A study on the role of the molecular organisation of cell membranes in the development of obesity.

by

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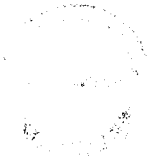
To my mother, father and brothers
with much love and thanks.



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ABSTRACT

FACULTY OF SCIENCE

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Doctor of Philosophy

A STUDY IN THE ROLE OF MOLECULAR ORGANISATION OF CELL MEMBRANES IN THE DEVELOPMENT OF OBESITY.

by Paul Andrew Hyslop

The molecular organisation of the adipocyte plasma membrane from lean and genetically obese (ob/ob) mice has been investigated. No difference was detected in the protein or cholesterol to phospholipid ratio between the membranes from the two phenotypes. The location of phospholipid classes in the two halves of the membrane bilayer has been determined for the adipocyte plasma membrane, and again no difference was apparent in either the phospholipid composition or the assymetric arrangement of these phospholipid classes between the membranes from both phenotypes. Using fluorescence depolarisation by probe labelled membranes, the fluidity of obese mouse adipocyte plasma membranes was found to be greatly increased compared to lean controls. The fluidity difference was largely due to an increase in long chain polyunsaturated fatty acids esterified to carbon atom 2 of phosphatidylethanolamine (PE). This phospholipid is confined to the inner half of the bilayer. The major fatty acid responsible for the fluidity change in obese mouse membranes is docosahexaenoic acid. Evidence is presented that the presence of this fatty acid in membranes regulates a number of metabolic processes, particularly the hormonal stimulation of adenylate cyclase. This concept is extended to form a general hypothesis that the presence of increased docosahexaenoyl PE in membranes forms the basis of many of the observed metabolic changes in the obese condition. As obesity is a disorder of energy balance, the locus of the regulation of cellular metabolic efficiency is postulated to reside at the level of the phospholipid composition of the plasma membrane.

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Introduction.

1.1. What is obesity?

Obesity is a term used loosely to describe the condition of excess storage of body fat in an individual, that is superfluous to forseeable need. Obesity becomes a problem to an animal only when the excess fat impairs its survival chances either directly by reducing mobility, or indirectly through altered endocrine funcion and cardiovascular changes. Indeed obesity has been identified as a major cause of premature death (Garrow, 1978).

Obesity can only result if energy intake exceeds energy expenditure, the excess being stored as fat. The generation of a positive energy balance could simply reflect an excessive food intake. However, many obese persons eat less then non-obese individuals (Garrow, 1978). On the other hand, obesity is not due to reduced physical activity (Björntorp et al, 1975). Therefore it may be concluded that obesity is caused primarily by the metabolic conservation of energy (Bray, 1978). Energy balance in animals is a homeostatic process with stable body weight hovering around the mean for an individuals height and sex. As the concept of an 'ideal body mass' is a rather arbitary one, the dividing line between lean and obese individuals is not well defined.

Energy intake, especially in humans varies considerably. Environmental, social and psychological factors, as well as growth and pregnancy all alter food intake in humans. As the energy taken into an animal must equal the energy it expends plus the energy stored in its tissues, some homeostatic mechanism must control energy stored to maintain energy balance. In recent years, it has become clear that energy balance is regulated by the hypothalamus, although the mechanisms involved in this process are completely unknown. Understanding the hypothalamic neurophysiology and also the effector mechanisms that control energy balance in man will be a lever for pharmacological or surgical aleviation of the obese syndrome. Research into obesity also provides information on how our bodies adjust their overall metabolic efficiency, such that stored energy can be wasted or conserved. Such information may be of use in helping people suffering from long term energy malnutrition.

1.2. Animal models of obesity.

Obesity may be classified into a number of categories: hypothalamic, endocrine, genetic and dietary. Hypothalamic obesity is rare in man and has been mostly associated with other hypothalamic diseases (Bray, 1974). A major factor in the genesis of this obesity is an enhanced secretion of insulin. The increased lipid is accommodated through fat cell hypertrophy (Hirsch and Han, 1969). Animal models of this obesity are the hypothalamically lesioned rat and chemically lesioned (gold thioglucose) mouse, in both of which the ventromedial nucleus of the hypothalamus is damaged.

Endocrine obesity in humans is associated with Cushing's syndrome, insulinomas, castration, or may remain after pregnancy. Animal models exist for these types through corticosteroid or insulin injections, or by castration (Bray et al., 1974).

There are general genetically transmitted obesities in animal models which exhibit different modes of inheritance (Bray and York, 1971). The yellow obese mouse results from an autosomal dominant gene with three varieties, in which the degree of obesity is proportional to the yellow pigmentation. Three types of genetic obesity exhibit Mendelain recessive characteristics: the obese mouse (ob/ob), the allelomorphic diabetic mouse (db/db), the adipose mouse (ad/ad) and the fatty rat (fa/fa).

Dietary manipulation may also be used to induce obesity although the success of each protocol is often dependant upon the strain of rats. Among the manipulations used are high fat diets, sucrose feeding and the currently popular "Cafeteria" type diets (Rothwell and Stock, 1979).

1.3. Characteristics of the obese mouse (ob/ob).

The ob/ob mouse strain, originated from the C57BL/6J background. It was first described by Ingalls et al. (1950). The ob/ob mouse inherits its obesity as an autosomal recessive mutation on chromosome 6. The gene defects have been maintained in the University of Southampton animal house on a strain of mice

originating from the Institute of Genetics at Edinburgh, that was chosen for its better breeding performance.

