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THE OXIDATION OF LINOLEATE AND
OTHER LONG-CHAIN FATTY ACIDS
IN RAT AND SHEEP LIVER
MITOCHONDRIA

A thesis presented in partial fulfilment
of the requirement for the degree of
Doctor of Philosophy
in Biochemistry
at
Massey University
New Zealand

John Campbell William Reid
1986
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Abstract

Sheep liver mitochondria oxidised palmitate, oleate and linoleate at slower rates than did rat liver mitochondria. Rat liver mitochondria oxidised linoleate at 1.2 to 1.7 times the rate observed with palmitate as the substrate. However, sheep liver mitochondria oxidised linoleate at 0.74 to 0.84 the rate observed when palmitate was the substrate. The biochemical basis of this difference is not understood.

The reaction catalysed by the enzyme carnitine acyltransferase I is believed to be an important regulatory step in the oxidation of long-chain fatty acids and is known to be competitively inhibited by malonyl-CoA. Both rat and sheep liver mitochondria were able to form acyl carnitine when palmitoyl-CoA and linoleate, coupled with an acyl-CoA generating system, were the acyl substrates.

Malonyl-CoA was very effective in inhibiting the CAT I reaction in sheep liver mitochondria. When linoleate, coupled with an acyl-CoA generating system, was the substrate for CAT I, 1 μM malonyl-CoA was found to inhibit the reaction by 90%. However, when the same substrate was assayed in rat liver mitochondria the inhibition was much less, 22 μM malonyl-CoA leading to only 50% inhibition of the CAT I enzyme. When palmitoyl-CoA was used as a substrate for the enzyme CAT I, little difference was seen between rat and sheep liver mitochondria in the extent of inhibition observed over the concentration range of 1 to 5 μM malonyl-CoA.

These experiments indicate that sheep liver mitochondria could oxidise palmitate rather than linoleate at low levels of malonyl-CoA, as one might expect in vivo. In contrast, in rat liver mitochondria, linoleate would be oxidised faster than palmitate at all concentrations of malonyl-CoA investigated.

It is suggested that this system may be an important means whereby sheep are able to conserve linoleate by preventing its oxidation.
In addition the mitochondrial glycerol 3-phosphate acyltransferase reaction was investigated with both sheep and rat liver mitochondria. With linoleate and an acyl-CoA generating system, rat liver preparations esterified 1.5 nmoles min/mg protein whereas sheep liver mitochondria esterified less than one tenth of this. It was concluded esterification of linoleate to glycerol 3-phosphate is not an important mechanism of conserving linoleate in sheep liver mitochondria.

Esterification of palmitate to glycerol 3-phosphate was studied using palmitoyl-CoA as the acyl donor. At maximal rates of esterification it was observed that rat liver mitochondria esterified palmitoyl-CoA at 2 nmoles/min/mg whereas sheep mitochondria esterified 0.8 nmoles/min/mg.
Acknowledgement

I wish to thank my supervisor Dr D.R. Husbands for his advice, encouragement and sense of humour throughout this work.

I would also like to thank Dr J.C. Hawke for his advice throughout this work especially in the writing of this thesis.

I am also grateful to Earl Stevens for his help with statistical analysis and for his general support.

An extended thanks is given to members of the Chem/Biochem department and to many other friends for their support throughout this work.
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<td>CAT</td>
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<tr>
<td>CPT</td>
<td>carnitine palmitoyltransferase</td>
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<tr>
<td>GPAT</td>
<td>glycerol 3-phosphate acyltransferase</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
</tr>
<tr>
<td>POPOP</td>
<td>1,4-bis[2-(5-phenyloxazolyl)]benzene</td>
</tr>
<tr>
<td>NbS$_2$</td>
<td>5,5'-dithiobis-(2-nitrobenzoic acid)</td>
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<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis-($\beta$-amino-ethyl ether) N,N'-tetra-acetic acid</td>
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<td>g</td>
<td>gravitational force</td>
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INTRODUCTION

1.1 The requirements of animals for linoleic acid

In simple stomached animals an intake of 1-2% of the total dietary energy as essential fatty acids is sufficient to prevent the appearance of deficiency signs, e.g. scaly paws, skin and tail, fatty livers and decreased growth rate (Holman, 1968). In the ruminant, although the dietary intake of linoleic acid usually exceeds this value (Scott, 1971), the hydrogenation of unsaturated fatty acids by the rumen microflora (Dawson and Kemp, 1970) considerably reduces the amount of linoleic acid available to the animal. Leat and Harrison (1972) have shown that, as a result of this biohydrogenation of unsaturated fatty acids in the rumen, the digesta leaving the abomasum and entering the duodenum contains only 0.3 to 0.5% of the total energy as linoleic acid. Although these levels of linoleic acid available to the ruminant would produce deficiency signs in non-ruminants, essential fatty acid deficiency has not been reported in the adult ruminant and no limitation on growth and production recorded. This ability of the ruminant animal to survive and grow on such low levels of linoleic acid suggests that these animals have a highly efficient method of conserving these fatty acids.

1.2 Synthesis of long-chain fatty acids by animal cells

Monounsaturated and saturated fatty acids can be synthesized de novo in the animal cell. The cytoplasmic fatty acid synthetase complex (Wakil et al., 1983) produces palmitic acid as a major product which can be elongated by the microsomal malonyl-CoA-dependent elongation system (Jeffcoat, 1979) to stearic acid and longer-chain saturated acids. These saturated fatty acids can in turn be desaturated to form monoenoic acids by the microsomal desaturase (Jeffcoat, 1979). In contrast, many polyunsaturated fatty acids found in mammalian tissues are derived from linoleic acid or α-linolenic acid (Fig. 1) which the animal must obtain from its diet due to its
Fig. 1 The biosynthesis of long-chain polyunsaturated fatty acids from linoleic acid by a combination of desaturation and elongation reactions (Brenner, 1981).
inability to synthesize these acids de novo. These fatty acids in the linoleate and linolenate family, which are essential for the animal's well being, are termed essential fatty acids (Burr and Burr, 1929; Mead, 1981; Lough and Garton, 1958; Reiser and Reddy, 1956; Shorland et al., 1955).

1.3 Hydrogenation of polyunsaturated fatty acids in the rumen

The major lipid constituents of pasture grasses are galactosyl-glycerides and phospholipids and the constituent fatty acids are predominantly linolenic, linoleic and palmitic acids (Garton, 1967). The acyl moieties are released as free fatty acids in the rumen by the action of lipolytic enzymes secreted by rumen micro-organisms (Garton et al., 1958; Faruque et al., 1974). These unsaturated fatty acids are rapidly hydrogenated by the rumen microflora (Reiser, 1951; Garton, 1964; Girard and Hawke, 1978). This biohydrogenation of unsaturated fatty acids is thought to proceed in a stepwise manner, with the rapid hydrogenation of the trienoic to monoenoic acids, followed by a slower conversion of the monoenoic to saturated acids (Singh and Hawke, 1979; Kepler and Tove, 1967). As a result of this process of hydrogenation the major reaction products are monoenoic and saturated acids with only trace amounts of polyunsaturated acids.

Rumen protozoa are known to absorb linoleic acid (Gutierrez et al., 1962) which is reflected in the high proportions of this fatty acid in protozoal phospholipids (Katz and Keeney, 1967).

It has been suggested that dietary linoleic acid may escape hydrogenation and thereby be conserved by its rapid uptake and incorporation into protozoal lipids (Keeney, 1970; Girard and Hawke, 1978).

During the hydrogenation of polyunsaturated fatty acids, considerable positional and geometrical isomerisation
occurs which leads to the formation of 18 carbon dienoic acids that are quite distinct from linoleic acid (Shorland et al., 1957; Ward et al., 1964). This isomerisation is reflected in the occurrence of these unsaturated C\textsubscript{18} isomers in ruminant tissue lipids (Shorland, 1962).

1.4 Function of long-chain polyunsaturated fatty acids in cell membranes

Cell membranes contain a variety of lipids, the fatty acid composition of which determines many of the properties exhibited by the membrane. These membranes must contain polyunsaturated fatty acids if they are to serve their physiological function in the animal cell (Singer and Nicholson, 1972; Fry and Green, 1981; Harb et al., 1981). Membranes within cells have a variety of functions (Chapman, 1972) and these functions are dependent on the careful control of the physical properties of the membrane, which are influenced by the lipid components. In order to maintain an effective functional environment, the fatty acid composition of the membrane lipids must be regulated within narrow limits.

The essential fatty acids which are associated with the structural lipids of the membranes occupy the two-position of glycerophospholipids (Hill and Lands, 1970) and these phospholipid components of the membrane show a species- and tissue-specific pattern (Crawford et al., 1981). The presence of polyunsaturated acyl groups is thought to maintain a fluid-crystalline environment within the membrane by disrupting the close packing exhibited by saturated acyl chains (Houslay and Stanley, 1982). As illustrated in Fig. 2 the incorporation of double bonds in the \textit{cis} configuration introduces a bend in the acyl chain and prevents the close packing that is seen with saturated acyl chains.

In addition to the structural role played by essential fatty acids they can also be metabolized to prostacyclin, prostaglandins and thromboxananes which have important
Fig. 2 Schematic representation of the fluid mosaic model for membrane structure proposed by Singer and Nicholson (1972).

The above illustration shows the orientation of membrane proteins, both integral (those embedded in the lipid bilayer) and peripheral proteins (those associated with the membrane surface), with the charged groups of the protein molecule facing the aqueous medium on either side of the lipid bilayer and the non-polar groups associated with the hydrophobic core of this bilayer. As well as proteins the membrane phospholipids are also orientated so that the charged groups (i.e. phosphate and amine groups) are facing the aqueous medium while the fatty-acyl chains esterified to positions 1 and 2 of the glycerol molecule are orientated towards the hydrophobic core. The fluidity of the membrane structure is controlled by the fatty acid composition of these phospholipids. The above diagram illustrates the effect of introducing a cis double bond on the close packing seen with the saturated acyl chains.
physiological effects on cellular metabolism (Bergstrom et al., 1964; Moncada et al., 1976).

It is now believed that it is the longer chain derivatives, synthesized from these essential acids, rather than the essential fatty acids themselves that are important in correcting essential fatty acid deficiency signs (Rivers et al., 1975).

1.5 Plasma entry rates of fatty acids in sheep

Lindsay and Leat (1977) observed that in sheep, the entry rate of linoleic acid and its contribution to CO₂ production was markedly less than that observed using stearic acid. Under starvation conditions sheep were found to preferentially mobilize palmitic, stearic and oleic acids rather than linoleic acid. However, the biochemical mechanism by which linoleic acid is selectively retained in sheep is not yet known.

Results from many laboratories suggest that, in general, dietary essential fatty acids are not conserved as they are absorbed from the small intestine, but like non-essential fatty acids have an equal chance of being oxidatively degraded in the intestinal mucosa. Mead et al. (1956) have shown that the recovery of 14C in expired CO₂ after feeding carboxyl-labelled stearate, oleate, and linoleate to mice was higher for linoleate and oleate than for stearate, when measured up to 10 hrs after feeding. As discussed below this has been confirmed in the present study (Reid and Husbands, 1985). However, it is thought that after uptake by the tissues, essential fatty acids may be protected by virtue of the types of compounds and structures in which they are located.

1.6 Metabolism of linoleic acid in the animal cell

The cellular metabolism of linoleic acid is diagrammatically represented in Fig. 3. Linoleic acid is made available to the liver cell by its release from
lipoprotein complexes circulating in the plasma, where it exists mainly as cholesterol ester (Lewis, 1971; Scanu, 1971). The cholesterol ester is taken up by the cell, as part of the partially degraded lipoprotein complex, and hydrolyzed to free linoleic acid and cholesterol by the enzyme cholesterol esterase (Sabine, 1977; Stokke, 1972), found in the cytoplasm of most mammalian cells. Linoleic acid, made available by the enzyme cholesterol esterase, is bound by a fatty acid binding protein or Z protein (Fournier and Rahim, 1983) present in the cytoplasm. The first step in the metabolism of linoleic acid and other fatty acids is the formation of their CoA esters by the enzyme fatty acyl-CoA synthetase (Groot et al., 1976), which is a membrane-bound enzyme located in the membrane fractions of mitochondria (Van Tol and Hulsmann, 1970), endoplasmic reticulum (Suzue and Marcel, 1972) and peroxisomes (Shindo and Hashimoto, 1978). The fatty acyl chain is now available as a substrate for three major pathways, namely, \(\beta\)-oxidation, esterification into lipids and modification to other fatty acids.

1.6.1 \(\beta\)-oxidation

The enzymes of \(\beta\)-oxidation are located in both the peroxisomes (Lazarow, 1978) and mitochondria (Fritz, 1961). This oxidative pathway degrades the hydrocarbon backbone of the long-chain fatty acid to smaller chain acyl derivatives, in the case of peroxisomal metabolism (Lazarow, 1978), and complete degradation to acetyl-CoA when considering mitochondrial metabolism. The acetyl-CoA units produced during mitochondrial oxidation can in turn be further metabolized to ketone bodies (Middleton, 1978) or completely oxidized to \(\text{CO}_2\), \(\text{H}_2\text{O}\), with the release of mechanical and thermal energy.

1.6.2 Esterification

Linoleoyl-CoA, and other fatty acyl thioesters, can be esterified to either glycerol 3-phosphate (Kornberg and Pricer, 1953; Van Golde and Van den Bergh, 1977) or dihydroxyacetone phosphate (La Belle and Hajra, 1972) to
Fig. 3 Metabolism of linoleic acid in the mammalian hepatocyte

Most of the linoleic acid taken up from the plasma by the hepatocyte is as cholesterol ester and phospholipid. The linoleic acid produced following the breakdown of these lipid components acts as a substrate for a number of biochemical pathways within the cell.
form phosphatidic acid by the esterifying enzymes of the endoplasmic reticulum (Yamashita et al., 1973) and peroxisomes (Jones and Hajra, 1977). Phosphatidic acid is then converted to a variety of phospholipids. These phospholipids can be directly incorporated into the membrane structure (Jos and Den Kamp, 1979), where they form more stable complexes as structural lipids.

1.6.3 Elongation/Desaturation

Linoleoyl-CoA can be converted to other members of the linoleic acid family by the malonyl-CoA dependent elongation system followed by direct oxidative desaturation, both enzymatic activities being associated with the microsomes (Jeffcoat, 1979). Polyunsaturated fatty acids derived from linoleic acid are then available for incorporation into phospholipids which may be utilized as structural lipids of the membranes.

It is possible to hypothesize that the acylating enzymes involved in the biosynthesis of phosphatidic acid exhibit a preference for polyunsaturated fatty acids, in particular linoleic or other polyunsaturated fatty acids derived from linoleic acid. This could act as a mechanism for conserving essential fatty acids by removing them from the pool of fatty acids that would otherwise be available for β-oxidation.

However, there appears to be no evidence in the literature for the preferential acylation of essential fatty acids into phospholipids by ruminants. Instead, the enzymes responsible for the acylation of glycerol 3-phosphate show similar properties when isolated from a range of animals (Daae, 1973).

Alternatively the pathway of β-oxidation could provide a mechanism for conserving essential fatty acids. This pathway is potentially of great importance in regulating both the quantity and, by selective degradation, the variety of fatty acids available to the animal. The β-oxidation pathway could therefore serve as a possible mechanism by
which essential fatty acids are conserved through the preferential oxidation of saturated and monoene fatty acids.

1.7 Free fatty acids as mitochondrial substrates

Long-chain fatty acids are oxidized by the mitochondrial β-oxidative pathway by successive removal of acetyl-CoA units from the fatty acyl chain. Compared with other mitochondrial substrates, they have a number of unique properties which make their metabolism different. Free long-chain fatty acids are potent uncouplers of mitochondrial respiration. This uncoupling action becomes stronger with increasing chain length and is particularly potent in the case of unsaturated fatty acids (Van den Bergh, 1966). In addition, long-chain fatty acids must first be converted to their CoA esters before they can be oxidized via the β-oxidative pathway as the enzymes of the β-oxidation pathway will only accept the CoA esters of the acyl substrate (Stadtman, 1954; Lynen, 1955).

