

4.3.5 Effect of pH on phospholipid methylation in sheep and rat tissue microsomal fractions

Figure 4.3 indicates that the overall methylation of phosphatidylethanolamine to phosphatidylcholine in sheep and rat liver microsomal fractions has a pH optimum of about 10 using 200μM-

Figure 4.1: Phospholipid methylation in sheep and rat liver microsomal fractions as a function of microsomal protein

Livers were collected and the microsomal fractions prepared as described in Sections 4.2.2 and 4.2.3. Phospholipid methylation in liver microsomal fractions was assayed in duplicate as described in Section 4.2.5 using microsomal protein contents of 0.5 - 5.0 mg in the incubation medium. Microsomal protein was determined in duplicate as described in Section 4.2.4. The data points are for one animal of each species.

Key to the figure:

● = sheep liver microsomal fraction

o = rat liver microsomal fraction

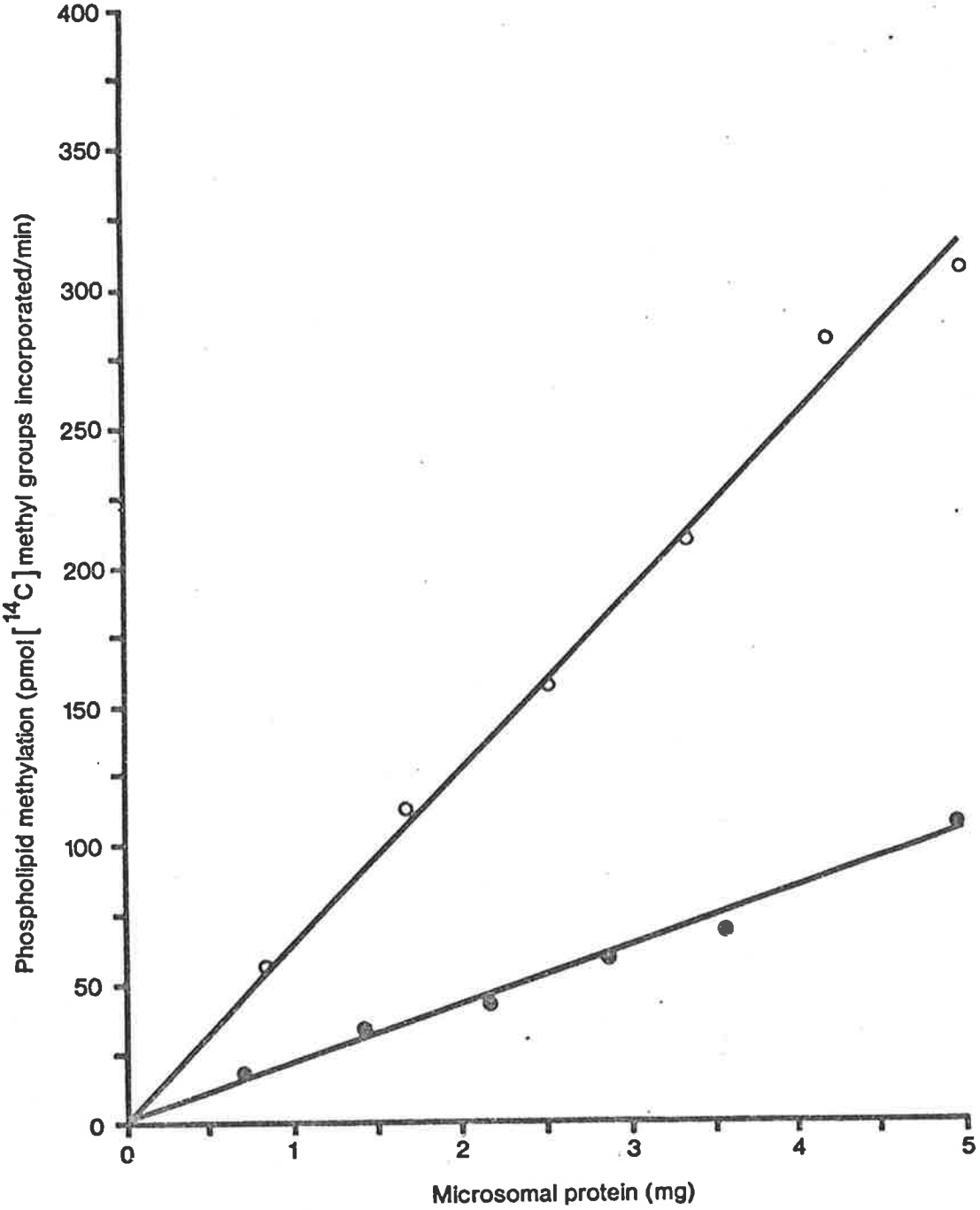

400
350
300
250
200
150
100
50
0
0
1
2
3
4
5
Phospholipid methylation (pmol [14C] methyl groups incorporated/min)
Microsomal protein (mg)

Figure 4.2: Phospholipid methylation in sheep and rat liver microsomal fractions as a function of time

Livers were collected and the microsomal fractions prepared as described in Sections 4.2.2 and 4.2.3. Phospholipid methylation in liver microsomal fractions was assayed in duplicate as described in Section 4.2.5 using incubation times of 10 - 60min. Microsomal protein was determined in duplicate as described in Section 4.2.4. The data points are for one animal of each species.

Key to the figure:

● = sheep liver microsomal fraction

o = rat liver microsomal fraction

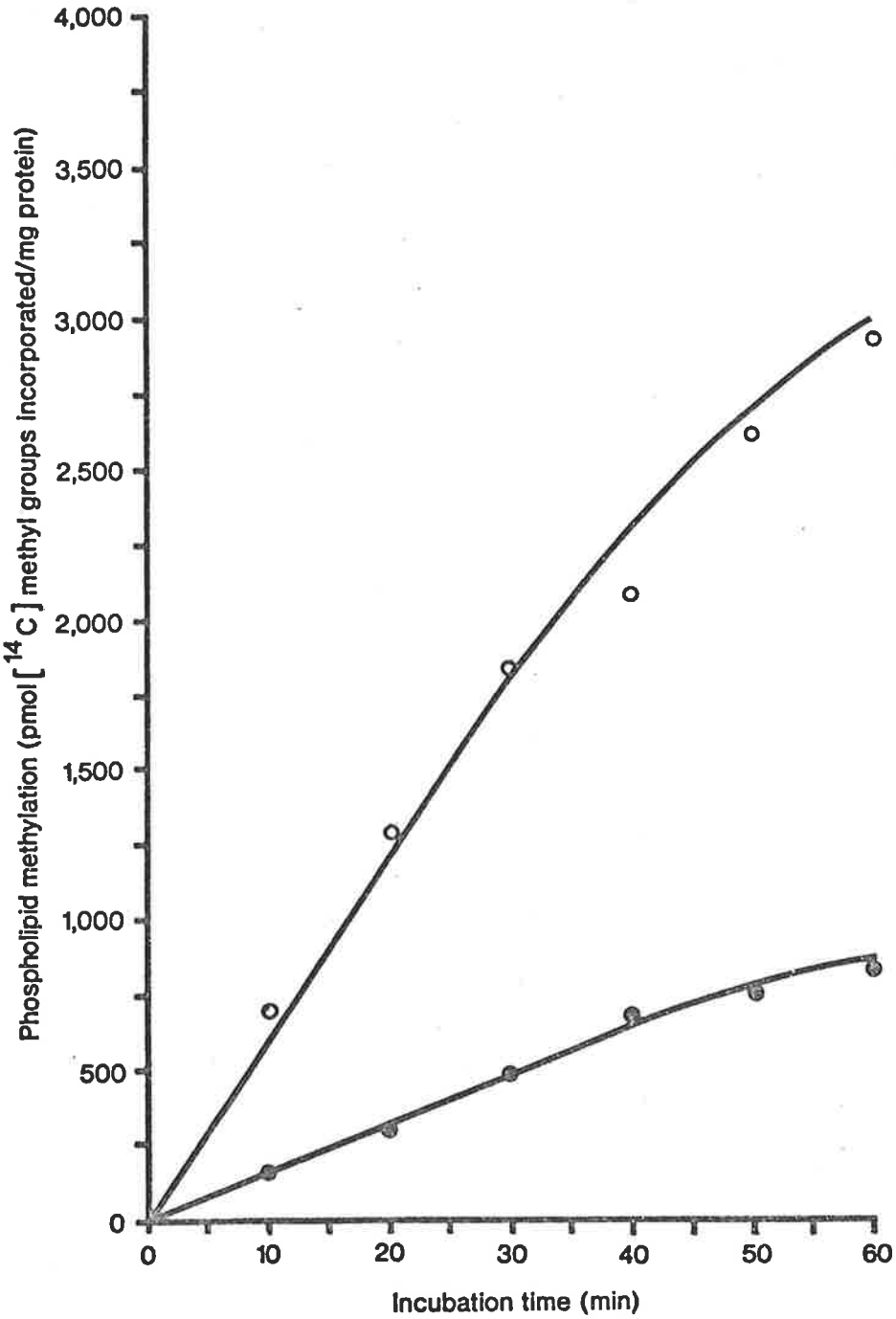

4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
Phospholipid methylation (pmol [14C] methyl groups incorporated/mg protein)
0
10
20
30
40
50
60
Incubation time (min)

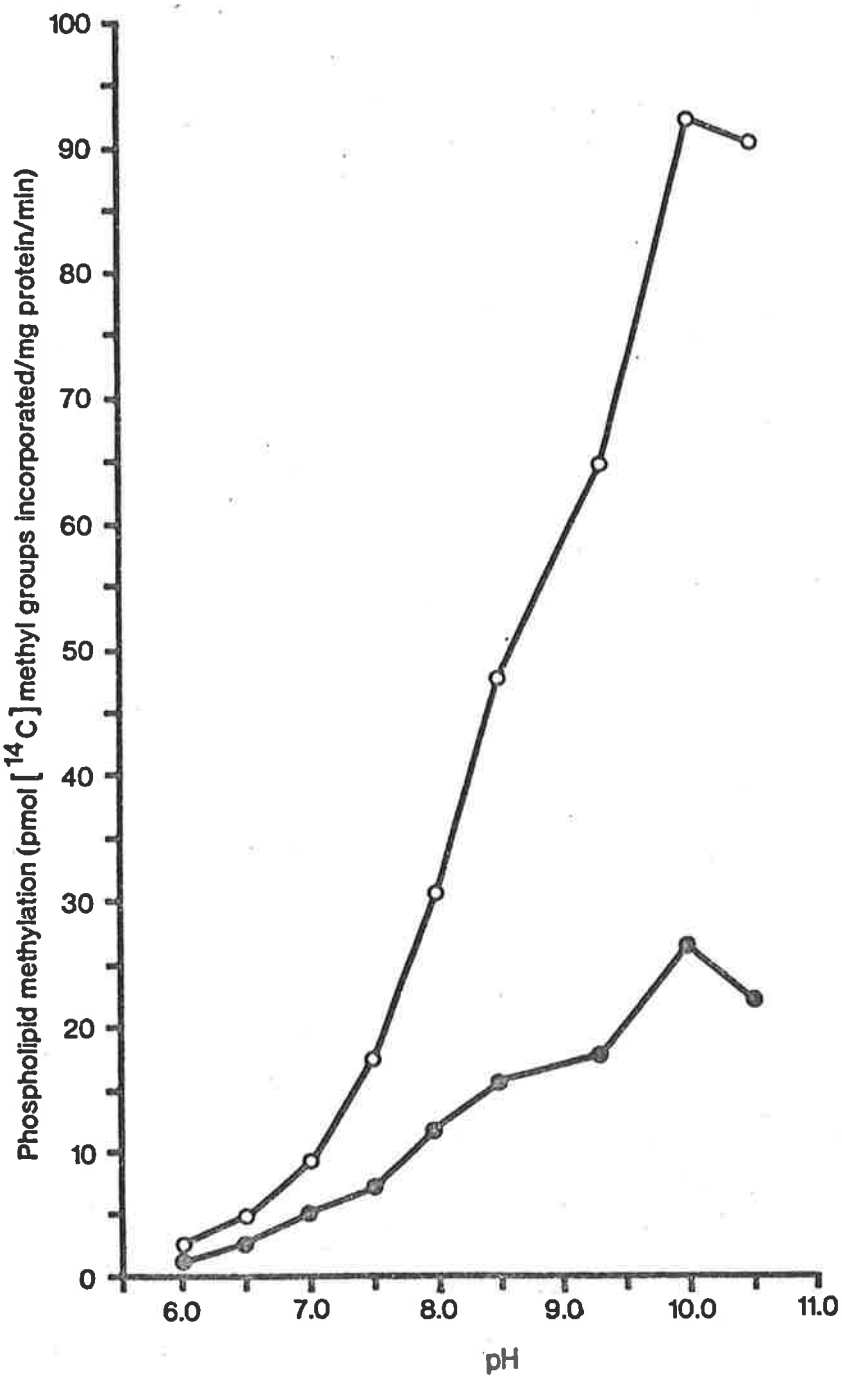
Figure 4.3: Phospholipid methylation in sheep and rat liver microsomal fractions as a function of pH

Livers were collected and the microsomal fractions prepared as described in Sections 4.2.2 and 4.2.3. Phospholipid methylation in liver microsomal fractions was assayed in duplicate as described in Section 4.2.5 using buffers at pH 6.0 - 10.5 in the incubation medium. The buffers used were:
125mM-sodium phosphate for pH 6.0 - 6.5,
125mM-Tris/HCl for pH 7.0 - 9.5, and
125mM-glycine/NaOH for pH 10.0 - 10.5.
Microsomal protein was determined in duplicate as described in Section 4.2.4. The data points are for one animal of each species.

Key to the figure:

● = sheep liver microsomal fraction

o = rat liver microsomal fraction


Phospholipid methylation (pmol [14C] methyl groups incorporated/mg protein/min)
100
90
80
70
60
50
40
30
20
10
0
6.0
7.0
8.0
9.0
10.0
11.0
pH

S-adenosyl-L-methionine in the incubation medium. A pH optimum of approximately 10 for phosphatidylcholine synthesis by the methylation of phosphatidylethanolamine was previously observed in rat liver microsomes (Bremer & Greenberg, 1961; Hoffman & Cornatzer, 1981; Sastry *et al.*, 1981). Audubert & Vance (1983) found that the three intermediate methylation steps of the overall reaction each had a pH optimum of 10 - 10.5 in this tissue subcellular fraction. This high pH optimum of the methylation pathway is a true optimal pH for the enzyme activity and not due to alkaline degradation of *S*-adenosyl-L-methionine or enzyme denaturation (Audubert & Vance, 1983). Phospholipid methylation was assayed at pH 9.3 in the microsomal fraction of various sheep and rat tissues as a compromise between the low methylation at physiological pH and the slight degradative effects on *S*-adenosyl-L-methionine at the higher pH.

4.3.6 Dependence of phospholipid methylation in sheep and rat tissue microsomal fractions on *S*-adenosyl-L-methionine

The overall methylation of phosphatidylethanolamine to phosphatidylcholine in sheep and rat liver microsomal fractions was highly dependent on the concentration of the methyl donor *S*-adenosyl-L-methionine up to approximately 50μM, as shown in Figure 4.4. The Vmax ± S.E. (pH 9.3) of phospholipid methylation using *S*-adenosyl-L-methionine was about 4 times higher in the microsomal fraction of rat liver than of sheep liver, being 72.1 ± 3.1 and 19.2 ± 0.8 pmol [^{14}C] methyl groups incorporated into phospholipids/mg protein/min respectively. The apparent Km ± S.E. (pH 9.3) of phospholipid methylation for *S*-adenosyl-L-methionine was of the same order of magnitude in sheep and rat liver microsomal fractions, being 20.9 ±

Figure 4.4: Phospholipid methylation in sheep and rat liver microsomal fractions as a function of *S*-adenosyl-L-methionine concentration

Livers were collected and the microsomal fractions prepared as described in Sections 4.2.2 and 4.2.3. Phospholipid methylation in liver microsomal fractions was assayed in duplicate as described in Section 4.2.5 using *S*-adenosyl-L-methionine concentrations of 5-400 μM in the incubation medium. Microsomal protein was determined in duplicate as described in Section 4.2.4. The data points are for one animal of each species. Kinetic parameter estimates for each species were calculated using the iterative Gauss-Newton method to provide a least-squares fit of the data directly to the non-linear form of the Michaelis-Menten equation (Cleland, 1967). The analyses were conducted on a Hewlett-Packard 9825A computer (Hewlett-Packard Co., Fort Collins, Colorado, U.S.A.).

Key to the figure:

● = sheep liver microsomal fraction

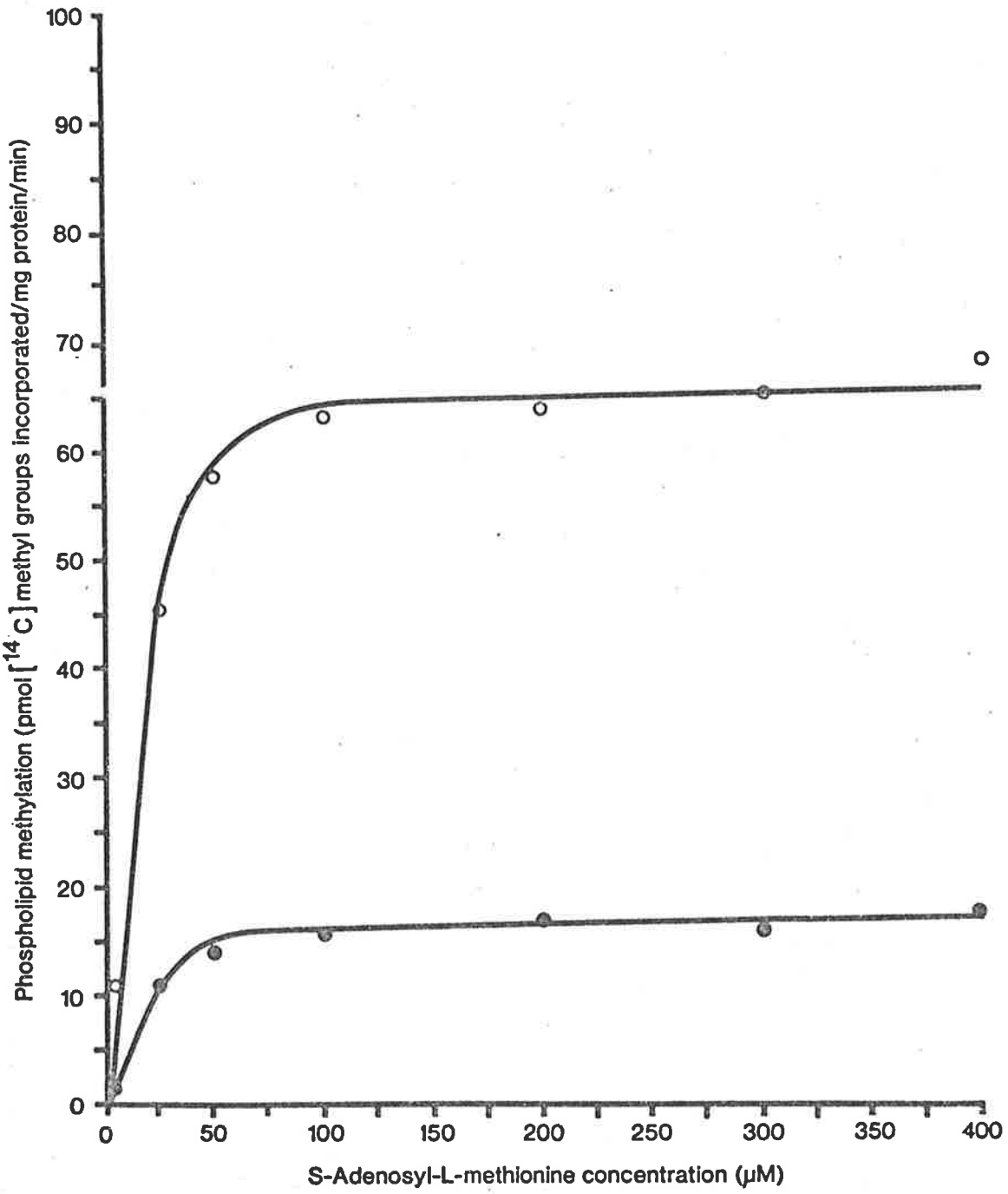
Apparent K_m ± S.E. (pH 9.3) = 20.9 ± 4.1 μM.

V_{max} ± S.E. (pH 9.3) = 19.2 ± 0.8 pmol [^{14}C] methyl groups incorporated into phospholipids/mg protein/min.

o = rat liver microsomal fraction

Apparent K_m ± S.E. (pH 9.3) = 16.4 ± 3.5 μM.

V_{max} ± S.E. (pH 9.3) = 72.1 ± 3.1 pmol [^{14}C] methyl groups incorporated into phospholipids/mg protein/min.


