THE CONTROL OF FATTY ACID COMPOSITION OF MEMBRANE PHOSPHOLIPIDS IN THE GENETICALLY OBESE (ob/ob) MOUSE

by

Stephen James Hughes

A thesis presented for the degree of

Doctor of Philosophy

at the

University of Southampton

Department of Nutrition
University of Southampton

March 1985

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

DEPARTMENT OF NUTRITION

Doctor of Philosophy

THE CONTROL OF FATTY ACID COMPOSITION OF MEMBRANE PHOSPHOLIPIDS IN THE GENETICALLY OBESE (ob/ob) MOUSE

by Stephen James Hughes

The fatty acyl composition of membrane phospholipids of lean and obese mice was analysed by gas liquid chromatography. Alterations to the hepatic mitochondrial, microsomal and brown adipose tissue(BAT) mitochondrial phospholipid compositions were observed in obese mice, and involved reduced linoleic acid content, increased oleic acid content and increased polyunsaturated fatty acid components.

Essential fatty acid desaturation was investigated as a possible mechanism for controlling membrane lipid composition. Hepatic Δ6-desaturase activity was primarily located in the mitochondrial fraction in mice. Both Δ6- and Δ5-desaturase activity were increased in the liver of obese mice. The increase in Δ6-desaturase activity did not occur until weaning. Restriction of food intake reduced but did not normalise the hepatic Δ6-desaturase activity of the obese mice. Both cold acclimation and tri-iodothyronine injection reduced hepatic Δ6-desaturase of obese mice to levels observed in lean mice. The elevated enzyme activity in the obese animals was maintained after the induction of hypothyroidism.

Some changes in fatty acid desaturation could be related to membrane lipid compositional changes in obese mice. However, thyroid hormone treatment was only partially successful in normalising the hepatic mitochondrial phospholipid-fatty acyl composition. It was concluded that changes in desaturase activity could not be used to predict changes in membrane lipid composition.

Activity of lysophosphatidylcholine (lysoPC) acyltransferase in obese mice exhibited normal substrate selectivity with regard to oleic and linoleic acids. In experimental conditions paralleling the triglyceride fatty acid composition of obese mice, hepatic microsomal lysoPC acyltransferase from lean or obese mice incorporated increased levels of oleic acid and reduced levels of linoleic acid into phosphatidylcholine.

Consequences of altered membrane lipid composition on membrane protein function were investigated. The Arrhenius break temperature of ATP-Pi exchange activity was increased in hepatic mitochondria of the obese mouse. This was interpreted to suggest that a protein component of the ATP-Pi exchange complex was sensitive to the membrane compositional changes. However, the Arrhenius characteristics of hepatic mitochondrial cytochrome oxidase and succinate-cytochrome C reductase were similar in lean and obese animals.

BAT mitochondrial thermogenesis, assessed by the GDP binding assay, was similar in lean and obese mice housed at room temperature, or exposed to a cold environment for 1 hour. It was concluded that altered membrane lipid composition was not involved in the defective BAT mitochondrial thermogenesis of obese mice.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. David York for his guidance and encouragement in the production of this thesis. I would also like to thank Professor T. G. Taylor for the provision of research facilities. I am grateful to Karen Platt for performing the GLC analysis of samples, and to Colin Bunce and his staff for maintaining and providing the animals used in this work.

Finally, I would like to thank Jo, for typing the manuscript and for her support and encouragement.

This work was carried out with the financial support of the British Diabetics Association.

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
ATP	adenosine 5' triphosphate
ADP	adenosine 5' diphosphate
BAT	brown adipose tissue
BSA	bovine serum albumin
Bmax	maximum binding
cAMP	cyclic adenosine monophosphate
coA	coenzyme A
DIT	diet induced thermogenesis
DPH	diphenyl hexa-1,3,5-triene
DSC	differential scanning calorimetry
EDTA	ethylenediaminetetra-acetic acid
EFA	essential fatty acid
ESR	electron spin resonance
FAD	flavine adenine nucleotide
FCCP	carbonyl cyanide m-chlorophenylhydrazone
GDP	guanosine 5' diphosphate
Gpp(NH)p	guanylylimidodiphosphate
GLC	gas liquid chromatography
HEPES	2-(N-2-hydroxyethylpiperazine-N-2) ethanesulphonic acid
K_D	dissociation constant
LPL	lipoprotein lipase
LysoPC	lysophosphatidylcholine
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
NST	non shivering thermogenesis
Pi	inorganic phosphate
POPOP	dimethyl bis phenyloxazoyl benzene
PPO	diphenyloxazole
PUFA	polyunsaturated fatty acid
PMF	proton motive force
SNS	sympathetic nervous system
T_3	tri-iodothyronine
TLC	thin layer chromatography

Tris	tris(hydroxymethyl)amino-methane
VHM	ventromedial hypothalmus

Fatty Acids

14:0	myristic acid
16:0	palmitic acid
16:1ω9	palmitoleic acid
18:0	stearic acid
18:1ω9	oleic acid
18:2ω6	linoleic acid
18:3ω6	γ-linolenic acid
18:3ω3	linolenic acid
18:4ω3	octadecatetraenoic acid
20:3ω6	eicosatrienoic acid
20:4ω6	arachidonic acid
20:5ω3	eicosapentaenoic acid
22:6ω3	docosahexaenoic acid

CONTENTS

CHAPTER 1 INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 Obesity in Man

Obesity arises from an accumulation of superfluous body energy resulting either from an excessive energy intake and/or from a reduced energy expenditure. In western societies, obesity is prevalent. Most authorities define obesity as a 10% increase in body weight over the ideal or norm (Craddock, 1978). Ideal body weights are arbitrary standards such as those prepared by the Metropolitan Life Insurance Company (USA) which distinguish people of small, medium and large frame size for a given height. A more recently devised standard is the Body Mass Index (BMI) which is calculated by dividing body weight (kg) by the square of height (m^2). Once more an arbitrary value must be assigned to define obesity (Taylor, 1982). Extreme obesity may be considered as a 20% increase in weight over the ideal or standard (Craddock, 1978). In men and women aged between 20 and 40 years, between 12% and 25% of the population fall into this category. The consequences of extreme obesity include increased incidence of cardovascular disease, respiratory insufficiency and diabetes, and may result in premature death (Craddock, 1978).

Many factors may contribute to the occurrence of obesity in man. Excessive consumption of very palatable food in western societies, reduced metabolic expenditure, lack of exercise, psychological and social factors, may all be involved. Due to the problems of studying the development of obesity in humans, many workers use animal models for research purposes. Various models exist, including hypothalmic, endocrine, genetic and dietary obesities. These have been reviewed previously (Bray, 1974; Bray and York, 1979; York, 1983).

Feeding very palatable "cafeteria" or "supermarket" diets to certain strains of rats leads to the development of obesity (Sclafani and Springer, 1976). This may have particular relevance to certain causes of obesity in western society. Endocrine models of obesity, resulting from corticosteroid or insulin injection are useful in the study of obesity associated with Cushing's Syndrome and insulinomas (Bray, 1974). Several genetic animal models of obesity exist which exhibit mendelian recessive characteristics, including the obese mouse (ob/ob), the diabetic mouse(db/db) and the Zucker fatty rat (fa/fa). Lesions to the ventromedial part of the hypothalmus (VHM) by electrolytic techniques, knife cuts, or by injection of gold

thioglucose or monosodium glutamate all produce hypothalmic (VHM) obesity (Bray and York, 1979).

It is evident that extreme obesity in man results from several contributing factors. Recently, increasing emphasis has been placed on the role of metabolic efficiency and decreased energy expenditure (James and Trayhurn, 1976). In this respect, hypothalmic and genetic animal models are useful tools in the study of obesity.

1.2 Genetic Obesity

The animal model used in the studies reported in this thesis is the genetically obese (ob/ob) mouse. The Southampton colony is of undetermined background derived from the original stock provided by the Institute of Animal Genetics (Edinburgh). The obesity is characterised by hyperphagia, hyperinsulinemia, insulin resistance, hyperglycemia, hypothermia and an increase in adipose tissue size. (Bray and York, 1979). Most of these abnormalities are present in other genetic animal models of obesity to varying degrees of severity. The obesity is inherited as a single recessive mutation on chromosome 6 and provides a model for juvenile onset obesity and for insulin independant diabetes in man (Coleman, 1978).

1.3 Energy Balance and Thermogenesis

Obesity is caused by the development of a positive energy balance. The obese mouse has an increased energy intake, (is hyperphagic) and exhibits a decrease in energy expenditure compared to lean littermates, i.e. it shows an increased efficiency of energy utilisation. Hyperphagia is mainly attributed to a loss of the normal diurnal variation of feeding observed in lean mice (Bailey et al., 1975). Food intake in rodents is normally highest during the night whereas obese mice continue to eat throughout the day as well. The control of food intake is complex and both neural and endocrine factors are involved (Bray, 1978). The precise mechanism causing the hyperphagia of the obese mouse is uncertain, although alteration in several endocrine hormone functions may be important.

It is well documented that hyperphagia is not necessary for the maintenance of the obesity. In experiments in which obese mice were

fed reduced quantities of food (similar or lower than ad libitum fed lean mice) (Dubuc, 1976a) or were pair weight-gained with lean littermates (Wetton et al., 1973), their body fat content as a percentage of body weight remained unaltered whereas protein deposition was further depressed when compared to ad libitum fed obese animals. In more recent studies (Dubuc et al., 1984), the weight gain of obese mice was reduced by restricted feeding and/or voluntary exercise. Under either of these regimes, or with the two combined, obese mice maintained a higher body fat content than lean animals. It was concluded that obese mice would rather restrict their growth rate and reduce protein deposition than alter their body fat content.

Obese mice exhibit a reduction in energy expenditure and this can be monitored by measuring a reduced oxygen consumption in these animals (Boissoneault et al., 1978). Obese mice are less physically active than lean animals (Joosten and Van der Kroon, 1974 c). This reduces the energy requirement for work and hence contributes to the decreased energy expenditure of these animals. The resting metabolic rate (RMR) of obese mice is normal or slightly increased when measured at their thermoneutral temperature (34°C) (Trayhurn and James, 1978). It has been reported that obese mice have lower maintenance energy requirement than lean animals (i.e. the energy intake required for maintenance of body weight) (Dubuc, 1976 a). However, this has been contested (Miller et al., 1979).

The main contributor to the increased metabolic efficiency of the obese mouse is defective thermogenesis. In mammals, maintaining a constant body temperature at lower ambient temperatures requires the expenditure of energy to produce heat. The processes involved are shivering and non-shivering thermogenesis (NST). Thus, the thermogenic processes are functioning in mice housed at normal room temperature (22°-26°C) since the thermoneutral temperature of these animals is approximately 34°C. Normally, intake of large quantities of food (in excess of requirements) also stimulates thermogenic mechanisms to maintain energy balance. This has been termed diet induced thermogenesis (DIT). In the obese mouse, both non-shivering and diet induced thermogenic mechanisms are defective. Obese mice fed palatable "cafeteria" diets failed to stimulate thermogenesis to the extent observed in over feeding lean animals (Trayhurn et al., 1982). Most studies on defective thermogenesis in obese mice have centred on

NST. Obese mice had lower core temperatures (by 1 - 2°C) than lean animals when housed at normal room temperature, and failed to maintain these body temperatures when exposed to a 4°C environment unless previously acclimated to 12°C (Trayhurn et al., 1977; Trayhurn and James, 1978). Obese mice housed for one hour across a range of temperatures from 10 to 25°C had lower core temperatures and reduced oxygen consumption compared to lean animals. Housing at thermoneutrality normalised both parameters. The capacity of lean and obese mice for NST has been assessed by monitoring oxygen consumption after noradrenaline injection. Trayhurn and James (1978) and Hogan and Himms-Hagen (1980) reported an impaired response to noradrenaline injection in obese mice whereas Macdonald and Stock(1979) reported similar responses in lean and obese animals.

Thurlby and Trayhurn (1979) concluded that the defective thermoregulatory thermogenesis of the obese mouse contributes significantly to their reduced energy expenditure and high metabolic efficiency.

The increased metabolic efficiency of the obese mouse results in a massive accumulation of storage fat; both an increase in fat cell size and fat cell number are observed (Bray and York, 1979). In twelve week old animals, the body weight of the obese mouse is more than double that of its lean littermate, whereas the adipose tissue weight may be ten times larger (Rath and Thenen, 1980). The syndrome of the genetically obese mouse is very complex. One author concludes that the metabolic abnormalities of the obese mouse not only cause the surplus energy (resulting from depressed energy expenditure) to be stored as fat, but cause body fat to be regulated to a much higher level than in the lean animal (Dubuc et al., 1984).

1.4 Endocrine Status

1.4.1 Insulin

Hyperinsulinemia is characteristic of many genetic animal models of obesity. It is not thought to be a primary defect in the obese mouse, since the earliest appearance of hyperinsulinemia at 17 days (Dubuc, 1976 b) is preceded by the thermogenic defect and by increased fat deposition at 10 days (Thurlby and Trayhurn, 1978; Boissonneault et al., 1978).

By measuring the incorporation of radiolabelled leucine into proinsulin, in collagenase isolated pancreatic cells stimulated by glucose, high rates of in vitro insulin synthesis and secretion have been observed (Berne, 1975). An increase in the number of ß cells was also apparent in the pancreas of the obese mouse (Bray and York, 1979). Both of these observations are consistent with the hyperinsulinemia of the obese mouse. Beloff-Chain et al. (1979) have suggested that an increased stimulation of insulin secretion by pituitary factors may also be involved. They tested the ability of pituitary perfusates from lean and obese mice to stimulate insulin release from pancreatic islets. Highest insulin release was observed using obese mouse pituitary perfusates. A gene dosage effect of insulin releasing activity was obtained from pituitary perfusates of obese, heterozygote and homozygote lean animals. In studies to further characterize the insulin releasing activity (Billingham et al., 1982), the pituitary factor was identified as ß-cell trophin, a peptide component of ACTH.

Insulin resistance develops in many tissues of the obese mouse as the serum insulin concentrations increase with age. It can be shown to be a secondary response to the hyperinsulinemia by starving obese mice, which reduces serum insulin levels and restores insulin sensitivity (Le Marchand-Brustel et al., 1977). Insulin resistance can be partially attributed to a down regulation of the number of insulin receptors. This is apparent in liver, adipose tissue and muscle plasma membrane. (Kahn et al., 1973; Soll et al., 1975; Le Marchand-Brustel and Freychet, 1978). Post receptor defects also contribute to peripheral insulin resistance. In skeletal muscle from obese mice, glycogen synthesis activity remained depressed in the presence of maximally stimulating doses of insulin (Le Marchand-Brustel and Freychet, 1977). Glucose transport was elevated and showed an increased responsiveness to insulin in adipose tissue from obese mice aged between 4 and 7 weeks. By 12 weeks of age, glucose utilisation in adipose tissue was impaired although hexose transport was still fully sensitive to insulin (Czech et al., 1977).

1.4.2 Glucagon

The concentration of serum glucagon is increased in the adult obese mouse (Dubuc et al., 1982). This may result from insensitivity of

pancreatic ∝cells to insulin (Flatt et al., 1982).

1.4.3 Adrenal Corticosteroids

Obese mice exhibit increased serum corticosterone levels which are consistent with their adrenal hypertrophy (Naeser, 1974; Dubuc, 1977). Indeed, a gene dosage effect of serum corticosterone concentrations has been observed in obese, heterozygote and homozygote lean mice (Herberg and Kley, 1975). Increased serum corticosterone may be the result of increased adrenal stimulation by ACTH. ACTH injection stimulated corticosterone release from the adrenal cortex to a larger extent in obese mice than in lean animals (Naeser, 1974). In addition, pituitaries from obese mice contained more ACTH than those from lean animals. In experiments in which isolated pituitaries were perfused, increased rates of ACTH secretion were observed from the pituitaries of obese animals. (Edwardson and Hough, 1975). However, the precise role of ACTH in the elevated corticosterone secretion of the obese mice is unclear since pituitaries from 5 week old obese animals contained normal amounts of ACTH whereas elevated serum corticosterone was apparent at the much earlier age of 17 days (Edwardson and Hough, 1975; Dubuc, 1977).

It is of interest to note that many of the observed metabolic defects of the obese mouse are attenuated or corrected by adrenalectomy. Adrenalectomy normalised weight gain, reduced serum insulin and glucose levels, normalised ^{131}I uptake into thyroid, restored $[Na^+ K^+]$-ATPase sensitivity to thyroid hormones, increased protein deposition and normalised glucose uptake into muscle in obese mice (Naeser, 1976; Yukimara and Bray, 1978; Shimomura et al., 1981; Oshima et al., 1984; Saito and Bray, 1984).

1.4.4 Thyroid Hormones

Considerable controversy exists as to whether the obese mouse is hypothyroid. Several characteristics of hypothyroidism can be observed in the suckling preobese mouse. These include depressed serum T_4 levels in mice aged between 10 and 18 days (Mobely and Dubuc, 1979), delayed eye and ear opening, and delayed lower incisor tooth eruption (Van der Kroon et al., 1982.) However, in the adult

animal serum T_3 concentrations are normal, although uptake and turnover of ^{131}Iodine are reduced (Joosten and Van der Kroon, 1974b; York et al., 1978a). Certain tissues may be insensitive to thyroid hormones in the obese mouse. In liver, tri-iodothyronine (T_3) injection elicited a reduced stimulation of fatty acid synthetase activity (Volpe and Marasa, 1975), whereas hepatic $\left[Na^+ K^+\right]$- ATPase activity was unresponsive to stimulation at all concentrations of T_3 administered to obese animals(Shimomura et al., 1981). In adipose tissue from obese mice, catecholamine induced lipolysis was also insensitive to thyroid hormones (Otto et al., 1976; York et al., 1978a).

Pharmacological doses of thyroxine administered to obese mice normalised their reduced core temperatures but did not prolong their resistance to 4°C cold exposure (Thenen and Carr, 1980; Hogan and Himms-Hagen, 1981). These observations confirm previous reports that thyroid malfunction does not cause hypothermia in the obese mouse (Ohtake et al., 1977).

1.5 Development of Obesity

The obese mouse exhibits a complex series of metabolic abnormalities, many of which are secondary to the obesity. In order to define the primary locus of the obese syndrome, attempts have been made to determine the earliest recognisable defect during the development of the obesity. Considerable emphasis has been placed on developmental studies. However, the problem is complicated by the inability to recognise pre-obese mice at a very early age. Obese mice only become physically recognisable as such at around 3 weeks, at the time of weaning (Bray and York, 1979).

The earliest known defect in the pre-obese mouse is a reduction in thermogenesis. This has been indicated by measuring the enhanced fall in rectal temperature on mild cold exposure (Trayhurn et al., 1977) or by measuring the depressed oxygen consumption of pre-obese pups (Kaplan and Leveille, 1974). These methods have been utilized as tests for recognition of pre-obese mice. Depressed oxygen consumption has been measured in pre-obese mice aged 11 days and older (Boissonneault et al., 1978) whereas reduced thermogenesis as indicated by the depression of rectal temperature has been observed

as early as 10 days of age (Thurlby and Trayhurn, 1978). Brown adipose tissue (BAT) thermogenesis has been shown to be defective in pre-obese mice aged 14 days and may be reduced in animals aged 10 days (Goodbody and Trayhurn, 1982). In agreement with this, sympathetic control of BAT, as indicated by noradrenaline turnover measurements, is also reduced in 14 day old pre-obese pups housed at 24°C (Knehans and Romsos, 1983).

Carcass analysis of litters reveals an increase in body fat content of pre-obese mice, which may be apparent at 7 days of age (Boissonneault et al., 1978). Joosten and Van der Kroon (1974a) have observed an increase in fat cell size in adipose tissue from 12 to 14 day old pre-obese mice, which correlates well with the above observations. In this study epididymal adipocytes were removed during surgery and the obese animals identified subsequently at 6 weeks of age.

Van der Kroon et al. (1982) have reported delayed eye and ear opening and delayed lower incisor tooth eruption in pre-obese mice, indicative of a reduced thyroid function. These observations are consistent with the depressed serum T_4 concentrations observed in pre-obese mice aged between 10 and 18 days (Mobley and Dubuc, 1979) and a reduced number of $\left[Na^+ K^+\right]$- ATPase enzyme units in muscle of 14 day old pups (Lin et al., 1979).

Through the development of a non-invasive method for measuring milk intake (which involves the equilibration of tritiated water into the dams' milk), Rath and Thenen (1979) have shown that pre-obese pups are not hyperphagic. Immediately prior to weaning at 17 days, serum corticosterone concentrations are elevated and remain high in obese mice, whereas the levels in the lean animals fall during the post weaning period (Dubuc, 1977). Also at this time (17 days), a moderate (two fold) hyperinsulinemia can be detected in pre-obese mice and is accompanied by a mild hypoglycemia (Dubuc, 1976b; Rath and Thenen, 1980). This suggests that insulin sensitivity is normal in pre-weaned obese mice.

After weaning, the rate of weight gain of the obese mouse increases dramatically so that they can be physically identified as such before 4 weeks of age (Bray and York, 1979). Hyperphagia first becomes

apparent between 4 and 5 weeks in obese animals (Lin et al., 1977). A reduction in hepatic $[Na^+ K^+]$-ATPase activity paralleled by an apparent loss of enzyme units is first observed after weaning (Hughes and York,1983; Lin et al., 1979). Between 3 and 4 weeks of age, reduced protein deposition has been reported in the hind limb muscle of obese mice (Bergen et al., 1975). Using the protein content and urinary output of 3 methyl-histidine, Trostler et al. (1979) calculated that muscle degradation was increased in obese mice whereas fractional synthesis rates were normal. Thus the reduced protein depostion of the obese mouse results from increased protein catabolism.

Obese mice exhibit a moderate hyperinsulinemia prior to weaning, although serum insulin concentrations increase dramatically after weaning. At 23 days of age, serum insulin concentrations were elevated four fold in the obese animals and by five weeks of age had increased six fold (Dubuc, 1976b). Hyperglycemia first becomes apparent at 4 weeks of age in obese mice and serum glucose levels have almost doubled by 5 weeks (Dubuc,1976b). The hyperglycemia is caused partly by the developing insulin resistance of peripheral tissues.

1.6 Brain Development

The development of the brain in obese mice is abnormal. An increase in catecholamine concentration but not turnover has been measured in the hypothalmus of the obese mouse (Lorden et al., 1976). Bereiter and Jeanrenaud (1979) have reported decreased brain weights of obese animals. Van der Kroon and Speijers (1979) confirm this and also report a reduction in DNA content of obese mouse brains. They suggest that these abnormalities may result from congenital hypothyroidism of obese mice.

1.7 Carbohydrate and Lipid Metabolism

Hyperglycemia is observed in the obese mouse after weaning (Dubuc, 1976b). Several factors may contribute to this state. These include the rapidly developing insulin resistance of many tissues in the face of increasing hyperinsulinemia (Soll et al., 1975; Le Marchand-Brustel and Freychet, 1978), reduced uptake of glucose by soleus muscle of

obese mice which is independant of the hyperinsulinemia and is not improved by fasting (Cuendet et al., 1976), and an increase in gluconeogenesis in obese mouse liver. This is probably caused by elevated glucocorticoid stimulation. Appearance of hyperglycemia is coincident with maximally elevated corticosterone concentrations in the obese mouse (Dubuc, 1977). Increased gluconeogenesis may also result from elevated serum glucagon which can be measured at 5 weeks of age in obese animals (Dubuc et al., 1982). Elliot et al. (1971) studied carbohydrate metabolism in isolated perfused livers from lean and obese mice. They observed faster rates of glycogenolysis in perfused livers from obese animals and concluded that these would contribute to the hyperglycemia. In agreement with this, Das and Hems (1974) reported increased activity of glycogen phosphorylase but not glycogen synthase in liver and heart from obese mice. Katyare and Howland (1974) have shown that the activity of phosphofructokinase (PFK) is increased in the liver of the obese mouse. They also report the defective allosteric inhibition of this enzyme by citrate and ATP and suggest that this condition may indirectly augment the increased lipogenic activity in obese animals. It is proposed that this could occur by increasing glycolytic flux and hence increasing the supply of citrate and acetylcoA substrates for fatty acid synthesis.

Glucose transport and metabolism in adipose tissue is elevated and shows an increased responsiveness to insulin in obese animals aged between 4 and 7 weeks (Czech et al., 1977). This may reduce their hyperglycemia. By 12 weeks of age, glucose metabolism in adipose tissue is impaired although transport is still fully sensitive to insulin (Czech et al., 1977)

An increase in lipogenesis is observed in liver, adipose tissue and carcass of adult obese mice. Lipogenic activity may be normal or increased in skin of obese animals, depending on the strain studied (Loten et al., 1974; Rath and Thenen, 1980). In a developmental study in which $[^3H]$-water incorporation into lipid was measured, lipogenic activity was shown to be similar in both the liver and adipose tissue of lean and pre-obese pups at 18 days of age (Rath and Thenen, 1980).

It can therefore be concluded that both the increased body fat content and increased fat cell size observed in pre-obese mice, (Boissonneault et al., 1978; Joosten and Van der Kroon, 1974a) result from increased uptake of dietary fat into tissues mediated by lipoprotein lipase activity (LPL). Increased LPL activity has been measured in heart, skeletal muscle and white adipose tissue, although not in lung and kidney of obese mice (Rath et al., 1974). Increased lipogenesis first becomes apparent after weaning in obese animals (Rath and Thenen, 1980). This was shown to be much more pronounced in adipose tissue than in liver. By five weeks of age, lipogenic activity was clearly elevated in both tissues of obese mice. Increased fatty acid synthesis in the carcass of obese animals was only apparent after weaning (Rath and Thenen, 1980).

The increase in lipogenesis of the obese mouse is probably caused by the hyperinsulinemia. Insulin injection of lean animals increased fatty acid synthesis whereas streptozoticin diabetic obese mice had markedly reduced lipogenic activity at all sites of synthesis (Loten et al., 1974).

In brown adipose tissue (BAT), lipogenesis is similar in 14 day old lean and pre-obese pups. Lipogenic activity rises during development and a transient increase is observed in obese animals between 21 and 28 days of age. By 5 weeks of age, activity is similar once more. The transient increase has been attributed to the initial hyperinsulinemia observed before weaning (Dubuc,1976b) and a very rapidly developing insulin resistance in BAT (Mercer and Trayhurn, 1983). Increased accumulation of triglyceride in BAT from adult obese mice can therefore be attributed to elevated LPL activity in this tissue (Rath et al., 1974).

1.8 Defective Thermogenesis and Brown Adipose Tissue (BAT)

The obese mouse exhibits a defective thermogenesis. This is indicated by the reduced rectal temperature and depressed oxygen consumption of obese animals housed at temperatures between 10 and 25°C (Trayhurn and James, 1978). Obese mice fail to maintain their body temperatures when exposed to a 4°C environment, unless previously acclimated to 12°C (Trayhurn and James, 1978) and also fail to respond to cafeteria overfeeding which promotes DIT in lean animals (Trayhurn et al., 1982).

It has been suggested that the defective thermogenesis is the main contributor to the increased metabolic efficiency of the obese mouse (Trayhurn and James, 1979).

In rodents, the primary site of adaptive thermogenesis has been assigned to brown adipose tissue (BAT) (Foster and Friedman, 1978). Studies using radiolabelled microspheres to measure regional blood flow to tissues during noradrenaline stimulated thermogenesis, discount liver and muscle and implicate BAT as the main site of defective thermogenesis in the obese mouse (Thurlby and Trayhurn, 1980).

The mechanism of BAT thermogenesis is briefly outlined below. This process has been extensively reviewed in the literature (Nicholls, 1979; Nicholls and Locke, 1984). The thermogenic mechanism of BAT involves the uncoupling of oxidative phosphorylation in the mitochondria and is controlled by the sympathetic nervous system (SNS). Release of noradrenaline from nerve endings located in the tissue, stimulates intracellular cyclic adenosine monophosphate (cAMP) production which in turn activates hormone sensitive lipase. Triglyceride is hydrolysed releasing fatty acids, which it is suggested (Locke et al., 1982a; 1982b) are the messengers responsible for opening the proton conductance pathway or proton channel across the inner mitochondrial membrane. This dissipates the mitochondrial proton motive force (PMF) and prevents ATP synthesis. Normal respiratory control is lost and respiration increases to a maximum. Thus, the mitochondrion is uncoupled. Dissipation of the PMF produces heat. BAT is highly vascularised which allows transfer of the heat to the body of the animal as a whole. Fatty acids released on triglyceride breakdown also act as fuel for respiration and thermogenesis, via ß-oxidation, after transport into the mitochondrion.

A 32K dalton membrane protein unique to BAT mitochondria is believed to be responsible for the proton channel. Its function is inhibited by purine nucleotide binding. Indeed, ADP is believed to be the physiological inhibitor that binds to the 32K dalton protein and recouples oxidation and ATP synthesis in the mitochondria. Since the *in vivo* intracellular concentration of ADP is high, the transient messenger must overcome the purine nucleotide blockage to uncouple the mitochondria and stimulate thermogenesis. These observations have lead to the development of an *in vitro* assay system which involves the binding

of purine nucleotides (normally GDP) to isolated BAT mitochondria. The extent of binding is proportional to the activity of the proton conductance pathway and hence reflects the thermogenic capacity of the tissue (Nicholls, 1976; 1979).

Himms-Hagen and Desautels (1978) have shown that purine nucleotide binding to BAT mitochondria from obese mice was reduced. In addition, binding to obese mouse BAT mitochondria was unresponsive to the normal stimuli. Thus the normal increase in GDP binding observed after cold exposure (Hogan and Himms-Hagen, 1980), after overfeeding a cafeteria diet (Trayhurn et al., 1982) or after chronic treatment with noradrenaline (Hogan and Himms-Hagen, 1981) were all absent in the obese mouse. Sympathetic control of BAT thermogenesis can be assessed by measuring noradrenaline turnover in the tissue, after equilibration of injected radiolabelled noradrenaline into nerve terminal pools. At normal housing temperature (22 - 26°C), sympathetic activity in BAT (as indicated by noradrenaline turnover) was reduced in obese mice (Knehans and Rosmos, 1982; Young and Landsberg, 1983; Zaror-Behrens and Himms-Hagen, 1983). This correlates well with the reduced thermogenic capacity of BAT from these animals as indicated by GDP binding studies. However, noradrenaline turnover in BAT from these animals was responsive to cold exposure at 14°C and 4°C, unlike GDP binding (Knehans and Rosmos, 1982; Young and Landsberg, 1983; Zaror-Behrens and Himms-Hagen, 1983). Zaror-Behrens and Himms-Hagen (1983) have suggested that BAT from obese mice may be refractory to sympathetic stimulation.

In summary, both diet and cold induced BAT mitochondrial thermogenesis are defective in the obese mouse. It has been suggested that this defect is the main contributing factor which causes the high metabolic efficiency and subsequent obesity of these animals (Thurlby and Trayhurn, 1979, 1980). Other defective thermogenic mechanisms possibly include the reduced $\left[Na^{+}K^{+}\right]$ -ATPase activity of liver and muscle in obese mice (York et al., 1978b, Lin et al., 1979).

1.9 Altered Membrane Composition and Function in Obese Mice

The development of the syndrome of the genetically obese mouse involves a complex series of metabolic alterations. It is difficult to understand how a single gene defect (and hence a defect in one protein)

can cause the multiplicity of alterations observed. The developmental studies indicate that the primary lesion is close to the defective thermogenesis of the obese mouse. However, it is unclear how a defect in the thermogenesis could cause the reduced brain weights (Bereiter and Jeanrenaud, 1979), defective thyroid function (Joosten and Van der Kroon, 1974b), reduced protein deposition (Dubuc, 1976b), and the altered pituitary-adrenal function (Naeser, 1974; Edwardson and Hough, 1975). It is more plausible to suggest that a generalised change occurs in the tissues of the obese mouse which results in the wide spectrum of alterations observed.

Many of the defective processes of the obese mouse are associated with membrane functions. These include the defects in glucose transport in muscle and adipose tissue (Cuendet et al., 1976; Czech et al., 1977); BAT mitochondrial thermogenesis (Himms-Hagen and Desautels, 1978); hormone binding to liver plasma membranes (Chang et al., 1975); control of adipocyte adenylate cyclase (Dehaye et al., 1978; French and York, 1984 ; 1985); liver and muscle $[Na^+K^+]$-ATPase (York et al., 1978b; Lin et al., 1979); and hepatic microsomal enzymes (Rouer et al., 1980; Hyslop et al., 1982). Several of these defective processes,(for example $[Na^+K^+]$-ATPase, glucose transport and hepatic microsomal enzymes) are not apparent in other genetic animal models of obesity (Bray and York, 1979) so are unlikely to be secondary to the obese state. It has been suggested that there may be a generalised membrane defect in the tissues of the obese mouse. This hypothesis was initially proposed by Chang et al., (1975). These workers reported a reduction in hormone and lectin binding to receptors in liver plasma membranes which indicated the possibility of a generalised defect in membrane glycoprotein. Subsequently, Dehaye et al.,(1978) studying the control of adipocyte adenylate cyclase, reported different responses to ethanol stimulation in preparations from lean and obese mice. They suggested that this may result from an altered membrane lipid environment in the obese animals. A generalised membrane defect might then give rise to the wide variety of altered membrane functions observed.

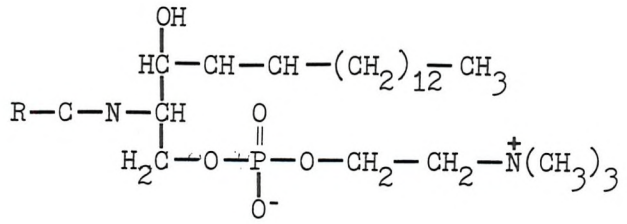
1.10 Fluid Mosaic Model of Biological Membranes

The current concept of the structure of biological membranes is

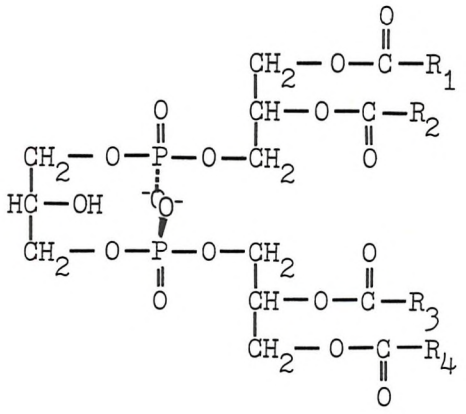
best represented by the fluid mosaic model proposed by Singer and Nicholson (1972). This envisages the membrane as a fluid bilayer of phospholipid molecules. Intrinsic membrane proteins are embedded in or span the bilayer. Extrinsic membrane proteins are more loosely associated with the surface by electrostatic charges. The intrinsic proteins are asymmetrically distributed and include metabolite transporters, ion pumps, and hormone receptors. The bilayer is in a fluid state and proteins and phospholipids can spin or move freely in the plane of the membrane. Lateral motion may be restricted by the presence of other protein molecules. The fluid nature of the bilayer allows interaction to take place between proteins within the membrane. For example, the glucagon or adrenergic hormone receptor complex interacts with the regulatory and catalytic subunits of adenylate cyclase.

The phospholipids are ampipathic molecules containing both polar and non polar components. The structure of the polar head group defines the phospholipid subclass. The head group may be charged or neutral and interacts with the surrounding aqueous environment (the internal cytoplasm or extracellular space). The structures of the main phospholipids are set out in figure 1.1.

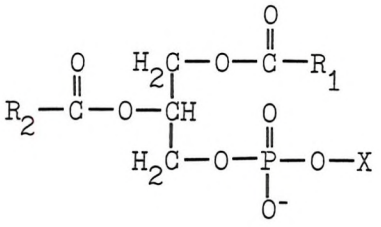
The hydrophobic part of phospholipids, which constitute the interior of the membrane bilayer, are fatty acyl chains. These vary in length from 16 to 22 carbon atoms long and may have up to 6 carbon-carbon double bonds. It is this degree of unsaturation that determines in part the fluidity of the membrane. Saturated fatty acids are able to pack together well in the interior of the bilayer and confer a high melting temperature (or transition temperature) on the membrane. The introduction of a 'cis' double bond produces a bend in the carbon chain creating a steric hinderance to the tight packing. This allows a greater mobility to the acyl chains, increases fluidity, with a consequent reduction in melting temperature (Wahle, 1983). It has been suggested that some poikilotherms alter their membrane fatty acyl composition and degree of unsaturation in order to adapt to their environmental temperature. In this way, it is suggested,they are able to maintain a constant optimum membrane viscosity or fluidity at all environmental temperatures (Sinensky, 1974; Sellner and Hazel, 1982). This mechanism has been termed "homoviscous adaptation" (Sinensky 1974).



Sphingomyelin

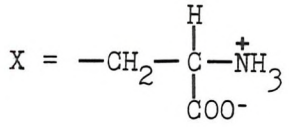


Cardiolipin

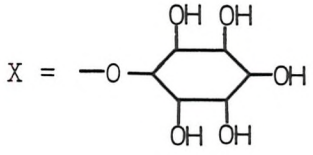


X = $-CH_2-CH_2-\overset{+}{N}H_3$ Phosphatidylethanolamine

X = $-CH_2-CH_2-\overset{+}{N}(CH_3)_3$ Phosphatidylcholine



Phosphatidylserine



Phosphatidylinositol

Fig 1.1 The Major Phospholipid Subclasses of Mammalian Cell Membranes.

However, Stubbs and Smith (1984) have pointed out that the relationship between membrane unsaturation and melting temperature is not a simple one. The length of the fatty acyl chains and the positions on the chain at which the double bonds occur are important factors in determining the fluidity of the membrane. Other factors which may affect the melting temperature of the bilayer include the cholesterol content of the membrane and the nature of the phospholipid head groups (Wahle, 1983; Thompson, 1980).

In mammals at physiological temperature, phospholipid acyl chains are liquid (above the melting temperature) whereas the head groups are in a rigid conformation. Hence the phospholipid bilayer is described as being in a liquid-crystalline state.

Phospholipids are asymmetrically distributed in the membrane. For example, in liver plasma membrane, phosphatidylcholine is mainly located in the outer half of the bilayer, phosphatidylserine and phosphatidylinositol are almost exclusively found in the inner leaf whilst sphingomyelin and phosphatidylethanolamine are evenly distributed between the two (Higgins and Evans, 1978). Phospholipids also have a heterogenous distribution within the cell. For instance, cardiolipin is mainly found in the inner mitochondrial membrane whereas sphingomyelin is located principally in the plasma membrane (Wahle, 1983).

1.11 Lipid-Protein Interactions

Many intrinsic membrane proteins are dependent on specific phospholipids and/or acyl chain environments for activity (Fourcain and Jain, 1974). Interactions occur between the protein and phospholipid molecules which make up its boundary layer or lipid annulus (Jost, 1973). Phospholipid asymmetry is believed to be important for the orientation of intrinsic proteins into the correct half of the membrane bilayer(Houslay et al., 1976; Wahle, 1983; Stubbs and Smith, 1984).

Detailed investigation of lipid-protein interactions have been carried out for several membrane associated processes. For example, Kimelberg and Papahadjopoulos (1974) purified and delipidated the

intrinsic plasma membrane protein $[Na^+K^+]$-ATPase from rabbit kidney. They then reactivated the enzyme by adding back various phospholipids and found a requirement for the presence of phosphatidylserine. By reconstituting the enzyme into liposomes of phosphatidylglycerols with different fatty acyl composition, they found that the characteristics of the $[Na^+K^+]$-ATPase could be modified. In addition, incorporation of cholesterol into the liposomes reduced the ability of phospholipids to reactivate the enzyme. It was proposed that the mechanism of this effect occurred through a reduction in fluidity of the liposomes caused by the cholesterol. Further evidence is derived from studies with cultured tumour cells. Poon et al. (1981) measured $[Na^+K^+]$ -ATPase in plasma membrane fractions from EL4-T cells which had been cultured in media supplemented with different fatty acid substrates. They found that activity was increased in membranes from cells supplemented with unsaturated fatty acids (18:1, 18:2). In contrast, activity was depressed by saturated fatty acid (19:0, 17:0) supplementation.

In addition to intrinsic membrane enzymes, lipid-protein interactions for various hormone receptors have also been studied. Gould et al. (1982) purified insulin receptors from turkey erythrocytes and reconstituted them into phosphatidylcholine vesicles of different fatty acyl compositions. They chose a saturated lipid environment (rich in dimyristoyl-phosphatidylcholine) and an unsaturated environment (mainly oleoyl-phosphatidylcholine and linoleoyl-phosphatidylcholine). A comparison of insulin binding to the receptors reconstituted into the two types of lipid vesicles was made by Scatchard analysis. The results indicated that receptors incorporated into the saturated lipid environment had a higher affinity for insulin than those incorporated into the unsaturated lipid environment. A similar result was obtained in parallel studies with cultured Friend erythroleukemia cells. Ginsberg et al. (1981) analysed insulin binding to cells cultured in media supplemented with saturated or unsaturated fatty acids. They concluded that insulin receptor function was modified by the membrane lipid environment.

It is evident that lipid-protein interactions are very important for many membrane associated processes. In this respect it becomes

clear that the modified function of many intrinsic membrane proteins observed in the genetically obese mouse, could be the result of a generalised membrane defect.

1.12 Altered Membrane Compostion in the Obese Mouse

1.12.1 Fluorescence Polarisation Studies

Several studies have utilized a fluorescence polarisation technique to probe the hydrophobic interior of membrane bilayers from various tissues of lean and obese mice (Hyslop and York, 1980; Rouer et al., 1980; York et al., 1982; French et al., 1983). The technique involves the use of a small hydrophobic fluorescent molecule such as diphenylhexatriene (DPH) which partitions into the membrane bilayer. DPH is a planar molecule that is excited by polarised light, only if it is orientated in the correct plane. Hence, if it partitions into a lipid environment which is very fluid, its rate of rotation is fast (relative to a predefined standard) resulting in a low steady state fluoresence polarisation. This can be readily monitored using a spectrophotofluorimeter. The function of DPH as a specific membrane probe is aided by its inability to fluoresce in aqueous media. Using this fluorescence polarisation technique, membrane preparations from various tissues of the obese mouse have been examined. These include, adipocyte plasma, red blood cell plasma, hepatic microsomal, hepatic plasma, salivary gland plasma, muscle sarcolemma, brown adipose tissue mitochondrial, hepatic mitochondrial, and pancreatic plasma membranes (Hyslop and York, 1980; York et al., 1982; French et al., 1983). In virtually all cases, fluorescence polarisation values were reduced compared to those for the corresponding membrane preparations from lean animals. It was proposed that the DPH probe partitioned into a different lipid environment in the membranes of obese mice. It was also suggested that this lipid environment was more "fluid" than that of lean animals. The red blood cell plasma membrane was the only exception. Here, increased polarisation values were reported for obese mouse preparations. This was thought to result from an increased equilibration of cholesterol into the membranes from hypercholesterolaemic serum (York et al., 1982). Rouer et al. (1980), however, found that fluorescence polarisation at 37°C was

normal in hepatic microsomal membranes from obese mice. However, these authors failed to separate microsomal membranes from hepatic glycogen granules, which would greatly enhance light scattering and might result in erroneous data.

Recently, the interpretation of data from fluorescence polarisation studies has been questioned. McVey et al. (1981) have suggested that the DPH probe may partition into selected lipid domains in the bilayer. Kleinfeld et al. (1981) have shown that steady state fluorescence is not related to the lateral movement of proteins in the membrane. In cell culture studies, in which cell membranes were modified by fatty acid supplementation, large changes in polarisation parameters were shown to be artefactual. This was because the probe molecule selectively partitioned into cytoplasmic lipid droplets (McVey et al., 1981; Stubbs and Smith, 1984). However, Kleinfeld et al. (1981) conclude that steady state fluorescence probes still remain sensitive indicators of local lipid packing. The studies reported above in genetically obese mice did not involve fatty acid supplementation, and therefore the data remain relevant on a comparative basis.

1.12.2 Membrane Phospholipids and Fatty Acids

In order to understand the apparent increases in membrane fluidity (indicated by fluorescence polarisation studies), work has been undertaken to analyse the fatty acid compositions of obese mouse phospholipids. This has involved purifying cell organelles, extracting and separating the phospholipid subclasses and then performing gas liquid chromatography on the transmethylated fatty acids of phospholipids. Due to the complexity of preparation and requirement for sufficient purified phospholipid material for analysis, work in this area has been less extensive. However, results have shown that the fatty acid compositions of obese mouse phospholipids are altered, particularly with regard to the nature and distribution of the essential fatty acids (EFA) and the polyunsaturated fatty acids (PUFA). This can be illustrated by considering the fatty acid profiles of phospholipids prepared from adipocyte plasma membranes (York et al., 1982). In phosphatidylethanolamine, there was a considerable reduction in the proportion of linoleic acid (from

21.7% to 10.8% of the total) which was compensated for by an increase in docoshaxaenoic acid (from 13.8% to 20.7% of the total). In phosphatidylcholine, a decrease in linoleic acid content was observed, which was compensated for mainly by an increase in oleic acid.

In a study on total phospholipid composition from lean and obese mouse hepatic microsomes, Rouer et al. (1980) reported similar changes. Linoleic acid had decreased from 17% to 7% of total phospholipid fatty acid, compensated for mainly by an increase in oleic acid (from 10% to 18% of total) and in this case a small increase in arachidonic acid. Hyslop et al. (1982) extended this study by separating out the phospholipid subclasses. However, these authors found only small reductions in linoleic acid content of phosphatidylcholine and phosphatidylethanolamine with the largest changes given by an increase in docoshexaenoic acid at the expense of palmitic acid in phosphatidylethanolamine.

French et al. (1983) purified liver plasma membranes from lean and obese mice and subsequently separated the phospholipid subclasses. In phosphatidylcholine, a reduction in linoleic acid and an increase in oleic acid were apparent. Phosphatidylethanolamine also showed a small reduction in the proportion of linoleic acid, whereas sphingomyelin showed large changes in composition mainly associated with saturated fatty acids.

In conclusion, the most widespread alteration in fatty acid composition of obese mouse membranes is a reduction in the proportion of linoleic acid. This is compensated for mainly by an increase in oleic acid content. Recently, Cunnane et al. (1985), confirmed the data on hepatic membrane changes in obese mice. They analysed total liver phospholipid and reported that linoleic acid content was reduced and oleic acid (primarily) and arachidonic acid (slightly) were increased. As the reduction in linoleic acid is often paralleled by an increase in one PUFA (either arachidonic acid or docoshexaenoic acid), the double bond number or unsaturation index is usually increased in membranes from obese mice. This correlates well with the increased fluidity of these membranes as indicated by fluorescence polarisation techniques.

1.12.3 Alteration in Membrane Processes in Obese Mice

Considerable emphasis has been placed on attempts to correlate the changes in fatty acid composition of phospholipids in the obese mouse, with altered membrane associated processes. This has usually involved the presentation of enzyme kinetic data as Arrhenius plots. Arrhenius plots are widely used to investigate protein-lipid interactions. The rate of enzyme activity is measured as a function of temperature, and data is plotted

$$\log_e \text{(activity)} \quad \text{versus} \quad \frac{1}{T^{o}K}$$

This is derived from the Arrhenius equation,

$$K = Ae^{-(E/RT)}$$

therefore,

$$\log_e K = \log_e A - \frac{E}{R} \times \frac{1}{T}$$

where, K = rate constant
A = constant (frequency factor)
E = energy of activation
R = gas constant
T = temperature (K^{o})

Hence the energy of activation can be derived from the slope of the Arrhenius plot. Many Arrhenius plots of intrinsic membrane proteins exhibit breaks or discontinuities at given temperatures (Fourcain and Jain, 1974). These are attributed to reversible phase transitions in the surrounding lipid which interchanges between gel and liquid crystalline states. Discontinuities are also associated with lipid phase separations between heterogeneous molecular species in the membrane bilayer (Sandermann, 1978). Kimmelberg and Papahadjopoulos (1974) reconstituted purified $\left[Na^{+}K^{+}\right]$-ATPase into diacylglycerol lipid micelles of different fatty acid composition. They showed that the Arrhenius characteristics of the $\left[Na^{+}K^{+}\right]$-ATPase activity were responsive to the differences in chain length and degree of unsaturation of the fatty acids in the lipid micelles. Hence, the Arrhenius characteristics of an intrinsic membrane protein may respond to changes in the organisation or composition of the

surrounding lipid environment.

However, Sandermann (1978) advises caution when analysing Arrhenius plot data. He documents several examples in which Arrhenius plot discontiniuties do not result from lipid-protein interactions. For example, the Arrhenius discontinuity of the $\Delta 9$-desaturase was caused by a temperature induced change in the catalytic mechanism of the enzyme. In addition, the Arrhenius discontinuity of cytochrome b_5 reductase was caused by substrate concentrations becoming rate limiting at lower temperatures.

Despite these reservations, most of the studies outlined in section 1.12.2 have investigated lipid-protein interactions in lean and obese mouse membranes by Arrhenius plot analysis. In obese mouse liver microsomes, the Arrhenius characteristics of NADPH:cytochrome P_{450} oxidoreductase activity exhibit a reduction in break temperature of $3^{\circ}C$ (Hyslop et al., 1982) . Plots of ethoxycoumarin - O - deethylase activity were linear across the range of temperatures examined in obese mouse hepatic microsomes, whereas the lean preparation exhibited a discontinuity at $20^{\circ}C$ (Rouer et al., 1980). This was associated with a defect in substrate binding to mixed function oxidases in the obese animals (Rouer and Leroux, 1980). Arrhenius characteristics of obese mouse $\left[Na^{+}K^{+}\right]$-ATPase activity in liver and brain exhibited reduced break temperatures by $3^{\circ}C$ and $5.5^{\circ}C$ respectively (Hughes and York, 1983). The abnormality in the brain was abolished by deoxycholate treatment which has been shown to disrupt the membrane and stimulate $\left[Na^{+}K^{+}\right]$-ATPase activity (Dahl and Hokin,1974). Normalisation of the data by this treatment supports the view that lipid -protein interactions determine Arrhenius plot characteristics.

In contrast to these studies, Arrhenius characteristics of 5'-nucleotidase from obese mouse liver plasma membrane exhibit an increase in break temperature from $23^{\circ}C$ to $32^{\circ}C$ (French et al., 1983). The change here was correlated with alterations in saturated fatty acid content of sphingomyelin. 5'-nucleotidase can be purified as a complex with sphingomyelin (Widnell, 1974).