1.7.1 Activation of long-chain fatty acids

The activation of long-chain fatty acids is catalyzed by the membrane-bound enzyme fatty acyl-CoA synthetase, an enzyme whose location has been the subject of extensive studies (Van Tol and Hulsmann, 1970; Norum et al., 1966; Lippel and Beattie, 1970; Aas, 1971; Aas and Bremer, 1968). It is now generally agreed that the enzyme has a dual location in the mitochondria, both on the inner-surface of the outer membrane (Nimmo, 1979) as well as on the matrix side of the inner membrane (Skrede and Bremer, 1970; Groot et al., 1974), inside the carnitine barrier (Fig. 4). Van den Bergh et al. (1969) have shown that the bulk of the synthetase activity is associated with the mitochondrial outer membrane. Van Tol and Hulsmann (1970) have attributed approximately 90–95% of the total synthetase activity of rat liver mitochondria to the outer mitochondrial membrane. The remainder is attributed to the inner-membrane/matrix compartment. The synthetase activity associated with both the inner and outer mitochondrial membranes exhibits
Fig. 4 Schematic representation of the metabolism of long-chain fatty acids in liver mitochondria.
different apparent Km's for both the fatty acid substrates and ATP (Van Tol and Hulsmann, 1970). The synthetase associated with the outer membrane has a lower Km for palmitate, but a higher Km for ATP, compared with the inner membrane enzyme. These kinetic properties of the enzymes are affected by the nature of the acyl substrate as well as the presence or absence of added carnitine (Van Tol and Hulsmann, 1970).

Aas and Bremer (1968) have reported that, aside from these two long-chain fatty acyl synthetases, there are also ATP-dependent medium and short-chain synthetases localized in the matrix of rat liver mitochondria. However, more research is needed to fully differentiate between these different enzymes responsible for activating the various chain lengths of the fatty acyl substrates.

Furthermore, it has been found that long-chain fatty acids can also be activated by a GTP-dependent acyl-CoA synthetase present in the matrix fraction of rat liver mitochondria (Rossi et al., 1967). In this reaction GTP is hydrolyzed to GDP and inorganic phosphate. In comparison, the ATP-dependent activity hydrolyses ATP to AMP and pyrophosphate (Kornberg and Pricer, 1953(b)).

1.8 Oxidation and esterification of acyl-CoA in mitochondria

The long-chain fatty acyl thioesters formed by the outer membrane synthetase are substrates for two alternative reactions, the acylation of carnitine by the enzyme carnitine acyltransferase (CAT) I (Norum, 1965; Brosnan et al., 1973; Norum and Bremer, 1967; Bremer and Norum, 1967; West et al., 1971) and the acylation of glycerol 3-phosphate to form lysophosphatidate by the membrane bound enzyme glycerol 3-phosphate acyltransferase (GPAT) (Daae, 1972(a); Bjerve et al., 1976; Sanchez et al., 1973). This branch point in the metabolism of long-chain acyl-CoA within the mitochondria is diagrammatically illustrated in Fig. 5.
The control of the flux of acyl-CoA through the pathways of mitochondrial oxidation and esterification determines the metabolic fate of the acyl-CoA formed in the intermembrane space.

The partition of acyl-CoA's between oxidation and esterification has been the subject of much research (Sugden and Williamson, 1981; Mayes and Laker, 1981; Van Tol, 1974). The activities of carnitine palmitoyl-transferase and acyl-CoA:sn-glycerol 3-phosphate acyl-transferase in rat liver homogenates were found by Aas and Daae (1971) to vary inversely during fasting. In addition to this, the overall hormonal status of the animal alters the balance between the pathways of oxidation and esterification in mitochondria (Poledne and Mayes, 1970; Heimberg et al., 1969; Mayes and Felts, 1976).

1.8.1 The esterification of acyl-CoA to glycerol 3-phosphate in liver mitochondria

Although mitochondria are believed to lack the enzymatic machinery for the de novo synthesis of most complex phosphoglycerides (McMurray and Dawson, 1969; Van Golde et al., 1971; and Kennedy, 1961), with the exception of cardiolipin (Kennedy, 1961), they contribute about one-half of the total glycerol 3-phosphate acylation capacity of the cell (Daae and Bremer, 1970; Lloyd-Davies and Brindley, 1975). The enzyme responsible for this acylation of glycerol 3-phosphate in mitochondria is the mitochondrial glycerolphosphate acyltransferase, located on the inner side of the outer mitochondrial membrane (Daae, 1972(a)).

The mitochondrial enzyme has a similar specific activity to that of the microsomal enzyme, when assayed in rat, mouse, guinea pig, rabbit and beef liver (Haldar et al., 1979). This is in contrast to that observed in other organs where the microsomal enzyme is at least 10 times more active. The mitochondrial enzyme shows a strong preference for palmitoyl-CoA compared to oleoyl- or linoleoyl-CoA (Daae, 1972(a); Monroy et al., 1972; Monroy et al., 1973), is insensitive to N-ethylmaleimide, and produces mono-
Fig. 5 Metabolism of long-chain acyl-CoA formed within the inter-membrane space of liver mitochondria.
acylglycerolphosphate (lysophosphatidate) as a major product (Haldar et al., 1979). In contrast, the microsomal enzyme catalyzes the acylation of glycerol 3-phosphate with both palmitoyl-CoA and oleoyl-CoA, is almost completely inhibited by N-ethylmaleimide, and produces diacylglycerolphosphate (phosphatidate) as the chief assay product (Haldar et al., 1979).

Although evidence for the involvement of mitochondrial acyltransferases in non-mitochondrial glycerolipid synthesis is inconclusive (Stern and Pullman, 1978), preference of the enzyme for palmitoyl-CoA, as compared to oleoyl- or linoleoyl-CoA as acyl substrate, suggests that this enzyme may be responsible for the predominance of saturated fatty acids in position 1 of liver glycerophospholipids (Arvidson, 1968; Daae, 1972(b)).

1.8.2 The esterification of acyl-CoA to carnitine in liver mitochondria

The acylation of carnitine and subsequent transfer of the acylcarnitine across the inner membrane requires two separate pools of carnitine acyltransferase activity (Fritz and Yue, 1963; Yates and Garland, 1970; Hoppel and Tomec, 1972). In intact mitochondria these enzymes are located on the outer surface (overt activity) and matrix side (latent activity) of the inner-mitochondrial membrane. Many of the schemes put forward to explain the role of carnitine in the transport of long-chain fatty acids have postulated that, although impermeable to coenzyme A and CoA esters, the inner membrane would be permeable to carnitine and acylcarnitine (Fritz and Yue, 1963; Yates and Garland, 1970). However, many investigators now believe that the mitochondrial inner membrane is as impermeable to carnitine and acylcarnitine as it is to coenzyme A and its esters and that carnitine and acylcarnitine traverse the membrane by a process of exchange diffusion, facilitated by the presence of a mitochondrial translocase system (Fig. 6) (Pande, 1975; Ramsey and Tubbs, 1975; Idell-Wenger, 1981).

On the basis of acyl group specificity, at least three acylcarnitine transferases exist; the carnitine short-chain
acyltransferase (Freidman and Fraenkel, 1955; Fritz et al., 1963), the carnitine long-chain acyltransferase (Fritz and Yue, 1963; Bremer, 1963; Norum, 1963), and a recently discovered carnitine medium-chain acyltransferase (Solberg, 1972; Solberg et al., 1972; Kopec and Fritz, 1971). There is also evidence for the existence of a carnitine branched-chain acyltransferase present in the mitochondria (Choi et al., 1977; Solberg and Bremer, 1970). Although the patterns of acyl group specificity of the two pools of carnitine acyltransferase differ (Solberg, 1974), it is not known whether all three activities show latent and overt activities and whether each acyltransferase has a unique translocase system or if several different transferases can use the same translocase. At present, there is no evidence that different translocases are involved in the transport of differing chain length substrates. Control of this particular transport process may serve to regulate the utilization of acyl-CoA units formed in the inter membrane space, but the regulation of this transferase-translocase process is not yet clear.

As well as being located on opposite sides of the inner mitochondrial membrane, the two carnitine acyltransferase activities also differ with respect to their kinetic parameters. Kopec and Fritz (1973) state that carnitine palmitoyltransferase II catalyzes the reaction in the direction towards long-chain acyl-CoA formation, suggesting this transferase-translocase system is only involved in the transport of long-chain acyl groups into the mitochondrial matrix. Houslay and Stanley (1982) propose that although the unidirectional transfer of long-chain acyl groups into the mitochondrial matrix for oxidation is the primary object of this transport system, the transferasetranslocase exchange transporter allows the exchange of carnitine and acylcarnitine in both directions (Fig. 6). This apparently 'futile' cycle serves an important role in the oxidation of long-chain fatty acids by maintaining an active metabolic pool of unacylated carnitine and coenzyme A, the latter being required as an acceptor molecule in the β-oxidation
Fig. 6 Transport of long-chain fatty acids across the inner-membrane of mitochondria.

The above schematic representation outlines the transfer of acyl-CoA across the inner-membrane of the mitochondria adapted from Houslay and Stanley (1982).
cycle (Houslay and Stanley, 1982). The kinetics of this exchange transport system allows control of the net flux of acyl substrate whilst not affecting the overall $V_{\text{max}}$ of the system. The rate of influx and efflux through this system is determined not only by the kinetics of the individual enzyme steps but by the concentration of acyl groups on either side of the inner-membrane. This concentration is, in turn, influenced by the overall physiological status of the animal (Houslay and Stanley, 1982).

It was initially thought the rate limiting factor for the pathways of oxidation and esterification was the availability of the co-substrates, carnitine (Parvin and Pande, 1979; McGarry et al., 1975) and glycerol 3-phosphate (Bortz and Lynen, 1963). However, studies by Mayes and Felts (1967), using perfused livers from starved rats, showed clearly that glycerol 3-phosphate availability could not be rate limiting, since the liver was able to maintain a constant fractional rate of esterification in the face of an increasing influx of fatty acid. This observation was confirmed by McGarry et al. (1973), who showed that under conditions where the operation of CAT I was inhibited, the esterification pathway was still fully functional in the starved animal. These results suggest that the enzymes involved in glycerolipid biosynthesis were not limiting under the conditions described, although it has been shown that the mitochondrial GPAT is capable of regulatory activity (Bates et al., 1977). Similar conclusions have been drawn concerning the availability of carnitine as highlighted by the work of McGarry et al. (1975), Christiansen et al. (1976), and Whitelaw and Williamson (1977), who showed that in fed rats the addition of carnitine in vivo led to ketogenic rates that were only 50% of those found in the livers of starved rats.
1.9 Effect of malonyl-CoA on CAT I activity

The activity of CAT I has been shown to be competitively inhibited by malonyl-CoA (see review by McGarry and Foster, 1980), an intermediate of fatty acid biosynthesis, the concentration of which varies depending on the nutritional state of the animal. Recently it has been shown that the inhibition of CAT I by malonyl-CoA is extremely sensitive to a variety of controlling factors. These include the pH at which the enzyme is assayed (Stephens et al., 1983), the nutritional state of the animal (Cook et al., 1980), the animal's hormonal status (Saggerson et al., 1982), the pre-existing levels of malonyl-CoA in the tissue (Zammit, 1983(a)) as well as the particular tissue under examination (Long et al., 1982; McGarry et al., 1983).

Zammit (1983(a)&(b)) and Robinson and Zammit (1982) have shown that it is possible to induce reversible changes in the sensitivity of CAT I to malonyl-CoA in vitro, provided appropriate conditions are used. Pre-incubation of liver mitochondria, prepared from fed virgin rats or starved animals, with 65 μM-malonyl-CoA resulted in a marked increase in sensitivity of CAT I to malonyl-CoA compared to that shown by the enzyme pre-incubated in the absence of malonyl-CoA (Zammit, 1983(a)). The concentration of malonylCoA required to induce maximal sensitization of CAT I was found to be within the physiological range of concentrations previously found to occur in freeze-clamped liver samples (Zammit, 1981). On the other hand, pre-incubation of mitochondria with NbS2, and subsequent removal of excess reagent with reduced glutathione, resulted in desensitization of CAT I to malonyl-CoA inhibition (Zammit, 1983(b)). When these desensitized mitochondria were then exposed to an additional period of incubation with 8 μM malonyl-CoA, the NbS2-induced desensitization of CAT I to malonyl-CoA was reversed and the restored sensitivity of the enzyme was almost the same as that of the native enzyme (Zammit, 1983(b)).

These observations are of great physiological relevance when considering CAT I as an important regulatory step in
the oxidation of long-chain fatty acids. Not only do they show that changes in sensitivity of CAT I can be reproduced in vitro, but these observations suggest that such a sensitization/desensitization cycle may operate in vivo to amplify the response of the enzyme to small changes in hepatic malonyl-CoA concentrations.

Although in vitro experiments provide strong in vitro evidence to support the concept of regulation at the CAT I step, by malonyl-CoA, the operation of such a regulatory mechanism in vivo has yet to be demonstrated.

1.10 Oxidation of polyunsaturated fatty acids

The acyl-CoA product of the CAT II enzymatic step is available for β-oxidation, since all the enzymes of β-oxidation, namely acyl-CoA dehydrogenase, enoyl-CoA hydratase or 'crotonase', β-hydroxyacyl dehydrogenase and thiolase, are localized in the inner membrane/matrix fraction of the mitochondria (Lynen, 1953; Tubbs, 1978). β-oxidation of both saturated and unsaturated acids is believed to proceed essentially by the same mechanism, that is, the hydrocarbon chain is shortened by the successive removal of two carbon units until the entire carbon backbone is degraded (Fig. 7). The occurrence of a cis double bond at odd numbered carbon atoms, as in oleic and linoleic acid, leads to the removal of acetyl units by the 'classical' β-oxidation pathway until the chain shortening produces cis-3-enoyl-CoA (Kunau and Dommes, 1978). This intermediate is converted to trans-2-enoyl-CoA by the action of cis, trans-3,2, enoyl-CoA isomerase, an enzyme whose activity has been detected in mitochondria (Stoffel et al., 1964; Davidoff and Korn, 1965; and Struijk and Beerthuis, 1966). The trans-2-enoyl-CoA produced can then be further degraded by the enzymes of β-oxidation, as this is an intermediate encountered in the oxidation of saturated acids. Thus the oxidation of oleic acid may be accomplished by the enzymes of β-oxidation working in conjunction with the above mentioned cis, trans isomerase.
Fig. 7  β-oxidation of saturated fatty acids in mitochondria.

Palmitoyl-CoA is degraded to acetyl-CoA by the enzymes of the β-oxidation cycle, namely, acyl-CoA dehydrogenase, enoyl-CoA hydratase, β-OH acyl-CoA dehydrogenase and thiolase, located in the inner-membrane/matrix fraction of the mitochondria. The complete degradation of palmitoyl-CoA is achieved after seven cycles of the above oxidation pathway.
When the cis double bond is situated at even carbon atoms, it is thought that β-oxidation continues until a cis double bond is encountered at carbon two. This intermediate is the same as that produced after the first dehydrogenation step in the 'classical' β-oxidation pathway except it is of opposite geometrical configuration. The enoyl-CoA hydratase is not specific concerning the geometrical configuration of the double bond and will catalyze the hydration of either the cis or trans double bond (Stoffel and Caesar, 1965; Wakil and Mahler, 1954). The products of this reaction are both the L- and D- isomers of the β-OH acyl-CoA ester depending on whether the stereochemistry of the double bond is trans or cis respectively. The oxidation of this hydroxyl group to a ketone is catalyzed by a β-OH acyl-CoA dehydrogenase which is specific for the L-isomeric configuration of the hydroxyl group (Wakil, 1963). Any β-OH acyl esters in the D-configuration are converted to the L-isomer by the action of an epimerase (Stoffel et al., 1964; Stoffel and Caesar, 1965). According to this concept the complete oxidation of most polyunsaturated fatty acids could be accomplished by the enzymes associated with the 'classical' β-oxidation pathway as well as two additional enzyme activities, as described.

Although the enzymatic activities described above have all been detected in mitochondria, the operation of such a proposed pathway has yet to be substantiated by experimental data.

The studies of Kunau and Bartnik (1974) and Kunau and Dommes (1978) on the partial degradation of polyunsaturated fatty acids suggests a more complex picture.

The oxidative degradation of cis-4-decenoyl-CoA has been studied by Kunau and Dommes (1978), using a buffer-soluble extract from beef liver. The crude extract was capable of oxidising decanoyl-CoA with the production of the expected intermediates, except 3-oxodecanoyl-CoA, in the form of their methyl esters. When radioactive cis-4-decenoyl-CoA was incubated with the crude extract, methyl
octanoate and not methyl cis-2-octenoate was detected among the methyl esters. Furthermore, the degradation of cis-4-decenoyl-CoA was found to be dependent on NADPH, a cofactor not required by the four enzymes of β-oxidation. These results suggest a direct reduction of the double bond at carbon four and not reduction of cis-2-octenoyl-CoA after the subsequent removal of one acetyl-CoA unit.