Phospholipid methylation (pmol [14C] methyl groups incorporated/mg protein/min)
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
350
400
S-Adenosyl-L-methionine concentration (μM)

4.1 and 16.4 ± 3.5μM respectively. Apparent Km values of 100, 18.2 and 67μM for *S*-adenosyl-L-methionine have previously been reported for the overall conversion of phosphatidylethanolamine to phosphatidylcholine in rat liver microsomes (Bremer & Greenberg, 1961; Hoffman & Cornatzer, 1981; Sastry *et al.*, 1981). Audubert & Vance (1983) reported that the apparent Km for *S*-adenosyl-L-methionine for the conversion of phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine was 58μM, phosphatidyl-*N*-monomethylethanolamine to phosphatidyl-*N*, *N*-dimethylethanolamine was 65μM, and phosphatidyl-*N*, *N*-dimethylethanolamine was 96μM in rat liver microsomes. The observed Km value of phospholipid methylation for *S*-adenosyl-L-methionine is obviously dependent on the purity of this substrate, the microsomal preparation and other conditions used in the assay. An *S*-adenosyl-L-methionine concentration of 200μM was used in the assay of phospholipid methylation in the microsomal fraction of various sheep and rat tissues to ensure a saturating level of this substrate.

4.3.7 Influence of *S*-adenosyl-L-homocysteine on phospholipid methylation in sheep and rat tissue microsomal fractions

Figure 4.5 shows that the overall conversion of phosphatidylethanolamine to phosphatidylcholine in sheep and rat liver microsomal fractions was decreased by the addition of *S*-adenosyl-L-homocysteine, a known competitive inhibitor of the methylation pathway (Hirata *et al.*, 1978; Schneider & Vance, 1979; Schanche *et al.*, 1981; Hoffman *et al.*, 1981*b*). The concentration of *S*-adenosyl-L-homocysteine required for half-maximal inhibition of phospholipid

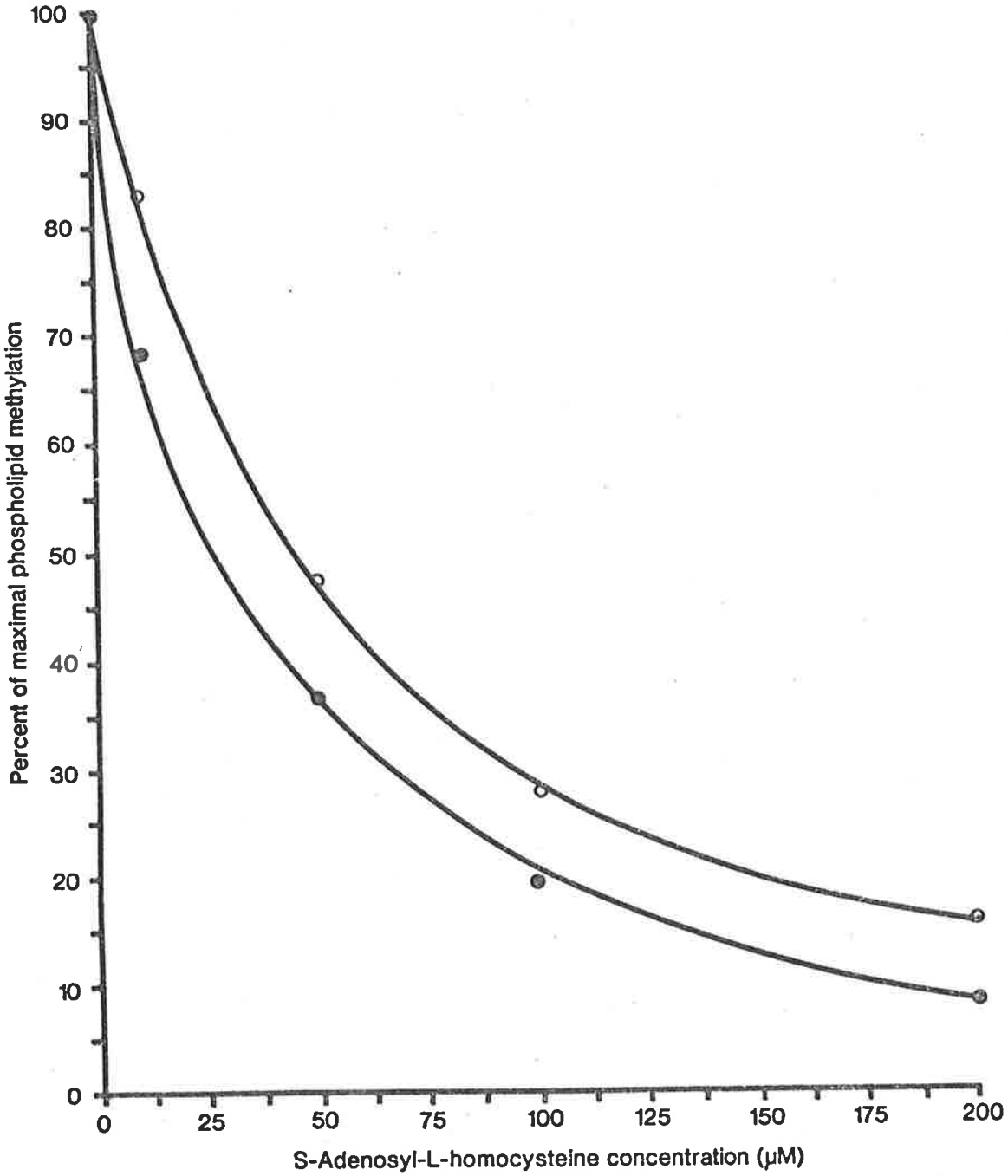
Figure 4.5: Effect of *S*-adenosyl-L-homocysteine on phospholipid methylation in sheep and rat liver microsomal fractions

Livers were collected and the microsomal fractions prepared as described in Sections 4.2.2 and 4.2.3. Phospholipid methylation in liver microsomal fractions was assayed in duplicate as described in Section 4.2.5 with *S*-adenosyl-L-homocysteine concentrations of 0-200μM included in the incubation medium. Microsomal protein was determined in duplicate as described in Section 4.2.4. The data points are for one animal of each species. One hundred percent phospholipid methylation corresponds to 16.8 and 63.8 pmol [^{14}C] methyl groups incorporated into phospholipids/mg protein/min for sheep and rat liver microsomal fractions respectively. The concentration of *S*-adenosyl-L-homocysteine at which 50% inhibition of phospholipid methylation occurs ($[I]_{50\%}$) in the presence of 200μM *S*-adenosyl-L-methionine at pH 9.3 was read directly from the curve for each species.

Key to the figure:

● = sheep liver microsomal fraction. $[I]_{50\%}$ ~ 26μM

o = rat liver microsomal fraction. $[I]_{50\%}$ ~ 45μM


Percent of maximal phospholipid methylation
100
90
80
70
60
50
40
30
20
10
0
0
25
50
75
100
125
150
175
200
S-Adenosyl-L-homocysteine concentration (μM)

methylation (in the presence of 200μM-*S*-adenosyl-L-methionine at pH 9.3) was approximately 26 and 45μM for sheep and rat liver microsomal fractions respectively. These observations provided further evidence that the assay used for the determination of phospholipid methylation in the microsomal fraction of various tissues was reliable.

4.3.8 Comparison of phospholipid methylation in sheep and rat tissue microsomal fractions

The overall methylation of endogenous phosphatidylethanolamine to phosphatidylcholine in the microsomal fraction of various sheep and rat tissues is presented in Table 4.2. In rats phospholipid methylation was many times higher in the microsomal fraction of the liver than in the microsomal fraction of extrahepatic tissues. Phospholipid methylation was slight in rat kidney, lung and stomach microsomal fractions and negligible in rat skeletal muscle, heart, brain, small intestine and large intestine microsomal fractions. The inner epithelial microsomal fraction of the rat stomach, small intestine and large intestine had approximately the same capacity to synthesize phosphatidylcholine as that of the entire wall of these regions. The distribution of phospholipid methylation in rat tissues observed here is in agreement with the findings of others (Bremer & Greenberg, 1961; Bjørnstad & Bremer, 1966; Skurdal & Cornatzer, 1975; Vance & De Kruijff, 1980). In sheep the methylation of phosphatidylethanolamine to phosphatidylcholine was highest in the microsomal fraction of the liver, but was also appreciable in the microsomal fraction of most extrahepatic tissues. Phospholipid methylation was substantial in the microsomal fraction of

Table 4.2: Phospholipid methylation in the microsomal fraction of various sheep and rat tissues

Tissues were collected and the microsomal fractions prepared as described in Sections 4.2.2 and 4.2.3. Phospholipid methylation in tissue microsomal fractions was assayed in duplicate as described in Section 4.2.5. Microsomal protein was determined in duplicate as described in Section 4.2.4. The values shown are means ± S.E.M. for three animals of each species. The significance of the difference between corresponding sheep and rat tissue microsomal fractions, as determined by Student's *t* test, is indicated. Abbreviations used: N.S. = not significant; N.D. = not detectable; - = not determined.

Phospholipid methylation (pmol [^{14}C] methyl groups incorporated/mg protein/min)			
Tissue	Sheep	Rat	Significance
Liver	17.2±1.0	64.0±2.7	$P<0.001$
Kidney	3.4±0.3	1.5±0.5	$P<0.01$
Skeletal muscle (M.biceps femoris)	0.9±0.1	N.D.	
Heart	1.4±0.1	0.4±0.2	$P<0.01$
Lung	4.0±0.3	1.4±0.3	$P<0.02$
Brain	1.1±0.6	0.8±0.3	N.S.
Abomasum or stomach	1.4±0.6*	1.0±0.2*	N.S.
Small intestine	2.7±0.3**	0.3±0.2*	$P<0.01$
Large intestine	2.1±0.1*	0.8±0.3*	$P<0.05$
Rumen	2.6±0.2*	-	
Pancreas	2.3±0.3	-	
Lymph node	2.3±0.2	-	
Bone marrow	N.D.	-	
Adrenal gland	2.0±0.6	-	
Salivary gland	2.3±0.4	-	
Spleen	1.8±0.1	-	
Inferior vena cava	N.D.	-	

* Value is for the whole tissue wall and is similar for the inner epithelium.

** Value is for the whole duodenal wall and is similar for the whole jejunal and ileal walls. The inner epithelium of the regions of the small intestine also had an equivalent value.

sheep lung and kidney and particularly important in that of skeletal muscle and various regions of the alimentary tract if the total tissue mass is considered. Phosphatidylcholine synthesis was similar in the microsomal fraction of the duodenum, jejunum and ileum of the sheep small intestine. The inner epithelial microsomal fraction of various alimentary tract regions had the same capacity for phospholipid methylation as that of the entire tissue wall. The microsomal fraction of sheep spleen, salivary gland, adrenal gland, lymph nodes, pancreas, brain and heart all showed reasonable ability to synthesize phosphatidylcholine in contrast to that of sheep bone marrow and inferior vena cava.

The methylation of phosphatidylethanolamine to phosphatidylcholine was approximately 4 fold higher in the liver microsomal fraction of rats as compared to that of sheep (Table 4.2). Phosphatidylcholine synthesis by the methylation pathway was previously found to be higher in rat liver compared to sheep liver (Bremer & Greenberg, 1961; Neill & Dawson, 1977; Henderson, 1978; Neill *et al.*, 1979). Sheep kidney, skeletal muscle, heart, lung, small intestine and large intestine microsomal fractions showed significantly higher phospholipid methylation than the corresponding rat extrahepatic tissue microsomal fractions (Table 4.2). Phosphatidylcholine synthesis by the methylation pathway was previously found to be higher in the small intestine of sheep than of rats (R.M.C. Dawson, personal communication). Table 4.2 indicates that phospholipid methylation was similar in brain and stomach (abomasum) microsomal fractions of sheep and rats.

4.4 Discussion

In this Chapter the capacity of corresponding sheep and rat tissues to synthesize choline was determined *in vitro* by measuring the overall methylation of endogenous phosphatidylethanolamine to phosphatidylcholine in tissue microsomal fractions using *S*-adenosyl-L-[*methyl*-^{14}C] methionine as the methyl donor. The microsomal fraction of tissues was used because it was found to be the principle intracellular site of phospholipid methylation and offered greater sensitivity and more economic use of labelled substrate than tissue slices, homogenates, isolated cells or whole animal studies. Hirata & Axelrod (1980) claim that the conversion of phosphatidylethanolamine to phosphatidylcholine in animal tissues involves two phosphatidylethanolamine methyltransferase enzymes but this has been severely questioned by some workers (Schneider & Vance, 1979; Vance *et al.*, 1982; Mato & Alemany, 1983; Audubert & Vance, 1983; Pajares *et al.*, 1984; Mato *et al.*, 1984). It was therefore pertinent in the present *in vitro* study to assay the overall methylation reaction rather than the specific enzymes which may or may not be involved. Phospholipid methylation in the microsomal fraction of various sheep and rat tissues was assayed at near optimum pH using a saturating level of *S*-adenosyl-L-[*methyl*-^{14}C] methionine and was linear with incubation time and microsomal protein and inhibited by *S*-adenosyl-L-homocysteine. Phosphatidylcholine was the major phospholipid product and radioactivity was exclusively associated with the choline moiety.

The results indicate that respective tissues of sheep and rats have markedly different capacities to synthesize choline by the

methylation of phosphatidylethanolamine to phosphatidylcholine. In sheep the liver is an important site of choline synthesis but most extrahepatic tissues also have appreciable capacity to synthesize choline. In rats the extrahepatic tissues have negligible capacity to synthesize choline and the liver is clearly the main organ of synthesis. Sheep liver is less effective at synthesizing choline than rat liver, however most sheep extrahepatic tissues have a higher capacity for choline synthesis than those of rats. Choline synthesis by the methylation pathway is particularly significant in sheep skeletal muscle and regions of the alimentary tract on the basis of total tissue mass but this is not so in the corresponding rat tissues. Presumably the substantial choline synthesis in sheep extrahepatic tissues in addition to the liver is sufficient to maintain the balance of the endogenous choline body pool of sheep. Conversely in rats the choline requirements of the body must be maintained by hepatic synthesis and supplemented by the dietary supply of choline. Aliev *et al.* (1980) reported that acetate is an important source for choline synthesis in various sheep tissues, but preliminary investigations found this not to be the case (G.-P. Xue, B.S. Robinson & A.M. Snoswell, unpublished results). Similar to choline synthesis, the hydroxylation of γ-butyrobetaine to carnitine occurs in skeletal muscle, heart, liver and kidney of sheep but is confined to the liver of rats (Erfle, 1975; Costa, 1977), and emphasizes the existence of species differences in the site of biosynthesis of metabolites.

In Chapter 3 it was shown in conscious chronically catheterized sheep that there is significant production of plasma unesterified

choline by the alimentary tract and upper- and lower-body regions drained by the venae cavae which is mainly supplied by skeletal muscle with minor contributions by brain and heart. It was suggested that the source of the unesterified choline was phosphatidylcholine synthesized by the methylation pathway in these tissues that is hydrolysed by phospholipases or undergoes base-exchange. The confirmation that the sheep alimentary tract and skeletal muscle are capable of substantial *de novo* choline synthesis in the present work makes this hypothesis more conceivable. Blusztajn *et al.* (1979) and Blusztajn & Wurtman (1981) reported that rat brain synaptosomal preparations can liberate unesterified choline from the phosphatidylcholine that is generated by the methylation of phosphatidylethanolamine, which is consistent with observations that there is a net efflux of unesterified choline from rat brain (Dross & Kewitz, 1972; Choi *et al.*, 1975; Spanner *et al.*, 1976). Blusztajn & Wurtman (1981) suggested that there may exist specific physical domains of phospholipids within synaptosomal membranes, such that the phosphatidylethanolamine methylation and phosphatidylcholine degradation occur in the same domain. There appears to be several phosphatidylcholine pools in rat brain synaptosomes: one with a half-life of 2 days, one with a half-life of 52.5 days (Pasquini *et al.*, 1973) and another which turns over much more rapidly that is formed by the methylation pathway (Blusztajn & Wurtman, 1981). Zeisel (1985) concluded that phospholipase A activity is the primary initiator of choline release from phosphatidylcholine in rat brain membranes. Similar phenomena may well exist in the microsomal fraction of sheep extrahepatic tissues that produce plasma unesterified choline. This important point obviously requires further investigation. In

Chapter 3 it was shown that the lower-body region drained by the inferior vena cava of rats does not produce plasma unesterified choline in contrast to sheep which probably reflects the inability of rat skeletal muscle to synthesize choline by the methylation pathway.

Recent *in vivo* experiments using intravenously and intramuscularly administered [^{14}C] ethanolamine suggested that sheep skeletal muscle is not a major site of choline synthesis by the methylation of phosphatidylethanolamine to phosphatidylcholine (R.M.C. Dawson, personal communication). This is difficult to reconcile with the observations reported here that sheep skeletal muscle produces substantial amounts of plasma unesterified choline in the whole animal and that sheep skeletal muscle microsomes have the ability to synthesize phosphatidylcholine from endogenous phosphatidylethanolamine *in vitro* using *S*-adenosyl-L-[*methyl*-^{14}C] methionine as the methyl donor. A similar discrepancy exists in the literature for choline synthesis in mammalian brain. Ansell (1973) and Freeman & Jenden (1976) concluded that the brain is unable to synthesize choline *in vivo* on the basis of evidence that it fails to convert intraventricularly administered [^{14}C] ethanolamine to the choline moiety of phosphatidylcholine, and that the fatty acid composition of rat brain phosphatidylethanolamine differs significantly from that of phosphatidylcholine. However, there are reports that there is a net efflux of choline from the brain (Dross & Kewitz, 1972; Aquilonius *et al.*, 1975; Choi *et al.*, 1975; Spanner *et al.*, 1976) and recent *in vitro* evidence that brain membranes can methylate endogenous phosphatidylethanolamine to phosphatidylcholine using

labelled *S*-adenosyl-L-methionine as the methyl donor (Blusztajn *et al.*, 1979; Mozzi & Porcellati, 1979; Crews *et al.*, 1980*a*; Blusztajn & Wurtman, 1981; Fonlupt *et al.*, 1981). Blusztajn *et al.* (1979) suggested that since no data are available concerning the metabolic heterogeneity of brain phosphatidylethanolamine, it is possible that a small phosphatidylethanolamine pool exists which is not formed from free ethanolamine but nevertheless does constitute a substrate for methylation to phosphatidylcholine. As referred to previously, Blusztajn & Wurtman (1981) subsequently demonstrated that brain synaptosomes contain a phosphatidylcholine pool synthesized by the methylation of phosphatidylethanolamine that turns over to free choline much more rapidly than two other phosphatidylcholine pools. There is evidence that the phosphatidylethanolamine involved in the synthesis of phosphatidylcholine and free choline in brain synaptosomes is derived by the decarboxylation of phosphatidylserine which is formed from free serine by a base-exchange pathway (Blusztajn & Wurtman, 1983). Further studies may reveal that this process also exists in the microsomal fraction of sheep skeletal muscle, brain and heart which would explain the failure of *in vivo* experiments involving [^{14}C] ethanolamine to detect choline synthesis by the methylation pathway in these tissues.

Methionine is the first limiting amino acid in the nutrition of sheep (Chalupa, 1972; Barry *et al.*, 1973; Schelling *et al.*, 1973) and its availability for transmethylation reactions is likely to be limited. The synthesis of choline by the methylation of phosphatidylethanolamine to phosphatidylcholine in the sheep liver and extrahepatic tissues would constitute a substantial drain on available tissue

methionine *via* the methyl group donor *S*-adenosyl-L-methionine. Dawson *et al.* (1981) calculated that the sheep must synthesize approximately 17.3 mmol of choline per day in order to maintain the endogenous choline body pool which would use about 52 mmol of methionine per day. The absorption of dietary methionine by the small intestine of sheep is no more than 13 mmol per day (Egan & Macrae, 1979) and is much less than the methionine required daily for choline synthesis plus the other reactions in body tissues. Presumably the tissue methionine is adequately replenished in the normal metabolic state through the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine by methyl B_{12} (methylcobalamin) dependent 5-methyltetrahydrofolate-homocysteine methyltransferase and the transfer of a methyl group from betaine to homocysteine by betaine-homocysteine methyltransferase. Xue & Snoswell (1985) recently found that the total body capacity of betaine-homocysteine methyltransferase for methionine synthesis in sheep is much lower than that in rats on a body weight basis. However the total body capacity for methionine synthesis by 5-methyltetrahydrofolate-homocysteine methyltransferase in sheep is considerably greater than that for rats. In sheep 5-methyltetrahydrofolate-homocysteine methyltransferase plays a significant role in hepatic methionine recycling along with betaine-homocysteine methyltransferase. In contrast, hepatic methionine recycling in the rat is virtually dependent on betaine-homocysteine methyltransferase (Xue & Snoswell, 1985). Finkelstein *et al.* (1982) and Barak & Tuma (1983) recently reported the important function of betaine-homocysteine methyltransferase for methionine synthesis in rat liver. Since sheep receive negligible amounts of dietary choline

(Neill *et al.*, 1979; Dawson *et al.*, 1981; results in Chapter 2) and betaine (Mitchell *et al.*, 1979) and the endogenous choline pool is conserved by slow oxidation to betaine *via* choline oxidase (Neill *et al.*, 1979; Dawson *et al.*, 1981), it is not surprising that betaine-homocysteine methyltransferase plays a minor role in methionine synthesis compared to 5-methyltetrahydrofolate-homocysteine methyltransferase. Presumably the methyl group of 5-methyltetrahydrofolate is derived from one-carbon sources such as formate, formaldehyde, serine, glycine and histidine through tetrahydrofolate intermediaries which are known to exist in sheep (Osborne-White & Smith, 1973; Gawthorne & Smith, 1973). In contrast, the important role of betaine-homocysteine methyltransferase for tissue methionine synthesis in rats is consistent with the high oxidation of choline to betaine (Sidransky & Farber, 1960; Neill *et al.*, 1979; Dawson *et al.*, 1981) and the high dietary choline (and betaine) requirement of this species (Lucas & Ridout, 1967).