In conclusion, the Arrhenius characteristics of several intrinsic membrane proteins are altered in the obese mouse. In all cases

the differences have been attributed to alteration in membrane lipid environment either in the bulk phase and/or the composition of annular phospholipids.

The control of adipocyte adenylate cyclase is defective in the obese mouse (Dehaye et al., 1978). Investigation into the possible involvement of altered lipid environment in this system has involved a different approach. Dehaye et al. (1978) reported a loss of coupling between hormone-receptor, regulatory and catalytic subunits. They implicated an alteration in membrane lipid environment from differential responses to ethanol stimulation. Houslay et al. (1976) have shown that the lipid environment is very important for the adenylate cyclase complex. However, ß-antagonist (dihydro-alprenalol) binding was reduced in obese mouse adipocyte plasma membranes, indicating a reduced receptor number (Begin-Heick, 1981; French and York, 1984). The possible involvement of this reduced receptor number in the defective system is unclear, since no shift in dose-response curve (of adenylate cyclase to isoprenaline) was apparent in obese mouse membranes (French and York, 1984).

More recently the defect in the obese mouse adenylate cyclase system has been attributed to the interaction between the hormone-receptor complex and the regulatory subunit. This suggestion was supported by the observation that normal adenylate cyclase activity was obtained by fluoride or guanylylimidodiphosphate (Gpp(NH)p) stimulation of the system. Gpp(NH)p stimulates adenylate cyclase activity after binding to and activating the regulatory subunit whereas fluoride bypasses the guanine nucleotide requirement for hormone-receptor-regulatory-catalytic subunit coupling(French and York, 1984).

Additional evidence comes from studies in which Arrhenius characteristics of the adipocyte adenylate cyclase systems of lean and obese mice were examined (French and York, 1985). In previous studies, it has been suggested that the Arrhenius discontinuity of glucagon stimulated adenylate cyclase activity reflects the sensitivity of the coupled adenylate cyclase system to lipid phase separations in the membrane (Houslay et al., 1976). Arrhenius characteristics of adenylate cyclase activity of obese

mouse preparations were abnormal only under conditions in which adenylate cyclase was coupled by isoprenaline binding to the hormone receptor. Arrhenius characteristics of fluoride or Gpp(NH)p stimulated adenylate cyclase activity of obese mice were similar to those of the lean animals. In the liver, both the Arrhenius characteristics and the activity of coupled adenylate cyclase were normal in the obese mouse (York et al., 1978b; French and York, 1985). Hence strong correlation is given between impaired coupling of the adenylate cyclase system, and the sensitivity of the system to abnormal membrane lipid composition or organisation.

1.13 Control of Fatty Acid Composition of Membrane Phospholipids

It is not known whether all of the defective membrane functions of the obese mouse result from membrane compositional changes (via protein-lipid interactions). The possible involvement of altered membrane composition in these processes awaits further investigation. However, the fatty acyl compositions of phospholipids in obese mouse membranes are altered. Hence, certain enzyme systems which control membrane fatty acid composition, may be defective in the obese mouse. De novo synthesis of phospholipids and the enzyme systems that may control membrane fatty acid composition are briefly reviewed below.

De novo synthesis of phospholipids occurs on the endoplasmic reticulum (Bell and Coleman, 1980) (see fig 1.2). Fatty acylcoA moieties are first esterified to glycerol 3-phosphate by acyltransferase enzymes to give phosphatidic acid. This lipid is a precursor for triglyceride or phosphoglycerides. In the case of the latter, for example, it is metabolised further to diacylglycerol and then conjugated with CDP-choline or CDP-ethanolamine to produce phosphatidylcholine or phosphatidylethanolamine. The acyltransferase enzymes of this pathway have substrate specificities for esterifying saturated fatty acids at the C_1 position and unsaturated fatty acids at the C_2 position of the glycerol-3-phosphate. However, the fatty acid compositions of phospholipids in various tissues and in the various cell organelles are markedly different (Wahle, 1983). Hence it is the subsequent modification of the

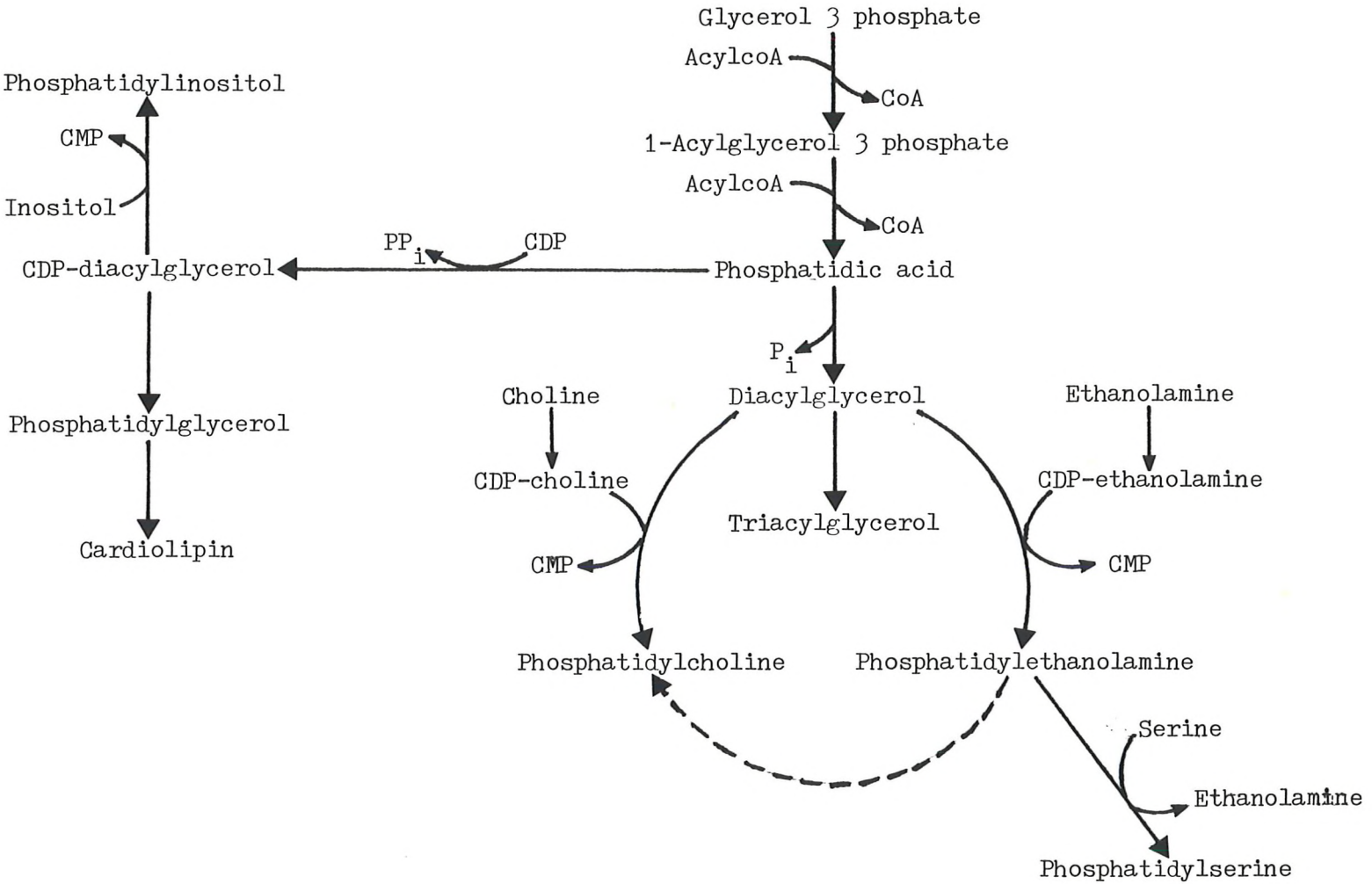


Fig 1.2 De Novo Synthesis of Phospholipids.

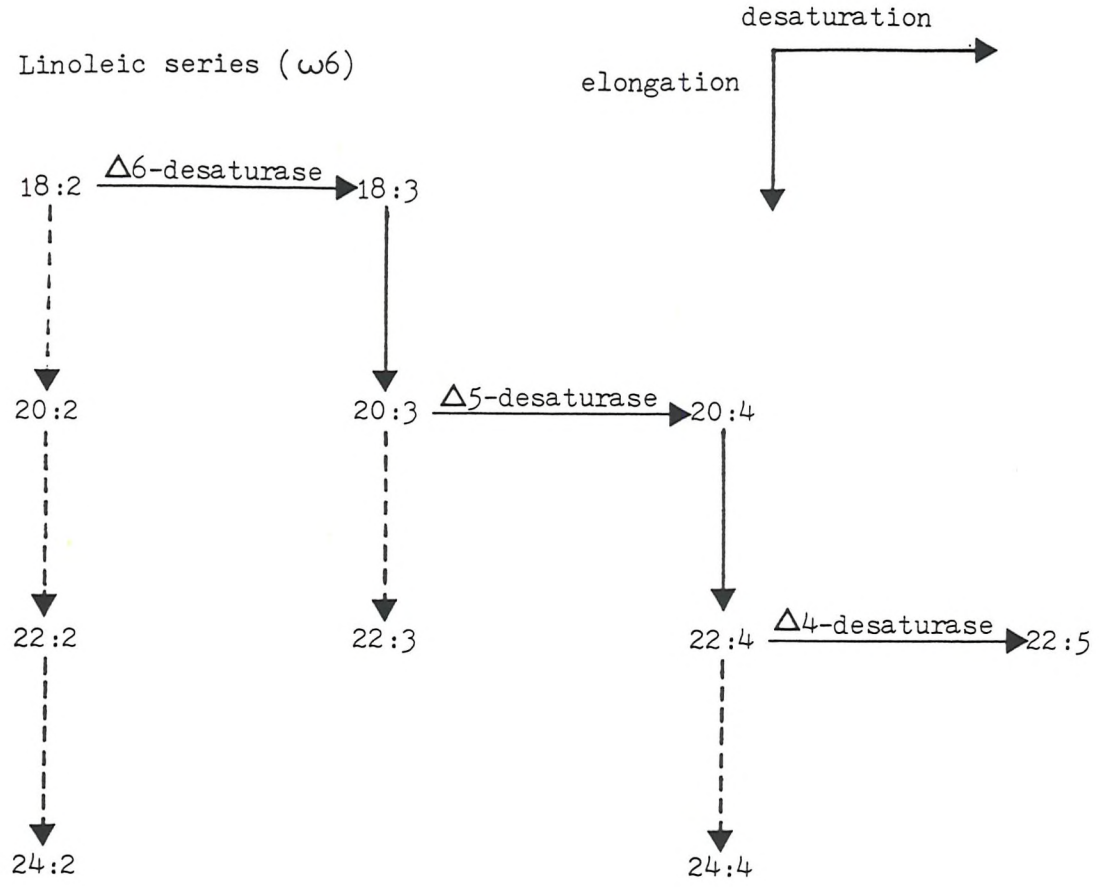
phospholipids that is important in the control of membrane fatty acid composition.

1.14 Desaturation and Elongation of Essential Fatty Acids(EFA)

Studies which report altered membrane fatty acid composition after hormonal treatment, or in disease states, often consider fatty acid desaturation as a possible controlling mechanism (Faas and Carter, 1981; 1982; 1983; Eck et al., 1979; Alam et al., 1984). Many of the changes observed in the obese mouse membranes involve essential fatty acids (EFA),and their metabolites polyunsaturated fatty acids (PUFA). EFA's, linoleic and linolenic acid, are so called because they can only be obtained in the diet. They are converted to PUFA prior to incorporation into phospholipids. This occurs by a sequential process of desaturation and elongation. The generally accepted pathways of PUFA synthesis in rat liver microsomes are outlined in figure 1.3. Elongation occurs from the carboxyl end of the fatty acid by the addition of a 2-carbon unit from malonylcoA. Three desaturase enzymes produce double bonds at positions $\Delta 6$-, $\Delta 5$-, and $\Delta 4$-, in turn on the acyl chain. In this way, the 'ω' number, which is the number of carbon atoms from the methyl terminal end to the first double bond, is maintained for each EFA (see figure 1.4). Two series of PUFA homologues result.

1.14.1 Requirements for PUFA Synthesis

The fatty acids are desaturated and elongated as acylcoenzyme A esters. Desaturation requires molecular oxygen, ATP, NADH, or NADH whilst elongation requires malonylcoA, ATP and NADPH (Naughton, 1981). Sprecher (1977) has shown that the rate of elongation is always faster than the preceeding desaturation step. Hence desaturation is rate limiting. The desaturase enzymes exhibit a preference for substrates with an increasing number of double bonds. For example, linolenic acid (18:3 ω3) is desaturated at a faster rate than linoleic acid (18:2 ω6) by the $\Delta 6$-desaturase (Brenner, 1977; Naughton, 1981). Product inhibition is also a feature of the enzymes in this system. The $\Delta 6$-desaturase is inhibited by γ-linolenic acid (18:3 ω6) and to a greater extent by docosapentaenoic acid (22:5 ω6). Increasing inhibition is given



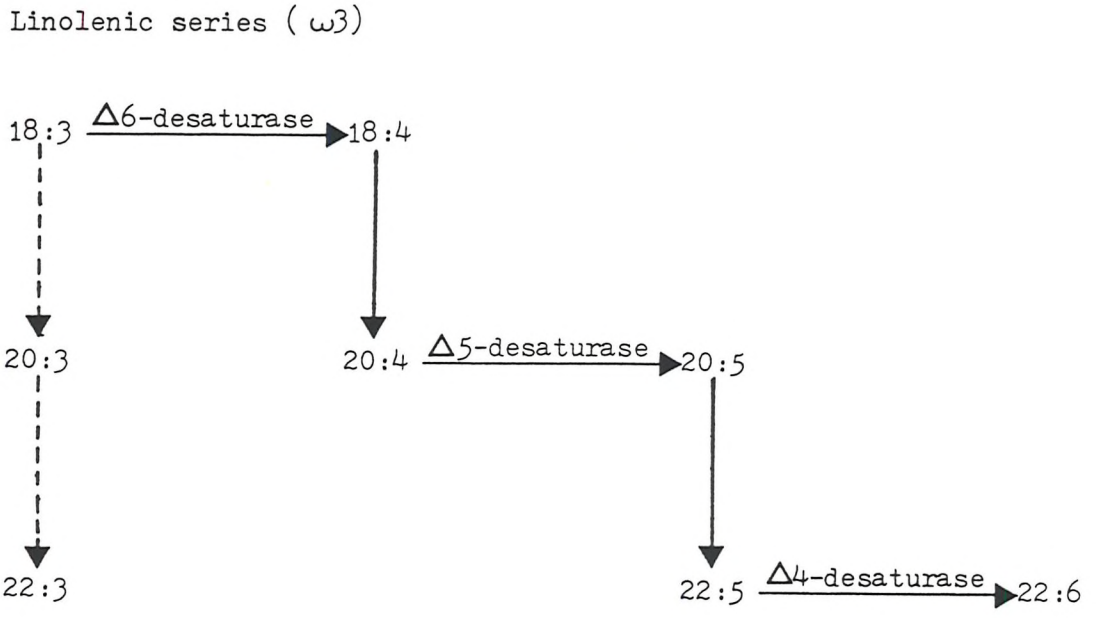


Fig 1.3 The Major Pathways of PUFA Synthesis in Rat Liver Microsomes.

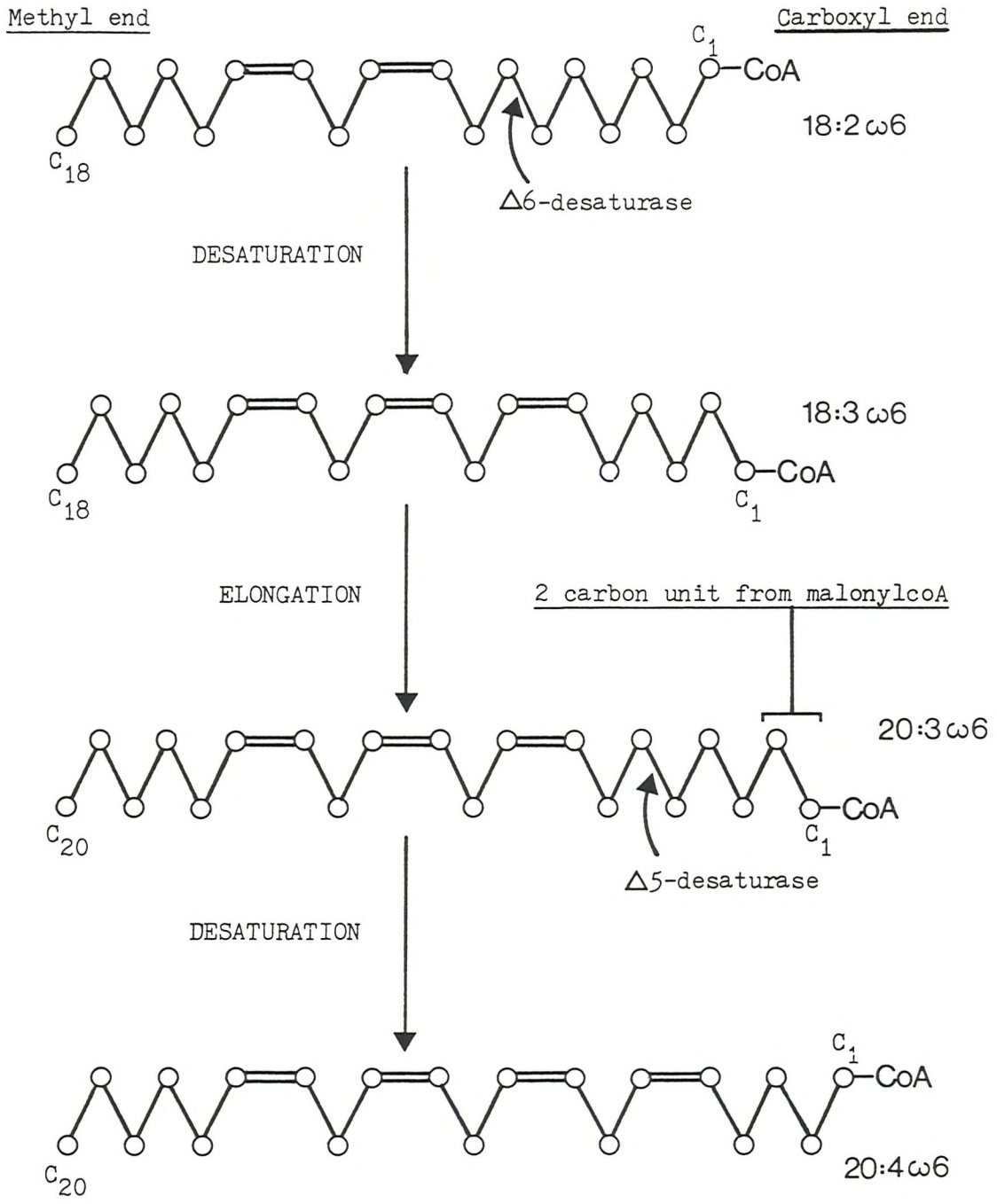


Fig 1.4 Desaturation and Elongation of Linoleic Acid to Produce Arachidonic Acid.

by higher homologues of the same series (Brenner, 1974).

The enzymes involved in essential fatty acid desaturation have been extensively investigated in the liver (Naughton, 1981), where activity is high in adult rodents. The brain is also an important site of PUFA synthesis at foetal (Sanders and Naismith, 1979) and neonatal (Cook, 1978) stages of development.

Most of the studies on PUFA synthesis have been carried out on liver microsomal fractions, although it has long been recognised that mitochondria are capable of EFA desaturation (Dahlen and Porter, 1968) and elongation (Harlan and Wakil, 1963). This latter process occurs by addition of a two-carbon unit from acetylcoA. Indeed, Strouve-Vallet and Pascaud (1971) have shown that mitochondrial activity was higher than microsomal activity in studies on rat brain desaturase. Recently, the Δ6-desaturase has been purified and characterised from rat liver microsomes (Okayasu et al., 1981). This has lead to evidence that the desaturase enzymes of PUFA synthesis are immunologically distinct. Mono-specific antibody prepared against the Δ6-desaturase did not inhibit Δ5-desaturase or Δ9-desaturase activity of rat liver microsomes (Fujwara et al., 1983).

1.14.2 Organisation of Desaturase Proteins

The molecular organisation of the Δ9-desaturase (which converts saturated fatty acids to mono-unsaturated fatty acids, for example stearic acid to oleic acid) has been determined. (Jeffcoat, 1977). The Δ6-desaturase has been postulated to have a similar molecular organisation (Brenner, 1977). The desaturase complex consists of three proteins. NADH-cytochrome b_5 reductase and cytochrome b_5, both of which are anchored in the endoplasmic reticulum by hydrophobic polypeptide tails, and the desaturase catalytic unit which is an intrinsic membrane protein buried in the bilayer. Evidence to support the view that the molecular organisation of the Δ6-desaturase is similar to that of the Δ9-desaturase, comes from studies in which the Δ6-desaturase was purified (Okayasu et al., 1981). Reconstituted Δ6-desaturase activity could be achieved only by the addition of cytochrome b_5, NADH-cytochrome b_5 reductase, NADH and lipid or detergent. In an immunological study,

specific antibody prepared against rat liver cytochrome b_5 blocked hepatic microsomal Δ6-desaturase activity (Lee et al., 1977). Hence it has been concluded that the Δ9-desaturase and Δ6-desaturase share common electron transport proteins (Jeffcoat and James, 1984).

The location of the desaturase catalytic unit within the bilayer has lead to speculation that the desaturase protein may be sensitive to the fluidity of its membrane lipid environment and hence provide a self regulating mechanism for controlling membrane composition. However, the evidence is inconclusive. Holloway and Holloway (1975) altered microsomal membrane fatty acid composition and microsomal Δ9-desaturase activity in rats by feeding diets of different fatty acid composition. They then delipidated the microsomal preparations and reconstituted the membranes with egg yolk phosphatidylcholine and oleic acid, but were unable to further modify the Δ9-desaturase activity. They concluded that the different microsomal desaturase activities derived from the rats on the different diets were not caused by altered membrane lipid environment. However, the possibility that changes in activity were caused by changes in the annular lipid tightly bound to the desaturase protein cannot be overlooked. Garda and Brenner (1984) used n-butyl alcohol and isoamyl alcohol to increase the fluidity of rat liver microsomal membranes. This was monitored by DPH steady state fluorescence polarisation. Isoamyl alcohol had differential effects on desaturase activity, decreasing both Δ6-desaturase and Δ9-desaturase activities but only when high substrate concentrations were used in the case of the latter enzyme. However, direct effects of the alcohol on the enzymes could not be ruled out in this study.

1.14.3 Physiological Control of Desaturase Enzymes

The Δ6-desaturase has been postulated as the key regulator of PUFA synthesis since it is very responsive to nutritional and hormonal changes and as the first enzyme in the pathway is rate limiting for PUFA synthesis (Brenner, 1977). This is in contrast to the Δ5-desaturase activity which is less responsive to diet and endocrine changes. Fasting decreased Δ6-desaturase activity

of rat liver microsomes which was restored to normal after refeeding. Restoration of the Δ6-desaturase activity was believed to involve protein synthesis since it was prevented by actinomycin D (Brenner, 1968). Rat liver Δ6-desaturase activity is sensitive to dietary EFA. EFA deprivation of rats caused an increase in hepatic microsomal Δ6-desaturase activity (Peluffo et al., 1976) whereas feeding diets containing PUFA, depressed the Δ6-desaturase activity (Wahle, 1983). These two processes occur presumably to maintain the optimum PUFA content of the cell.

Insulin has been shown to increase microsomal desaturation. The reduction in hepatic microsomal Δ6-desaturase activity of streptozoticin or alloxan diabetic rats was restored by insulin injection (Eck et al., 1979, Brenner, 1968). A number of hormones have been shown to depress Δ6-desaturase activity. These include thyroxine, adrenaline and glucagon (DeGomez-Dumm et al., 1975; 1976; 1977; Brenner, 1977). In the case of the latter two hormones, the mechanism is believed to be mediated through the adenylate cyclase-cyclic adenosine-3'5'-monophosphate system.

1.14.4 Δ9-desaturase Activity in the Obese Mouse

Enser (1979) has shown that the activity of the Δ9-desaturase is increased in liver and adipose tissue of obese mice. He has discounted hyperinsulinemia and the alleviation of linoleic acid inhibition of the enzyme as the causational factors of the increased activity. Recent studies indicate that the elevated Δ9-desaturase activity probably results from increased provision of fatty acid substrate either from the diet or as a result of increased lipogenic activity (Enser and Roberts, 1982).

1.15 Deacylation/Reacylation Pathway

A second possible control mechanism for membrane fatty acid composition involves the deacylation/reacylation pathway for phospholipids. After de novo synthesis on the endoplasmic reticulum, phospholipids are transported to the various cell organelles. It is now widely accepted that phospholipids are modified by deacylation/reacylation at their final subcellular

location as a process of fine adjustment (Stubbs and Smith, 1984). A fatty acid is first cleaved from the phospholipid by a membrane associated phospholipase (Van den Bosch, 1980). These enzymes have a widespread occurrence in cells and tissues of mammals. Phospholipase A_1 removes a fatty acid from the C_2 position on the glycero-backbone whereas phospholipase A_2 cleaves at the C_2 position (see figure 1.4). The resulting lysophospholipid can then be reacylated with a selected fatty acid by lysophospholipid acyltransferase.

Lysophospholipid acyltransferase activity has been reported in many tissues, including liver, brain, lung, kidney, heart and muscle (Van den Bosch et al., 1972; Blaise-Smith et al., 1982). Similarly, subcellular distribution of lysophospholipid acyltransferase activity would be expected to be widespread. The majority of studies have concentrated on microsomal fractions (Holub et al., 1979; Lands et al., 1982) although activity has also been reported in mitochondria (Waite et al., 1970). 1-acylglycerophosphorylcholine acyltransferase has been partially purified from rat liver microsomes (Hasegawa-sasaki and Ohno, 1980) and has been shown to be tightly bound to the membrane. It is probable that many lysophospholipid acyltransferases exist although it is not clear whether different enzymes are found for each fatty acid substrate (Holub and Kukis, 1978). Cofactor requirements of lysophospholipid acyltransferases are very similar to their counterparts in the de novo synthesis pathway. Both acyltransferases utilize fatty acylcoenzyme A esters as substrates.

1.15.1 Developmental Studies on Lysophospholipid Acyltransferases

Changes in lysophosphatidylcholine acyltransferase activity during development have been followed in rat skeletal muscle. However, results are conflicting. Sarzala and Pilarska (1976) found high levels of activity at 2-10 days after birth which fell to lower levels in the adult. In comparable experiments Blaise-Smith et al. (1982) could find no difference between neonatal and adult activity.

1.15.2 Dietary Studies on Lysophospholipid Acyltransferases

Pugh and Kates (1984) studied the effect of different dietary

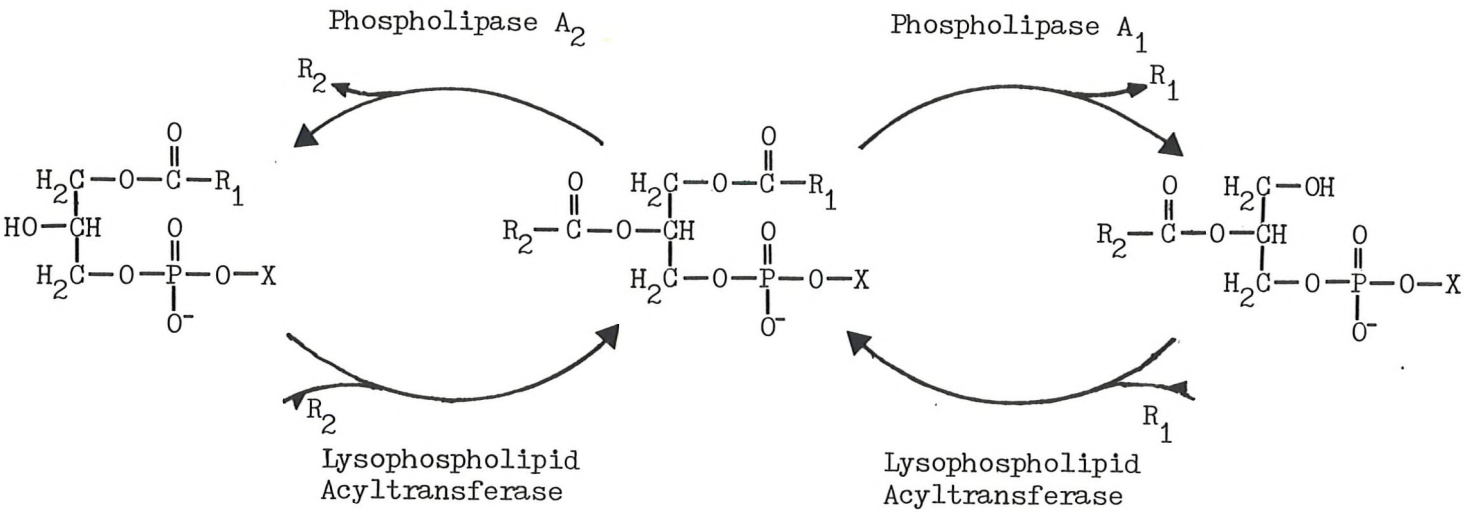


Fig 1.4 The Deacylation/Reacylation Pathway of Phospholipids

lipids on rat liver microsomal lysophosphatidylcholine acyltransferase activity. Although total phospholipid fatty acid composition changed markedly, no difference in acyltransferase activity between the dietary groups was apparent. However, the earliest measurement in their study was made after six weeks on the dietary regimes. Van Golde et al.(1968) fed EFA deficient rats a corn oil diet and found that the total liver phosphatidylcholine fatty acid composition changed dramatically during the first seven days. The 18:2 ω6 component had reached a maximum after three days and the appearence of 20:4 ω6 (at the C_2 position) mirror imaged the loss of 20:3 (at this position) during the course of the study. However, these authors could not distinguish turnover and de novo synthesis of phosphatidylcholine from deacylation/reacylation activity.

1.15.3 Studies on Substrate Specificity

Most studies on lysophospholipid acyltransferases have been concerned with their specificity for acylcoA substrates. Deacylation/reacylation enzymes are similar to de novo synthesis acyltransferases in that saturated fatty acids are preferentially incorporated at the C_1 position and unsaturated fatty acids at the C_2 position of the lysophospholipid (Holub and Kukis, 1978). Much work has been carried out on the substrate specificity for the C_2 position. In an early study, Hill and Lands(1968) investigated acyltransferase activity in rat liver microsomes. Using lysophosphatidylcholine (175 μM) as acceptor and employing a spectrophotometric assay they obtained a series of rates for the various acyl coA substrates. From these, an order of preference for the various substrates was obtained as follows:-

$$20{:}5\,\omega3 = 20{:}3\,\omega6 > 20{:}4\,\omega6 = 18{:}2\,\omega6 > 20{:}2\,\omega9$$
$$> 18{:}3\,\omega3 > 18{:}1\,\omega9 > 22{:}6\,\omega3 > 16{:}0 > 18{:}0$$

Later work has shown that at much lower acceptor concentrations (for example 16 μM), preference for arachidonic acid (20:4 ω6) was increased (Holub and Kukis, 1978; Holub et al., 1979). More recently, Lands et al.(1982) have studied substrate competition for rat liver microsomal 1-acyl-glycerophosphorylcholine(GPC) acyltransferase using $\left[^{14}C\right]$ -arachidonoylcoA (36 μM) and

$[^{3}H]$ -lysophosphatidylcholine. Varying the concentration of competing (unlabelled) fatty acylcoA substrate, plots of $[^{14}C]$ -arachidonate incorporation into phosphatidylcholine against concentration of competing substrate were obtained. They then determined the concentration of competing substrate required to give 50% $[^{14}C]$ -arachidonate incorporation (i.e. equal incorporation of arachidonate and competing substrate). Comparing several different fatty acylcoA substrates, the order of preference obtained was as follows:-

$$20{:}5\,\omega3 > 18{:}3\,\omega3 > 20{:}4\,\omega6 > 20{:}3\,\omega6 > 18{:}2\,\omega6 \gg 18{:}1\,\omega9 > 22{:}6\,\omega3 \gg 16{:}0 = 18{:}0$$

This series is similar to that described in the earlier study although the preference for arachidonic acid (20:4 ω6) and linolenic acid (18:3 ω3) have increased. In contrast to these results, 1-acyl GPC:acyltransferase has a low specificity for the acyl acceptor species. Miki et al. (1977) tested a wide range of fatty acyl groups at the C_1 position of lysophosphatidylcholine (including 14:0, 16:0, 18:0, 18:1 ω9, 18:2 ω6, 20:4 ω6) and found that only 20:4 ω6 and 18:0 acted as poor acceptors. This was confirmed for 18:0 by Holub et al. (1979).

The substrate specificity of 1-acylglycerphosphorylethanolamine acyltransferase is believed to be very similar to that of the lysophosphatidylcholine acyltransferases just described (Holub and Kukis, 1978). These observations, in conjuction with extensive in vivo studies (in which radiolabelled fatty acids or lysophospholipids are injected into animals, followed by extraction and analysis of tissue phospholipids) suggest that 18:1 ω9 and 18:2 ω6 are incorporated in phospholipids partly by de novo synthesis whereas 20:4 ω6 incorporation occurs almost exclusively by the deacylation/reacylation pathway (Holub and Kukis, 1978; Van den Bosch, 1980).

1.16 Objectives

The aim of the present research was to investigate the causes of the altered membrane lipid composition in tissues from obese mice. GLC analysis indicated that these alterations involved

changes in oleic acid, essential fatty acid and polyunsaturated fatty acid content. Activity of the Δ9-desaturase, (which converts stearic acid to oleic acid) is increased in liver and adipose tissue of the obese mouse, although no data on essential fatty acid desaturation in obese animals is available in the literature. Hence, essential fatty acid desaturation, which provides a possible control mechanism for membrane lipid composition has been investigated. Deacylation/reacylation activity has also been studied in lean and obese animals.

In addition to the investigation on control of membrane lipid composition in lean and obese mice, studies to further understand the consequences of altered membrane lipid environment on membrane protein function have been undertaken. Lipid-protein interactions have been investigated primarily by Arrhenius plot characterisation of membrane enzymes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Atractyloside, bovine serum albumin (BSA) (fraction 5), coenzyme A, Coomassie Brilliant Blue G 250, cytochrome C (horse heart), fatty acids (linoleic, linolenic, oleic and eicosatrienoic acids), guanosine diphosphate (GDP), insulin, lysophosphatidylcholine, reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and tri-iodothyronine (T_3) were all supplied by Sigma Chemicals, Poole, Dorset, UK.

Disodium adenosine 5'-triphosphate (ATP) was purchased from Boehringer Corporation Ltd., Lewes, East Sussex, UK, and diphenylhexatriene (DPH) from Aldrich Chemicals, Gillingham, Dorset, UK.

Radiochemicals, [1-^{14}C]- linoleic acid (specific activity : 58.0 Ci/mol), [1-^{14}C]-linolenic acid (56.2 Ci/mol), [2-^{14}C]- eicosa -8,11,14,-trienoic acid (55 Ci/mol), [1-^{14}C]-oleic acid (56.8 Ci/mol), [^{32}P]-orthophosphate (carrier free), [8-^{3}H]- guanosine 5' -diphosphate, ammonium salt (10.8 Ci/mmol) and [U-^{14}C]-sucrose (552 Ci/mol), and NCS tissue solubiliser were supplied by the Radiochemical Centre, Amersham, UK.

2.2 Animals

Male and female obese (ob/ob) mice and their lean litter mates (ob/+ or (+/+) were bred from heterozygote parents in the department's animal facility. The adult mice used in the experiments were aged between 6 and 8 weeks. 17 day old pre-obese (ob/ob) mice were identified by their enhanced fall in rectal temperature after a 15 minute exposure to 14°C environment (Trayhurn et al., 1977) This selection was confirmed by measuring inguinal fat pad weights, after sacrifice. All animals were fed a commercial mouse chow (Christopher Lee, Poole, UK.). The normal housing temperature was 23-24°C with a 12 hour light/dark cycle (0800-2000 hours).

Hyperthyroidism was induced either by tri-iodothyronine (T_3) injection, subcutaneously, twice a day, at a dose of 30 µg/kg body wt./day for 14 days or by feeding ad libitum a diet containing 0.02% (w/w) thyroid powder (Sigma, full strength) reconstituted with mouse chow, for 14 days.

Hypothyroidism was induced by feeding an iodine deficient diet (Special Diet Services, Ltd., Essex, UK.) ad libitum and providing a 0.5% (w/v) perchlorate drinking solution for 14 days.

Insulin treated animals were injected subcutaneously with 40IU/kg body wt. porcine insulin for 3 days prior to sacrifice.

A restricted feeding regime was maintained in some experiments by providing chow for only $2\frac{1}{2}$ hours per day (0900-11.30) for 13 days. Mice were sacrificed at the end of the feeding period on the last day.

Temperature acclimation was performed by housing mice at 34°C for 2 weeks, at 12°C for 2 weeks or at 12°C for 1 week followed by 1 week at 4°C prior to sacrifice. In all conditions mice were housed in pairs and provided with food ad libitum.

A severe cold challenge was performed by exposing 6 week old lean and obese mice, housed 2 per cage to 3°C for 1 hour.

2.3 Preparation of Liver Subcellular Fractions

1 g of liver was homogenised in a teflon/glass homogeniser with 5 stokes in 10 ml buffer (10mM Tris, pH 7.4, 250 mM sucrose, 1 mM EDTA) at 4°C. Homogenates were centrifuged at 1000 g for 10 minutes to sediment the nuclear fraction. The pellet was resuspended at approximately 10 mg protein/ml in buffer. The supernatant was subsequently centrifuged at 15,000 g for 15 minutes to sediment the mitochondrial pellet and this supernatant at 105,000 g for 1 hour to obtain the microsomal fraction. The mitochondrial pellet was resuspended at approximately 10 mg/ml in buffer and the microsomal pellet at approximately 5 mg/ml. All steps were performed at 4°C in either a MSE 21 centrifuge or in a MSE Pegasus ultracentrifuge.

2.4 Preparation of Brown Adipose Tissue Mitochondria

The procedure was based on the method of Cannon and Lindberg (1979). The buffer used at all stages in the preparation contained 250 mM sucrose, 0.2 mM EDTA and 1 mM HEPES pH 7.2 at 4°C. All steps were carried out at 4°C in a MSE 21 centrifuge. Mice were killed by cervical dislocation, interscapular brown adipose tissue was removed

and placed in buffer. Associated white adipose tissue was carefully removed and the tissue homegenised in buffer (0.5 g in 12 ml). The homogenate was centrifuged at 8,500 g for 15 minutes, the fat cake and supernatant were discarded and the pellet gently resuspended in the original volume of buffer. This was then centrifuged at 700 g for 10 minutes to pellet nuclei and cell debris. The supernatant was decanted off and the pellet gently resuspended in 10 ml buffer and recentrifuged at 700 g for 10 minutes. The 700 g supernatants were pooled and centrifuged at 8,500 g for 15 minutes. The resulting mitochondrial pellet was resuspended and washed in 10 ml buffer containing 2% defatted bovine serum albumin (BSA) (to remove endogenous free fatty acids) by centrifugation at 9,500 g for 20 minutes. This mitochondrial pellet was washed and centrifuged once more in buffer to give the final mitochondrial pellet. This was resuspended at protein concentrations between 2 and 4 mg/ml in 0.25 M sucrose and used immediately.

2.5 AcylcoA Synthetase Activity (EC 6.2.1.3)

The conversion of fatty acids to coenzyme A esters was assayed by the method of Blaise-Smith et al.(1982). Incubation conditions were similar to those of the desaturase of lysophosphatidylcholine acyltransferase assays. Reactions were started by addition of 1 mg protein (microsomal or mitochondrial) to preincubated flasks containing 100 mM phosphate buffer, pH 7.4 (or 50 mM Tris-HCl buffer pH 7.4) , 0.2 mM reduced CoA, 2 mM $MgCl_2$, 2 mM ATP, 0.05% (w/v) Triton WR1339 and 25 µM [^{14}C]-labelled fatty acid substrate (2.8 Ci/mol) in 2 ml at 37°C. Reactions were stopped by transferring 0.5 ml aliquots at timed intervals to test tubes containing 1 ml of Dole reagent (isopropyl alcohol : heptane : 3.5 M H_2SO_4, 40:10:1, v/v/v) to which 1 ml volumes of heptane were added with vigorous mixing. On separation, the upper heptane layer was discarded and the lower phase re-extracted three times with 1 ml heptane to remove any remaining free fatty acids. 0.5 ml volumes of the aqueous phase containing acylcoA esters were counted in duplicate on a Phillips PW 4700 liquid scintillation counter after addition of "Tritoscint" scintillation fluid [Xylene : Symperonic detergent(I.C.I.), 2:1(v/v) containing scintillants 0.4%(v/v) diphenyloxazole(PPO) and 0.3%(w/v) dimethyl bisphenyloxazoylbenzene (POPOP)]. The counting efficiency was 73%.

2.6 Fatty Acid Desaturase Activity (EC 1.14.99.5)

The measurements of Δ6- and Δ5- desaturase activity were based on the method of Cook(1978). Incubations were carried out using freshly prepared homogenates or subcellular fractions. Reactions were initiated by addition of 1 mg of protein to preincubated flasks containing 100 mM phosphate buffer pH 7.4, 0.2 mM reduced coenzyme A, 2 mM $MgCl_2$, 2 mM ATP, 0.5 mM NADH, 0.05% (w/v) triton WR 1339 and 25 uM ^{14}C -labelled fatty acid substrate (2.8 Ci/mol) in a final volume of 2 ml at 37°C. The substrate fatty acid for Δ6-desaturase assays was [1-^{14}C]- linoleic acid (18:2 ω6) or [1-^{14}C]- linolenic acid (18:3 ω3) and for Δ5-desaturase assays was [2-^{14}C]-eicosatrienoic acid (20:3 ω6). 1 mg protein samples were used, as preliminary experiments showed that desaturase activity was not linear with protein concentration. Reactions were stopped after 3 minutes by transferring 1.6 ml aliquots to test tubes containing 3.5 ml 10% (w/v) KOH in 95% (w/v) methanol. Mixtures were heated at 65°C for 30 minutes, acidified with 1.1 ml 7 M HCl and the fatty acids extracted twice with 3 ml diethyl ether:petroleum ether (1:1 v/v). The extracts were dried over anhydrous Na_2SO_4, evaporated to dryness under a stream of nitrogen gas and the fatty acids methylated in 500 µl 1 mM diazomethane in diethyl ether at 4°C for 1 hour. Diazomethane was prepared freshly each time, prior to use from nitrosomethylurea which was chemically synthesised in the department (Blatt,1961). Excess diazomethane was removed by evaporation under nitrogen. The fatty acyl methyl esters were separated by thin layer chromatography (TLC) on silica gel G plates containing 5% (w/w) silver nitrate using a solvent system of ethylacetate:toluene (70:30 v/v). Typical R_F values were:-

Fatty acid methyl ester	R_F
linoleic (18:2 ω6) methyl ester)	0.78
ɣ-linolenic (18:3 ω6) methyl ester)	
linolenic (18:3 ω3) methyl ester)	0.52
eicosatrienoic (20:3 ω6) methyl ester)	
arachidonic (20:4 ω6) methyl ester)	0.30
octadecatetraenoic (18:4 ω3) methyl ester)	

Radioactive peaks were monitored on a TLC scanner and the required sections of silica gel were scraped into scintillation vials. The samples were counted on a Phillips PW 4700 liquid scintillation counter after addition of Beckman Ready Solv scintillation fluid at 80% efficiency. Recovery of radioactivity from the plates was 80% and all results were corrected for recovery.

2.7 Lysophosphatidylcholine Acyltransferase Activity (EC 2.3.1.23)

This procedure was adapted from the method of Sarzala and Pilarska(1976). 200 to 400 µg of microsomal or mitochondrial protein was preincubated in 50 mM Tris-HCl pH 7.4 at 37°C, 0.2 mM reduced CoA, 2 mM $MgCl_2$, 2 mM ATP, 0.05% (w/v) triton X100 and 30 µM [^{14}C]-labelled linoleic acid or oleic acid substrate (3.3 Ci/mole), in a final volume of 0.5 ml. Preincubations were carried out for 3 minutes to allow conversion of substrate fatty acid to the coenzyme A ester by endogenous acylcoA synthetase activity. The lysophosphatidylcholine acyltransferase reaction was initiated by addition of (final concentration) 150 µM acceptor phospholipid (lysophosphatidylcholine). Reaction rates were linear for approximately 9 minutes. The reaction was stopped by addition of 3 ml chloroform:methanol (1:2 v/v). Substrate fatty acids and phosphatidylcholine product were extracted by the Bligh and Dyer procedure (see section 2.13.1). This allowed removal of the reaction intermediate fatty acid coenzyme A esters in the methanol:water wash. Radiolabelled fatty acids and phosphatidylcholine were separated by thin layer chromatography on silica gel H plates developed in chloroform : acetone : methanol : acetic acid : water (6:8:2:2:1, v/v/v/v/v). Radioactive peaks were located using a TLC scanner. Typical R_F values were:-

Lipid	R_F
fatty acylcoA	0.0
phosphatidylcholine	0.18
linoleic acid, oleic acid	0.80

The radioactive bands were scraped into scintillation vials, Beckman Ready Solv scintillation fluid added and the vial counted on a Phillips PW 4700 liquid scintillation counter. Efficiency of counting was 80%

and recovery of radioactivity from the TLC plate approximately 90%.

Competition Experiments

In these experiments, microsomes were incubated with 30 μM [^{14}C]-linoleic acid (6 Ci/mol) and increasing concentrations of [^{14}C]-oleic acid (6 Ci/mol). Incubation conditions were identical to those described above, except that the coenzyme A concentration was increased to 0.5 mM (to account for the higher substrate concentrations used). The extraction procedure was identical to that described before except that the 2 ml chloroform extracts were washed twice with 3.8 ml methanol : water (1:1 v/v) to remove all fatty acid coenzyme A esters. [^{14}C]-fatty acids incorporated into phosphatidylcholine were then transmethylated by the procedure outlined in section 2.13.3. The methyl esters of linoleic and oleic acids were then separated by TLC on silica gel G plates containing 5% (w/w) silver nitrate developed in petroleum ether : diethylether (80:20 v/v) (Privett et al 1963). Typical R_F values obtained were:-

Fatty acid methyl ester	R_F
linoleic (18:2ω6) methyl ester	0.33
oleic (18:1ω9) methyl ester	0.50

The radioactive bands were scraped into scintillation vials, Beckman Ready Solv scintillation fluid added and the vials counted as before. The transmethylation procedure used does not methylate free fatty acids, only fatty acid esters, for example phospholipids or triacylglycerols. These free fatty acids remain at the origin during the thin layer chromatography stage and are thus easily distinguishable from those incorporated into phosphatidylcholine by the acyltransferase activity.

2.8 Mitochondrial Succinate - Cytochrome C Oxidoreductase Activity (EC 1.3.99.1)

The assay of succinate - cytochrome C reductase was based on the method of Tisdale (1967) and follows the appearance of reduced cytochrome C spectrophotometrically at 550 nm using succinate as the electron donor.

A Pye-Unicam SP8-400 dual beam spectrophotometer linked to a Grant Flow FH15 heater/FC 200 cooler unit was used. 1 ml cuvettes containing (final concentrations) 10 mM potassium phosphate buffer pH 7.4 at 23°C, 1 mM NaN_3, 0.2 mM EDTA, 0.1% (w/v) cytochrome C, 0.5% (w/v) bovine serum albumin(BSA) and 30-50 µg of mitochondrial protein were preincubated at the chosen temperature for 3 minutes. The cuvettes were transferred to the spectrophotometer and the baseline allowed to stabilize. The reaction was started by addition of 5 mM sodium succinate. The volume of the blank cuvette was adjusted with water. The reaction rate was linear for 2 minutes at temperatures up to 37°C. Reaction rates were calculated using the molecular extinction coefficient for cytochrome C 18.5 x 10^6 M^{-1} cm^{-1} (at 550 nm).

2.9 Microsomal NADPH : Cytochrome C Reductase Activity(EC 1.6.2.4)

The assay of NADPH : cytochrome C reductase was based on the method of Phillips and Langdon (1962) and followed the appearance of reduced cytochrome C at 550 nm.

A Pye Unicam SP8-400 spectrophotometer was used.

3 ml cuvettes containing 100 mM NaCl, 20 mM KCl, 25 mM HEPES buffer, pH 7.4 at 37°C, 0:1% (w/v) cytochrome C, 1 mM KCN and 30-50 µg of protein were preincubated at 37°C for 3 mins. The cuvettes were transferred to the spectrophotometer and the reaction started by addition of 0.004% (w/v) NADPH to the test cuvette. The volume of the blank cuvette was adjusted with water. Under these conditions reaction rates were linear for approximately 2 minutes after mixing.

The molar extinction coefficient for cytochrome C used in calculations was 18.5 x 10^6 M^{-1} cm^{-1}.

2.10 Mitochondrial Cytochrome Oxidase Activity. (EC 1.9.3.1)

The assay of mitochondrial cytochrome oxidase was based on the method of Wharton and Tzagoloff (1967) and follows the disappearance of reduced cytochrome C spectrophotometrically at 550 nm.

$$2 \text{ reduced cytochrome C} + \tfrac{1}{2}O_2 + 2H^+ \longrightarrow 2 \text{ oxidised cytochrome C} + H_2O$$

A dual beam spectrophotometer was used. 1 ml cuvettes containing 0.1% reduced cytochrome C, 0.5% (w/v) bovine serum albumin, and 10 mM KH_2PO_4 buffer pH 7.2 at 23°C, were incubated at the chosen temperature for 3 minutes. The cuvettes were transferred to the spectrophotometer and the baseline allowed to stabilize. The reaction was started by the addition of 10-16 µg mitochondrial protein to the test cuvette. The reaction rate was linear for approximately 2 minutes after mixing at temperatures up to 37°C. Reaction rates were calculated using the molecular extinction coefficient for cytochrome C as $18.5 \times 10^6 \ M^{-1} \ cm^{-1}$.

Reduced cytochrome C was prepared as follows; 10 ml of 1% (w/v) cytochrome C in 10 mM KH_2PO_4 buffer pH 7.2, was reduced by the addition of 100 µl of 1 mM potassium ascorbate. The excess ascorbate was removed by dialysis against the 10 mM KH_2PO_4 buffer for 24 hours at 4°C with three changes of buffer using size 8 visking tubing.