The ob/ob mouse may provide a good model for juvenile-onset obesity in humans since several characteristics of each syndrome are similar (Bray et al., 1974). The characteristics of the ob/ob mouse have been fully discussed by Bray and York, (1979), and only a brief summary of the complex metabolic changes that occur in this animal model will be discussed here. Fuller discussions on the relevance of these changes to experiments described in this thesis are in the introductions to each chapter.

1.3.a. Development

A decrease in oxygen consumption and a fall in core temperature in the ob/ob mouse are apparent as early as 10 days of age (Trayhurn et al., 1977). Shortly after this (17-21 days) there is a small increase in serum insulin with a consequent hypoglycaemia (Dubuc, 1976). Obesity as indicated by increased carcass fat, is detectable by days 17-21. This is accompanied by a decreased muscle protein deposition (Dubuc, 1976). After weaning, hypophagia is observed till day 28, when the animals become hyperphagic (Liu and Yin, 1974). Hyperplasia and hypertrophy of the adipose tissue is observed, with increasing hyperinsulinaemia and hyperglycaemia. Brown adipose tissue becomes refractory to stimulation (Hogan and Himms-Hagan, 1979), and cold tolerance becomes impaired. With advancing age (over 6 months) weight gain stops.

1.3.b. Brain and hypothalamus.

A generalised reduction in weight of most areas of the brain of ob/ob mice has been observed by Bereiter and Jeanrenaud, (1979) the concentration of neurotransmitters in the hypothalamus of ob/ob mice is altered (Lorden et al., 1975). The altered morphology of the brain in this animal is suggestive of congenital hypothyroidism, (Joosten and Van der Kroon, 1976).

1.3.c. Endocrine status.

Serum insulin rises to levels 50 fold higher in the ob/ob mouse compared to lean animals. Basal hyperinsulinaemia contributes to the obesity but is unlikely to be the sole, primary cause of obesity (York and Bray, 1979). Insulin resistance follows hyperinsulinaemia. The mechanisms underlying hyperinsulinaemia are unclear, although the autonomic nervous system (Bray et al., 1981) and a pituitary factor (Beloff-Chain, 1979) have been implicated in excessive insulin secretion by the β cell. Down regulation of tissue insulin receptors results from the hyperinsulinaemia, and this contributes to the developing insulin resistance (Bray and York, 1979). Glucagon secretion by the pancreas is also increased (Le Marchand-Brustel et al., 1977). Adrenal hypertrophy and increased serum corticosterone levels are observed in ob/ob mice (Bray and York, 1979) in response to increased serum ACTH secretion from the pituitary. Catecholamine resistance is also observed in adipose tissue from ob/ob mice (Dehaye et al., 1978) in vitro.

Thyroid hormone metabolism has been found to be normal in ob/ob mice (Bray and York, 1979) although thyroid hormone resistance by the tissues has been observed. For example both the thyroid hormone stimulation of Na^+/K^+ ATPase (Bray and York, 1979) and catecholamine-induced lipolysis (Otto et al., 1976) are deficient in ob/ob mice. These observations, consistant with a functional hypothyroidism may account for the altered brain morphology, hypometabolism and impaired thermogenic ability of the ob/ob mouse (Bray and York, 1979).

1.3.d. Carbohydrate, Lipid and Protein metabolism.

Fasting hyperglycaemia in the ob/ob mouse is probably the result of insulin resistance, impaired glucose transport into muscle (Cuendot et al., 1976) and increased rates of gluconeogenesis and glycogenolysis (Bray and York, 1979). Impaired carbohydrate metabolism may be secondary to the increase in glucagon and glucocorticoid secretion (Bray and York, 1979).

The failure of ob/ob mice to deposit normal amounts of muscle protein may contribute to the hypometabolism of this animal, as it has been suggested that the regulation of protein turnover may contribute to energy balance (Yousef and Chaffee, 1970). Also, Miller et al. (1979) have observed a lack of dietary regulation of hepatic protein turnover in the ob/ob mouse, whereas in lean littermates, a sigmoidal relationship between dietary energy and hepatic protein turnover was observed.

Increased lipogenesis in both liver and adipose tissue occurs from an early age in the ob/ob mouse (Bray and York, 1979). The control of fatty acid synthesis is relatively normal in ob/ob mice, the observed hyperlipogenesis probably resulting from the hyperinsulinaemia. The increased turnover rate of glycerol in adipose tissue from ob/ob mice suggests that the lipolytic function is not impaired. However, the response of lipolysis to catecholamines may be impaired. This is secondary to the impaired elevation of intracellular cAMP produced by an impaired response of the adenylate cyclase to hormones (Dehaye et al., 1978).

1.3.e. Energy conserving mechanisms in the ob/ob mouse.

The failure to demonstrate any major defect in lipid metabolism in the ob/ob mouse suggests that lipid accumulation is secondary to a metabolic lesion involved in the conversion of dietary energy to waste products. James and Trayhurn (1981) have suggested that less energy is spent on thermoregulatory thermogenesis in ob/ob mice compared to lean because the core temperature of the ob/ob mouse is reduced and that this may account for the increased efficiency of food utilisation.