CHAPTER 5

THE RETENTION AND REUTILIZATION OF BILE CHOLINE IN SHEEP

5.1 Introduction

The data of Adams & Heath (1963) and Noble (1978) together with the results in Chapter 2 showed that sheep secrete 11-17 mmol of lipid choline per day (mainly as phosphatidylcholine) into the intestinal lumen through bile which originates in the liver. This quantity of lipid choline exported in bile represents approximately 75% of the total lipid choline content of sheep liver (calculated from the data in Chapter 3), and rapid replacement would be required to maintain steady-state conditions. Sheep derive about 0.1 mmol of dietary choline per day (Neill *et al.*, 1979; Dawson *et al.*, 1981; results in Chapter 2) which would make a negligible contribution to the high daily secretion rate of bile phosphatidylcholine. The sheep liver can synthesize about 5 mmol of phosphatidylcholine/day by the methylation of phosphatidylethanolamine (Neill *et al.*, 1979) and even if the majority was used for bile secretion this would be inadequate. In addition, Balint *et al.* (1967) and Treble *et al.* (1970) demonstrated in rat liver that phosphatidylcholine destined for bile is preferentially synthesized from unesterified choline *via* the CDP-choline and base-exchange pathways, rather than by the methylation pathway. The study of the uptake and output of various forms of choline by organs of the conscious chronically catheterized sheep in Chapter 3 showed there was significant production of plasma unesterified choline by the alimentary tract which was approximately

balanced by the plasma unesterified choline taken up by the liver, and in addition was almost equal to the amount of choline secreted in bile. Collectively the results implied that the sheep must have the capacity for extensive retention and recycling of bile choline in order to maintain the daily rate of secretion in bile and the balance of the endogenous choline pool. The work in this Chapter investigated this possibility and involved measuring the amounts of physiologically infused radioactively-labelled choline compounds incorporated into the bile lipid choline of a surgically prepared sheep. Care was taken to sustain the enterohepatic circulation of bile constituents to maintain favourable conditions for bile lipid choline synthesis and secretion by the liver. The results obtained for the sheep are discussed in relation to those previously reported for non-ruminant species.

5.2 Methods and Materials

5.2.1 Animals

A Merino ewe (*Ovis aries*) obtained from the flock of The Waite Agricultural Research Institute, The University of Adelaide, approximately 2 years old and weighing 35-40kg, was acclimatised indoors in a metabolism crate for at least 1 week. It was provided with chaffed lucerne hay and water *ad libitum*. Prior to surgery the animal was starved for 48h.

5.2.2 Surgical preparation and post-operative treatment of sheep

The sheep was surgically prepared with:

(a) a portal vein catheter for the infusion of [^{3}H] un-

esterified choline;

(b) a duodenal catheter for the infusion of [^{3}H] lipid choline bile and the infusion of unlabelled bile in order to replace bile salts and bile choline during continuous bile collection;

(c) a gallbladder catheter and ligated bile duct for the collection of [^{3}H] bile as it was produced by the liver.

Surgery was performed aseptically under general anaesthesia induced with intravenous sodium pentobarbitone (25mg/kg body wt.) and, after endotracheal intubation with a cuffed Magill tube, maintained with a mixture of 0.5 - 2% cyclopropane and balance oxygen using a closed-circuit anaesthetic apparatus (The Medishield Division of The Commonwealth Industrial Gases Ltd., Adelaide, S.Aust., Australia).

The sheep was placed on its left side and the right flank shaved and sterilized with a benzalkonium chloride antiseptic solution. A paracostal incision was made 4-5cm behind the last rib and extending from the sternum to the midflank region. The common bile duct was exposed and ligated between the points of entry of the pancreatic and cystic ducts. A silicone rubber tube (1.98mm i.d., 3.18mm o.d. and 1m in length; "Silastic", Dow Corning Corporation Medical Products, Midland, Mich., U.S.A.) with a side-hole near the tip was inserted about 5cm into the lumen of the gallbladder through a stab incision in the blind-end wall (fundus) and secured with a purse-string suture (medium 0.3mm "Vetafil Bengen" synthetic suture, Messrs. A.E. Stansen and Co., Pty. Ltd., Mt. Waverley, Vic., Australia).

A loop of small intestine was exposed and a silicone rubber tube (0.635mm i.d., 1.19mm o.d. and 1m in length) was inserted about 10cm through a stab incision into a small mesenteric tributary of the portal vein and retained in place with a double knot. The length of the portal vein catheter was cuffed with a polyethylene tube (1.5mm i.d. and 2.5mm o.d.; "Sterivac Cannula", Allen and Hanburys Ltd., London, U.K.) to prevent it from kinking inside the abdominal cavity.

The duodenum was catheterized about 10cm from the pylorus of the abomasum using a similar silicone rubber tube and technique as for the gallbladder.

All catheter tubing was sterilized for at least 1h in a benzalkonium chloride antiseptic solution and rinsed in a sterile 0.9% (w/v) sodium chloride solution containing 50 units of heparin/ml and 3,000 units of procaine penicillin/ml before insertion into respective sites. The free ends of the catheters were exteriorized through stab incisions in the right flank wall and sutured to the skin and the abdominal wound was closed in layers using plain 4-0 surgical gut (Davis and Geck American Cyanamid Company, New York., U.S.A.).

The surgery was completed in 2h and on recovery the sheep was returned to its metabolism crate. The bile draining continuously from the gallbladder catheter was collected into a plastic bottle. Bile was returned to the animal by infusing fresh sheep gallbladder bile collected from the local abbatoirs (South Australian Meat Corporation, Gepps Cross, S.Aust., Australia) *via* the duodenal catheter at a physiological rate of 35ml/h using a peristaltic pump

(Gilson Minipuls 2, Villiers, Le Bel, France). The infused gall-bladder bile had a bile salt concentration of 149 µmol /ml and contained 16.1 µmol /ml of lipid choline and a negligible amount of water-soluble choline. Thus the sheep duodenum received 13.5 mmol of bile lipid choline and 125 mmol of bile salts per day which were physiological levels. Replacement bile was stored as 250ml aliquots at -15°C and warmed to room temperature prior to duodenal infusion.

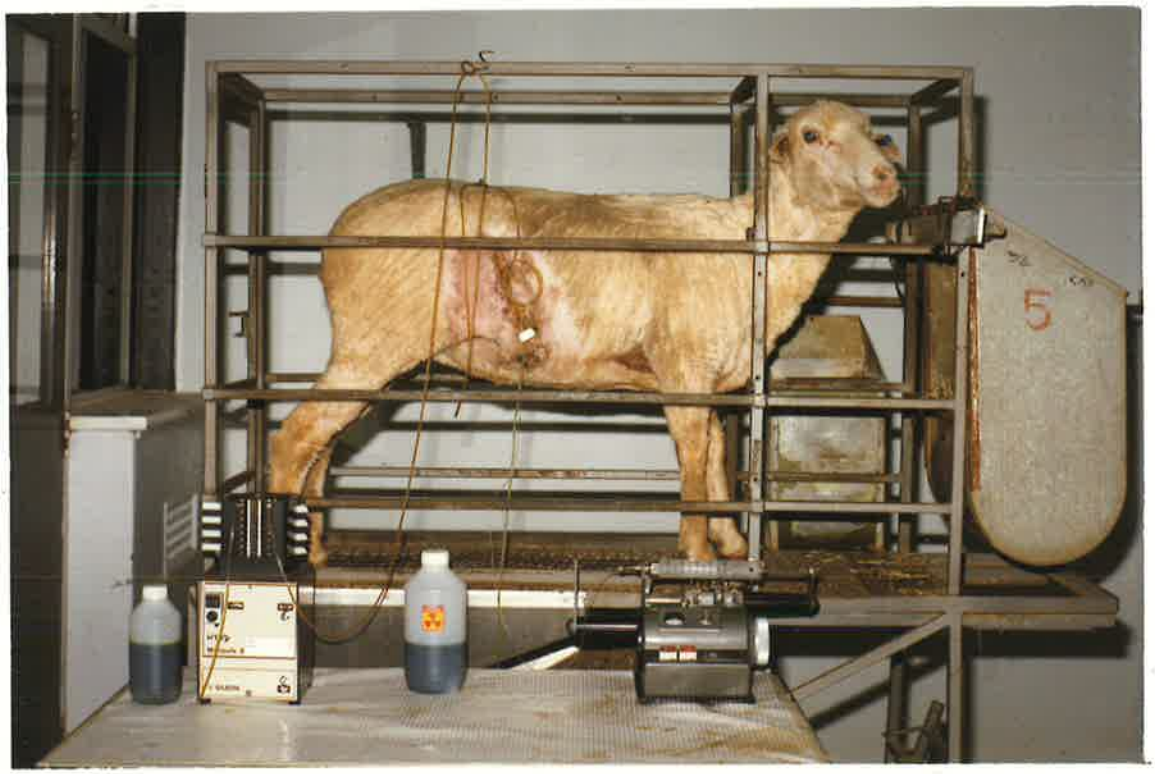
Post-operative care included a 3 day course of procaine penicillin and the application of antiseptic cream to the skin around the points of entry of the catheters to minimize infection. The portal vein catheter was kept patent by flushing twice each day with a sterile isotonic saline solution containing 50 units of heparin/ml and 3,000 units of procaine penicillin/ml. The positions of the three catheters were checked by post-mortem examination and in all cases were found to be correct. The sheep was allowed to stabilize on full feed intake and replacement bile for 3 days post-operatively before the infusion of [^{3}H] choline compounds and subsequent collection of bile samples. The surgically prepared sheep remained functional for 30 days and is shown in Figure 5.1.

5.2.3 Portal vein infusion of [^{3}H] unesterified choline and collection of bile samples

[*methyl*-^{3}H] choline chloride and unlabelled carrier (2mCi; 47µCi/µmol) in 22.5ml of sterile isotonic saline adjusted to pH 7.4 was infused into the sheep *via* the portal vein catheter at a constant rate of 0.375ml/min over 1h using a syringe pump (B. Braun, Apparatebau melsungen, West Germany). The absolute amount of choline chloride

Figure 5.1: The surgically prepared sheep used for the study of retention and reutilization of bile choline

The sheep was fitted with portal vein, duodenal and gallbladder catheters and maintained as described in Sections 5.2.1 and 5.2.2.


5

infused over the 1h period was only 5% of the portal vein return of plasma unesterified choline in sheep (calculated from the data in Chapter 3) to ensure that physiological levels were taken up by the liver. Bile was collected continuously at various intervals for a total period of 120h (5 days), immediately the portal vein infusion commenced. Thereafter spot-samples of bile were collected for analysis every 12h for 12 days. Bile samples were collected from the gallbladder catheter by gravity drainage into plastic bottles on ice and stored at -15°C until analysed. The duodenal infusion of replacement bile was continued throughout the entire experimental procedure to sustain the enterohepatic circulation of bile choline and bile salts to promote normal bile production by the liver.

5.2.4 Duodenal infusion of [^{3}H] lipid choline bile and collection of bile samples

[^{3}H] bile samples collected from the sheep 1-4h after the portal vein infusion of [^{3}H] unesterified choline had the highest specific radioactivity and fractions were pooled to give a total volume of 39ml. The pooled [^{3}H] bile sample contained 12.3 µmol /ml of lipid choline and a negligible amount of water-soluble choline and a bile salt concentration of 43.9 µmol /ml. The total radioactivity was 117µCi with a specific radioactivity of 244µCi/mmol of lipid choline and 99% of the radioactivity was associated with phosphatidylcholine (determined by acid hydrolysis and thin-layer chromatography as described in Section 5.2.5.3). The [^{3}H] lipid choline was specifically of bile origin which has a different fatty acid composition than lipid choline from other sources and is solubilized in micelles with bile salts and lipids (Christie, 1978).

The [^{3}H] lipid choline bile sample was physiologically reinfused into the sheep *via* the duodenal catheter at a constant rate of 0.650ml/min over 1h using a peristaltic pump (Gilson Minipuls 2, Villiers, Le Bel, France) 17 days after the portal vein infusion of [^{3}H] unesterified choline. Bile samples were collected continuously at various intervals from the gallbladder catheter by gravity drainage into plastic bottles on ice and stored at -15°C until analysed. Bile was collected during the 1h infusion period and for a total of 120h (5 days). The infusion of replacement bile into the duodenum of the sheep was maintained during the entire experimental procedure to ensure physiological conditions for hepatic bile synthesis.

5.2.5 Analysis of bile samples

5.2.5.1 Extraction of bile samples

1.5ml portions of bile samples (pH 6.5 - 7.0) were acidified (pH 4.0 - 4.5) with approximately 10µl of 5M-HCl. Acidic bile (1ml) was then extracted with 19ml of ice-cold chloroform/methanol (2:1, v/v) containing the antioxidant 2,6-di-*tert*-butyl-4-methylphenol (50mg/l) followed by 4ml of distilled water in a 35ml plastic centrifuge sample tube (Kayline Plastics, Adelaide, S.Aust., Australia), essentially by the method of Folch *et al*. (1957). The extract was shaken for 5min and then centrifuged at 1,000g for 15min which gave a 10ml water-soluble phase and a 14ml lipid phase.

5.2.5.2 Determination of bile choline

A 1ml portion of the lipid phase of extracted bile samples containing lipid choline (choline phospholipids) was

evaporated to dryness under N_2 and hydrolysed to unesterified choline with 3ml of 6M-HCl at 110°C for 24h in a sealed test tube. The fraction was taken to dryness *in vacuo* at 30-40°C and stored for at least 12h in a vacuum desiccator containing KOH pellets to remove traces of acid. The residue was redissolved in 10ml of 5 mM-Tris/HCl buffer (pH 7.8) and assayed for unesterified choline as described below.

A 7ml portion of the water-soluble phase of extracted bile samples (containing glycerophosphocholine, phosphocholine and unesterified choline) was evaporated to dryness *in vacuo* at 30-40°C and hydrolysed to unesterified choline with 3ml of 3M-HCl at 123°C for 24h in a sealed test tube. The fraction was taken to dryness *in vacuo* and stored in a desiccator containing KOH pellets. The residue was redissolved in 250µl of 5 mM-Tris/HCl buffer (pH 7.8) and transferred to a 1ml stoppered plastic centrifuge tube (Eppendorf Gerätebau, Netheler and Hinz G.m.b.H., Hamburg, Germany) and about 25mg of finely ground charcoal added to reduce the green pigmentation. The fraction was shaken for 5min and centrifuged for 2min at 8,000g in an Eppendorf microcentrifuge 3200 (Eppendorf Gerätebau, Netheler and Hinz G.m.b.H., Hamburg, Germany) and assayed for unesterified choline.

Unesterified choline in the bile fractions was assayed by a modification of the spectrophotometric method described by Takayama *et al.* (1977) and Hise & Mansbach II (1983). A colour reagent consisting of 3 units of choline oxidase, 6 units of peroxidase and 3mg of 2,2'-azino-di-(3-ethylbenzthiazoline

sulfonic acid) in 3ml of 50 mM-Tris/HCl buffer (pH 7.8, containing 2g Triton X -100/l) was prepared just before use and placed on ice. Colour reagent (75µl) was added to 275µl of 5 mM-Tris/HCl buffer (pH 7.8) containing 0-5 nmol of bile sample unesterified choline or standard choline chloride in 7mm x 50mm plastic tubes (Disposable Products Pty. Ltd., Adelaide, S.Aust., Australia) and incubated at room temperature for 60min. The incubation mixture (350µl) was immediately transferred to a 1ml semi-micro cuvette and the absorbance read at 420nm in a Zeiss PMQII spectrophotometer (Carl Zeiss, Oberkocken, Germany) against a blank containing no added choline. Time related incubation studies showed that the colour intensity was maximal at 60min and declined thereafter. The choline chloride standard curve was linear over the 0-5 nmol range, and 1 nmol of choline chloride had an absorbance at 420nm of 0.252 ± 0.006 units (mean ± S.E.M. of 8 curves). A standard curve was prepared for every batch of samples assayed.

5.2.5.3 Determination of bile radioactivity

The general recommendations of Peng (1977) for sample preparation and quench correction in liquid scintillation counting were followed.

The radioactivity in all bile fractions was determined using a toluene/Triton X -100 scintillation fluid [7g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(4-methyl-5-phenyl-2-oxazolyl) benzene per litre of toluene and Triton X -100 (2:1, v/v)] and counting for 10min in a Packard TRI-CARB 460 CD liquid scintillation system (Packard Instrument Co. Inc.,

Downers Grove, Il., U.S.A.) set with external standardization and automatic efficiency control to correct for quenching.

Whole bile samples (200µl) were mixed with 300µl of distilled water and 4ml of scintillation fluid in polyethylene scintillation vials (Packard Instrument Co. Inc., Downers Grove, Il., U.S.A.) and the radioactivity measured. The radioactivity was determined in the water-soluble and lipid phases of extracted bile samples after 1ml portions were evaporated to dryness at 80°C for 1h in scintillation vials and the residues taken up in 4ml of scintillation fluid respectively.

The radioactivity associated with each type of choline-containing phospholipid in the bile lipid phase was measured as follows: A 2ml portion of the radioactive lipid phase was dried in lots under N_2 in a 1ml plastic centrifuge tube and the residue redissolved in 100µl of chloroform. A 20µl aliquot of the extract along with 10µg of choline-containing phospholipid standards (phosphatidylcholine, lysophosphatidylcholine and sphingomyelin) were applied to a 0.2mm x 20cm x 20cm pre-coated silica gel 60 thin-layer chromatography plate (E. Merck, Darmstadt, Germany) that had previously been chromatographically washed with diethyl ether. The plate was first developed in the solvent chloroform/methanol (9:1, v/v) to move pigments and neutral lipids to the top and, after air-drying, developed in the solvent chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) in the same direction to separate phospholipids. The phosphatidylcholine, lysophosphatidylcholine and sphingomyelin strips were located with I_2 vapour, scraped off, and extracted

with 3ml of chloroform/methanol (1:1, v/v) respectively. A 1ml portion of each radioactive extract was evaporated to dryness in a scintillation vial and then taken up in 4ml of scintillation fluid and counted.

In some cases, a 1-2ml portion of the phosphatidylcholine extract was dried and the residue hydrolysed with 3ml of 6M-HCl at 110°C for 24h in a sealed test tube. The fraction was then dried *in vacuo* and redissolved in 3ml of distilled water and extracted 3 times with 1ml of diethyl ether. The radioactivity of the water-soluble phase (containing unesterified choline, glycerol and inorganic phosphate) and the combined ether phases (containing free fatty acids) were determined. A concentrated portion of the water-soluble phase and 20µg of choline chloride standard were applied on a silica gel 60 thin-layer chromatography plate (E. Merck, Darmstadt, Germany) which was developed in the solvent methanol/acetone/11M-HCl (45:5:2, by vol.). The choline spot was detected with I_2 vapour, scraped off, and eluted with 3ml of methanol. A 2ml portion was evaporated in a scintillation vial and then counted in 4ml of scintillation fluid. This showed if bile [^{3}H] radioactivity was associated specifically with the choline portion of the phospholipid molecule.

The radioactivity in aliquots of bile samples collected after the infusion of [^{3}H] unesterified choline was fitted to an exponential curve with respect to time and extrapolated over the period of bile collection after the infusion of [^{3}H] lipid choline bile. This allowed the residual bile radioactivity

from the initial infusion to be subtracted from the radioactivity in aliquots of bile samples collected at various intervals after the infusion of [^{3}H] lipid choline bile.

5.2.5.4 Determination of bile salts

Total bile salt concentration in bile samples was measured essentially by the spectrophotometric method described by Talalay (1960). Bile samples were prepared for analysis by diluting 10-50µl of whole bile to a final volume of 1ml with methanol. The reaction mixture contained 33 mM-sodium pyrophosphate buffer (pH 9.5), 0.33M-hydrazine sulphate (pH 9.5), 0.33 mM-β-NAD, 30µl of methanolic bile and 0.2 units of 3α-hydroxysteroid dehydrogenase (in 10 mM-potassium phosphate buffer, pH 7.2) in a final volume of 1ml. The reaction mixture containing 30µl of methanol instead of methanolic bile was used as a blank. The reaction was started by the addition of enzyme and the change in absorbance at 340nm continuously monitored in semi-micro cuvettes using a Zeiss PMQII spectrophotometer fitted with an automatic sample changer and transmittance extinction converter (TE) (Carl Zeiss, Oberkocken, Germany) connected to a Rikadenki model B16 chart recorder (Rikadenki Kogyo Co. Ltd., Tokyo, Japan). The instrument was fitted with a temperature-controlled cuvette-holder and the temperature was maintained at 25°C. The reaction reached completion after approximately 20min.