DEAE-cellulose fractionation and gel chromatography on Sephadex G-150 were used by Kunau and Dommes (1978) to separate, almost completely, the NADPH-dependent enoyl-CoA reductase activity measured with cis-4-decenoyl-CoA, and substrates possessing a 2,4-diene structure, from that measured with trans-2-decenoyl-CoA. The latter of these substrates is reduced by a 2-enoyl-CoA reductase, which is known to exist in beef liver (Podack and Seubert, 1972).

Using affinity chromatography Kunau and Dommes (1978) were able to separate the acyl-CoA dehydrogenase from the 4-enoyl-CoA reductase. They observed that when intermediates possessing a 2,4-diene structure, but not cis-4-decenoyl-CoA, were used as substrate for the reductase the reaction oxidized NADPH.

From their observations Kunau and Dommes (1978) concluded that in the reaction catalyzed by the 4 enoyl-CoA reductase only one double bond of the 2,4-diene thioester is reduced and that the double bond in the four position is absent after this reduction step. The results obtained in these experiments however, do not distinguish between a 1,2-addition (carbon atoms 4 and 5) and a 1,4-addition (carbon atoms 2 and 5) of the two hydrogen atoms. Therefore, the products of such a reduction could be trans-2-decenoyl-CoA or 3-decenoyl-CoA respectively.

From these results Kunau and Dommes (1978) have proposed a series of reactions for the degradation of cis-4-decenoyl-CoA. The proposed pathway incorporates the 4-enoyl-CoA reductase as described above with the four enzyme steps involved in the 'classical' β-oxidation of
saturated fatty acids. If 3-decenoyl-CoA is the product of the reductase step then it is proposed that this is converted to trans-2-decenoyl-CoA by the action of cis, trans-3,2 enoyl-CoA isomerase, as discussed previously. This proposed series of reactions is supported by the work of Cuebas and Schulz (1982) which investigated the enzymatic degradation of 2,4 decadienoyl-CoA, an assumed metabolite of linoleic acid oxidation. Therefore it would appear that 2 trans, 4 cis-decadienoyl-CoA cannot be degraded according to the pathway proposed by Stoffel and Caesar (1965) for the oxidation of linoleic acid.

A modified pathway for the degradation of linoleic acid, as proposed by Kunau and Dommes (1978) and supported by the observations of Cuebas and Schulz (1982), is presented in Fig. 8.

Although the in vitro evidence supports the proposal of such a pathway operating, the use of crude extracts, supplemented with semi-pure protein fractions, needs refining. Only when the above system can be reconstituted from its isolated components and shown to catalyze the complete oxidation of linoleic acid will it be possible to confirm the proposed reaction scheme.

The major reaction product of the β-oxidative pathway is acetyl-CoA which in turn has a number of metabolic fates once formed within the matrix (Fig. 9). The levels of acetyl-CoA within the mitochondria are regulated by: the rate of acetyl-CoA production via β-oxidation; utilization via either the Krebs cycle and electron transport chain or ketone-body production, when considering liver metabolism; or by its transportation out of the mitochondria either as citrate or via the carnitine acetyl-CoA transferase exchange system.
Fig. 8 Oxidation of linoleic acid in mitochondria.

Although it is generally accepted that the basic mechanism for the oxidation of unsaturated fatty acids is essentially the same as for saturated fatty acids, the introduction of cis double bonds in the acyl chain leads to intermediates not encountered in the β-oxidation of saturated fatty acids. The above scheme illustrates the two proposed pathways for the oxidation of linoleic acid (See section 1.10).
Acetyl-CoA synthesized within the mitochondrial matrix can either be completely degraded to CO₂ and H₂O within the mitochondria or transported out of the mitochondria (indicated by broken line) either as ketone bodies (Sugden and Williamson, 1981; Middleton, 1978; Mayes and Laker, 1981), citrate (Watson and Lowenstein, 1970), or possibly as free acetyl-CoA via the carnitine acetyl-CoA transferase reaction (Freidman and Fraenkel, 1955; Fritz et al., 1963). However, the involvement of this last mechanism in the export of acetyl-CoA from the mitochondria has yet to be proven.
1.11 Peroxisomal oxidation

Lazarow and de Duve (1976) have shown that rat liver peroxisomes are capable of oxidizing long-chain acyl-CoA esters. The oxidation of these long-chain acyl substrates proceeds by an almost identical series of reactions as described for mitochondrial β-oxidation (Lazarow, 1978), with the generation of acetyl-CoA through successive steps of dehydrogenation, hydration, dehydrogenation, and thiolytic cleavage. In contrast to mitochondrial β-oxidation, however, the first dehydrogenation step involves the reduction of O₂ to H₂O₂, while the second dehydrogenation step, as in mitochondria, reduces NAD⁺ to NADH. When mitochondrial and peroxisomal fatty acid oxidation were compared in whole liver homogenates (Mannaerts et al., 1979) it was observed that, in contrast with mitochondrial oxidation, peroxisomal oxidation did not produce ketone bodies, was not dependent on carnitine, and remained unchanged during starvation and diabetes. In addition to this, peroxisomal oxidation is not coupled to a phosphorylating system and is insensitive to inhibition by cyanide (Lazarow and de Duve, 1976).

The degradation of the acyl chain by the enzymes of β-oxidation in mitochondria is thought to be essentially complete with the formation of acetyl-CoA, equivalent to the number of acetyl units contained in the fatty acid. This, however, does not appear to be the same in peroxisomes. Lazarow (1978) observed that purified peroxisomes would catalyze the oxidation of palmitoyl-CoA with the generation of only 5 acetyl-CoA units per equivalent of palmitoyl-CoA. Peroxisomes were less active with octanoyl-CoA than with lauroyl-CoA or palmitoyl-CoA and were inactive when short chain acyl-CoA esters such as butyryl-CoA were used as substrates.

The incomplete oxidation of long-chain fatty acids and inactivity towards shorter chain acyl-CoAs raises questions as to the fate of these short chain derivatives. The presence of a carnitine transfer system (Markwell et al.,
1973), although not needed for oxidation, suggests that these shorter chain acyl-CoAs may be transported elsewhere in the cell. The possibility of the further oxidation of these short chain substrates by mitochondria is still a topic of discussion.

Although the results, obtained thus far, suggest that the peroxisomes are a major site for the β-oxidation of long chain fatty acids (Lazarow and de Duve, 1976; Mannaerts et al., 1979), the relative contribution of both the peroxisomal and mitochondrial pathways to the overall oxidation of fatty acids in the cell is uncertain.

When considering maximal enzyme activity, the peroxisomal β-oxidation capacity in mouse liver is about the same as that observed in the mitochondrial system (Murphy et al., 1979). In contrast, the hepatic peroxisomal β-oxidation capacity in rats accounts for about 25-33% of the total activity (Krahling et al., 1978).

A role for peroxisomal oxidation has been proposed by Neat et al., (1981) as regards the oxidation of fatty acids during periods of sustained high hepatic influx of these substrates, and especially in relation to the metabolism of fatty acids that are poorly oxidized by mitochondrial β-oxidation.

The overall regulation of fatty acid oxidation is a very complex picture with a wide variety of contributing factors. However, as in many other metabolic schemes, various enzyme steps seem to act as important control points.
level in this thesis, using the rat and the sheep as model animal systems.
CHAPTER 2
MATERIALS AND METHODS

2.1 Chemical reagents

Reagents purchased from Sigma Chemical Company, St. Louis (USA), were: palmitic acid, stearic acid, oleic acid, linoleic acid, palmitoyl-CoA, oleoyl-CoA, linoleoyl-CoA, malonyl-CoA, coenzyme A, L-carnitine, DL-α-glycerol 3-phosphate, ATP, ADP, DTT, NAD⁺, antimycin D, albumin (Cohn Fraction V), EGTA.

DL-[methyl-¹⁴C]carnitine hydrochloride and L-[U-¹⁴C]glycerol 3-phosphate (ammonium salt) were purchased from Amersham International, Amersham, Bucks, U.K.

Other reagents were analytical grade.

Butanol was washed free of impurities with distilled water, dried with CaCl₂ overnight and re-distilled twice, the initial 50 ml and the final 100 ml from a volume of 21 were discarded.

2.2 Preparation of reagents

Solutions were prepared using deionised-distilled water. Micellar solutions of saturated fatty acids were prepared by titrating aqueous solutions of the potassium salts to between pH 7 to 8 with HCl. For micellar solutions of palmitate and stearate this operation was carried out at 70°C to ensure the solubility of these fatty acids.

Unsaturated fatty acids, present as the free acid, were titrated with an equimolar amount of KOH to form a micellar solution of the potassium salt of the fatty acid. Oxygen was excluded at all stages by flushing with N₂ and solutions were then stored under nitrogen. The concentrations of the fatty acid micellar solutions were determined by adjusting the pH of the solution to 2, using a pH meter, with HCl and titrating the free fatty acids with NaOH using Nile Blue A as an indicator (Chen, 1967).
Radioactive substrates were prepared as follows;

2.2.1 \([^{14}C]\)glycerol 3-phosphate

50 µCi of L-\([U-^{14}C]\)glycerol 3-phosphate, ammonium salt (171 mCi/mmol) was made up to a final concentration of 10.94 mM with unlabelled DL \(\alpha\)-glycerol 3-phosphate.

2.2.2 \([^{14}C]\)carnitine

50 µCi of DL[methyl-\(^{14}C\)]carnitine hydrochloride (51.4 mCi/mmol) was made up to a final concentration of 4.32 mM with unlabelled L-carnitine.

Both radioactive substrates were stored in 1 cm\(^3\) aliquots at -20°C until required.

Glassware used in the preparation of mitochondria and mitochondrial solutions was cleaned by soaking in alkali then acid-washing before finally rinsing with deionised water. No detergents were used for cleaning glassware associated with the isolation of mitochondria or measurement of mitochondrial activity.

2.3 Preparation of intact liver mitochondria

To isolate intact functional mitochondria from tissues the osmolarity of the suspending medium must be such that the mitochondria retain their physiological shape and size. The mitochondrial membrane, like many other membranes, has elastic properties which allows it to shrink or expand to within certain limits depending on the osmolarity of the medium surrounding it (Whittacker, 1965) or the presence of agents that promote swelling or contraction (Kuttis et al., 1968). Although this swelling and contracting process is an in vivo response, the isolation of mitochondria in a prolonged swollen or contracted state, due to incorrect osmolarity of the preparation medium, has a marked effect on the metabolic activity of these organelles (Vignais and Vignais, 1965). It is therefore important to ensure that, during the isolation of mitochondria, their physiological shape and size is maintained.
Besides maintaining the physiological size and shape of the mitochondria, it is important that intact mitochondria are released from the liver cell. This is achieved by using shearing forces and, in order to carry out this process successfully, material that is resistant to shearing must be removed (Nedergaard and Cannon, 1979). One common method of shearing intact cells is by a hand-operated, or motor-driven, homogenizer. The function of the homogenizer is to shear the cell membranes and release the intact cell organelles into the homogenization medium. The particle size of the tissue to be homogenized in relation to the clearance of the homogenizer is critical in achieving successful shearing. If the particles of tissue are too large, the pressure exerted by the homogenizer pestle may cause the cells to burst and thereby damage the cell organelles, resulting in an increase in the percentage of broken mitochondria. On the other hand, if the particles are too small in relation to the homogenizer clearance, the lack of shearing drastically reduces the yield of intact mitochondria. In both instances, the proportion of intact mitochondria released is lowered. Particles in the range of 1.5 to 3.0 mm³ diameter were found to be suitable for an optimal yield of mitochondria using a homogenizer clearance of 0.25 mm.

Most published methods of preparing liver mitochondria sediment the mitochondria at 9,000-15,000 g for 5-10 min, forming a compact pellet of broken and intact mitochondria (Johnson and Lardy, 1967; Otto and Ontko, 1978). The broken, and less dense, mitochondria are then carefully removed from the top of the pellet and the remaining intact mitochondria resuspended in fresh preparation medium. If the mitochondrial pellet is too compact, resuspension is often incomplete and an increase in broken mitochondria is observed after further centrifugation.

The method described here follows the same procedure as described by Johnson and Lardy (1967) but uses a lower force of 4,500 g for 20 mins which leads to the formation of a softer pellet. After centrifugation, the broken mito-
chondria form an opaque fluffy layer and this is easily removed with the supernatant by a gentle rocking of the centrifuge tube. The ease of resuspension of the relatively soft mitochondrial pellet leads to the isolation of mitochondria with minimal damage.

2.3.1 Treatment of animals and liver tissue prior to the preparation of rat and sheep mitochondria

Female Sprague-Dawley rats weighing between 150-200 g were kept at 25°C with daily cycles of artificial illumination between 6.00 a.m. and 6.00 p.m. and fed ad libitum on a stock laboratory diet consisting of 21.61% protein, 2.39% fat, 5.17% fibre, 5.02% ash, 0.79% Ca2+, 0.5% P, 3.46% total nitrogen and 12.81% moisture.

Rats were given free access to fresh water at all times. Food was withheld 18-20 h prior to slaughter, the animals were killed by decapitation between 9.00 a.m. and 10.00 a.m. and the livers removed and placed in an ice-cold beaker.

Sheep livers were removed and placed on ice within 10 min of slaughter of the animal at the local abattoir. The livers remained on ice for 20-30 min while being transported to the laboratory.

The hepatic cells of ovine liver are enclosed by a fibro-connective covering known as the liver capsule. As well as this, the liver contains an infiltration of blood vessels and sinusoids. In preparing mitochondria from sheep liver, the liver capsule was removed with a razor blade and a section of liver chosen in which large blood vessels were not likely to hinder homogenization of the tissue.

2.3.2 Preparation of mitochondria

All procedures were carried out at 2-4°C and solutions and glassware were chilled prior to use. Twelve to fifteen g portions of either rat or sheep liver were rinsed in cold preparation medium (0.255 mannitol, 0.075 M sucrose, 0.05 mM EGTA, pH 7.4). Care was taken to ensure
that handling did not damage the liver tissue. Special care was needed during removal of the soft hepatic tissue from the rat. The preparation medium used was identical to that of Tyler and Gonze (1967) except that EGTA replaced EDTA. EGTA, like EDTA, was used in order to chelate Ca$^{2+}$ ions, which can function as uncouplers and are also cofactors for certain phospholipases (Nedergaard and Cannon, 1979). EGTA, however, was selected because it has a lower affinity for Mg$^{2+}$ ions, which are important ions in maintaining the stability of mitochondrial membranes (Hommes, 1965).

The preparation medium was decanted and the liver tissue finely chopped into 2-3 mm$^3$ pieces with a razor blade on a cooled glass plate. Alternatively, ovine liver tissue was passed through a hand operated tissue press (1.5 mm diameter holes) after being cut into 1 cm cubes. The chopped tissue was then washed twice with preparation medium in order to remove contaminating blood cells and transferred to a manually-operated Potter-Elvehjem homogenizer (0.25 mm clearance). The tissue was homogenized in 60 cm$^3$ of cold preparation medium by three passes of the pestle or until there was no resistance from intact tissue. Care was taken to ensure that the pestle reached the bottom of the homogenizing tube on each pass and that the procedure was carried out slowly with even pressure on the pestle. The homogenate was centrifuged at 800 g for 5 min in a SS-34 Sorvall rotor to remove red blood cells, whole cells, nuclei and other cellular debris. The supernatant was carefully decanted without disturbing the sediment and then centrifuged at 4500 g for 20 min to precipitate the mitochondria as a soft pellet which was easily resuspended. The supernatant was decanted and any lipid present was removed by wiping the inside of the centrifuge tube with a clean tissue. The mitochondrial pellet was gently resuspended in fresh medium by careful stirring with a test tube filled with ice. A smooth suspension could only be made if the pellet was gently stirred before the addition of fresh medium. The dispersed pellet was then suspended in a total volume of 30 cm$^3$ of preparation medium and again
centrifuged at 4500 g for 20 min. Any broken mitochondria were removed with the supernatant by a gentle rocking of the tube prior to decantation of the supernatant. The centrifugation-resuspension procedure was repeated a second time and the final washed mitochondrial pellet was resuspended in 3-4 cm³ of preparation medium giving a final yield of 6-8 mg mitochondrial protein/g of liver.

Although this method of isolating mitochondria did not recover all the mitochondria present in the homogenate it led to the isolation of intact mitochondria with little contamination from microsomal or lysosomal fractions.