Various metabolic processes have been postulated to contribute to non-shivering thermogenesis in mammals, e.g. ion pumping across membranes (Guernsey and Stevens, 1977; Bray and York, 1979); protein turnover (Miller et al., 1979); substrate cycling (Newsholme, 1978); uncoupling of brown adipose tissue mitochondria (Foster and Frydman, 1979). Although all of these processes are thermogenic, and certainly contribute to basal metabolic rate, identifying metabolic pathways that are impaired sufficiently to

change the energy requirements of the whole animal is an exceptionally difficult problem.

1.4. Possible importance of membrane defects in the aetiology of obesity.

All of the defective metabolic processes (described in the previous section), that occur in the ob/ob mouse are the net result of a single enzyme defect (Coleman, 1978). The type of enzyme that is mutated and its tissue and cellular location are completely unknown at present. The enzyme defect is not directly responsible for many of the observed metabolic changes in the ob/ob mouse, as fat cell tissue transplant studies from an obese donor to a lean recipient demonstrate that many of the metabolic abnormalities of the transplanted tissue disappear (Bray and York, 1979). Thus, many of the metabolic abnormalities in the ob/ob mice are secondary to the gene defect. The defects that have been described cover a wide range of metabolic changes and it is difficult to see how a single protein defect could explain such changes. For example, if the primary defect is the control of thermogenesis, how could such a defect explain the hyper secretion of insulin, the increase in food intake and the failure to deposit normal amounts of protein? It is easier to envisage that the defective thermogenesis is simply another expression of the gene defect. These considerations lead to the conclusion that there is perhaps a general change that occurs in the tissues of the ob/ob mouse which results in the alteration of cell metabolism dependant on the specialised function of that particular tissue. The search for a generalised alteration of cell metabolism that precipitates the obese condition is of interest because it is precisely at this level that pharmacological treatment of the obese syndrome may be useful, and also in establishing the nature of the generalised metabolic disturbance will certainly help in the identification of the mechanisms that regulate energy balance. What then could be the locus of such a postulated, generalised metabolic change in the tissues that precipitate obesity? A defect in membrane composition could be the link between all of these metabolic changes. Without exception, all of the known metabolic

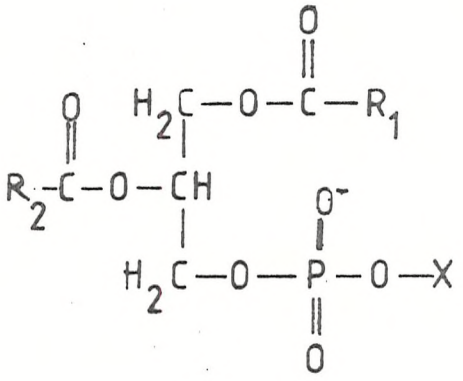
defects, at some stage or another, are associated with some membrane related process. For example, insulin secretion by the β cell is under both metabolite and nervous control. Both of these effector mechanisms involve the transducing of a 'signal' across the β cell plasma membrane. A defect at the plasma membrane level may give rise to the enhanced insulin secretion by this tissue in the ob/ob mouse. The hormone resistance observed in many tissues of the ob/ob mouse could be explained by a plasma membrane defect, as the cellular response to hormone binding to the plasma membrane is mediated by various transmembrane signalling processes. Also, the impaired thermogenic response could be a failure of hormone activation of thermogenic tissue at the plasma membrane level. Finally, the altered neurotransmitter levels observed in the hypothalamus of the ob/ob mouse may result from an impaired secretion and uptake of neurotransmitters by synaptosomal membrane.

A possible defect in tissues at the level of the cell plasma membrane of ob/ob mice may be a more generalised membrane defect, and other membranes such as the endoplasmic reticulum and the mitochondrial membranes may also be defective in the ob/ob mouse.

1.5. Structure and composition of biological membranes.

It has been suggested that the properties of the cell membranes may be altered in the ob/ob mouse. In order to identify possible components that may have undergone change, the structure of the biological membrane is considered. The current concept of the structure of biological membranes is that of the model proposed by Singer and Nicholson (1972). The model proposes that the membrane consists of a bilayer of fluid lipid, with proteins either integrated with the membrane matrix, or associated with the surface of the bilayer. The proteins are free to rapidly migrate in the plane of the bilayer. The protein components of the membrane comprise the hormone receptors, metabolite transporters, redox enzymes, etc. The structure and activity of these membrane proteins are dependant on their lipid environment (Lenaz, 1977). The bilayer itself is comprised mainly of amphipathic molecules called phospholipids. These phospholipids have specific head-groups that define the class of phospholipid, esterified via a phosphate group to the 3 carbon position of glycerol (Fig 1.1).