2.11 Measurement of ATP-P_i Exchange Activity of Mitochondria

The assay is based on the method of Pulman (1967) as described by Innis and Clandinin (1981) and follows the incorporation of radioactive $[^{32}P]$ - phosphate into ATP by intact mitochondria.

150-250 µg of mitochondrial protein were preincubated at the chosen temperature for 5 minutes in 0.3 ml containing 20 mM PO_4^{3-} buffer pH 7.4 at 23°C, 10 mM $MgCl_2$, 0.2 M sucrose, 0.5% bovine serum albumin and 10^6 cpm $[^{32}P]$ -phosphate. Reactions were started by addition of 10 mM ATP (buffered with HEPES to pH 7.4). Preliminary experiments showed that the reaction rate was linear for at least 8 minutes, so 6 minutes was chosen as a suitable incubation time. The reactions were stopped by addition of 30 µl of 35% (w/v) perchloric acid with immediate cooling to 4°C. After standing for

10 minutes, the samples were centrifuged for 5 minutes at maximum speed in a MSE Minor centrifuge to remove precipitated protein, The supernatants were then analysed for incorporation of $^{32}P_i$ into ATP as follows; 0.2 ml aliquots were added to test tubes containing 4 ml 1.25 M perchloric acid and 5 ml isobutanol : benzene (1:1 v/v). Immediately, 1.0 ml volumes of 5% (w/v) ammonium molybdate were added and the tubes vortexed vigorously for 10 seconds. The mixing was repeated after 1 minute to clear all yellow, acid-phosphate-molybdate complex from the lower aqueous phase. The upper organic phase was discarded and the aqueous phase was re-extracted twice with 4 ml of isobutanol. Finally, all traces of isobutanol were removed by shaking with 1-2 ml diethyl ether. 1 ml volumes of the remaining aqueous phase were counted in duplicate on a Phillips PW 4700 liquid scintillation counter after addition of Tritoscint scintillation fluid. All solvents were saturated with water prior to use.

2.12 GDP Binding Assay

The assay for purine nucleotide binding to brown adipose tissue (BAT) mitochondria was based on the method of Nicholls(1976). Incubations were carried out in 400 µl polyethylene microfuge tubes in final volumes of 250 µl. Mitochondria were incubated in 20 mM Tris-HCl pH 7.1 at 24°C, 10 mM choline chloride, 1 mM EDTA, 5 µM rotenone, 100 µM potassium atractyloside and 0.1 µCi [U-^{14}C]-sucrose in 100 mM sucrose. Mitochondrial protein (100-200 µg) was preincubated in the reaction mixtures for 2 minutes at 24°C and the reactions started by addition of 0.625 µCi [8-^{3}H]-guanosine diphosphate(GDP) (final concentration 5 or 10 µM). The reactions were stopped after 8 minutes by centrifugation in a Beckman microfuge (for 2 minutes) and the supernatants removed from the sedimented mitochondria by suction on a vacuum line. Specific binding was assessed by the ability to displace [^{3}H]-GDP by 120 µM GDP. The mitochondrial pellets were then dissolved in 0.5 ml NCS tissue solubiliser for 1 hour at 50°C. The extracts were neutralised with (18 µl) glacial acetic acid and Beckman Ready Solv scintillant added. The radioactivity in the samples was counted using a dual label programme on a Phillips PW 4700 liquid scintillation counter. [U-^{14}C]-sucrose was included in order to calculate the volume of buffer trapped in the mitochondrial pellet. The counting efficiency

for [^{3}H]-GDP was 27% and for [U-^{14}C]- sucrose was 62%.

2.13 Extraction, Separation and Analysis of Mitochondrial and Microsomal Phospholipids

The procedures were based on those outlined by Veerkamp and Broekhuyse (1976). Phospholipids were extracted from membrane preparations and separated by TLC. The fatty acid components of the phospholipids were then transmethylated so that analysis by gas liquid chromatography could take place. All glassware used was chromic acid washed and solvents distilled prior to use.

2.13.1 Bligh and Dyer Extraction of Membrane Phospholipids

Each 0.8 ml sample containing 3-5 mg protein was extracted with 3 ml chloroform : methanol (1:2 v/v) over a 20 minute period with frequent mixing. The mixture was centrifuged at 1000 g in a MSE Minor centrifuge for 5 minutes, the supernatant decanted off and the pellet re-extracted with 1 ml chloroform. The pooled extracts were washed with 1 ml 100 mM KCl, and the upper aqueous / methanol phase discarded after separation. The chloroform phase was evaporated down under nitrogen to 200 µl and stored in small brown glass vials at -20°C.

2.13.2 Separation of Phospholipids

The extracted phospholipids were separated by thin layer chromatography on silica gel H plates containing 3% (w/w) magnesium hydroxycarbonate using chloroform : methanol : water (65:25:4, v/v/v) as developing solvent. The solvent tank contained 10 mg butylated hydroxytoluene to minimize oxidation. Phospholipid bands were visualised and marked under U.V. light after staining with 0.01% (w/v) diphenyl hexatriene (DPH) in petroleum ether (60-40°C) (Hyslop and York 1980). Typical R_F values obtained were :-

Phospholipid	R_F
phosphatidylcholine	0.15
phosphatidylethanolamine	0.28
cardiolipin	0.45

The DPH stain was eluted by rechromatographing the TLC plates in petroleum ether and the phospholipids extracted from the silica gel with chloroform : methanol (1:1 v/v). The samples were washed with KCl and evaporated down as described above.

2.13.3 Transmethylation

Before determination of fatty acid composition, phospholipids were transmethylated as follows. Sodium methoxide reagent was prepared by dissolving 460 mg sodium in 50 ml methanol which had been dried over Molecular Sieve (type 4A BDH, Poole, Dorset, UK.). Phospholipid samples were evaporated to dryness, 20 µl methanol and 20 µl sodium methoxide reagent added and the samples incubated under nitrogen at 23°C for 30 minutes. 20 µl acetyl chloride : methanol (1:19 v/v) was then added and the fatty acid methyl esters extracted twice into dry hexane (100 µl) before analysis by gas liquid chromatography.

2.13.4 Gas Liquid Chromatography Analysis of Fatty Acid Methyl Esters

The fatty acid methyl esters were separated by gas liquid chromatography (GLC) on a Pye-Unicam Series 106 liquid chromatograph. The temperature programme used was 160°C for 3 minutes at 3°C per minute and 210°C for 5 minutes. The column was obtained from Applied Science and was a Silar 5CP Quadrex capillary column (50 metres). The size of the fatty acid methyl peaks was obtained by computer integration.

2.14 Delipidation of Bovine Serum Albumin (BSA)

35 g of activated charcoal was washed overnight in 400 ml distilled water. Any residue floating on the surface was removed by suction on a vacuum line. 70 g of BSA (fraction V) was dissolved in the charcoal suspension and the mixture transferred to an ice bath. The pH was reduced over a one hour period to pH 3.0 by slow addition of 0.2 M HCl with stirring and maintained there for a further one hour. The mixture was then centrifuged at 32,000 g for 1 hour at 4°C to remove the charcoal.

The supernatant containing the defatted BSA was decanted off and any remaining particles of charcoal removed by filtration through millipore filters (HAWP - 0025, size 0.45 μm, Millipore Filter Corporation, Bedford, Mass. USA). Finally, the pH was neutralized (pH 7.0) by addition of 0.2 M NaOH dropwise while the solution was stirred in an ice bath. The protein concentration of the defatted BSA was determined prior to storage at - 20°C in 10 ml aliquots.

2.15 Radioimmuno Assay (RIA) of Serum Insulin

Serum insulin concentrations were measured by the method of Hales and Randle (1963), using the Wellcome RIA kit. This employs a double antibody technique. 0.1 ml duplicate serum samples and rat insulin standards (6.3 to 200 μ units / ml in 0.1 ml of 40 mM sodium phosphate buffer pH 7.4 at 4°C, 0.9% (w/v) NaCl, 0.5% (w/v) BSA and 0.1% (w/v) NaN_3) were added to tubes containing 0.1 ml of Wellcome Insulin Binding Reagent, mixed and incubated for 6 hours at 4°C. The Wellcome Insulin Binding Reagent contains a functional immunoprecipitate comprising guinea-pig anti-insulin serum and rabbit anti-guinea-pig globulin serum in 40 mM sodium phosphate buffer pH 7.4, at 4°C, 20 mM EDTA, 0.1% (w/v) NaN_3 and 0.5% (w/v) BSA. 0.1 ml volumes of the working solution of tracer $[^{125}I]$-insulin (0.25 μCi/ml) were then added to all tubes, mixed and incubated for 18 hours at 4°C. After dilution by 1 ml sodium phosphate buffer pH 7.4 at 4°C containing 0.9% (w/v) NaCl, 0.5% (w/v) BSA and 0.1% (w/v) NaN_3, the tubes were centrifuged at 2,000 g for 20 minutes at 4°C to separate the $[^{125}I]$-insulin bound to immune precipitate and free insulin. The supernatants were carefully decanted and the tubes blotted dry. They were then counted in a Beckman γ-counter (at 80% efficiency for ^{125}I) for 1 minute.

In addition to the samples and standards, "zero", "blank" and "total" duplicate incubation tubes were also assayed. "Zero" tubes contained no unlabelled insulin whilst "blanks" contained no insulin binding reagent (only the appropriate volume of buffer) and served as controls for the washing procedure. "Total" incubation tubes contained only $[^{125}I]$-insulin and were not diluted with buffer after the 18 hour incubation. These tubes were counted and provided values for the total radioactivity added to the incubation tubes.

Bound radioactivity was expressed as a percentage of the total radioactivity added, after the background count was subtracted.

$$\frac{\text{Bound - blank}}{\text{Total - blank}} \times 100$$

A standard curve was plotted (percentage bound versus log. concentration of standard insulin) from which the concentration of insulin in the serum samples was determined.

2.16 Phospholipid Assay

The phospholipid assay used was that of Raheja et al. (1973). Chromogenic reagent was prepared as follows. 1 ml mercury was mixed with 8 ml 13.3% (w/v) ammonium molybdate and 4 ml concentrated HCl. The mixture was shaken vigorously before filtering through Whatman No. 1 filter paper. 20 ml concentrated H_2SO_4 was added to 4 ml 13.3% (w/v) ammonium molybdate and this was then pooled with the filtrate. 25 ml of this solution was then added to 45 ml methanol, 5 ml chloroform and 20 ml distilled water to give the chromogenic reagent. This was stored at $4^{\circ}C$ until used and was viable for several months.

The assay was performed as follows. Phospholipid samples and standards (10 - 250 n moles egg phosphatidylcholine) were evaporated to dryness, and dissolved in 0.4 ml chloroform. 0.1 ml chromogenic solution was added and the samples heated in a boiling water bath for $1\frac{1}{2}$ minutes. The samples were allowed to cool, 2.5 ml aliquots of chloroform added and the mixtures shaken gently. Colour was allowed to develop for 15 minutes in the chloroform layer, and the samples were read at 710 nm on a spectrophotometer.

2.17 Protein Assay

Protein determination was carried out by the method of Bradford (1976). Coomassie Blue Reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G 250 dye in 50 ml of 95% (v/v) ethanol.

After addition of 100 ml orthophosphoric acid, and 850 ml distilled water, the solution was filtered through Whatman No. 1. filter paper and stored in the dark, ready for use.

The assay was performed as follows. 5 ml of reagent was added to samples (containing between 10 and 50 µg of protein) and standards (10 - 90 µg bovine serum albumin, fraction V) and mixed gently. Colour was allowed to develop for five minutes and the samples read at 595 nm on a dual beam spectrophotometer.

2.18 Statistics

All results were assessed for statistical significance by Students 'T' test for unpaired or where appropriate, paired data.

CHAPTER 3

ESSENTIAL FATTY ACID DESATURATION IN LEAN AND OBESE MICE

3.1 Introduction

The fatty acid compositions of membrane phospholipids in the obese mouse have been shown to be abnormal (Rouer et al., 1980; Hyslop et al., 1982; French et al., 1983), although the biochemical basis for these changes is not clear. However, since membrane lipid composition may be controlled by the availability of fatty acids for incorporation into phospholipids, one possible mechanism for controlling membrane lipid composition is through the regulation of fatty acid desaturation. In the liver, EFA desaturation is subject to control by many hormonal and nutritional states and the Δ6-desaturase has been suggested as the rate limiting step in PUFA synthesis (Brenner, 1977; Naughton, 1981). Hence, in the present study, the control of Δ6-desaturase activity has been investigated with regard to the hyperphagia and abnormal endocrine status of the obese mouse. Before the results of this work are presented, studies undertaken to characterise murine hepatic Δ6-desaturase activity are described.

Results

3.2 Characterisation of Hepatic Δ6-desaturase Activity

The time courses of Δ6-desaturation of linoleic acid (18:2 ω6) in brain and liver homogenates from neonatal and adult lean mice respectively are shown in figure 3.1. Enzyme activity was determined in brain homogenates from neonates as previous studies have shown that Δ6-desaturase activity is highest in brain immediately after birth (Cook, 1978). Δ6-desaturase activity in liver homogenates was linear for only 5 minutes (figure 3.1). Hence 3 minutes was chosen as a suitable incubation time in all subsequent experiments. In contrast, Δ6-desaturase activity was much lower in brain homogenates and was linear for at least 10 minutes. From the data, rates of conversion of 18:2 ω6 to 18:3 ω6 were calculated. These were 0.882 nmoles/min/mg protein for liver, and 0.086 nmoles/min/mg protein for brain homogenates.

Figure 3.2 shows the effect of protein concentration on the assay of Δ6-desaturase activity in liver homogenates from lean and obese

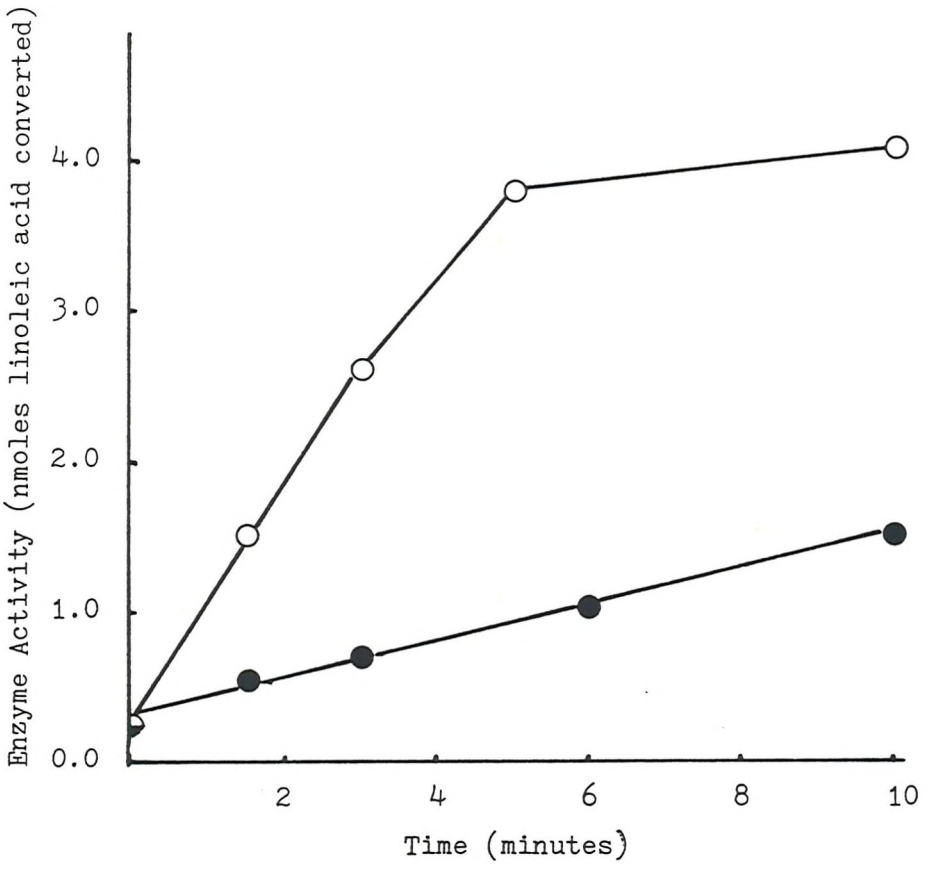


Fig 3.1 The Time Course of Δ6-desaturase Activity in Liver and Brain Homogenates.

Homogenates from adult mouse liver (1 mg ○) and neonatal mouse brain (1.5 mg ●) were incubated with 25μM linoleic acid for various times and assayed for Δ6-desaturase activity as described in section 2.6.

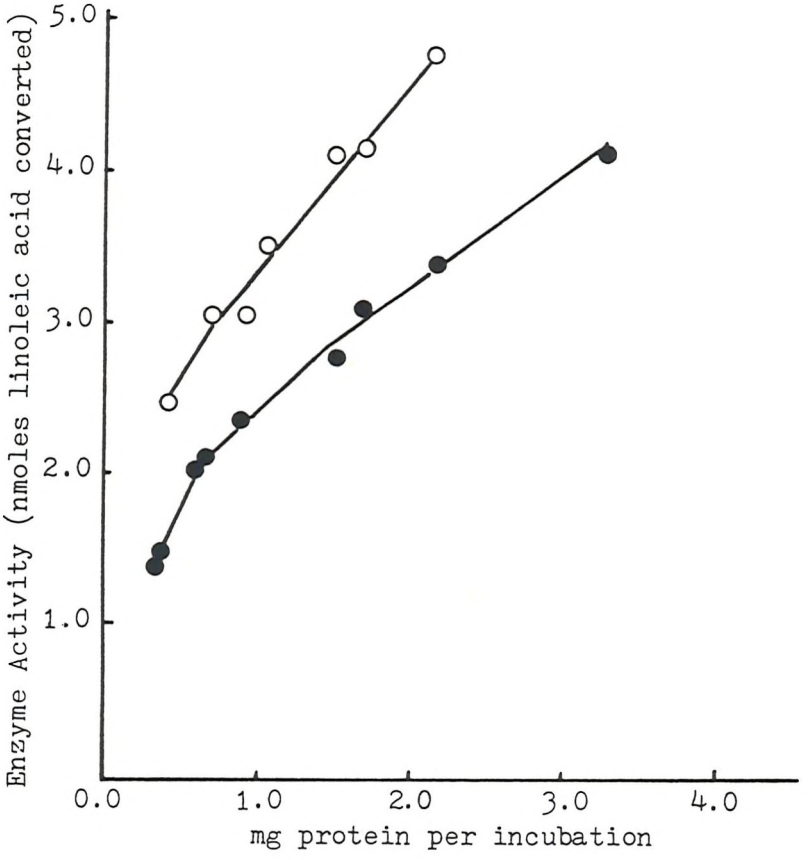


Fig 3.2 The Effect of Protein Concentration on Δ6-desaturase Activity in Lean and Obese Mouse Liver Homogenates.

Δ6-desaturase activity was assayed in liver homogenates from lean (●) and obese (○) mice as described in section 2.6 using linoleic acid as the substrate. The data presented is from 3 experiments.

mice, using linoleic acid (18:2 ω6) as substrate and a 3 minute incubation period. The activity in liver homogenates from obese mice was consistently higher than the activity in those from lean animals. However, enzyme activity was not linear with protein concentration in either preparation. The non-linearity of Δ6-desaturase activity with protein concentration has been observed previously (Jeffcoat, 1977) and may result from either product inhibition of the enzyme and/or incorporation of substrate into phospholipids at high protein concentrations. In subsequent experiments, 1 mg liver homogenate protein was chosen as a suitable concentration for desaturase assays. The hepatic Δ6-desaturase activities at this protein concentration were 0.80 and 1.12 nmoles/min/mg protein in lean and obese mice respectively. Enzyme activity (conversion of 18:2 ω6 to 18:3 ω6) was prevented by boiling the liver homogenates prior to the Δ6-desaturase assay. In this situation, Δ6-desaturase activity was negligible at less than 0.05 nmoles/min/mg 18:2 ω6 converted, which corresponded to approximately 6% of the enzyme activity in the control homogenates.

The substrate specificity of the Δ6-desaturase was investigated using liver homogenates from lean and obese mice (table 3.1). In agreement with Naughton (1981), faster rates of enzyme activity were obtained with increasing unsaturation of the fatty acid substrate. Δ6-desaturase activity was increased by 22% when linolenic acid (18:3 ω3) rather than linoleic acid (18:2 ω6) was used as the substrate in both lean and obese mouse liver homogenates. In addition, Δ6-desaturase activity using both substrates was 20% to 30% higher in liver homogenates from obese mice when compared to the Δ6-desaturase activity in lean mouse preparations. In all subsequent experiments, linoleic acid (18:2 ω6) was used as the substrate for Δ6-desaturase assays.

3.3 Δ6-desaturase Activity During Development in Liver and Brain

Δ6-desaturase activity has been shown to be elevated in the liver of the obese mouse. In order to determine the age at which this defect in fatty acid metabolism develops, Δ6-desaturase activity was measured in liver homogenates of lean and obese mice aged between

Substrate	Δ6-desaturase Activity (nmoles/min./mg) Lean	Obese	
Linoleic acid (18:2ω6)	0.82 ± 0.07	1.02 ± 0.04	$p<0.05$
Linolenic acid (18:3ω3)	1.07 ± 0.12	1.30 ± 0.08	$p<0.05$

Table 3.1 Δ6-desaturase Activity in Liver Homogenates of Lean and Obese Mice Using Various Substrates

Liver homogenates from lean and obese mice were assayed for Δ6-desaturase activity as described in section 2.6 using either linoleic acid or linolenic acid as the substrate. The values represent the means ± SEM for 5 individual preparations.

7 and 56 days. Enzyme activity was also examined in brain homogenates of these animals in order to investigate the possibility of a similar increase in Δ6-desaturation in this tissue of obese mice.

Reciprocal changes in Δ6-desaturase activity of liver and brain homogenates were observed during development (figure 3.3). This confirms the previous observations of Cook (1978) who studied Δ6-desaturase activity in the rat. In mouse brain, Δ6-desaturase activity was highest at the neonatal stage (assayed here at 7 days of age) and fell to basal level before weaning (less than 1% conversion of 18:2 ω6 during a 6 minute incubation period). The high neonatal enzyme activity may reflect the rapid development of the brain at this time. In contrast, Δ6-desaturase activity in liver homogenates was low in the suckling mouse but increased after weaning and had doubled by 56 days. At this age, Δ6-desaturase activity in liver was 30 fold greater than in brain, in agreement with observations of Cook (1978). No differences in Δ6-desaturase activity were apparent in the livers of 18 day old suckling lean and pre-obese mice, distinguished by the cold challenge test of Trayhurn et al. (1977). However, after weaning there was a greater increase in enzyme activity in the obese mice with the result that their hepatic Δ6-desaturase activity was 30% higher than in lean animals by 56 days of age.

Δ6-desaturase activity in brain homogenates of lean and obese mice was apparently similar at all stages during development. However, pre-obese mice could not be identified by the cold challenge test at 7 days of age when enzyme activity in the brain was highest.

Preliminary studies using adipose tissue revealed low levels of Δ6-desaturase activity in fat-free, crude membrane preparations (30,000 g pellet preparation of adipose tissue homogenates). Activity was similar at 0.23 ± 0.02 and 0.24 ± 0.05 nmoles/min/mg protein (mean ± SEM) for 4 adult lean and 4 adult obese mice respectively.

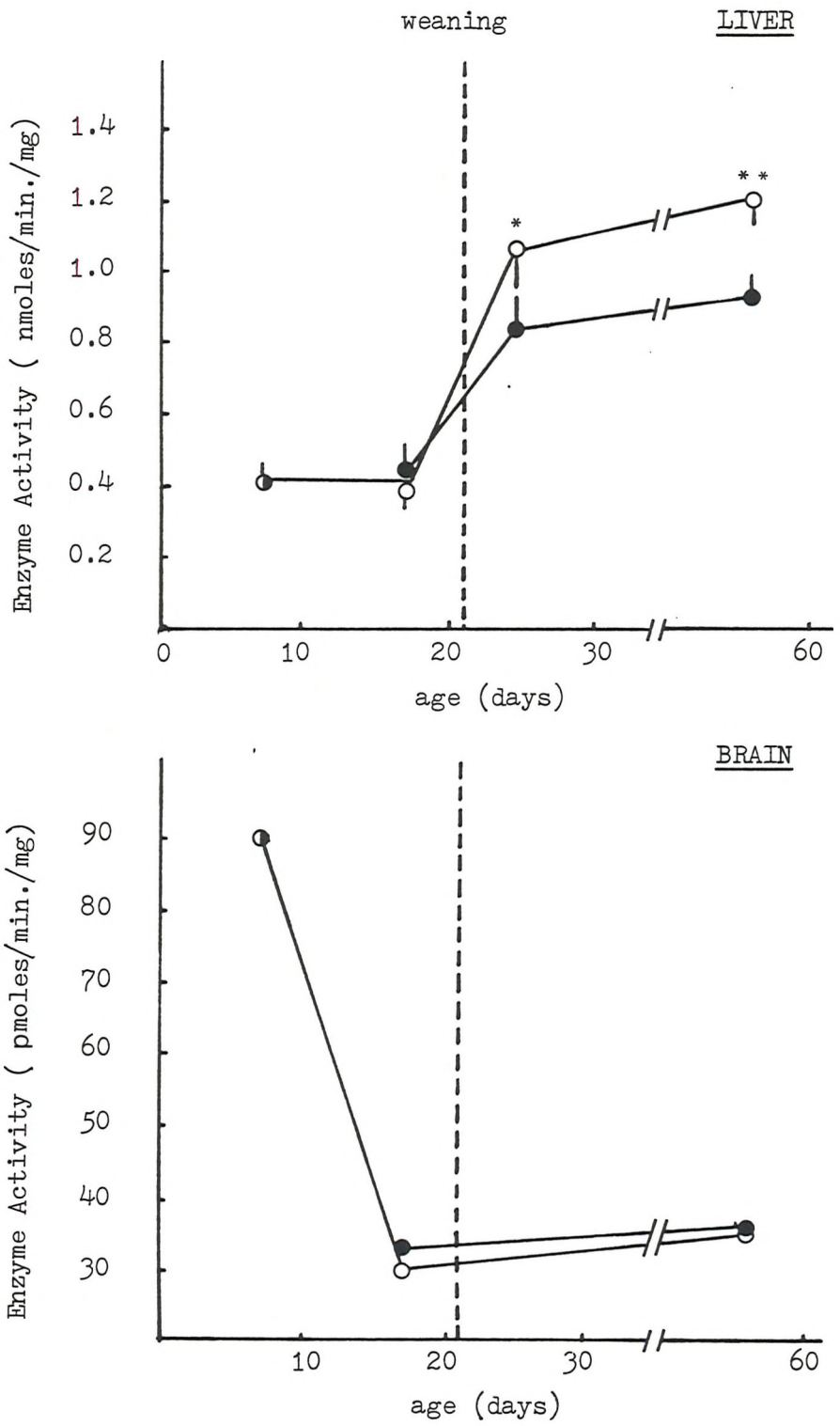


Fig. 3.3 Δ6-desaturase Activity in Homogenates of Liver and Brain of Lean and Obese Mice at Differing Ages

Δ6-desaturase activity was assayed as described in section 2.6 in brain homogenates from 2 lean (●) and 2 obese (○) mice or liver homogenates from 4 lean (●) and 4 obese (○) mice at each age. It was not possible to distinguish lean and pre-obese mice at 8 days of age.

* $p<0.05$; ** $p<0.005$ compared to lean mice

3.4 Subcellular Distribution of Hepatic Δ6-desaturase Activity

Subcellular fractions of liver were prepared by differential centrifugation and assayed for Δ6-desaturase activity. The distribution of hepatic Δ6-desaturase activity in cell organelles is shown in table 3.2. The highest enzyme activity observed was in the mitochondrial fraction. This was two fold greater than the Δ6-desaturase activity in the microsomes. Even allowing for the presence of significant microsomal contamination of the mitochondrial fraction as indicated by the activity of the microsomal marker enzyme, NADPH cytochrome P_{450} reductase, the data suggests that Δ6-desaturase activity was located principally in the mitochondria of mouse liver. The presence of significant mitochondrial succinate-cytochrome C reductase activity in the low speed nuclear pellet suggested that the Δ6-desaturase activity in this fraction probably resulted from mitochondrial contamination.

The high Δ6-desaturase activity located in the mitochondrial fraction was unexpected. Previous studies on desaturase activity have used principally hepatic microsomal preparations (Cook, 1978; Naughton, 1981; Faas and Carter, 1981; 1982). To be certain that the apparent distribution of Δ6-desaturase activity between microsomal and mitochondrial fractions was correct, experiments were undertaken to confirm that the incubation conditions used were optimal for enzyme activity in both subcellular fractions. Thus Δ6-desaturase activities in hepatic mitochondrial and microsomal fractions from lean and obese mice were measured under various incubation conditions (table 3.3). The data suggests that Δ6-desaturase activities of both the mitochondrial and microsomal fractions were optimal under the conditions described. Enzyme activities were not significantly altered by two fold increases in concentrations of ATP, $MgCl_2$, reduced coA or NADH, added either individually or collectively to the assay media. In addition, no further stimulation in activity could be achieved for either fraction by addition of a 100,000 g liver supernatant factor, as has been described previously (Cook, 1978; Jeffcoat, 1979).

Δ6-desaturase activity in hepatic mitochondria and microsomes from obese mice were 40% higher than in the comparative liver fractions

	Δ6-desaturase		Succinate-cytochrome C reductase	NADPH-cytochrome P_{450} reductase
	(nmoles/mg/min.)	(Total nmoles/min.)	(nmoles/mg/min.)	(nmoles/mg/min.)
Nuclei	0.52 ± 0.02	24.4 ± 2.2	163.4 ± 45.4	18.7 ± 9.2
Mitochondria	1.08 ± 0.11	28.3 ± 3.9	180.8 ± 56.2	71.0 ± 30.2
Microsomes	0.53 ± 0.02	6.1 ± 0.7	5.2 ± 0.3	150.6 ± 33.2
105,000g supernatant	0.18 ± 0.02	10.8 ± 2.7	4.5 ± 0.7	11.1 ± 6.2

Table 3.2 Subcellular Distribution of Hepatic Δ6-desaturase Activity

Hepatic subcellular fraction were prepared as described in section 2.3 and assayed for Δ6-desaturase activity as described in section 2.6. Mitochondrial (succinate-cytochrome C reductase) and microsomal (NADPH-cytochrome P_{450} reductase) marker enzymes were measured in the fractions as described in sections 2.8 and 2.9 respectively. The values represent the means ± SEM for 3 individual preparations.

	Δ6-desaturase (nmoles/min./mg protein)			
	Mitochondria		Microsomes	
Additions	Lean	Obese	Lean	Obese
none	0.95 + 0.02 (4)	1.34 + 0.02 (4)***	0.40 + 0.05 (4)	0.61 + 0.11 (4)
+ 2 mM ATP	1.02 (2)		0.38 (2)	
+ 2 mM Mg^{++}	1.13 (2)		0.43 (2)	
+ 2 mM Mg^{++}/ATP	0.91 + 0.02 (4)	1.23 + 0.10 (4)**	0.36 (2)	
+ 0.5 mM NADH	0.88 (2)		0.44 (2)	
+ 0.2 mM CoA-SH	1.15 (2)		0.36 (2)	
+ all	1.12 + 0.08 (4)	1.50 + 0.11 (4)**	0.41 (2)	
+ 0.70 mg supernatant protein	0.90 (2)		0.46 (2)	

Table 3.3 Δ6-desaturase Activity in Hepatic Mitochondria and Microsomes of Lean and Obese Mice

Enzyme activity was assayed under the incubation conditions described in section 2.6, or in the presence of additional concentrations of cofactors as shown. Values represent means for the number of observations shown in parentheses, SEM are presented when the number of observations exceeded 3. ** $p < 0.01$; *** $p < 0.001$ compared to lean group.

from lean mice, although the increase in microsomal activity was not statistically significant. The elevated mitochondrial Δ6-desaturase activity in obese mice was maintained under all the incubation conditions tested.

Mitochondrial preparations were used in all subsequent studies on the regulation of hepatic Δ6-desaturase activity in lean and obese mice. Mitochondrial Δ6-desaturase activity was low and similar in 17 day old suckling lean and pre-obese mice (0.65 ± 0.02 and 0.58 ± 0.05 nmoles/min/mg for 3 lean and 3 pre-obese mice respectively), the enzyme activity being only 56% of that observed in the weaned mice. These results confirm the previous data obtained using hepatic homogenates.

3.5 Mitochondrial AcylcoA Synthetase Activity

Prior to desaturation and elongation, free fatty acids must be activated by conversion to coenzyme A esters. To confirm that mitochondrial Δ6-desaturase activity was not limited by the rate of the conversion of linoleic acid to linoleoylcoA, endogenous acylcoA synthetase activity was measured in lean and obese mouse hepatic mitochondria. Figure 3.4 shows the conversion of linoleic acid to linoleoyl coenzyme A by mitochondrial acylcoA synthetase under identical conditions to those used in desaturase enzyme assays. Clearly, acylcoA formation was not rate limiting for the desaturase reactions. 80% of the linoleic acid substrate had been converted in the first minute under these conditions by either lean or obese hepatic mitochondrial preparations. From the data, the initial rates of conversion of linoleic acid to linoleoylcoA by hepatic mitochondrial acylcoA synthetase were estimated. These were 44 nmoles/min/mg and 66 nmoles/min/mg for lean and obese mice respectively. Thus, the activity of acylcoA synthetase was approximately 50 times greater than that of Δ6-desaturase activity in liver mitochondria of lean or obese mice. In the absense of added coenzyme A, no linoleoylcoA was formed. In addition, previous studies have shown that the hepatic mitochondrial acylcoA synthetase has a wide specificity for fatty acid substrates (Tanaka et al., 1979). Hence it is unlikely that formation of other unsaturated fatty acylcoA substrates was rate limiting for desaturase reactions.

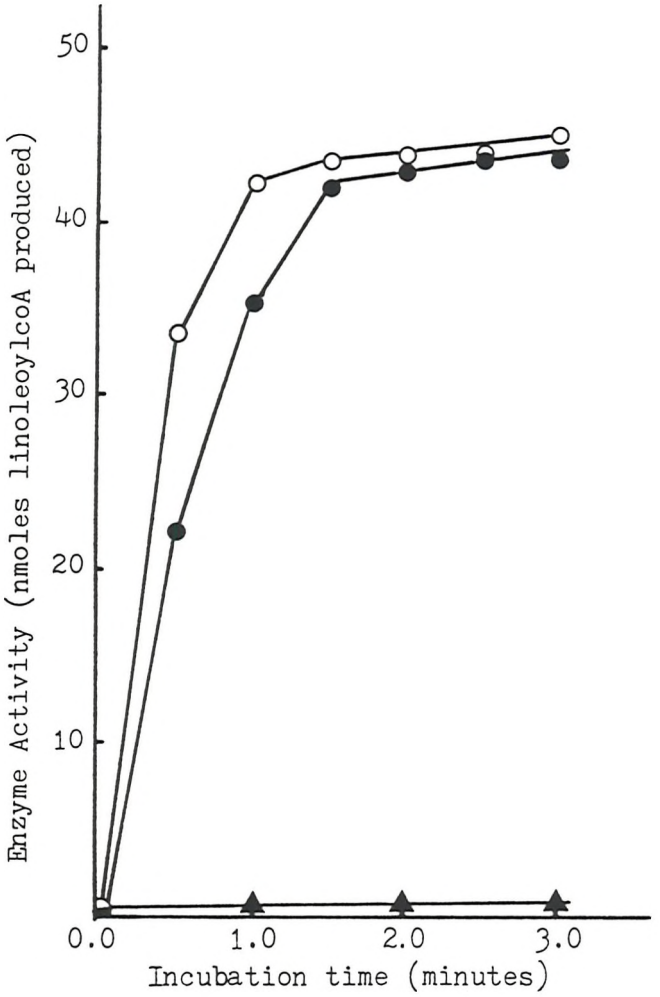


Fig 3.4 The Time Course of AcylcoA Synthetase Activity in Hepatic Mitochondria of Lean and Obese Mice.

Hepatic mitochondria were prepared from lean (●) and obese (O) mice as described in section 2.3 and assayed for AcylcoA Synthetase activity as described in section 2.5 using linoleic acid as the substrate. Enzyme activity was also assayed in the absence of reduced coA (▲).

3.6 Effect of Diet on Hepatic Mitochondrial Δ6-desaturase Activity

Hyperphagia does not appear to be present before weaning in pre-obese mice (Rath and Thenen, 1979), but develops immediately after weaning (Lin et al., 1977). To investigate whether the increase in hepatic Δ6-desaturase activity observed only after weaning was secondary to this development of hyperphagia, enzyme activity was measured under conditions of pair-feeding and of starvation. Obese mice were pair fed to the food intake of lean littermates by a meal time feeding regime. Animals were allowed access to food for only $2\frac{1}{2}$ hours each day to ensure a similar pattern of meal eating in both groups. The meal time feeding regime was chosen in preference to the method of pair feeding each obese mouse to the previous day's food intake of a lean animal. Without the use of automatic feeding machines, it was found that obese mice gorged their food pellets or that the animals scattered and soiled the pellets on the floor of the cage when feeding regimes other than meal time feeding were employed.

Meal time feeding reduced the food intake of lean mice by 30% and prevented the hyperphagia in the obese animals (table 3.4). Pair feeding to the food intake of lean mice, reduced the weight gain of obese animals by 75% when compared to that of obese mice fed ad libitum, whereas it had no significant effect on weight gain in lean animals. Mitochondrial Δ6-desaturase activity was reduced in both lean and obese mice by the pair feeding regime. However, the Δ6-desaturase activity remained significantly higher (by 19%) in the obese restricted feeding group than in the corresponding lean animals. Table 3.4 also shows the activity of hepatic mitochondrial Δ6-desaturase in lean and obese mice which were starved for 17 hours. Enzyme activity was increased as a result of short term food deprivation. Hepatic mitochondrial Δ6-desaturase activity in starved lean animals almost doubled (90% increase) whereas enzyme activity in the obese mice starved for 17 hours increased significantly by 30% when compared to the ad libitum fed obese group. The difference in Δ6-desaturase activity between lean and obese starved animals was not significant.

Animals	Dietary Status	Food Intake (grms.)	Body wt. (grms.) Before	Body wt. (grms.) After	Δ6-desaturase (nmoles/min/mg)	
Lean (4)	ad libitum	4.96 ± 0.17	26.0 ± 1.3	27.5 ± 1.6	0.87 ± 0.04	$p<0.05$
	restricted	3.51 ± 0.15	26.1 ± 1.4	27.0 ± 1.5	0.67 ± 0.04	
Obese (4)	ad libitum	6.76 ± 0.25	36.6 ± 1.6	44.5 ± 1.4 $p<0.05$	1.10 ± 0.04 *	$p<0.01$
	restricted	3.48 ± 0.19	36.4 ± 1.5	38.3 ± 1.4	0.80 ± 0.03 *	
Lean (3)	ad libitum	4.90 ± 0.24	- -	- -	0.80 ± 0.05	$p<0.001$
(4)	starved	- -	- -	- -	1.52 ± 0.06	
Obese (3)	ad libitum	6.65 ± 0.23	- -	- -	1.29 ± 0.04 ***	$p<0.001$
(4)	starved	- -	- -	- -	1.65 ± 0.04	

Table 3.4 The Effect of Food Intake on Hepatic Mitochondrial Δ6-desaturase of Lean and Obese Mice

6 week old mice were either fed ad libitum or were pair fed during a 2.5 hour restricted feeding period for 13 days. Mice were sacrificed at the end of the 2.5 hour feeding period. Mice fed ad libitum were starved for 17 hours prior to sacrifice. Hepatic mitochondria were prepared as described in section 2.3 and Δ6-desaturase activity assayed as described in section 2.6. Values represent the means ± SEM for the number of animals(n) shown in parentheses. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to equivalent lean mouse group (students 't' test for paired data).

Insulin has been shown to be an important regulator of liver microsomal Δ6-desaturase activity in the rat. Alloxan diabetic rats had reduced Δ6-desaturase activity which was prevented by insulin injection (Brenner et al., 1968). To investigate whether the hyperinsulinemia of the obese mouse was responsible for the elevated hepatic mitochondrial Δ6-desaturase activity, lean animals were injected with 40IU insulin/Kg body wt./day for 3 days and their Δ6-desaturase activity compared to that of saline injected control mice. Chronic treatment of lean mice did not increase hepatic mitochondrial Δ6-desaturase activity (0.76 ± 0.06 and 0.77 ± 0.03 nmoles/min/mg protein for control and insulin treated mice respectively) despite a 57% increase in serum insulin from 59.7 ± 3.7 to 93.9 ± 6.0 μ units ml^{-1} (mean ± SEM for 4 mice in each group).

3.7 Effect of Temperature Acclimation on Hepatic Δ6-desaturase Activity

The obese mouse has a reduced body temperature when housed at temperatures between 22°C to 25°C. Body temperatures were normalised and the altered membrane lipid compositions of obese mice were partially rectified when the animals were housed at the thermoneutral temperature of 34°C (Hyslop and York, 1980b; French et al., 1983). Hence it was of interest to investigate the effect of housing temperature on hepatic mitochondrial Δ6-desaturase activity. Animals were housed at different temperatures between 4°C and 34°C for 14 days prior to the assay of hepatic Δ6-desaturase activity (figure 3.5). Housing at 34°C did not affect the increased Δ6-desaturase activity of obese mouse liver mitochondria which remained 35% higher than in lean mice. In contrast, after housing at 12°C, the enzyme activity in obese mouse preparations was reduced towards that of lean mice (which remained unchanged). Exposure to extreme cold, which involved housing at 4°C for 7 days after pre-acclimation at 12°C for 7 days, reduced both the lean and obese mouse hepatic Δ6-desaturase activities to similar levels (0.891 ± 0.040 and 0.893 ± 0.093 nmoles/min/mg protein for 4 lean and 4 obese animals respectively). The difference in enzyme activity in hepatic mitochondria from lean control and cold acclimated lean animals was not statistically significant.

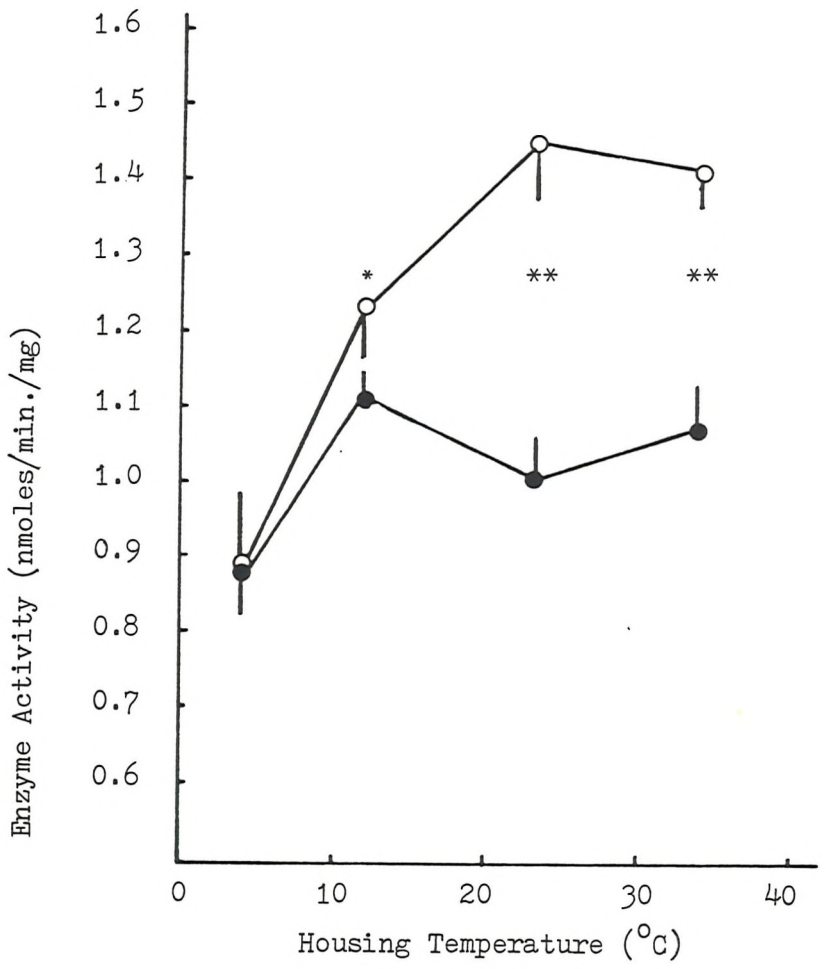


Fig 3.5 The Effect of Housing Temperature on Hepatic Mitochondrial Δ6-desaturase Activity of Lean and Obese Mice

4 lean (●) and 4 obese (○) mice were housed at each temperature for 14 days prior to sacrifice. Mice acclimated to a 4°C environment were housed at 14°C for the preceding 7 days to prevent mortality in the obese mouse group. Hepatic mitochondrial Δ6-desaturase activity was assayed as described in section 2.6. Values represent means ± SEM
* $p<0.05$; ** $p<0.005$ compared to the lean mouse group.

3.8 Changes in Δ6-desaturase Activity With Thyroid Status

The observation that hepatic Δ6-desaturase activity in obese mouse mitochondria was normalised by cold acclimation, a process which involves increased thyroid function (Goglia et al., 1983), prompted an investigation into the role of thyroid status in Δ6-desaturase activity of lean and obese mice. Lean and obese mice were injected with tri-iodothyronine (T_3) subcutaneously for 14 days at a dose of 30 μg/kg body wt./day. In agreement with Faas and Carter (1981) who injected rats with T_3, ad libitum food intake was significantly increased (by 26%) in T_3 treated lean mice. Food intake of T_3 treated obese animals was unchanged (table 3.5). In contrast, T_3 treatment normalised the increased % weight gain of obese mice but had no significant effect on the weight gain of lean animals. The effect of T_3 treatment on hepatic mitochondrial Δ6-desaturase and Δ5-desaturase activities is shown in figure 3.6. The activity of the Δ5-desaturase was lower than the Δ6-desaturase in both control lean and obese mice, in agreement with previous studies (Sprecher, 1977). In addition to the increase in hepatic mitochondrial Δ6-desaturase activity, the hepatic mitochondrial Δ5-desaturase activity of obese mice was also increased in comparison to the enzyme activity present in lean animals. The increases in enzyme activity in obese mice were 32% and 40% for Δ6- and Δ5-desaturase respectively. T_3 treatment normalised both Δ6- and Δ5-desaturase activities in obese mice but had no effect on the activities of the enzymes in lean animals (figure 3.6). Induction of hypothyroidism increased hepatic mitochondrial Δ6-desaturase activity by 40% in both lean and obese mice. Enzyme activity in the obese hypothyroid mice remained significantly higher (by 29%) than the activity in the lean hypothyroid animals. T_3 treatment of hypothyroid mice again reduced Δ6-desaturase activities to similar levels in lean and obese mice.

3.9 Fatty Acid Compositions of Hepatic Mitochondrial Phospholipids

Fatty acyl compositional changes in obese mouse liver plasma membranes and hepatic microsomes have previously been investigated (French et al., 1983; Rouer et al., 1980; Hyslop et al., 1982).

	Food Intake (grms.)	Body weight(grms.) Before	After	% Increase in body weight
Lean control	4.22 ± 0.10	23.1 ± 0.9	24.2 ± 1.0	5.2 ± 2.4
	$p<0.001$			
Lean T_3	5.33 ± 0.14	22.6 ± 0.4	24.5 ± 0.9	8.3 ± 1.1
Obese control	6.92 ± 0.17 ***	36.0 ± 1.1	41.5 ± 2.0	16.6 ± 2.4 ***
				$p<0.005$
Obese T_3	6.67 ± 0.18 ***	39.0 ± 2.3	41.7 ± 2.8	7.3 ± 1.3
	(n=8)	(n=5)	(n=5)	(n=5)

Table 3.5 The Effect of T_3 Injection on Food Intake and Weight Gain of Lean and Obese Mice

Lean and obese mice were injected with T_3 subcutaneously, twice a day, at a dose of 30 µg/kg. body wt./day for 14 days. Mice were housed in pairs and food intake was estimated each day. The values represent the means ± SEM for the number of animals (n) shown in parentheses. * $p<0.05$; ** $p<0.005$; *** $p<0.001$; compared to the lean control group.

Fig 3.6 The Effect of Thyroid Status on Hepatic Mitochondrial Δ5- and Δ6-desaturase Activity of Lean and Obese Mice

6 week old mice were injected subcutaneously with either saline vehicle (0.9% w/v) or T_3 (30 μg/kg) twice daily for 14 days. A hypothyroid state was induced by provision of a low iodine diet and perchlorate drinking solution (0.5% w/v) for 14 days, saline vehicle or T_3 being injected subcutaneously on days 8-14. Hepatic mitochondria were prepared as described in section 2.3 and Δ5- and Δ6-desaturase activity assayed as described in section 2.6. Values represent mean ± SEM for 5 mice in each group. * $p<0.05$; ** $p<0.01$; *** $p<0.005$ compared to lean mice.