2.4 Preparation of peroxisomes from liver tissue

The preparation of peroxisomes from a liver homogenate is achieved either by prolonged differential and equilibrium density centrifugation, to give pure peroxisomes (Leighton et al., 1968; Baudhuin, 1974), or by the rapid preparation of a peroxisome-enriched fraction where any contaminating mitochondrial activity is inhibited by cyanide or some other inhibitor of respiration (Thomassen et al., 1982). A rapidly prepared peroxisome-enriched fraction was chosen for experiments here because it allowed peroxisomes to be isolated and assayed under similar conditions to mitochondria. Prior to the isolation of peroxisomes from a particular tissue the animals are often injected with either clofibrate, a hypo-lipidaemic drug known to increase the number of hepatic peroxisomes (Hess et al., 1965), or Triton WR-1339 which sufficiently decreases the equilibrium density of lysosomes so that the peroxisomes are not significantly contaminated by these organelles upon isolation (Lazarow and de Duve, 1976). Triton WR-1339, like clofibrate, is also known to affect lipid metabolism (Thor nearly) and even low doses, as used to separate lysosomal contamination from a pure peroxisomal fraction, might affect the enzymes under investigation. Hence, in this study, animals were not subjected to any treatment to increase peroxisomal activity.
2.4.1 Preparation of a peroxisome-enriched fraction from liver

Livers were removed and homogenized as described for the preparation of mitochondria (Section 2.3.2) except that 0.1% ethanol was added to the preparation medium in order to prevent the inactivation of catalase (Leighton et al., 1968). The pellet of nuclei and cell debris obtained from the 800 g spin was rehomogenized in 35 cm$^3$ of preparation medium and recentrifuged at 800 g. The supernatants from the two 800 g centrifugations were then centrifuged at 4,500 g for 20 min in a Sorvall SS-34 rotor. The pellet contained most of the mitochondria and was discarded. The supernatant was centrifuged at 27,000 g for 20 min to sediment a peroxisome-enriched fraction. This pellet was then resuspended in 2-3 cm$^3$ of preparation medium.

2.5 Preparation of long-chain fatty acid-free albumin

Albumin which was essentially free of fatty acids, was prepared from commercial grade bovine serum albumin by the method of Chen (1967). The lyophilized solution was dialyzed as described by Hanson and Ballard (1968) and the product assayed for long-chain fatty acids by the method of Chen (1967).

The albumin, prepared according to the above methods, was found to contain 0.056 mole FFA/mole albumin and no activity was detectable when this was used as a substrate for the $\beta$-oxidation assay.

2.6 Assay conditions

2.6.1 Rate of $\beta$-oxidation

The rate of $\beta$-oxidation of long-chain fatty acyl substrates was assayed at 25°C by measuring the ADP-stimulated oxygen uptake from the reaction mixture using a Clark-type oxygen electrode (Yellow Springs Instruments, Ohio) (refer Fig. 10).
Fig. 10 The Clark-type oxygen electrode (Yellow Spring Instrument, Ohio) used to assay the rate of oxidation of fatty acids, as described on page 37.
mitochondria plus substrate.

ADP added

substrate related O₂ consumption. (Y)

returns to endogenous rate after added ADP has been metabolised.

chart movement (known rate of x mm/min).

O₂ consumed = \( \frac{Y}{X} \times \text{nmoles } O₂ \text{ (in air saturated distilled water)} \) / time (min).

Fig. 11 Oxygen electrode trace.

The interpretation and analysis of an oxygen electrode trace is explained in the above diagram.
The standard reaction buffer, which consisted of 50 mM sucrose, 15 mM KCl, 2 mM EGTA, 50 mM Tris-HCl (pH 7.4), 25 mM KH$_2$PO$_4$ (pH 7.4), was supplemented with 0.6 mM L-carnitine, 1.5 mM ATP, 2 mM malate and 1 mM DTT. In addition, the reaction mixture contained 25 μM and 5 μM coenzyme A in assays using rat liver mitochondria and sheep liver mitochondria, respectively (refer Fig. 16). These conditions ensured that the activation and transport of fatty acids to the enzymes of β-oxidation was not limited by cofactor requirements. Free fatty acids were added with fatty acid-free albumin in a 7:1 (FFA/albumin) molar ratio. The addition of 2 mM L-malate to the reaction ensured that the acetyl-CoA formed from the oxidation of the fatty acyl substrates was further metabolised via the Krebs cycle and electron transport chain rather than utilized for ketone body production (Van den Bergh, 1967).

The oxygen electrode was calibrated using air-saturated distilled H$_2$O (Gazzotti et al., 1979) and adjusted to zero percent oxygen by the addition of dithionite crystals. The reaction medium was saturated with air prior to use and after the addition of 1.5-4 mg mitochondrial protein an endogenous rate of oxygen uptake was measured.

The reaction was started by the addition of 10 μl ADP to give a final ADP concentration of 0.2 mM in a total reaction volume of 3 cm$^3$. The rate of oxygen uptake was calculated from the difference between the two slopes and was expressed as μmoles O$_2$ consumed/mg mitochondrial protein/h (refer Fig. 11).

2.6.2 Assay of fatty acid oxidation by liver peroxisomes

The oxidation of acyl-CoA substrates by the peroxisome-enriched liver fraction, prepared as described above, was measured using a Clark type oxygen electrode.

The assay measured the overall oxygen uptake which accompanies the shortening of the acyl chain as described in the assay scheme below.
The rate of oxygen uptake measured by the electrode has been adjusted to allow for the oxygen produced from the catalase reaction.

The reaction medium contained 30 mM KH₂PO₄ (pH 7.4), 0.2 mM NAD⁺, 0.1 mM coenzyme A, 12 mM DTT, 0.01% Triton X-100, antimycin D (66 μg in 20 μl ethanol - to inhibit any mitochondrial respiratory activity), fatty acyl-CoA/albumin in a 7:1 molar ratio, 0.2 cm³ of a peroxisome-enriched fraction and H₂O to make a final reaction volume of 3 cm³. This assay mixture was essentially the same as that described by Lazarow and de Duve (1976).

The reaction was carried out at 37°C and each reaction mixture was incubated for 2 min before the reaction was started by the addition of NAD⁺. Oxidation rates for the acyl-CoA substrates were expressed as μmoles O₂/h/μunit catalase activity. The peroxisomal extract was diluted with Triton X-100 (0.1 g/l), 1 mM EGTA and 1 mM NaHCO₃ when determining catalase activity. This was assayed by measuring the production of O₂ from added H₂O₂ using the oxygen electrode (Baudhuin, 1974).

2.6.3 Assay for carnitine acyltransferase (CAT) I in liver mitochondria

The activity of CAT I is usually measured in the direction of acylcarnitine formation either spectro-
photometrically following the release of CoASH with NbS$_2$ or by following the incorporation of labelled carnitine into acylcarnitine.

In the experiments reported in this thesis, hepatic mitochondrial CAT I activity was assayed essentially as described by Saggerson et al. (1982). This assay follows the production of [$_{14}$C]acylcarnitine by the reaction:

\[
_{[14}C]-L\text{-carnitine} + \text{acyl-CoA} \xrightarrow{\text{CAT I}} \text{acyl}_{[14}C\text{]carnitine} + \text{CoASH}
\]

The standard reaction conditions were as follows; 0.225 M mannitol, 0.07 M sucrose, 1 mM EGTA, 0.01 M phosphate buffer (pH 7.4), 5 mM MgCl$_2$, fatty acyl substrates as indicated in 7:1 molar ratio with fatty acid-free albumin, 1 mM DTT, ATP and coenzyme A concentrations as used for the oxygen uptake assay, and 0.6 mM carnitine in a total reaction volume of 0.35 cm$^3$. When palmitoyl-CoA was used as a substrate for the reaction, ATP and coenzyme A were omitted from the reaction mixture. The reaction mixture was pre-incubated at 30°C for 1 min with 0.17 to 0.40 mg mito-chondrial protein and the reaction started by the addition of [$_{14}$C]-carnitine (0.077 μCi).

The reaction was allowed to proceed for 3 min and it was then stopped by the addition of 2 cm$^3$ butanol and 10 cm$^3$ 0.1 M boric acid which was saturated with butanol. The phases were separated by centrifugation and the upper butanol phase, which contained the [$_{14}$C]acylcarnitine, was quantitatively transferred to another glass centrifuge tube. The butanol phase was then washed twice with 10 cm$^3$ of the butanol-saturated boric acid solution to remove unreacted [$_{14}$C]-carnitine. The washed butanol was then transferred to a scintillation vial, care being taken to avoid any contamination from the lower aqueous phase, and 10 cm$^3$ of scintillation fluid (2 L re-distilled toluene, 1 L Triton X-100, 12 g PPO, 0.3 g POPOP) added. The samples were counted in a Beckman LS 8000 Liquid Scintillation System with an applied quench correction. The results were expressed as nmoles acyl[$_{14}$C]carnitine formed/min/mg mitochondrial protein.
2.6.4 Assay for glycerol 3-phosphate acyl transferase (GPAT) in liver mitochondria

GPAT activity was assayed in intact mitochondria by following the formation of the butanol-soluble reaction product using [14C]-glycerol 3-phosphate as the acyl-acceptor molecule. The standard reaction conditions were as follows: 0.225 M mannitol, 0.07 M sucrose, 1 mM EGTA, 0.01 M phosphate buffer (pH 7.4), 5 mM MgCl₂, fatty acyl substrates, as indicated, in 7:1 molar ratio with fatty acid-free albumin, 1 mM DTT, ATP and coenzyme A concentrations, as used for oxygen uptake studies, and 2.5 mM glycerol 3-phosphate, in a total reaction volume of 0.35 cm³. These conditions were identical to those for assaying CAT I activity, except that 2.5 mM glycerol 3-phosphate replaced carnitine. After a 1 min pre-incubation period, which followed the addition of 0.1 to 0.5 mg mitochondrial protein, the reaction was started by the addition of [14C]-glycerol 3-phosphate (0.041 μCi). After 3 min at 30°C the reaction was stopped by the addition of 2 cm³ of butanol and 10 cm³ of 0.1 M boric acid, which was saturated with butanol. The isolation of the labelled product was carried out in an identical manner to that described for CAT I and the results expressed as nmoles glycerol 3-phosphate converted to lipid soluble product/min/mg mitochondrial protein.

2.7 Preparation of electron micrographs

Electron micrographs of mitochondrial preparations from both rat and sheep liver were prepared by Mr D. Hopcroft of the DSIR, Palmerston North, New Zealand using Karnovsky's fixative.

The mitochondrial pellet was suspended in a primary fixative consisting of 3% glutaraldehyde and 2% formaldehyde in 0.1 M KH₂PO₄ buffer (pH 7.2). This was then washed three times with 0.1 M KH₂PO₄ buffer with washings lasting 5, 10 and 30 min respectively. The pellet was then resuspended in a secondary fixative of 1% O₅S₀₄ in 0.05 M KH₂PO₄ buffer.
Following centrifugation the pellet was washed three times before undergoing the dehydration stage. It was then embedded in resin prior to sectioning and photography.

2.8 Determination of protein

Mitochondrial protein was determined by the method of Lowry et al. (1951) with bovine serum albumin (Cohn Fraction V) as standard. The concentration of the standard albumin solution was determined by measuring the absorbance at 280 nm using the extinction coefficient of 6.8 for a 1% albumin solution. A new standard curve was prepared for each experiment.
CHAPTER 3
3.1 Integrity of Mitochondria

The integrity of mitochondria may be assessed in vitro by their ability to catalyze the phosphorylation of ADP to ATP in a tightly coupled manner. Rat liver mitochondria prepared by the method described above exhibited respiratory control ratios of 4 to 7 with 10 mM succinate, that is, the rate of oxygen consumption, following the addition of ADP, expressed as a ratio of the endogenous rate of oxygen consumption, prior to the addition of ADP (Johnson and Lardy, 1967). Mitochondria from rat liver oxidized 10 mM succinate at a rate of 4-6 μmoles O2/mg/h which is slightly higher than that reported in the literature (Eastbrook, 1967). Sheep liver mitochondria oxidized 10 mM succinate at a rate of 2-3 μmoles O2/mg/h and showed respiratory control ratios for the same substrate of 3 to 4. In both rat and sheep liver mitochondria the rate of oxidation of 10 mM succinate was independent of the concentration of ADP (results not shown) and respiratory control ratios were maintained at those ratios stated above for several additions of ADP in both rat and sheep liver mitochondrial preparations (refer Figs. 12 & 13).

From electron micrographs of rat and sheep liver mitochondria, both preparations showed 90-100% intactness of the mitochondrial membranes (Figs. 14 & 15). Using marker enzymes, the mitochondrial preparations from both rat and sheep liver were shown to be predominantly mitochondrial with little or no contamination by microsomal or lysosomol fractions (Table 1).

3.2 Oxidation of long-chain free fatty acids

The rate of oxidation of long-chain free fatty acids, in both rat and sheep liver mitochondria, was found to be dependent on the concentrations of coenzyme A and carnitine (Figs. 16 & 17). Both of these cofactors are involved in the activation and transport of free fatty acids across the
Fig. 12  Oxygen electrode tracing using 10 mM succinate as a substrate with rat liver mitochondria.

Following the addition of ADP, the rate of oxidation of succinate by intact mitochondria increases until all the ADP has been converted to ATP. After this the rate of oxygen consumption returns to the same endogenous rate seen prior to the addition of ADP. If the mitochondrial inner-membrane is damaged, the ATP produced is reconverted to ADP and the endogenous rate of oxygen consumption is increased.

The above tracing is representative of 20 rat liver mitochondrial preparations.
Fig. 13  **Oxygen electrode tracing using 10 mM succinate as a substrate in sheep liver mitochondria.**

For details see Fig. 12.

The above tracing is representative of 10 sheep liver mitochondrial preparations.
Fig. 14 Electron micrographs of rat and sheep liver mitochondrial preparations.
(magnification = 8,200 times)
Fig. 15 Electron micrographs of rat and sheep liver mitochondrial preparations. 
(magnification = 19,200 times)
Table 1  Characterisation of mitochondria from rat and sheep liver

<table>
<thead>
<tr>
<th>Marker enzymes</th>
<th>Rat liver mitochondria</th>
<th>Sheep liver mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>standard preparation</td>
<td></td>
</tr>
<tr>
<td>cytochrome oxidase</td>
<td>25 μmoles O₂/mg/h</td>
<td>11.7 μmoles O₂/mg/h</td>
</tr>
<tr>
<td>(mitochondrial)</td>
<td></td>
<td>no detectable activity</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase (microsomal)</td>
<td>0.0234 mmoles cytochrome c reduced/mg/h</td>
<td>no detectable activity</td>
</tr>
<tr>
<td>Acid phosphatase (lysosomal)</td>
<td>no determined activity</td>
<td>no detectable activity</td>
</tr>
<tr>
<td>(Schnaitman et al., 1967)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Sottocasa et al., 1967)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gianetto and de Duve, 1955)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rat liver mitochondria</th>
<th>Sheep liver mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>organelles isolated on</td>
<td></td>
</tr>
<tr>
<td>sucrose gradient</td>
<td></td>
</tr>
<tr>
<td>mitochondria</td>
<td>29 μmoles O₂/mg/h</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>microsomes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.62 mmoles cytochrome c reduced/mg/h</td>
</tr>
<tr>
<td>lysosomes</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

After separation of the mitochondrial preparation from sheep liver by sucrose density fractionation, no protein bands corresponding to microsomal or lysosomal fractions were detectable, hence marker enzyme activity associated with these fractions were not determined.
Fig. 16  The influence of coenzyme A concentration on the rate of oxygen uptake by rat (●) and sheep (▲) liver mitochondria using 0.01 mM palmitate as the acyl substrate.

Assay conditions employed were as described on page 37. Each point represents the mean of a duplicate assay. The same mitochondrial preparation was used throughout the experiment.
Fig. 17 The influence of carnitine concentration on the rate of oxygen uptake by rat (○) and sheep (▲) liver mitochondria using 0.01 mM palmitate as the acyl substrate.

Assay conditions employed were as described on page 37. Each point represents the mean of a duplicate assay. The same mitochondrial preparation was used throughout the experiment.
inner mitochondrial membrane (Fig. 6). Sheep liver mitochondria were susceptible to inhibition by free coenzyme A at concentrations greater than 5 μM, exhibiting oxidation rates at 40 μM coenzyme A that were 48% those observed at 5 μM coenzyme A. Coenzyme A levels above 25 μM also inhibited oxygen uptake by rat liver mitochondria, but this inhibition was less pronounced than in sheep. The rate of oxygen uptake at 100 μM coenzyme A by rat liver mitochondria was 84% of the maximal rate observed at 25 μM coenzyme A. This inhibitory effect of coenzyme A, observed in both rat and sheep liver mitochondria, was not investigated further.