GLYCEROPHOSPHOLIPIDS

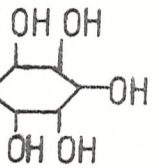


PE : X = $-CH_2-CH_2-NH_3^+$

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

PS : X = $-CH_2-\underset{\underset{COO^-}{|}}{\overset{H}{\overset{|}{C}}}-\overset{+}{N}H_3$

PI : X =



SPHINGOMYELIN

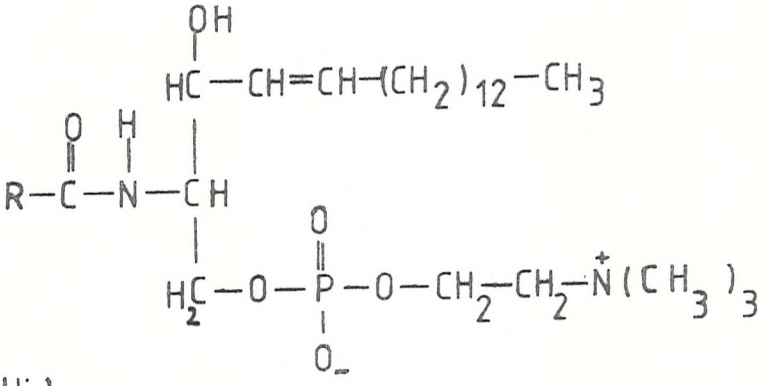


Fig 1.1. Major phospholipid classes of mammalian cell membranes.

Abbreviations:- PE = Phosphatidylethanolamine
PC = Phosphatidylcholine
PS = Phosphatidylserine
R = Fatty acyl group

PI = Phosphatidylinositol

The 1 and 2 carbon positions of glycerol are occupied by esterified fatty acyl groups. These fatty acyl moieties are usually liquid (above their melting temperature) when esterified to the polar phosphoryl glycerol, which is itself in a rigid quasi-crystalline structure (Lenaz, 1975). The phospholipid molecule is thus described as being in a liquid crystalline state. If the hydrocarbon chains are cooled to below their melting temperature, the phospholipids are said to be in the gel phase.

Biological membranes consist of two sheets of these molecules stacked on top of each other, forming a bilayer hydrocarbon matrix. The phospholipid headgroups separate the aqueous medium so as to form a diffusional barrier for water soluble molecules, and therefore the membrane compartmentalises the aqueous medium. If the membrane matrix is continuous, a vesicle or cell is formed, and thus the membrane delineates an 'internal' and 'external' environment. Proteins incorporated into these membranes are themselves amphipathic. Hydrophobic regions on these proteins (containing non polar amino acids) interact with the hydrocarbon matrix. Hydrophilic regions of the proteins (containing polar amino acids) interact with both the headgroups of the phospholipids, and the aqueous medium. Protein-lipid interactions play a key role in the structural and functional properties of membranes (Lenaz,1977). These interactions can be classified as two types. There are specific interactions between proteins and lipids that affect the structural and functional properties of both components. At the same time, the lipid as a bulk phase is important in orienting proteins to the compartments separated by the membrane. The proteins described above are integral membrane proteins, that is they either penetrate the bilayer either completely (span the membrane) or partially, so that the protein is oriented permanently to just one aqueous compartment. Extrinsic membrane proteins are only loosely associated with the membrane, and will not be considered here.

The function of membrane proteins can be modified either by the class of polar headgroup of the phospholipid interacting with the protein, or by the physical state, or conformation of the

phospholipid fatty acyl chains. Mammalian membranes contain a wide variety of fatty acid species, which complicates the study of membrane protein interaction. Fatty acid species differ from each other in their chain length and degree of unsaturation. The degree of unsaturation of fatty acyl moieties determines the viscosity of the membrane matrix directly, and may directly alter protein mobility, activity and interaction with other proteins or membrane components (Lenaz, 1977).

Several techniques have shown that lateral motion of the individual molecules is very rapid in liquid-crystalline lipid matrices, (Lenaz, 1977) and the translocation of amphipathic molecules from one half of the bilayer to the other is slow (Kornberg and McConnell, 1971), partly because movement of a hydrated, polar region through a hydrophobic hydrocarbon matrix is thermodynamically unfavourable. The biological membrane, then produces a diffusion barrier for cells and is composed of many phospholipid species, which provides a matrix for membrane bound proteins to be vectorially oriented. The dynamic nature of membrane organisation and the wide variety of different membrane components accounts for the wide variety of metabolic processes that can be regulated by the cell membranes. Thus, changes that may occur in the membrane matrix may be expected to modify metabolic processes associated both directly and indirectly with the cell membranes.

1.6. Proposed investigations into cell membranes from lean and obese mice.

As the lipid matrix is so important in membrane related processes, detailed compositional analysis of membranes from lean and obese mice was carried out in order to ascertain if there were any gross structural differences between membranes from the two phenotypes. The protein content to phospholipid ratio was measured, as well as the proportion of each phospholipid class. Also, physical techniques were utilized to measure the internal viscosity of the hydrocarbon matrix of several membranes from lean and obese mice. Information concerning membrane viscosity is related to the fatty acid composition of the phospholipids, the protein

to phospholipid ratio and also to the amount of cholesterol within the lipid matrix. The presence of protein and cholesterol in phospholipid bilayers reduces the mobility of the phospholipid fatty acyl chains, and thus increases the viscosity of the membrane hydrocarbon matrix (Shinitzky and Inbar, 1976). The effects of protein on the viscosity of the hydrocarbon matrix of membranes from lean and ob/ob mice was assessed by extracting the bulk lipid fraction and reconstituting the extract into artificial membrane vesicles called liposomes. The components comprising the reconstituted liposomes will of course be 'scrambled'. In other words the bilayer halves of the liposomes will contain equal proportions of all clasees of lipid present, and thus any assymetric arrangement of the lipid species originally present in the membrane will be destroyed by this procedure. The effects of removing both protein and cholesterol can also be assessed in a similar manner, by removing cholesterol from the bulk lipid extract. Also each phospholipid class can be isolated and reconstituted into liposomes for viscosity measurements. Such information builds up a concise picture of the contribution of each component to the bulk lipid viscosity.