5.2.6 Chemicals

[*Methyl*-^{3}H] choline chloride (specific radioactivity 78 Ci/mmol

and radiochemical purity 97% was purchased from Amersham Australia Pty. Ltd., Sydney, N.S.W., Australia. Choline chloride (3x crystallized), L-α-phosphatidylcholine (type V-E, from egg yolk), L-α-lysophosphatidylcholine (type I, from egg yolk), sphingomyelin (from bovine brain), 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (crystalline diammonium salt), 2,5-diphenyloxazole, 1,4-bis-(4-methyl-5-phenyl-2-oxazolyl) benzene, β-NAD (grade III, from yeast), choline oxidase (from *Alcaligenes* sp.), peroxidase (type I, from horseradish) and hydroxysteroid dehydrogenase (grade II, from *Pseudomonas testosteroni* cells, containing both α and β enzyme activities) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Other chemicals were obtained as follows: 2,6-di-*tert*-butyl-4-methylphenol from Calbiochem, Los Angeles, Calif., U.S.A.; hydrazine sulphate from Hopkin and Williams, Ltd., London, U.K.; 0.9% (w/v) sodium chloride injection B.P. from Travenol Laboratories Pty. Ltd., Sydney, N.S.W., Australia; heparin sodium injection B.P. [mucous] 5,000 units/ml from David Bull Laboratories Pty. Ltd., Mulgrave, Vic., Australia; sodium pentobarbitone ("Nembutal") from Ceva Chemicals Australia Pty. Ltd., Hornsby, N.S.W., Australia; benzalkonium chloride antiseptic solution ("Zephiran") from Winthrop Laboratories, Division of Sterling Pharmaceuticals Pty. Ltd., Sydney, N.S.W., Australia; procaine penicillin ("Vetspen injection") from Glaxovet, Division of Glaxo Australia Pty. Ltd., Boronia, Vic., Australia and post-operative antiseptic cream ("Hibitane") from ICI Australia Operations Pty. Ltd., Pharmaceutical Division, Villawood, N.S.W., Australia. Other chemicals were reagent-grade or the best commercially available grade. Glass double-distilled water was used throughout this study.

5.3 Results

5.3.1 Bile flow rates

The flow rates of bile over various collection intervals for total periods of 120h after the sheep was infused consecutively *via* the portal vein with [^{3}H] unesterified choline and *via* the duodenum with [^{3}H] lipid choline bile are shown in Figure 5.2. Bile production fluctuated during the first 24h of collection and then remained reasonably constant for both infusions. The mean ± S.E.M. bile flow rates were 60.0 ± 1.64ml/h (15 x 8h collection periods) and 73.9 ± 2.26ml/h (10 x 12h collection periods) after the portal vein infusion of [^{3}H] unesterified choline and duodenal infusion of [^{3}H] lipid choline bile respectively. The flow rates of bile collected from the gallbladder were about twice the physiological 35ml/h rate of infusion of replacement bile into the duodenum of the animal. Bile was collected from the gallbladder of the sheep almost immediately it was produced by the liver and therefore would have had little time to be concentrated. The bile flow rate values obtained here are higher than those previously reported for sheep (Harrison, ,1962; Adams & Heath, 1963; Heath *et al.*, 1970; Phillis, 1976; Noble, 1978) which is almost certainly due to differences in the methods of bile collection from various regions of the bile tract maintaining the enterohepatic circulation of bile constituents and feeding.

5.3.2 Bile lipid choline secretion rates

Figure 5.3 shows the bile lipid choline secretion rates over various bile collection intervals for total periods of 120h after the sheep was consecutively given a portal vein infusion of [^{3}H] unesterified choline and a duodenal infusion of [^{3}H] lipid choline

Figure 5.2: Bile flow rates of sheep after consecutive infusions of [^{3}H] unesterified choline and [^{3}H] lipid choline bile

The sheep was consecutively infused *via* the portal vein with [^{3}H] unesterified choline and *via* the duodenum with [^{3}H] lipid choline bile and bile samples collected as described in Sections 5.2.3 and 5.2.4.

Key to the figure:

●——● = portal vein infusion of [^{3}H] unesterified choline.

●-----● = duodenal infusion of [^{3}H] lipid choline bile.

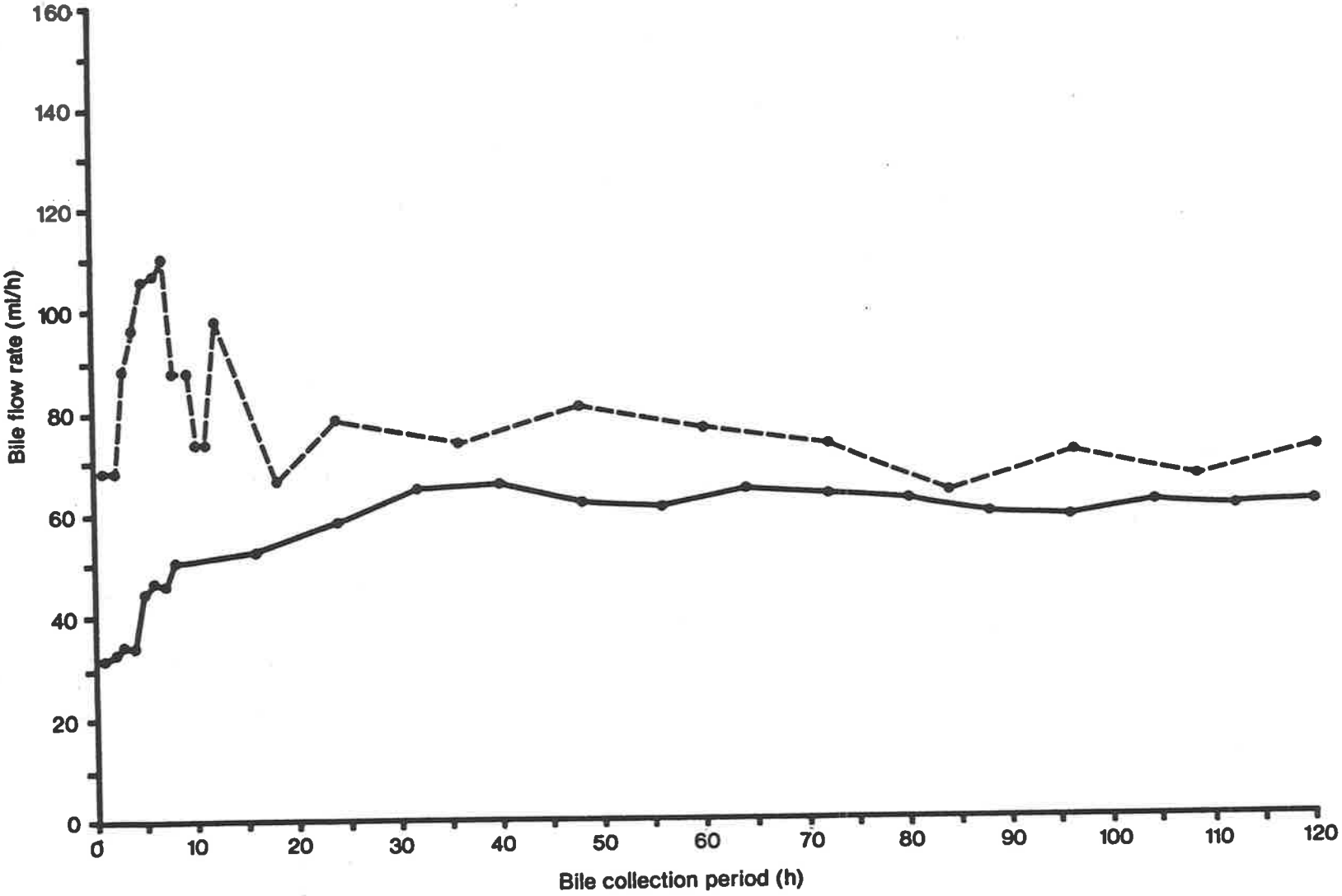

Bile flow rate (ml/h)
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
110
120
Bile collection period (h)

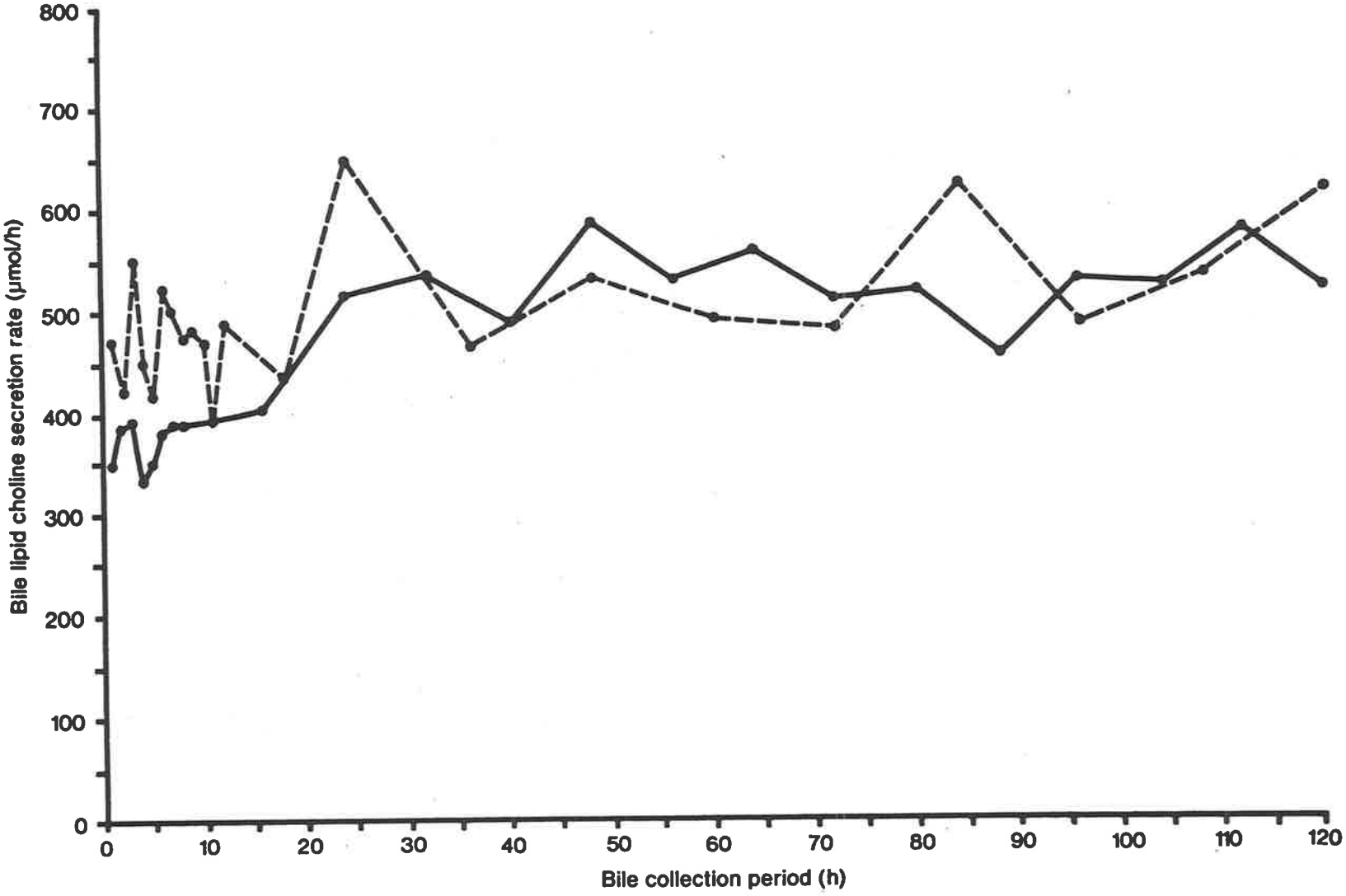
Figure 5.3: Bile lipid choline secretion rates of sheep after consecutive infusions of [^{3}H] unesterified choline and [^{3}H] lipid choline bile

The sheep was consecutively infused *via* the portal vein with [^{3}H] unesterified choline and *via* the duodenum with [^{3}H] lipid choline bile and bile samples collected as described in Sections 5.2.3 and 5.2.4. Bile lipid choline was determined as described in Section 5.2.5.2 and bile flow rates are shown in Figure 5.2.

Key to the figure:

●——● = portal vein infusion of [^{3}H] unesterified choline.

●------● = duodenal infusion of [^{3}H] lipid choline bile.


800
700
600
500
400
300
200
100
0
Bile lipid choline secretion rate (µmol/h)
0
10
20
30
40
50
60
70
80
90
100
110
120
Bile collection period (h)

bile. Bile lipid choline output increased during the first 24h of bile collection and then remained steady after the [^{3}H] unesterified choline infusion. Bile lipid choline production was reasonably stable throughout the 120h bile collection period after infusion of [^{3}H] lipid choline bile. The mean ± S.E.M. bile lipid choline secretion rates were 511 ± 15.4 µmol /h (15 x 8h collection periods) and 526 ± 18.3 µmol /h (10 x 12h collection periods) after infusion of [^{3}H] unesterified choline and [^{3}H] lipid choline bile respectively. These values are consistent with the 13.5 mmol of lipid choline/day infused into the sheep duodenum in replacement bile and the levels calculated from the results in Chapter 2 and the data of Adams & Heath (1963) and Noble (1978) for sheep.

The mean ± S.E.M. bile lipid choline concentration was 8.53 ± 0.172 µmol /ml (15 x 8h collection periods) after infusion of [^{3}H] unesterified choline and 7.21 ± 0.418 µmol /ml (10 x 12h collection periods) after infusion of [^{3}H] lipid choline bile. The sheep bile contained negligible amounts of water-soluble choline which is comparable with the results obtained in Chapter 2. The bile lipid choline concentration values obtained here are about half those previously found for sheep (results in Chapter 2; Leat & Harrison, 1975; Christie, 1978) and is almost certainly related to the high bile flow rates mentioned in Section 5.3.1.

5.3.3 Bile salt secretion rates

The bile salt secretion rates over 120h bile collection periods after the sheep was consecutively infused with [^{3}H] unesterified choline and [^{3}H] lipid choline bile are presented in Figure 5.4. The secretion of bile salts became stable after 30h of bile collec-

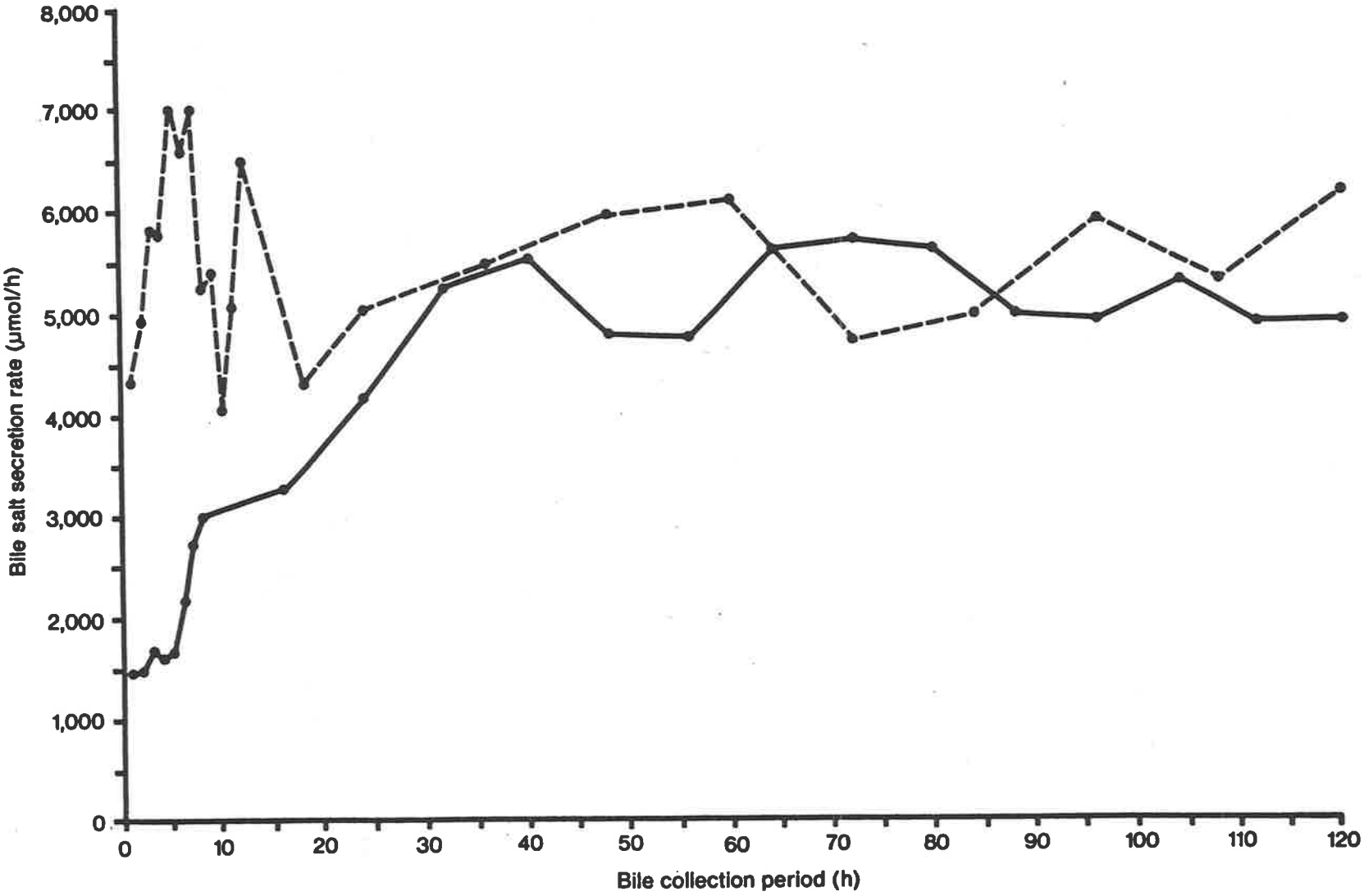
Figure 5.4: Bile salt secretion rates of sheep after consecutive infusions of [^{3}H] unesterified choline and [^{3}H] lipid choline bile

The sheep was consecutively infused *via* the portal vein with [^{3}H] unesterified choline and *via* the duodenum with [^{3}H] lipid choline bile and bile samples collected as described in Sections 5.2.3 and 5.2.4. Bile salts were determined as described in Section 5.2.5.4 and bile flow rates are shown in Figure 5.2.

Key to the figure:

●——● = portal vein infusion of [^{3}H] unesterified choline.

●------● = duodenal infusion of [^{3}H] lipid choline bile.


8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
Bile salt secretion rate (µmol/h)
0
10
20
30
40
50
60
70
80
90
100
110
120
Bile collection period (h)

tion for both infusions. The mean ± S.E.M. bile salt secretion rates were 4,800 ± 260 µmol /h (for 15 x 8h collection periods) and 5,520 ± 177 µmol /h (for 10 x 12h collection periods) after infusion of [^{3}H] unesterified choline and [^{3}H] lipid choline bile respectively which are comparable with the 125 mmol of bile salts/day infused into the sheep duodenum *via* replacement bile. The values are also in the range reported for sheep by Heath *et al.* (1970).

The mean ± S.E.M. bile salt concentrations were 79.0 ± 2.82 µmol /ml (for 15 x 8h collection periods) after the [^{3}H] unesterified choline infusion and 75.0 ± 2.56 µmol /ml (for 10 x 12h collection periods) after the [^{3}H] lipid choline bile which are lower than those found for sheep by Coleman *et al.* (1979). The lower bile salt concentrations are likely to be associated with the high bile flow rates reported fo the sheep in Section 5.3.1.

5.3.4 Recovery of infusate radioactivity in bile

Of the radioactivity in bile collected from the sheep after the portal vein infusion of [^{3}H] unesterified choline and the duodenal infusion of [^{3}H] lipid choline bile, 99.5% was recovered in the lipid phase and only 0.5% in the water-soluble phase. The low amount of radioactivity in the water-soluble phase corresponds with the negligible amount of water-soluble choline in bile. In the lipid phase of bile, 95.0% of the radioactivity was recovered in total lipid choline of which 99.0% was in phosphatidylcholine and only traces in lysophosphatidylcholine and sphingomyelin. The radioactivity in bile phosphatidylcholine was exclusively associated with the choline portion of the molecule.