The oxidation of 0.01 mM palmitate, in both rat and sheep liver, was stimulated by the addition of carnitine up to 0.3 mM. Above this concentration there was little increase in oxidation rate which indicated that the enzyme was saturated with respect to this substrate (Fig. 17).

Mitochondria from both sheep and rat liver were assayed with optimal coenzyme A and carnitine to ensure that the activation and transport of the fatty acyl groups across the inner membrane were not limited by cofactor requirements. Although the endogenous levels of ATP, either pre-existing or formed from oxidative phosphorylation of ADP, were sufficient for the operation of the synthetase reaction, the assay medium was supplemented with 1.5 mM ATP.

The rate of oxidation of fatty acid substrates by isolated liver mitochondria has been observed to vary considerably depending on chain length, degree of unsaturation, substrate concentration, as well as amount of mitochondrial protein present in the assay system (Lopes-Cardozo and Van den Bergh, 1974).

Mitochondria from both rat and sheep liver oxidized each of the fatty acyl substrates investigated in a tightly coupled manner over a concentration range of 0.0016 to 0.1 mM. Assuming that 23 moles of oxygen are consumed during the oxidation of 1 mole of palmitate to CO₂ and H₂O, the observed rates for palmitate oxidation in the rat were
comparable with those reported by Lopes-Cardozo and Van den Bergh (1974). Maximal rates of oxidation for palmitate by sheep and rat liver mitochondria were observed at 0.01 mM palmitate in both animals (Fig. 18), however, the individual rates were dependent on the amount of mitochondrial protein. In contrast to the results of Lopes-Cardozo and Van den Bergh (1974) it was found that, in the reaction system supplemented with carnitine and coenzyme A, palmitate was inhibitory at higher concentrations. The oxidation profile obtained, was similar in shape to that reported by Vaartjes and Van den Bergh (1978) when assaying palmitate oxidation in a carnitine-free system. In addition, maximal rates of oxidation occurred at palmitate concentrations some 10 to 20 times lower than that reported in the literature (Lopes-Cardozo and Van den Bergh, 1974; Vaartjes and Van den Bergh, 1978).

The oxidation of stearate by rat liver mitochondria, like palmitate, produced maximal rates of oxidation at around 0.01 mM stearate and was also found to be inhibitory at higher concentrations. In sheep liver mitochondria, however, the oxidation of stearate was not inhibited at substrate concentrations above 0.01 mM. When the concentration of stearate was increased above the concentrations at which maximal rates of oxidation were observed, the oxidative pathway was shown to be saturated with respect to this substrate (Fig. 19).

The oxidation of oleate, linoleate and linolenate by rat liver mitochondria exhibited two peaks of maximal activity for each substrate, one at 0.005 mM and the other at 0.06 mM (Fig. 20). Although this has been observed by Vaartjes and Van den Bergh (1978) in a carnitine-free assay system the reason for two peaks of activity is uncertain.

In order to obtain a better comparison for the rates of oxidation of various long-chain fatty acids, the rates of oxidation of palmitate, oleate and linoleate were measured at the same substrate concentrations in both rat and sheep experiments. In addition, the oxidation of each substrate
Fig. 18 The effect of palmitate concentration on the uptake of oxygen by rat (●) and sheep (▲) liver mitochondria.

Assay conditions were as described on page 37.

Rat 3 mg mitochondrial protein gave a rate of oxidation for 10 mM succinate = 5.6 μmoles O₂/mg/h

Sheep 4 mg mitochondrial protein gave a rate of oxidation for 10 mM succinate = 2.6 μmoles O₂/mg/h

Each point represents the mean of a duplicate assay. The same mitochondrial preparation was used throughout the experiment.
Fig. 19 The effect of stearate concentration on the uptake of oxygen by rat (○) and sheep (▲) liver mitochondria.

Assay conditions employed were as described on page 37.

**Rat**
2.5 mg mitochondrial protein gave a rate of oxidation for 10 mM succinate = 4 μmoles O₂/mg/h

**Sheep**
3.7 mg mitochondrial protein gave a rate of oxidation for 10 mM succinate = 2 μmoles O₂/mg/h

Each point represents the mean of a duplicate assay. The same mitochondrial preparation was used throughout the experiment.
Fig. 20 The effect of the concentration of C\textsubscript{18} unsaturated fatty acids on the uptake of oxygen by rat and sheep liver mitochondria

Assay conditions employed were as described on page 37.

Rat 1.5 mg mitochondrial protein gave a rate of oxidation for 10 mM succinate = 7.6 \textmu moles O\textsubscript{2}/mg/h

Sheep 3.75 mg mitochondrial protein gave a rate of oxidation for 10 mM succinate = 2.2 \textmu moles O\textsubscript{2}/mg/h

Each point represents the mean of a duplicate assay. The same mitochondrial preparation was used throughout the experiment.
was assayed using the same mitochondrial preparation thus effectively standardising the amount of mitochondrial protein per assay.

The rates of oxidation of oleate and linoleate, shown in Tables 2 and 3, are expressed as a ratio of the rate obtained with palmitate at each concentration examined, thus enabling a comparison to be made of the rates of oxidation of these two unsaturated free fatty acids in both sheep and rat liver. Rat liver oxidized both oleate and linoleate at a rate 1.2 to 1.7 times that of palmitate, whereas sheep liver mitochondria oxidized oleate and linoleate at a rate 0.74 to 0.84 times that observed for palmitate. Thus, sheep liver mitochondria exhibit a preference for palmitate, whereas rat mitochondria oxidize unsaturated acids at considerably higher rates (P<0.05) than for palmitate. This suggests that sheep mitochondria exhibit a sparing effect with respect to the oxidation of unsaturated fatty acids.

3.3 The oxidation of long-chain acyl-CoA esters by rat and sheep liver peroxisomes

As discussed above the oxidation of long-chain fatty acids and their coenzyme A esters was generally thought to be associated with the enzymes of β-oxidation localized exclusively in mitochondria.

By applying spectroscopic techniques used to study mitochondrial β-oxidation, Lazarow (1978) was able to show that peroxisomes could also oxidize palmitoyl-CoA by β-oxidation. The results obtained by Lazarow and other workers in this field (Lazarow and de Duve, 1976; Thomassen et al., 1982) suggests that the peroxisomes are a major site for the β-oxidation of long-chain fatty acids in liver.

Oxygen uptake associated with the oxidation of palmitoyl-CoA, oleoyl-CoA and linoleoyl-CoA in a rat liver peroxisome-enriched fraction averaged 2.3 μmoles O₂/h/unit of catalase for all three acyl-CoA substrates over the concentration range of 0.01 to 0.8 mM.
Table 2  A comparison of the rates of oxidation of palmitic, oleic and linoleic acids in rat liver mitochondria.

<table>
<thead>
<tr>
<th>Free fatty acid conc (mM)</th>
<th>Rate of oxidation (μmoles O₂/mg/h)</th>
<th>18:1 16:0 ratio</th>
<th>18:2 16:0 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0</td>
<td>18:1</td>
<td>18:2</td>
</tr>
<tr>
<td>0.0016</td>
<td>1.42</td>
<td>1.66</td>
<td>2.21</td>
</tr>
<tr>
<td>0.0082</td>
<td>1.61</td>
<td>2.54</td>
<td>2.73</td>
</tr>
<tr>
<td>0.0165</td>
<td>1.88</td>
<td>2.46</td>
<td>2.95</td>
</tr>
<tr>
<td>0.0495</td>
<td>2.21</td>
<td>2.55</td>
<td>2.58</td>
</tr>
</tbody>
</table>

P values compared with 16:0 &lt; 0.02 &lt; 0.0001

The values are the means for two separate experiments, i.e. two separate mitochondrial preparations.

Data was subjected to a students t test.
Table 3  A comparison of the rates of oxidation of palmitic, oleic and linoleic acids in sheep liver mitochondria.

<table>
<thead>
<tr>
<th>Free fatty acid conc (mM)</th>
<th>Rate of oxidation (umoles O₂/mg/h)</th>
<th>18:1/16:0 ratio</th>
<th>18:2/16:0 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0</td>
<td>18:1</td>
<td>18:2</td>
</tr>
<tr>
<td>0.0016</td>
<td>1.39</td>
<td>1.10</td>
<td>1.03</td>
</tr>
<tr>
<td>0.0082</td>
<td>1.26</td>
<td>0.99</td>
<td>1.04</td>
</tr>
<tr>
<td>0.0165</td>
<td>1.26</td>
<td>1.04</td>
<td>1.08</td>
</tr>
<tr>
<td>0.0495</td>
<td>1.29</td>
<td>1.08</td>
<td>1.08</td>
</tr>
</tbody>
</table>

P values compared with 16:0: < 0.02 < 0.0001

The values are the means for two separate experiments, i.e. two separate mitochondrial preparations.

Data was subjected to a students t test.
When the same concentrations of palmitoyl-CoA, oleoyl-CoA and linoleoyl-CoA were used as substrates the peroxisome-enriched fraction from sheep liver exhibited average rates of oxidation for the three substrates of 1.0 μmoles O₂/h/unit of catalase. Although the average rate of oxidation for all three acyl-CoA substrates in sheep was half of that observed in rats, no difference in oxygen consumption was observed with the three acyl substrates in either animal.

The coenzyme A esters of the long-chain fatty acyl substrates, rather than the free acids, were used in the experiments reported above even though it has been shown that the palmitoyl-CoA synthetase activity in peroxisomes is sufficient to keep pace with a maximally functioning peroxisomal β-oxidation system (Krisans et al., 1980).

3.4 Fatty acyl substrates for CAT I and GPAT assays

Palmitoyl-CoA and linoleate were used as examples of saturated and unsaturated fatty acids in CAT I and GPAT assays using mitochondria from both rat and sheep liver.

Initially the activity of CAT I and GPAT was assayed using coenzyme A esters of both palmitate and linoleate, however, no esterification of linoleoyl-CoA to either [1⁴C]carnitine or [1⁴C]glycerol 3-phosphate was observed, suggesting that the enzymes involved were inhibited by an impurity in the linoleoyl-CoA. The experiment was repeated using the free acid and an acyl-CoA generating system and linoleate was readily esterified to [1⁴C]carnitine under conditions which were optimised for the β-oxidation of fatty acids determined in previous experiments measuring oxygen uptake (refer p. 37).

To ensure linoleate was converted to linoleoyl-CoA in the experiments which measured GPAT activity, CAT I activity, as well as malonyl-CoA inhibition of CAT I activity, the addition of 1.5 mM ATP, 5 μM coenzyme A in sheep liver mitochondria and 25 μM coenzyme A in rat liver mitochondria was included in the assay mixture.
McGarry et al. (1978) have shown, using [1-\textsuperscript{14}C]-palmitate and [1-\textsuperscript{14}C]palmitoyl-CoA as substrates in the production of ketone bodies, that the inhibitory effect of malonyl-CoA is virtually the same irrespective of which substrate is used, indicating that malonyl-CoA affects only the CAT I site. This was confirmed using palmitoyl carnitine as a substrate where no inhibition due to malonyl-CoA was observed (McGarry et al., 1978).

In addition, earlier work by McGarry et al. (1977), studying the oxidation of oleate to ketone bodies, showed that inhibition of oleate oxidation by malonyl-CoA was accompanied by a marked increase in the incorporation of fatty acids into both the phospholipid and triglyceride fractions without any change in the quantity of oleate utilized. This implies that malonyl-CoA exerts its inhibitory effect at the CAT I step.

Although the use of unlike substrates is undesirable, the observations of McGarry and co-workers support a comparison of palmitoyl-CoA and linoleate, coupled with an acyl-CoA generating system, as substrates for GPAT and CAT I assays.

3.5 Glycerol 3-phosphate acyltransferase activity in liver mitochondria

Mitochondrial glycerolphosphate acyltransferase (GPAT) is located on the inner surface of the outer mitochondrial membrane (Nimmo, 1979). The enzyme catalyzes the esterification of acyl-CoA, produced by the fatty acyl synthetase reaction, to glycerol 3-phosphate forming lysophosphatidic acid which is detected as the major assay product (Monroy et al., 1972; Haldar et al., 1979; Yamada and Okuyama, 1978).

Rat liver mitochondria were found to actively esterify both palmitoyl-CoA and linoleate, supplemented by an acyl-CoA generating system, as acyl substrates. Over the substrate concentration range of 10 to 90 \(\mu\)M, the enzyme
Fig. 21. The effect of palmitoyl-CoA concentration on the activity of glycerol 3-phosphate acyltransferase (GPAT) in rat (●) and sheep (▲) liver mitochondria.

Assay conditions employed were as described on page 43. The values are the means ± S.D.

Each point represents the mean of duplicate assays from two separate experiments, i.e. two separate mitochondrial preparations.

* determined as $^{14}$C-lipid soluble product formed from $^{14}$C-glycerol 3-phosphate
Fig. 22  The effect of linoleate, coupled to an acyl-CoA generating system, on the activity of glycerol 3-phosphate acyltransferase (GPAT) in rat (○) and sheep (▲) liver mitochondria.

Assay conditions employed were as described on page 43.

The values are the means ± S.D.

Each point represents the mean of duplicate assays from two separate experiments, i.e. two separate mitochondrial preparations.

* determined as 14C-lipid soluble product from 14C-glycerol 3-phosphate
showed a greater activity with palmitoyl-CoA than with linoleate (Figs. 21 & 22). This observation is in agreement with the work of Monroy et al. (1972) which showed that the mitochondrial GPAT enzyme esterifies saturated fatty acids in preference to unsaturated fatty acids.

Sheep liver mitochondria, although catalyzing the esterification of palmitoyl-CoA to glycerol 3-phosphate, did not catalyze the esterification of linoleate, in the presence of an acyl-CoA generating system (Figs. 21 & 22). The rate of acyl incorporation into ester lipid was linear with up to 0.5 mg of rat mitochondrial protein while in sheep, the activity of GPAT was linear up to 1.9 mg of mitochondrial protein (Fig. 23). The rate of esterification of palmitoyl-CoA to glycerol 3-phosphate was approximately linear for 3 min when incubated at 30°C (Fig. 24). Sheep and rat GPAT showed similar palmitoyl-CoA dependency and both showed maximal activity at 35 μM palmitoyl-CoA (Fig. 21).

At optimum palmitoyl-CoA the specific activity of mitochondrial GPAT from sheep liver was just under half that for the enzyme in rat liver mitochondria (Fig. 21). With both sheep and rat liver mitochondria, palmitoyl-CoA concentrations above 35 μM caused marked inhibition of mitochondrial GPAT activity with the enzymes of rat and sheep exhibiting only 60% of maximal activity at 90 μM palmitoyl-CoA.

Maximal rates of esterification for linoleate, supplemented with an acyl-CoA generating system, were also observed between 30 to 40 μM for rat liver mitochondrial GPAT. Above 40 μM, the esterification of linoleoyl-CoA to glycerol 3-phosphate was also inhibited. However, this inhibitory effect was less pronounced than that observed with palmitoyl-CoA.
Fig. 23 The relationship between glycerol 3-phosphate acyltransferase (GPAT) and the amount of mitochondrial protein in rat (○) and sheep (▲) liver mitochondria.

Assay conditions employed were as described on page 48 and 35 μM palmitoyl-CoA was used as the acyl substrate in each assay.

The values are the means and the error bars indicate the range of the observed values.

Each point represents the mean of a duplicate assay using one mitochondrial preparation.

* determined as ¹⁴C lipid soluble product formed from ¹⁴C-glycerol 3-phosphate
The effect of incubation time on the activity of glycerol 3-phosphate acyltransferase (GPAT) in rat liver mitochondria.

Assay conditions employed were as described on page 43 and 35 μM palmitoyl-CoA was used as the acyl substrate in each assay.

The values are the means and the error bars indicate the range of the observed values.

Each point represents the mean of a duplicate assay. The same mitochondrial preparation was used throughout the experiment.

* determined as 14C lipid soluble product formed from 14C-glycerol 3-phosphate
3.6 Carnitine acyltransferase activity in liver mitochondria

Carnitine acyltransferase (CAT) I catalyzes the first obligatory step in the oxidative breakdown of long-chain fatty acids and is believed to play an important role in regulating the oxidation of long-chain fatty acids (McGarry and Foster, 1980).