As the viscosity of the hydrocarbon matrix is dependant on the fatty acyl composition, the fatty acid profile of each membrane phospholipid from both lean and obese mice membranes was assessed by Gas Liquid chromatography, in order to correlate viscosity measurements of lipid systems with their fatty acyl composition. The distribution of phospholipids on either side of the membrane was measured, to establish whether membranes from lean and obese mice have the same assymetric distribution of phospholipid classes.

Finally, the activities of certain membrane proteins were examined in both lean and ob/ob mice, and correlated with the composition of their lipid matrix. Thus, a complete study of cell membranes from both phenotypes may establish the hypothesis that membrane perturbations are involved in the aetiology of obesity.

1.7. Probing of the dynamic and molecular organisation of biological membranes.

Many techniques have been used to probe biological membranes. A probe of a biological system is considered to be a molecule, small relative to that system, providing information about the limited volume in which it is found. A molecular probe gives direct imformation only about itself, but provides much indirect evidence about their environment (Badley, 1976). The major techniques that have been used to probe biological membranes are:-

a) Electron spin resonance (ESR)
b) nuclear magnetic resonance (NMR), and
c) fluorescent probes.

ESR techniques utilise a nitroxide free radical bound to various sites of membranes or membrane components. The spin label absorbs electromagnetic radiation in the presence of an external magnetic field, and the interaction of the unpaired electron with nuclear spin produces a characteristic absorption spectra. The shape of the spectra reflects the orientation and motion of the spin label, as well as the polarity of its environment (Smith, 1972).
Although spin labels are popular methods of probing lipid mobility in bilayers and membranes, NMR has also been used extensively to probe the physical states of lipids in bilayers and membranes (Chapman, 1973). Any nucleus with a non zero magnetic moment will interact with electromagnetic radiation in the presence of an external magnetic field, and absorb energy. The degree of shielding of the nucleus by other local nuclei will change the value of the resonance energy. Another more sophisticated technique utilises the relaxation properties of nuclei that have been excited by external radiation. These techniques have been utilised to measure properties of various regions of the hydrocarbon matrix and also those of the polar headgroups of phospholipids.

In this thesis, much use is made of fluorescent probes to investigate the properties of biological membranes and lipid mixtures. In particular, the technique of measurement of polarised intensities of fluorophores is used, hence a discussion of this technique follows. Electronic absorption of an incident photon

by a fluorophore promotes an electron from an originally occupied bonding or non bonded orbital to an originally unoccupied molecular orbital, to form an excited state. As there are usually several unoccupied orbitals, several elctronically excited states are possible for each molecule, giving rise to unique absorption spectra for each fluorophore. It is the relaxation of these excited state electrons back to the ground state that gives rise to photophysical phenonena such as fluorescence. Relaxation occurs by more than one process, energy also being lost by non-radiative processes. This results in a Stokes-shift of the emission radiation to longer wavelengths than the excitation wavelength, and a characteristic quantum yield of the fluorescent process, which is a measure of the rate of photon emission as fluorescence compared to the rate of absorption. Fluorescence lifetimes are typically in the order of 10^{-7} to 10^{-9}sec.

The photophysical event of absorption and emission of a fluorophore is modified by many processes. Excited state lifetimes, quantum yield, and Stokes shift are all modulated by, for example, the polarity of the solvent environment of the fluorophore. In the fluorescence of aromatic compounds the process of absorption and emission of electromagnetic radiation are associated with transition dipoles of a well defined orientation on the molecular frame. Both the excitation and emission dipoles lie on the plane of the molecule. In the finite time between absorption and emission of radiation, the orientation of the molecular frame will not have changed if the molecule is in 'frozen' solution. However, if the molecule is undergoing axial rotation, the orientation of the molecule changes over the fluorescence time period. The technique of fluorescence polarisation utilizes this event to give information concerning the freedom for molecule motion, as measured by the degree of angular displacement of the transition dipole during the lifetime of the excited state. Experimentally, this can be achieved by photoselecting molecules in a fixed orientation with respect to defined laboratory axis, by exciting the fluorophores with polarised light. The emission dipole displacement is proportional to the ratio of the intensities observed through two orientations of a polarising lens (Fig 1,2). OX, OZ and OY are the laboratory axis, defined by the