Figure 5.5 shows the cumulative recovery of radioactivity in bile lipid choline over 120h collection periods after the sheep received the portal vein infusion of [^{3}H] unesterified choline and the duodenal infusion of [^{3}H] lipid choline bile consecutively. The recovery of infused [^{3}H] unesterified choline in bile lipid choline was 48.4% after 24h and 71.2% after 120h. The recovery of infused [^{3}H] lipid choline bile in bile lipid choline was 25.1% after 24h and 72.3% after 120h. Recovery of radioactivity was initially more rapid for the portal vein infusion than the duodenal infusion but was virtually identical at the end of the 120h collection period. Radioactivity recovery would have been greater for the duodenal infusion of [^{3}H] lipid choline bile during a more prolonged period of study. The amount of choline reincorporated into bile lipid choline observed here for sheep appears to be much higher than the levels reported for rats (Robins, 1975; Robins & Brunengraber, 1982) and man (Saunders, 1970).

5.3.5 Specific radioactivity of bile lipid choline

Bile lipid choline specific radioactivity rapidly reached a sharp peak between 2-4h of bile collection after the sheep was given the portal vein infusion of [^{3}H] unesterified choline, as shown in Figure 5.6. After the sheep received the duodenal infusion of [^{3}H] lipid choline bile the specific radioactivity of bile lipid choline reached a maximum after 12h of bile collection and then declined slowly as shown in Figure 5.7.

5.4 Discussion

Sheep secrete 11-17 mmol of lipid choline into the intestinal

Figure 5.5: Cumulative recovery of radioactivity in bile lipid choline of sheep after consecutive infusions of [^{3}H] unesterified choline and [^{3}H] lipid choline bile

The sheep was consecutively infused *via* the portal vein with [^{3}H] unesterified choline and *via* the duodenum with [^{3}H] lipid choline bile and bile samples collected as described in Sections 5.2.3 and 5.2.4. Bile lipid choline and bile radioactivity were determined as described in Sections 5.2.5.2 and 5.2.5.3 respectively.

Key to the figure:

●——● = portal vein infusion of [^{3}H] unesterified choline.

●-----● = duodenal infusion of [^{3}H] lipid choline bile.

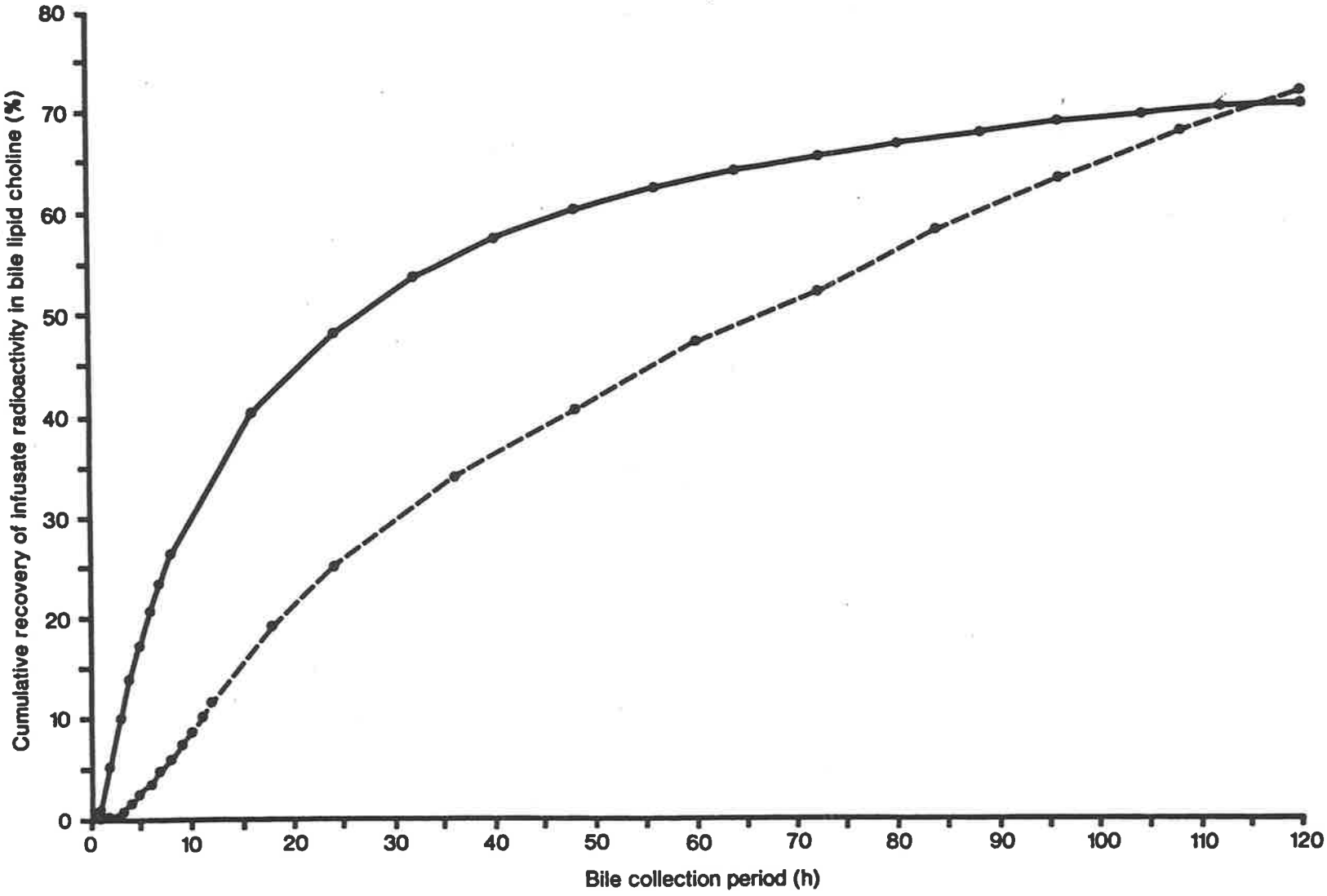

Cumulative recovery of infusate radioactivity in bile lipid choline (%)
0
10
20
30
40
50
60
70
80
0
10
20
30
40
50
60
70
80
90
100
110
120
Bile collection period (h)

Figure 5.6: Specific radioactivity of bile lipid choline of sheep after infusion of [^{3}H] unesterified choline

The sheep was infused *via* the portal vein with [^{3}H] unesterified choline and bile samples collected as described in Section 5.2.3. Bile lipid choline and bile radioactivity were determined as described in Sections 5.2.5.2 and 5.2.5.3 respectively.

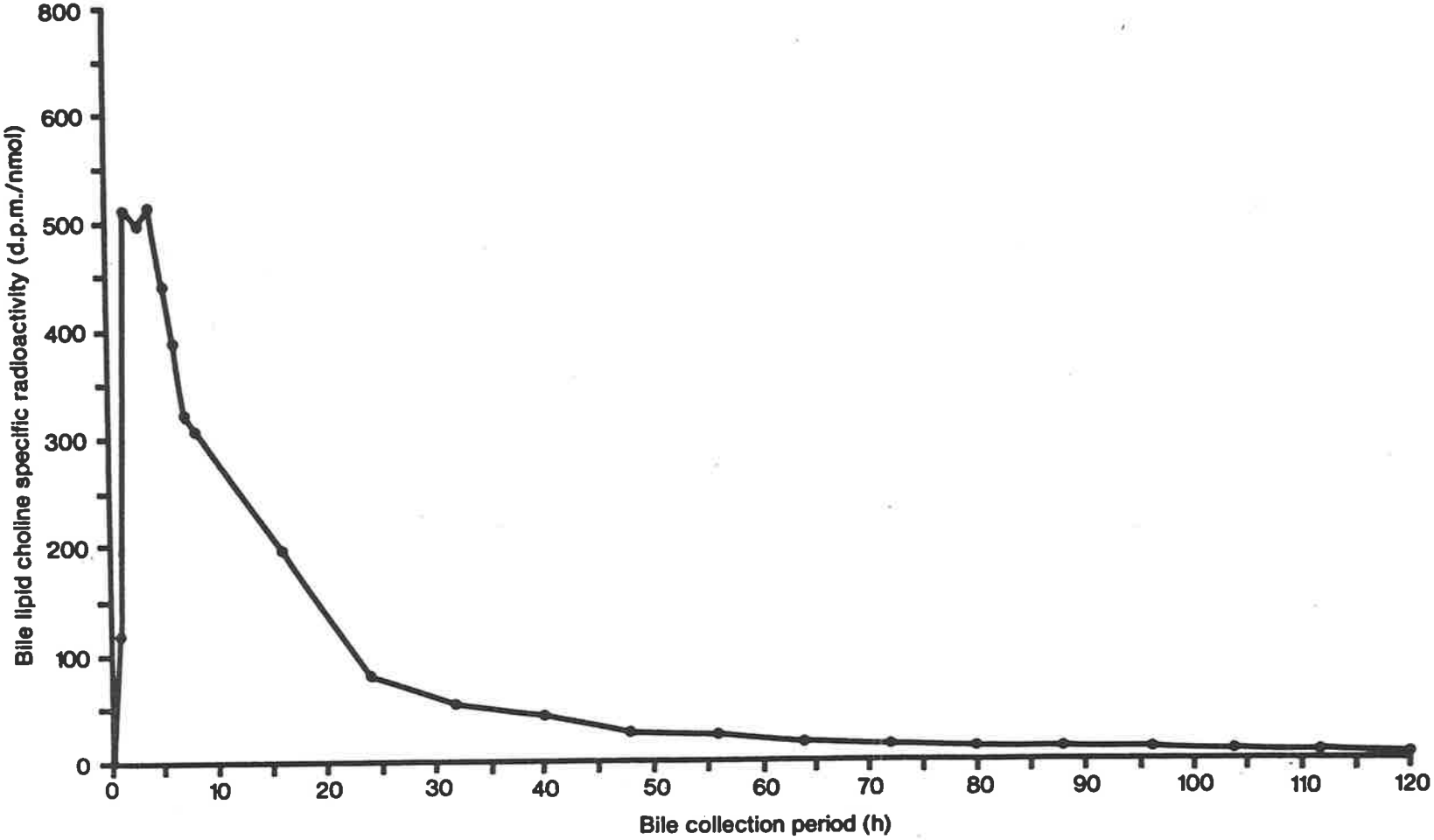
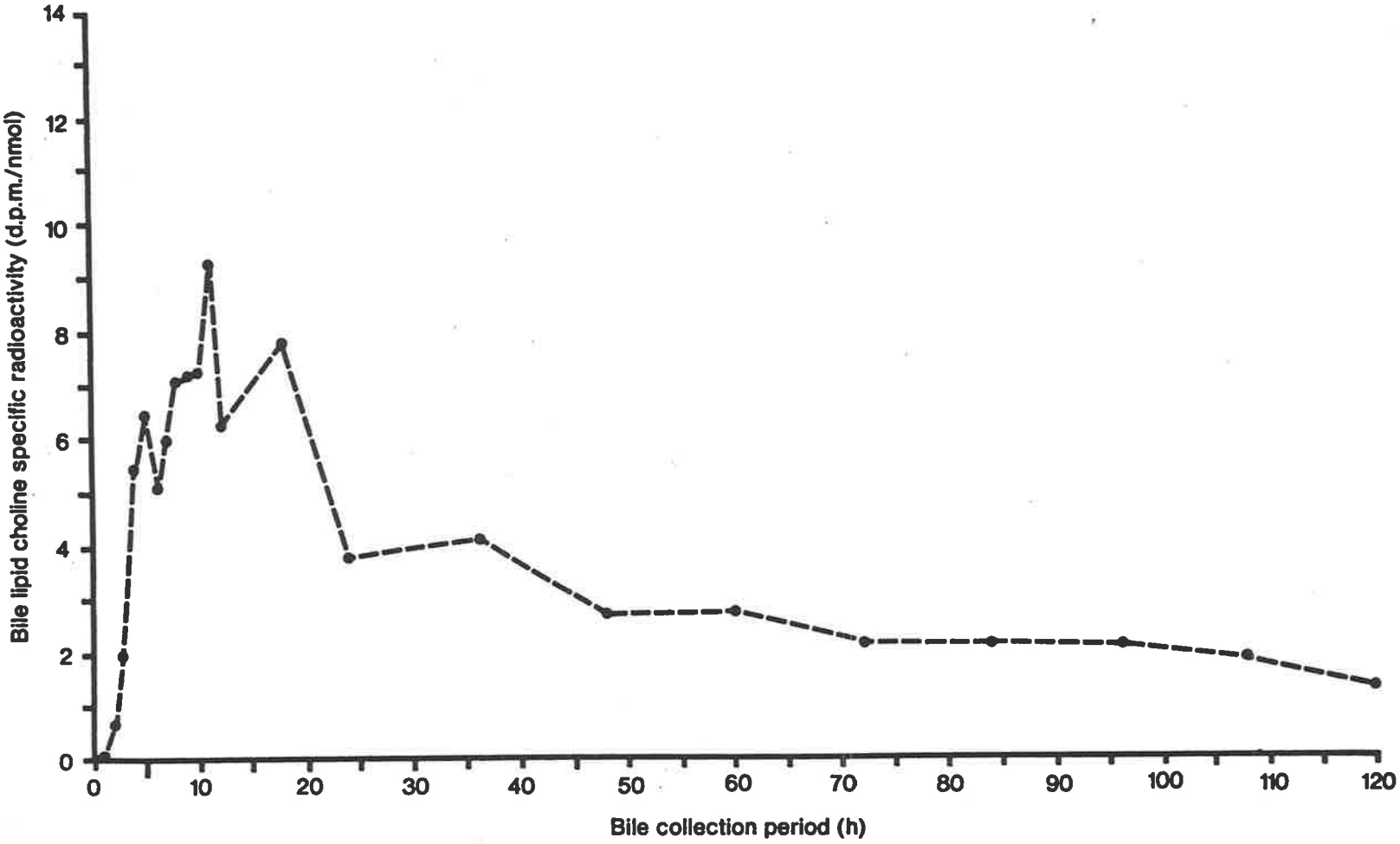

Bile lipid choline specific radioactivity (d.p.m./nmol)
800
600
500
400
300
200
100
0
0
10
20
30
40
50
60
70
80
90
100
110
120
Bile collection period (h)

Figure 5.7: Specific radioactivity of bile lipid choline of sheep after infusion of [^{3}H] lipid choline bile

The sheep was infused *via* the duodenum with [^{3}H] lipid choline bile and bile samples collected as described in Section 5.2.4. Bile lipid choline and bile radioactivity were determined as described in Sections 5.2.5.2 and 5.2.5.3 respectively.


Bile lipid choline specific radioactivity (d.p.m./nmol)
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60
70
80
90
100
110
120
Bile collection period (h)

lumen through bile which is produced in the liver (Adams & Heath, 1963; Noble, 1978; results in Chapter 2). Bile lipid choline secretion in sheep has previously been shown to be dependent upon the constant presence of digesta in the reticulo-rumen, the continuous passage of digesta through the abomasum into the duodenum and the maintenance of the enterohepatic circulation of bile salts (Noble, 1978). In this study such conditions were selected to ensure optimal bile lipid choline synthesis and secretion by the liver of the sheep, as demonstrated by the stable bile flow rates and physiological bile lipid choline and bile salt secretion rates. The [^{3}H] unesterified choline and [^{3}H] lipid choline bile infusates were of high radiochemical purity and administered to the sheep at physiological levels. The [^{3}H] lipid choline infusate was specifically of bile origin, and therefore most suitable to assess the extent of reabsorption and recycling of bile choline in the sheep.

In conscious chronically catheterized sheep (Chapter 3), the mean plasma unesterified choline production by the alimentary tract was11.2 mmol /day which was much higher than the 0.1 mmol of choline/day received from the diet (Neill *et al.*, 1979; Dawson *et al.*, 1981; results in Chapter 2) and almost equal to the 11-17 mmol of lipid choline/day secreted into the intestinal lumen through bile (Adams & Heath, 1963; Noble, 1978; results in Chapter 2). The mean uptake of plasma unesterified choline by the liver was 15.1 mmol /day and almost balanced the amount of plasma unesterified choline produced by the alimentary tract. This data, together with the low dietary choline intake (Neill *et al.*, 1979; Dawson *et al.*, 1981; results in Chapter 2) and limited hepatic choline synthesis

(Neill *et al.*, 1979), suggested that the sheep has the potential for efficient enterohepatic retention and reutilization of bile choline in order to maintain the daily rate of secretion in bile and balance of the endogenous choline pool. In the present experiments with a surgically prepared sheep, the very substantial incorporation of [^{3}H] unesterified choline infused *via* the portal vein, and [^{3}H] lipid choline bile infused *via* the duodenum into bile lipid choline confirms this conclusion.

The recovery of [^{3}H] unesterified choline infused *via* the portal vein in bile lipid choline of the sheep was 48.4% and 71.2% after 24h and 120h of bile collection respectively. In order for this high incorporation to be achieved, there must have been almost total extraction of unesterified choline from the portal vein blood followed by exclusive and rapid conversion to lipid choline destined for bile by the CDP-choline and base-exchange pathways in the sheep liver. The high uptake of unesterified choline from the portal vein by the liver supports the data obtained for conscious chronically catheterized sheep in Chapter 3. The very efficient conversion of unesterified choline to bile lipid choline is probably a consequence of the low oxidation of choline to betaine and slow turnover of lipid choline reported in sheep liver (Henderson, 1978). The results imply that in sheep liver, bile lipid choline is mainly formed from unesterified choline by the CDP-choline and base-exchange pathways rather than by the methylation pathway, which corresponds with the findings of Balint *et al.* (1967) and Treble *et al.* (1970) in rat liver. Sheep bile phosphatidylcholine predominately contains monoenoic and dienoic types of fatty acids (Christie, 1978). There

is evidence in rat liver that the monoenoic and dienoic molecular classes of phosphatidylcholine are synthesized largely *via* the CDP-choline 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 phosphatidylcholine (Van den Bosch, 1974; MacDonald & Thompson, 1975). The bile lipid choline specific radioactivity rapidly reached a sharp peak between 2-4h of bile collection after the sheep was given the portal vein infusion of [^{3}H] unesterified choline. This indicates that lipid choline formed from unesterified choline in sheep liver was secreted into bile almost immediately. It is therefore feasible that lipid choline destined for bile was not in equilibrium with the lipid choline in liver membranes and that used for lipoprotein synthesis. Thus two hepatic lipid choline pools may exist in sheep; a structural pool with a low turnover rate and a metabolic pool with a higher turnover rate for exportation into bile. A different hepatic pool for bile lipid choline is also suspected because sheep bile phosphatidylcholine contains significant amounts of fatty acids that are not normally present in sheep plasma and tissues, i.e. linolenic acid and two fatty acids containing conjugated double bond systems, 9-*cis*, 11-*trans*-octadecadienoic acid and 9-*cis*, 11-*trans*, 15-*cis*-octadecatrienoic acid, that are known to be intermediates or by-products of bihydrogenation of dietary poly-unsaturated fatty acids by rumen microorganisms (Christie, 1978).

The recovery of [^{3}H] lipid choline bile infused *via* the duodenum in bile lipid choline of the sheep was 25.1% and 72.3% after 24h and 120h of bile collection respectively. There must have been almost

complete absorption of bile lipid choline from the intestinal tract and then very significant enterohepatic recycling and reincorporation into bile. Dawson *et al.* (1981) found that digesta loses the very active microbial system for degrading choline after it reaches the abomasum, thus an insignificant amount of bile choline would have been converted to trimethylamine and methane gas in the sheep small intestine. There were negligible amounts of lipid choline in the faeces (results in Chapter 2) and Leat & Harrison (1975) and Noble (1978) reported little phospholipid present in the digesta of the ileum, indicating effective reabsorption of bile lipid choline in the upper part of the small intestine of sheep. It is extremely unlikely that the infused bile lipid choline recirculated to the liver intact. In the sheep intestinal lumen bile phosphatidylcholine is rapidly hydrolysed by phospholipases A_1 and A_2 secreted in pancreatic juice to 1- and 2-acyl lysophosphatidylcholine which is necessary for optimal absorption by the intestinal mucosa (Arienti *et al.*, 1975). From the data obtained with chronically catheterized sheep in Chapter 3, it appears that most of the absorbed bile lysophosphatidylcholine is hydrolysed to unesterified choline in the intestinal mucosa, which is then transported in the portal vein to the liver for reincorporation into bile lipid choline *via* the CDP-choline and base-exchange pathways. There is evidence that some of the bile lysophosphatidylcholine is reacylated to phosphatidylcholine in the intestinal mucosa, which then enters chylomicrons and very low density lipoproteins of lymph (Leat & Harrison, 1975, 1977, 1984; Noble, 1978). This phosphatidylcholine would enter the systemic circulation and may be taken up directly by the liver for incorporation into bile. After the sheep received the duodenal

infusion of [^{3}H] lipid choline bile, the specific radioactivity of the bile lipid choline showed a broad peak and declined slowly, which reflects the time taken for bile lipid choline absorption and hydrolysis by the intestinal mucosa and recycling to the liver *via* the portal vein and lymphatic system. The work in Chapters 3 and 4 provided evidence that extrahepatic tissues of sheep are capable of synthesizing choline which may make minor contributions to the recycling bile choline pool to account for any losses.