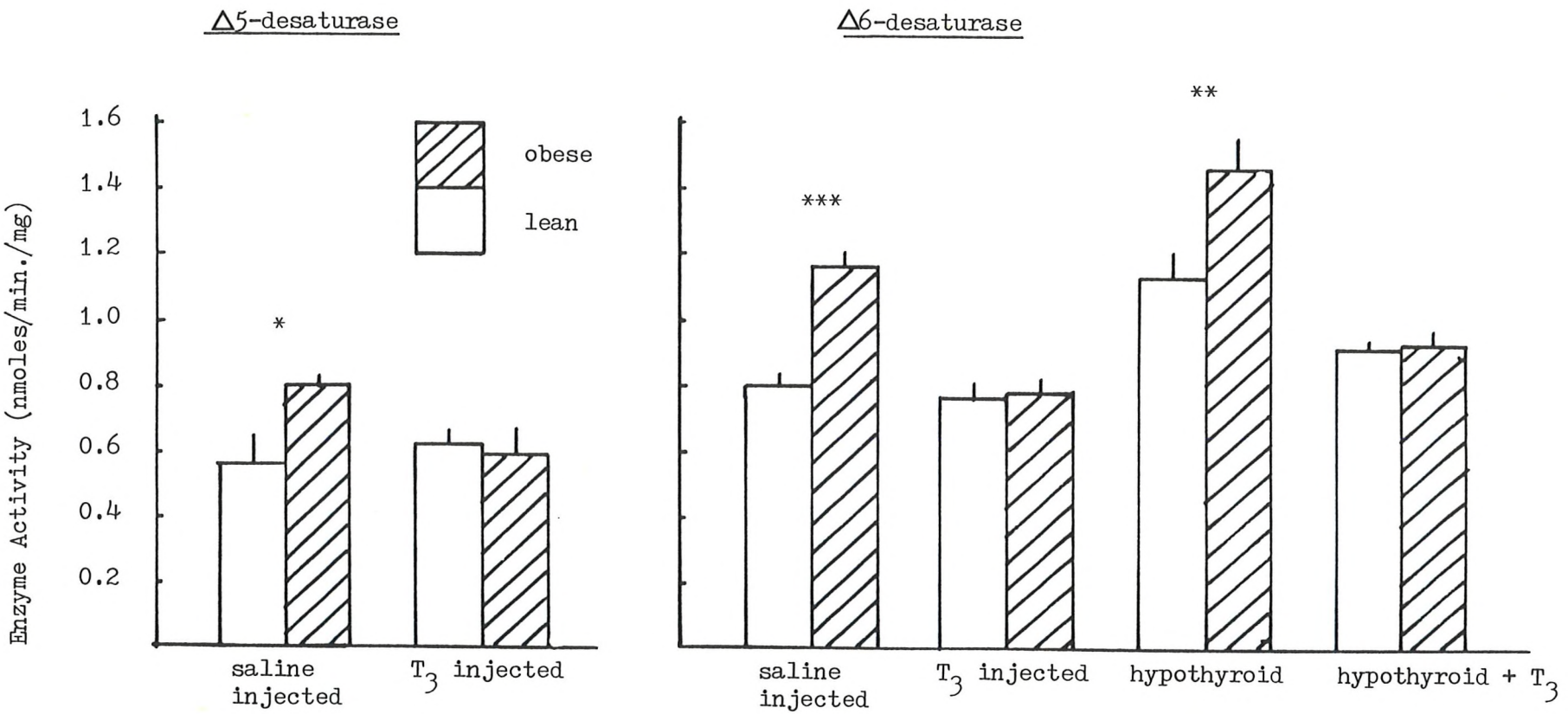


Fig 3.6 The Effect of Thyroid Status on Hepatic Mitochondrial Δ5- and Δ6-desaturase Activity of Lean and Obese Mice

In view of the increased desaturase activity in predominately hepatic mitochondrial fractions, it was of interest to determine fatty acid composition of phospholipids extracted from lean and obese mouse liver mitochondria. The fatty acid profiles of hepatic mitochondrial phospholipids are shown in figure 3.7. Alterations in the fatty acyl compositions were apparent in all of the phospholipid subclasses extracted from hepatic mitochondria of the obese mouse. The changes followed a similar pattern in each phospholipid, namely a reduction in the proportion of 18:2 ω6 which was compensated for by an increased 18:1 ω9 and usually by an increased PUFA component (either 22:6 ω3 or 20:4 ω6). In phosphatidylcholine, the level of 18:2 ω6 was reduced from 16.2% to 11.0% of the total, whereas 18:1 ω9 had increased from 13.5% to 17.5% of the total and 22:6 ω3 from 10.1% to 13.9% of the total. In cardiolipin, 18:2 ω6 had decreased (although not significantly), the proportion of 18:1 ω9 was elevated from 16.3% to 23.2% of the total and the minor component 20:4 ω6 was increased from 3% to 6.3% of the total. Finally in phosphatidylethanolamine, a similar pattern of compositional changes was observed. However, only the reduction in the 18:2 ω6 component from 6.5% to 3.9% of the total was statistically significant when the data was analysed on a paired basis. The reduction in 18:2 ω6 content and increased 18:1 ω9 content of hepatic mitochondrial phospholipids of the obese mouse becomes clearer by presenting the data as a ratio of the 18:1 ω9 content to that of the 18:2 ω6 content. Thus the 18:1 ω9/18:2 ω6 ratio was increased in each of the phospholipid subclasses of the obese mouse (table 3.6).

As a result of the compositional changes, there was a small but not significant increase in the unsaturation indices of all the phospholipid subclasses of the obese mouse hepatic mitochondria (table 3.6). The contribution of the three phospholipid subclasses to the total phospholipid content was the same in hepatic mitochondria from lean and obese animals (table 3.7).

3.10 Fatty Acid Compositions of Hepatic Mitochondrial Phospholipids of Thyroid Hormone Treated Mice

As chronic T_3 treatment normalised hepatic Δ6- and Δ5-desaturase

Figure 3.7 The Fatty Acid Profiles of Hepatic Mitochondrial Phospholipids of Lean and Obese Mice

Hepatic mitochondria were prepared as described in section 2.3 and phospholipids extracted, separated and analysed by GLC as described in section 2.13. The data represents the means ± SEM of four separate hepatic mitochondrial preparations from lean and obese mice.
* $p<0.05$; ** $p<0.01$; *** $p<0.005$; NS not significant (students 't' test for paired data).

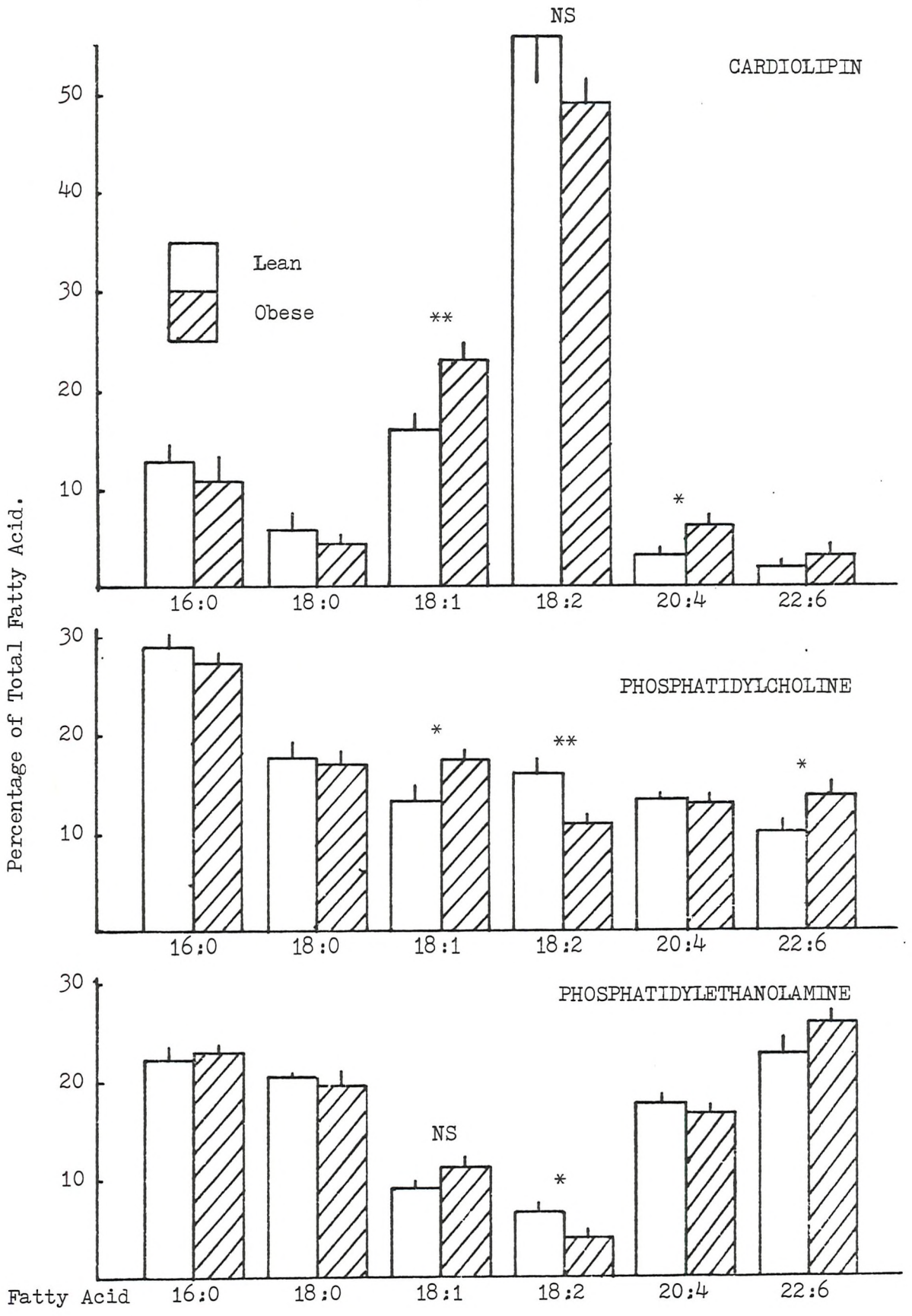


Fig 3.7 The Fatty Acid Profiles of Hepatic Mitochondrial Phospholipids of Lean and Obese Mice

activity in the obese mouse, it was of interest to determine the effect of a similar treatment on mitochondrial membrane lipid composition. To help clarify any changes that might occur, a second group of mice were treated with thyroid hormones for a prolonged period of 5 weeks. Figure 3.8 and figure 3.9 show the fatty acid profiles of hepatic mitochondrial phospholipids prepared from lean and obese mice treated with thyroid hormones for 2 and 5 weeks respectively. The data from control animals in these experiments are included in figure 3.7. Thyroid hormone treatment normalised some of the compositional changes in the hepatic mitochondria of the obese mouse whereas others persisted even after 5 weeks of treatment. In all of the phospholipids subclasses, the elevated levels of 18:1 ω9 in the obese animals were normalised after 2 weeks of thyroid hormone treatment. The PUFA component, 22:6 ω3 in phosphatidylcholine was also normalised after 2 weeks whereas 20:4 ω6 in cardiolipin remained elevated in the obese mouse mitochondria even after 5 weeks of thyroid hormone treatment (3.2% and 5.2% of total for hyperthyroid lean and obese mice respectively). The reduced levels of 18:2 ω6 content in phosphatidylcholine and phosphatidylethanolamine from obese mouse hepatic mitochondria persisted throughout the thyroid hormone treatment.

In addition, thyroid hormone treatment changed the levels of some fatty acyl components of hepatic mitochondrial phospholipids of both lean and obese animals. In phosphatidylcholine, 18:2 ω6 content decreased with duration of thyroid hormone treatment, from 16.2% in lean control animals, to 14.1% after 2 weeks and to 11.8% of total after 5 weeks, by which time 18:2 ω6 had fallen to 7.8% of the total in the corresponding obese mouse phospholipid. Similarly in phosphatidylethanolamine, 18:2 ω6 content decreased with duration of the thyroid hormone treatment. In control hepatic mitochondria of lean mice, the percentage contribution of 18:2 ω6 was 6.5% and had fallen to 3.8% of the total after 5 weeks of thyroid hormone treatment by which time the 18:2 ω6 component was 2.4% of the total in the corresponding obese mouse phospholipid.

While the 18:2 ω6 content of hepatic mitochondrial phospholipids decreased, so the 20:4 ω6 content increased with duration of thyroid hormone treatment. In phosphatidylcholine from euthyroid lean mice,

Fig 3.8 The Fatty Acid Profiles of Hepatic Mitochondrial Phospholipids of Lean and Obese Mice Treated with Thryoid Hormones for Two Weeks

Lean and obese mice were injected with T_3 subcutaneously twice a day at a dose of 30 µg/kg body wt./day for 14 days. Hepatic mitochondria were prepared as described in section 2.3 and phospholipids extracted, separated and analysed by GLC as described in section 2.13. The data represents the mean ± SEM of three separate hepatic mitochondrial preparations.
* $p < 0.05$; NS not significant (students 't' test for paired data.

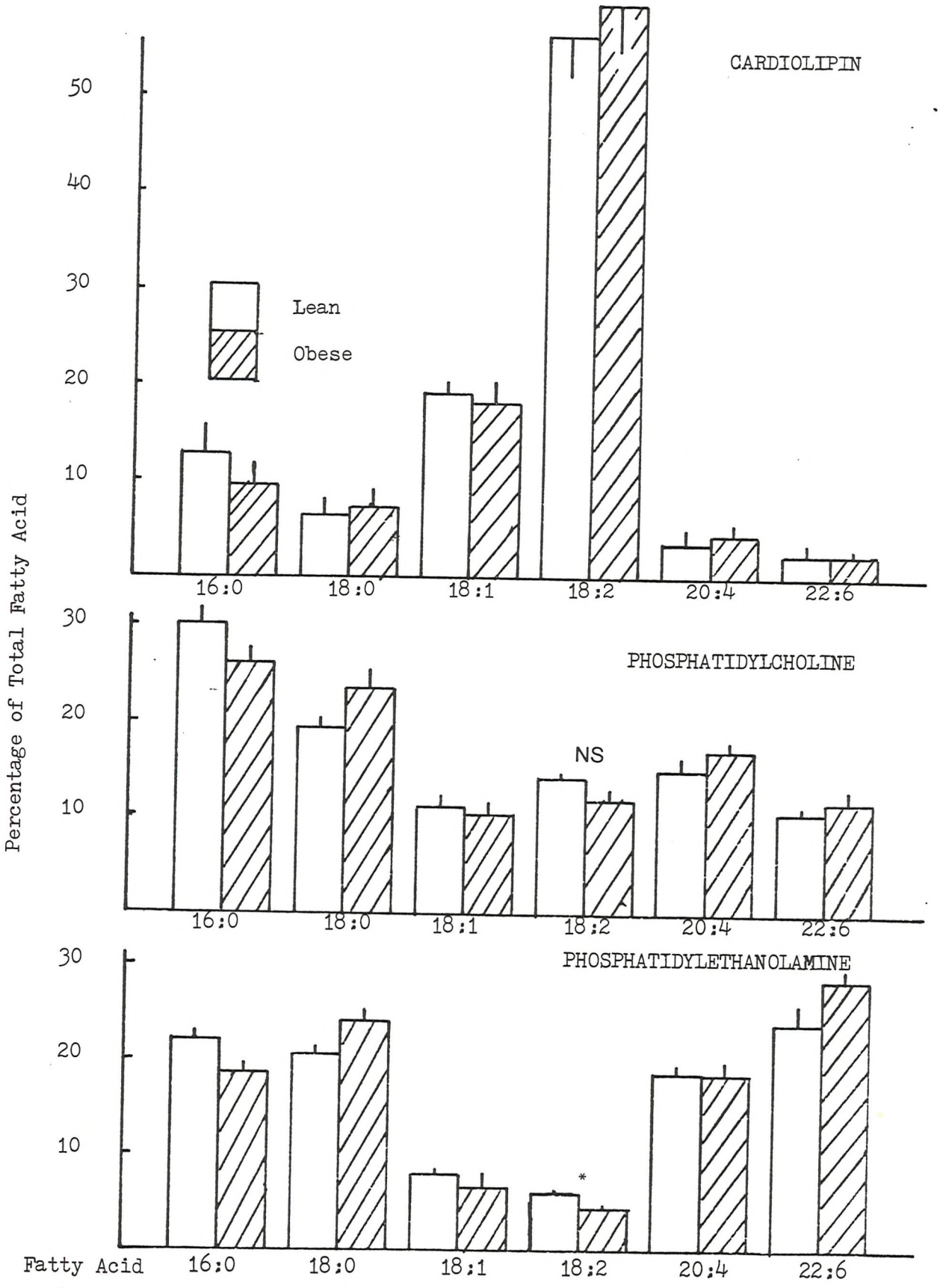


Fig 3.8 The Fatty Acid Profiles of Hepatic Mitochondrial Phospholipids of Lean and Obese Mice Treated With Thyroid Hormones for Two Weeks

Fig 3.9 The Fatty Acid Profiles of Hepatic Mitochondrial Phospholipids of Lean and Obese Mice Treated with Thyroid Hormones for Five Weeks

Lean and obese mice were fed ad libitum a diet containing 0.02%(w/w) thyroid powder for 5 weeks. Hepatic mitochondria were prepared as described in section 2.3 and phospholipids extracted, separated and analysed by GLC as described in section 2.13. The data represents the means from two separate mitochondrial preparations.

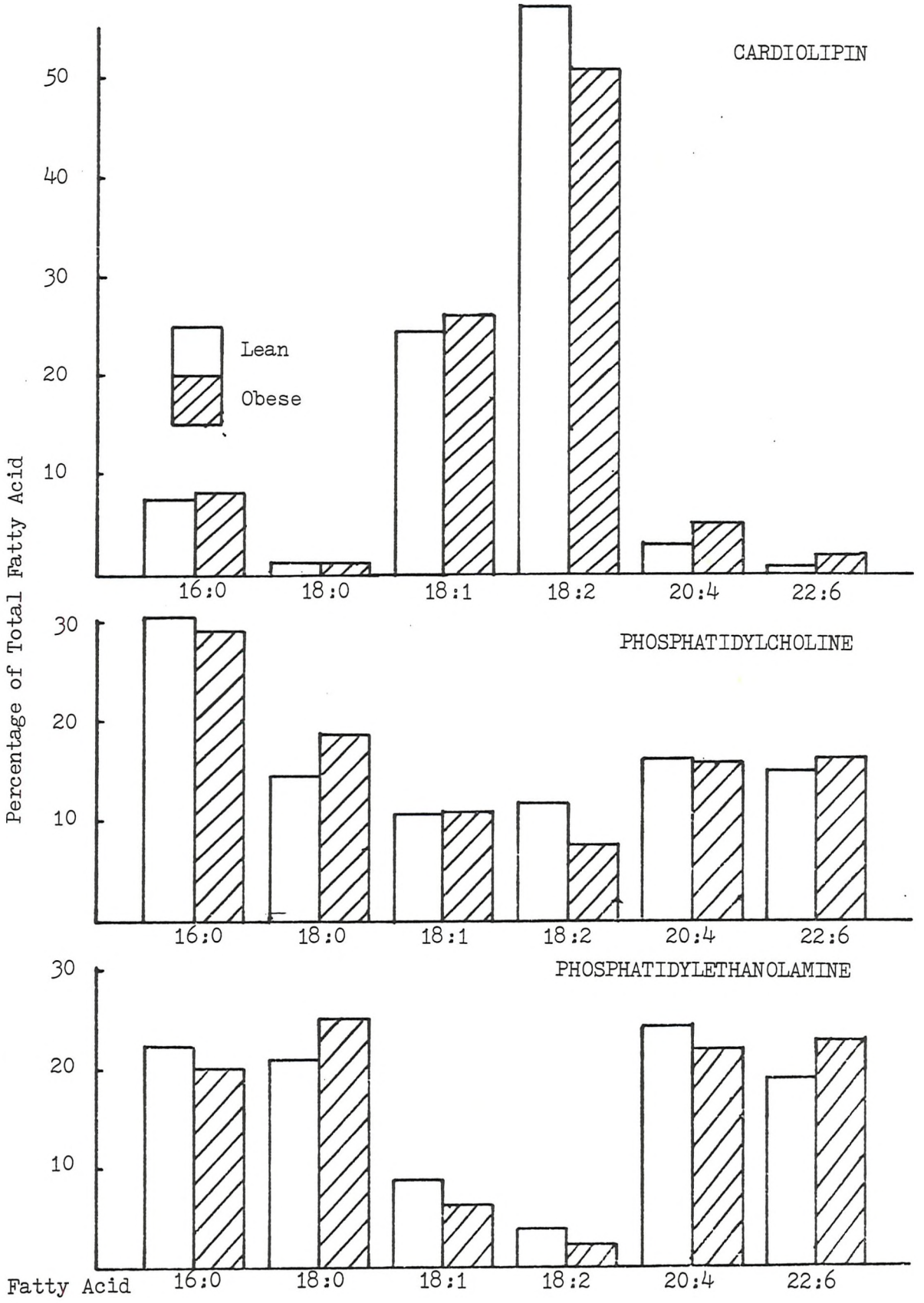


Fig 3.9 The Fatty Acid Profiles of Hepatic Mitochondrial Phospholipids of Lean and Obese Mice Treated with Thyroid Hormones for Five Weeks

20:4 ω6 content was 13.5% of the total and had increased to 16.5% of the total after 5 weeks of thyroid hormone treatment. In phosphatidylethanolamine, the increase in 20:4 ω6 content was more pronounced from 17.5% (in control) to 24.3% of the total after 5 weeks of thyroid hormone treatment.

The proportion of 18:0 was also increased in hepatic mitochondrial phospholipids after thyroid hormone treatment. This was more pronounced in the phospholipids of obese mice. For example, the percentage contribution of 18:0 in phosphatidylethanolamine from euthyroid lean and obese mice was 19.0% and 19.3% and had increased to 21.3% and 25.4% after 5 weeks of thyroid hormone treatment in lean and obese mice respectively.

As a consequence of these compositional changes, thyroid hormone treatment altered the 18:1 ω9/18:2 ω6 ratios of hepatic mitochondrial phospholipids of lean and obese mice in the following ways. After 2 weeks of thyroid hormone treatment, the elevated 18:1 ω9/18:2 ω6 ratios of obese mouse phosphatidylcholine and cardiolipin were normalised to lean control levels and that of obese mouse phosphatidylethanolamine significantly reduced. These changes were brought about mainly by the normalisation of oleic acid content of obese mouse phospholipids after 2 weeks T_3 treatment. The 18:1 ω9/18:2 ω6 ratios of lean mouse phospholipids were unchanged after 2 weeks T_3 treatment. After 5 weeks of thyroid hormone treatment, the 18:1 ω9/18:2 ω6 ratios of cardiolipin remained similar in lean and obese mice, whereas those of phosphatidyl-ethanolamine had increased in both lean and obese animals. In phosphatidylcholine, only the obese mouse 18:1 ω9/18:2 ω6 ratio had increased after 5 weeks thyroid hormone treatment (table 3.6).

Thyroid hormone treatment had no effect on the double bond numbers of the phospholipids, except in the case of phosphatidylcholine. Here, the unsaturation index had increased to 190 after 5 weeks thyroid hormone treatment in both lean and obese mice.

Mice/treatment		(n)	Phosphatidylcholine		Phosphatidylethanolamine		Cardiolipin	
			Lean	Obese	Lean	Obese	Lean	Obese
control	Unsat.Index	(4)	161 ± 9	176 ± 9	230 ± 12	241 ± 4	153 ± 14	169 ± 6
	18:1/18:2 ratio		0.88 ± 0.17	1.63 ± 0.20**	1.25 ± 0.05	2.52 ± 0.30***	0.30 ± 0.04	0.53 ± 0.06**
T_3,2 wks.	Unsat.Index	(3)	160 ± 8	171 ± 6	236 ± 10	256 ± 5	160 ± 8	167 ± 6
	18:1/18:2 ratio		0.83 ± 0.37	0.89 ± 0.05	1.33 ± 0.05	1.60 ± 0.1*	0.39 ± 0.1	0.33 ± 0.09
T_3,5 wks.	Unsat.Index	(2)	191	190	228	240	166	172
	18:1/18:2 ratio		0.93	1.43	2.37	2.71	0.40	0.47

Table 3.6 18:1ω9/18:2ω6 Ratios and Unsaturation Indices of Hepatic Mitochondrial Phospholipids From Control and Thyroid Hormone Treated Lean and Obese Mice

Hepatic mitochondria were prepared as described in section 2.3 and phospholipids extracted, separated and analysed by GLC as described in section 2.13. The data summarises parameters of figures 3.7, 3.8 and 3.9 on control and thryoid hormone treated mice (for 2 and 5 weeks). Values represent means ± SEM for the number of preparations (n) shown in parentheses. * $p<0.05$; ** $p<0.01$; *** $p<0.005$ (students 't' test for paired data).

Phospholipid (nmoles/mg protein)	Lean (n=7)	Obese (n=3)
Phosphatidylcholine	23.0 ± 2.9	23.3 ± 4.0
Phosphatidylethanolamine	21.7 ± 2.8	19.5 ± 1.3
Cardiolipin	8.5 ± 1.8	6.3 ± 1.1
Total	53.2	49.1

Table 3.7 Phospholipid Content of Hepatic Mitochondria From Lean and Obese Mice

Hepatic mitochondria from lean and obese mice were prepared as described in section 2.3 and phospholipids extracted and separated as described in section 2.13. The phospholipids were measured as described in section 2.16. Values represent the means ± SEM for the number of observations (n) shown in parentheses.

3.11 EFA Desaturation in Lean and Obese Mice

In the present study, desaturation of essential fatty acids has been shown to be elevated in the livers of obese mice. A similar finding, concerning the saturated fatty acid desaturase (Δ9-desaturase) has been reported by Enser (1979). Preliminary studies in adipose tissue reveal that Δ6-desaturase activity was normal in adult obese animals, as was the activity in the brain. In contrast, Δ9-desaturase activity was also increased in adipose tissue of obese mice (Enser, 1979).

The finding that mouse hepatic Δ6-desaturase activity is located primarily in the mitochondria is unusual. In the rat, Δ6-desaturase activity is normally assigned to the microsomal fraction (Brenner, 1977). Experiments in which incubation conditions were varied, indicated that Δ6-desaturase activity was optimal in hepatic microsomes and mitochondria. Previous reports have suggested that the presence of some cytosolic factor may be required for maximum Δ6-desaturase activity (Cook, 1978; Jeffcoat, 1979). However, in the present study, addition of 100,000g supernatant protein to the assay media gave no further stimulation of activity in either microsomal or mitochondrial fractions. The primarily mitochondrial activity may reflect a species difference, although Strouve-Valet and Pascaud (1971) reported that Δ6-desaturase activity associated with the mitochondrial fraction was higher than that of the microsomes of developing rat brain. Indeed, the activity reported in the present study in mouse liver microsomes is higher than has been previously reported in rat liver microsomes (Cook, 1978; Faas and Carter, 1981; 1982).

Hepatic Δ6-desaturase activity is very responsive to food intake. Short term fasting for 17 hours doubled the hepatic mitochondrial enzyme activity in lean animals. In contrast to this observation, Brenner et al. (1968) reported that fasting for 48 hours depressed rat liver microsomal Δ6-desaturase activity by 50%. However, the duration of fasting my be important in this process. The initial response to food deprivation may be an increase in

Δ6-desaturase activity to maintain an adequate supply of PUFA, followed by a reduction in enzyme activity when available EFA substrate has been utilised.

Hepatic Δ6-desaturase activity was also responsive to a reduction in food intake. Meal time feeding reduced the food intake of lean and obese mice and depressed their hepatic mitochondrial Δ6-desaturase activity by a similar amount.

Hepatic Δ6-desaturase activity was normal in suckling pre-obese animals, although at 14 days of age pre-obese mice already exhibit several characteristics of the syndrome. These include increased body fat content, increased inguinal fat cell size, reduced oxygen consumption and defective NST (Boissonneault et al., 1978; Joosten and Van der Kroon, 1974a; Goodbody and Trayhurn, 1982). Hence the increase in hepatic Δ6-desaturase activity of obese mice was secondary to the metabolic changes associated with the obese state since elevated enzyme activity was not apparent before weaning.

In liver after weaning, Δ6-desaturase activity increases in response to the increased provision of dietary EFA substrate (Cook, 1978; Naughton, 1981). It is significant that at this time, the difference in Δ6-desaturase activity between lean and obese animals became apparent. The characteristic hyperphagia of the obese mouse develops after weaning (Lin et al., 1977; Rath and Thenen, 1979), hence it was possible that increased food intake was responsible for the elevated Δ6-desaturase activity. Restricting food intake by meal time feeding obese mice abolished their hyperphagia and prevented the excess weight gain in obese animals in agreement with previous studies (Dubuc, 1976a; Jagot et al., 1982). However, it did not normalise hepatic mitochondrial Δ6-desaturase activity, despite the similar food intake of lean and obese animals. A similar finding was reported by Enser (1979) who measured elevated hepatic Δ9-desaturase activity in obese mice. Food restriction was only partially successful in normalising this enzyme activity.

Insulin has been shown to be an important regulator of hepatic microsomal Δ6-desaturase in the rat. Alloxan diabetic rats had reduced Δ6-desaturase activity which was normalised by insulin

injection (Brenner et al., 1968). In a study where streptozoticin diabetic rats were injected with high doses of insulin, hepatic microsomal Δ6-desaturase activity increased, to levels 2 fold greater than in control (non-diabetic) animals (Eck et al., 1979). Obese mice are hyperinsulinemic (Bray and York, 1979). In addition, it has been shown that meal time feeding or restricting the food intake of obese mice reduces but does not normalise their elevated serum insulin concentrations (Dubuc, 1976a; Enser, 1979; Jagot et al., 1982). Hence it was possible that the elevated Δ6-desaturase activity observed in control and food restricted obese mice was caused by their hyperinsulinemia. However, chronic treatment of lean mice with insulin did not increase hepatic mitochondrial Δ6-desaturase activity to the levels observed in obese mice. In addition to this, short term food deprivation increased hepatic mitochondrial Δ6-desaturase activity to similar levels in lean and obese mice although fasting for 16 hours has been shown to markedly reduce serum insulin concentrations in lean and obese animals (Cuendet et al., 1975). Hence it seems unlikely that the increased hepatic Δ6-desaturase activity of obese mice in caused by their hyperinsulinemia or that insulin regulates mitochondrial Δ6-desaturase activity.

Enser (1979) concluded that hyperinsulinemia was not responsible for the elevated hepatic Δ9-desaturase activity of obese mice. He reported that the induction of a diabetic state which reduced serum insulin concentrations, did not normalise the elevated Δ9-desaturase activity. He suggested the increase in Δ9-desaturase activity was probably caused by the increase in provision of stearate and palmitate substrates from dietary sources and/or from increased lipogenic activity (Enser and Roberts, 1982). In the case of EFA desaturation, increased provision of substrate from dietary sources has already been discounted as causing the elevated Δ6-desaturase activity. In addition, EFA substrates cannot be synthesised by lipogenic enzymes in situ unlike stearic or palmitic acid.

Obese mice exhibit many characteristics indicative of hypothyroidism (Joosten and Van der Kroon, 1974b;Thenen and Carr, 1980) although serum T_3 concentrations are normal in the adult animal(York et al., 1978a).

In the present study, T_3 treatment normalised the elevated hepatic mitochondrial Δ6- and Δ5-desaturase activity of obese mice without affecting their hyperphagia. This provides further evidence that the changes in desaturase activity in the obese mouse are unrelated to dietary intake. The observation that cold exposure normalised hepatic mitochondrial Δ6-desaturase activity in the obese mouse is consistent with the physiological regulation of the enzyme by thyroid hormones. The increase in hepatic Δ6-desaturase given on starvation, a status associated with reduced serum T_3 concentrations (Donati et al., 1963; Ingbar and Galton, 1975) is also consistent with thyroid hormone regulation of mitochondrial desaturases. However, T_3 injection did not alter hepatic mitochondrial Δ6-desaturase activity in lean animals, whereas in previous studies thyroid hormone treatment has been shown to depress hepatic microsomal Δ6-desaturase activity in the rat (DeGomez-Dumm et al., 1977; Faas and Carter, 1981). This apparent discrepancy may be resolved by considering the opposing effects of thyroid hormones and the increased food intake of hyperthyroid lean mice on the activity of the hepatic mitochondrial Δ6-desaturase. Thyroid hormone treatment may tend to depress the enzyme activity whereas the increase in food intake may counter this effect. Thus the Δ6-desaturase activity was unchanged in T_3 treated lean mice in the present study.

The findings discussed above indicate that the increased hepatic mitochondrial Δ6-desaturase activity of obese mice may result from their hypothyroid state. However, when a hypothyroid state was induced, hepatic mitochondrial Δ6-desaturase activity increased in both lean and obese mice by a similar amount so that the enzyme activity in the obese animals remained elevated above that of the lean animals. In addition, suckling pre-obese mice exhibit many characteristics of hypothyroidism (Van der Kroon et al., 1982) and serum T_3 concentrations are depressed in animals up to 20 days old (Mobley and Dubuc, 1979) yet hepatic Δ6-desaturase activity was normal in pre-obese animals at this age. These observations suggest that factors other than thyroid status may be primarily responsible for the increased Δ6-desaturase activity observed in obese mice.

The mechanism by which thyroid hormones control desaturase enzyme activity is unknown (DeGomez-Dumm et al., 1977). It is not thought

to involve changes in intracellular cAMP concentrations, unlike noradrenalin and glucagon mediated effects on Δ6-desaturase activity (DeGomez-Dumm et al., 1975; 1976). DeGomez-Dumm et al. (1977) injected rats chronically with T_4 and reported no alteration to hepatic cAMP levels despite showing a significant reduction in microsomal Δ6-desaturase activity. Many changes in enzyme activity and metabolism mediated by thyroid hormones are thought to require protein synthesis in order to take place (Bernal and Refetoff, 1977). Hence changes in desaturase activity may involve synthesis (or breakdown) of new desaturase proteins.

It is unlikely that other endocrine hormones, such as increased serum glucagon cncentrations (Flatt et al., 1982) would increase hepatic Δ6-desaturase activity in the obese mouse. DeGomez-Dumm et al. (1975) have shown that glucagon injection depresses rat liver microsomal Δ6-desaturase activity. However, reduced sympathetic activity in the obese mouse (Young and Landsberg, 1983) is consistent with elevated Δ6-desaturase activity since catecholamine injection has been shown to depress rat microsomal Δ6-desaturase activity (DeGomez-Dumm et al., 1976). However, the relationship between the reduced sympathetic activity and the altered membrane lipid composition of the obese mouse is unclear since Emilsson and Gudbjarnason (1983) have reported that rats treated with noradrenaline had reduced 18:2 ω6 content and increased PUFA content in heart miocardium phospholipids i.e changes in fatty acyl composition which parallel those observed in the obese mouse. (see section 3.12).

Another possible factor not investigated in this study involves the effect of EFA substrate concentration on hepatic desaturase activity. The livers of obese mice contain more than three times as much storage fat than their lean counterparts (Winand et al., 1973; Enser and Roberts, 1982). This would dilute out EFA substrates and may lead to a situation of apparent EFA deficiency in obese mice, despite their hyperphagia. This could induce Δ6-desaturase activity (Peluffo et al., 1976).

3.12 Fatty Acyl Composition of Hepatic Mitochondrial Phospholipids of Lean and Obese Mice.

The fatty acid profiles of hepatic mitochondrial phospholipids are

altered in the obese mouse following a similar pattern to the changes previously reported in phospholipid acyl compositions of plasma and microsomal membranes (Rouer et al., 1980; Hyslop et al., 1982; French et al., 1983). A similar pattern of compositional changes was observed in all three mitochondrial phospholipids in the obese mouse, namely a reduction in the proportion of 18:2 ω6 which was compensated for mainly by an increase in 18:1 ω9 and by a small increase in a PUFA component. The compositional changes were less pronounced in phosphatidylethanolamine than in phosphatidylcholine or cardiolipin.

Some of the compositional changes reported in the present study are consistent with alterations reported previously. Rouer et al. (1980) analysed total phospholipid extracted from hepatic microsomes and found that a decrease in the 18:2 ω6 content was compensated for by an increase in 18:1 ω9 and a small increase in 20:4 ω6. Hyslop et al. (1982) separated the phospholipid subclasses of hepatic microsomes and also found a small decrease in 18:2 ω6 content in phosphatidylcholine, whereas in phosphatidylethanolamine the 16:0 component was greatly reduced. In liver plasma membranes, reduction in the 16:0 and 18:2 ω6 components were paralleled by an increase in 18:1 ω9 content in phosphatidylcholine. In phosphatidylethanolamine 18:2 ω6 content was only slightly depressed. Phosphatidylserine from obese mouse plasma membrane contained many compositional changes including decreased 18:0 and increased 18:1 ω9 and 22:6 ω3 components (French et al., 1983). Finally, York et al. (1982) analysed the fatty acyl compositions of purified phospholipids from adipocyte plasma membranes of lean and obese mice. In phosphatidylcholine, the 18:2 ω6 content was reduced whereas in phosphatidylethanolamine both the 18:2 ω6 and 18:1 ω9 components were reduced. Clearly, the large increases in 22:6 ω3 content observed in phosphatidylethanolamine extracted from hepatic microsomes and adipocyte plasma membranes (Hyslop et al., 1982; York et al., 1982) are not apparent in any of the phospholipids extracted from hepatic mitochondrial membranes. In conclusion, the consistent reduction in 18:2 ω6 content of membrane phospholipids extracted from various organelles in different tissues of the obese mouse is often paralleled by an increase in 18:1 ω9 content. Thus the 18:1 ω9/18:2 ω6 ratio is increased, consistently in membrane phospholipids of the

obese mouse as has been observed in the present study.

The changes in fatty acyl components of hepatic mitochondrial phospholipids can be considered in relation to the altered desaturase activities of the obese mouse. This assumes that the desaturase activities control the fatty acid composition of the fatty acid pool available for incorporation into phospholipids.

The reduction in 18:2 ω6 content of phospholipids is consistent with the increased Δ6-desaturase activity. Elevated hepatic Δ9-desaturation, reported by Enser (1979) may be responsible for the increased 18:1 ω9 content in mitochondrial phospholipids. In cardiolipin, the increase in the 20:4 ω6 component may be caused by the elevated Δ5-desaturase activity and the increase in 22:6 ω3 content of phosphatidylcholine is consistent with an increase in desaturase enzyme activity in general. However, not all of the membrane PUFA's are altered in hepatic mitochondria of the obese mouse. For example, the 20:4 ω6 components of phosphatidyl-ethanolamine or phosphatidylcholine are normal. Some fatty acyl components are maintained at normal levels in the obese mouse wherease others reflect the changes expected by increased hepatic desaturase activity. The data suggests that during the process of fatty acid incorporation into phospholipids in the obese mouse, normal selection of some of EFA's and PUFA's takes place. This occurs despite the abnormal fatty acyl composition of storage triglyceride which is produced by increased lipogenic and desaturase activity (Enser and Roberts, 1982).

The involvement of desaturase enzymes in determining phospholipid fatty acyl composition may be clarified by considering the effect of thyroid hormone treatment on membrane lipid composition. Thyroid hormone treatment normalised hepatic mitochondrial Δ6- and Δ5-desaturase activity in the obese mouse. Obese animals that were injected with T_3 for 2 weeks had normal levels of 18:1 ω9 in their hepatic mitochondrial phospholipids. However, the reduced levels of 18:2 ω6 in phosphatidylcholine and phosphatidylethanolamine were still apparent after 5 weeks thyroid hormone treatment. Hence the normalisation of fatty acyl composition predicted from the changes in Δ6-desaturase activity in the obese mouse, did not occur.

However, the fatty acid composition of the storage triglyceride was not examined in thyroid hormone treated obese mice. Hence it is possible that T_3 injection failed to normalise the 18:2 ω6 content of the depot lipid during the limited experimental period. This might then account for the failure of thyroid hormone treatment to normalise membrane lipid composition in the obese mouse.

In lean and obese mice, thyroid hormone treatment reduced the 18:2 ω6 content and increased the 20:4 ω6 content of hepatic mitochondrial phospholipids in agreement with previous studies (Patton and Platner, 1971). Faas and Carter (1981) analysed total hepatic microsomal phospholipid from T_3 treated rats and also reported reduced 18:2 ω6 content and increased 20:4 ω6 content despite showing a reduction in Δ6-desaturase activity in hyperthyroid animals. Membrane lipid composition changed in the opposite direction to that predicted from observations of desaturase activity. Hence, these authors concluded that most of the membrane compositional changes in hyperthyroid animals were unrelated to changes in fatty acid desaturation.

In contrast to the present study and those outlined above, French et al. (1983) found that thyroid hormone treatment increased 18:1 ω9 and 18:2 ω6 content and reduced PUFA content in phosphatidylcholine extracted from liver plasma membranes of lean and obese mice. No changes were apparent in phosphatidylethanolamine, whereas in phosphatidylserine, thyroid hormone treatment increased 20:4 ω6 content in both lean and obese animals. Hence, thyroid hormone treatment may increase or decrease fatty acyl components of membrane phospholipids, depending on their subcellular location.

The present study indicates that changes in desaturase activity cannot be used to predict alterations in membrane lipid composition. Desaturase activity may determine the level of some membrane lipid components by regulating the concentrations of available EFA's and PUFA's. However, other mechanisms, for example lysophospholipid acyltransferases operating at the subcellular location of the phospholipids, may ultimately control the membrane fatty acid composition.

CHAPTER 4

LYSOPHOSPHATIDYLCHOLINE ACYLTRANSFERASE IN HEPATIC MICROSOMES OF LEAN AND OBESE MICE

4.1 Introduction

In the previous chapter, the regulation of EFA desaturation was examined as a possible mechanism for controlling membrane lipid composition in lean and obese mice. Desaturase enzymes may control membrane lipid composition by regulating the composition of fatty acids available for incorporation into phospholipids. Thus the spectrum of fatty acids in the hepatic storage lipid of obese mice was severely altered (Winand et al., 1973; Enser and Roberts, 1982) and the activities of the hepatic Δ9-desaturase (Enser, 1979), Δ6- and Δ5-desaturase were elevated in the livers of obese animals. It was concluded that the changes in desaturase activity could be correlated with some of the changes in membrane lipid composition in the obese mouse.

In this chapter, studies on hepatic lysophospholipid acyltransferases activity in lean and obese mice are reported. Lysophospholipid acyltransferases are responsible for modifying the fatty acyl composition of membrane phospholipids, after their de novo synthesis on the endoplasmic reticulum. Previous studies have mainly investigated the lysophosphatidylcholine acyltransferase activity (lysoPC acyltransferase) of rat liver microsomes (Hill and Lands, 1968; Holub et al., 1979; Lands et al., 1982). In view of the alteration to membrane lipid composition in hepatic microsomes of obese mice (Rouer et al., 1980; Hyslop et al., 1982), hepatic microsomal lysoPC acyltransferase activity in lean and obese animals has been studied. The most consistent membrane compositional changes in the obese mouse were a reduction in linoleic acid (18:2 ω6) content, usually paralleled by an increase in oleic acid content (18:1 ω9) (see chapter 3). Changes in hepatic storage triglyceride composition in the obese mouse primarily involved these two fatty acids (Winand et al., 1973; Enser and Roberts, 1982). Hence hepatic microsomal lysoPC acyltransferase activity in lean and obese animals has been studied using these two fatty acids as substrates.

Results

4.2 Fatty Acid Composition of Hepatic Storage Triglyceride of Lean and Obese Mice

Fig 4.1 The Fatty Acid Composition of Hepatic Storage Triglyceride of Lean and Obese Mice

Triglyceride was extracted from the supernatant fat cake of 100,000 g liver homogenate pellets from lean and obese mice using 6 ml chloroform. The triglyceride extracts were transmethylated and analysed by GLC as described in section 2.13.

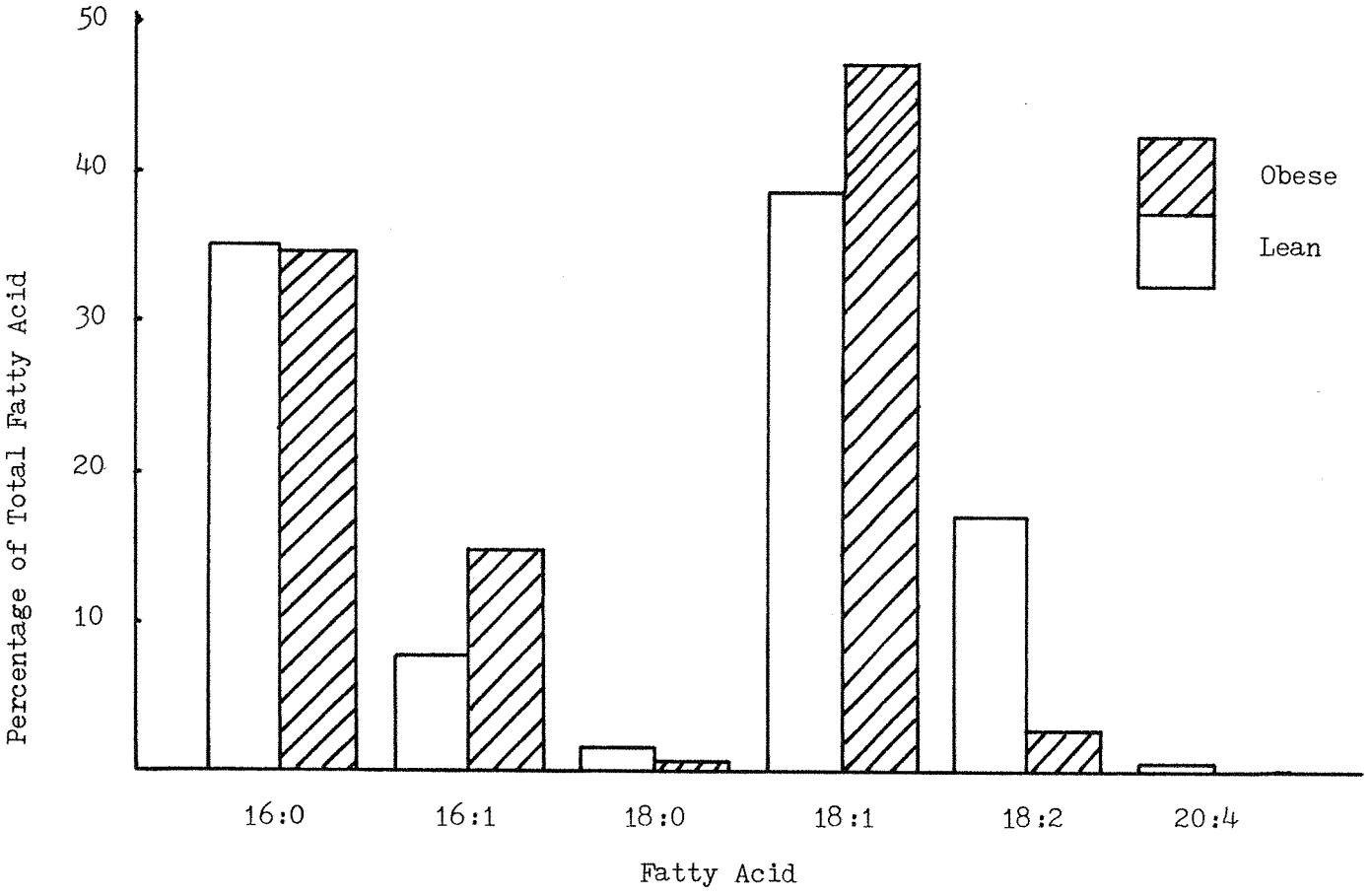


Fig 4.1 The Fatty Acid Composition of Hepatic Storage Triglyceride of Lean and Obese Mice

The fatty acid compositions of hepatic storage triglyceride of lean and obese mice are shown in figure 4.1. In agreement with previous studies (Winand et al., 1973; Enser and Roberts, 1982), increases in monenic fatty acid content and a reduction in linoleic acid content were apparent in the hepatic storage triglyceride of obese mice. The contribution of 16:1 ω9 had increased from 7.6% to 14.9% of the total whereas 18:1 ω9 content had increased from 38.5% to 47.1% of the total. Conversely, linoleic acid content was reduced from 16.9% to 2.7% of the total in hepatic storage triglyceride of the obese mouse. As a result of these compositional changes, the 18:1 ω9/18:2 ω6 ratio of hepatic storage triglyceride had increased from 2.3 in lean mice to 17.4 in obese animals.

4.3 Fatty Acid Composition of Hepatic Microsomal Phospholipids of Lean and Obese Mice

The fatty acid composition of hepatic microsomal phospholipids of lean and obese mice have previously been investigated (Rouer et al., 1980; Hyslop et al., 1982). In the present study, fatty acid analysis of the two main microsomal phospholipids (phosphatidylcholine and phosphatidylethanolamine) has been carried out on duplicate hepatic microsomal preparations of lean and obese mice. The data is presented in figure 4.2. In agreement with the previous report (Rouer et al., 1980) changes in the fatty acid composition of both microsomal phospholipids of obese animals involved an increase in the oleic acid content and a reduction in the linoleic acid content. In phosphatidylcholine, oleic acid content was increased from 10.3% to 13.7% of the total whereas the level of the linoleic acid component was reduced from 14.2% to 10.5% of the total. Similarly in phosphatidylethanolamine, oleic acid content was increased from 8.0% to 10.1% of the total, whereas linoleic acid content was reduced from 7.0% to 5.0% of the total. None of the other small compositional changes in obese mouse phospholipids were consistent in the two microsomal preparations analysed except the palmitic acid (16:0) content of phosphatidylethanolamine. Here, the palmitic acid content was reduced from 26.2% to 21.8% of the total in the obese mouse. As a result of the compositional changes, the ratio of the 18:1 ω9 content to 18:2 ω6 content in both microsomal phospholipids was increased in the obese animals

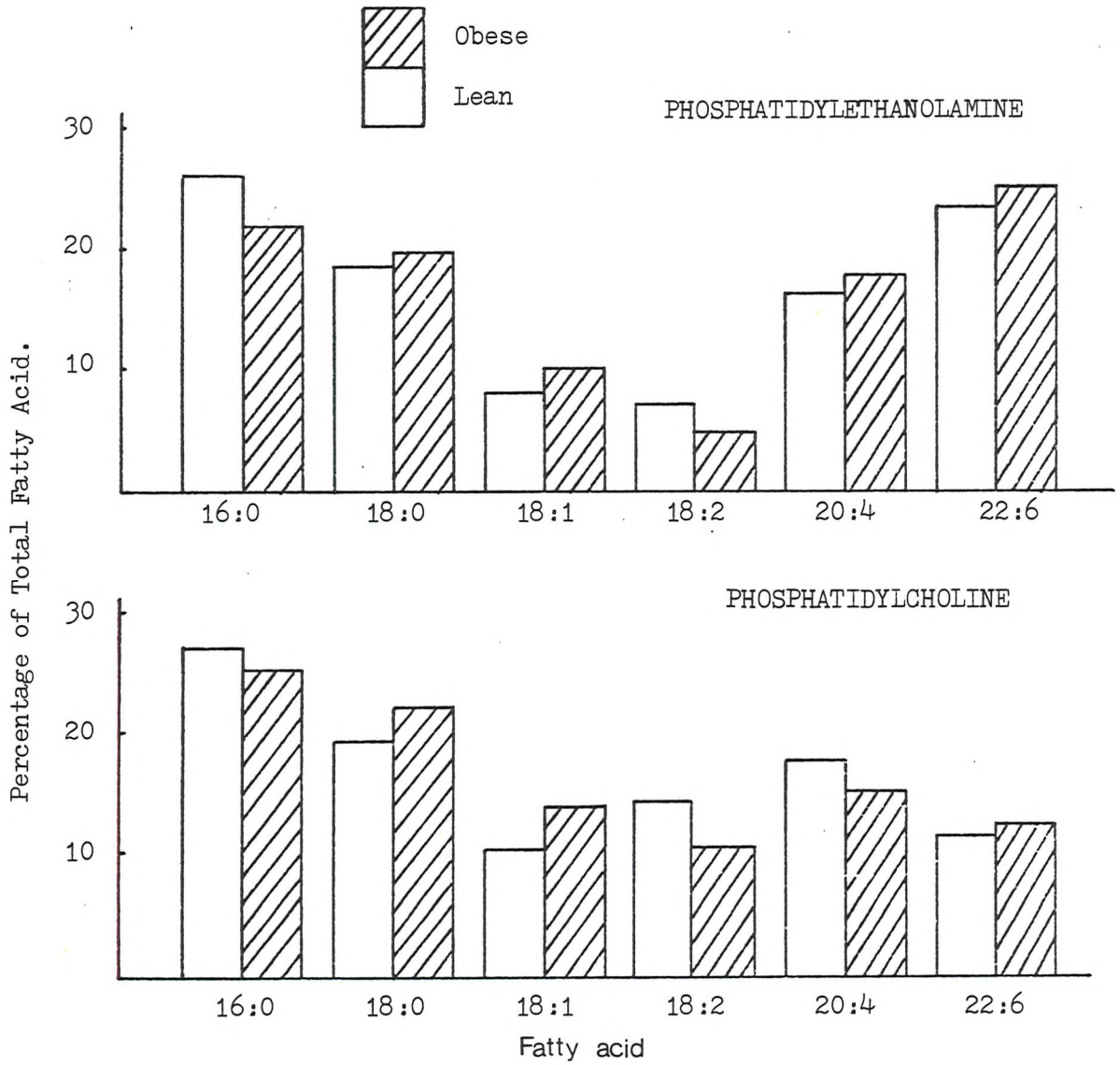


Fig 4.2 The Fatty Acid Profiles of Hepatic Microsomal Phospholipids of Lean and Obese Mice

Hepatic microsomal phospholipids from lean and obese mice were extracted, separated and analysed by GLC as described in section 2.13. The data represents the means of two separate preparations.