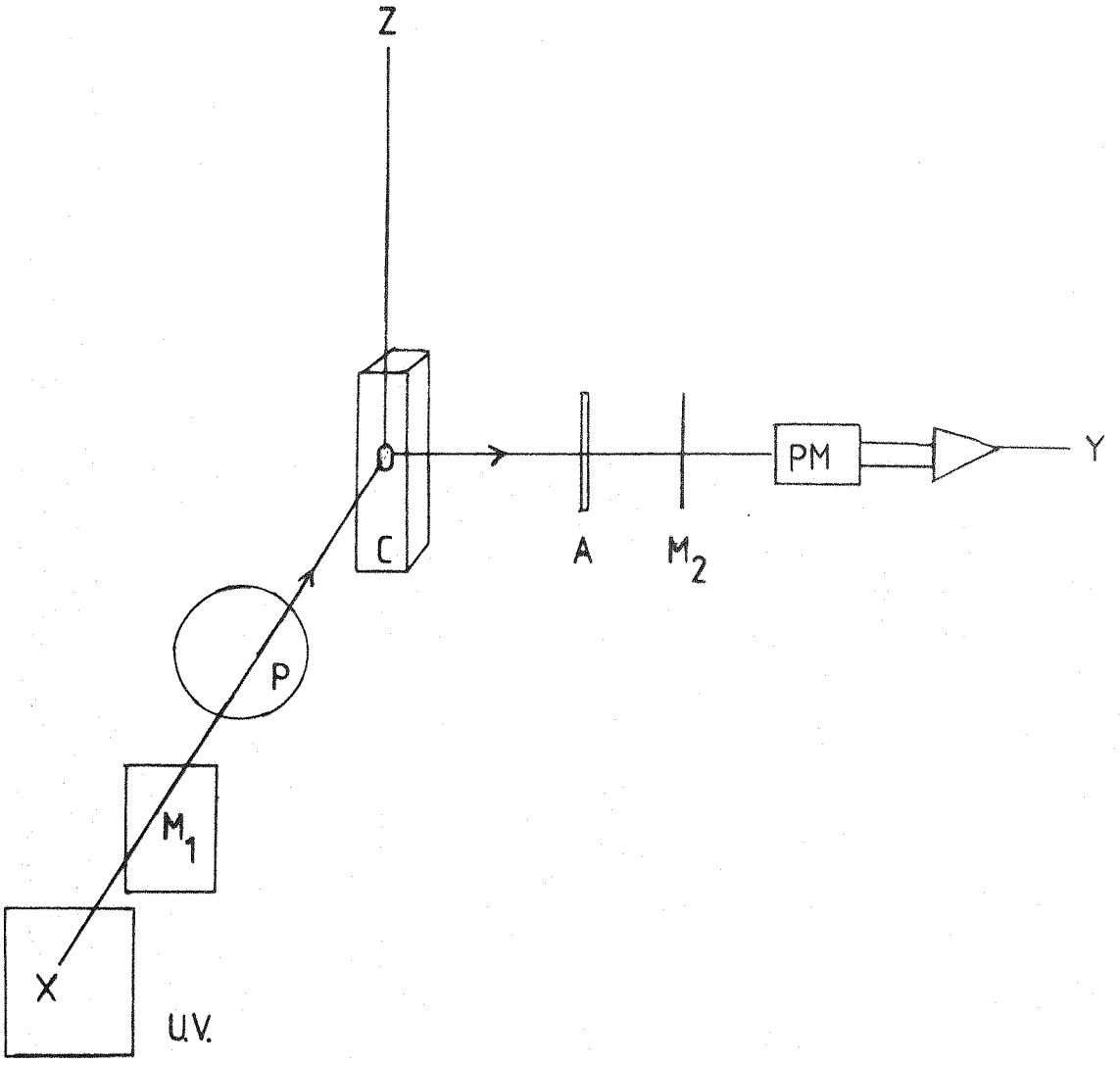


Fig 1.2. Light Path for measurement of polarised intensities.
M = monochromator, P = polarising lens. C = cuvette.
A = analysing lens. PM = photomultiplier.
OX, OY, OZ = laboratory axis. U.V. = Light source.

plane of polarisation of the exciting radiation (ZY plane) propagated along XO, then if the emission intensity is detected through a polarising lens (analyser) in the XZ plane between OY oriented parallel and perpendicular to the direction of polarisation of the excitation light then the degree of fluorescence polarisation of the emission dipole is defined as the difference divided by the sum of the two measured intensities. Because the degree of polarisation is a ratio of two intensities, the units of measurement are unimportant.

If the degree of rotation of the fluorophore is in the same order (10^{-9} rad sec^{-1}) as the fluorescence lifetime of the excited state, then the degree of depolarisation of the excitation dipole is proportional to the degrees of freedom of molecular rotation of the fluorophore about its axis. This in turn is governed by the number of degrees of freedom for molecular interaction between the probe and its environment. The equation which describes the general relation between the fluorescence depolarisation by a fluorophore and the hydrodynamic properites of the surrounding medium is known as the Perrin equation. This allows direct calculation of the 'viscous drag' imposed upon the rotating fluorophore by the solvent (see Shinitzky and Barenholz, 1978). The equation described by these authors allows calculation of the viscosity of the solvent from measurement of polarised intensities providing that factors pertaining to the molecular shape, precise location of the transition dipoles and the fluorescence lifetime of the fluorophore are known. Since the latter two factors are not constant for most fluorophores, the probes must be calibrated in a defined medium. The validity of this calibration for isotropic (ramdomly ordered) solvents for use in anisotropic system like biological membranes has been questioned by Hare and Lussan, (1977). Therefore the value of a rigourous determination of viscosity is not very high under certain situations, and therefore it suffices to regard the value of polarisation ratio obtained experimentally as a factor containing some information on the viscosity of the solvent medium. Values for polarisation measurements become increasingly more useful when two similar, but not identical solvent systems are employed for

study. The values of P serve to differentiate hydrodynamic properties of the two solvent systems. Under these conditions, the actual value of the viscosity callibration becomes less important and we can use the terms 'more viscous' or 'less viscous' to qualitatively differentiate physical properties of solvents as measured by fluorescence polarisation techniques.