The activity of CAT I was assayed in rat and sheep liver mitochondria under conditions which were optimal for the β-oxidation of both saturated and unsaturated fatty acids (Section 2.6.1). Palmitoyl-CoA and linoleate were chosen for the comparison between saturated and unsaturated acyl substrates. When linoleate was the substrate, CAT I activity was determined in the presence of 25 μM and 5 μM coenzyme A in rat and sheep liver mitochondria, respectively. These concentrations of coenzyme A were chosen so that a direct comparison could be made between CAT I activity and oxygen uptake studies when linoleate, coupled with an acyl-CoA generating system, was the substrate. Under these conditions both CAT I activity and oxygen uptake were measured at coenzyme A concentrations that ensured β-oxidation was operating at maximal activity in mitochondria from both animals.

Rates of acylcarnitine formation were approximately linear for up to 3 min incubation at 30°C in rat liver mitochondria. This incubation period was chosen for studying CAT I activity in subsequent experiments (Fig. 25). The rate of acylcarnitine formation, using 90 μM palmitoyl-CoA as the acyl donor, was linear with respect to mitochondrial protein in the reaction medium up to 0.45 mg in both rat and sheep liver assays (Figs. 26 & 27). The use of higher concentrations of mitochondrial protein decreased CAT I activity, most probably due to the non-specific binding of unreacted acyl-CoA to the added protein (Taketa and Pogell, 1966), which would effectively reduce the concentration of acyl-CoA. When palmitoyl-CoA was the acyl substrate, in a concentration range of 45 to 270 μM, CAT I,
The effect of incubation time on the activity of carnitine acyltransferase (CAT) I in rat liver mitochondria.

Assay conditions employed were as described on page 41 and 35 μM palmitoyl-CoA was used as the acyl substrate in each assay.

The values are the means and the error bars represents the range of the observed values.

Each point represents the mean of a duplicate assay. The same mitochondrial preparation was used throughout the experiment.
Fig. 26 The relationship between carnitine acyltransferase (CAT) I activity and the amount of mitochondrial protein in rat liver mitochondria.

Assay conditions employed were as described on page 41 and 90 μM palmitoyl-CoA was used as the acyl substrate in each assay.

The values are the means of a duplicate assay and the error bars represent the range of the observed values.

The same mitochondrial preparation was used throughout the experiment.
Fig. 27 The relationship between carnitine acyltransferase (CAT) I activity and the amount of mitochondrial protein in sheep liver mitochondria.

Assay conditions employed were as described on page 41 and 90 μM palmitoyl-CoA was used as the acyl substrate in each assay.

The values are the means of a duplicate assay and the error bars represent the range of the observed values.

The same mitochondrial preparation was used throughout the experiment.
Fig. 28  The influence of palmitoyl-CoA concentration on the activity of carnitine acyltransferase (CAT) I in rat (○) and sheep (▲) liver mitochondria.

Assay conditions employed were as described on page 41.

The values are the means ± S.D.

Each point represents the mean of duplicate assays from two separate experiments i.e. two separate mitochondrial preparations.
in both rat and sheep liver mitochondria, exhibited maximal activity at 135 \( \mu \text{M} \) palmitoyl-CoA. At palmitoyl-CoA concentrations higher than this, CAT I activity was inhibited; the activity of both rat and sheep enzymes of 270 \( \mu \text{M} \) palmitoyl-CoA was only 60\% of the maximal activity (Fig. 28). However, the specific activity of the rat enzyme, with palmitoyl-CoA as substrate, was twice that observed for the sheep enzyme at most concentrations tested.

When linoleate, supplemented by an acyl-CoA generating system (Section 3.4) was the substrate, CAT I in mitochondria from both species showed a similar dependency on substrate concentration over the range 45 to 270 \( \mu \text{M} \) (Fig. 29). The specific activity of the rat enzyme, with linoleate as substrate, was three times that observed for the sheep enzyme. CAT I activity, in both rat and sheep liver mitochondria, was greater using linoleate, coupled with an acyl-CoA generating system, as substrate, than with palmitoyl-CoA at all concentrations tested. In the case of the rat liver enzyme, this is consistent with the difference in the rates of oxidation of palmitate and linoleate in the studies of oxygen uptake. However, the lower rates of oxidation of linoleate compared to palmitate by sheep liver mitochondria does not appear to arise from differences in CAT I activity in isolated mitochondria. This suggests that some other step(s) in the \( \beta \)-oxidation pathway may discriminate against the degradation of linoleate in sheep liver mitochondria.

As well as this, the activity of CAT I in the intact liver may show different kinetic properties to that observed in isolated mitochondria. This could be due to modification of CAT I activity upon isolation or by the presence of in vivo regulators such as malonyl-CoA, an intermediate of fatty acid biosynthesis, which has been shown to competitively inhibit the oxidation of long-chain fatty acids by its action at the CAT I site (McGarry and Foster, 1980).
Fig. 29 The influence of linoleate, coupled to an acyl-CoA generating system, on the activity of carnitine acyltransferase (CAT) I in rat (○) and sheep (▲) liver mitochondria.

Assay conditions employed were as described on page 41.

The values are the means ± S.D.

Each point represents the mean of duplicate assays from two separate experiments i.e. two separate mitochondrial preparations.
3.7 **Competition for acyl-CoA between glycerol phosphate acyltransferase (GPAT) and carnitine acyltransferase (CAT) I in rat liver mitochondria**

When mitochondrial GPAT activity was determined in the presence of an operational oxidation pathway, there was no change in the GPAT activity detected with mitochondria isolated from overnight fasted rats using 0 to 1.5 mM carnitine (Fig. 30). However, when between 1.5 and 3 mM carnitine was included in the assay (Fig. 30) a 34% decrease in GPAT activity was observed, indicating that under these conditions fatty acid oxidation could successfully compete with mitochondrial GPAT for acyl-CoA formed by the fatty acyl synthetase reaction.

Varying the pre-incubation period, prior to starting the reaction by the addition of ADP, from 0.3 to 5 min, in the presence of glycerol 3-phosphate, had no significant effect on the rate of oxidation of 0.01 mM palmitate (Table 4). This concentration of palmitate was chosen because palmitate oxidation was found to be maximal at 0.01 mM (see Section 3.2). Hence, any change in the oxidation rate due to a lowering of the acyl-CoA available to the oxidation pathway would be best observed at this concentration (Fig. 18). At higher palmitate concentrations the rate of oxidation would show no obvious response to a small decrease in acyl-CoA concentration.

Although the results for mitochondrial GPAT activity, presented above, provide no clear-cut solution to the metabolic fate of acyl-CoA formed by the fatty acyl synthetase reaction, they do suggest that, in sheep liver mitochondria, the lower rates of linoleate oxidation do not result from preferential esterification to glycerol 3-phosphate by GPAT. Instead, rather the reverse was observed; GPAT from sheep liver mitochondria exhibited negligible rates of esterification when linoleate, coupled with an acyl-CoA generating system, was used as a substrate.
The influence of carnitine concentration on the activity of glycerol 3-phosphate acyltransferase (GPAT) in rat liver mitochondria

Assay conditions as described on page 43, were supplemented with 0.45 mM ADP, 35 μM palmitoyl-CoA and carnitine as indicated above.

The values are means ± S.D.

Each point represents the mean of duplicate assays from two separate experiments, i.e. two separate mitochondrial preparations.

* determined as $^{14}$C lipid soluble product formed from $^{14}$C-glycerol 3-phosphate
Table 4  The effect of incubation time, prior to the addition of ADP, on the rate of oxidation of 0.01 mM palmitate, in the presence of 2.5 mM glycerol 3-phosphate, in rat liver mitochondria.

<table>
<thead>
<tr>
<th>Incubation time prior to addition of ADP (min)</th>
<th>Rate of oxidation of 0.01 mM palmitate (µmoles O₂/mg/h)</th>
<th>prep. 1</th>
<th>prep. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>2.24</td>
<td>2.20</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>2.24</td>
<td>2.17</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>2.24</td>
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<td></td>
</tr>
<tr>
<td>2.0</td>
<td>1.90</td>
<td>2.06</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
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<td></td>
</tr>
<tr>
<td>5.0</td>
<td>1.90</td>
<td>1.80</td>
<td></td>
</tr>
</tbody>
</table>

Assay conditions were as described on page 37.
3.8 Effect of malonyl-CoA on CAT I in both rat and sheep liver mitochondria

Although the effects of malonyl-CoA on CAT I activity are well established, its physiological role as a regulatory molecule is still the subject of much discussion. CAT I activities in both rat and sheep liver mitochondria were found to be inhibited by micromolar concentrations of malonyl-CoA using both palmitoyl-CoA and linoleate, coupled with an acyl-CoA generating system, as acyl substrates. However, the sensitivity of this enzyme to malonyl-CoA was dependent not only on the species studied, but also on the acyl substrate used (Figs. 31-34). When 90 μM palmitoyl-CoA was the acyl substrate, CAT I activity in rat liver mitochondria was 50% inhibited at 2.5 μM malonyl-CoA whereas the sheep enzyme, at the same substrate concentration, was 50% inhibited at approximately 1 μM malonyl-CoA. Although CAT I activity was 50% inhibited at these concentrations of malonyl-CoA, the maximal percentage inhibition observed over the concentration range of malonyl-CoA tested, using palmitoyl-CoA as the acyl substrate, was only marginally higher than 50% in both animals.

When 90 μM linoleate, coupled with an acyl-CoA generating system, was used as the substrate in similar experiments, a much larger difference in sensitivity to malonyl-CoA was observed between rat and sheep liver enzymes. The activity of the rat liver enzyme was 50% inhibited at 22 μM malonyl-CoA, while the CAT I activity in sheep liver mitochondria was 91% inhibited at only 1 μM malonyl-CoA. This suggests that in sheep especially this enzymatic step might play an important role in discriminating against the oxidation of linoleate.
Fig. 31 The inhibition of carnitine acyltransferase (CAT) I by malonyl-CoA in mitochondria from rat liver mitochondria using palmitoyl-CoA as the acyl substrate.

Assay conditions employed were as described on page 41 and 90 μM palmitoyl-CoA was the substrate concentration used throughout the experiment.

The values represent the means ± S.D. of duplicate assays from two separate mitochondrial preparations.

preparation 1 = 0.17 mg mitochondrial protein/assay
preparation 2 = 0.18 mg mitochondrial protein/assay.
Fig. 32 The inhibition of carnitine acyltransferase (CAT) I by malonyl-CoA in mitochondria from rat liver using linoleate, coupled to an acyl-CoA generating system, as the acyl substrate.

Assay conditions employed were as described on page 41 and 90 μM linoleate was the substrate concentration used throughout the experiment.

The values represent the means ± S.D. of duplicate assays from two separate mitochondrial preparations.

preparation 1 = 0.17 mg mitochondrial protein/assay
preparation 2 = 0.18 mg mitochondrial protein/assay.
Fig. 33 The inhibition of carnitine acyltransferase (CAT) I by malonyl-CoA in mitochondria from sheep liver using palmitoyl-CoA as the acyl substrate.

Assay conditions employed were as described on page 41 and 90 μM palmitoyl-CoA was the substrate concentration used throughout the experiment.

The values represent the means ± S.D. of duplicate assays from two separate mitochondrial preparations.

preparation 1 = 0.28 mg mitochondrial protein/assay
preparation 2 = 0.40 mg mitochondrial protein/assay.
Fig. 34 The inhibition of carnitine acyltransferase (CAT) I by malonyl-CoA in mitochondria from sheep liver using linoleate, coupled with an acyl-CoA generating system, as the acyl substrate.

Assay conditions employed were as described on page 41 and 90 μM linoleate was the substrate concentration used throughout the experiment.

The values represent the means ± S.D. of duplicate assays from two separate mitochondrial preparations.

preparation 1 = 0.28 mg mitochondrial protein/assay
preparation 2 = 0.4 mg mitochondrial protein/assay.
CHAPTER 4
DISCUSSION

4.1 Metabolism in the rumen

Of the main food components in the ruminant diet, carbohydrates are by far the most important quantitatively because of their superiority as an energy source under anaerobic conditions (Hungate, 1955). The micro-organisms present in the rumen degrade these carbohydrates to volatile fatty acids, namely acetate, propionate and butyrate (Weston and Hogan, 1968). The concentration of the individual volatile fatty acids is regulated by the microbial activity in the rumen. The extent of this microbial activity is influenced by several factors, including the type of diet, as well as the quantity of food ingested (Hungate, 1966).

4.1.1 The limited availability of polyunsaturated fatty acids to ruminants

Reiser (1951) first reported that the rumen micro-organisms are capable of hydrogenating unsaturated fatty acids. Comparison of the compositions of the fatty acids in the diet and rumen contents of sheep and goats provided additional evidence of the hydrogenation of dietary unsaturated fatty acids (Shorland et al., 1955; Dawson and Kemp, 1970). The micro-organisms present in the rumen produce enzymes that rapidly hydrolyse the acyl ester linkages of galactosylglycerides, phospholipids, triglycerides, sterol esters, methyl and ethyl esters (Garton et al., 1958, Clarke and Hawke, 1966). As a result of this lipolysis, unesterified fatty acids constitute a large fraction of the total fatty acids in the rumen. There is considerable evidence that the hydrogenation of polyunsaturated fatty acids in the rumen occurs after lipolysis (Hawke and Silcock, 1969, 1970). Of a number of pure cultures of rumen bacteria examined, only Butyrivibrio fibrisolvens exhibited hydrogenating activity and this organism was capable of hydrogenating cis-9, cis-12 18:2 to a C18 monoenoic but not to stearic acid. The first step in the hydrogenation of cis-9, cis-12, cis-15 18:3 by mixed
rumen micro-organisms (Kemp and Dawson, 1968) and by B. fibrisolvens (Kepler and Tove, 1967) involved an isomerization and the primary reaction product was identified as cis-9, trans-11, cis-15 18:3. The subsequent steps involved the hydrogenation of the cis-9 and cis-15 double bonds respectively resulting in the formation of trans-11 18:1 (Mills et al., 1970). However, there is still considerable uncertainty about the extent of hydrogenation of polyunsaturated fatty acids in the rumen in vivo.

4.2 Conservation of linoleic acid by ruminants

It has been estimated that linoleic acid should comprise 1-2% of the total dietary energy (Holman, 1968) in order to prevent deficiency signs in non-ruminants. Although the dietary intake of linoleic acid in the ruminant usually exceeds this level, the microbial hydrogenation of unsaturated fatty acids results in linoleic acid accounting for only 0.3 to 0.5% of the total energy available to the ruminant in the duodenum (Leat and Harrison, 1972). This limited supply of linoleic acid would be expected to cause severe deficiency signs in ruminants, but no limitations on growth and production have been recorded. Thus, it appears that there is a highly efficient usage of this essential fatty acid in ruminants (for review see Noble, 1984).

A high retention of linoleic acid in ruminants, when compared with non-ruminants, could be attained by a number of different biochemical mechanisms, such as, preferential esterification to form stable lipid structures, elongation and desaturation to form other members of the linoleic acid family and/or decreased oxidation of linoleic acid. There appears to be no experimental evidence for the preferential esterification of essential fatty acids into tissue phospholipids; rather the enzymes responsible for the acylation of glycerol 3-phosphate show similar properties when isolated from a range of animal tissues, including calf liver (Daae, 1973).

Therefore, it was decided to test the possibility that ruminants preferentially oxidize saturated and monoenoic
fatty acids, thereby conserving linoleic acid. Such a comparison has not been made previously because of the difficulty in isolating intact sheep liver mitochondria capable of oxidizing free fatty acids.

4.3 Oxidation of long-chain fatty acids in rat and sheep liver mitochondria

Intact mitochondria were isolated from both rat and sheep liver so that the rates of oxidation of palmitate, oleate and linoleate could be compared in liver mitochondria from a non-ruminant and ruminant species. Oxygen uptake was measured under optimum conditions in which the requirement for coenzyme A and carnitine were not rate limiting.

Palmitate was chosen to represent saturated fatty acid substrates because it is the most abundant saturated fatty acid in the mammalian cell and because much of the earlier work in this area has used palmitate as a substrate.