	Unsaturation Index		18:1 ω9/18:2 ω6 Ratio	
	Lean	Obese	Lean	Obese
Phosphatidylcholine	176	169	0.73	1.30
Phosphatidylethanolamine	230	243	1.15	2.02

Table 4.1 The Unsaturation Indices and 18:1 ω9/18:2 ω6 Ratios of Hepatic Microsomal Phospholipids of Lean and Obese Mice

Hepatic microsomal phospholipids from lean and obese mice were extracted, separated and analysed by GLC as described in section 2.13. The data represents the means of two separate preparations.

(table 4.1). However no change in the overall unsaturation of microsomal phsopholipids, as indicated by the double bond number, was apparent in the obese mice.

4.4 Characterisation of Hepatic Microsomal LysoPC Acyltransferase Activity

4.4.1 Time Course of Hepatic Microsomal LysoPC Acyltransferase Activity

The time course of incorporation of linoleic acid (18:2 ω6) into phosphatidylcholine by hepatic microsomal lysoPC acyltransferase of lean mice is shown in figure 4.3. Enzyme activity was linear for 6 minutes. Hence this time period was chosen as a suitable incubation time in subsequent experiments. From the data, the lysoPC acyltransferase activity of hepatic microsomes in lean animals was calculated. This was 0.70 nmoles/min./mg microsomal protein.

4.4.2 Effect of Protein Concentration on Hepatic Microsomal LysoPC Acyltransferase Activity

Figure 4.4 shows the effect of microsomal protein concentration on the assay of lysoPC acyltransferase activity in hepatic microsomes of lean mice, using linoleic acid (18:2 ω6) as the substrate and a 6 minute incubation period. Enzyme activity was linear with protein concentration between 200 and 400 μg protein (per 0.5 ml incubation volume). However, lysoPC acyltransferase activity was negligible at protein concentrations of 100 μg or below. The reason for the failure of hepatic microsomes to incorporate linoleic acid into phosphatidylcholine at low protein concentrations is unclear. In subsequent experiments, 250 to 300 μg of microsomal protein was used in the assay of lysoPC acyltransferase activity. Enzyme activity using 250 μg protein was calculated from the data shown in figure 4.4, and was 0.86 nmoles/min./mg protein. The incorporation of linoleic acid into phosphatidylcholine by lysoPC acyltransferase activity was prevented by boiling the microsomal protein prior to the assay, or by the omission of coenzyme A or lysophosphatidylcholine from the assay media. Under all of these

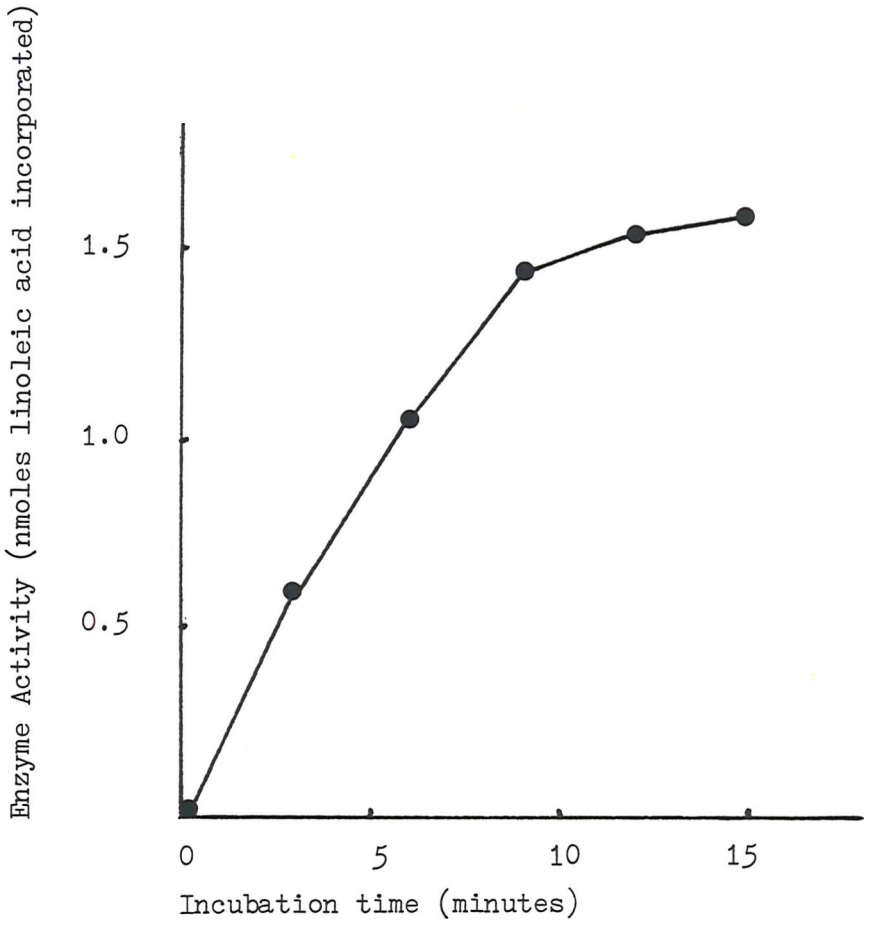


Fig 4.3 The Time Course of Lysophosphatidylcholine Acyltransferase Activity in Lean Mouse Hepatic Microsomes

Hepatic microsomes (250 µg) from lean mice were isolated as described in section 2.3 and assayed for lysoPC acyltransferase activity at the incubation times shown, as described in section 2.7 using linoleic acid as the substrate.

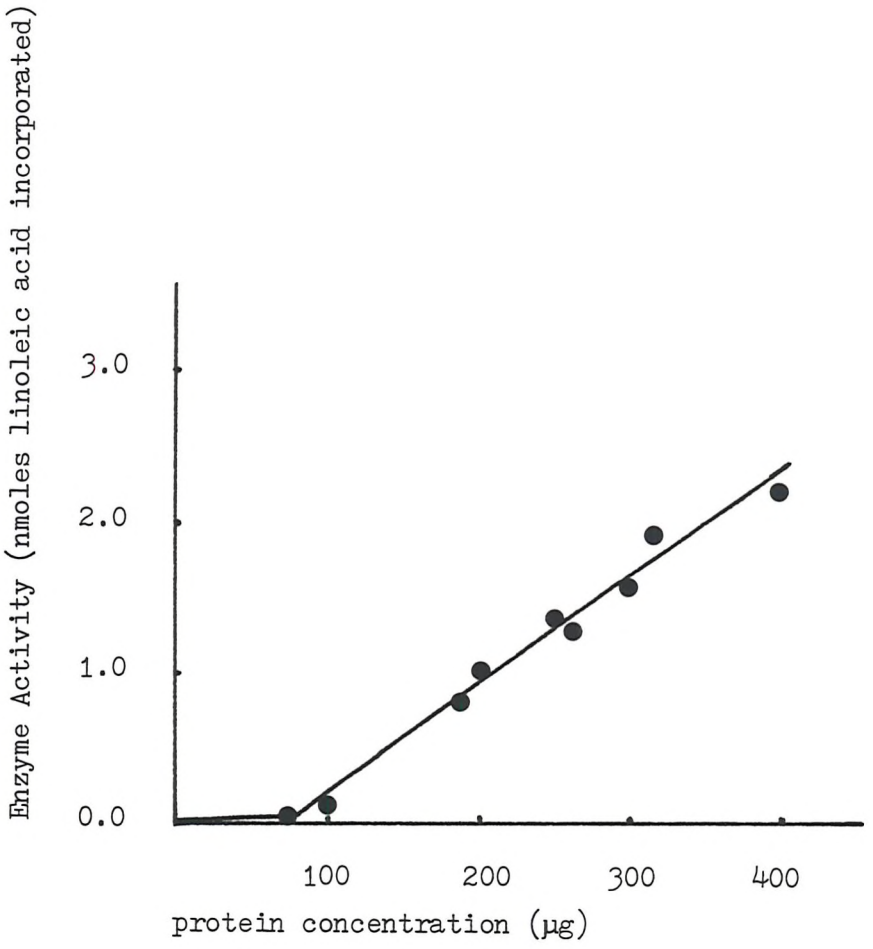


Fig 4.4 The Effect of Protein Concentration on Hepatic Microsomal Lysophosphatidylcholine Acyltransferase Activity of Lean Mice

Hepatic microsomes were isolated from lean mice as described in section 2.3 and assayed for lysoPC acyltransferase activity as described in section 2.7 using linoleic acid as the substrate, with a 6 minute incubation time. The data presented is from two experiments.

conditions, lysoPC acyltransferase activity was less than 0.05 nmoles/min./mg, which corresponded to approximately 5% of the enzyme activity in lean mouse microsomes.

4.5 Microsomal AcylcoA Synthetase Activity in Lean and Obese Mice

Prior to incorporation into phospholipid by lysophospholipid acyltransferase activity, fatty acids must first be converted to coenzyme A esters. To confirm that the hepatic microsomal lysoPC acyltransferase activity was not limited by the rate of conversion of fatty acid to acylcoA, endogenous acylcoA synthetase activity was measured in hepatic microsomes of lean and obese mice. Figure 4.5 shows the hepatic microsomal acylcoA synthetase activity of lean and obese mice, using linoleic acid as the substrate, under identical conditions to those used in lysoPC acyltransferase assays. Enzyme activity was similar in hepatic microsomes from lean and obese mice and 90% of the linoleic acid substrate had been converted in the first two minutes of the assay. From the data, the initial rate of acylcoA synthetase activity was estimated at 48 nmoles/min./mg protein. The activity of acylcoA synthetase was almost 50 times greater than that of lysoPC acyltransferase activity in hepatic microsomes of lean mice and hence is clearly not rate limiting for the incorporation of linoleic acid into phosphatidylcholine. In addition, previous studies have shown that hepatic microsomal acylcoA synthetase has a wide specificity for fatty acid substrates (Bar-Tana et al., 1971; Normann et al., 1981). Hence it is unlikely that the formation of other fatty acylcoA substrates was rate limiting for lysophospholipid acyltransferase reactions.

4.6 Hepatic Microsomal LysoPC Acyltransferase Activity in Lean and Obese Mice Using Different Fatty Acid Substrates

The substrate specificity of hepatic microsomal lysoPC acyltransferase activity of lean and obese mice was investigated using both linoleic acid (18:2 ω6) and oleic acid (18:1 ω9) as substrates. These fatty acids were chosen for the study since fatty acid analysis of membrane lipid composition of hepatic mitochondria (in chapter 3),

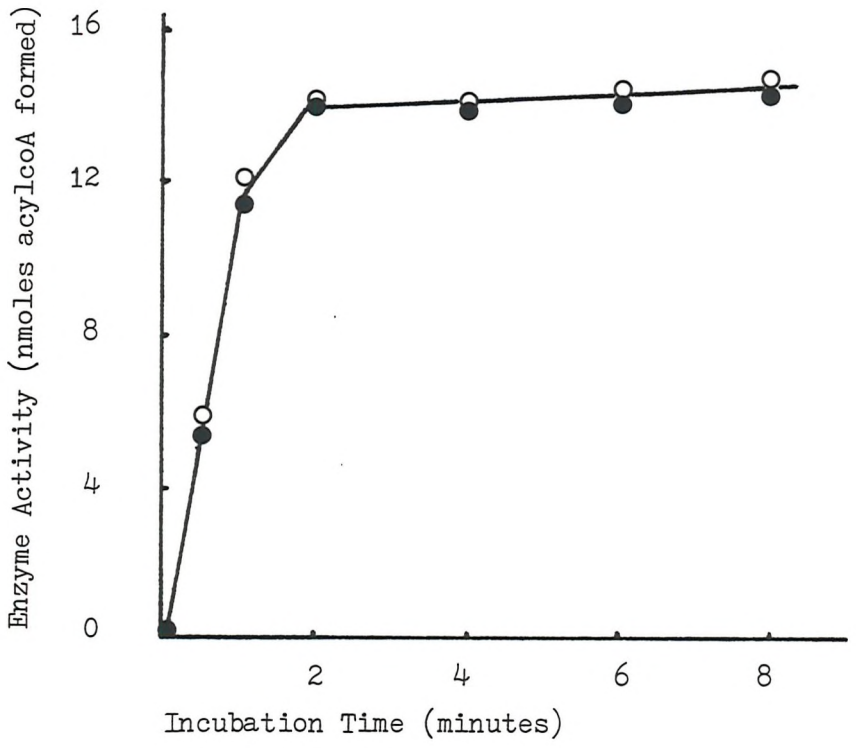


Fig 4.5 The Time Course of AcylcoA Synthetase Activity in Hepatic Microsomes From Lean and Obese Mice

Hepatic microsomes were prepared from lean (●) and obese (○) mice as described in section 2.3, and assayed for acylcoA synthetase activity as described in section 2.5 at the incubation times shown using linoleic acid as the substrate.

Substrate		Enzyme Activity (nmoles/min/mg) Lean	Obese	
Oleic acid	(n=4)	0.15 ± 0.04	0.20 ± 0.04	NS
Linoleic acid	(n=6)	0.72 ± 0.08	1.03 ± 0.12	$p < 0.05$

Table 4.2 Hepatic Microsomal LysoPC Acyltransferase Activity of Lean and Obese Mice Using Various Substrates

Hepatic microsomes were isolated from lean and obese mice as described in section 2.3 and assayed for lysoPC acyltransferase activity as described in section 2.7 using either linoleic acid or oleic acid as the substrate. The values represent the means ± SEM for the number of preparations (n) shown in parentheses. NS = not significant.

hepatic microsomes (Rouer et al., 1980), and liver plasma membrane (French et al., 1983), indicated that linoleic acid content was consistently reduced and oleic acid content was consistently increased in hepatic membranes of the obese mouse. LysoPC acyltransferase activity of lean and obese animals using either linoleic acid or oleic acid as the substrate is shown in table 4.2. In agreement with previous studies (Lands et al., 1982; Holub et al., 1979), faster rates of enzyme activity were obtained when linoleic acid rather than oleic acid was used as the substrate. LysoPC acyltransferase activity was approximately 5 times greater using linoleic acid as the substrate in hepatic microsomes from both lean and obese mice. In addition, lysoPC acyltransferase activity using both substrates was 30% to 40% higher in hepatic microsomes from obese mice when compared to the enzyme activity in lean mouse preparations. However, the increase in lysoPC acyltransferase activity in the obese mouse using oleic acid as the substrate was not statistically significant. LysoPC acyltransferase activity was also measured in hepatic mitochondria of lean and obese mice using linoleic acid as the substrate. Mitochondrial enzyme activity was lower than that of hepatic microsomes and similar in lean and obese animals (0.30 ± 0.05 and 0.26 ± 0.04 nmoles/min/mg protein for 3 lean and 3 obese mice respectively) .

4.7 Competition of Linoleic Acid and Oleic Acid as the Substrate for Incorporation into Phosphatidylcholine by LysoPC Acyltransferase

The finding that incorporation of linoleic acid into phosphatidylcholine by lysoPC acyltransferase activity was increased in hepatic microsomes of obese mice was unexpected since the linoleic acid content of microsomal phosphatidylcholine was reduced in obese animals (figure 4.2 and Hyslop et al., 1982). However, measurement of lysoPC acyltransferase activity in the presence of only one substrate is probably an over simplified model of the *in vivo* situation, since the enzyme *in vivo* may have to select from a pool of different fatty acids of varying concentrations. GLC analysis of fatty acid composition indicated that the relative proportion of oleic aicd (18:1 ω9) to linoleic acid (18:2 ω6) was increased at least seven fold in the hepatic storage triglyceride of obese mice

Fig 4.6 Competition of Linoleic Acid and Oleic Acid as the Substrate for Hepatic Microsomal Lysophosphatidylcholine Acyltransferase of Lean and Obese Mice

The incorporation of linoleic acid (●) and oleic acid (○) into phosphatidylcholine by hepatic microsomal lysoPC acyltransferase of lean and obese mice was assayed as described in section 2.7. Incubations were carried out in the presence of 30 μM linoleic acid and similar or greater concentrations of oleic acid (up to 300 μM). Total incorporation of fatty acid (▲) into phosphatidylcholine was obtained by addition of the linoleic acid and oleic acid incorporation.

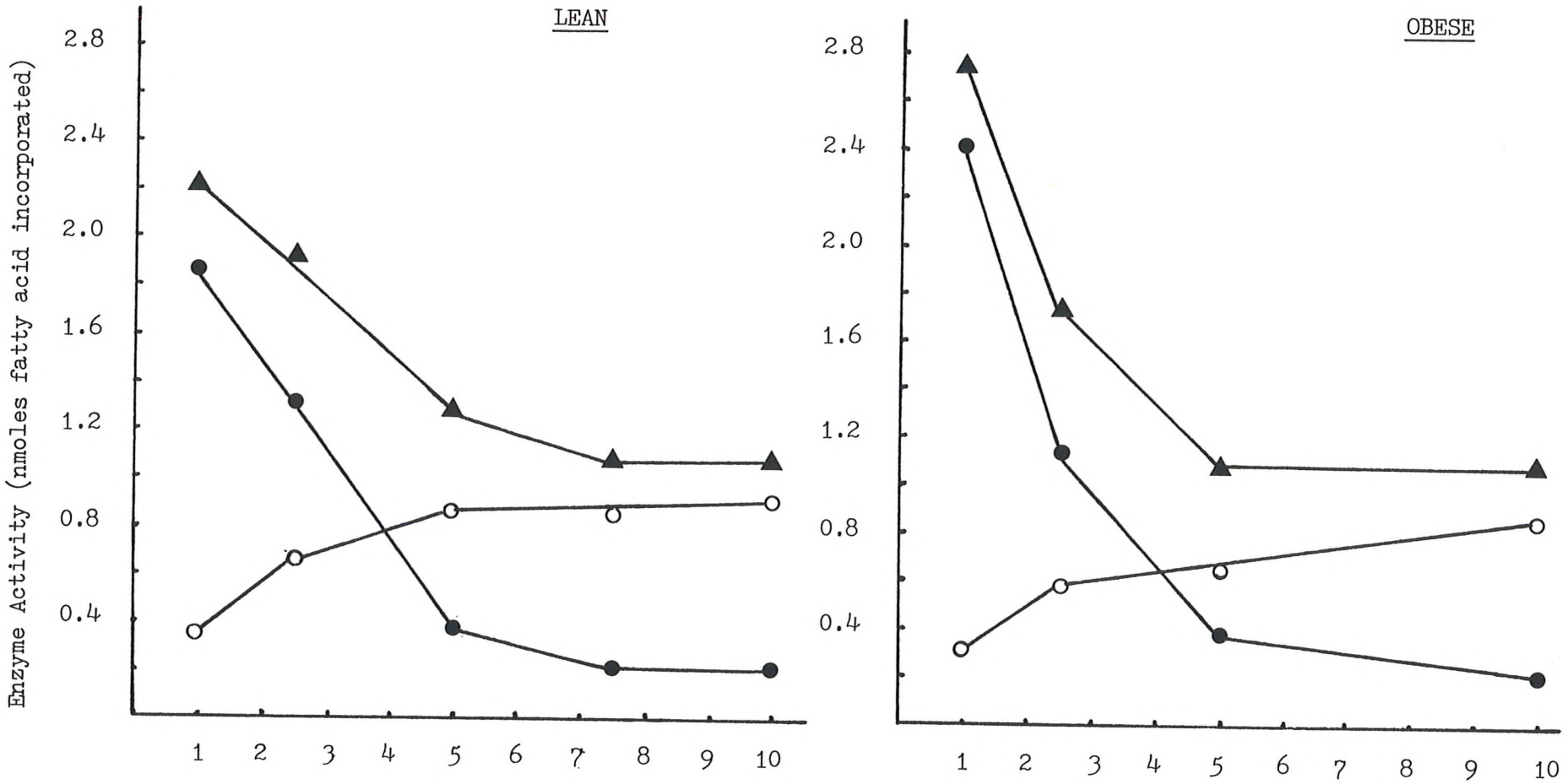


Fig 4.6 Competition of Linoleic Acid and Oleic Acid as the Substrate for Hepatic Microsomal Lysophosphatidylcholine Acyltransferase of Lean and Obese Mice

when compared to that of lean animals (Enser and Roberts, 1982; figure 4.1). Hence it was of interest to investigate the incorporation of linoleic acid and oleic acid into phosphatidylcholine by hepatic microsomal lysoPC acyltransferase of lean and obese mice under conditions in which the enzyme had varying proportions of these fatty acid substrates to choose from. Incubation conditions were set up such that the linoleic acid concentration was constant (30 μM) in the presence of similar or increasing concentrations of oleic acid (up to 300 μM). In this way, the lysoPC acyltransferase activity was examined over a range of substrate ratios of oleic acid to linoleic acid from 1 to 10. The data from these experiments is presented in figure 4.6. At similar substrate concentrations, the incorporation of linoleic acid into phosphatidylcholine was 6 to 7 times greater than that of oleic acid incorporation into into phosphatidylcholine by hepatic microsomal lysoPC acyltransferase of both lean and obese mice. Increasing the substrate ratio reduced the incorporation of linoleic acid and increased the incorporation of oleic acid into phosphatidylcholine by the lysoPC acyltransferase activity. However, a four fold excess of oleic acid substrate was required to give similar incorporation of linoleic acid and oleic acid into phosphatidylcholine by both lean and obese mouse preparations. At the highest substrate ratio (ten fold excess of oleic acid), oleic acid incorporation into phosphatidylcholine by lysoPC acyltransferase activity was 4.5 times greater than that of linoleic acid incorporation for both lean and obese animals. Increasing the concentration of oleic acid present in the assay media inhibited the total incorporation of fatty acid into phosphatidylcholine by lysoPC acyltransferase in both lean and obese animals. At 300 μM oleic acid (ten fold excess of oleic acid to linoleic acid), total incorporation of fatty acid into phosphatidylcholine was approximately 50% of the total fatty acid incorporation obtained using 30 μM oleic acid and 30 μM linoleic acids.

4.8 Fatty Acid Composition of Hepatic Storage Triglyceride and Microsomal and Mitochondrial Phospholipids of 18 Day Old Lean and Pre-obese Mice

Some of the fatty acid compositional changes of hepatic mitochondrial and microsomal phospholipids in the obese mouse were similar, although

less severe than the fatty acyl compositional changes in hepatic storage triglyceride (figure 4.1). Studies on lysoPC acyltransferase activity in lean and obese mice supported the view that the changes in membrane lipid composition were secondary to the compositional changes in the storage triglyceride of obese animals. Hence, it would be expected that compositional changes in the storage triglyceride would precede those of the membrane phospholipids during the development of the obese mouse. In order to clarify the relationship between the altered fatty acid composition of storage triglyceride and that of the membrane phospholipids of obese animals, the fatty acid compositions of these lipids were analysed in 18 day old lean and pre-obese mice, (prior to the onset of most of the abnormal metabolic characteristics of the obese mouse).

The fatty acid composition of hepatic storage triglyceride of 18 day old lean and pre-obese mice is shown in table 4.3. At 18 days of age pre-obese animals exhibited a reduction in linoleic acid content, from 29.2% to 14.9% of the total. The oleic acid content of storage triglyceride was similar in lean and pre-obese mice (at 18.5% and 19.7% of the total respectively) whereas the palmitoleic acid content was substantially increased from 0.2% to 6.4% of the total in the pre-obese animals.

In contrast, hepatic microsomal and mitochondrial phospholipids of pre-obese mice exhibited none of the fatty acid compositional changes characteristic of the 8 week old animal. Thus, oleic acid and linoleic acid contents were normal in phosphatidylcholine and phosphatidylethanolamine of both hepatic mitochondria and microsomes of 18 day old pre-obese mice. The characteristic increase in arachidonic acid observed in hepatic mitochondrial cardiolipin of obese mice was not apparent in the corresponding phospholipid of suckling pre-obese animals. Similarly, the elevated docosahexaenoic acid content of mitochondrial phosphatidylcholine was absent in pre-obese mice at 18 days of age. Consequently the double bond numbers and 18:1 ω9/18:2 ω6 ratios were similar in microsomal and mitochondrial phospholipids of 18 day old lean and pre-obese mice (tables 4.4 and 4.5).

Fatty Acid	Lean	Obese
16:0	34.0	37.2
16:1	0.2	6.4
18:0	8.6	11.1
18:1	18.5	19.7
18:2	29.2	14.9
20:4	4.5	6.2
22:6	5.2	4.4

Table 4.3 The Fatty Acid Composition of Hepatic Storage Triglyceride of 18 Day Old Lean and Pre-obese Mice

2 pre-obese mice were identified at 18 days of age by the cold challenge test (Trayhurn et al., 1977). Triglyceride was extracted from the supernatant fat cake of 100,000g liver homogenate pellets using 6 ml chloroform. The triglyceride extracts were transmethylated and analysed by GLC as described in section 2.13.

Fatty Acid	Phosphatidylcholine		Phosphatidylethanolamine			Cardiolipin	
	Lean	Obese	Lean	Obese		Lean	Obese
16:0	46.7	46.0	26.4	23.2	16:0	12.6	12.7
18:0	10.4	10.9	19.7	18.2	16:1	12.7	16.0
18:1	6.4	6.1	5.2	7.0	18:0	8.0	6.1
18:2	18.1	20.0	7.0	9.6	18:1	3.4	3.4
20:4	9.0	10.8	16.4	20.0	18:2	60.3	57.6
22:6	9.1	8.2	25.2	21.3	20:4	3.1	4.2
Unsat Index	133.2	138.5	236	234		149	152
18:1/18:2 Ratio	0.35	0.31	0.74	0.73		0.05	0.06

Table 4.4 The Fatty Acid Profiles of Hepatic Mitochondrial Phospholipids of 18 Day Old Lean and Pre-Obese Mice

Hepatic mitochondria were prepared from 2 lean and 2 pre-obese mice as described in section 2.3 and phospholipids extracted, separated and analysed by GLC as described in section 2.13.

Fatty Acid	Phosphatidylcholine		Phosphatidylethanolamine	
	Lean	Obese	Lean	Obese
16:0	40.7	38.9	35.3	33.3
18:0	11.3	11.4	15.0	15.3
18:1	4.9	4.4	5.7	3.6
18:2	19.4	25.0	10.5	16.0
20:4	11.2	9.8	10.3	11.9
22:6	12.5	10.3	22.2	20.0
Unsat. Index	163.5	155.0	201	203
18:1/18:2 Ratio	0.25	0.18	0.54	0.23

Table 4.5 The Fatty Acid Profiles of Hepatic Microsomal Phospholipids of 18 Day Old Lean and Pre-obese Mice

Hepatic microsomes were prepared from 2 lean and 2 pre-obese mice as described in section 2.3 and phospholipids extracted, separated and analysed by GLC as described in section 2.13.

4.9 Discussion

In order to further understand the biochemical basis of the altered membrane lipid composition in the obese mouse, the activity of hepatic lysophospholipid acyltransferases have been examined in lean and obese animals. In agreement with previous studies (Waite et al., 1970), hepatic microsomal lysoPC acyltransferase activity is 2-3 times greater than that of the hepatic mitochondrial enzyme. Hence most of the work described in the present chapter has been carried out on microsomal lysoPC acyltransferase activity. The fatty acid compositions of hepatic microsomal phospholipids of lean and obese mice were re-examined in order to relate changes in lysoPC acyltransferase activity to changes in membrane lipid composition. These findings confirm the previous report of Rouer et al. (1980) who analysed total phospholipid fatty acids from hepatic microsomes of lean and obese animals. Reduced levels of linoleic acid were apparent in both of the microsomal phospholipids of the obese mouse and these were parelleled by increases in oleic acid content. The reduction in palmitic acid content of phosphatidylethanolamine of the obese mice (figure 4.2) has been reported previously by Hyslop et al (1982) although these authors did not observe the increased oleic acid content of phosphatidylcholine or phosphatidylethanolamine seen in the present study. Similarly, the large increase in docosahexaenoic acid(22:6ω3) in hepatic microsomal phosphatidylethanolamine of obese animals reported by Hyslop et al.(1982) was not observed in the present study. It can be concluded that the most widespread alteration to membrane phospholipid composition involves reduced linoleic acid content usually accompanied by an increase in oleic acid content (see chapter 3). These compositional changes reflect the severe alteration in linoleic acid and oleic acid content of the hepatic storage triglyceride of obese mice (figure 4.1 and Enser and Roberts, 1982). Hence studies on lysoPC acyltransferase activity in lean and obese animals have been carried out using linoleic acid and oleic acid as the substrates.

Hepatic microsomal lysoPC acyltransferase of both lean and obese mice preferentially incorporated linoleic acid rather than oleic acid into phosphatidylcholine in agreement with previous reports (Holub et al., 1979; Lands et al., 1982). Thus the rate of

incorporation of linoleic acid into phosphatidylcholine was five times greater than that of oleic acid when the hepatic microsomal enzyme was incubated with 30 μM fatty acid substrates either individually or collectively. However, the elevated incorporation of linoleic acid into phosphatidylcholine by lysoPC acyltransferase activity of the obese animals was unexpected since the linoleic acid content of their hepatic microsomal phosphatidylcholine was reduced. This apparently paradoxical situation may be explained by considering the inhibitory effect of high concentrations of oleic acid on the incorporation of linoleic acid into phosphatidylcholine by lysoPC acyltransferase activity (figure 4.6). The elevated concentration of hepatic storage triglyceride of the obese mouse contains an increased proportion of oleic acid, when compared to that of lean animals (Enser and Roberts, 1982), which may result in an increased concentration of free oleic acid *in vivo*. This has been shown to inhibit lysoPC acyltransferase activity *in vitro*. Hence if this inhibitory effect also occurs *in vivo*, the increased incorporation of linoleic acid into phosphatidylcholine by lysoPC acyltransferase activity of the obese mouse may not be apparent. Indeed, the increased lysoPC acyltransferase activity of the obese mouse observed *in vitro*, may therefore result from a compensatory mechanism, inducing enzyme activity in the presence of the high inhibitory concentrations of oleic acid *in vivo*. The inhibitory effect of various fatty acids on lysoPC acyltransferase activity has been reported previously by Lands et al., (1982), although these authors found no oleic acid inhibition of arachidonic acid incorporation into phosphatidylcholine.

In competition experiments, hepatic microsomal lysoPC acyltransferase activity was examined under substrate conditions which reflected the relative contents of oleic acid and linoleic acid in storage triglyceride of lean and obese mice. This experimental approach assumed that the composition of the pool of fatty acids available for incorporation into phospholipids reflected the storage triglyceride composition. Under conditions which reflected the hepatic storage triglyceride composition of lean mice (i.e 2 to 3 fold excess of oleic acid to linoleic acid), the incorporation of linoleic acid into phosphatidylcholine by lysoPC acyltransferase

activity of both lean and obese animals was greater than the incorporation of oleic acid by the enzyme. Thus the substrate selectivity of the lysoPC acyltransferase activity under conditions which paralleled the triglyceride storage composition of lean mice, generally reflected the hepatic microsomal phosphatidylcholine 18:1 ω9/18:2 ω6 ratio of the lean animals (table 4.1). In contrast, under conditions which reflected the hepatic storage triglyceride composition of the obese mice (i.e at least a 10 fold excess of oleic acid to linoleic acid), the incorporation of linoleic acid into phosphatidylcholine by lysoPC acyltransferase activity of both lean and obese animals was lower than that of oleic acid. Thus the substrate selectivity of lysoPC acyltransferase activity under conditions which paralleled the triglyceride storage composition of obese mice, generally reflected the hepatic microsomal phosphatidylcholine 18:1 ω9/18:2 ω6 ratio of the obese animals (table 4.1).

These observations suggest that the altered membrane lipid composition of obese mice may result from the inability of the lysophospholipid acyltransferases to compensate for the pronounced changes in the fatty acid composition of the storage triglyceride. The substrate selectivity of the hepatic microsomal lysoPC acyltransferase activity of obese animals was apparently normal. This was indicated by the concentration of substrate required to obtain a similar incorporation of linoleic acid and oleic acid into phosphatidylcholine. Thus to obtain equal incorporation of oleic acid and linoleic acid, the concentration of oleic acid required by the lysoPC acyltransferase of both lean and obese animals was four times greater than that of linoleic acid. Lands (1980) points out that there is no clear evidence to suggest that a mechanism exists to alter the substrate selectivity of lysophospholipid acyltransferases, beyond changing the supply of fatty acid substrates.

Closer inspection of the data on substrate selectivity of hepatic microsomal lysoPC acyltransferase activity of lean and obese mice reveals that the substrate selectivities of the enzyme did not exactly parallel the 18:1 ω9/18:2 ω6 ratios of microsomal phosphatidylcholine in lean and obese animals. Under conditions which reflected the triglyceride storage composition of lean

animals (i.e 2-3 fold excess of oleic acid to linoleic acid), the incorporation of oleic acid into phosphatidylcholine was less than half that of linoleic acid incorporation, whereas the 18:1 ω9 /18:2 ω6 ratio of hepatic microsomal phosphatidylcholine in lean mice was 0.73. Similarly, under conditions which reflected the triglyceride composition of obese animals, the incorporation of oleic acid into phosphatidylcholine was four times higher than that of linoleic acid, whereas in microsomal phosphatidylcholine of obese mice, the 18:1 ω9/18:2 ω6 ratio was only 1.3.

These anomalies may be artefactual, since in the competition experiments it was assumed the triglyceride storage composition reflected the composition of the fatty acid pool available for incorporation into phospholipids. This need not necessarily be the case. For example, carnitine acyltransferase mediated transport of fatty acids into mitochondria may selectively utilise fatty acids of defined chain length or unsaturation. Kopec and Fritz (1971) have reported that calf liver carnitine acyltransferase preferentially utilized palmitic and myristic acids rather than stearic acid. Hence this may alter the spectrum of fatty acids available for incorporation into phospholipids in the mitochondria. Alternatively, the observation that the substrate selectivities of lysoPC acyltransferase activity did not exactly parallel the 18:1 ω9/18:2 ω6 ratio of microsomal phosphatidylcholine, may indicate that other processes, in addition to lysophospholipid acyltransferases, control membrane lipid composition.

Membrane associated phospholipase activity begins the modification of phospholipid fatty acyl composition by removing fatty acyl groups from the phospholipids in the membrane. The resulting lysophospholipid is now a potential substrate for re-acylation by lysophospholipid acyltransferase activity. In the present study, the possibility of substrate selectivity by phospholipase activity has not been considered. Most studies on phospholipase A_2 substrate selectivity have been carried out on platelet preparations. Here, phospholipase A_2 activity, preferentially releases PUFA's which can be metabolised further to produce prostaglandins and other eicosanoids (Van den Bosch, 1980; Lagarde et al., 1982). In the obese mouse, hepatic phospholipases may

selectively remove oleoyl groups,which are present at abnormally high levels in the phospholipids. Acyltransferase activity may then reacylate the lysophospholipid, selecting linoleic acid from the pool of fatty acids available.

Thus the changes in membrane lipid composition that are present in the obese mouse may develop in the following way. Increases in lipogenesis and desaturase enzyme activity (which utilize both saturated fatty acids and EFA's as substrates) may alter the composition of fatty acids in the storage triglyceride. This may alter the composition of the fatty acid pool available for incorporation into phospholipids. De novo synthesis on the endoplasmic reticulum produces phospholipids with abnormal fatty acid compositions. Subsequently, the processes of deacylation and reacylation at the subcellular location of the phospholipid, modify the abnormal fatty acid composition. However, the reacylation process, selecting from the pool of fatty acids available in the obese mouse, is unable to rectify the altered membrane lipid composition of obese animals.

Evidence to support this hypothesis comes from the analysis of hepatic membrane lipid composition in 18 day old suckling lean and pre-obese mice. At 18 days of age, the hepatic storage triglyceride of pre-obese animals exhibited some of the characteristic compositional changes found in the adult obese mouse. Thus linoleic acid content was reduced and palmitoleic acid content was increased in the triglyceride of pre-obese mice. However, the mitochondrial and microsomal phospholipids in these animals did not exhibit the reductions in linoleic acid content (or increases in oleic acid content) which were characteristic of phospholipids in the adult obese mouse. In addition, arachidonic acid content of cardiolipin and docosahexaenoic acid content of phosphatidylcholine were both normal in the mitochondrial phospholipids of the pre-obese mouse. Hence the changes in fatty acid composition of storage triglyceride precede the changes in fatty acid composition of hepatic membrane phospholipids during development in the obese mouse. This might be expected if the sequence of events described above were to take place.

The altered plasma membrane lipid composition of obese mouse adipose tissue can be considered in relation to the abnormal fatty acid composition of the storage triglyceride in this tissue of obese animals. In the storage triglyceride, the levels of oleic acid were normal although linoleic acid content was reduced in obese animals (Winand et al., 1973; Enser, 1979). Similarly, in plasma membrane phosphatidylcholine and phosphatidylethanolamine, the levels of oleic acid were approximately normal, whereas linoleic acid content was reduced in both phospholipids of the obese mouse (York et al., 1982). Hence the levels of these fatty acids in the phospholipids of obese mice seem to parallel the fatty acid levels in the storage triglyceride of the tissue.

The PUFA components of membrane phospholipids in lean and obese mice have not yet been considered in this chapter. The PUFA content of storage lipid is low whereas that of membrane phospholipids is much higher. However, lysophospholipid acyltransferases preferentially incorporate PUFA's into membrane phospholipids and hence selectively increase their PUFA content (Hill and Lands, 1968).

However, the reason for the selective increase in docosahexaenoic acid content of phosphatidylethanolamine but not of phosphatidylcholine in adipocyte plasma membrane of obese mice (York et al., 1982) is unclear. Similarly, the reason for the selective increase in arachidonic acid content of cardiolipin or docosahexaenoic acid content of phosphatidylcholine in hepatic mitochondria of obese animals is also unclear. It would appear that additional mechanisms for modifying the fatty acid compositions of selected phospholipids are present in the obese mouse. Pugh and Kates (1977) have reported the direct $\Delta 5$-desaturation of eicosatrienoyl-phosphatidylcholine to arachidonoyl-phosphatidylcholine by rat liver microsomes. In addition, under conditions of dietary restriction of the animals, the activity of this desaturase system was increased. These authors postulate a physiological role for this desaturase system under nutritional conditions where the levels of normal desaturase substrates are depressed. Hence this system provides one independant mechanism by which phospholipid-fatty acyl composition can be altered apart from changing the supply of fatty acid substrates available for incorporation into

phospholipids.

Another mechanism by which membrane lipid composition may be modified involves the activity of phospholipid transfer proteins. A series of different phospholipid transfer proteins exist catalysing the exchange of the individual classes of phospholipids (Schulze et al., 1977). Bovine liver phospholipid transfer protein preferentially transported phosphatidylcholine to unsaturated egg-phosphatidylcholine liposomes rather than saturated dimyristoyl-phosphatidylcholine liposomes (Helmkamp, 1983). Schulze et al.(1977) examined the substrate selectivity of rat liver phospholipid transfer protein using radiolabelled and chemically defined phosphatidylcholine vesicles as the donor lipid and rat liver mitochondria as the acceptor. Increased rates of exchange were observed when the donor phosphatidylcholine vesicles were subtituted with oleoyl or linoleoyl groups rather than stearoyl or palmitoyl groups. Thus it appears that the ability of phospholipid transfer proteins to use phospholipids of defined fatty acyl composition provides another mechanism by which membrane lipid composition may be altered. However, it is important to note that after transport to the new subcellular location by phospholipid transfer proteins, phospholipid-fatty acyl groups are potential substrates for modification by deacylation/reacylation enzymes.

To summarise, it is suggested that some of the changes in membrane lipid composition of the obese mouse result from the alteration in the composition of the pool of fatty acids available for incorporation into phospholipids, and the failure of the deacylation/reacylation enzymes to compensate for the changes in this lipid pool. However, the reason for the increase in some of the PUFA components of membrane phospholipids in the obese mouse is unclear.

CHAPTER 5

THE EFFECT OF ALTERED MEMBRANE LIPID ENVIRONMENT ON HEPATIC MITOCHONDRIAL MEMBRANE PROTEINS IN LEAN AND OBESE MICE

5.1 Introduction

In chapter 3, the fatty acyl compositions of phospholipids extracted from lean and obese mouse hepatic mitochondria were shown to be different. The consequence of these alterations in membrane lipid composition in the obese mouse for intrinsic membrane protein function has been examined, and the results are presented in this chapter. Lipid-protein interactions have been investigated by the presentation of data as Arrhenius plots. As has been outlined previously, many intrinsic protein are responsive to the lipid composition or molecular organisation that occur in their membrane lipid environment. Discontinuities or 'breaks' in Arrhenius plots are usually attributed to lipid-phase transitions or lipid separations in the surrounding membrane lipid (Sandermann, 1978). Hence, the Arrhenius plots of intrinsic membrane proteins in hepatic mitochondrial membranes isolated from obese mice are expected to have different characteristics to those from lean animals, provided that the intrinsic membrane proteins are responsive to the particular alterations in membrane lipid composition. Intrinsic mitochondrial membrane proteins involved in electron transport and oxidative phosphorylation have been investigated. Succinate-cytochrome C reductase is a protein complex spanning the inner mitochondrial membrane. It transports reducing equivalents from succinate, inside the mitochondrion, to cytochrome C on the outside of the inner mitochondrial membrane. It is comprised of succinate dehydrogenase (which contains FAD and two iron-sulphur centres), cytochrome b, ubiquinone, cytochrome C_1 , another iron-sulphur centre and various intrinsic membrane proteins (Nicholls, 1982). Yu et al. (1973) have demonstrated that succinate-cytochrome C reductase has a lipid requirement for activity and McMurchie et al.(1983c) have shown that Arrhenius characteristics of this protein complex can be changed by dietary modulation of membrane fatty acid composition. Hence this protein complex is suitable for the study of lipid-protein interactions.

Similarly, cytochrome oxidase is transmembranous. This protein complex transports electrons from cytochrome C, outside the inner mitochondrial membrane, to molecular oxygen, inside the matrix.

Cytochrome oxidase contains 6-8 polypeptide chains, two haem groups (cytochrome a and cytochrome a_3) and 2 copper atoms. Cytochrome oxidase has a requirement for tightly bound cardiolipin for activity (Aswachi et al., 1971) and has been widely used to study lipid-protein interactions because of its associated boundary lipid layer (Jost et al., 1973; 1977; Kang et al., 1979; Semin et al., 1984). Hence, Arrhenius plots of this respiratory enzyme complex have also been investigated in the present study.

Finally, the temperature profiles of ATP-Pi exchange activity in lean and obese mouse hepatic mitochondria have also been characterised. The ATP-Pi exchange reaction requires coupled mitochondria or sub-mitochondrial particles for activity. Kagawa et al. (1973) have demonstrated the requirement of this process for phospholipid whilst Innis and Clandinin (1981) have shown that Arrhenius characteristics of ATP-Pi exchange can be altered by dietary modulation of membrane lipid composition. Before the Arrhenius plots of these processes are presented, studies undertaken to characterise hepatic mitochondrial ATP-Pi exchange activity in lean and obese mice, are described.

Results

5.2 Characterisation of Hepatic Mitochondrial ATP-Pi Exchange Activity in Lean and Obese Mice

The mitochondrial preparations used in the experiments described in this chapter of the thesis were isolated as outlined in section 2.3 with the addition of an extra centrifugation step. Resuspending and washing the mitochondrial pellet by centrifugation at 15,000g for 15 minutes at 4°C, enhanced the specific activities of the mitochondrial enzymes studied. Hence, more purified mitochondrial preparations were used for determining Arrhenius characteristics of mitochondrial enzymes.

The time courses of mitochondrial ATP-Pi exchange activity at 37°C, using various mitochondrial protein concentrations, are shown in figure 5.1. ATP-Pi exchange activity was linear for at least 8 minutes at all protein concentrations used , hence

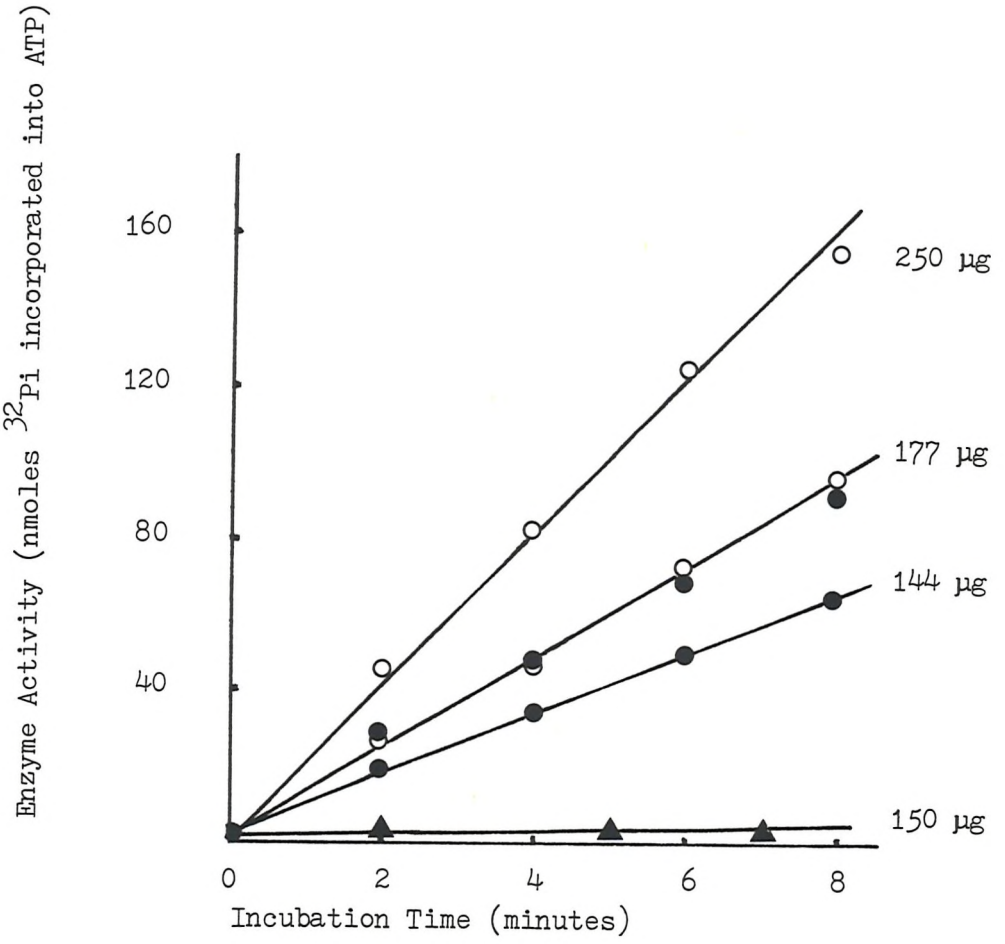


Fig 5.1. The Time Courses of Hepatic Mitochondrial ATP-Pi Exchange Activity in Lean and Obese Mice

Hepatic mitochondria were prepared from lean (●) and obese (○) mice as described in section 2.3 with the modifications outlined in section 5.2. Mitochondria were assayed for ATP-Pi exchange activity at 37°C as described in section 2.11 for the incubation times shown using various mitochondrial protein concentrations. Mitochondrial ATP-Pi exchange activity was also assayed in the presence of 2 mM azide (▲).

6 minutes was chosen as a suitable incubation time. The inclusion of 2 mM sodium azide in the incubation cocktail inhibited 95% of the ATP-Pi exchange activity in obese mouse hepatic mitochondria.

The data indicated that ATP-Pi exchange activity in hepatic mitochondria from lean and obese mice was similar. The rates of exchange of Pi into ATP at 37°C were 63 nmoles/min./mg and 72 nmoles/min./mg for hepatic mitochondria isolated from lean and obese mice respectively.

5.3 Effect of Protein Concentration on Hepatic Mitochondrial ATP-Pi Exchange Activity of Lean and Obese Mice

Figure 5.2 shows the effect of mitochondrial protein concentration on the assay of ATP-Pi exchange activity at 37°C using a 6 minute incubation period. ATP-Pi exchange activity was linear over a wide range of protein concentrations, from 0.1 to 0.35 mg of protein. The ATP-Pi exchange activity of hepatic mitochondria from lean and obese animals was similar. Routinely, incubations were carried out using 0.15 mg to 0.2 mg protein in subsequent experiments.