In rats (Robins, 1975) and humans (Saunders, 1970) bile phosphatidylcholine is extensively hydrolysed in the intestinal lumen as well as after absorption, and less than 10% of the liberated choline is reutilized for new bile phosphatidylcholine synthesis in the liver. Robins & Brunengraber (1982) showed that the bulk of the bile phosphatidylcholine is mobilized from a preformed hepatic pool which seems to be repleted by the uptake of exogenous lipids from the circulation. Bile phosphatidylcholine production and secretion in rat liver is dependent on dietary choline (Robins, 1974; Robins & Armstrong, 1976), which can be attributed to the high turnover of choline and phosphatidylcholine through oxidation to betaine and lipoprotein synthesis in this tissue (Coleman, 1973; Zeisel, 1981).

Thus the process of retention and reutilization of bile choline is much more efficient in sheep compared to non-ruminant species because of the decreased loss of choline from the system and the increased opportunities for recycling. This supports the results in Chapters 3 and 4 which explain the insensitivity of the normal sheep to an almost complete microbial destruction of dietary choline before alimentary tract absorption. Of course any disturbance of

the conservation of bile choline (e.g. scouring in the intestine; lack of pancreatic phospholipases) could cause the ruminant to become choline deficient and develop pathological lesions.

GENERAL DISCUSSION

One of the main findings of the work in this thesis was that in sheep a large proportion of total body choline synthesis occurs in tissues other than the liver. The significant production of plasma unesterified choline by the alimentary tract, brain, heart, hindlimb muscle and the upper- and lower-body regions drained by the venae cavae of conscious chronically catheterized sheep provided good evidence for this phenomenon. There was no significant uptake of lipid choline and no uptake or output of glycerophosphocholine and phosphocholine in the plasma across the upper- and lower-body of these sheep which implied that production of unesterified choline does not arise from breakdown of choline esters that are delivered to these regions in the blood. It was concluded that the most likely source of the unesterified choline is from hydrolysis of phosphatidylcholine that has been newly synthesized by the methylation of phosphatidylethanolamine in upper- and lower-body tissues of the sheep in order to maintain the endogenous choline body pool. In rats there was insignificant plasma unesterified choline production by the lower-body drained by the inferior vena cava which suggested that there was negligible choline synthesis in this region, in contrast to sheep. Unesterified choline can be released from phosphatidylcholine that is synthesized by the methylation of phosphatidylethanolamine in rat brain synaptosomes (Blusztajn *et al.*, 1979; Blusztajn & Wurtman, 1981), which is in accordance with the finding that there is a net efflux of unesterified choline from rat brain (Dross & Kewitz, 1972; Choi *et al.*, 1975; Spanner *et al.*, 1976). Zeisel (1985) suggested that phospholipase A activity

mediates the initial release of unesterified choline from phosphatidylcholine in rat brain. Further research may well reveal that the mechanism of unesterified choline production by the rat brain also exists in the upper- and lower-body tissues of the sheep.

The *in vitro* studies on the overall methylation of endogenous phosphatidylethanolamine to phosphatidylcholine in isolated microsomal preparations using *S*-adenosyl-L-[*methyl*-^{14}C] methionine as the methyl donor further supported the concept that substantial choline synthesis occurs in a variety of sheep tissues. It was found that the liver is the main site of choline synthesis in sheep, however appreciable production also occurs in other tissues, particularly the lungs, kidneys, alimentary tract and skeletal muscle. In contrast to sheep, choline synthesis was found to be essentially confined to the liver in rats.

Since methionine is the first limiting amino acid in the nutrition of sheep (Chalupa, 1972; Barry *et al.*, 1973; Schelling *et al.*, 1973), its availability for choline synthesis and other transmethylation reactions *via* the methyl donor *S*-adenosyl-L-methionine in tissues is likely to be restricted. There is evidence that the methyl group of methionine is used preferentially for carnitine synthesis at the expense of choline synthesis in sheep liver in times of metabolic stress such as starvation, alloxan-diabetes and pregnancy toxaemia (Henderson, 1978). Sheep may well become choline deficient under these stress conditions. Xue & Snoswell (1985) reported that vitamin B_{12} (cobalamin) dependent 5-methyltetrahydrofolate-homocysteine methyltransferase is an important enzyme for methionine synthesis in sheep tissues. During cobalt deficiency the activity of

5-methyltetrahydrofolate-homocysteine methyltransferase would be decreased resulting in reduced availability of methionine for choline synthesis. Indeed, Lough *et al.* (1982) observed an increased hepatic phosphatidylethanolamine : phosphatidylcholine ratio in cobalt deficient sheep which suggests a reduction in the methylation of phosphatidylethanolamine to phosphatidylcholine.

Another major finding of the present investigation was that sheep have the capacity for extensive retention and reutilization of bile choline. In conscious chronically catheterized sheep there was production of plasma unesterified choline by the alimentary tract which was approximately balanced by the uptake of plasma unesterified choline by the liver, and was almost equal to the amount of choline secreted in bile. Since there was production of glycerophosphocholine and phosphocholine and no uptake or output of lipid choline in the plasma across the alimentary tract of these animals, it seemed unlikely that the unesterified choline arises from the hydrolysis of choline esters as the blood flows through this region. The amount of unesterified choline produced by the alimentary tract was much higher than that received from the diet. It was thus concluded that there is considerable reabsorption and enterohepatic recirculation of the unesterified choline moiety of bile lipid choline in sheep in order to maintain the rate of choline secretion in bile and the balance of the endogenous choline pool. This was subsequently confirmed by infusing a surgically prepared sheep with portal vein, duodenal and gallbladder catheters and ligated bile duct with radioactively-labelled choline compounds. The amount of choline reincorporated into bile lipid choline for the sheep was much higher than that reported for rats and humans. If anything interferes with the efficient

recycling of bile choline in sheep, such as prolonged scouring, the animal could rapidly pass into a choline deficient state.

This study also showed that there is a high concentration of glycerophosphocholine in sheep liver. There was production of plasma glycerophosphocholine by the liver and uptake by the lungs and kidneys of conscious chronically catheterized sheep. The activity of glycerophosphocholine phosphodiesterase which liberates unesterified choline from glycerophosphocholine is known to be very low in sheep liver (Dawson, 1956; R.M.C. Dawson, personal communication). These factors could limit the oxidation of the liver choline pool in sheep in contrast to rats. The significance of glycerophosphocholine metabolism in relation to the overall choline status of sheep needs to be elucidated. In addition, there was negligible excretion of choline in the urine and faeces of sheep which would help to conserve the endogenous choline body pool.

The synthesis of choline in extrahepatic tissues, the efficient retention and reutilization of bile choline, and the slow turnover of the endogenous choline body pool collectively explain the low requirement of sheep for dietary choline in contrast with non-ruminant species. Thus it is unlikely that sheep suffer from dietary choline deficiency, even when grazing on dried summer pastures which were found to contain low levels of choline and in a form highly susceptible to rapid microbial breakdown in the rumen. Metabolic disorders may prove to be the only way sheep become choline deficient which would pose important considerations in production.

BIBLIOGRAPHY

Acara, M. (1975) *Am. J. Physiol.* 228, 645-649.

Acara, M. & Rennick, B. (1972) *J. Pharmacol. Exp. Ther.* 182, 1-13.

Acara, M. & Rennick, B. (1976) *J. Pharmacol. Exp. Ther.* 199, 32-40.

Acara, M., Kowalski, M., Rennick, B. & Hemsworth, B. (1975) *Br. J. Pharmacol.* 54, 41-48.

Adams, E.P. & Heath, T.J. (1963) *Biochim. Biophys. Acta* 70, 688-690.

Agricultural Research Council (1980) *The Nutrient Requirements of Ruminant Livestock*, Commonwealth Agricultural Bureaux, Slough, England.

Åkesson, B. (1978) *FEBS Lett.* 92, 177-180.

Åkesson, B. (1983) *Biochim. Biophys. Acta* 752, 460-466.

Åkesson, B., Fehling, C. & Jägerstad, M. (1978) *Br. J. Nutr.* 40, 521-527.

Åkesson, B., Fehling, C. & Jägerstad, M. (1979) *Br. J. Nutr.* 41, 263-274.

Akiyama, M., Minari, O. & Sakagami, T. (1967) *Biochim. Biophys. Acta* 137, 525-532.

Alemany, S., García Gil, M. & Mato, J.M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 6996-6999.

Alemany, S., Varela, I., Harper, J.F. & Mato, J.M. (1982) *J. Biol. Chem.* 257, 9249-9251.

Aliev, A.A., Burkova, L.M., Blinov, V.I. & Nagdaliev, F.A. (1980) *Sel'skokhozyaĭstvennaya Biologiya* 15, 731-735 [cited in *Nutr. Abstr. Rev. - Series B* (1982) 52, 86].

Allden, W.G. (1984) in *International Congress on Dryland Farming Proceedings, Adelaide, South Australia 1980*, pp. 342-378, Department of Agriculture, South Australia.

Ansell, G.B. (1973) in *Form and Function of Phospholipids* (Ansell, G.B., Hawthorne, J.N. & Dawson, R.M.C., eds.), 2nd rev. edn., B.B.A. Library vol.3, pp. 377-422, Elsevier Scientific Publishing Company, Amsterdam.

Ansell, G.B. & Spanner, S. (1971) *Biochem. J.* 122, 741-750.

Aquilonius, S.-M., Ceder, G., Lying-Tunell, U., Malmlund, H.O. & Schuberth, J. (1975) *Brain Res.* 99, 430-433.

Arienti, G., Leat, W.M.F. & Harrison, F.A. (1975) *Q1. J. Exp. Physiol.* 60, 15-24.

Artom, C. (1965) *Fed. Proc. Fed. Am. Soc. Biol.* 24, 477.

Artom, C. (1968) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 27, 457.

Artom, C. (1969) *Proc. Soc. Exp. Biol. Med.* 132, 1025-1030.

Asatoor, A.M. & Simenhoff, M.L. (1965) *Biochim. Biophys. Acta* 111, 384-392.

Audubert, F. & Vance, D.E. (1983) *J. Biol. Chem.* 258, 10695-10701.

Baer, E. & Robinson, R. (1967) *Can. J. Biochem.* 45, 1747-1754.

Baldwin, E. (1953) *Dynamic Aspects of Biochemistry*, 2nd edn., pp. 328-329, Cambridge University Press, Cambridge.

Baldwin, J.J. & Cornatzer, W.E. (1968) *Biochim. Biophys. Acta* 164, 195-204.

Baldwin, J.J. & Cornatzer, W.E. (1969) *Biochim. Biophys. Acta* 176, 193-195.

Balint, J.A., Beeler, D.A., Treble, D.H. & Spitzer, H.L. (1967) *J. Lipid Res.* 8, 486-493.

Barak, A.J. & Tuma, D.J. (1983) *Life Sci.* 32, 771-774.

Barclay, L.L., Blass, J.P., Kopp, U. & Hanin, I. (1982) *N. Engl. J. Med.* 307, 501.

Barnholz, Y., Roitman, A. & Gatt, S. (1966) *J. Biol. Chem.* 241, 3731-3737.

Barry, T.N., Fennessy, P.F. & Duncan, S.J. (1973) *N.Z. J. Agric. Res.* 16, 64-68.

Bauchop, T. & Clarke, R.T.J. (1976) *Appl. Environ. Microb.* 32, 417-422.

Benevenga, N.J. & Haas, L.G. (1979) *J. Anim. Sci.* 49, (Suppl.1), 94.

Bergman, E.N. & Wolff, J.E. (1971) *Am. J. Physiol.* 221, 586-592.

Bernheim, F. & Bernheim, M.L.C. (1933) *Am. J. Physiol.* 104, 438-440.

Bernheim, F. & Bernheim, M.L.C. (1938) *Am. J. Physiol.* 121, 55-60.

Biezenski, J.J., Carrozza, J. & Li, J. (1971) *Biochim. Biophys. Acta* 239, 92-97.

Bjerve, K.S. (1973) *Biochim. Biophys. Acta* 296, 549-562.

Bjerve, K.S., Daae, L.N.W. & Bremer, J. (1974) *Anal. Biochem.* 58, 238-245.

Bjørnstad, P. & Bremer, J. (1966) *J. Lipid Res.* 7, 38-45.

Blumenstein, J. (1964) *Can. J. Biochem.* 42, 1183-1194.

Blunt, M.H. & Huisman, T.H.J. (1975) in *The Blood of Sheep : Composition and Function* (Blunt, M.H., ed.), pp. 155-183, Springer-Verlag, Berlin, Heidelberg and New York.

Blusztajn, J.K. & Wurtman, R.J. (1981) *Nature (London)* 290, 417-418.

Blusztajn, J.K. & Wurtman, R.J. (1983) *Science* 221, 614-620.

Blusztajn, J.K., Zeisel, S.H. & Wurtman, R.J. (1979) *Brain Res.* 179, 319-327.

Borchardt, R.T. (1980) *J. Med. Chem.* 23, 347-357.

Brady, R.O., Bradley, R.M., Young, O.M. & Kaller, H. (1965) *J. Biol. Chem.* 240, PC3693-PC3694.

Bremer, J. (1969) in *Methods in Enzymology* (Lowenstein, J.M., ed.) vol. 14, pp. 125-128, Academic Press Inc., New York.

Bremer, J. (1977) *Trends Biochem. Sci.* 2, 207-209.

Bremer, J. & Greenberg, D.M. (1960) *Biochim. Biophys. Acta* 37, 173-175.

Bremer, J. & Greenberg, D.M. (1961) *Biochim. Biophys. Acta* 46, 205-216.

Broad, T.E. & Dawson, R.M.C. (1975) *Biochem. J.* 146, 317-328.

Broad, T.E. & Dawson, R.M.C. (1976) *J. Gen. Microbiol.* 92, 391-397.

Bygrave, F.L. & Dawson, R.M.C. (1976) *Biochem. J.* 160, 481-490.

Cantoni, G.L. (1951) *J. Biol. Chem.* 189, 745-754.

Cantoni, G.L. (1952) *J. Am. Chem. Soc.*74, 2942-2943.

Cantoni, G.L. (1953) *J. Biol. Chem.* 204, 403-416.

Cantoni, G.L. (1975) *Annu. Rev. Biochem.* 44, 435-451.

Cantoni, G.L. (1977) in *The Biochemistry of Adenosylmethionine* (Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H.G. & Schlenk, F., eds.), pp. 557-577, Columbia University Press, New York.

Case, G.L. & Benevenga, N.J. (1976) *J. Nutr.* 106, 1721-1736.

Castaño, J.G., Alemany, S., Nieto, A. & Mato, J.M. (1980) *J. Biol. Chem.* 255, 9041-9043.

Chalupa, W. (1972) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 31, 1152-1164.

Chap, H.J., Zwaal, R.F.A. & Van Deenen, L.L.M. (1977) *Biochim. Biophys. Acta* 467, 146-164.

Chiang, P.K. & Cantoni, G.L. (1979) *Biochem. Pharmacol.* 28, 1897-1902.

Chittenden, C.G., Laidman, D.L., Ahmad, N. & Wyn Jones, R.G. (1978) *Phytochemistry* 17, 1209-1216.

Choi, R.L., Freeman, J.J. & Jenden, D.J. (1975) *J. Neurochem.* 24, 735-741.

Chou, T.-C., Coulter, A.W., Lombardini, J.B., Sufrin, J.R. & Talalay, P. (1977) in *The Biochemistry of Adenosylmethionine* (Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H.G. & Schlenk, F., eds.), pp. 18-36, Columbia University Press, New York.

Choy, P.C. & Vance, D.E. (1978) *J. Biol. Chem.* 253, 5163-5167.

Choy, P.C., Lim, P.H. & Vance, D.E. (1977) *J. Biol. Chem.* 252, 7673-7677.

Choy, P.C., Paddon, H.B. & Vance, D.E. (1980) *J. Biol. Chem.* 255, 1070-1073.

Christie, W.W. (1978) *Prog. Lipid Res.* 17, 111-205.

Church, D.C. (1979*a*) *Digestive Physiology and Nutrition of Ruminants*, 2nd edn. vol. 2, pp. 230-257, O & B Books, Inc., Oregon.

Church, D.C. (1979*b*) *Digestive Physiology and Nutrition of Ruminants*, 2nd edn., vol. 1, pp. 99-114, D.C. Church, Oregon.

Church, D.C. (1979*c*) *Digestive Physiology and Nutrition of Ruminants*, 2nd edn., vol. 1, pp. 61-68, D.C. Church, Oregon.

Church, D.C. (1979*d*) *Digestive Physiology and Nutrition of Ruminants*, 2nd edn., vol. 1, pp. 115-132, D.C. Church, Oregon.

Cleland, W.W. (1967) *Adv. in Enzymol.* 29, 1-32.

Clifford, A.J., Goodrich, R.D. & Tillman, A.D. (1967) *J. Anim. Sci.* 26, 400-403.

Cohen, E.L. & Wurtman, R.J. (1975) *Life Sci.* 16, 1095-1102.

Coleman, R. (1973) in *Form and Function of Phospholipids* (Ansell, G.B., Hawthorne, J.N. & Dawson, R.M.C., eds.), 2nd rev. edn., B.B.A. Library, vol. 3, pp. 345-375, Elsevier Scientific Publishing Company, Amsterdam.

Coleman, R., Iqbal, S., Godfrey, P.P. & Billington, D. (1979) *Biochem. J.* 178, 201-208.

Coleman, G.S., Dawson, R.M.C. & Grime, D.W. (1980) *Proc. Nutr. Soc.* 39, 6A.

Collins, F.D. & Shotlander, V.L. (1961) *Biochem. J.* 79, 316-320.

Corredor, C., Mansbach, C. & Bressler, R. (1967) *Biochim. Biophys. Acta* 144, 366-374.

Costa, N.D. (1977) Ph.D. Thesis, University of Adelaide.

Coughlan, S.J. & Wyn Jones, R.G. (1982) *Planta* 154, 6-17.

Crews, F.T., Hirata, F. & Axelrod, J. (1980*a*) *J. Neurochem.* 34, 1491-1498.

Crews, F.T., Morita, Y., Hirata, F., Axelrod, J. & Siraganian, R.P. (1980*b*) *Biochem. Biophys. Res. Commun.* 93, 42-49.

Crewther, W.G., Fraser, R.D.B., Lennox, F.G. & Lindley, H. (1965) *Adv. Protein Chem.* 20, 191-346.

Cromwell, B.T. & Rennie, S.D. (1953) *Biochem. J.* 55, 189-192.

Davies, W.L. (1936) *J. Dairy Res.* 7, 14-24.

Dawson, R.M.C. (1955*a*) *Biochem. J.* 59, 5-8.

Dawson, R.M.C. (1955*b*) *Biochem. J.* 60, 325-328.

Dawson, R.M.C. (1956) *Biochem. J.* 62, 689-693.

Dawson, R.M.C. (1959) *Nature (London)* 183, 1822-1823.

Dawson, R.M.C. (1973) in *Form and Function of Phospholipids* (Ansell, G.B., Hawthorne, J.N. & Dawson, R.M.C., eds.), 2nd rev. edn., B.B.A. Library vol. 3, pp. 97-116, Elsevier Scientific Publishing Company, Amsterdam.

Dawson, R.M.C. & Hemington, N. (1974*a*) *Br. J. Nutr.* 32, 327-340.

Dawson, R.M.C. & Hemington, N. (1974*b*) *Biochem. J.* 143, 427-430.

Dawson, R.M.C. & Kemp, P. (1967) *Biochem. J.* 105, 837-842.

Dawson, R.M.C. & Kemp, P. (1969) *Biochem. J.* 115, 351-352.

Dawson, R.M.C., Hemington, N. & Lindsay, D.B. (1960) *Biochem. J.* 77, 226-230.

Dawson, R.M.C., Grime, D.W. & Lindsay, D.B. (1981) *Biochem. J.* 196, 499-504.

Decker, K. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.U., ed.), 2nd edn., vol. 4, pp. 1988-1993, Academic Press, New York and London.

De la Haba, G. & Cantoni, G.L. (1959) *J. Biol. Chem.* 234, 603-608.

De la Huerga, J. & Popper, H. (1951) *J. Clin. Invest.*30, 463-470.

De la Huerga, J. & Popper, H. (1952) *J. Clin. Invest.*31, 598-603.

De Ridder, J.J.M. & Van Dam, K. (1975) *Biochim. Biophys. Acta* 408, 112-122.

Dinning, J.S., Keith, C.K. & Day, P.L. (1949) *Arch. Biochem. Biophys.* 24, 463-464.

Diringer, H., Marggraf, W.D., Koch, M.A. & Anderer, F.A. (1972) *Biochem. Biophys. Res. Commun.* 47, 1345-1352.

Domanski, A., Lindsay, D.B. & Setchell, B.P. (1974) *J. Physiol. (London)* 242, 28P-29P.

Dross, K. & Kewitz, H. (1972) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 274, 91-106.

Dubnoff, J.W. (1949) *Arch. Biochem. Biophys.* 24, 251-262.