Pronounced differences were found in the ability of mitochondria isolated from the two species to oxidize the saturated and the unsaturated fatty acids. Sheep liver mitochondria oxidized oleate and linoleate at a rate which was 74–85% (P<0.05) that observed for palmitate when the fatty acids were at concentrations of 0.0016 to 0.00495 mM. In contrast, rat liver mitochondria oxidized oleate and linoleate at a rate which was 110–170% (P<0.05) that observed for palmitate. This indicates that sheep liver may preferentially oxidize saturated long-chain fatty acids such as palmitate at greater rates than unsaturated fatty acids and thereby conserve essential fatty acids. Sheep liver mitochondria oxidized both oleate and linoleate at lower rates than observed for palmitate. Therefore, it appears that the lower rates of oxidation observed for linoleate is not restricted to essential fatty acids but could be characteristic of unsaturated fatty acids in general.
4.4 Comparison of relative rates of oxidation of both saturated and unsaturated fatty acids

Lopes-Cardozo and Van den Bergh (1974) have shown that the oxidation of fatty acids by rat liver mitochondria is dependent on not only the amount of micellar fatty acid suspension added but also on the amount of mitochondrial protein and the concentrations of reaction components such as albumin, CoASH and carnitine present in the reaction medium. For this reason, reported rates of fatty acid oxidation by liver mitochondria vary considerably. Lopes-Cardozo and Van den Bergh (1974) have reported rates of oxidation for palmitate, oleate and linoleate in the presence of 100 μM CoASH and 1 mM carnitine of 2.15, 2.12 and 2.25 μmoles O₂/mg/h, respectively, in rat liver mitochondria. In the present study, the rates of oxidation for the same fatty acid substrates in rat liver mitochondria are 1.61, 2.54 and 2.73 μmoles O₂/mg/h, respectively, in the presence of 25 μM CoASH and 0.6 mM carnitine. Although the results from the present study are in favourable agreement with those results reported by Lopes-Cardozo and Van den Bergh (1974), a strict comparison cannot be made because of the different cofactor levels used in the two studies, as well as the different levels of fatty acid substrate at which maximum rates of oxidation were observed.

No literature values for the rates of oxidation of free fatty acids by intact sheep liver mitochondria are available. Although Koundakjian and Snoswell (1970) have reported that sheep liver mitochondria could oxidize the L-carnitine esters of lauric, myristic, palmitic and stearic acids at rates of 0.054, 0.049, 0.043 and 0.039 μmoles O₂/mg/h protein, respectively, their mitochondrial preparation was unable to oxidize the free fatty acids. However, in the same study Koundakjian and Snoswell (1970) reported that, in contrast, their preparations of rat liver mitochondria were able to oxidize the same free fatty acids, as mentioned above, at rates of 0.142, 0.122, 0.095 and 0.015 μmoles O₂/mg/h, respectively. The present study shows that intact sheep liver mitochondria oxidize palmitate at
half the rate obtained for palmitate by rat liver mitochondria. This suggests that the sheep liver mitochondria prepared by Koundakjian and Snoswell (1970) had damaged membranes that lacked the fatty acyl-CoA synthetase activity or contained an inactivated carnitine acyltransferase system. The rates of oxidation of the L-carnitine esters of palmitic and stearic acids by sheep liver mitochondria reported by Koundakjian and Snoswell (1970) are extremely low when compared to the rates observed in the present study, which were 1.5 and 1.3 μmoles O₂/mg/h protein for palmitate and stearate, respectively. In addition, the rates reported by Koundakjian and Snoswell (1970) for the oxidation of palmitic and stearic acids by rat liver mitochondria are considerably lower than those rates reported in the literature (Reid and Husbands, 1985; Lopes-Cardozo and Van den Bergh, 1974). This further suggests that the sheep liver mitochondria prepared by Koundakjian and Snoswell (1970) were, in some way, damaged and that the true rates of oxidation in both rat and sheep liver mitochondria were not observed due to an impaired assay system.

The absolute rates of oxidation of palmitate, oleate and linoleate by rat liver mitochondria observed in the present study are in agreement with those reported by Lopes-Cardozo and Van den Bergh (1974), and the substrate dependency profiles for the same fatty acids are similar in shape to those presented by Vaartjes and Van den Bergh (1978) in a carnitine-free system. Comparing the rate of oxidation of palmitate as a function palmitate concentration Vaartjes and Van den Bergh (1978) observed that, in contrast to a carnitine supplemented reaction medium, concentrations of palmitate higher than those producing maximum rates of oxidation were inhibitory. In contrast, earlier work by Lopes-Cardozo and Van den Bergh (1974) reported that palmitate, at higher concentrations, did not inhibit its own oxidation. Interpreting their data, Vaartjes and Van den Bergh (1978) have suggested that the decline in the rate of oxidation of palmitate at concentrations higher than
210 nmol/mg protein, in the carnitine-free system, is due to partial uncoupling of respiratory chain phosphorylation. The decline in the rate of oxidation of linoleate as the concentration of linoleate gradually increases has also been interpreted to be due to uncoupling of the respiratory chain at these concentrations.

4.5 Long-chain free fatty acids as uncouplers of oxidative phosphorylation

For many years free fatty acids have been known to be uncouplers of respiratory chain phosphorylation (Pressman and Lardy, 1956; Lehninger and Remmert, 1959; Hulsman et al., 1960; Borst et al., 1962). This uncoupling action of fatty acids becomes stronger with increasing chain length and is especially powerful in the case of unsaturated fatty acids (Van den Bergh, 1966). Respiratory uncouplers allow electron transport to continue with subsequent oxygen uptake, but prevent the phosphorylation of ADP to ATP. According to this definition, if fatty acid substrates partially uncouple phosphorylation, then one would expect to observe a decrease in the respiratory control ratios at higher fatty acid concentrations because, at uncoupling concentrations of free fatty acids, the initial rate of oxygen consumption, prior to the addition of ADP, would be greater (Fig. 11). It has been demonstrated that, at concentrations of fatty acids which cause uncoupling, the generation of ATP in the respiratory chain is prevented and fatty acid oxidation is inhibited at the level of fatty acid activation (Van den Bergh, 1966).

The results reported in this present study also demonstrate a decline in the rate of oxidation of palmitate as the concentration of this fatty acid is increased. However, at all concentrations of fatty acids tested, the rate of oxidation prior to the addition of ADP remained constant. This suggests that respiratory uncoupling was not responsible for the decline in the rates of oxidation of palmitate, oleate, linoleate and linolenate. Only at higher free fatty acid concentrations, above 0.1 mM, and well
beyond the concentration at which maximum rates of oxygen uptake were observed, were signs of uncoupling evident with noticeable increases in the rates of oxidation prior to the addition of ADP.

4.6 Long-chain fatty acids as inhibitors of enzyme activity

Palmitoyl-CoA and other long-chain acyl-CoA derivatives inhibit a wide variety of enzymes, some related and others unrelated to lipid metabolism (Taketa and Pogell, 1966; Volpe and Vagelos, 1976). These include acetyl-CoA carboxylase (Bortz and Lynen, 1963), fatty acid synthetase from yeast (Lynen et al., 1964), from pigeon liver (Dorsey and Porter, 1968), from rat liver (Tubbs and Garland, 1963) and human liver (Roncari, 1975), along with citrate synthase (Wieland and Weiss, 1963; Hansel and Powell, 1984). Lust and Lynen (1968) demonstrated that the inhibition of the yeast fatty acid synthetase complex by long-chain acyl-CoA compounds was competitive with respect to malonyl-CoA. The concept of feedback inhibition, as a regulatory mechanism for fatty acid synthetase, received support from observations that liver concentrations of long-chain acyl-CoA are elevated during starvation and high-fat feeding when hepatic fatty acid synthetase activity is low (Bortz and Lynen, 1963; Tubbs and Garland, 1963). However, the effect of palmitoyl-CoA on the pigeon liver enzyme was questioned by Dorsey and Porter (1968). Inhibition by palmitoyl-CoA depended on the molar ratio of palmitoyl-CoA to protein, suggesting that palmitoyl-CoA acts as a detergent and is not a site specific inhibitor. Similar findings were reported by Roncari (1975) with purified human liver synthetase.

Whether fatty acyl-CoA inhibition, in general, is due to a non-specific detergent effect or is actually due to a site-specific interaction of physiological significance has been a controversial topic. However, Hansel and Powell (1984) have reported that citrate synthase responds to long-chain acyl-CoA selectively, in a manner distinguishable from its response to synthetic detergents.
The decline in the rates of oxidation for palmitate, oleate, linoleate and linolenate with increasing concentration could, therefore, be due to a specific inhibitory effect by the long-chain acyl-CoA on a single, or variety of, enzyme(s) involved in the oxidation of long-chain fatty acids, rather than a non-specific uncoupling effect as stated by Vaartjes and Van den Bergh (1978).

4.7 Fatty acid concentrations producing maximum rates of oxidation

In a carnitine-free reaction medium, Vaartjes and Van den Bergh (1978) observed maximum rates of oxidation for palmitate, oleate, linoleate and linolenate at fatty acid concentrations of 0.55, 0.07, 0.07 and 0.07 mM, respectively. In contrast, the fatty acid concentrations at which maximum rates of oxidation were observed in the present study, where 0.6 mM carnitine was included in the reaction medium, were 0.01, 0.005, 0.005 and 0.005 mM for palmitate, oleate, linoleate and linolenate respectively. The markedly different fatty acid concentrations required to give maximum rates of oxidation in the experiments of Vaartjes and Van den Bergh (1978), compared to those recorded in this study, may be related to the inclusion of carnitine in the current reaction medium.

In the carnitine-supplemented reaction medium, the acyl-CoA produced by the fatty acyl-CoA synthetase reaction, associated with the outer mitochondrial membrane, would be rapidly transported across the inner membrane as acylcarnitine to the enzymes of β-oxidation. Therefore, in the presence of carnitine, the concentration of acyl-CoA within the matrix should reflect more closely the acyl-CoA concentration outside the carnitine barrier due to the nature of the carnitine acyltransferase system proposed by Houslay and Stanley (1982). In a carnitine-free system this transport pathway is non-operational, unless endogenous carnitine is present. Groot et al. (1974) demonstrated that in rat liver mitochondria, in the absence of carnitine, the
activation of long-chain fatty acids is catalysed by the medium-chain acyl-CoA synthetase located in the matrix. In the absence of carnitine it is this activation step that is rate limiting in the oxidation of long-chain fatty acids (Vaartjes and Van den Bergh, 1978). The long-chain fatty acid concentration needed for half-maximal velocity of fatty acid oxidation by isolated rat liver mitochondria is about 1 μM in the presence of carnitine and 100-200 μM in the absence of carnitine (Van Tol and Hulsmann, 1970). Therefore, the effective concentration of free acyl groups required to produce a particular level of acyl-CoA within the matrix is considerably higher in a carnitine-free system compared to that observed when carnitine is present in the reaction medium.

4.8 Mitochondrial oxidation and esterification of long-chain fatty acids

Many of the substrates that are metabolised by mitochondria require no modification prior to their metabolism. In contrast, long-chain fatty acids must first be converted to their CoA esters, by the fatty acyl-CoA synthetase reaction, before mitochondrial metabolism of the acyl substrates can proceed (Van Tol and Hulsmann, 1970). Once formed, the acyl-CoA can act as a substrate for mitochondrial esterification, catalyzed by glycerol 3-phosphate acyltransferase (GPAT), as well as β-oxidation, which involves carnitine acyltransferase (CAT)I as the first step. Conversion of the free acid to its thioester enables the acyl substrate to be transported across the inner membrane, in conjunction with the carnitine acyltransferase/translocase pathway, to thus become available to the enzymes of β-oxidation. Under normal conditions, where the carnitine transport system is operational, the majority of fatty acids entering the mitochondria are activated via the fatty acyl-CoA synthetase associated with the outer mitochondrial membrane (Norum et al., 1966; Groot et al., 1974).

The activities of CAT I and GPAT were investigated
using palmitoyl-CoA and linoleate, supplemented with an acyl-CoA generating system, as substrates in experiments with both rat and sheep liver mitochondria. Although it would have been preferable to use linoleoyl-CoA as the acyl substrate, the lack of activity observed with the linoleoyl-CoA in the early stages of this investigation (see section 3.4) necessitated the use of an acyl-CoA generating system. The use of an acyl-CoA generating system requires the addition of coenzyme A to the reaction medium. However, the use of linoleate, coupled to an acyl-CoA generating system, relies on the operation of the fatty acyl-CoA synthetase to produce linoleoyl-CoA, the true substrate for the CAT I and GPAT enzymes. Consequently, any differences in the activity of either CAT I or GPAT towards linoleate, supplemented with an acyl-CoA generating system, in rat and sheep liver mitochondria may be due to different acyl-CoA levels resulting from the different concentrations of coenzyme A used in rat and sheep liver assays.

4.9 Mitochondrial glycerol 3-phosphate acyltransferase activity in rat and sheep liver mitochondria

Mitochondrial GPAT activity was assayed to determine whether linoleate oxidation in sheep liver mitochondria was decreased because of increased, and preferential, esterification of linoleate to glycerol 3-phosphate. However, linoleoyl-CoA, formed from linoleate in the presence of an acyl-CoA generating system, was esterified to glycerol 3-phosphate in rat liver mitochondria at slower rates than palmitoyl-CoA, the rates of incorporation being 1.5 and 2.1 nmoles/min/mg protein respectively. GPAT in sheep liver mitochondria showed negligible activity towards linoleate, supplemented with an acyl-CoA generating system, compared to palmitoyl-CoA which was incorporated at a rate of 0.9 nmoles/min/mg protein.
4.10 Carnitine acyltransferase I activity in rat and sheep liver mitochondria

Carnitine acyltransferase (CAT) I is the first obligatory step in the oxidation of acyl-CoA substrates in mitochondria and is thought to have an important regulatory function in the oxidation of long-chain fatty acids (McGarry and Foster, 1980). CAT I is inhibited by malonyl-CoA concentrations that are within the range of concentrations found in vivo (Zammit, 1981). The sensitivity of CAT I to malonyl-CoA is the basis for the proposed regulatory role of CAT I (refer Section 1.9).

Kopec and Fritz (1973) compared the properties of carnitine palmitoyltransferase (CPT) I and CPT II using a partially purified preparation from liver. This study provided evidence that, in contrast to CPT I, CPT II catalyzes the reaction only in the direction towards long-chain acyl-CoA formation. If this is true, then once across the permeability barrier imposed by the inner-mitochondrial membrane, the acyl-CoA would be committed to the β-oxidation pathway as there would be no transport mechanism operating by which the acyl-CoA could pass back across the inner membrane. Consequently, any discrimination against the use of linoleate as a substrate for oxidation would have to occur at the CAT I step.

In the presence of CoASH, ATP and Mg²⁺, palmitate is activated at the mitochondrial outer membrane with a maximal rate of 70 nmoles/min/mg protein, which is 7-fold greater than the maximal rate of palmitate oxidation (Van Tol and Hulsmann, 1970). Therefore, under the above conditions the fatty acyl-CoA synthetase reaction is not rate limiting.

Houslay and Stanley (1982) have proposed that, rather than a unidirectional transport of acyl-CoA from the intermembrane space into the matrix, the carnitine-acyl carnitine exchange mechanism operates in a cyclic manner with continual mixing of the two acyl-CoA pools on either side of the inner membrane. According to Houslay and Stanley (1982), the flux through this bidirectional
transport cycle is considerably faster than the flux through the \( \beta \)-oxidation pathway. Therefore, if CAT II and/or the first enzymatic step of the \( \beta \)-oxidation pathway have a lower affinity for linoleoyl-CoA than for other acyl-CoA's, this would allow linoleoyl-CoA to be effectively preserved and transported back across the inner membrane.

The overall rates of oxidation of palmitate and linoleate suggested that, in contrast to the rat, sheep oxidize linoleate at a lower rate than palmitate. However, this was not reflected in the activity of CAT I when assayed in sheep liver mitochondria using palmitoyl-CoA and linoleate, supplemented with an acyl-CoA generating system, as the substrates. In both rat and sheep liver experiments the rate of linoleoylcarnitine formation was consistently higher than that observed for palmitoylcarnitine formation.

There is no evidence for the restricted oxidation of linoleate, as observed in the oxygen uptake studies, being achieved by substrate specificity of the CAT I enzyme. However, other reaction steps in the \( \beta \)-oxidation pathway, namely CAT II and/or the acyl-CoA dehydrogenase reaction, might be points of regulation.

In the present study CAT I activity was determined using washed mitochondria isolated from rat and sheep liver. Although the results allow a comparison of the in vitro activity of the enzymes towards palmitoyl-CoA and linoleate, supplemented with an acyl-CoA generating system, other factors may affect the in vivo activity of the enzyme. One such factor could be the concentration of malonyl-CoA.