5.4 pH Profile of ATP-Pi Exchange Activity of Lean Mouse Hepatic Mitochondria

The ATP-Pi exchange activity of hepatic mitochondria from lean mice was dependant on the pH of the incubation media (figure 5.3a). Increasing pH increased ATP-Pi exchange activity. In order to present data as an Arrhenius plot, enzyme activity must be measured over a range of temperatures. Arrhenius plots are then constructed by plotting the logarithm of enzyme activity against the reciprocal of the temperature (in °K). The Arrhenius characteristics of membrane enzymes in this study were examined over a range of temperatures from 8°C to 37°C. The pH of many buffers vary with temperature and in view of the marked pH dependence of ATP-Pi exchange activity, the pH of the phosphate buffer system used in the present study was examined over a range of temperatures from 8° to 37°C. Figure 5.3b shows that the pH of the phosphate buffer increased with increasing temperature. It was estimated that the increase in pH caused by the change in temperature from 8°C

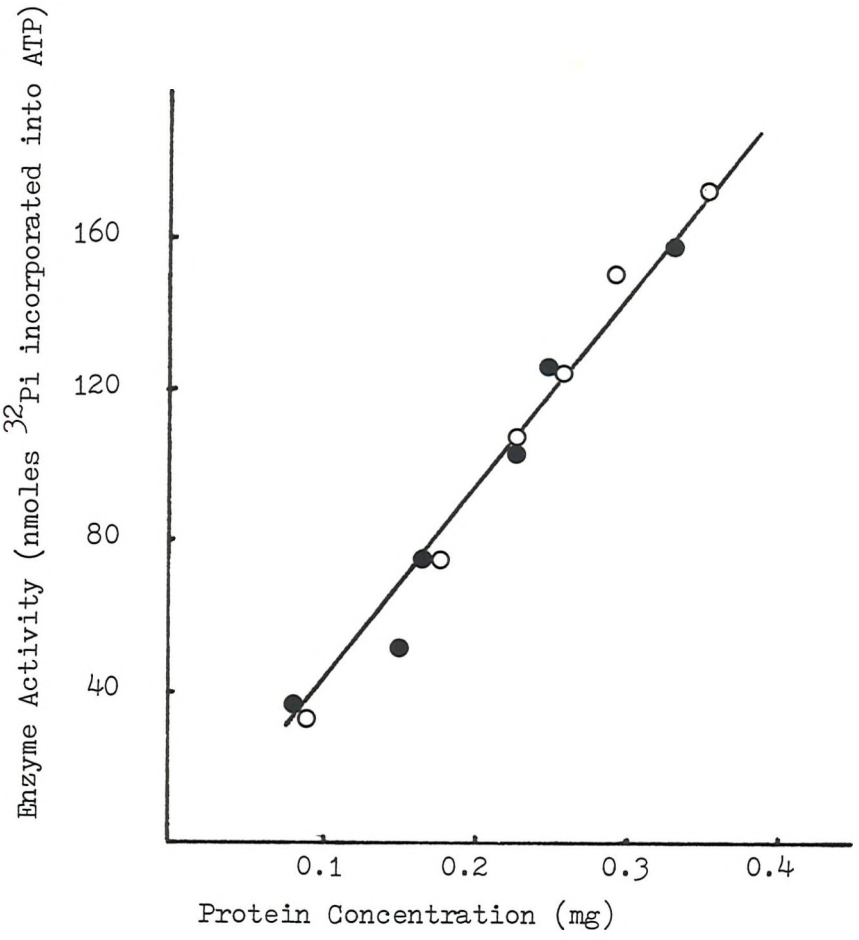


Fig 5.2 The Effect of Protein Concentration on Hepatic Mitochondrial ATP-Pi Exchange Activity of Lean and Obese Mice

Hepatic mitochondria from lean (●) and obese (○) mice were prepared as described in section 2.3 with the modifications outlined in section 5.2 as assayed for ATP-Pi exchange activity at 37°C as described in section 2.11, using a 6 minute incubation period. The data presented is from two experiments.

Fig 5.3a The pH Profile of Hepatic Mitochondrial ATP-Pi Exchange Activity of Lean Mice

Hepatic mitochondria were prepared as described in section 2.3 with the modifications outlined in section 5.2 and assayed for ATP-Pi exchange activity at 37°C as described in section 2.11. Incubations were carried out under the pH conditions shown.

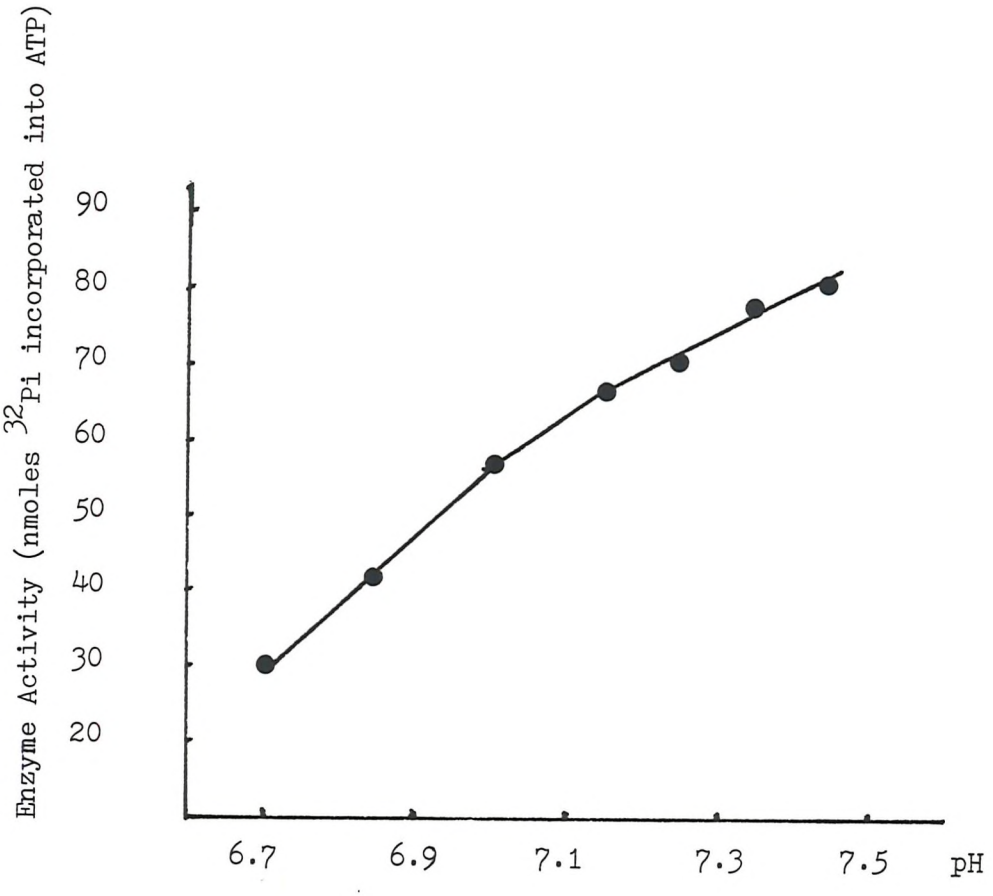


Fig 5.3a The pH Profile of Hepatic Mitochondrial ATP-Pi Exchange Activity of Lean Mice

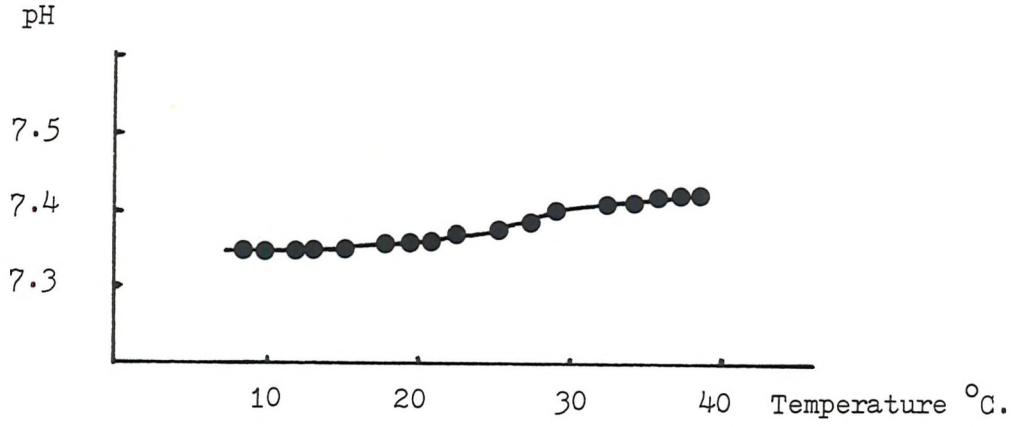


Fig 5.3b The Effect of Temperature on the pH of the HEPES-phosphate Buffer Used in Mitochondrial ATP-Pi Exchange Assays

The pH of the HEPES-phosphate buffer was measured across the temperature range from 8 to 39°C.

to 37°C would increase ATP-Pi exchange activity by not more than 5%. This small increase in ATP-Pi exchange activity was considered acceptable especially in view of the comparative nature of the work (i.e Arrhenius characteristics of lean and obese hepatic mitochondrial ATP-Pi exchange activity were compared under identical conditions of temperature and pH.).

5.5 Arrhenius Characteristics of ATP-Pi Exchange Activity in Lean and Obese Mouse Hepatic Mitochondria

Typical Arrhenius characteristics of hepatic mitochondrial ATP-Pi exchange activity in lean and obese mice are shown in figure 5.4 and the parameters summarised in table 5.1. For all Arrhenius plots in this study, lines through points were drawn free-hand with an estimation of the break temperature by inspection. Arrhenius characteristics of enzyme activity were confirmed using a modified computer programme based on that described by Bogartz (1968).

The Arrhenius plots of hepatic mitochondrial ATP-Pi exchange activity of obese animals exhibited an increase in break temperature when compared to those of the lean animals (figure 5.4). The break temperature had increased by 3°C, from 19.2°C in the lean mice to 22.1°C in the obese animals (table 5.1). This difference in break temperature may be interpreted as suggesting that some component of the ATP-Pi exchange reaction senses a change in the molecular organisation or composition of the mitochondrial membrane lipid environment in the obese mouse. Associated with the differences in break temperature, the ATP-Pi exchange activity of obese mice had a small but significant reduction in activation energy above the break temperature. The rates of Pi exchange into ATP at physiological temperatures in lean and obese animals were not significantly different, confirming previous observations (section 5.2).

The observation that thyroid treatment of lean and obese mice modified hepatic mitochondrial membrane fatty acid compositions, prompted an investigation into the effects of a similar treatment on the Arrhenius characteristics of ATP-Pi exchange activity in

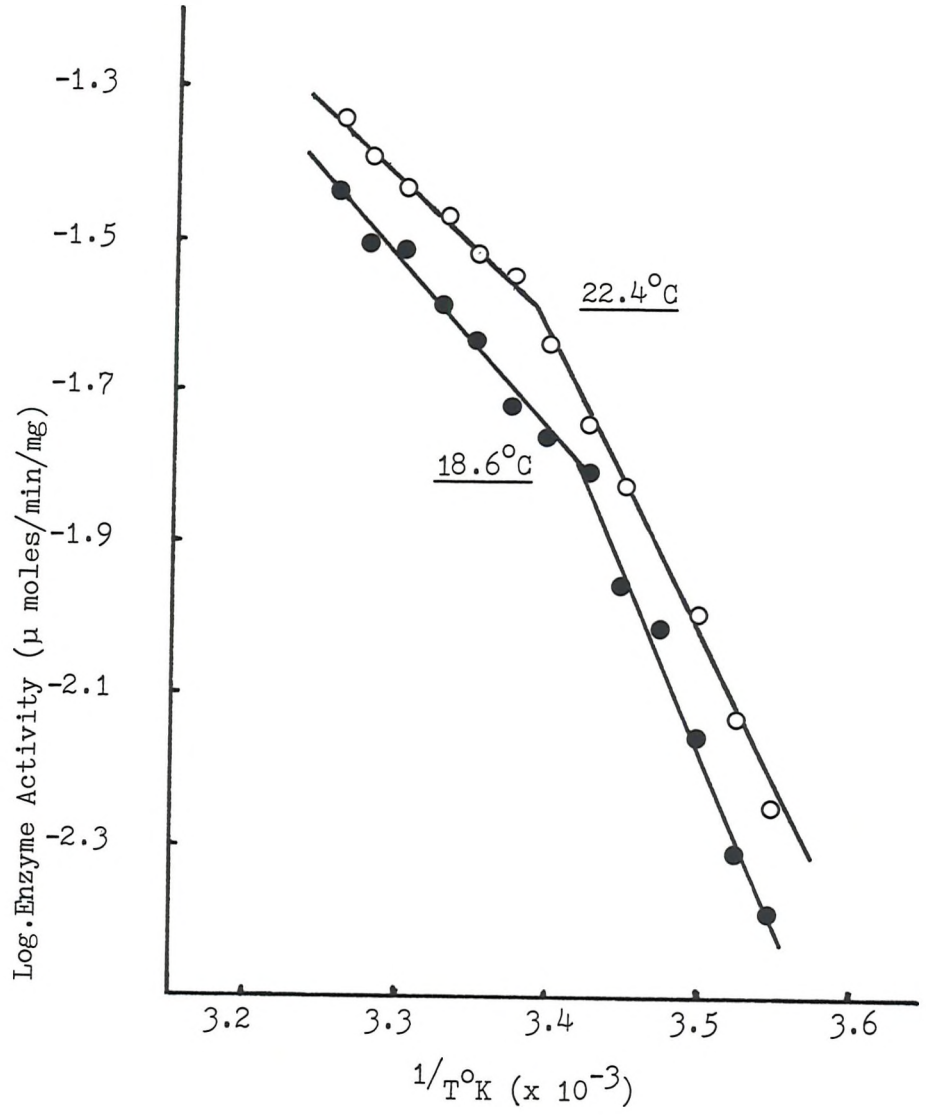


Figure 5.4 Arrhenius Plots of ATP-Pi Exchange Activity From Lean and Obese Mouse Liver Mitochondria

Hepatic mitochondria were prepared from lean (●) and obese (○) mice as described in section 2.3 with the modifications outlined in section 5.2 and assayed from ATP-Pi exchange activity over the temperature range 8-37°C as described in section 2.11. The lines of the Arrhenius plots were drawn by inspection and confirmed using a modified computer programme based on that described by Bogartz (1968). The numbers underlined are the Arrhenius break temperatures.

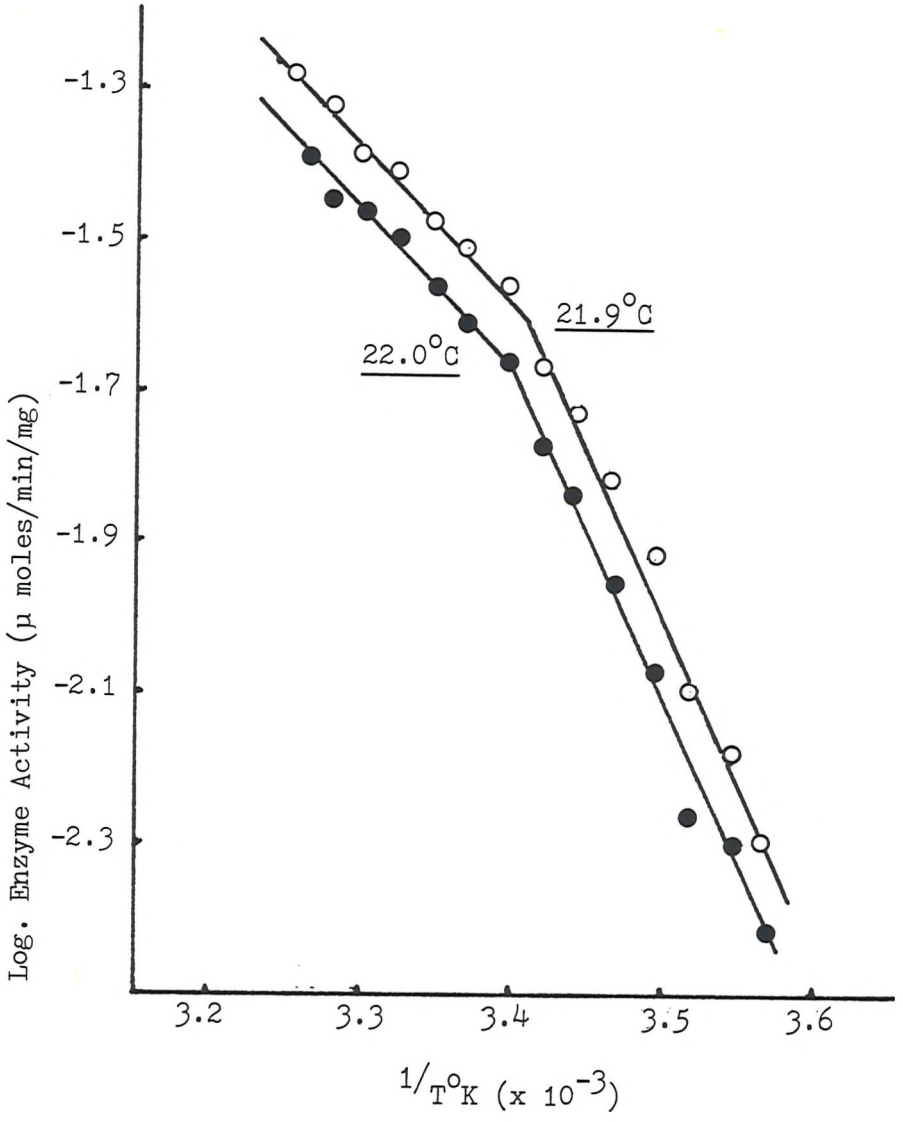


Fig 5.5 Arrhenius Plots of Hepatic Mitochondrial ATP-Pi Exchange Activity of Lean and Obese Mice Treated with T_3 for Two Weeks

Hepatic mitochondria from lean (●) and obese (○) mice injected subcutaneously with T_3 (30 μg/kg) twice daily for 14 days were prepared as described in section 2.3 with the modifications outlined in section 5.2. Mitochondria were assayed for ATP-Pi exchange activity over the temperature range, 8-37°C as described in section 2.11. The lines of Arrhenius plots were drawn by inspection and confirmed using a modified computer programme (Bogartz, 1968).

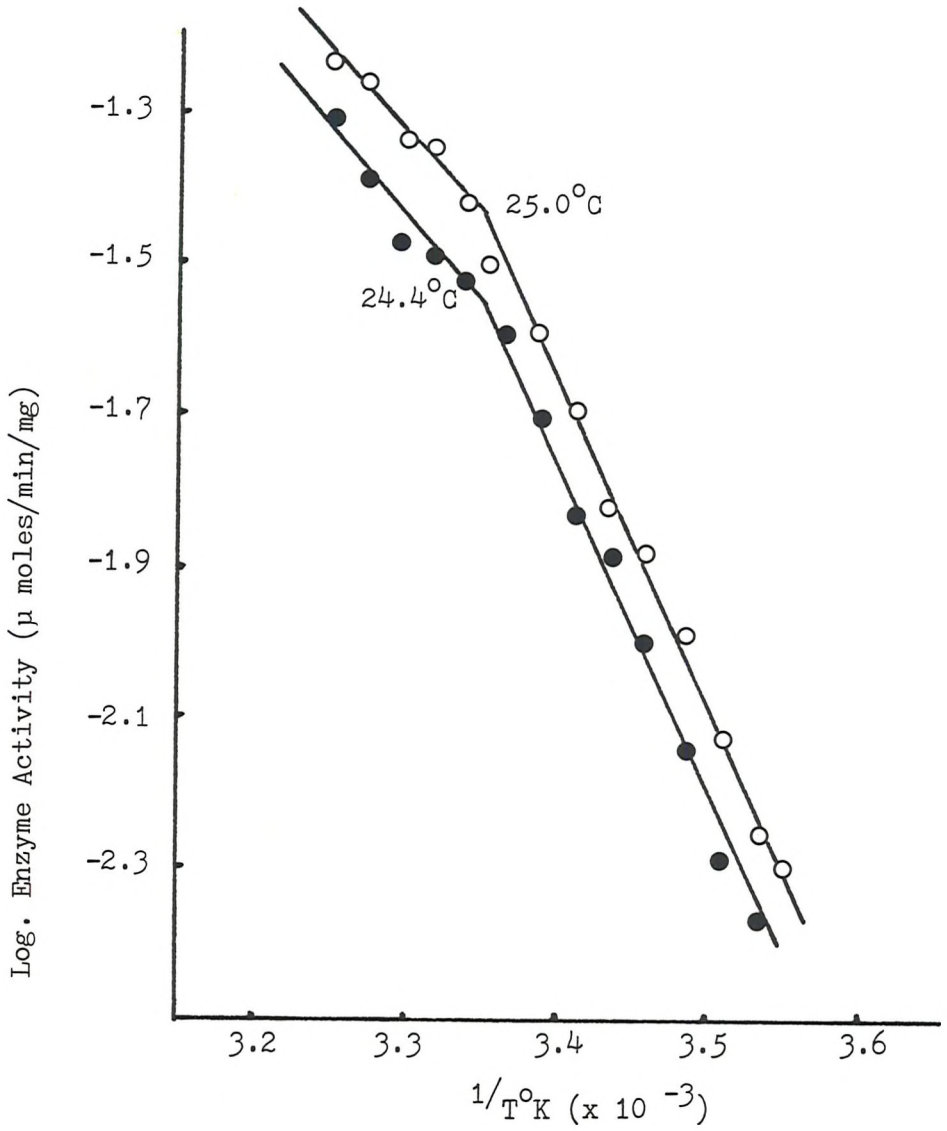


Fig 5.6 Arrhenius Plots of Hepatic Mitochondrial ATP-Pi Exchange Activity of Lean and Obese Mice Treated with Thyroid Hormones for 5 Weeks

Hepatic mitochondria from lean (●) and obese (○) mice fed ad libitum a diet containing 0.02%(w/w) thyroid powder for 5 weeks were prepared as described in section 2.3 with the modifications outlined in section 5.2. Mitochondria were assayed for ATP-Pi exchange activity over the temperature range, 8-37°C as described in section 2.11. The lines of the Arrhenius plots were drawn by inspection and confirmed using a modified computer programme (Bogartz, 1968).

these animals. Figure 5.5 and figure 5.6 show the Arrhenius characteristics of hepatic mitochondrial ATP-Pi exchange of lean and obese mice treated with thyroid hormones for 2 and 5 weeks respectively. T_3 treatment increased the Arrhenius break temperatures of ATP-Pi exchange activity. After 2 weeks T_3 treatment, the break temperature in lean mouse preparations had risen by 2°C to 21.2°C and after 5 weeks thyroid hormone treatment the break temperatures had increased further to 24.4°C and 25.2°C for lean and obese preparations respectively (table 5.1). The difference in activation energy above the break temperature observed in lean and obese control mice was abolished by thyroid hormone treatment. In contrast mitochondrial ATP-Pi exchange activity at 37°C was unaffected by thyroid hormones (table 5.1).

5.6 Arrhenius Characteristics of Respiratory Enzyme Complex Activities in Lean and Obese Mouse Hepatic Mitochondria

Typical Arrhenius characteristics of succinate-cytochrome C reductase (complex 2-3) and cytochrome oxidase (complex 4) in hepatic mitochondrial preparations from lean and obese mice are shown in figures 5.7 and 5.8. Arrhenius plot parameters are summarised in table 5.1. Arrhenius plots of both cytochrome oxidase activity and succinate-cytochrome C reductase activity were similar in lean and obese mice. Cytochrome oxidase activity exhibited Arrhenius break temperatures around 20°C for both lean and obese mouse hepatic mitochondrial preparations while enzyme activities were similar at physiological temperatures (1.7 and 1.8 μ moles/min./mg protein for lean and obese mouse hepatic mitochondria respectively). Succinate-cytochrome C reductase activity exhibited a small but not significant reduction in Arrhenius break temperature from 25.3° to 23.8°C in obese mice and enzyme activity at 37°C was similar to that of the lean mouse preparations at 0.36 μ moles/min./mg protein. These data may be interpreted to suggest that the respiratory enzyme complexes sense similar lipid environments in hepatic mitochondrial membranes of lean and obese mice.

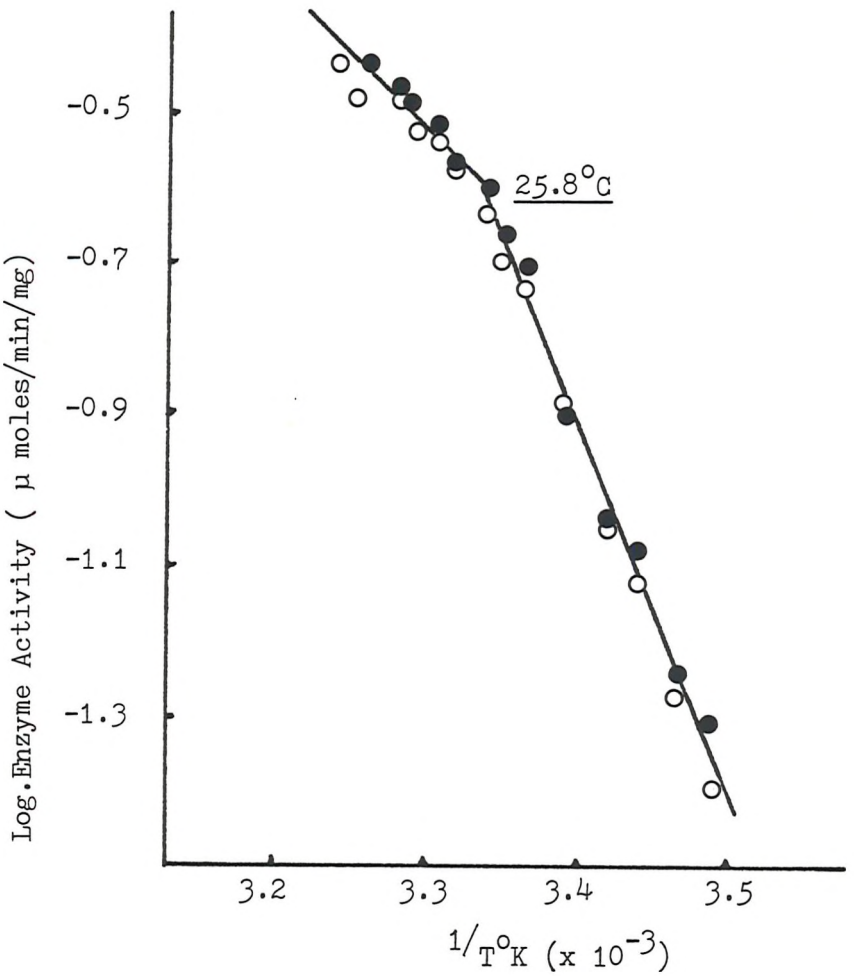


Fig 5.7 Arrhenius Plots of Hepatic Mitochondrial Succinate-Cytochrome C Reductase Activity from Lean and Obese Mice

Hepatic mitochondria from lean (●) and obese (○) mice were prepared as described in section 2.3 with the modifications outlined in section 5.2, and assayed for succinate-cytochrome C reductase activity over the temperature range 13-37°C as described in section 2.8. The lines of the Arrhenius plots were drawn by inspection and confirmed using a modified computer programme (Bogartz 1968). The number underlined is the Arrhenius plot break temperature.

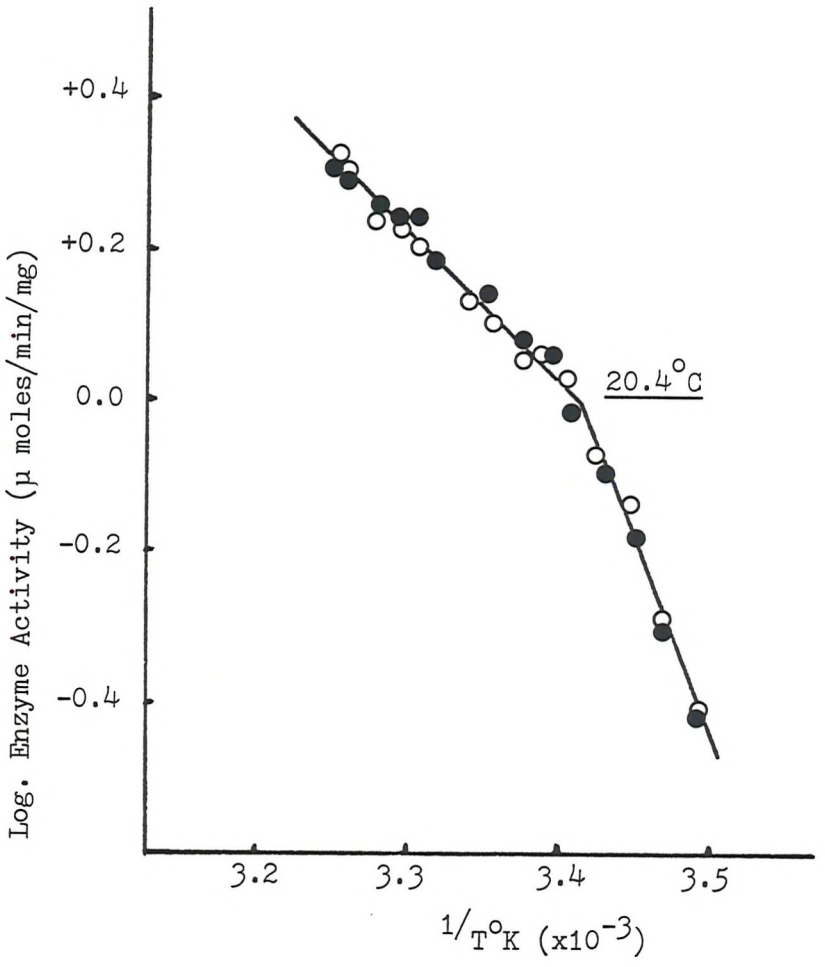


Fig 5.8 Arrhenius Plots of Hepatic Mitochondrial Cytochrome Oxidase Activity from Lean and Obese Mice

Hepatic mitochondria from lean (●) and obese (○) mice were prepared as described in section 2.3 with the modifications outlined in section 5.2 and assayed for cytochrome oxidase activity over the temperature range 12-37°C as described in section 2.10. The lines of the Arrhenius plots were drawn by inspection and confirmed using a modified computer programme (Bogartz, 1968). The number underlined is the Arrhenius plot break temperature.

Table 5.1 Summary of Arrhenius Plot Parameters of Hepatic Mitochondrial Enzymes From Lean and Obese Mice

Hepatic mitochondria from lean and obese mice were prepared as described in section 2.3 with the modifications outlined in section 5.2. Hepatic mitochondria were prepared from control animals, mice injected with T_3 subcutaneously twice daily for 14 days (30 µg/kg/day) ,or mice fed ad libitum a diet containing 0.02% thyroid powder for 5 weeks. Mitochondria were assayed for ATP-Pi exchange activity, succinate-cytochrome C reductase activity and cytochrome oxidase activity as described in sections 2.11, 2.8, and 2.10 respectively. The lines of Arrhenius plots were drawn by inspection and confirmed using a modified computer programme based on that described by Bogartz (1968). Values represent the means $\pm$ SEM for the number of preparations (n) shown in parentheses. * $p<0.05$; ** $p < 0.01$; *** $p<0.005$.

			Activity 37°C (μmoles/min./mg)	Break Temp. °C	Activation Energy Above (break) (Kcal/mole)	Below
Succinate-Cytochrome C Reductase	(n=6)	Lean	0.361 ± 0.042	25.3 ± 0.5	10.4 ± 1.3	25.3 ± 1.1
		Obese	0.351 ± 0.050	23.8 ± 0.9	8.3 ± 0.7	25.5 ± 2.4
Cytochrome Oxidase	(n=4)	Lean	1.818 ± 0.218	20.2 ± 0.5	7.4 ± 0.4	24.8 ± 3.6
		Obese	1.692 ± 0.228	19.3 ± 0.6	7.3 ± 0.7	29.2 ± 2.8
ATP-Pi Exchange	(n=4)	Lean	0.075 ± 0.012	19.2 ± 0.4 ***	11.5 ± 0.3 *	23.8 ± 3.1
		Obese	0.087 ± 0.012	22.1 ± 0.2	9.6 ± 1.0	21.2 ± 1.2
ATP-Pi Exchange T_3 Treatment for 2 wks	(n=2)	Lean	0.051	21.2	8.6	19.5
		Obese	0.069	22.1	9.8	19.2
ATP-Pi Exchange, T_3 Treatment for 5 wks	(n=2)	Lean	0.056	24.4	10.7	21.8
		Obese	0.068	25.2	10.1	20.4

Table 5.1 Summary of Arrhenius Plot Parameters of Hepatic Mitochondrial Enzymes From Lean and Obese Mice

5.7 Discussion

Many studies have attempted to correlate alterations in mitochondrial membrane fatty acid composition (induced by dietary modulation) with changes in intrinsic membrane enzyme activity or characteristics (Clandinin, 1979; Innis and Clandinin, 1981; McMurchie et al., 1983c; Mak et al., 1983). Hence it might be expected that the changes reported in obese mouse mitochondrial membranes would give rise to alterations in membrane associated processes. Katyare and Howland (1978) reported increased oxidative metabolism in hepatic mitochondria of obese mice. They observed increased levels of succinate oxidation, succinate dehydrogenase activity, ATPase activity and increased mitochondrial cytochrome content. The data was considered consistent with the increased production of ATP necessary for elevated hepatic lipogenic activity in the obese mouse. In the present study however, oxidative phosphorylation as measured by ATP-Pi exchange activity and by the specific activities of respiratory enzyme complexes (succinate-cytochrome C reductase and cytochrome oxidase) was similar in lean and obese animals. The reason for the apparent discrepancy between this data and that of Katyare and Howland (1978) is unclear.

The rates of ATP-Pi exchange activity in the present study were similar or lower than those reported previously (Ida Chen and Hoch, 1977; Sandoval et al., 1970). This may reflect relatively impure or less well coupled mitochondrial preparations. Kagawa and Racker (1971) have demonstrated that mitochondrial ATP-Pi exchange activity is abolished by uncouplers (e.g. FCCP) whereas in the present study complex IV inhibitor, sodium azide has been shown to block ATP-Pi exchange activity. These observations indicate that mitochondria must be intact or coupled in order that ATP-Pi exchange can take place. The specific activity of succinate-cytochrome C reductase in the present study was similar to that reported previously (McMurchie et al., 1983c), whereas the Arrhenius characteristics and 'break temperatures' of mitochondrial enzymes in these experiments were quite different to those reported by other workers (Innis and Clandinin, 1981; Raison and McMurchie, 1983c). Thus, McMurchie et al. (1983c) reported that the Arrhenius break temperature of rat liver mitochondrial succinate-cytochrome C

reductase was 23°C, which is 2° lower than observed in the present study, whereas, Innis and Clandinin (1981) reported that the Arrhenius break temperature of rat liver mitochondrial ATP-Pi exchange was 14.8°C, 4.4°C lower than observed in the present study. These variations may result from a species difference. Raison and McMurchie (1974) have shown that Arrhenius characteristics of succinate oxidation from sheep and rat liver mitochondria are different. Arrhenius break temperatures were observed at 29°C and 17°C in sheep liver mitochondrial preparations and much lower, at 24°C and 8°C, in the rat liver mitochondrial preparations. Coincident with these Arrhenius break temperatures in sheep and rat liver mitochondrial succinate oxidation, Raison and McMurchie (1974) reported changes in the molecular ordering of the mitochondrial membranes as indicated by electron spin label studies.

Arrhenius characteristics of various membrane associated processes of hepatic mitochondria have been compared in lean and obese mice. However, only the Arrhenius characteristics of one process, ATP-Pi exchange activity, were significantly different in the obese mice. The Arrhenius characteristics of cytochrome oxidase and succinate-cytochrome C reductase were similar in lean and obese mice.

Yu et al. (1973) have demonstrated that mitochondrial succinate-cytochrome C reductase from bovine heart requires phospholipids for activity. The activity of purified and delipidated succinate-cytochrome C reductase could only be fully restored by adding back mitochondrial phospholipids and ubiquinone. They found that adding back cardiolipin and phosphatidylethanolamine with ubiquinone restored 98% of the original activity, whereas cardiolipin or phosphatidylethanolamine alone gave only 65% or 45% of the activity respectively. McMurchie et al. (1983c) altered the fatty acid composition of rat liver mitochondrial phospholipids by feeding diets of different fatty acid composition. Feeding an unsaturated fatty acid diet caused widespread changes in total mitochondrial phospholipid-fatty acid composition although the largest changes were associated with an increase in the arachidonic acid (20:4 ω6) component and a decrease in palmitic acid (16:0) content. Conversely, feeding a saturated fatty acid diet produced changes in virtually all of the phospholipid fatty acyl components.

These were, a substantial reduction in the arachidonic acid (20:4ω6) content, reduced levels of palmitic acid (16:0) and linoleic acid (18:2ω6) and, increases in stearic acid content (18:0), oleic acid content (18:1ω9) and docosahexaenoic acid content (22:6ω3). These authors also reported that the Arrhenius characteristics of succinate-cytochrome C reductase activity were altered dramatically in hepatic mitochondrial preparations isolated from the animals fed the different diets. The Arrhenius break temperature for succinate-cytochrome C reductase from animals fed the saturated fatty acid diet had increased by 8.5° to 31.7°C, compared to that of the control animals. McMurchie et al.(1983c) correlated changes in the Arrhenius characteristics with the ω6/ω3 fatty acid ratio and concluded that the temperature profiles of succinate-cytochrome C reductase activity were sensitive to changes in membrane fatty acid composition . In the present study,the Arrhenius characteristics of succinate-cytochrome C reductase in the obese mouse were unaffected by membrane lipid compositional changes. Presumably, this is because mitochondrial membrane lipid composition in the obese mouse was less extensively altered than in the animals studied by McMurchie et al. (1983c).

Mitochondrial cytochrome oxidase activity from beef heart has been shown to be dependent on the presence of very tightly bound cardiolipin, (at about 5-6 mol. of cardiolipin per mol. of enzyme complex) (Aswachi et al., 1971; Robinson et al., 1980). Vik and Capaldi (1977) incorporated purified cytochrome oxidase into lipid vesicles of defined composition and found that the enzyme did not require specific phospholipid head groups for activity. Cytochrome oxidase activity was similar in phosphatidylcholine and phosphatidylethanolamine lipid vesicles. However, activity was found to vary depending on the nature of the fatty acyl chains of the phospholipids used. Cytochrome oxidase incorporated into dioleoyl-phosphatidylcholine vesicles had higher activity compared to cytochrome oxidase reconstituted with distearoyl-phosphatidyl-choline or dipalmitoyl-phosphatidylcholine. Vik and Capaldi (1977) concluded that cytochrome oxidase exhibited a preference for an unsaturated membrane lipid environment. In the present study, alteration in obese mouse membrane lipid composition involved predominantly linoleic and oleic acid components (section 3.12).

Presumably, these small changes were insufficient to affect the Arrhenius characteristics of cytochrome oxidase.

The ATP-Pi exchange reaction requires coupled mitochondria for activity (Kagawa and Racker, 1971), and is catalysed by the oligomysin sensitive coupling device of mitochondria. The coupling device is comprised of at least 4 soluble and 2 hydrophobic proteins, and has been shown to require phospholipids for activity (Kagawa et al., 1973). Beef heart mitochondria were depleted of phospholipid and respiratory enzyme complexes. The coupling device was then reconstituted with chemically defined phospholipids. The resulting vesicles catalysed the ATP-Pi exchange reaction. Phosphatidylcholine and phosphatidylethanolamine were both necessary for activity and maximal ATP-Pi exchange activity was obtained with equimolar phosphatidylcholine and phosphatidylethanolamine, with the presence of a trace amount of cardiolipin. It was reported that phospholipids containing unsaturated fatty acyl groups were essential for activity. Innis and Clandinin (1981) found that Arrhenius plots of ATP-Pi exchange activity responded to changes in membrane fatty acid composition resulting from dietary manipulation. Arrhenius characteristics of mitochondrial ATP-Pi exchange isolated from rats fed a diet rich in unsaturated fatty acids exhibited an increase in break temperature of $4^{o}C$. This could be reduced by switching the animals to a diet rich in mono-unsaturated fatty acids.

In the present study, Arrhenius characteristics of hepatic mitochondrial ATP-Pi exchange isolated from obese mice exhibited an increase in break temperature with an associated reduction in activation energy above this temperature. These Arrhenius parameters have been shown to change in a reciprocal manner in previous studies (Innis and Clandinin, 1981; McMurchie et al., 1983c). The increase in break temperature in obese mouse preparations may be interpreted as suggesting that a protein, or protein components, of the ATP-Pi exchange reaction sense a change in the molecular organisation or composition of the surrounding membrane lipid. An increase in break temperature is usually associated with an increase in saturation of membrane phospholipid fatty acids. However, in the obese mouse, mitochondrial fatty acid composition was slightly

more unsaturated (see chapter 3, table 3.6). Thyroid hormone treatment, which increased the break temperature of ATP-Pi exchange Arrhenius plots in lean and obese animals, had no effect on the overall unsaturation index of the mitochondrial membrane lipids, except in the case of phosphatidylcholine, which became more unsaturated (table 3.6). Hence, it seems unlikely that the changes in Arrhenius characteristics of mitochondrial ATP-Pi exchange activity of obese mice result from gross changes in membrane lipid unsaturation or membrane fluidity.

An alternative explanation for the occurence of break temperatures in Arrhenius plots of ATP-Pi exchange activity and other mitochondrial processes requiring adenosine nucleotides, has been proposed (Heldt and Klingengerg, 1968; Kemp et al., 1969; Lee and Gear, 1974). It is suggested that the protein catalysing mitochondrial ATP/ADP translocation has a different temperature dependence to ATP synthetase and respiratory chain enzyme complexes. Thus, at lower temperatures, below the Arrhenius break temperatures, ATP-ADP translocation is rate limiting for mitochondrial processes requiring adenosine nucleotides. At temperatures above the Arrhenius discontinuity, ADP-ATP translocation is no longer rate limiting for these processes. If this explanation is correct, mitochondrial ATP-ADP translocation in obese mice is rate limiting at higher temperatures than in lean animals. This might result from a lower ATP-ADP translocase activity in hepatic mitochondria of the obese mice. Thyroid hormones have been shown to regulate the level of ATP-ADP translocase activity in rat liver mitochondria (Mowbray and Corrigoll, 1984) and the apparent hypothyroid state of the obese mouse (Joosten and Van der Kroon, 1974b;Thenen and Carr, 1980) is consistent with a reduced ATP-ADP translocase activity. However, evidence which opposes this argument comes from experiments in which ATP-Pi exchange activity was measured in thyroid hormone treated mice. Thyroid hormone treatment increased the Arrhenius break temperature of ATP-Pi exchange activity in lean and obese mice. Increased ATP-ADP translocase activity associated with hyperthyroidism (Barbior et al., 1973) would be expected to reduce Arrhenius break temperatures of adenosine nucleotide requiring processes. Hence, it seems unlikely that the changes in Arrhenius characteristics of ATP-Pi exchange activity in the obese mouse result from changes

in ATP-ADP translocase activity.

The difference in Arrhenius characteristics of hepatic mitochondrial ATP-Pi exchange activity of lean and obese mice and the changes in the Arrhenius break temperature caused by thyroid hormone treatment may be correlated (inversely) to the linoleic acid content of phosphatidylcholine or phosphatidylethanolamine. These phospholipids contain reduced levels of linoleic acid in obese mouse hepatic mitochondria, whereas the Arrhenius breakpoint of ATP-Pi exchange is increased. Thyroid hormone treatment reduces linoleic acid content of phosphatidylcholine and phosphatidylethanolamine in lean and obese mice and increases the ATP-Pi exchange Arrhenius break temperature. Raison and McMurchie (1983c) altered the fatty acyl composition of rat liver mitochondrial phospholipids by dietary manipulation. They showed that the Arrhenius characteristics of succinate-cytochrome C reductase were different in the animals on the various dietary regimes, whilst the overall unsaturation of the membrane phospholipids remained constant. Hence, the Arrhenius characteristics of this enzyme complex were sensitive to changes in individual fatty acyl components in the membrane.

Arrhenius plot break temperatures were originally associated with the gel to crystalline lipid phase transitions in the membrane. Blazyk and Steim (1972) observed, by differential scanning calorimetry, that broad lipid phase transitions occurred in rat liver mitochondrial and microsomal membranes at temperatures centred around $0^{o}C$. However, most Arrhenius break temperatures of intrinsic proteins in these membranes occur between $20^{o}C$ and $30^{o}C$ (Lenaz et al., 1972; Lee and Gear, 1974; McMurchie et al., 1983a). It was subsequently proposed (Stubbs and Smith, 1984; Sandermann, 1978; Lee et al., 1974; Wunderlich et al., 1975) that Arrhenius break temperatures may result from lipid phase separations or the formation of transient quasi-crystalline lipid clusters which occur in the membrane between $20^{o}C$ and $30^{o}C$.

Studies employing more sensitive differential scanning calorimetry (DSC) reported the occurrence of a second (less pronounced) lipid phase transition centred at $30^{o}C$ in mitochondrial and microsomal membranes (Bach et al., 1977; 1978). It was suggested that this

high melting temperature lipid might be responsible for Arrhenius plot break temperatures. However, McMurchie et al. (1983b) reported that the lipid phase transition of this small pool of lipid was unaffected by dietary induced changes in mitochondrial membrane lipid composition. (Their previous studies revealed that similar dietary induced changes markedly altered mitochondrial enzyme Arrhenius break temperatures (McMurchie and Raison, 1979; McMurchie et al., 1983c)). Hence, the relationship between Arrhenius plot discontinuities and the bulk membrane-lipid phase transition remains unclear.

In the present study, the Arrhenius break temperatures of mitochondrial enzymes in the same membrane, occurred at different temperatures. The break temperature for succinate-cytochrome C reductase was 25°C, for cytochrome oxidase was 20°C , and for ATP-Pi exchange was 19°C in lean mouse hepatic mitochondria. This phenomenom has been observed previously (Lenaz et al., 1972; Lee and Gear, 1974; McMurchie et al., 1983a). It is not consistent with the theory that the break temperature of the enzyme is related to the lipid phase transition of the bulk membrane. It has been suggested that intrinsic proteins in the same membrane may have different lipid compositions in their annular lipid or boundary lipid regions (Lenaz et al., 1972; Innis and Clandinin, 1981). The fatty acyl composition of the boundary phospholipid need not reflect the bulk lipid composition. Hence, if membrane enzymes are sensitive to the lipid phase transitions or changes in molecular organisation that occur in their boundary lipid regions, Arrhenius discontinuities of intrinsic proteins in the same membrane may occur at different temperatures (Lenaz et al., 1972; Silvius and McElhaney, 1980; Innis and Clandinin, 1981).

Direct evidence that boundary lipid regions contain fatty acids of different composition to the bulk bilayer is scarce. The boundary lipid theory was originally proposed by Jost et al.(1973), after electron spin resonance (ESR) studies on reconstituted lipid: cytochrome oxidase vesicles. These authors reported that at low phospholipid:protein ratios, the spin label was immobilized by the protein. At higher phospholipid:protein ratios, the characteristic spectra of fluid lipid bilayers became apparent. Their data was consistent with a single layer of immobilized phospholipid surrounding

the protein. Subsequently, several comparable studies have reported the presence of boundary lipid for various intrinsic membrane proteins. These include myelin apoprotein (Curaloto et al., 1977), cytochrome P_{450} reductase (Stier and Sackmann, 1973), glycorphin (Brulet at McConnell, 1976), cytochrome b_5 (Dehlinger et al., 1974) and $\left[Ca^{2+}Mg^{2+}\right]$-ATPase (Hesketh et al., 1976).

However, conflicting evidence has been produced by ESR and NMR techniques to suggest that the boundary layer surrounding cytochrome oxidase (Jost et al., 1977; Kang et al., 1979) and $\left[Ca^{2+}Mg^{2+}\right]$-ATPase (Hesketh et al., 1976; Rice et al., 1979) is either more or less ordered than the bulk lipid bilayer. Chapman, (1983) has attempted to resolve this apparent controversy by considering the relative time scales of the techniques employed. He pointed out that boundary lipid molecules measured on one time scale e.g. 10^{-8}sec. using ESR techniques, may appear to be rigid whilst viewed on another time scale e.g. 10^{-5}sec. using NMR methods, the same molecules would be mobile or fluid.

Most of these studies have investigated the effect of the intrinsic protein on the adjacent lipid and have not considered the preference of the membrane protein for annular lipid of defined fatty acyl composition. Indirect evidence for the presence of specific phospholipid-fatty acyl groups in the annular lipid, comes from a study on beef heart mitochondria using differential scanning calorimetry (Blazyk and Newman, 1980). These authors reported the presence of a latent pool of high melting lipid in the membrane which was only observed after release by protein denaturation. Thus, it was suggested that this latent pool was associated with the proteins. It was further suggested that this latent pool corresponded to boundary layer lipid which was of different fatty acid composition to the bulk bilayer lipid. Using $\left[^{13}C\right]$-labelled dipalmitoyl phosphatidylcholine as a NMR probe, Brulet and McConnell (1976) identified the boundary lipid region of glycorphin reconstituted into lipid vesicles. Introduction of unsaturated fatty acyl-phosphatidylcholine into the vesicles, modified the NMR spectra. This could be interpreted by suggesting that the unsaturated lipid was preferentially associated with the protein in the boundary layer. Evidence,by differential scanning calorimetry,

that cytochrome oxidase may segregate phospholipids and preferentially select cardiolipin for its boundary layer was reported by Semin et al. (1984).