Duerre, J.A. & Walker, R.D. (1977) in *The Biochemistry of Adenosylmethionine* (Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H.G. & Schlenk, F., eds.), pp 43-57, Columbia University Press, New York.

Du Vigneaud, V. & Rachele, J.R. (1965) in *Transmethylation and Methionine Biosynthesis* (Shapiro, S.K. & Schlenk, F., eds.), pp. 1-20, University of Chicago Press, Chicago and London.

Eckernäs, S.-Å. & Aquilonius, S.-M. (1977) *Scand. J. Clin. Lab. Invest.* 37, 183-187.

Egan, A.R. & Macrae, J.C. (1979) *Ann. Rech. Vet.* 10, 376-378.

Erbland, J.F. & Marinetti, G.V. (1965) *Biochim. Biophys. Acta* 106, 128-138.

Erfle, J.D. (1975) *Biochem. Biophys. Res. Commun.* 64, 553-557.

Faichney, G.J. (1972) *Aust. J. Agric. Res.* 23, 859-869.

Faichney, G.J. & Weston, R.H. (1971) *Aust. J. Agric. Res.* 22, 461-468.

Fallon, H.J., Gertman, P.M., Kemp, E.L. (1969) *Biochim. Biophys. Acta* 187, 94-104.

Farrell, P.M., Lundgren, D.W. & Adams, A.J. (1974) *Biochem. Biophys. Res. Commun.* 57, 696-701.

Faruque, A.J.M.O., Jarvis, B.D.W. & Hawke, J.C. (1974) *J. Sci. Fd. Agric.* 25, 1313-1328.

Feldman, D.A., Kovac, C.R., Dranginis, P.L. & Weinhold, P.A. (1978) *J. Biol. Chem.* 253, 4980-4986.

Finkelstein, J.D. (1974) *Metabolism* 23, 387-398.

Finkelstein, J.D. (1978) in *Transmethylation* (Usdin, E., Borchardt, R.T. & Creveling, C.R., eds.), pp. 49-58, Elsevier/North-Holland, New York, Amsterdam, Oxford.

Finkelstein, J.D. & Mudd, S.H. (1967) *J. Biol. Chem.* 242, 873-880.

Finkelstein, J.D., Kyle, W.E. & Harris, B.J. (1971) *Arch. Biochem. Biophys.* 146, 84-92.

Finkelstein, J.D., Harris, B.J., Martin, J.J. & Kyle, W.E. (1982) *Biochem. Biophys. Res. Commun.* 108, 344-348.

Flower, R.J., Pollitt, R.J., Sanford, P.A. & Smyth, D.H. (1972*a*) *J. Physiol. (London)* 222, 146P-147P.

Flower, R.J., Pollitt, R.J., Sanford, P.A. & Smyth, D.H. (1972*b*) *J. Physiol. (London)* 226, 473-489.

Folch, J., Lees, M. & Sloane-Stanley, G.H. (1957) *J. Biol. Chem.* 226, 497-509.

Fonlupt, P., Rey, C. & Pacheco, H. (1981) *Biochem. Biophys. Res. Commun.* 100, 1720-1725.

Fonnum, F. (1969*a*) *Biochem. J.* 113, 291-298.

Fonnum, F. (1969*b*) *Biochem. J.* 115, 465-472.

Fonnum, F. (1973) *Brain Res.* 62, 497-507.

Freeman, J.J. & Jenden, D.J. (1976) *Life Sci.* 19, 949-962.

Frosolono, M.F. (1977) in *Lipid Metabolism in Mammals* (Snyder, F., ed.), vol. 2, pp. 1-38, Plenum Press, New York.

Fujino, Y. (1952) *J. Biochem. (Tokyo)* 39, 55-62.

Gardiner, R.M. (1980) *J. Physiol. (London)* 301, 429-438.

Gawthorne, J.M. & Smith, R.M. (1973) *Biochem. J.* 136, 295-301.

Gawthorne, J.M. & Smith, R.M. (1974) *Biochem. J.* 142, 119-126.

Geelen, M.J.H., Groener, J.E.M., De Haas, C.G.M. & Van Golde, L.M.G. (1979) *FEBS Lett.* 105, 27-30.

Gemmell, R.T. & Heath, T. (1972) *Anat. Record.* 172, 57-70.

Gibson, K.D., Wilson, J.D. & Udenfriend, S. (1961) *J. Biol. Chem.* 236, 673-679.

Glenn, J.L. & Austin, W. (1971) *Biochim. Biophys. Acta* 231, 153-160.

Glick, D. (1945) *Cereal Chem.* 22, 95-101.

Glomset, J.A. (1968) *J. Lipid Res.* 9, 155-167.

Glomset, J.A. (1979) in *Blood Lipids and Lipoproteins : Quantitation, Composition, and Metabolism* (Nelson, G.J., ed.), pp. 745-787, Robert E. Krieger Publishing Co., Inc., Huntington, New York.

Goldberg, A.M. & McCaman, R.E. (1973) *J. Neurochem.* 20, 1-8.

Griffith, W.H., Nyc, J.F., Harris, R.S., Hartroft, W.S., Lucas, C.C. & Best, C.H. (1954) in *The Vitamins* (Sebrell, W.H., Jr. & Harris, R.S., eds.), vol. 2, pp. 1-130, Academic Press Inc., New York.

Griffith, W.H., Nyc, J.F., Harris, R.S., Hartroft, W.S. & Porta, E.A. (1971) in *The Vitamins* (Sebrell, W.H., Jr. & Harris, R.S., eds.), 2nd edn., vol. 3, pp. 1-154, Academic Press, New York and London.

Grubb, D.J. & Jones, A.L. (1971) *Anat. Record.* 170, 75-80.

Guha, S. & Wegmann, R. (1963) *Nature (London)* 200, 1218-1219.

Haines, D.S.M. (1966) *Can. J. Biochem.* 44, 45-57.

Hales, J.R.S. (1973) *Pflügers Arch.* 344, 119-132.

Hamprecht, B. & Amano, T. (1974) *Anal. Biochem.* 57, 162-172.

Handler, P. (1949) *Proc. Soc. Exp. Biol. Med.* 70, 70-73.

Handler, P. & Bernheim, F. (1949) *Proc. Soc. Exp. Biol. Med.* 72, 569-571.

Hanin, I. (1974) *Choline and Acetylcholine : Handbook of Chemical Assay Methods* Raven Press, New York.

Hanin, I., Merikangas, J.R., Merikangas, K.R. & Kopp, U. (1979) *N. Engl. J. Med.* 301, 661-662.

Hanson, A.D. & Nelsen, C.E. (1978) *Plant Physiol.* 62, 305-312.

Hanson, A.D. & Rhodes, D. (1983) *Plant Physiol.* 71, 692-700.

Hanson, A.D. & Scott, N.A. (1980) *Plant Physiol.* 66, 342-348.

Hanson, A.D. & Wyse, R. (1982) *Plant Physiol.* 70, 1191-1198.

Harfoot, C.G. (1978) *Prog. Lipid Res.* 17, 21-54.

Harrison, D.G., Beever, D.E. & Osborn, D.F. (1979) *Br. J. Nutr.* 41, 521-527.

Harrison, F.A. (1962) *J. Physiol. (London)* 162, 212-224.

Harrison, F.A. & Leat, W.M.F. (1972) *J. Physiol. (London)* 225, 565-576.

Hatefi, Y. & Stiggall, D. (1976) *The Enzymes*, 3rd edn. 13, 260-263.

Haubrich, D.R., Wang, P.F.L. & Wedeking, P.W. (1975a) *J. Pharmacol. Exp. Ther.* 193, 246-255.

Haubrich, D.R., Wang, P.F.L., Clody, D.E. & Wedeking, P.W. (1975b) *Life Sci.* 17, 975-980.

Haubrich, D.R., Wang, P.F.L., Chippendale, T. & Proctor, E. (1976) *J. Neurochem.* 27, 1305-1313.

Haubrich, D.R., Gerber, N., Pflueger, A.B. & Zweig, M. (1981) *J. Neurochem.* 36, 1409-1417.

Hazlewood, G.P. & Dawson, R.M.C. (1975) *J. Gen. Microbiol.* 89, 163-174.

Hazlewood, G.P. & Dawson, R.M.C. (1976) *Biochem. J.* 153, 49-53.

Heath, T., Caple, I.W. & Redding, P.M. (1970) *Q1. J. Exp. Physiol.* 55, 93-103.

Hebb, C., Mann, S.P. & Mead, J. (1975) *Biochem. Pharmacol.* 24, 1007-1011.

Heller, M. & Shapiro, B. (1966) *Biochem. J.* 98, 763-769.

Henderson, G.D. (1978) Ph.D. Thesis, University of Adelaide.

Hicks, P., Rolsten, C., Taylor, D. & Samorajski, T. (1982) *Gerontology* 28, 104-107.

Hill, E.E. & Lands, W.E.M. (1970) in *Lipid Metabolism* (Wakil, S.J., ed.), pp. 185-277, Academic Press, New York.

Hinton, B.T. & Setchell, B.P. (1980) *J. Reprod. Fertil.* 58, 401-406.

Hirata, F. & Axelrod, J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* 75, 2348-2352.

Hirata, F. & Axelrod, J. (1980) *Science* 209, 1082-1090.

Hirata, F., Viveros, O.H., Diliberto, E.J., Jr. & Axelrod, J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* 75, 1718-1721.

Hirata, F., Strittmatter, W.J. & Axelrod, J. (1979*a*) *Proc. Natl. Acad. Sci. U.S.A.* 76, 368-372.

Hirata, F., Corcoran, B.A., Venkatasubramanian, K., Schiffmann, E. & Axelrod, J. (1979*b*) *Proc. Natl. Acad. Sci. U.S.A.* 76, 2640-2643.

Hise, M.K. & Mansbach, C.M., II (1983) *Anal. Biochem.* 135, 78-82.

Hitz, W.D. & Hanson, A.D. (1980) *Phytochemistry* 19, 2371-2374.

Hitz, W.D., Rhodes, D. & Hanson, A.D. (1981) *Plant Physiol.* 68, 814-822.

Hitz, W.D., Ladyman, J.A.R. & Hanson, A.D. (1982) *Crop Sci.* 22, 47-54.

Hoffman, D.R. & Cornatzer, W.E. (1981) *Lipids* 16, 533-540.

Hoffman, D.R., Cornatzer, W.E. & Duerre, J.A. (1979) *Can. J. Biochem.* 57, 56-65.

Hoffman, D.R., Marion, D.W., Cornatzer, W.E. & Duerre, J.A. (1980*a*) *J. Biol. Chem.* 255, 10822-10827.

Hoffman, D.R., Uthus, E.O. & Cornatzer, W.E. (1980*b*) *Lipids* 15, 439-446.

Hoffman, D.R., Haning, J.A. & Cornatzer, W.E. (1981*a*) *Int. J. Biochem.* 13, 745-748.

Hoffman, D.R., Haning, J.A. & Cornatzer, W.E. (1981*b*) *Lipids* 16, 561-567.

Holman, R.T. (1967) *Prog. Chem. Fats Other Lipids* 9, 3-12.

Hopper, J.H. & Johnson, B.C. (1956) *Proc. Soc. Exp. Biol. Med.* 91, 497-499.

Hübscher, G., West, G.R. & Brindley, D.N. (1965) *Biochem. J.* 97, 629-642.

Hynd, P.I. (1982) Ph.D. Thesis, University of Adelaide.

Illingworth, D.R. & Portman, O.W. (1972) *J. Chromatogr.* 73, 262-264.

Illingworth, D.R. & Portman, O.W. (1973) *Physiol. Chem. Phys.* 5, 365-373.

Infante, J.P. (1977) *Biochem. J.* 167, 847-849.

Infante, J.P. & Kinsella, J.E. (1978) *Biochem. J.* 176, 631-633.

Ishizaka, T., Foreman, J.C., Sterk, A.R. & Ishizaka, K. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 5858-5862.

Ishizaka, T., Hirata, F., Ishizaka, K. & Axelrod, J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 1903-1906.

Itzhaki, R.F. & Gill, D.M. (1964) *Anal. Biochem.* 9, 401-410.

Jelsema, C.L. & Morré, D.J. (1978) *J. Biol. Chem.* 253, 7960-7971.

John, A. & Ulyatt, M.J. (1979) *Proc. Nutr. Soc.* 38, 144A.

Johnson, B.C., Hamilton, T.S. & Mitchell, H.H. (1945) *J. Biol. Chem.* 159, 5-8.

Johnson, B.C., Mitchell, H.H. & Pinkos, J.A. (1951) *J. Nutr.* 43, 37-48.

Jope, R.S. (1982) *J. Pharmacol. Exp. Ther.* 220, 322-328.

Jope, R.S., Jenden, D.J., Ehrlich, B.E., Diamond, J.M. & Gosenfeld, L.F. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 6144-6146.

Jorswieck, I. (1974) *N.S. Arch Pharmakol. Suppl.* 282, R42.

Jukes, T.H. (1980) *Trends Biochem. Sci.* 5, 307-308.

Kanfer, J.N. (1980) *Can. J. Biochem.* 58, 1370-1380.

Katyal, S.L. & Lombardi, B. (1976) *Lipids* 11, 513-516.

Katz, M.L. & Bergman, E.N. (1969) *Am. J. Physiol.* 216, 946-952.

Kennedy, E.P. (1962) *Harvey Lect.* 57, 143-171.

Kennedy, E.P. & Weiss, S.B. (1956) *J. Biol. Chem.* 222, 193-214.

Kensler, C.J. & Langemann, H. (1954) *Proc. Soc. Exp. Biol. Med.* 85, 364-367.

Kon, S.K. & Porter, J.W.G. (1954) *Vitam. Horm.* 12, 53-68.

Kramer, R., Jungi, B. & Zahler, P. (1974) *Biochim. Biophys. Acta* 373, 404-415.

Kuksis, A. & Mookerjea, S. (1978) *Nutr. Rev.* 36, 201-207.

Kutzbach, C., Galloway, E. & Stokstad, E.L.R. (1967) *Proc. Soc. Exp. Biol. Med.* 124, 801-805.

Ladyman, J.A.R., Hitz, W.D. & Hanson, A.D. (1980) *Planta* 150, 191-196.

Lands, W.E.M. (1960) *J. Biol. Chem.* 235, 2233-2237.

Lands, W.E.M. & Merkl, I. (1963) *J. Biol. Chem.* 238, 898-904.

Leat, W.M.F. & Harrison, F.A. (1974) *Q1. J. Exp. Physiol.* 59, 131-139.

Leat, W.M.F. & Harrison, F.A. (1975) in *Digestion and Metabolism in the Ruminant* (McDonald, I.W. & Warner, A.C.I., eds.), pp. 481-495, The University of New England Publishing Unit, Armidale.

Leat, W.M.F. & Harrison, F.A. (1977) *Proc. Nutr. Soc.* 36, 70A.

Leat, W.M.F. & Harrison, F.A. (1984) *Proc. Nutr. Soc.* 43, 132A.

Leat, W.M.F., Kubasek, F.O.T. & Buttress, N. (1976) *Q1. J. Exp. Physiol.* 61, 193-201.

Le Kim, D., Betzing, H. & Stoffel, W. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* 354, 437-444.

Lester, R.L. & White, D.C. (1967) *J. Lipid Res.* 8, 565-568.

Lindsay, D.B. & Leat, W.M.F. (1975) in *The Blood of Sheep : Composition and Function* (Blunt, M.H., ed.), pp. 45-62, Springer-Verlag, Berlin, Heidelberg and New York.

Lindsay, D.B. & Setchell, B.P. (1972) *J. Physiol. (London)* 226, 51P-52P.

Lindsay, D.B. & Setchell, B.P. (1974) *J. Physiol. (London)* 242, 29P-30P.

Ling, E.R., Kon, S.K. & Porter, J.W.G. (1961) in *Milk : The Mammary Gland and Its Secretion* (Kon, S.K. & Cowie, A.T., eds.), vol.2, pp. 195-263, Academic Press, New York and London.

Lloyd-Davies, K.A., Michell, R.H. & Coleman, R. (1972) *Biochem. J.* 127, 357-368.

Lombardi, B. (1971) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 30, 139-142.

Lombardi, B., Pani, P., Schlunk, F.F. & Shi-Hua, C. (1969) *Lipids* 4, 67-75.

Lough, A.K., Duncan, W.R.H., Earl, C.R.A. & Coutts, L. (1982) *Proc. Nutr. Soc.* 41, 16A.

Lucas, C.C. & Ridout, J.H. (1967) *Prog. Chem. Fats Other Lipids* 10, 1-150.

Luecke, R.W. & Pearson, P.B. (1944) *J. Biol. Chem.* 153, 259-263.

Luecke, R.W. & Pearson, P.B. (1945) *J. Biol. Chem.* 158, 561-566.

Lyman, R.L., Sheehan, G. & Tinoco, J. (1973) *Lipids* 8, 71-79.

MacDonald, G. & Thompson, W. (1975) *Biochim. Biophys. Acta* 398, 424-432.

Macfarlane, M.G., Patterson, L.M.B. & Robison, R. (1934) *Biochem. J.* 28, 720-724.

Mann, P.J.G. & Quastel, J.H. (1937) *Biochem. J.* 31, 869-878.

Mann, S.P. (1975) *Experientia* 31, 1256-1258.

Mannervik, B. & Sörbo, B. (1970) *Biochem. Pharmacol.* 19, 2509-2516.

Marggraf, W.-D. & Anderer, F.A. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* 355, 803-810.

Marinetti, G.V., Erbland, J., Witter, R.F., Petix, J. & Stotz, E. (1958) *Biochim. Biophys. Acta* 30, 223.

Massoulié, J. (1980) *Trends Biochem. Sci.* 5, 160-164.

Mather, L.E., Runciman, W.B. & Ilsley, A.H. (1982) *Reg. Anesth. Suppl.* 7, S24-S33.

Mato, J.M. & Alemany, S. (1983) *Biochem. J.* 213, 1-10.

Mato, J.M., Pajares, M.A. & Varela, I. (1984) *Trends Biochem. Sci.* 9, 471-472.

May, N.D.S. (1970) *The Anatomy of the Sheep*, 3rd. edn., University of Queensland Press.

McGilvery, R.W. (1970) *Biochemistry : a functional approach*, pp. 409-438, W.B. Saunders Company, Philadelphia, London, Toronto.

McMurray, W.C. (1973) in *Form and Function of Phospholipids* (Ansell, G.B., Hawthorne, J.N. & Dawson, R.M.C., eds.), 2nd rev. edn., B.B.A. Library vol. 3, pp. 205-251, Elsevier Scientific Publishing Company, Amsterdam.

Messmer, K., Sunder-Plassmann, L., Klovekorn, W.P. & Holper, K. (1972) *Adv. Microcirc.* 4, 1-77.

Michel, M. (1956) *C.R. Hebd. Séances Acad. Sci.* 242, 2883-2886.

Mitchell, A.D. & Benevenga, N.J. (1978) *J. Nutr.* 108, 67-78.

Mitchell, A.D., Chappell, A. & Knox, K.L. (1979) *J. Anim. Sci.* 49, 764-774.

Mogelson, S. & Sobel, B.E. (1981) *Biochim. Biophys. Acta* 666, 205-211.

Mookerjea, S. (1971) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 30, 143-150.

Moore, S.L., Godley, W.C., Van Vliet, G., Lewis, J.P., Boyd, E. & Huisman, T.H.J. (1966) *Blood* 28, 314-329.

Morell, P. & Braun, P. (1972) *J. Lipid Res.* 13, 293-310.

Mozzi , R. & Porcellati, G. (1979) *FEBS Lett.* 100, 363-366.

Mudd, S.H. (1973) in *The Enzymes* (Boyer, P.D., ed.), 3rd edn., vol.8, pp. 121-154, Academic Press, New York and London.

Mudd, S.H. & Cantoni, G.L. (1964) in *Comprehensive Biochemistry* (Florkin, M. & Stotz, E.H., eds.), vol. 15, pp. 1-47, Elsevier Publishing Company, Amsterdam, London, New York.

Mudd, S.H. & Poole, J.R. (1975) *Metabolism* 24, 721-735.

Mudd, S.H., Ebert, M.H. & Scriver, C.R. (1980) *Metabolism* 29, 707-720.

Nachmansohn, D. & Machado, A.L. (1943) *J. Neurophysiol.* 6, 397-403.

Nash, D., Paleg, L.G. & Wiskich, J.T. (1982) *Aust. J. Plant Physiol.* 9, 47-57.

National Research Council (1974) *Nutrient Requirements of Domestic Animals No.8.Nutrient Requirements of Dogs*, Rev. edn., Nat. Acad. Sci., Washington, D.C..

National Research Council (1975) *Nutrient Requirements of Domestic Animals No.5. Nutrient Requirements of Sheep*, 5th rev. edn., Nat. Acad. Sci., Washington, D.C.

National Research Council (1976) *Nutrient Requirements of Domestic Animals No. 4. Nutrient Requirements of Beef Cattle*, 5th rev. edn., Nat. Acad. Sci., Washington, D.C.

National Research Council (1977*a*) *Nutrient Requirements of Domestic Animals No.1. Nutrient Requirements of Poultry*, 7th rev. edn., Nat. Acad. Sci., Washington, D.C.