4.11 Effect of malonyl-CoA on CAT I activity in rat and sheep liver mitochondria

Malonyl-CoA is an intermediate in the de novo synthesis of fatty acids. The concentration of malonyl-CoA depends on the physiological status of the animal (Cook et al., 1980; Ontko and Johns, 1980; Saggerson and Carpenter, 1981; Robinson and Zammit, 1982; McGarry and Foster, 1981;
Stakkestad and Bremer, 1983). Malonyl-CoA competitively inhibits fatty acid oxidation at the CAT I enzymatic step by a mechanism which, as yet, is not fully understood. The sensitivity of the CAT I enzyme to inhibition by malonyl-CoA is strongly dependent on the in vivo concentrations of malonyl-CoA in the tissue from which the mitochondria are isolated (Saggersson and Carpenter, 1981). For example, the inhibitory potency of malonyl-CoA towards carnitine palmitoyltransferase (CPT) I activity from rat heart and skeletal muscle, respectively, is about 40- and 200-times that observed with rat liver mitochondria (Saggersson and Carpenter, 1981; McGarry et al., 1983). Zammit (1983(a)) has been able to mimic this in vivo response by pre-incubating mitochondria with malonyl-CoA in vitro prior to measuring the sensitivity of the CAT I to this inhibitor. As well as being sensitized by pre-incubation with malonyl-CoA, the CAT I enzyme has also shown to be reversibly desensitized in vitro by the thiol-group reagent NbS₂ (Zammit, 1983(b)). The operation of a sensitization/desensitization cycle in vivo could have profound physiological relevance to the concept of CAT I being a major regulatory site in the oxidation of long-chain fatty acids. Such a sensitization/desensitization cycle could amplify the response of the enzyme to small changes in malonyl-CoA concentration.

Other factors such as intracellular tissue pH (Stephens et al., 1983), levels of other CoA esters, e.g. succinyl-CoA, methylmalonyl-CoA, acetyl-CoA and free coenzyme A (Mills et al., 1983; McCormick et al., 1983), as well as the experimental conditions employed (McGarry and Foster, 1981), alter the kinetic properties of CAT I and the response of CAT I to inhibition by malonyl-CoA.

The role of malonyl-CoA in the regulation of long-chain fatty acid oxidation in ruminants has not been extensively investigated. In the present study, the sensitivity of CAT I to inhibition by malonyl-CoA was shown to depend on the animal tissue under examination and on the acyl substrate used to assay the activity of the enzyme
(Section 3.8). This difference in the degree of inhibition by malonyl-CoA on CAT I with different acyl substrates has not previously been reported. The activity of CAT I in rat liver mitochondria, with 90 μM palmitoyl-CoA, was 50% inhibited at 2.5 μM malonyl-CoA (see Section 3.8). In contrast, the activity of CAT I was 50% inhibited at about 1 μM malonyl-CoA in sheep liver mitochondria with 90 μM palmitoyl-CoA (see Section 3.8). When the sensitivity of CAT I to inhibition by malonyl-CoA was investigated using 90 μM linoleate, supplemented with an acyl-CoA generating system, the rat enzyme was 50% inhibited at 22 μM malonyl-CoA while the sheep enzyme was 91% inhibited at only 1 μM malonyl-CoA.

The above observations suggest that, under in vivo conditions, the tissue levels of malonyl-CoA present in rat liver (Zammit, 1981) are likely to alter the activity of CAT I so that linoleate could be preferentially transported as opposed to palmitate. In contrast, sheep liver mitochondria show quite the opposite picture, with linoleoylcarnitine formation almost completely inhibited at very low levels of malonyl-CoA and palmitate preferentially transported under these conditions. However, one possible reason for the greater inhibition of CAT I by low levels of malonyl-CoA, observed with linoleate as substrate, in sheep liver mitochondria, compared to that observed in rat liver mitochondria, may be due to different acyl-CoA concentrations in rat and sheep experiments, as a consequence of the different concentrations of coenzyme A supplied to the acyl-CoA generating system.

Mills et al. (1983) have reported that malonyl-CoA is not the only physiological compound capable of suppressing carnitine palmitoyltransferase (CPT) I, since chemically related compounds, e.g. succinyl-CoA, methylmalonyl-CoA, acetyl-CoA and free CoA found in mammalian cells, also inhibit the CAT I step. Malonyl-CoA, and its analogues, appear to interact with the same site as palmitoyl-CoA on CAT I and it appears that the degree of occupancy of that site by inhibitors governs the activity of CPT I (Mills et
Inhibition of CPT I activity by CoA-related compounds was particularly significant in tissues where the enzyme shows the greatest sensitivity to malonyl-CoA (Mills et al., 1983).

In contrast with the CoA esters, free coenzyme A inhibited the CPT I reaction to a similar degree in rat liver, heart and skeletal muscle (Mills et al., 1983). Furthermore, CoA is a product of the CPT I reaction. These observations suggest that free CoA may work through mass action, rather than by the malonyl-CoA type of mechanism. If CoA exerted its effect on CPT I activity via mass action, it is possible that free CoA may protect CPT I by displacing malonyl-CoA from the mitochondrial enzyme. That other factors contribute to this effect of free CoA is suggested by the experiments of Mills et al. (1983) in which CPT I activity of skeletal muscle mitochondria was measured over a range of malonyl-CoA concentrations in the presence, and absence, of free CoA. The concentrations of free CoA used were such that they should have displaced a large fraction of the malonyl-CoA without causing a marked inhibition of CAT I activity, thereby protecting the CPT I enzyme. However, no protection was observed.

The incorporation of different levels of free CoA in the reaction medium when linoleate was used as the acyl substrate for CAT I in rat and sheep liver experiments raises questions as to the contribution by free CoA to the inhibition of CAT I activity observed in the present study.

4.12 Regulation of the oxidation of linoleic acid in sheep

Lindsay and Leat (1977) have shown that both the entry rate of linoleic acid in sheep and its contribution to CO₂ production was considerably less than stearic acid. They observed that infused ¹⁴C-labelled linoleic acid was initially incorporated into plasma phospholipids, exhibiting maximal radioactivity in this fraction at about 1 day post-infusion, and subsequently incorporated into plasma cholesterol esters. Furthermore, the incorporation of
linoleic acid into phospholipid and cholesterol ester was considerably greater than observed with stearic acid (Lindsay and Leat, 1977).

Based on this observation Lindsay and Leat (1977) advanced several possible reasons for ruminants being able to survive on levels of essential fatty acids which would produce deficiency signs in non-ruminants.

(i) the levels of linoleic acid in the plasma-free fatty acid fraction are low and during starvation the amount of linoleic acid entering this free fatty acid pool is smaller compared to non-essential fatty acids.

(ii) the amount of linoleic acid entering cells from the circulation is much smaller than for other non-essential fatty acids. Therefore, the amount of linoleic acid available for energy production is smaller compared to stearic and palmitic acids.

(iii) the greater incorporation of linoleic acid than stearic acid into the plasma phospholipids and cholesterol esters may serve as a protective mechanism, thereby conserving linoleic and other essential fatty acids.

Therefore, although the incorporation of linoleic acid into phospholipids and cholesteryl esters in plasma may serve to protect this essential fatty acid during its transport from the small intestine, once taken up by the liver cell linoleic acid may still re-enter the free fatty acid pool within the cell as a consequence of its release from cholesterol ester.

The results observed in the present study suggest that in sheep, the small contribution of linoleic acid to CO₂ production, compared to stearic acid, observed by Leat and Lindsay (1977) could result from the modification of carnitine acyltransferase (CAT) I activity towards linoleoyl-CoA as a consequence of the inhibitory action of malonyl-CoA. However, it would seem likely that the
conservation of linoleic acid in ruminants is the result of the complex interaction of a number of protective mechanisms, rather than the result of total protection at one step in the metabolism of linoleic acid. Therefore, the results reported in the present study could compliment the proposals of Leat and Lindsay (1977) concerning the conservation of linoleic acid in ruminants.

4.13 Inhibition of CAT I by malonyl-CoA in ruminants

The involvement of malonyl-CoA in the regulation of long-chain fatty acids, as proposed by McGarry and Foster (1980), implies that a high rate of lipogenesis, with subsequent elevated levels of malonyl-CoA, is a prerequisite for the inhibition of CAT I. Brindle and Zammit (refer Brindle et al., 1985) have observed that rates of lipogenesis in the ruminant liver are less than 10% of those rates observed in rat liver. Although the capacity for fatty acid synthesis in the ruminant liver is poorly expressed (Ballard et al., 1969), it is known that the enzymes involved in the synthesis of malonyl-CoA from acetate, namely acetyl-CoA synthetase (Cook et al., 1969; Knowles et al., 1974; Quraishi and Cook, 1972) and acetyl-CoA carboxylase (Ballard et al., 1972), are present in the ruminant liver. These could produce concentrations of malonyl-CoA which would be sufficient to severely inhibit CAT I activity and hence linoleate oxidation.

In contrast, in the non-ruminant system, the concentrations of hepatic malonyl-CoA are dependent mainly on the production of acyl-CoA from glucose metabolism and are directly related to the nutritional status of the animal (McGarry and Foster, 1981).
4.14 Metabolism of acetate in the ruminant

Much of the energy requirement of ruminants is met by the metabolism of volatile fatty acids, namely acetate, propionate and butyrate, produced in the rumen following the fermentation of ingested herbage (Warner, 1964). The rumen epithelium is capable of metabolizing butyrate and propionate, and to some extent acetate (Pennington, 1952; Pennington and Sutherland, 1956) and the mixture of volatile fatty acids entering the portal blood is depleted in butyrate compared to that produced in the rumen (Annisson, et al., 1957). Pennington (1952) has shown that liver slices from sheep are capable of metabolizing acetate, propionate and butyrate. However, using perfused goat liver in the presence of all three acids it was found that propionate and butyrate, but not acetate, are extensively consumed (Holter et al., 1963).

Therefore, acetate is the only short-chain volatile fatty acid present in significant concentration in the peripheral blood in sheep (Reid, 1950), with propionate and butyrate being largely removed during its passage through the liver. Even in the starved ruminant, the portal circulation is continually supplied with acetate arising from the fermentation process in the rumen (Lindsay and Ford, 1964). The control of acetate uptake by ruminant tissues is poorly understood and the question of how much acetate is metabolized by the liver in vivo is not resolved. Holter et al. (1963) found that acetate was not metabolised by perfused goat liver when propionate and butyrate were present, whereas Cook and Miller (1965), working with anaesthetized sheep, found that more acetate than propionate was removed by the liver in some cases. Acetate and propionate penetrate sheep liver mitochondria very readily (Smith and Osborne-White, 1971), but there is a strong inhibition of acetate metabolism in the presence of propionate (Smith, 1971). It would appear, from the above, that the peripheral tissues of ruminants utilize most of the acetate, but at the same time, in both fed and fasted animals, a substantial amount of endogenous acetate is produced (Bergman and Wolff, 1971).
Despite the low rates of acetate utilization by the ruminant liver, it is possible that malonyl-CoA is synthesized independently of hepatic lipogenesis. The enzyme malonyl-CoA decarboxylase has been isolated from a number of non-ruminant animals (Kim and Kolattukudy, 1978(a); Kim and Kolattukudy, 1978(b); Kim et al., 1979) but, as yet, not ruminants. It is possible that the enzymes acetyl-CoA synthetase, acetyl-CoA carboxylase and malonyl-CoA decarboxylase operate in conjunction with each other to synthesize and degrade malonyl-CoA independently of fatty acid synthesis. The amounts of acetate utilized by this synthesis/degradation cycle, if it is operational in the ruminant liver, would only need to be minimal to maintain tissue levels of malonyl-CoA which would severely limit the oxidation of linoleic acid.

4.15 Effect of methylmalonyl-CoA on CPT I activity in sheep liver

In contrast to non-ruminants, the fed ruminant is characterized by a high rate of gluconeogenesis from propionate. Methylmalonyl-CoA is an intermediate of this pathway of propionate metabolism to glucose in the ruminant liver and has been shown to specifically inhibit carnitine palmitoyltransferase I activity in sheep (Brindle et al. 1985). Scaife et al. (1978) have observed that in sheep adipose tissue methylmalonyl-CoA acts as a primer for the synthesis of odd-chain fatty acids, which implies that some methylmalonyl-CoA is present in the cytosol. Brindle et al. (1985) propose that in species which are characterized by high rates of gluconeogenesis from propionate in the fed state, the integration of carbohydrate and fatty acid metabolism in the liver may be mediated by methylmalonyl-CoA in conjunction with malonyl-CoA.

Mills et al. (1983) have shown that carnitine palmitoyltransferase I in rat liver, heart and skeletal muscle is inhibited by malonyl-CoA and other related compounds, namely, succinyl-CoA, methylmalonyl-CoA, propionyl-CoA, acetyl-CoA and free coenzyme A. The
possibility that these thioesters act to regulate CAT I activity in vivo, either individually, or in conjunction with malonyl-CoA needs clarification.

Conclusion

In conclusion, it would appear that the limited oxidation of linoleic acid observed in sheep liver mitochondria is not achieved by regulation at one particular step in the metabolism of linoleic acid. In contrast, the discrimination against the use of linoleic acid as an energy source, thereby conserving this acyl substrate, seems likely to result from a number of different regulatory mechanisms operating together. However, as in many metabolic pathways, certain enzymatic steps appear to play major regulatory roles. The results reported in this study provide evidence to support the involvement of CAT I in this regulation of long-chain fatty acid oxidation in the ruminant. The true regulator of this enzyme in vivo is unclear in the ruminant but, as observed in other animal tissues, it seems likely that malonyl-CoA could have an important role.

Summary

1. Mitochondria isolated from the livers of sheep and rats were shown to oxidize palmitate, oleate and linoleate in a tightly coupled manner, by monitoring the oxygen consumption associated with the degradation of these acids in the presence of 2 mM-L-malate.

2. Rat liver mitochondria oxidized linoleate and oleate at a rate 1.1-1.7 times that of palmitate.

3. Sheep liver mitochondria had a specific activity for the oxidation of palmitate that was 50-80% that of rats and a specific activity for the oxidation of oleate and linoleate that was 30-40% that of rats. This would indicate that sheep conserved linoleate by limiting its oxidation.

4. The possibility that either the mitochondrial esterification pathway, via glycerol 3-phosphate
acyltransferase, or oxidation pathway, via carnitine acyltransferase I, was involved in this protective mechanism was investigated.

5. Rat and sheep liver mitochondrial GPAT showed greater activity towards palmitoyl-CoA as an acyl substrate compared to linoleate, supplemented with an acyl-CoA generating system. Sheep liver mitochondrial GPAT showed negligible activity towards linoleate and an acyl-CoA generating system. This would suggest that the reduced rates of linoleate oxidation in sheep liver mitochondria compared to palmitate is not achieved by the preferential esterification of linoleate to glycerol 3-phosphate. However, the use of an acyl generating system complicates this comparison.

6. Carnitine acyltransferase (CAT) I actively esterified palmitoyl-CoA and linoleate, supplemented with an acyl-CoA generating system, in rat and sheep liver mitochondria. In both cases the rate of linoleoylcarnitine formation was greater than that observed for the formation of palmitoylcarnitine.

7. The CAT I reaction in both rat and sheep liver was inhibited by micromolar amounts of malonyl-CoA. With 90 μM palmitoyl-CoA as substrate, CAT I was inhibited by 50% with 2.5 μM malonyl-CoA in rats, and in sheep, 50% inhibition was found with all malonyl-CoA concentrations between 1 and 5 μM. With 90 μM linoleate, supplemented with an acyl-CoA generating system, as substrate for CAT I, a much larger difference in response to malonyl-CoA was observed. The rat enzyme was found to be 50% inhibited at 22 μM malonyl-CoA, whereas sheep liver CAT I was 91% and 98% inhibited at 1 μM and 5 μM malonyl-CoA respectively.

8. It is proposed that malonyl-CoA may act as an important regulator of β-oxidation in sheep, discriminating against the use of linoleate as an energy-yielding substrate.
Further research

In order to substantiate the proposed role of malonyl-CoA in the regulation of fatty acid oxidation it would be useful to establish whether a synthesis/degradation cycle exists in the ruminant liver that is capable of producing levels of malonyl-CoA of around 1 µM. This concentration of malonyl-CoA would enable ruminants to conserve linoleic acid by severely limiting its oxidation.

It would be important to investigate whether the inhibitory effect of malonyl-CoA on CAT I activity is the same with linoleoyl-CoA as that observed using linoleate and an acyl-CoA generating system. In addition, a study of the inhibitory effect of malonyl-CoA on CAT I using other long-chain fatty acyl-CoA substrates in both rat and sheep liver mitochondria could further elucidate the regulatory role of malonyl-CoA in the oxidation of long-chain fatty acids. Furthermore, there is a possibility that there are different CAT I enzymes for saturated and polyunsaturated fatty acids in sheep liver.
BIBLIOGRAPHY


Hanson, R.W. and Ballard, F.J. (1968). J. Lipid Res. 9, 667-668.


APPENDIX