Finally, studies on lipid-protein interactions of the $[Ca^{2+}Mg^{2+}]$-ATPase suggest a boundary layer or annulus of 30 lipids, which are necessary for enzyme activity (Warren et al., 1975). $[Ca^{2+}Mg^{2+}]$-ATPase was purified and reconstituted in dipalmitoyl-phosphatidylcholine vesicles, which undergo a normal lipid phase transition at 41°C (Hesketh et al., 1976). Using ESR techniques, it was reported that in vesicles of low lipid:protein ratios (less than 30:1), the dipalmitoyl-phosphatidylcholine phase transition was not observed. This only became apparent at higher lipid:protein ratios. It was suggested that the boundary lipid of the $[Ca^{2+}Mg^{2+}]$ATPase could not undergo the normal phase transition at 41°C. Arrhenius characteristics of $[Ca^{2+}Mg^{2+}]$-ATPase activity exhibited break temperatures at around 30°C and 38°C. These were attributed to co-operative structural interactions of annular lipid with the $[Ca^{2+}Mg^{2+}]$-ATPase (Hesketh et al., 1976). Recently, the annular lipid selectivity of the $[Ca^{2+}Mg^{2+}]$-ATPase has been examined (East and Lee, 1982). Using purified enzyme reconstituted with defined phospholipids, the selectivity of lipid binding was determined by fluorescence quenching of the protein by a synthesised brominated phospholipid. These authors reported little selectivity for acyl chain length, although previous studies have suggested that cholesterol is excluded from the boundary lipid (Warren et al., 1975).

In summary, direct evidence for the hypothesis that the boundary lipid regions of intrinsic proteins contain lipid of different chemical composition to the bulk bilayer is scarce, although most authors agree that the phospholipids in the boundary layer region exchange with those in the bulk bilayer (Hesketh et al., 1976; Jost et al., 1977; Brulet and McConnell, 1976; Rice et al., 1979; Chapman, 1983). However, the implications of this hypothesis for the present study on obese mice may still be considered. In this study, the fatty acid compositions of phospholipids in obese mouse liver mitochondria were altered, whilst Arrhenius characteristics of membrane enzymes were unaffected. Arrhenius characteristics may only be changed if abnormal fatty acyl groups penetrate the

boundary layer region of the protein. This will depend on the degree of selectivity the intrinsic protein has for phospholipids or fatty acyl groups.

In a recent study, Abeywardena et al. (1984) altered the composition of rat heart muscle sarcoplasmic reticulum membranes by dietary modulation, but were unable to change the specific activities or Arrhenius characteristics of ATPase enzymes associated with the membranes. One possible explanation was that the intrinsic proteins were buffered from the changes in composition of the bulk membrane by their boundary layer regions. Hence, for intrinsic proteins that are able to select out phospholipids or fatty acyl groups, change in activity or Arrhenius characteristics may only occur if membrane compositional changes are extensive.

CHAPTER 6

ALTERED MEMBRANE LIPID ENVIRONMENT AND BROWN ADIPOSE TISSUE MITOCHONDRIAL THERMOGENESIS IN LEAN AND OBESE MICE

6.1 Introduction

In the previous chapter, the effects of alterations to membrane lipid composition on hepatic mitochondrial intrinsic membrane proteins of obese mice were investigated. Lipid-protein interactions were examined by the construction of Arrhenius plots. However, most of the enzymes studied were unresponsive to the changes in their membrane lipid environment.

The work presented in this chapter is concerned with brown adipose tissue (BAT) which has been suggested as the primary site of defective thermogenesis in the obese mouse (Thurlby and Trayhurn, 1980). The mechanism of BAT thermogenesis and the evidence for a defect in BAT thermogenesis of obese mice have been briefly reviewed in this thesis (see section 1.8). Fluorescence polarisation studies indicated that BAT mitochondrial membranes of obese mice had altered lipid composition or different molecular organisation to those from lean animals (York et al., 1982). GLC analysis of phospholipid fatty acid composition has been performed to further characterise this abnormality. BAT mitochondrial thermogenesis in lean and obese mice has been assessed by the GDP binding technique. Previous studies have indicated that GDP binding is reduced in obese mice housed at room temperature and fails to respond either to acute or chronic stimuli (Himms-Hagen and Desautels, 1978; Trayhurn et al., 1982; Holt and York, 1984). Chronic stimulation of BAT in normal rats or mice (e.g. cold acclimation for 2 weeks) results in tissue hypertrophy, increased mitochondrial number and protein synthesis and also specific synthesis of the 32K dalton mitochondrial protein (Desautels et al., 1978; Ashwell et al., 1983). Chronic stimulation may also lead to changes in the fatty acid composition of BAT mitochondrial phospholipids (Ricquier et al., 1975), although this finding has been contested (Cannon et al., 1975).

The mechanism by which BAT mitochondrial thermogenesis responds to acute stimulation is unclear. This process involves a rapid increase in the number of GDP binding sites and is independent of protein synthesis (Desautels and Himms-Hagen, 1979). The possible involvement of membrane compositional changes in this mechanism has been investigated in the present study. In addition, GDP binding

to BAT mitochondria of lean and obese mice has been characterised by Scatchard analysis. Previous studies with the insulin receptor system indicate that insulin binding was responsive to membrane lipid compositional changes. Gould et al. (1982) reconstituted purified insulin receptors into phospholipid vesicles comprised of, either saturated dimyristoyl phosphatidylcholine, or unsaturated soy phosphatidylcholine. They characterised insulin binding to the different receptor preparations by Scatchard analysis and reported that the receptors incorporated into the saturated lipid environment had a higher affinity for insulin than those incorporated into the unsaturated lipid environment. Thus, membrane compositional changes may alter the binding parameters of BAT mitochondrial GDP binding in obese mice.

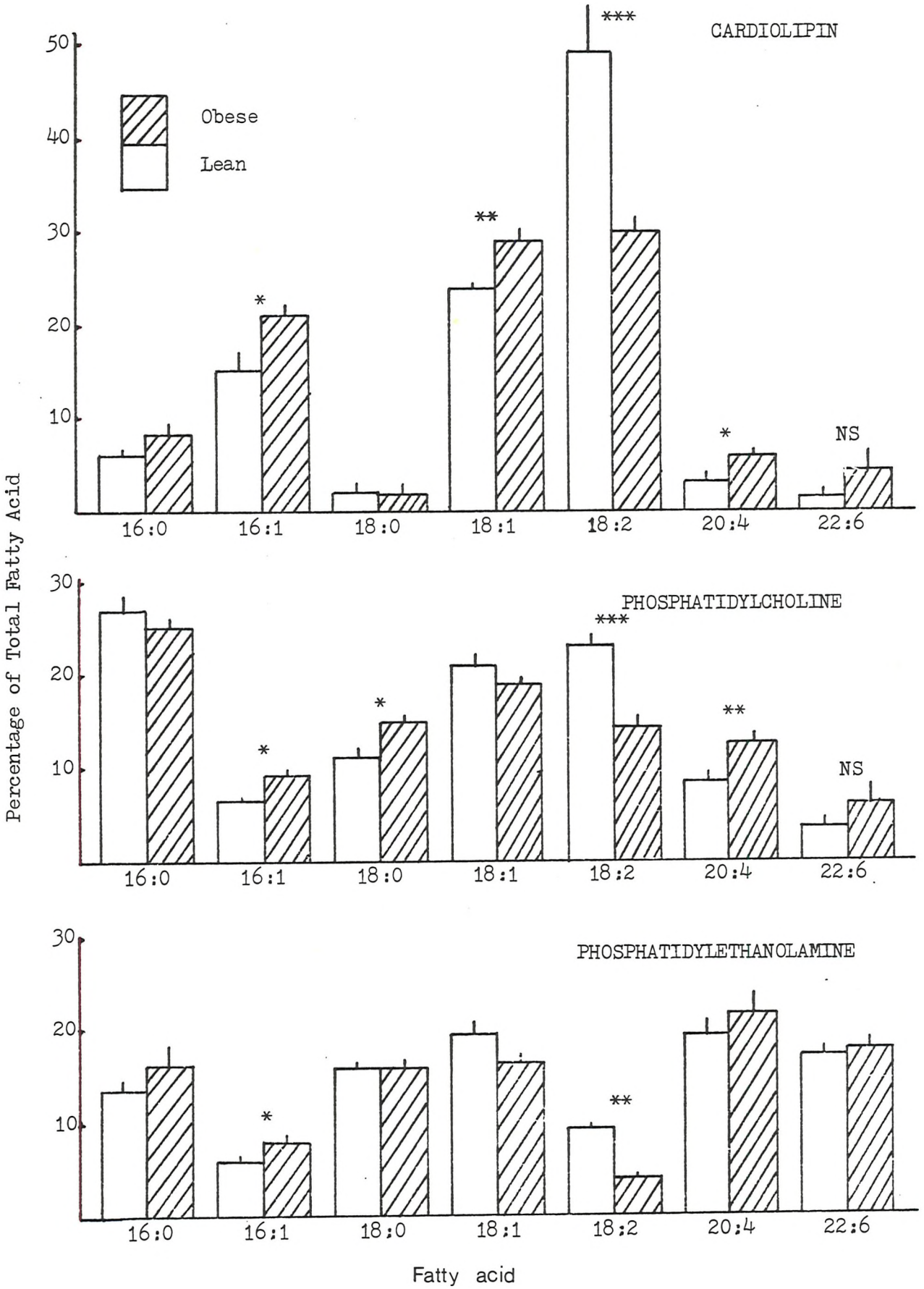
Results

6.2 Fatty Acid Composition of BAT Mitochondrial Phospholipids

The fatty acid composition of phospholipids extracted from lean and obese mouse BAT mitochondria are shown in figure 6.1. The fatty acid components of BAT mitochondrial phospholipids were similar to those from hepatic mitochondria except for the presence of singificant amounts of palmitoleic aicd (16:1 ω9) in all phospholipid subclasses of BAT mitochondria. Alteration in fatty acyl profiles were apparent in all of the phospholipid subclasses from obese mouse BAT mitochondria. In all the phospholipids, the changes followed a similar pattern. Reduced linoleic acid (18:2 ω6) content in obese mouse phospholipids was compensated for mainly by an increase in monoenoic fatty acid components and arachidonic acid (20:4 ω6). In phosphatidylcholine, the linoleic acid (18:2 ω6) content was reduced from 23% to 14% of the total whereas the level of arachidonic acid (20:4 ω6) had increased from 8.2% to 12.5% of total. Palmitoleic acid (16:1 ω9) content was also increased (from 6.4% to 9.2% of total) as was the stearic acid (18:0) content (from 11.1% to 14.8% of total) in BAT mitochondrial phosphatidylcholine from obese mice. In phosphatidylethanolamine, the changes were less pronounced ; linoleic acid (18:2 ω6) content was reduced (from 9.3% to 3.9% of total) and palmitoleic acid (16:1 ω9) content increased (from 5.8% to 8% of total). The largest alterations in fatty acyl composition of BAT

Fig 6.1 The Fatty Acid Profiles of BAT Mitochondrial Phospholipids from Lean and Obese Mice

BAT mitochondria were prepared from 4 lean and 4 obese mice as described in section 2.4 and phospholipids extracted, separated and analysed by GLC as described in section 2.13. The data represents the means $\pm$ SEM of three separate BAT mitochondrial preparations. * $p<0.05$; ** $p<0.01$; *** $p<0.005$ (students 't' test for paired data).


CARDIOLIPIN
Obese
Lean
50
40
30
20
10
***
**
*
*
NS
16:0
16:1
18:0
18:1
18:2
20:4
22:6
PHOSPHATIDYLCHOLINE
30
20
10
*
*
***
**
NS
16:0
16:1
18:0
18:1
18:2
20:4
22:6
PHOSPHATIDYLETHANOLAMINE
30
20
10
*
**
16:0
16:1
18:0
18:1
18:2
20:4
22:6
Percentage of Total Fatty Acid
Fatty acid

	18:1 ω9/18:2 ω6 Ratio		Unsaturation Index	
	Lean	Obese	Lean	Obese
Phosphatidylcholine	0.95 ± .12	1.25 ± .14	127 ± 2	142 ± 6 *
Phosphatidylethanolamine	1.96 ± .18	3.48 ± .50 ***	222 ± 4	225 ± 7
Cardiolipin	0.46 ± .03	0.92 ± .05 ***	157 ± 1	159 ± 4

Table 6.1 18:1 ω9/18:2 ω6 Ratios and Unsaturation Indices of BAT Mitochondrial Phospholipids from Lean and Obese Mice

BAT mitochondria were prepared from 4 lean and 4 obese mice as described in section 2.4 and phospholipids extracted, separated and analysed by GLC as described in section 2.13. The data represents the means ± SEM of three separate BAT mitochondrial preparations. * $p<0.05$; ** $p<0.01$; *** $p<0.005$ (students 't' test for paired data)

Phospholipid (nmoles/mg)	Lean (n=8)	Obese (n=6)
Phosphatidylcholine	31.7 ± 2.7	32.5 ± 4.3
Phosphatidylethanolamine	46.4 ± 5.4	49.5 ± 5.6
Cardiolipin	26.4 ± 3.0	18.6 ± 2.0
Total	104.5	100.6

Table 6.2 Phospholipid Content of BAT Mitochondria from Lean and Obese Mice

BAT mitochondria were prepared from lean and obese mice as described in section 2.4 and phospholipids extracted and separated as described in section 2.13. The phospholipids were measured as described in section 2.16. Values represent the means ± SEM for the number of preparations (n) shown in parentheses.

mitochondrial phospholipids of the obese mouse were apparent in cardiolipin where the level of linoleic acid was reduced from 49% to 30% of total. This was compensated for by increases in several fatty acyl components including palmitoleic acid (increased from 14.9% to 21% of total), oleic acid (increased from 23.5% to 29% of total) and arachidonic acid (increased from 2.9% to 5.7% of total).

Despite these changes, the double bond numbers for phospholipid fatty acid content were similar in lean and obese animals, except in the case of phosphatidylcholine. Here, the double bond number had increased in the obese mouse from 127 to 142 (table 6.1). In contrast, the 18:1 ω9/18:2 ω6 ratio was increased in all of the phospholipids of the obese mouse, (table 6.1) mainly because of the reduction in 18:2 ω6 levels. Table 6.2 shows that the contribution of the individual phospholipid subclasses to the total phospholipid content was similar in lean and obese mouse BAT mitochondria.

6.3 Characterisation of GDP Binding to Murine BAT Mitochondria

The quantity of BAT mitochondria that can be isolated from mice is low (approximately 0.5 mg. mitochondrial protein per animal). In order to facilitate economic use of animals, the incubation volume in GDP binding assays were reduced by 50%. Characterisation of purine nucleotide binding to BAT mitochondria has been well documented (Nicholls,1976 ; Sundin and Cannon, 1980 ; Holt, 1984). However, in view of the modification made in the present study, preliminary work was carried out to examine the effect of mitochondrial protein and purine nucleotide concentration on the assay of GDP binding to BAT mitochondria. Figure 6.2 shows the equilibrium binding of 10 μM GDP at different BAT mitochondrial protein concentrations. BAT mitochondria were isolated from lean mice housed either under control conditions (22-24°C) or exposed to cold (4°C) for 17 hours. For both mitochondrial preparations, GDP binding was linear over the protein concentration range from 50 to 150 μg protein. As expected, GDP binding was substantially higher in the mitochondrial preparation isolated from the cold exposed mice. The GDP binding values obtained were 140 and 290 pmol/mg. mitochondrial protein for control and 17 hour cold exposed mice respectively. In subsequent experiments, between 80 and 140 μg of mitochondrial protein was used.

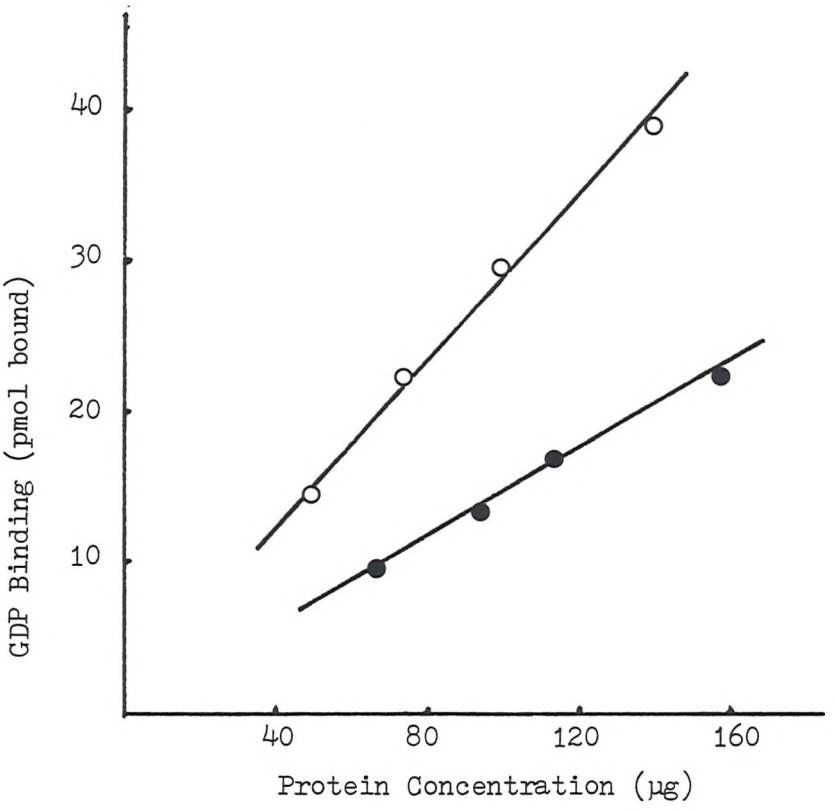


Fig 6.2 The Effect of Protein Concentration on BAT Mitochondrial GDP Binding of Lean Mice Housed at Room Temperature and Lean Mice Cold Exposed Over Night

BAT mitochondria were prepared from 4 lean mice housed at room temperature (●) or housed at a 4°C environment for 17 hours (○) as described in section 2.4. BAT mitochondrial GDP binding was assessed as described in section 2.12 using the mitochondrial protein concentrations shown.

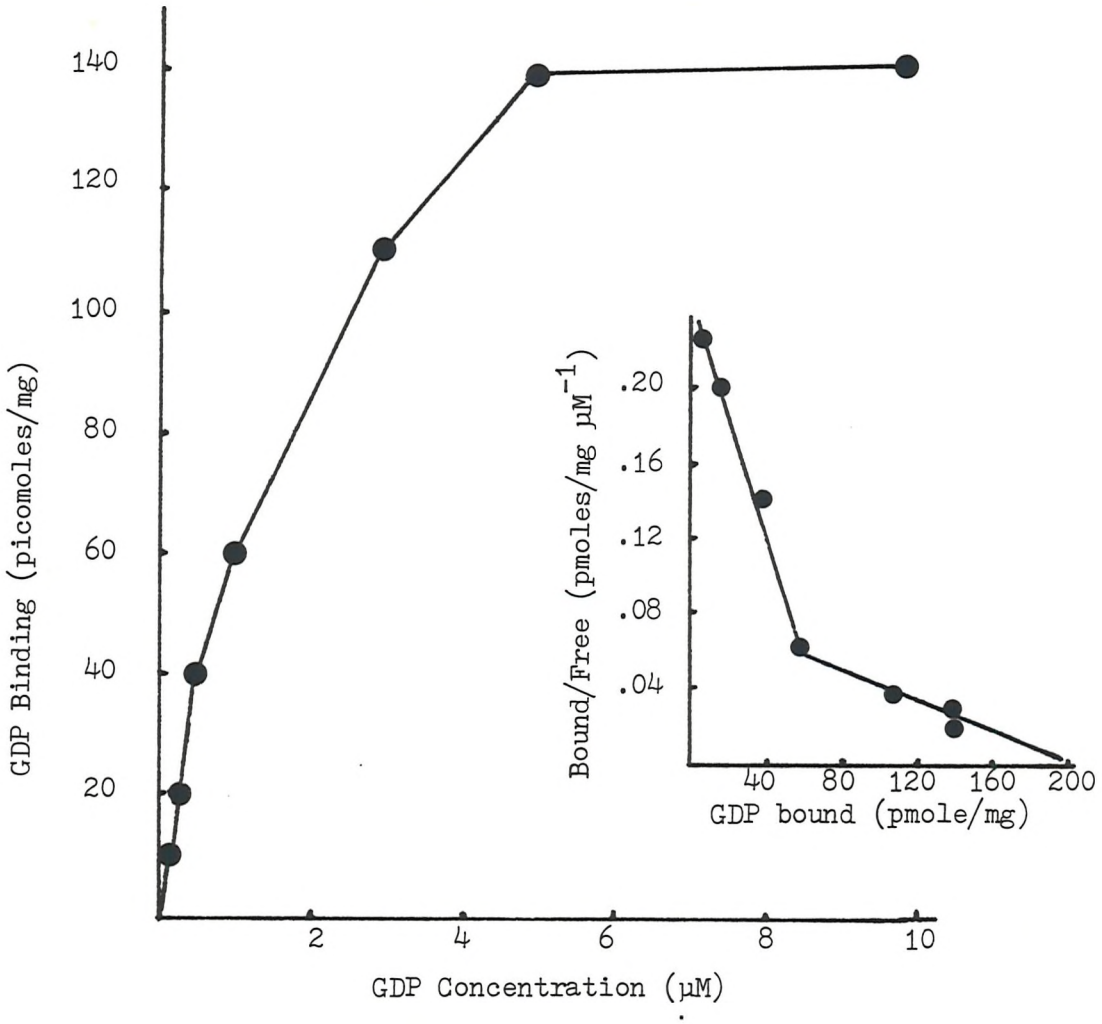


Fig 6.3 The Effect of GDP Concentration on BAT Mitochondrial GDP Binding of Lean (control) Mice

BAT mitochondria were prepared from 6 lean mice as described in section 2.4. BAT mitochondrial GDP binding was assessed over a range of GDP concentrations from 0.05 μM to 10 μM as described in section 2.12. Scatchard analysis was performed on this data (inset).

Figure 6.3 shows the specific GDP binding to BAT mitochondria (0.1 mg) from mice housed under control conditions over a range of purine nucleotide concentrations from 0.05 to 10 μM. Specific GDP binding was saturable and maximum at concentrations of 5 μM GDP and above. At these concentrations the amount of GDP bound was 140 picomoles per mg. protein. In subsequent experiments, the concentration of GDP routinely used was 5 μM. This data was analysed by Scatchard determination (figure 6.3, inset), which produced a biphasic Scatchard plot indicative of two types of GDP binding sites. Detailed examination of Scatchard plots of GDP binding to lean and obese mouse BAT mitochondria is presented later in section 6.6.

6.4 Effect of 4°C Cold Exposure on BAT Mitochondrial GDP Binding and the Fatty Acid Composition of Phospholipids

Ricquier et al.(1975) have reported that the fatty acid composition of total phospholipid is altered in BAT mitochondrial membranes from chronically cold exposed rats. In view of the extensive alteration to fatty acid composition of BAT mitochondrial phospholipids in the obese mouse, an investigation was undertaken to examine the possible involvement of membrane compositional changes in the mechanism of increased GDP binding in BAT mitochondria on cold exposure. Large increases in GDP binding occur after a few hours of cold exposure in the apparent absence of any increases in the amount of 32K dalton protein (Desautels and Himms-Hagen, 1979). The precise mechanism of this 'unmasking' effect is unclear.

In the present investigation, lean mice were exposed to a 4°C environment for 0, 4 and 8 hours. BAT mitochondria were isolated from the animals, and these were assayed for their GDP binding capacity and used for determination of phospholipid fatty acid composition. In confirmation of previous studies, the GDP binding capacity of lean mouse BAT mitochondria increased dramatically within a few hours of exposure to a 4°C enviroment (table 6.3). After 4 hours cold exposure, GDP binding had increased by 50%. Exposure for a further 4 hours, gave an additional but less pronounced increase in GDP binding to BAT mitochondria (63% higher than in control animals).

During the period of cold exposure, no substantial changes in fatty

	Duration of Cold Exposure(hrs)		
	0	4	8
Phosphatidylcholine			
18:1 ω9/18:2 ω6	0.86	0.93	0.86
Unsaturation Index	128	115	121
Phosphatidylethanolamine			
18:1 ω9/18:2 ω6	2.0	2.0	1.9
Unsaturation Index	199	214	210
Cardiolipin			
18:1 ω9/18:2 ω6	0.51	0.65	0.56
Unsaturation Index	149	152	148
BAT Mitochondrial GDP Binding (pmol/mg)	135	203	220

Table 6.3 The Effect of Cold Exposure on BAT Mitochondrial GDP Binding, and Unsaturation Indices and 18:1 ω9/18:2 ω6 Ratios of BAT Mitochondrial Phospholipids of Lean Mice

6 lean mice were exposed to a 4°C environment for 0, 4 or 8 hours. BAT mitochondria were prepared as described in section 2.4 and BAT mitochondrial GDP binding assessed as described in section 2.12. Phospholipids were extracted from the mitochondria, separated and analysed by GLC as described in section 2.13.

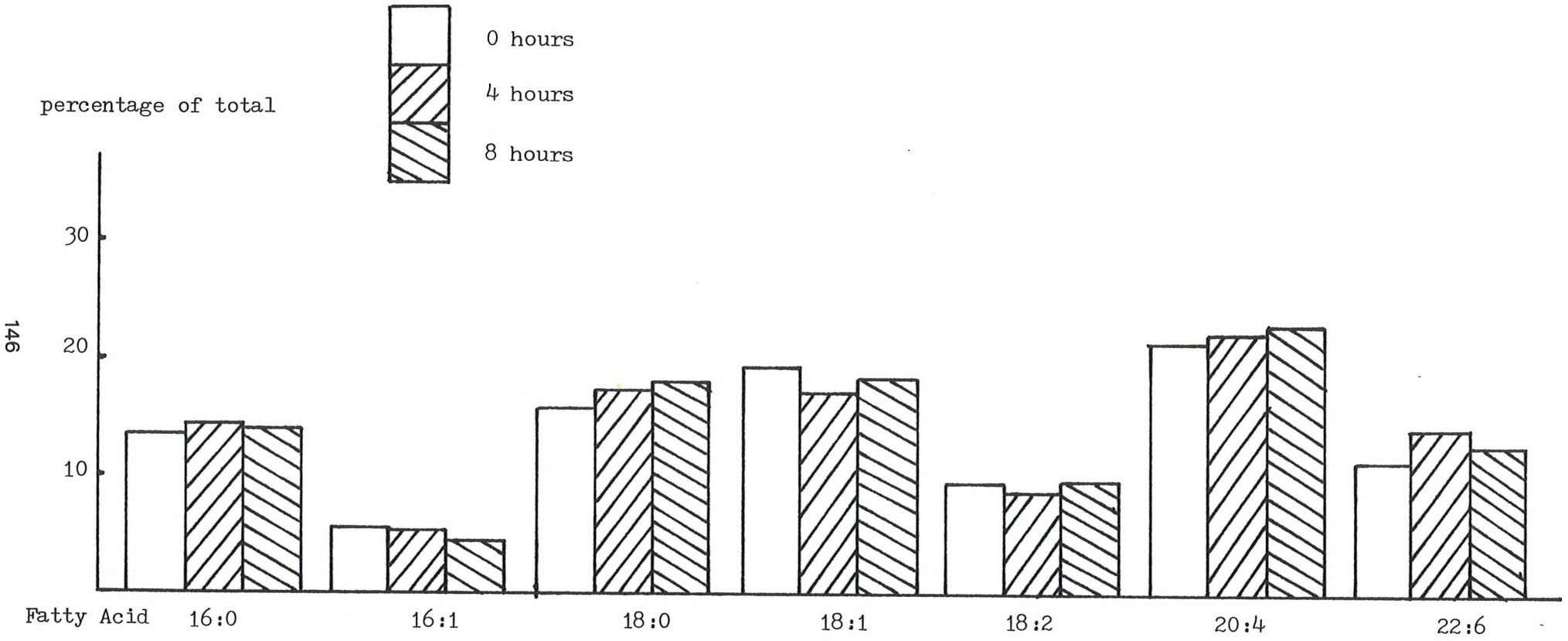


Fig 6.4a The Effect of Cold Exposure on the Fatty Acid Profiles of BAT Mitochondrial Phosphatidylethanolamine from Lean Mice.

6 lean mice were exposed to a 4°C environment for either 0, 4 or 8 hours. BAT mitochondria were prepared as described in section 2.4 and phospholipids extracted, separated and analysed by GLC as described in section 2.13.

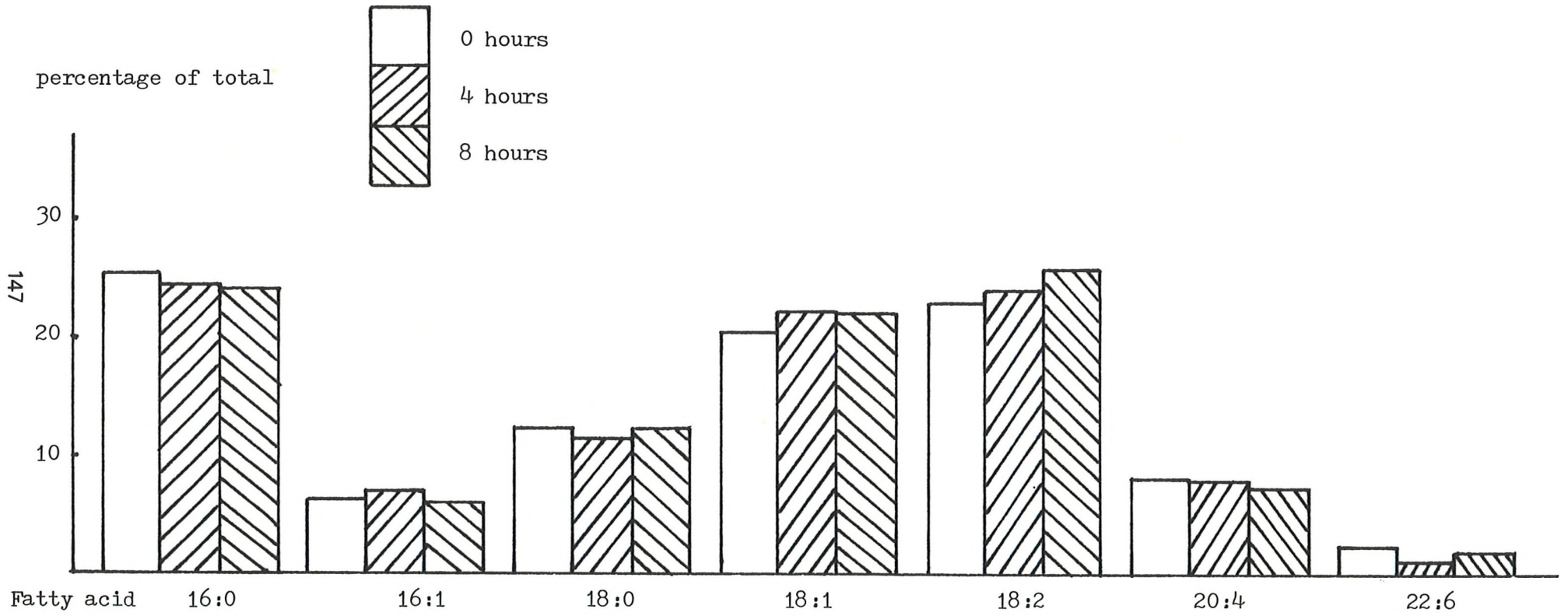


Fig 6.4b The Effect of Cold Exposure on the Fatty Acid Profiles of BAT Mitochondrial Phosphatidylcholine from Lean Mice.

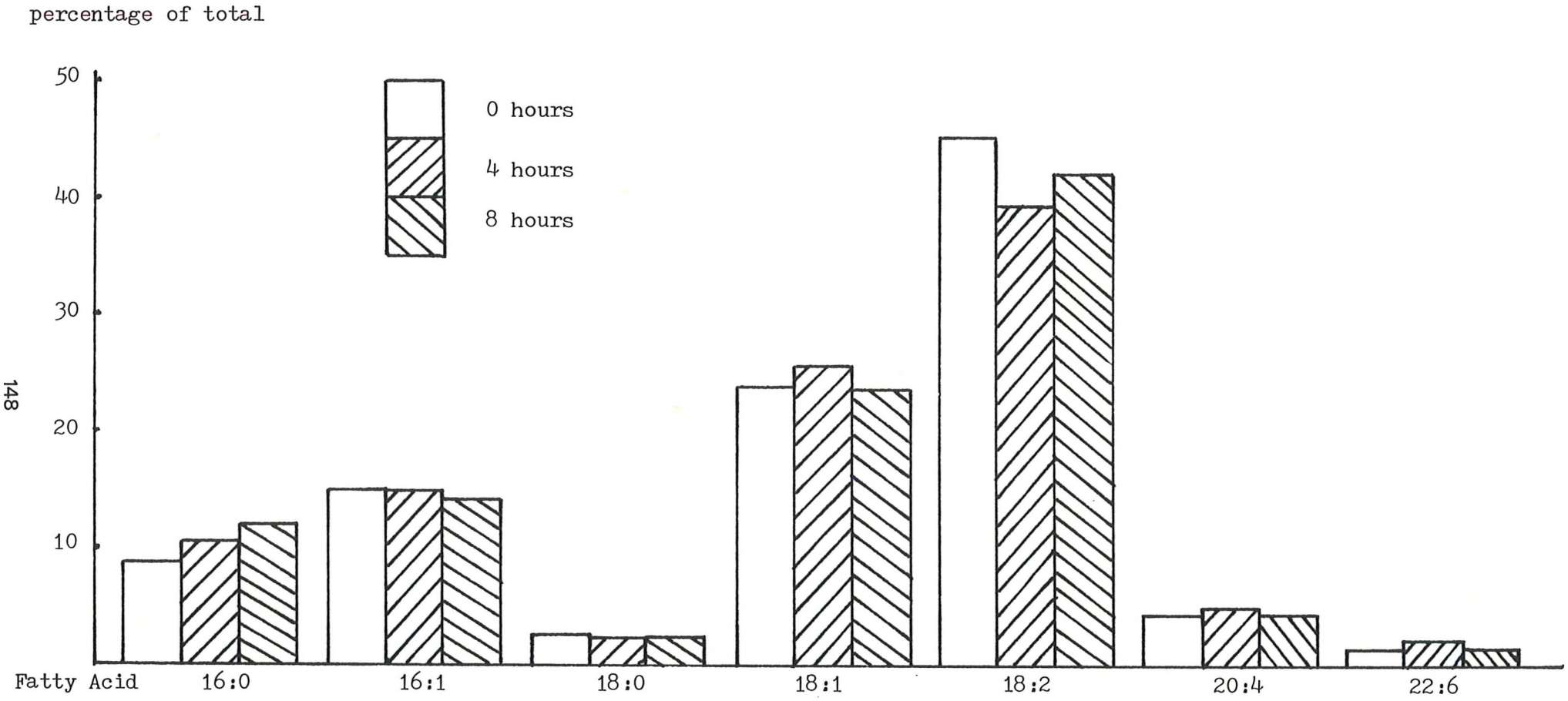


Fig. 6.4c The Effect of Cold Exposure on the Fatty Acid Profiles of BAT Mitochondrial Cardiolipin from Lean Mice.

acid composition were observed in phospholipids from the BAT mitochondria (figure 6.4). Small time dependent changes were apparent in the palmitic acid content (6% reduction after 8 hours exposure) and linoleic acid content (12% increase after 8 hours) of phosphatidylcholine, the stearic acid content (13% increase after 8 hours) of phosphatidylethanolamine and the palmitic acid content (30% increase after 8 hours) of cardiolipin. However, it seems unlikely that these small changes were involved in the 63% increase in BAT mitochondrial GDP binding observed in the lean mice after 8 hours of cold exposure. No substantial changes in the unsaturation indices of any of the mitochondrial phospholipids was observed during the period of cold exposure (table 6.3).

6.5 Comparison of GDP Binding and Succinate-cytochrome C Reductase Activity in BAT Mitochondria from Lean and Obese Mice Housed Under Control Conditions

The purine nucleotide binding capacity of BAT mitochondria isolated from lean and obese mice housed at 23°C is shown in table 6.4. No significant difference was apparent in GDP binding between lean and obese mouse preparations whether the data was expressed per mg. mitochondrial protein or when calculated to obtain the binding capacity per brown fat tissue depot. This finding was unusual since previous studies have reported that GDP binding to BAT mitochondria was reduced in obese mice housed at room temperature (Himms-Hagen and Desautels, 1978; Trayhurn et al., 1982; Holt and York, 1984). Measurement of mitochondrial marker enzyme activity (succinate-cytochrome C reductase) in BAT homogenates and mitochondrial preparations indicated that the mitochondria from the obese animals were less contaminated (with protein) than the corresponding lean mouse preparations. However, the difference in purification was not statistically significant. Calculation of GDP binding capacity per tissue depot takes into account the percentage recovery of mitochondrial succinate-cytochrome C reductase activity. GDP binding per BAT depot indicated no apparent difference between lean and obese animals. Rectal temperature measurements indicated that obese animals were hypothermic when housed at 23°C. A significant reduction in rectal temperature of 0.8°C was apparent in obese mice when compared to lean animals.

		Lean (n=13)	Obese (n=13)
Succinate-Cytochrome C Reductase (μmoles/min/mg)			
	Homogenates	0.09 ± 0.01	0.07 ± 0.01
	Mitochondria	0.36 ± 0.03	0.31 ± 0.02
	Purification	4.20 ± 0.40	4.80 ± 0.40
GDP Binding			
	(pmol/mg)	120 ± 10	127 ± 8
	(pmol/BAT depot)	298 ± 21	282 ± 26
Rectal Temperature		35.0 ± 0.3	34.2 ± 0.2 **

Table 6.4 Succinate-Cytochrome C Reductase Activity and GDP Binding in BAT Mitochondria from Lean and Obese Mice

BAT mitochondria were prepared from lean and obese mice as described in section 2.4 and BAT mitochondrial GDP binding assessed as described in section 2.12. Succinate-cytochrome C reductase activity was assayed in BAT homogenates and mitochondrial preparations as described in section 2.8. Rectal temperatures of the mice were measured 1 hour prior to sacrifice. The values represent the mean ± SEM for the number of preparations (n) shown in parentheses.
** $p < 0.01$

6.6 Scatchard Analysis of GDP Binding to BAT Mitochondria From Lean and Obese Mice

Purine nucleotide binding to BAT mitochondria isolated from lean and obese mice was further characterised by Scatchard analysis. Binding assays were carried out over a range of GDP concentrations from 0.05 to 10 μM and typical Scatchard plots obtained are shown in figure 6.5. Scatchard analysis binding parameters are summarised in table 6.5.

Scatchard plots of GDP binding to BAT mitochondria were biphasic. This indicates the presence of two populations of GDP binding sites in the mitochondrial membrane. One type exhibits a high affinity and the other a low affinity for purine nucleotides. Scatchard plots of GDP binding to BAT mitochondria from lean and obese mice were similar. As has been indicated above, using one concentration GDP binding assays, the maximum numbers of low affinity binding sites were similar in lean and obese animals (at 165 and 189 picomoles/mg for lean and obese mice respectively). The maximum number of high affinity binding sites was also similar at 70 and 88 picomoles/mg. for lean and obese mice respectively. The dissociation constant for GDP binding to the high affinity site was increased (by 45%) in BAT mitochondrial preparations from obese mice. However, this increase was not statistically significant. The dissociation constants for the low affinity binding sites were similar, at 1.61 and 1.77 μM in lean and obese mouse mitochondrial preparations respectively.

6.7 GDP Binding to BAT Mitochondria in Control and 1 Hour Cold Exposed Lean and Obese Mice

In view of the similar BAT mitochondrial GDP binding capacity in lean and obese mice housed at 23°C (as indicated above) experiments were undertaken to evaluate the response of GDP binding to cold exposure in lean and obese animals. 5-6 week old lean and obese mice were exposed to a 4°C environment for 1 hour, BAT mitochondria were isolated and GDP binding capacity compared to that from control animals, housed at 23°C. More prolonged cold exposure has been shown to cause hypothermia and death of obese animals (Trayhurn and James, 1978), hence the period of cold exposure was restricted to 1 hour.

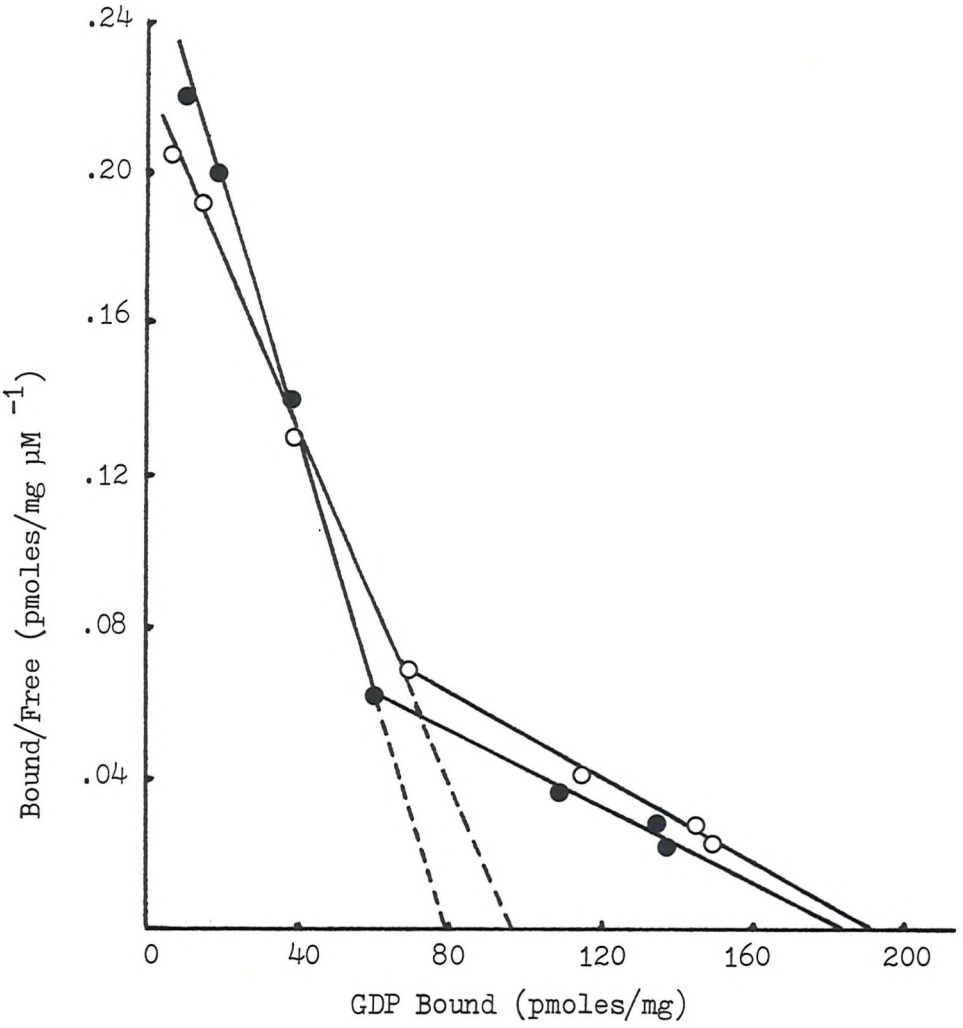


Fig 6.5 Scatchard Plots of GDP Binding to BAT Mitochondria from Lean and Obese Mice

BAT mitochondria were prepared from 6 lean (●) and 6 obese (○) mice as described in section 2.4. BAT mitochondrial GDP binding was assessed over the range of GDP concentrations from 0.05 µM to 10 µM as described in section 2.12. Scatchard analysis was then performed on this data.

	Binding Site			
	High Affinity		Low Affinity	
	K_D (μM)	Bmax(pmol/mg)	K_D (μM)	Bmax(pmol/mg)
Lean (n=5)	0.33 ± 0.04	70 ± 10	1.61 ± 0.28	165 ± 19
	NS			
Obese (n=3)	0.48 ± 0.09	88 ± 18	1.77 ± 0.22	187 ± 12

Table 6.5 Summary of Scatchard Analysis Parameters of BAT Mitochondrial GDP Binding of Lean and Obese Mice

BAT mitochondria were prepared from 6 lean and 6 obese mice as described in section 2.4. BAT mitochondrial GDP binding was assessed over the range of GDP concentrations from 0.05 μM to 10 μM as described in section 2.12. Scatchard analysis was then performed on this data. The values represent the means ± SEM of the number of BAT mitochondrial preparations (n) shown in parentheses. NS not significant.

Succinate-Cytochrome C Reductase Activity (μmoles/min/mg)		Lean (n=4)		Obese (n=4)	
		Control	4°C Exposed	Control	4°C Exposed
	Homogenates	0.12 ± 0.01	0.11 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
	Mitochondria	0.41 ± 0.04	0.36 ± 0.03	0.32 ± 0.04	0.33 ± 0.06
	% Recovery of Activity	24 ± 4	20 ± 2	25 ± 1	25 ± 1
GDP Binding	Per mg	123 ± 4	170 ± 9 ***	125 ± 12	171 ± 18 *
	Per depot	298 ± 46	430 ± 44 *	284 ± 7	383 ± 33 *

Table 6.6 GDP Binding and Succinate-Cytochrome C Reductase Activity in BAT Mitochondria of Lean and Obese Mice Under Control Conditions and Cold Exposed for 1 Hour

Lean and obese mice, housed in pairs, were maintained at room temperature or exposed to a 4°C environment for 1 hour. BAT mitochondria were prepared as described in section 2.4 and BAT mitochondrial GDP binding assessed as described in section 2.12. Succinate-cytochrome C reductase activity was assayed in the BAT homogenates and mitochondrial preparations as described in section 2.8. The values represent the means ± SEM for 4 BAT mitochondrial preparations in each group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$ compared to the corresponding control group.

GDP binding to BAT mitochondria in control lean and obese mice was similar at 123 pmol/mg. (table 6.6). Exposure to a 4°C environment for 1 hour elicited a similar increase (38%) in GDP binding per mg. protein in lean and obese animals (table 6.6). From the standard errors of this data, it is evident that a much more variable response was given by the obese mice on cold exposure. BAT homogenates from obese mice exhibited a 38% reduction in succinate-cytochrome C reductase activity. However, when GDP binding capacity was calculated per tissue depot, no statistical differences in GDP binding were observed between lean and obese control mice, or between lean and obese mice exposed to a 4°C environment. The data indicates that BAT thermogenesis in obese mice, assessed by the GDP binding assay, responds normally to a one hour (4°C) cold exposure.

6.8 Discussion

Analysis of the fatty acid composition of BAT mitochondrial phospholipids indicated extensive alteration to EFA, PUFA and monoenoic fatty acid components, in the obese mouse. The changes observed in fatty acid components of BAT mitochondrial phospholipids were similar to the changes found in hepatic mitochondrial and microsomal phospholipids also reported in this thesis. However, in BAT, increases in monoenoic fatty acid were mainly associated with palmitoleic acid (16:1 ω9) rather than with oleic acid (18:1 ω9) as found in hepatic mitochondria of the obese mouse. The changes in PUFA composition in the obese mouse varied in severity, depending on tissue or subcellular location, whereas the linoleic acid (18:2 ω6) content was consistently reduced in virtually all membrane phospholipids studied. This seems to be the case for BAT and hepatic mitochondrial membranes, hepatic microsomal membranes, adipocyte plasma membranes and liver plasma membranes (Rouer et al., 1980; Hyslop et al., 1982; York et al., 1982; French et al., 1983).

The observation that the fatty acid compositions of BAT mitochondrial phospholipids were extensively altered, prompted an investigation into the possible involvement of these membrane compositional changes in the defective thermogenic mechanism of BAT mitochondria in obese mice. The capacity of BAT mitochondria for thermogenesis

in lean and obese mice was assessed by the GDP binding technique. Previous studies have shown that GDP binding to BAT mitochondria of obese mice housed at room temperature was reduced (Himms-Hagen and Desautels, 1978; Trayhurn et al., 1982; Holt and York, 1984). However, in the present investigation, similar levels of binding were observed for preparations from lean and obese animals. The reason for the apparent discrepancy between the data presented in this thesis and that from other studies is unclear. It is unlikely that this discrepancy results from a difference in the strains of obese mice used, since previous studies from this laboratory have reported reduced BAT mitochondrial GDP binding in obese animals (Holt and York, 1984).

Himms-Hagen found that GDP binding in lean mice aged 8 weeks housed at 28°C was of the order of 80-100 picomoles/mg. (Himms-Hagen and Desautels, 1978; Hogan and Himms-Hagen, 1980) whereas Trayhurn et al. (1982) using 7 week old mice housed at 23°C reported a value for lean control GDP binding of 345 picomoles/mg. The reason for this large difference in BAT mitochondrial GDP binding in the two studies is unclear. It is unlikely that the difference in housing temperature is the cause of the large difference in GDP binding values reported by these two groups since Hogan and Himms-Hagen (1980) found that exposing lean mice to a 14°C environment for 2 weeks only increased binding to approximately 160 picomoles/mg. It would appear that the thermogenic capacity of BAT mitochondria as assessed by GDP binding varies greatly in the different obese mouse strains.

The similarity in BAT mitochondrial thermogenesis of lean and obese mice as indicated by GDP binding in the present study, may reflect a less severe hypothermia of these obese animals. This may be indicated by their less pronounced reduction in rectal temperature when compared to the reduced rectal temperatures of obese animals in other studies. Trayhurn and James (1978) reported that the rectal temperatures of obese mice housed at 23°C were 2°C lower than their lean counterparts, whereas in this study the reduction in temperature was much smaller (less than 1°C). The normal BAT thermogenic capacity of obese mice and their reduced rectal temperatures at 23°C reported in this study, indicates the presence of some other defective thermogenic mechanism in the animals. Possible candidates

for this role could include the reduced protein turnover (Miller et al., 1979) or reduced $\left[Na^{+}K^{+}\right]$-ATPase activity in liver and muscle (York et al., 1978a; Lin et al., 1979) of obese animals.

Scatchard analysis confirmed the similarity in GDP binding between BAT mitochondria from lean and obese mice. Scatchard plots were biphasic, indicating 2 populations of binding site characterised by high or low affinity GDP binding. Previous studies have reported linear Scatchard plots of GDP binding to BAT mitochondria suggesting only one type of binding site (Sundin and Cannon, 1980; Goodbody and Trayhurn, 1982). More recently, using a wider range in GDP binding concentrations, two classes of binding site have been discerned (Bryant et al., 1983; 1984; Holt, 1984). The present work indicates that specific GDP binding is saturated at 5 µM GDP, in agreement with Bryant et al. (1983; 1984). Bryant et al.(1983;1984) have reported that the number and affinity of both classes of binding site vary with the thermogenic status of the BAT mitochondria. Acute noradrenaline treatment increased both the dissociation constants (K_D)and maximum binding site number (Bmax) of the high and the low affinity site. Their work also indicated that the two types of binding site were interconvertible. Incubation of BAT mitochondria at 37°C for 30 minutes prior to the assay (at 24°C) lead to the disappearence of the high affinity binding site on Scatchard analysis. The high affinity site could be partially recovered by returning the mitochondria to 0°C for 15 minutes prior to the assay. Interconversion of the two types of binding site suggests that they are possibly differentiated by a protein conformational change. Alternatively, the differences in GDP binding affinities may result from changes in the membrane lipid environment of the receptor. However, evidence to discount this second possibility comes from the present study. Extensive alteration to the membrane lipid composition of BAT mitochondria of obese mice had no significant effect on the GDP binding parameters of either type of binding site, when the parameters were compared to those of the lean animals.