National Research Council (1977*b*) *Nutrient Requirements of Domestic Animals No.9. Nutrient Requirements of Rabbits*, 2nd rev. edn., Nat. Acad. Sci., Washington, D.C.

National Research Council (1978*a*) *Nutrient Requirements of Domestic Animals No.10. Nutrient Requirements of Laboratory Animals*, 3rd rev. edn., Nat. Acad. Sci., Washington, D.C.

National Research Council (1978*b*) *Nutrient Requirements of Domestic Animals No.13. Nutrient Requirements of Cats*, rev. edn., Nat. Acad. Sci., Washington, D.C.

National Research Council (1978c) *Nutrient Requirements of Domestic Animals No.3. Nutrient Requirements of Dairy Cattle*, 5th rev. edn., Nat. Acad. Sci., Washington, D.C.

National Research Council (1979) *Nutrient Requirements of Domestic Animals No.2. Nutrient Requirements of Swine*, 8th rev. edn., Nat. Acad. Sci., Washington, D.C.

Nayman, R., Thomson, M.E., Scriver, C.R. & Clow, C.L. (1979) *Am. J. Clin. Nutr.* 32, 1279–1289.

Neill, A.R. & Dawson, R.M.C. (1977) *Proc. Aust. Biochem. Soc.* 10, 54.

Neill, A.R., Grime, D.W. & Dawson, R.M.C. (1978) *Biochem. J.* 170, 529–535.

Neill, A.R., Grime, D.W., Snoswell, A.M., Northrop, A.J., Lindsay, D.B. & Dawson, R.M.C. (1979) *Biochem. J.* 180, 559-565.

Noble, R.C. (1978) *Prog. Lipid Res.* 17, 55-91.

Noble, R.C., Steele, W. & Moore, J.H. (1971) *Lipids* 6, 926-929.

Nomenclature Committee of the International Union of Biochemistry (1979) *Enzyme Nomenclature, Recommendations 1978*, Academic Press, Inc., New York.

Nomenclature Committee of the International Union of Biochemistry (1980) *Enzyme Nomenclature, Recommendations 1978, Supplement 1 : Corrections and Additions* *Eur. J. Biochem.* 104, 1-4.

Nomenclature Committee of the International Union of Biochemistry (1981) *Enzyme Nomenclature, Recommendations 1978, Supplement 2 : Corrections and Additions, Eur. J. Biochem.* 116, 423-435.

Osborne-White, W.S. & Smith, R.M. (1973) *Biochenm. J.* 136, 265-278.

Pajares, M.A., Alemany, S., Varela, I., Marin Cao, D. & Mato, J.M. (1984) *Biochem. J.* 223, 61-66.

Paleg, L.G., Douglas, T.J., Van Daal, A. & Keech, D.B. (1981) *Aust. J. Plant Physiol.* 8, 107-114.

Pappenheimer, J.R. & Setchell, B.P. (1972) *J. Physiol. (London)* 226, 48P-50P.

Pasquini, J.M., Krawiec, L. & Soto, E.F. (1973) *J. Neurochem.* 21, 647-653.

Peng, C.-T. (1977) *Sample Preparation in Liquid Scintillation Counting Review 17*, Amersham International Ltd., Amersham, U.K.

Peter, K., Van Ackern, K., Berend, D., Buchert, W., Kersting, K.-H., Kraatz, J., Lutz, H. & Schade, W. (1975) in *International Hemodilution, Biblthca Haemat., no.41*(Messmer, K. & Schmid-Scliobein, H., eds.), pp. 260-269, Karger, Basel.

Pethick, D.W. (1975) Honours Thesis, University of Adelaide.

Pethick, D.W. (1980) Ph.D. Thesis, University of Cambridge.

Pethick, D.W., Lindsay, D.B., Barker, P.J. & Northrop, A.J. (1981) *Br. J. Nutr.* 46, 97-110.

Phillis, J.W. (1976) in *Veterinary Physiology* (Phillis, J.W., ed.), pp. 416-459, Wright-Scientechnica, Bristol.

Pike, M.C., Kredich, N.M. & Snyderman, R. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 2922-2926.

Porcellati, G. (1972) *Adv. Enzyme Regul.* 10, 83-100.

Radcliffe, B.C. & Egan, A.R. (1974) *Aust. J. Biol. Sci.* 27, 465-471.

Radcliffe, B.C. & Egan, A.R. (1978) *Aust. J. Biol. Sci.* 31, 105-114.

Read, L.C. (1976) Honours Thesis, University of Adelaide.

Rehbinder, D. & Greenberg, D.M. (1965) *Arch. Biochem. Biophys.* 109, 110-115.

Reid, M.E. (1955) *J. Nutr.* 56, 215-229.

Reid, W.D., Haubrich, D.R. & Krishna, G (1971) *Anal. Biochem.* 42, 390-397.

Reis, P.J., Tunks, D.A. & Downes, A.M. (1973*a*) *Aust. J. Biol. Sci.* 26, 249-258.

Reis, P.J., Tunks, D.A. & Sharry, L.F. (1973*b*) *Aust. J. Biol. Sci.* 26, 635-644.

Rendina, G. & Singer, T.P. (1959) *J. Biol. Chem.* 234, 1605-1610.

Rennick, B., Acara, M. & Glor, M. (1977) *Am. J. Physiol.* 232, F443-F447.

Richardson, P.D.I. & Withrington, P.G. (1981) *Gastroenterology* 81, 159-173.

Robins, S.J. (1974) *Gastroenterology* 67, 823.

Robins, S.J. (1975) *Am. J. Physiol.* 229, 598-602.

Robins, S.J. & Armstrong, M.J. (1976) *Gastroenterology* 70, 397-402.

Robins, S.J. & Brunengraber, H. (1982) *J. Lipid Res.* 23, 604-608.

Robinson, B.S. (1980) Honours Thesis, University of Adelaide.

Rooney, S.A. (1979) *Trends Biochem. Sci.* 4, 189-191.

Rosenberry, T.L. (1975) *Adv. Enzymolog.* 43, 103-218.

Rothman, J.E. & Lenard, J. (1977) *Science* 195, 743-753.

Rothschild, H.A. & Barron, E.S.G. (1954) *J. Biol. Chem.* 209, 511-523.

Roughan, P.G. & Batt, R.D. (1969) *Phytochemistry* 8, 363-369.

Runciman, W.B. (1982) Ph.D. Thesis, Flinders University of South Australia.

Runciman, W.B., Ilsley, A.H., Mather, L.E., Carapetis, R. & Rao, M.M. (1984) *Br. J. Anaesth.* 56, 1015-1028.

Ryan, R.L. & McClure, W.O. (1979) *Biochemistry* 18, 5357-5365.

Rytter, D., Miller, J.E. & Cornatzer, W.E. (1968) *Biochim. Biophys. Acta* 152, 418-421.

Salerno, D.M. & Beeler, D.A. (1973) *Biochim. Biophys. Acta* 326, 325-338.

Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H.G. & Schlenk, F. (1977) *The Biochemistry of Adenosylmethionine*, Columbia University Press, New York.

Sastry, B.V.R., Olubadewo, J., Harbison, R.D. & Schmidt, D.E. (1976) *Biochem. Pharmacol.* 25, 425-431.

Sastry, B.V.R., Statham, C.N., Axelrod, J. & Hirata, F. (1981) *Arch. Biochem. Biophys.* 211, 762-773.

Saunders, D.R. (1970) *Gastroenterology* 59, 848-852.

Scarborough, G.A. & Nyc, J.F. (1967*a*) *Biochim. Biophys. Acta* 146, 111-119.

Scarborough, G.A. & Nyc, J.F. (1967*b*) *J. Biol. Chem.* 242, 238-242.

Schanche, J.-S., Schanche, T. & Ueland, P.M. (1981) *Mol. Pharmacol.* 20, 631-636.

Schelling, G.T., Chandler, J.E. & Scott, G.C. (1973) *J. Anim. Sci.* 37, 1034-1039.

Schmidt, G., Hecht, L., Fallot, P., Greenbaum, L. & Thannhauser, S.J. (1952) *J. Biol. Chem.* 197, 601-609.

Schmidt, G., Greenbaum, L.M., Fallot, P., Walker, A.C. & Thannhauser, S.J. (1955) *J. Biol. Chem.* 212, 887-895.

Schneider, W.J. & Vance, D.E. (1978) *Eur. J. Biochem.* 85, 181-187.

Schneider, W.J. & Vance, D.E. (1979) *J. Biol. Chem.* 254, 3886-3891.

Sethi, J.K. & Carew, D.P. (1974) *Phytochemistry* 13, 321-324.

Shea, P.A. & Aprison, M.H. (1973) *Anal. Biochem.* 56, 165-177.

Sidransky, H. & Farber, E. (1960) *Arch. Biochem. Biophys.* 87, 129-133.

Siliprandi, N. (1980) in *Carnitine Biosynthesis, Metabolism and Functions* (Frenkel, R.A. & McGarry, J.D., eds.), p. 204, Academic Press, Inc., New York.

Skurdal, D.N. & Cornatzer, W.E. (1974) *Proc. Soc. Exp. Biol. Med.* 145, 992-995.

Skurdal, D.N. & Cornatzer, W.E. (1975) *Int. J. Biochem.* 6, 579-583.

Smith, A.L. (1979) Ph.D. Thesis, University of Adelaide.

Smith, R.M., Osborne-White, W.S. & Gawthorne, J.M. (1974) *Biochem. J.* 142, 105-117.

Snoswell, A.M. & Henderson, G.D. (1970) *Biochem. J.* 119, 59-65.

Snoswell, A.M. & Henderson, G.D. (1980) in *Carnitine Biosynthesis, Metabolism and Functions* (Frenkel, R.A. & McGarry, J.D., eds.), pp. 191-205, Academic Press, Inc., New York.

Snoswell, A.M. & Koundakjian, P.P. (1972) *Biochem. J.* 127, 133-141.

Snoswell, A.M. & Mann, S.P. (1978) *Proc. Aust. Biochem. Soc.* 11, 37.

Snoswell, A.M. & McIntosh, G.H. (1974) *Aust. J. Biol. Sci.* 27, 645-650.

Snoswell, A.M., Henderson, G.D. & Dawson, R.M.C. (1978) *Proc. Aust. Nutr. Soc.* 3, 82.

Sottocasa, G.L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967) *J. Cell. Biol.* 32, 415-438.

Spanner, S., Hall, R.C. & Ansell, G.B. (1976) *Biochem. J.* 154, 133-140.

Spitzer, H.L., Norman, J.R. & Morrison, K. (1969) *Biochim. Biophys. Acta* 176, 584-590.

Stadtman, E.R. (1957) in *Methods in Enzymology* (Colowick, S.P. & Kaplan, N.O., eds.), vol. 3, pp. 931-941, Academic Press, Inc., New York.

Steele, R.D. & Benevenga, N.J. (1978) *J. Biol. Chem.* 253, 7844-7850.

Stein, O. & Stein, Y. (1969) *J. Cell Biol.* 40, 461-483.

Stoffel, W. (1971) *Ann. Rev. Biochem.* 40, 57-82.

Storey, R. (1976) Ph.D. Thesis, University of Wales, Cardiff.

Storey, R. & Wyn Jones, R.G. (1975) *Plant Sci. Lett.* 4, 161-168.

Storey, R. & Wyn Jones, R.G. (1977) *Phytochemistry* 16, 447-453.

Storey, R., Ahmad, N. & Wyn Jones, R.G. (1977) *Oecologia (Berl.)* 27, 319-332.

Strength, D.R., Yu, S.Y. & Davis, E.Y. (1965) in *Recent Research on Carnitine* (Wolf, G., ed.), pp. 45-56, Massachusetts Institute of Technology Press, Cambridge, Mass.

Streumer-Svobodová, Z. & Drahota, Z. (1977) *Physiol. Bohemoslov.* 26, 525-534.

Sundler, R. & Åkesson, B. (1975*a*) *Biochem. J.* 146, 309-315.

Sundler, R. & Åkesson, B. (1975*b*) *J. Biol. Chem.* 250, 3359-3367.

Sundler, R., Arvidson, G. & Åkesson, B. (1972) *Biochim. Biophys. Acta* 280, 559-568.

Sung, C.P. & Johnstone, R.M. (1969) *Biochim. Biophys. Acta* 173, 548-553.

Swingle, R.S. & Dyer, I.A. (1970) *J. Anim. Sci.* 31, 404-408.

Takayama, M., Itoh, S., Nagasaki, T. & Tanimizu, I. (1977) *Clin. Chim. Acta* 79, 93-98.

Talalay, P. (1960) *Methods Biochem. Anal.* 8, 119-143.

Tanaka, Y., Doi, O. & Akamatsu, Y. (1979) *Biochem. Biophys. Res. Commun.* 87, 1109-1115.

Taylor, P.H., Wallace, J.C. & Keech, D.B. (1971) *Biochim. Biophys. Acta* 237, 179-191.

Thompson, G.A., Jr. (1973) in *Form and Function of Phospholipids* (Ansell, G.B., Hawthorne, J.N. & Dawson, R.M.C., eds.), 2nd rev. edn., B.B.A. Library vol. 3, pp. 67-96, Elsevier Scientific Publishing Company, Amsterdam.

Thompson, G.A., Jr. (1980) *The Regulation of Membrane Lipid Metabolism*, pp. 75-103, CRC Press, Inc., Boca Raton, Florida.

Thompson, G.E., Bassett, J.M. & Bell, A.W. (1978) *Br. J. Nutr.* 39, 219-225.

Thompson, J.A. & Reitz, R.C. (1976) *Ann. N.Y. Acad. Sci.* 273, 194-204.

Thompson, W., MacDonald, G. & Mookerjea, S. (1969) *Biochim. Biophys Acta* 176, 306-315.

Toyosawa, I. & Nishimoto, U. (1967) *Agric. Biol. Chem.* 31, 275-283.

Treble, D.H., Frumkin, S., Balint, J.A. & Beeler, D.A. (1970) *Biochim. Biophys. Acta* 202, 163-171.

Tsielens, E. (1954) in *Vitaminnye Resursy i ikh Isopol'zovanie*, Sbornik 2, p. 136, Moscow : Akad. Nauk. S.S.S.R. [cited from *Dairy Sci. Abstr.* (1957) 19, 146*a*]

Tuček, S., Havránek, M. & Ge, I. (1978) *Anal. Biochem.* 84, 589-593.

Tuma, D.J., Barak, A.J., Schafer, D.F. & Sorrell, M.F. (1973) *Can. J. Biochem.* 51, 117-120.

Tyler, D.D., Gonze, J. & Estabrook, R.W. (1966) *Arch. Biochem. Biophys.* 115, 373-384.

Uthus, E.O., Skurdal, D.N. & Cornatzer, W.E. (1976) *Lipids* 11, 641-644.

Vance, D.E. & Choy, P.C. (1979) *Trends Biochem. Sci.* 4, 145-148.

Vance, D.E. & De Kruijff, B. (1980) *Nature (London)* 288, 277-278.

Vance, D.E., Trip, E.M. & Paddon, H.B. (1980) *J. Biol. Chem.* 255, 1064-1069.

Vance, D.E., Audubert, F. & Pritchard, P.H. (1982) in *Biochemistry of S-Adenosylmethionine and Related Compounds* (Usdin, E., Borchardt, R.T. & Creveling, C.R., eds.), pp. 119-128, McMillan Press, London.

Van den Bosch, H. (1974) *Annu. Rev. Biochem.* 43, 243-277.

Van den Bosch, H. (1980) *Biochim. Biophys. Acta* 604, 191-246.

Van den Bosch, H., Bonte, H.A. & Van Deenen, L.L.M. (1965) *Biochim. Biophys. Acta* 98, 648-651.

Van den Bosch, H., Van Golde, L.M.G. & Van Deenen, L.L.M. (1972) *Rev. Physiol. Biochem. Pharmacol.* 66, 13-146.

Van Golde, L.M.G. & Van den Bergh, S.G. (1977*a*) in *Lipid Metabolism in Mammals* (Snyder, F., ed.), vol. 1, pp. 1-33, Plenum Press, New York and London.

Van Golde, L.M.G. & Van den Bergh, S.G. (1977*b*) in *Lipid Metabolism in Mammals* (Snyder, F., ed.), vol. 1, pp. 35-149, Plenum Press, New York and London.

Wajda, I.J., Manigault, I., Hudick, J.P. & Lajtha, A. (1973) *J. Neurochem.* 21, 1385-1401.

Wall, J.S., Christianson, D.D., Dimler, R.J. & Senti, F.R. (1960) *Anal. Chem.* 32, 870-874.

Wallace, J.C. & White, I.G. (1965) *J. Reprod. Fertil.* 9, 163-176.

Wang, F.L. & Haubrich, D.R. (1975) *Anal. Biochem.* 63, 195-201.

Warner, H.R. & Lands, W.E.M. (1961) *J. Biol. Chem.* 236, 2404-2409.

Waugh, R.K., Hauge, S.M. & King, W.A. (1947*a*) *J. Dairy Sci.* 30, 457-461.

Waugh, R.K., Hauge, S.M. & King, W.A. (1947*b*) *J. Dairy Sci.* 30, 641-648.

Webster, G.R. & Cooper, M. (1968) *J. Neurochem.* 15, 795-802.

Webster, G.R., Marples, E.A. & Thompson, R.H.S. (1957) *Biochem. J.* 65, 374-377.

Weinhold, P.A. & Sanders, R. (1973) *Life Sci.* 13, 621-629.

Weinhold, P.A., Skinner, R.S. & Sanders, R.D. (1973) *Biochim. Biophys. Acta* 326, 43-51.

Weller, R.A. & Pilgrim, A.F. (1974) *Br. J. Nutr.* 32, 341-351.

Wells, I.C. (1971) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 30, 151-154.

Welsch, F. (1978) *Biochem. Pharmacol.* 27, 1251-1257.

Wheeler, J.L. & Hutchinson, K.J. (1973) in *The Pastoral Industries of Australia* (Alexander, G. & Williams, O.B., eds.), pp. 201-232, Sydney University Press, Sydney.

White, D.A. (1973) in *Form and Function of Phospholipids* (Ansell, G.B., Hawthorne, J.N. & Dawson, R.M.C., eds.), 2nd rev. edn., B.B.A. Library vol. 3, pp. 441-482, Elsevier Scientific Publishing Company, Amsterdam.

Williams, G.R. (1960) *J. Biol. Chem.* 235, 1192-1195.

Williams, J.N., Jr. (1952) *J. Biol. Chem.* 195, 37-41.

Wise, E.M., Jr. & Elwyn, D. (1965) *J. Biol. Chem.* 240, 1537-1548.

Wong, E.R. & Thompson, W. (1972) *Biochim. Biophys. Acta* 260, 259-271.

Wren, J.J. & Szczepanowska, A.D. (1964) *J. Chromatogr.* 14, 405-410.

Wurtman, R.J. (1979) in *Nutrition and the Brain* (Barbeau, A., Growdon, J.H. & Wurtman, R.J., eds.), vol. 5, pp. 73-81, Raven Press, New York.

Wykle, R.L. & Snyder, F. (1976) in *The Enzymes of Biological Membranes* (Martonosi, A., ed.), vol. 2., pp. 87-117, Plenum Press, New York.

Wyn Jones, R.G. & Storey, R. (1978) *Aust. J. Plant Physiol.* 5, 817-829.

Wyn Jones, R.G. & Storey, R. (1981) in *The Physiology and Biochemistry of Drought Resistance in Plants* (Paleg, L.G. & Aspinall, D., eds.), pp. 171-204, Academic Press, Sydney.

Xue, G.-P. & Snoswell, A.M. (1985) *Comp. Biochem. Physiol.* 80B, 489-494.

Yamamoto, A., Sano, M. & Isozaki, M. (1969) *J. Biochem.* (Tokyo) 65, 85-91.

Yue, K.T.N., Russell, P.J. & Mulford, D.J. (1966) *Biochim. Biophys. Acta* 118, 191-194.

Zatz, M., Dudley, P.A., Kloog, Y. & Markey, S.P. (1981) *J. Biol. Chem.* 256, 10028-10032.

Zatz, M., Engelsen, S.J. & Markey, S.P. (1982) *J. Biol. Chem.* 257, 13673-13678.

Zeisel, S.H. (1981) *Annu. Rev. Nutr.* 1, 95-121.

Zeisel, S.H. (1985) *Biochim. Biophys. Acta* 835, 331-343.

Zeisel, S.H. & Wurtman, R.J. (1981) *Biochem. J.* 198, 565-570.

Zeisel, S.H., Epstein, M.F. & Wurtman, R.J. (1980*a*) *Life Sci.* 26, 1827-1831.

Zeisel, S.H., Story, D.L., Wurtman, R.J. & Brunengraber, H. (1980*b*) *Proc. Natl. Acad. Sci. U.S.A.* 77, 4417-4419.

Zwaal, R.F.A., Fluckiger, T., Moser, S. & Zahler, P. (1974) *Biochim. Biophys. Acta* 373, 416-424.