1.8. Use of 1,6 diphenyl hexa 1,3,5 triene (DPH) as a fluorescent probe.

The fluorescent properties and the validity of steady-state polarisation measurements of biomembranes has been documented by Shinitzky and Barenholz. (1978). This fluorescent probe is rod-shaped, and does not appear to cause large perturbations in its environment, indeed it is probably this property that allows it to equally partition into both gel and liquid crystalline regions (Shinitzky and Barenholz, 1978). DPH is easily incorporated into lipid membranes, and gives highly reproducible polarisation data with standard lipid mixtures. The absorption and emission maxima are well separated, and thus this probe is ideal for use in steady state polarisation on relatively inexpensive equipment.

The majority of the experiments in this thesis concerned with the measurement of the viscosity of the hydrocarbon matrix of membranes and lipid mixtures are performed using DPH as fluorescent probe. The thermotropic properties of probe labelled membranes and lipid mixtures were determined over a wide temperature range. This not only gives very accurate viscosity data, but also gives added information about the nature of components present within the lipid matrix.

DPH was used as a fluorescent probe to investigate the viscosity parameters of whole membranes, liposomes of lipid mixtures and phospholipid liposomes derived from lean and obese mouse tissues. Viscosity parameters are expressed as the 'fluidity ratio' Φ_R, at a particular temperature, and is simply the viscosity calculated from the Perrin equation of one system divided by the other. This manipulation results in an easily recognizable fluidity change in one system compared to another. Since it is a ratio, errors in interpretation of the calibration factor used to calculate are not important.

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CHAPTER 2

MATERIALS AND METHODS

2.1. Preparation of hepatic microsomes from lean and obese mice.

Four lean and four obese mice were killed by cervical dislocation. The livers were pooled and minced with scissors in 5 volumes of 250mM sucrose, 10mM tris-HCl, 1mM EDTA pH 7.4 at 4°C (Buffer A). The tissue pieces were then washed in two changes of buffer A and then homogenised in 5 volumes of buffer A in a motor-driven 50ml Jencons Uniform teflon-glass homogeniser at 5,000rpm. The homogenate was then centrifuged at 17,000 X g (R_{av}) for 10 minutes at 4°C. The infranatant was carefully removed, leaving behind the fat cake and pellet. Microsomes were sedimented by centrifugation at 105,000 X g (R_{av}). The supernatant was decanted and the high speed pellet was then homogenised in 4ml of buffer A. Protein concentration was estimated by the method of Lowry et al., (1951), prior to dilution with buffer A to 1mg microsomal protein per ml.

The microsomal suspension was then stored under nitrogen at -50°C until required.

2.2. Preparation of hepatic plasma membranes.

Plasma membranes were purified from livers of lean and obese mice according to the method of Ray (1970). Four lean and four obese mice were killed and the livers from each group were pooled and minced with scissors in 5 volumes of 250mM sucrose, 10mM tris-HCl, 0.5mM $CaCl_2$, pH 7.4 at 4°C; (Buffer B). All subsequent steps were performed at 4°C. The liver pieces were washed twice with buffer B. 2.5g of the minced tissue was then homogenised in 40ml of buffer B in a 50ml motor-driven Jencons Uniform homogeniser at 5,000rpm. The homogenate was then filtered through two layers of fine mesh nylon and left to stand on ice for 5 minutes. The homogenate was diluted to 250ml with buffer B and centrifuged at 2,000rpm in an MSE 6 X 250ml rotor for 30 minutes. The pellet was re-homogenised in buffer B and diluted to 125ml with buffer B, and centrifuged for a further 15 minutes as before. This process was repeated twice. The final pellet was then homogenised in 2-3ml 250mM sucrose, 10mM tris-HCl, 10mM EDTA ph 7.4 and 4°C (Buffer C).

Plasma membranes were then purified on a 4 step discontinuous sucrose gradient by the following method. Enough 70% sucrose solution was mixed with the 2-3ml homogenate to bring the final sucrose concentration to 48%. This solution was then divided into three aliquots and placed in 3 25ml polycarbonate centrifuge tubes on ice. Over this was layered 8ml 45% sucrose, 8ml 41% sucrose, and finally the tubes were overlayed and balanced with 2-4ml 37% sucrose and centrifuged for 2 hours at 25,000rpm in an MSE 3 X 25ml S.O. rotor. The plasma membrane fraction appearing at the 41% - 37% interface was collected with a pipette, diluted to 40ml with buffer C and centrifuged at 30,000 X g for 15 minutes in and MSE 8 X 50ml rotor. The pellet was resuspended to 1mg plasma membrane protein ml^{-1} by twenty up and down strokes of a tight fitting glass homogeniser in buffer C, and stored in 200µl aliquots under nitrogen at -50°C until required.