Evidence presented above from studies on GDP binding to BAT mitochondria at a single concentration or over a range of concentrations indicate that the obese mice used in this study have a normal capacity for BAT mitochondrial thermogenesis when

housed at room temperature. However, it must be emphasised that BAT thermogenesis as indicated by GDP binding in these obese animals is impaired in relation to their food intake. The food intake of obese mice in the experiments presented in this thesis was 40% greater than that of their lean littermates. Hence the similar levels of GDP binding observed in lean and obese mice indicate that the obese animals failed to stimulate their BAT diet induced thermogenesis.

It was of interest, in the present study, to examine the response of BAT thermogenesis to "cold" stimulation in lean and obese mice. After exposure to a 4°C environment, BAT in rodents responds in a manner that can be divided into two phases. Initially, and within a few hours, a large increase in BAT mitochondrial GDP binding occurs. This has been termed an 'unmasking' of GDP binding sites (Desautels and Himms-Hagen, 1979). This does not appear to be a generalised phenomenon since it is not apparent in the guinea-pig (Rial and Nicholls, 1984). More prolonged cold exposure for 2 days or more results in BAT hypertrophy, an increase in the number of mitochondria, with concomitant synthesis of respiratory enzymes and specifically increased synthesis of the 32K dalton protein (Desautels et al., 1978; Ashwell et al., 1983). These responses clearly enhance the thermogenic capacity of the tissue. Both phases are apparently controlled by release of noradrenaline from sympathetic nerve terminals in the BAT since both responses can be mimicked by either acute or chronic noradrenaline treatment (Desautels and Himms-Hagen, 1979; Mory et al., 1984).

The mechanism involved in the unmasking effect is unknown. This process occurs very rapidly, within 1 hour of stimulation and is believed to be independent of protein synthesis, since it is not blocked by protein synthesis inhibitor, cycloheximide, unlike the chronic response (Desautels and Himms-Hagen, 1979). In addition, it is unlikely that protein synthesis could account for such a rapid increase in the number of GDP binding sites. Increase in GDP binding within the first day of cold exposure is accompanied by changes in the morphology of BAT mitochondria (Suter, 1969; Desautels and Himms-Hagen, 1980). It can be speculated that these morphological changes may be involved in the mechanism of the unmasking of GDP

binding sites. The process may involve unmasking of binding sites previously buried in the phospholipid bilayer. Conversely, unmasking may require phosphorylation to give activation of the 32K dalton protein. This might be given by an activated protein kinase in response to the elevated cyclic AMP levels given on noradrenaline stimulation of the tissue (Assimacopoulos-Jeannet et al., 1982). Another possibility could involve the rapid transfer of 32K dalton protein from another cellular location (e.g. the endoplasmic reticulum) to the mitochondrial membrane. This mechanism has been suggested for the rapid appearance of glucose transporters in the rat adipocyte plasma membrane which occurs after stimulation of isolated cells by insulin (Cushman and Warzalda, 1980).

Ricquier et al. (1975) reported that the fatty acid composition of BAT mitochondrial phospholipids was altered in rats chronically exposed to cold whereas Cannon et al.(1975) observed no such changes after cold adaptation. In the present study the possible involvement of membrane compositional changes in the unmasking of BAT mitochondrial GDP binding sites during acute cold exposure was examined. However, no substantial changes in phospholipid-fatty acyl composition occured over the time course in which the number of GDP binding sites was increased by 63%. Hence it is unlikely that membrane compositional changes are directly involved in the unmasking effect.

In the obese mouse, the lack of response of BAT mitochondrial thermogenesis to stimuli has been well documented. (Himms-Hagen and Desautels, 1978; Hogan and Himms-Hagen, 1980; 1981; Trayhurn et al., 1982). This defect apparently includes both aspects of the thermogenic mechanism, the unmasking effect and the long term response. Desautels and Himms-Hagen (1978) reported that obese mice failed to increase BAT mitochondrial GDP binding after 3 hours exposure to a 4°C environment. However, these authors found that GDP binding and respiratory enzyme activity did increase in obese animals acclimated to a more moderate 14°C environment for 2 weeks (Hogan and Himms-Hagen, 1980). Trayhurn et al. (1982) studied the effects of cafeteria overfeeding on energy balance and BAT thermogenesis in lean and obese mice. Although a similar increase was observed in BAT mitochondrial GDP binding per mg. protein in lean and obese mice after 3 weeks of cafeteria over feeding, no

adaptive increase in respiratory enzyme activity was apparent in the obese animals. This failure to respond to dietary stimuli resulted in deposition of the excess energy intake as fat in the obese mice.

In the present investigation, the capacity of BAT mitochondrial thermogenesis to respond to acute cold exposure was examined in lean and obese mice. Lean and obese animals exposed to a 4°C environment for 1 hour increased their BAT mitochondrial GDP binding by a similar amount (38%). Recently, Mercer and Trayhurn (1985) reported similar responses in BAT mitochondrial GDP binding in 26 day old lean and obese mice after 1 hour cold exposure at 4°C. Hence the mechanism of unmasking of GDP binding sites is normal in obese mice in response to certain stimuli. It was not determined in the present study whether this thermogenic response was sustained in the obese animals. Experiments were not carried out in which obese mice were exposed to cold for longer than 1 hour. It may be significant that Himms-Hagen and Desautels,(1978) found no BAT mitochondrial thermogenic response in obese animals after 3 hours cold exposure. Shortly after this period of cold exposure, obese mice die because of hypothermia (Trayhurn and James, 1978; Hogan and Himms-Hagen, 1980).

The present study demonstrates that obese mice are capable of normal BAT mitochondrial thermogenesis in response to acute stimulation. Trayhurn et al. (1982) observed similar responses in GDP binding (per mg. protein) to BAT mitochondria isolated from lean and obese animals after 3 weeks of cafeteria overfeeding. Hence it seems unlikely that the BAT mitochondria of obese mice are refractory to stimulation as has been suggested previously (Himms-Hagen and Desautels, 1978; Zaror-Behrens and Himms-Hagen, 1983). It is more plausible to suggest that it is the central control of BAT mitochondrial thermogensis which is defective in the obese mouse. In this respect the thermogenic defect may be similar to that found in the genetically obese Zucker rat. In these animals, BAT mitochondrial thermogenesis responds normally to cold exposure but is not stimulated by cafeteria or sucrose overfeeding (Holt et al., 1983; Triandafillou and Himms-Hagen, 1983). However, obese rats are generally less susceptible to cold than are obese mice.

In view of these findings, the possible involvement of altered membrane lipid composition in the defective thermogenesis of BAT mitochondria in the obese mouse may be considered. Despite large compositional changes, BAT mitochondrial thermogensis in obese mice is capable of responding to cold stimuli. Changes in membrane lipid composition do not appear to be directly involved in the unmasking of GDP binding sites. The failure of obese mice to respond to cafeteria overfeeding (Trayhurn et al., 1982) does not primarily involve a failure to increase GDP binding sites per mg. mitochondrial protein. Hence it may be concluded that changes in membrane lipid composition are not involved in the defective thermogenic mechanism of BAT mitochondria in the obese mouse.

CHAPTER 7

SUMMARY AND DISCUSSION

7. Summary and Discussion

Many tissues of the obese mouse exhibit a membrane defect which appears to involve an alteration to the fatty acid composition of the membrane phospholipids (York et al., 1982). Changes in fatty acid composition have been reported for membrane phospholipids from adipocyte plasma membrane, hepatic microsomes, and hepatic plasma membranes of obese mice (Rouer et al., 1980; York et al., 1982; French et al., 1983). In the present study, the number of tissue membranes in the obese mouse which exhibit lipid compositional changes has been extended to include hepatic and BAT mitochondrial membranes.

From the work presented in this thesis, some insight can be gained into the biochemical basis of this generalised membrane defect. The activities of the enzymes which desaturate saturated fatty acids and EFA's are increased in the liver of obese mice (see also Enser, 1979; Enser and Roberts, 1982). Desaturase enzymes may regulate membrane lipid composition by controlling the composition of the fatty acids available for incorporation into phospholipids. Thus, increased hepatic $\Delta 9$- and $\Delta 6$-desaturase activities were consistent with the elevated oleic acid content and depressed linoleic acid content of hepatic storage triglyceride and the corresponding changes in these fatty acid components of membrane phospholipids in the obese mouse.

An investigation has been undertaken into the cause of increased hepatic EFA desaturation in obese mice. The results of this study discount hyperinsulinemia and hyperphagia and implicate that the functional hypothyroidism of obese mice may be responsible for the elevated $\Delta 6$-desaturase activity. However, when a hypothyroid state was induced in lean and obese mice, the increased $\Delta 6$-desaturase activity was maintained in the obese animals. This suggests that factors other than thyroid status may be primarily responsible for the increased $\Delta 6$-desaturase activity observed in obese mice.

It is not clear to what extent changes in EFA desaturation contribute to the alteration in the fatty acid composition of hepatic storage triglyceride of obese mice. The linoleic acid content of hepatic triglyceride was reduced in 17 day old pre-obese mice (table 4.2), although hepatic $\Delta 6$-desaturase activity was normal in obese animals

of this age (figure 3.3). This would suggest that the initial factor responsible for the reduced linoleic acid content of storage lipid in suckling pre-obese mice involves increased accumulation of saturated and mono-unsaturated fatty acids, although the subsequent increase in Δ6-desaturase activity in obese animals after weaning probably exaggerates the changes in storage lipid fatty acid composition. The increased accumulation of storage fat in suckling pre-obese mice (Dubuc, 1976b; Thurlby and Trayhurn, 1978) does not result primarily from increased lipogenic activity. Fatty acid synthesis in liver and adipose tissue was similar in 18 day old lean and pre-obese mice (Rath and Thenen, 1980) although lipogenic activity was increased in the carcass of 15 day old pre-obese animals (Godbole et al., 1980). Thus, the initial mechanism for reducing the linoleic acid content of hepatic storage triglyceride of pre-obese mice is either increased uptake of circulating dietary fat by lipoprotein lipase activity, reduced hepatic lipolysis or reduced oxidation of saturated and mono-unsaturated fatty acids. The mechanism may also involve an increase in Δ9-desaturation to elevate monoenoic fatty acid content of hepatic storage triglyceride in pre-obese animals.

It is probable that membrane lipid composition is ultimately controlled by deacylation/reacylation enzymes, which modify phospholipids at their site of function (Stubbs and Smith, 1984). The substrate selectivity of hepatic microsomal lysoPC acyltransferase (with regard to oleic and linoleic acids) was normal in the obese mouse. Lands (1980) points out that there is no apparent mechanism known to alter the selectivity of lysophospholipid acyltransferases other than by changing the supply of fatty acid substrates available. Thus, despite a strong preference for linoleic acid, the enzyme was unable to incorporate normal levels of linoleic acid into phosphatidylcholine when a large excess of oleic acid was present, under experimental conditions which paralleled the triglyceride composition of the obese animals. This resulted in an increased incorporation of oleic acid and reduced incorporation of linoleic acid into phosphatidylcholine, a situation which reflected the fatty acyl composition of microsomal membrane phospholipids in the obese mouse.

The increases in membrane PUFA components in some phospholipid

subclasses, but not in others extracted from the same membrane preparation of the obese mouse, are unusual. Thus the increased docosahexaenoic acid content of only phosphatidylethanolamine in the adipocyte plasma membrane (York et al., 1982), or the increased docosahexaenoic acid content of hepatic mitochondrial phosphatidylcholine, or the increased arachidonic acid content of BAT mitochondrial phosphatidylcholine of the obese mouse, cannot be explained by changes in the composition of fatty acids available for incorporation into phospholipids. This is because the amount of PUFA available for incorporation into phospholipids is reduced in lipid storage depots of the obese mouse (Enser and Roberts, 1982; Winand et al., 1973). Hence some other mechanism must operate to increase the PUFA content of certain membrane phospholipids in the obese mouse. It is possible that this might occur by direct desaturation of the fatty acyl groups esterified to the phospholipids in the membrane. Pugh and Kates (1977) reported the direct desaturation of eicosatrienoyl-phosphatidylcholine to arachidonoyl phosphatidylcholine by rat liver microsomes. They suggested that this process might provide a mechanism to maintain the optimum fatty acid composition of the membrane under conditions where the normal levels of EFA and PUFA substrates were restricted.

It may be significant that the unsaturation indices of membrane phospholipids of the obese mouse are often unaltered or little changed, despite the large changes in the content of the individual fatty acid components. Hence the increase in PUFA content of some membrane phospholipids in the obese mouse may result from a compensatory mechanism which operates to maintain the optimum fluidity of the bulk membrane. However, it is important to remember that the relationship between membrane fluidity and membrane unsaturation is not simple (Stubbs and Smith, 1984). The position of the double bonds on the carbon backbone as well as the number of double bonds determines the mobility (or fluidity) of the fatty acyl chains in the membrane. In addition, Lands (1980) points out that there is no firm evidence to suggest that a compensatory mechanisms operates to alter membrane lipid composition in response to abnormal membrane fluidity.

Several studies have reported that the membrane lipid composition of poikilotherms change when the organisms are grown at different temperatures (Sinensky, 1974; Sellner and Hazel, 1982). However,

these membrane compositional changes may only take place to maintain the bulk membrane in the liquid crystalline state rather than to regulate membrane fluidity about some optimum level (East et al., 1984).

The second part of the work presented in this thesis was concerned with the consequences of altered membrane lipid composition on the function of intrinsic membrane proteins in the genetically obese mouse. Previous studies have reported the effects of altered membrane lipid composition on intrinsic membrane proteins in hepatic microsomes, hepatic plasma membrane and the adipocyte plasma membrane of the obese mouse (Rouer et al., 1980; Hyslop et al., 1982; French et al., 1983; French and York, 1984 ; 1985). In the present study, intrinsic membrane protein characteristics were examined in hepatic and BAT mitochondrial preparations of lean and obese mice. It was concluded that the abnormalities in the function of the GDP binding protein (associated with the proton conductance channel) of BAT mitochondria of obese mice, reported previously by other workers (Desautels and Himms-Hagen, 1978; Hogan and Himms-Hagen, 1980; Goodbody and Trayhurn, 1982; Trayhurn et al., 1982), were not associated with the extensive alteration to membrane lipid composition observed in this tissue of the obese animals.

In hepatic mitochondria, the Arrhenius characteristics of ATP-Pi exchange activity were altered in the obese mice. This could be interpreted to suggest that a protein component of the ATP-Pi exchange complex was sensitive to the changes in membrane lipid composition in hepatic mitochondria of the obese animals. However, other intrinsic proteins (cytochrome oxidase and succinate-cytochrome C reductase) in the same membrane were unresponsive to the membrane lipid compositional changes. Thus, it was possible that the various hepatic mitochondrial intrinsic membrane proteins were sensitive to different fatty acyl components in the membrane, or that some intrinsic membrane proteins were buffered by their boundary lipid regions to changes in the bulk membrane lipid composition.

Many intrinsic membrane proteins are dependent on phospholipids for activity (Sandermann, 1978; Fourcains and Jain, 1974). However, it is not yet clear whether certain membrane proteins require specific fatty acyl groups to maximise activity. Many studies have examined

lipid-protein interactions by characterising membrane enzyme activity after changing membrane lipid composition by feeding diets of different fatty acid composition (Clandinin, 1979; Innis and Clandinin, 1981; McMurchie et al., 1983a; 1983b; 1983c; Mak et al., 1983; Royce and Holmes, 1984; Abeywardena et al., 1984). However, the results have been inconclusive. In liver and heart mitochondria isolated from rats fed diets of different fatty acid composition, respiration rates were unchanged (Mak et al., 1983; McMurchie et al., 1983b; Royce and Holmes, 1984) although the Arrhenius characteristics of several intrinsic membrane proteins were responsive to the changes in membrane lipid composition (Innis and Clandinin 1981; McMurchie et al., 1983a; 1983c). Royce and Holmes (1984) suggested that mitochondrial membranes adapt to changes in dietary fatty acids in a way which prevents changes in their functional properties. McMurchie et al. (1983a) reported that intrinsic proteins in the same membrane responded differently to the changes in membrane lipid composition brought about by dietary modulation.

Clearly, some intrinsic membrane proteins are sensitive to changes in their membrane lipid environment whereas others are unresponsive to alterations in the bulk membrane composition. It may be that for certain membrane proteins, such as has been reported for the $\left[Ca^{2+}\right]$-ATPase (East et al., 1984), enzyme activity is unaffected by changes in membrane fluidity or membrane fatty acyl composition. Conversely, for more complex intrinsic protein systems, such as the adenylate cyclase system, which comprises of 3 protein subunits able to move independently in the membrane bilayer, membrane fluidity or phospholipid-fatty acyl composition may play an important role in regulating or coupling enzyme activity. This seems to be the case for the adipocyte adenylate cyclase system of the obese mouse which exhibits an impairment in hormone receptor-catalytic unit coupling (Dehaye et al., 1978; French and York, 1984 ; 1985).

In summary, the changes in membrane lipid composition that are present in the obese mouse may develop in the following way. In suckling pre-obese mice, increased hepatic $\Delta 9$-desaturase activity or increased accumulation of saturated and mono-unsaturated fatty acids increase the palmitoleic acid content (predominantly) and dilute out the linoleic acid content of the hepatic storage

triglyceride. After weaning, increased hepatic lipogenesis and increased EFA desaturation augment the changes in the fatty acid composition, increasing oleic acid content and further reducing linoleic acid content. These changes are subsequently transferred to the pool of fatty acids available for incorporation into phospholipids. De novo synthesis on the endoplasmic reticulum produces phospholipids with abnormal fatty acid compositions (increased oleic acid and reduced linoleic acid content). Subsequently, the deacylation/reacylation enzymes modify the abnormal fatty acid composition at the subcellular location of the phospholipid. However, the reacylation enzymes, selecting from the pool of fatty acids available in the obese mouse, are unable to rectify the altered membrane lipid composition of the obese animal.

Thus, hepatic membrane phospholipids in the obese mouse have increased levels of oleic acid and reduced levels of linoleic acid. However, the sequence of events outlined above cannot explain the increased PUFA content of membrane phospholipids of the obese mice, since the PUFA content of their hepatic storage lipid is reduced (Winand et al., 1973; Enser and Roberts, 1982). Future work could therefore include the identification of the mechanism which increases the PUFA content of membrane phospholipids in the obese mouse. This may involve an investigation into the substrate specificity of lysophospholipid acyltransferases in lean and obese animals, particularly with regard to PUFA substrates. Alternatively, the direct desaturation of phospholipid fatty acyl groups may provide the mechanism by which the PUFA content of membrane phospholipids is increased in the obese mouse.

It is clear that future work should also concentrate on the role of altered membrane lipid environment on the defective membrane functions of the obese mouse. Much evidence has been produced to suggest that alterations to phospholipid fatty acyl composition are involved in the defective adenylate cyclase system of adipocytes of obese mice. However, it may require the purification of the protein subunits and their reconstitution into defined lipid environments to elucidate the role of membrane phospholipids in the coupling of the adenylate cyclase system. Alternatively, the defective glucose transport systems of adipose tissue or muscle (Cuendet et al., 1976b;

Czech et al., 1977) provide an interesting area of study. Armatruda and Finch (1979) reported that insulin stimulated glucose transport in isolated adipocytes was sensitive to the membrane lipid environment. In addition, Pilch et al. (1980) have shown that unsaturated fatty acid stimulated, whereas saturated fatty acids depressed, glucose transport after their incorporation into adipocyte plasma membrane preparations. They further suggest that insulin may stimulate glucose transport in fat cells by modulating membrane fluidity.

REFERENCES

Abeywardena, M.Y., McMurchie, E.J., Russell, G.R., Sawyer, W.H., and Charnock, J.S., (1984) Biochim.Biophys. Acta, 776, 48-59.

Alam, S.Q., Alam, B.S., and Chen, T.W., (1984). Biochim. Biophys. Acta, 792, 110-117.

Armatruda, J.M., and Finch, E.D. (1979) J. Biol. Chem. 254, 2619-2625

Ashwell, M., Jennings, G., Richard, D., Stirling, D.M., and Trayhurn, P. (1983) FEBS. Lett. 161, 108-112.

Assimacopoulos-Jeannet, F., Giabobino, J.P., Seydoux, J., Girardier, L., and Jeanrenaud, B. (1982) Endocrinology, 110, 439-443.

Aswachi, Y.C., Chuang, T.F., Keenan, T.W., and Crane, F.L., (1971) Biochim. Biophys. Acta 226, 42-52.

Bach, D., Bursuker, I., and Goldman, R. (1977), Biochim. Biophys. Acta, 469, 171-179.

Bach, D., Bursuker, I., and Miller, I.R. (1978) Experientia, 34, 717-718.

Bailey, C. J., Atkins, T.W., Connor, M.J., Manley C. G., and Matty, A.J. (1975), Hormones, 6, 380-386.

Barbior, B.M., Creagan, S., Ingbar, S. H., and Kipness, R. S., (1973), Proc. Natl. Acad. Sci. USA, 70, 98-102.

Bar-Tana, J., Rose, G., and Shapiro, B., (1971), Biochem, J. 122, 353-362.

Begin-Heick, N., (1980) Can. J. Biochem. 58, 1033-1038.

Begin-Heick, N., (1981) Can. J. Biochem. 59, 816-820.

Bell R. M., and Coleman, R.A. (1980) Annu. Rev. Biochem. 49, 459-487.

Beloff-Chain, A., Bogdanovic, S., and Cawthorne, M.A. (1979) J. Endocrinology 81, 271-279.

Beriter, D. A., and Jeanrenaud, B., (1979) Brain Res. 165, 249-260.

Bergen, W.G., Kaplan, M.L., Merkel, R.A., and Leveille, G.A., (1975) Am. J. Clin. Nutr. 28, 157-161.

Bernal, J. and Refetoff, S., (1977) Clin. Endocrinology 6, 227-249.

Berne, C., (1975) Endocrinology, 97, 1241-1247.

Billingham, N., Beloff-Chain, A., and Cawthorne M.A. (1982) J. Endocrin. 94, 125-130.

Blaise-Smith, P., Reitz, R.C., and Kelley, D., (1982) Biochim. Biophys. Acta 713, 128-135.

Blatt, A.H., (1961), in "Organic Syntheses" vol. 2. John Wiley and Sons, London. pp 461.

Blazyk, J.F., and Newman, J.L., (1980) Biochim. Biophys. Acta 600, 1007-1011.

Blazyk, J. F. and Steim, J. M. (1972) Biochim. Biophys. Acta 266, 737-741.

Bogartz, R.S. (1968) Psychol. Bull. 70, 749-755.

Boissonneault, G.A., Hornshuh, M.J., Simons, J.W., Rosmos, D.R., and Leveille, G.A.,(1978) Proc. Soc. Exp. Bio. Med. 157, 402-406.

Bradford, M.M., (1976) Anal. Biochem. 72, 248-254.

Bray, G.A. (1974) in "Obesity" edited by Burland, W. L., Samuel, P.D., and Yudkin, J., Churchill Livingstone, Edinburgh.

Bray, G.A., (1978) Proc. Nutr. Soc. 37, 301-309.

Bray, G.A., and York, D.A., (1979) Physiol Rev. 59, 719-809.

Brenner, R.R. (1974) Molec. Cell. Biochem. 3, 41-53.

Brenner, R.R. (1977) Adv. Exp. Med. Biol. 83, 85-101.

Brenner, R.R., Peluffo, R.O., Mercuri, O., and Restelli, M.A., (1968) Am.J. Physiol. 215, 63-70.

Broekhuyse, J.H. and Veerkamp, R.M., (1976) in "Biochemical Analysis of Membranes" Chpt. 8. edited by Maddy, E.H. ; Chapman and Hall.

Brulet, P., and McConnell, H.M., (1976) Biochem. Biophys. Res. Commun. 68, 363-368.

Bryant, K.R., Rothwell, N.J., and Stock, M.J., (1984) Biosci. Reports. 4, 523-533.

Bryant, K.R., Rothwell, N.J., Stock, M.J., and Stribling, D. (1983) Biosci. Reports. 3, 589-598.

Cannon, B.C., and Lindberg, O. (1979), Methods Enzymol. 55, 65-78.

Cannon, B.C., Polncizek, C.F., Butler, K.W., Eriksson, L.E.G., and Smith, I.C.P. (1975) Ach. Biochem. Biophys. 167, 505-518.

Castuma, C.E. and Brenner, R.R. (1983) Biochim. Biophys. Acta 729, 9-16.

Chang, K.J., Huang, D. and Cuatrecases P.(1975) Biochem. Biophys. Res. Commun. 64, 566-573.

Chapman, D. (1983) chpt. 2. in "Membrane Fluidity in Biology" vol. 2. edited by Aloia, R. C. , Academic Press.

Clandinin, M.T., (1979) FEBS Lett. 102, 173-176.

Coleman, D.L. (1978) Diabetologia, 14, 141-148.

Cook, H.W. (1978) J. Neurochem. 30, 1327-1334.

Craddock, D., (1978) in "Obesity and its Management" 3rd edition. Churchill Livingstone.

Cuendet, G.S., Loten, E.G., Cameron, D.P., Renold, A.E., and Malbis, E.B. (1975) Am. J. Physiol. 228, 276-283.

Cuendet, G.S., Loten, E.G., Jeanrenaud, B., and Renold, A.E. (1976) J. Clin, Invest. 58, 1078-1088.

Cunnane, S.C., Mahku, M.S. and Horrobin, D.F. (1985) Br. J. Nutr. (in press).

Curatolo, W., Sakura, J. D., Small D.M. and Shipley, G.G., (1976) Biochemistry, 16, 2313-2319.

Cushman, S.W. and Warazalda, L.T., (1980) J. Biol. Chem. 255, 4758-4762.

Czech, M.P., Richardson, D.R. and Smith, D.J. (1977) Metabolism 26, 1057-1078.

Dahl, J.L. and Hokin, L.E. (1974) Ann. Rev. Biochem. 43, 327-356.

Dahlen, J.V. and Porter, J.W. (1968) Arch. Biochem. Biophys. 127, 207-223.

Das, I. and Hems, D.A., (1974) Horm. Metab. Res. 6, 40-44.

De Gomez-Dumm, I.N.T., DeAlaniz, M.J.T., and Brenner, R.R. (1975) J. Lipid. Res. 16, 264-268.

De Gomez-Dumm, I.N.T., De Alaniz, M.J.T. and Brenner, R.R. (1976) J. Lipid. Res. 17, 616-621.

De Gomez-Dumm, I.N.T., De Alaniz, M.J.T., and Brenner, R.R. (1977) Adv. Exp. Med. Biol. 83, 609-616.

Dehaye, J.P., Winand, J. and Christophe, J. (1978), Diabetologia, 15, 45-51.

Dehlinger, P.J., Jost, P.C. and Hayes-Griffith, O. (1974) Proc. Natl. Acad. Sci. USA 71, 2280-2284.

Desautels, M. and Himms-Hagen, J. (1979) Can. J. Biochem. 57, 968-976.

Desautels, M. and Himms-Hagen. J. (1980) Can. J. Biochem. 58, 1057-1068.

Desautels, M., Zaror-Behrens, G. and Himms-Hagen, J. (1978) Can. J. Biochem. 56, 378-383.

Donati, R.M., Warnecke, M.A., and Gallagher, N.I. (1963) Metabolism 12, 833-836.

Dubuc, P.U., (1976a) Am. J. Physiol. 230, 1474-1479.

Dubuc, P.U. (1976b) Metabolism, 25, 1567-1574.

Dubuc, P.U. (1977) Horm. Metab. Res. 9, 95-97.

Dubuc, P.U., Cahn, P.J., Ristumaki, S., and Willis, P.L. (1982) Horm. Metab. Res. 14, 532-535.

Dubuc, P.U., Cahn, P.J., and Willis, P.L. (1984) Int. J. Obesity, 8, 271-278.

East, J.M., Jones, O.T., Simmonds, A.C. and Lee, A.G., (1984) J. Biol. Chem. 259, 8070-8071.

East, J.M. and Lee, A.G. (1982) Biochemistry 21, 4144-4151.

Eck, M.G., Wynn, J.O., Carter, W.J., and Faas, F.J. (1979) Diabetes 28, 479-485.

Edwardson, J. and Hough, L.C. (1975) J. Endocrinol. 65, 99-107.

Elliot, J., Hems, D.A. and Beloff-Chain, A. (1971) Biochem. J. 125, 773-780.

Emilsson, A. and Gudbarnason, S. (1983) Biochim. Biophys. Acta 750, 1-6.

Enser, M. (1979) Biochem. J. 180, 551-558.

Enser, M. and Roberts, J.L. (1982) Biochem. J. 206, 561-570.

Faas, F.H. and Carter, W.J. (1981) Biochem. J. 193, 845-852.

Faas, F.J. and Carter, W.J. (1982) Biochem. J. 207, 29-35.

Faas, F.J. and Carter, W.J. (1983) Lipids 18, 339-342.

Flatt, P.R., Bailey, C.J. and Buchanan, K.D. (1982) J. Endocrinol. 95, 215-227.

Foster, D.O. and Frydman, M.L. (1978) Can. J. Biochem. 56, 110-122.

Fourcains, B. and Jain, M.K. (1974) Adv. Lipid Res. 12, 147-188.

French. R.R. and York, D.A. (1984) Diabetelogia 26, 466-472.

French, R.R. and York, D.A. (1985) Bioscience Reports (in press).

French, R.R., York, D.A., Portman, J.M. and Isaacs, K. (1983) Comp. Biochem. Physiol. 76B, 309-319.

Fujiwara, J., Okayasu, T., Ishibushi, T., and Imai, Y. (1983) Biochem. Biophys. Res. Commun. 110, 36-41.

Garda, H.A. and Brenner, R.R. (1984) Biochim. Biophys. Acta. 769, 160-170.

Ginsberg, B.H., Brown, T.J., Simon, I. and Spector, A.A. (1981) Diabetes 30, 773-780.

Godbole, V.Y., Grundleger, M.L. and Thenen, S.W. (1980) Am. J. Physiol. 239, E265-E268.

Goglia, F., Liverini, G., de Leo, T. and Barletta, A. (1983) Pflugers Arch. 396, 49-53.

Goodbody, A.E. and Trayhurn, P. (1982) Biochem. Biophys. Acta 680, 119-126.

Gould, R.J., Ginsberg, B.H. and Spector, A.A. (1982) J. Biol. Chem. 257, 477-484.

Hales, C.N. and Randle, P.J. (1963) Biochem. J. 88, 137-146.

Harlan, W.H. and Wakil, S.J. (1963) J. Biol. Chem. 238, 3217-3228.

Hasegawa-sasaki, H. and Ohno, K. (1980) Biochim. Biophys. Acta 617, 205-217.

Heldt, H.W. and Klingenberg, M. (1968) Eur. J. Biochem. 4, 1-8.

Helmkamp Jnr. G.M., (1983) chpt. 6 in "Membrane Fluidity in Biology" vol.2 ed. Aloia, R.C. (Academic Press).

Hems, D.A. and Ma, G.Y. (1976) Biochem. J. 160, 23-28.

Herberg, L. and Kley, H.K. (1975) Horm. Metab. Res. 7, 410-415.

Hesketh, T.R., Smith, G.A., Houslay, M.D., McGill, K. A., Birdsall, N.J.M., Metcalfe, J.C. and Warren, G.B., (1976) Biochemistry 15, 4145-4151.

Higgins, J.A. and Evans, W.E.(1978) Biochem. J. 174, 563-567.

Hill, E.E. and Lands, W.E.M. (1968) Biochim. Biophys. Acta 152, 645-648.

Himms-Hagen, J. and Desautels, M. (1978) Biochem. Biophys. Res. Commun. 83, 628-634.

Hogan, S. and Himms-Hagen, J. (1980) Am. J. Physiol. 239, E301-E309.

Hogan, S. and Himms-Hagen, J. (1981) Am. J. Physiol. 241, E436-E443.

Holloway, C.T. and Holloway, P.W. (1975) Arch. Biochem. Biophys. 167, 496-504.

Holt, S.J. (1984) Ph. D. Thesis, "The Control of Brown Adipose Tissue in the Genetically Obese Zucker (fa/fa) Rat". University of Southampton.

Holt, S.J. and York. D.A. (1984) Horm. Metab. Res. 16, 384-379.

Holt, S.J., York, D.A. and Fitzsimons, J.T. (1983) Biochem. J. 214, 251-223.

Holub, B.J. and Kuksis, A. (1978) Adv. Lipid Res. 16, 1-102.

Holub, B.J., McNaughton, J.A. and Piekarski, J. (1979) Biochim. Biophys. Acta 572, 413-422.

Hostetler, K.Y., Galesloot, J.M., Boer, P., and Van den Bosch, H. (1975) Biochim. Biophys. Acta 380, 382-389.

Houslay, M.D., Heskith, T.R., Smith, G.A., Warren, G.B. and Metcalfe, J.C.(1976) Biochim. Biophys. Acta. 436, 495-504.

Hughes, S.J. and York, D.A. (1983) Horm. Metab. Res. 15, 335-339.

Hyslop, P.A. and York, D.A. (1980a) Anal. Biochem. 101, 75-77.

Hyslop, P.A. and York, D.A. (1980b) Biochem. Biophys. Res. Commun. 92, 819-824.

Hyslop, P.A., York, D.A. and Corrina, D.L. (1982) Int. J. Obesity. 6, 279-289.

Ida-Chen, Y. and Hoch, F.L. (1976) Arch. Biochem. Biophys. 172, 741-744

Ida-Chen, Y. and Hoch, F.L. (1977) Arch. Biochem. Biophys. 181, 470-483

Ingbar, D.H. and Galton, V.A. (1975) Endocrinology 96, 1525-1532.

Innis, S.M. and Clandinin, M.T. (1981) Biochem. J. 198, 167-175.

Jagot, S.A., Dickerson, J.W.T. and Webb, G.P. (1982) Int. J. Obesity. 6, 443-456.

James, W.P.T. and Trayhurn, P. (1976) Lancet 2, 770-772.

Jeffcoat, R. (1977) Biochem. Soc. Trans. 5, 811-818.

Jeffcoat, R. (1979) Essays. Biochem. 15, 1-36.

Jeffcoat. R. and James, A.T. (1984) Chpt. 4. in "Fatty Acid Metabolism and its Regulation" ed. Numa, S. (Elsevier).

Joosten, H.F.P. and Van der Kroon, P.H.W. (1974a) Metabolism 23, 59-66.

Joosten, H.F.P. and Van der Kroon, P.H.W. (1974b) Metabolism 23, 425-436.

Joosten, H.F.P. and Van der Kroon P.H.W. (1974c) Metabolism 23, 1141-1147.

Jost, P.C., Hayes-Griffith, O., Capaldi, R.A. and Vanderkooi, G.(1973) Proc. Natl. Acad. Sci. USA 70, 480-484.

Jost, P.C., Nadakavukaran, K.K. and Hayes-Griffith, O. (1977) Biochemistry, 16, 3110-3114.

Kagawa, Y., Kandrach, A. and Racker, E. (1973) J. Biol. Chem. 248, 676-684.

Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477-5487.

Kahn, C.R., Neville, D.M. and Roth, J. (1973) J. Biol. Chem. 248, 244-250.

Kang, S.Y., Gutowsky, H.S., Hsung, J.C., Jacobs, R., King, T.E., Rice, D. and Oldfield, E. (1979) Biochemistry 18, 3257-3262.

Kaplan, M. and Leveille, G.A. (1974) Am. J. Physiol. 227, 912-915.

Katyare, S.S. and Howland, J.L. (1974) FEBS Lett. 43, 17-19.

Katyare, S.S. and Howland, J.L. (1978) Arch. Biochem. Biophys. 188, 15-20.

Kemp, A., Groot, G.S.P. and Reitsma, H.J. (1969) Biochim. Biophys. Acta 180, 28-34.

Kimelberg, H.K. and Papahadjopoulos, D. (1974) J. Biol. Chem. 249, 1071-1080.

Kleinfeld, A.M., Dragsten, P., Klausner, R.D., Pjura, W.J. and Matayoshi, E.D. (1981) Biochim. Biophys. Acta 649, 471-480.

Knehans, A.W. and Romsos, D.R. (1982) Am. J. Physiol. 242, E253-E261.

Knehans, A.W. and Romsos, D.R. (1983) Am. J. Physiol. 244, E567-E574.

Kopec, B. and Fritz, I.B. (1971) Can. J. Biochem. 49, 941-948.

Lagarde, M., Authi, K., and Crawford, N. (1982) Biochem. Soc. Trans. 10, 241-243.

Lands, W.E.M. (1980) Biochem. Soc. Trans. 8, 25-27.

Lands, W.E.M., Inoue, M., Sugiura, Y. and Okuyama, H. (1982) J. Biol. Chem. 257, 14968-14972.

Lee, T.C., Backer, R.C., Stephens, N. and Snyder, F. (1977) Biochim. Biophys. Acta 489, 25-31.

Lee, A.G., Birdsall, N.J.M., Metcalfe, J.C., Toon, P.A., and Warren, G.B. (1974) Biochemistry 13, 3699-3705.

Lee, M.P. and Gear, A.R.L. (1974) J. Biol. Chem. 249, 7541-7549.

Le Marchand-Brustel, Y. and Freychet, P. (1978) Metabolism 27, 1982-1993.

Le Marchand-Brustel, Y., Loten, E.G., Assimacopoulos-Jeannet, F., Forgue, M.E., Freychet, P. and Jeanrenaud, B. (1977) Diabetes 26, 582-590.

Lenaz, G., Sechi, A.M., Parenti-Castelli, G., Landi, L. and Bertolli, E. (1972)Biochem. Biophys. Res. Commun. 49, 536-542.

Lin, M.H., Romsos, D.R., Akera, T. and Leveille, G.A. (1979) Proc. Soc. Exp. Biol. Med. 161, 235-238.

Lin, M.H., Romsos, D.R. and Leveille, G.A. (1977) J. Nutr. 107, 1715-1723.

Locke, R.M., Rial, E. and Nicholls, D.G. (1982a) Eur. J. Biochem. 129, 381-387.

Locke, R.M. Rial, E., Scott, I.D. and Nicholls, D.G. (1982b) Eur. J. Biochem. 129, 373-380.

Lorden, J.F., Oltmans, G.A. and Margules, D.L. (1976) Brain Res. 117, 357-361.

Loten, E.G. Rabinovitch, A. and Jeanrenaud, B. (1974) Diabetologia 10, 45-52.

Macdonald, I.A. and Stock, M.J. (1979) Nutr. Metabolism 23, 250-255.

Mak, I.T., Shrago, E. and Elson, C.E. (1983) Lipids 18, 130-136.

McMurchie, E.J. Abeywardena, M.Y.,Charnock, J.S. and Cubson, R.A. (1983a) Biochim. Biophys. Acta 760, 13-24.

McMurchie, E.J., Abeywardena, M.Y., Charnock, J.S. and Gibson, R.A. (1983b) Biochim. Biophys. Acta 734, 114-124.

McMurchie, E.J., Gibson, R.A., Abeywardena, M.Y. and Charnock, J.S. (1983c) Biochim. Biophys. Acta. 727, 163-169.

McMurchie. E.J. and Raison, J.K. (1979) Biochim. Biophys. Acta 554, 364-374.

McVey, E., Yguerabide, J., Hanson, D.C., and Clark, W.R. (1981) Biochim. Biophys. Acta 642, 106-118.

Mercer, S.W. and Trayhurn, P. (1983) Biochem. J. 212, 393-398.

Mercer, S.W. and Trayhurn, P. (1985) Proc. Nutr. Soc. (in press).

Miki, Y., Hosaka, K., Yamashita, S., Handra, H. and Numa, S. (1977) Eur. J. Biochem. 81, 433-441.

Miller, B.G., Otto, W.R., Grimble, R.F., York, D.A. and Taylor, T.G. (1979) Br. J. Nutr. 42, 185-199.

Mobley, P.W. and Dubuc, P.C. (1979) Horm. Metabl. Res. 11, 37-39.

Mory, G., Bouillard, R., Combes-George, M. and Ricquier, D. (1984) FEBS Lett. 166, 393-396.

Mowbray, J. and Corrigoll, J. (1984) Eur. J. Biochem. 139, 95-99.

Naeser, P. (1973) Diabetologia 9, 376-379.

Naeser, P. (1974) Diabetologia, 10, 449-453.

Naughton, J.M. (1981) Int. J. Biochem. 13, 21-32.

Nicholls, D.G. (1976) Eur. J. Biochem. 62, 223-228.

Nicholls, D.G. (1979) Biochim. Biophys. Acta. 549, 1-29.

Nicholls. D.G. (1984) in "Bioenergetics" (Academic Press).

Nicholls, D.G. and Locke, R.M. (1984) Physiol. Rev. 64, 1-64.

Normann, P.T., Thomassen, M.S., Christiansen, E.N. and Flatmark, T. (1981) Biochim. Biophys. Acta 664, 416-427.

Ohshima, K., Shargill, N.S., Chan, T.M. and Bray, G.A. (1984) Am. J. Physiol. 246, E193-E197.

Ohtake, M., Bray, G.A. and Azukizawa, M. (1977) Am. J. Physiol. 233, R110-R115.

Okayasu, T., Nagao, M., Ishibashi, T. and Imai, Y. (1981) Arch. Biochem. Biophys. 206, 21-28.

Otto. W., Taylor, T.G. and York, D.A. (1976) J. Enodcrinol. 71, 143-155.

Patton, J.F. and Platner, W.S. (1971) Proc. Soc. Exp. Biol. Med. 137, 196-201.

Peluffo, R.O., Nervi, A.M. and Brenner, R.R. (1976) Biochim. Biophys. Acta 441, 25-31.

Phillips, A.H. and Langdon, R.G. (1962) J. Biol. Chem. 237, 2652-2660.

Pilch, P.F., Thompson, P.A., Czech, M.P. (1980) Proc. Natl. Acad. Sci. USA 77, 915-918.

Poon, R., Richards, J.M. and Clark, W.R. (1981) Biochim. Biophys. Acta 649, 58-68.

Privett, O.S., Blank, M.L. and Romanus, O. (1963) J. Lipid Res. 4, 260-265.

Pugh, E.L., and Kates, M. (1977) J. Biol. Chem. 252, 68-73.

Pugh, E.L. and Kates, M. (1984) Lipids 19, 48-55.

Pulman, M.E. (1967) Methods Enzymol. 10, 57-60.

Raheja, R.K., Paur, C., Singh, A. and Bhata, I.S. (1973) J. Lipid Res. 14, 695-697.

Raison, J.K. and McMurchie. E. J. (1974) Biochim. Biophys. Acta 363, 135-140.

Rath, E.A., Hems, D.A. and Beloff-Chain, A. (1974) Diabetologia 10, 261-265.

Rath, E.A. and Thenen, S.W. (1979) J. Nutr. 109, 840-847.

Rath, E.A. and Thenen, S.W. (1980) Biochim. Biophys. Acta 618, 18-27

Rial, E. and Nicholls, D.G. (1984) Biochem. J. 222, 685-693.

Rice, D.M., Meadows, M.D., Scheinman, A.O., Goni, F.M., Goniez-Fernandez, J.C., Moscari, M.A., Chapman, D. and Oldfield, E. (1979) Biochemistry 18, 5893-5903.

Ricquier, D., Mory, G.and Hernon, P. (1975) FEBS Lett. 53, 342-346.

Robinson, N.C. Steey, F. and Talbert, L. (1980) Biochemistry 19, 3656-3661.

Rouer, E., Dansette, P., Beaune, P. and Leroux, J.P. (1980) Biochem. Biophys. Res. Commun. 95, 41-46.

Rouer, E. and Leroux, J.P. (1980) Biochem. Pharmacol. 29, 1959-1962.

Royce, S.M. and Holmes, R.P. (1984) Biochim. Biophys. Acta 792, 371-375.

Saito, M. and Bray, G.A. (1984) Am. J. Physiol. 246, R20-R25.

Sandermann Jnr., H. (1978) Biochim. Biophys. Acta 515, 209-237.

Sanders, T.A. and Naismith, D.J. (1979) Proc. Nutr. Soc. 38, 94A.

Sandoval, F., Gomez-Puijou, A., Tuena, M., Chavez, E. and Pena, A. (1970) Biochemistry 9, 684-689.

Sarzala, M.G. and Pilarska, M. (1976) Biochim. Biophys. Acta 441, 81-92.

Schulze, G., Jung, K., Kunze, D. and Egger, E. (1977) FEBS Lett. 74, 220-224.

Sclafani, A. and Springer, D. (1976) Physiol. Behav. 17, 461-471.

Sellner, P.A. and Hazel, J.R. (1982) Am. J. Physiol. 243, R223-R228.

Semin, B.K., Saraste, M. and Wikstrom, M. (1984) Biochim. Biophys. Acta 769, 15-22.

Shimomura, Y., Bray, G.A. and York, D.A. (1981) Horm. Metab. Res. 13, 578-582.

Silvius, J.R. and McElhaney, R.N. (1980) Proc. Natl. Acad. Sci. USA 77, 1255-1259.

Sinensky, M. (1974) Proc. Natl. Acad. Sci. USA 71, 522-525.

Singer, S.J. and Nicolson, G.L. (1972) Science 175, 720-731.

Soll, A.H., Kahn, C.R. and Neville, D.M. (1975) J. Biol. Chem. 250, 4702-4707.

Sprecher, H. (1977) Adv. Exp. Med. Biol. 83, 35-50.

Stier, A. and Sackmann, E. (1973) Biochim. Biophys. Acta 311, 400-408.

Strouve-Vallet, C. and Pascaud, M. (1971) Biochimie 53, 699-703.

Stubbs, C.D., and Smith, A.D. (1984) Biochim. Biophys. Acta 779, 89-137.

Sundin, U. and Cannon, B. (1980) Comp. Biochem. Physiol. 65B, 463-471.

Suter, E.R. (1969) Experientia, 25, 286-287.

Tanaka, T., Hosaka, K., Hoshimaru, M. and Numa, S. (1979) Eur. J. Biochem. 98, 165-172.

Taylor, T.G. (1982) chpt. 3 in "Nutrition and Health", Studies in Biology no. 141. (Edward Arnold Ltd.).

Thenen, S.W. and Carr, R.H. (1980) J. Nutr. 110, 189-199.

Thompson, G.A. (1980) in "The Regulation of Membrane Lipid Metabolism" (Florida: CRC Press)

Thurlby, P.L. and Trayhurn, P. (1978) Br. J. Nutr. 39, 397-402.

Thurlby, P.L. and Trayhurn, P. (1979) Br. J. Nutr. 42, 377-385.

Thurlby, P.L. and Trayhurn, P. (1980) Pflugers Arch. 385, 193-201.

Tisdale, H.D.(1967) Methods Enzymol. 10, 213-215.

Trayhurn, P. and James, W.P.T. (1978) Pflugers Arch. 373, 189-193.

Trayhurn, P., James, W.P.T. and Gurr, M.I. (1977) Nature 266, 60-62.

Trayhurn, P., Jones, P.M., McGuckin, M.M. and Goodbody, A.E. (1982) Nature 295, 323-325.

Triandafillou, J. and Himms-Hagen, J. (1983) Am. J. Physiol. 244, E145-E150.

Trostler, N., Romsos, D.R., Bergen, W.G. and Leveille, G.A. (1979) Metabolism, 28, 928-933.

Van der Kroon, P.H.W., Boldewijn, H. and Langeveldsoeter, N. (1982) Int. J. Obesity 6, 83-90.

Van der Kroon, P.H.W. and Speijers, G.J.A. (1979) Metabolism 28, 1-3.

Van den Bosch, H. (1980) Biochim. Biophys. Acta 604, 191-246.

Van den Bosch, H. Van Golde, L.M.G., and Van Deenen, L.L.M. (1972) Rev. Physiol. 66, 13-145.

Van Golde, L.M.G., Pieterson, W.A. and Van Deenen, L.L.M. (1968) Biochim. Biophys. Acta 152, 84-95.

Vik, S.B. and Capaldi, R.A. (1977) Biochemistry 16, 5755-5759.

Volpe, J.L. and Marasa, J.C. (1975) Biochim. Biophys. Acta 409, 235-248.

Wahle, K.W.J. (1983) Proc. Nutr. Soc. 42, 273-287.

Waite, M., Sisson, P. and Blackwell, E. (1970) Biochemistry, 9, 746-753.

Warren, G.B., Houslay, M.D., Metcalfe, J.C. and Birdsall, N.J.M. (1975) Nature 255, 684-687.

Wetton, R.F., Marin, R.J. and Maurngardt, B.R. (1973) J. Nutr. 103, 1212-1219.

Wharton, D.C. and Tzagoloff, A. (1967) Methods Enzymol. 10, 245-246.

Widnell, C.C. (1974) Methods Enzymol. 32, 368-374.

Winand, J., Furnelle, J., Woden, C. Hebbelinck, M. and Christophe, J. (1973) Biochimie, 55, 63-73.

Wunderlich, F., Ronai, A., Speth , V., Seelig, J. and Blume, A., (1975) Biochemistry 14, 3730-3735.

York, D.A. (1983) Chpt. 3 in "Biochemical Pharmacology of Obesity" ed. Curtis-Prior, P.B. (Elsevier Press).

York, D.A., Bray, G.A. and Yukimura, Y. (1978b) Proc. Natl. Acad. Sci. USA 75, 477-481.

York, D.A., Hyslop, P.A. and French, R.R. (1982) Biochem. Biophys. Res. Commun. 106, 1478-1483.

York, D.A. Otto, W. and Taylor, T.G. (1978a) Comp. Biochem. Physiol. 59B, 59-65.

Young, J.B. and Landsberg, L. (1983) Am. J. Physiol. 245, E148-E154.

Yu, L., Yu, C.H. and King, T.E. (1973) Biochemistry 12, 540-546.

Yukimara, Y. and Bray, G.A. (1978) Proc. Soc. Exp. Biol. Med. 159, 364-367.

Zaror-Behrens, G. and Himms-Hagen, J. (1983) Am. J. Physiol. 244, E361-E366.