Sucrose solutions were prepared in the following manner. 500ml of approximately 72% sucrose was made by adding 50ml 100mM EDTA, 100mM tris-HCl pH 7.4 at 4°C to 380g sucrose with stirring. Enough distilled water was added to the dissolved sucrose to bring the final volume to 500ml. 5ml of this solution was then pipetted into a sack of dialysis tubing containing 5mg activated charcoal. The solution was then dialysed over night with the dialysis sack, with stirring at 4°C.

The solution was then adjusted to 70% (± 0.1%) at 25°C using a Zeiss refractometer with buffer C. A 200ml aliquot of this solution was then taken and the remainder adjusted to 45% (± 0.1%) and a further 200ml aliquot removed. In this manner, 200ml aliquots of 41% (± 0.1%) and 37% (± 0.1%) were obtained. All sucrose solutions were stored at -50°C until required.

EDTA was present in all gradient solutions, as we found that excessive 'clumping' of the membrane fractions took place in its absence.

2.3. Preparation of adipocytes from epididymal fat pads from lean and obese mice and rats.

The adopted procedure was essentially that of Gliemann (1967) with some modifications. Mice were killed by cervical dislocation and both epididymal fat pads were removed. Blood vessels and vas deferens were trimmed away and pooled tissue was minced with scissors to a fine consistency in buffer containing the following: 100 vol. 0.9% NaCl, 4 vol. 1.12% KCl, 3 vol. 1.22% $CaCl_2$, 1 vol. 3.8% $MgSO_4$, 1 vol. 2.11% KH_2PO_4, 21 vol. 3.4% HEPES, 4% BSA, RIA grade, pH 7.4 at 37°C. To this 1mg ml^{-1} collagenase was added and incubated at 1ml buffer gm^{-1} tissue. Digestion was carried out for 30 minutes, shaking at 120 cycles $minute^{-1}$. At the end of this period, liberated adipocytes were sieved through two layers of fine mesh nylon into a plastic 12ml centrifuge tube. The cell suspension was left to stand for 2 minutes. At the end of this period, the infranatant was removed via a length of plastic tubing that had been inserted to the bottom of the tube and held in place by acrylic glue. The plastic tubing was connected by means of a screw clip on the vacuum line. The cells were washed X 3 with Krebs-HEPES buffer containing 1% BSA minus collagenase. Cells were interspersed with washing medium by gently tipping the tube from side to side after being closed with a piece of sealing film.

2.4. Preparation of adipocyte plasma membranes from lean and obese mice.

This was essentially similar to the method of Jarret (1974) with some modifications. The isolated washed adipocytes were diluted to 5 vol. with 250mM sucrose, 1mM EDTA, 10mM tris-HCl pH 7.4 at 4°C containing 1mg ml^{-1} Bacitracin at 25°C. The cells were homogenised in a loose fitting 50ml Jencons homogeniser at 25°C with 10 up and down strokes at 2,000rpm. The homogenate was then immediately centrifuged at 1,000 X g on a MSE minor centrifuge at R.T. for 2 minutes. The infranatant was then left to stand on ice. This last manipulation increases the yield of plasma membrane, as the triglyceride separates easily from cell organelles in the liquid state at room temperature, whereas if allowed to cool prior to a brief centrifugation, the congealed

triglyceride traps plasma membrane. Bacitracin is present in the homogenisation medium to reduce proteolytic degradation of membrane components. The cooled homogenate was then centrifuged at 16,000 X g (R_{av}) for 10 minutes in an 8 X 50ml rotor. The pellet was then homogenised in 6ml of 250mM sucrose, 1mM EDTA, 10mM tris-HCl pH 7.4 at 4°C (Buffer E) and centrifuged at 500 X g for 2 minutes. The supernatant was decanted off and then layered over 15ml 9% Ficoll made up in buffer E, contained in an MSE 25ml polycarbonate centrifuge tube, and was then centrifuged at 59,000 X g for 45 minutes in an MSE 3 X 25 S.O. rotor. The purified plasma membrane fraction, appearing as a sharp band at the buffer E/9% Ficoll interface, was then collected with a pipette and diluted with buffer E to 40ml and centrifuged at 25,000 X g for 15 minutes in an MSE 8 X 50 rotor. The pellet was made up to 1mg protein ml^{-1} in buffer B, divided into 200µl aliquots and stored under nitrogen at -50°C until required.

2.5. Preparation of red blood cell plasma membranes from lean and obese mice.

The method employed was essentially that of Hanahan and Ekholm (1974). Animals were decapitated and blood was allowed to drip freely from the torso via a collecting funnel to a plastic 12ml heparinized centrifuge tube. Blood was collected from 12 animals of each phenotype. After collection, the tubes were centrifuged at 1,000 X g for 30 minutes in an MSE minor centrifuge installed in a cold room at 4°C. Plasma was decanted off and the layer of white cells gently washed from the underlying erythrocytes with 172ml tris-HCl pH 7.4 at 4°C which is isotonic with plasma. Erythrocytes were then suspended in 1 vol. of isotonic buffer by gentle homogenisation with a loose fitting homogeniser and the suspension re-centrifuged at 1,000 X g for 30 minutes. Erythrocytes were washed twice more in this fashion. Erythrocytes were then suspended in 1 vol. of isotonic buffer and diluted with 6 vol. 11.1mM tris-HCl (buffer F), with shaking. Buffer F is a hypotonic buffer, and after the tubes have been allowed to stand on ice for 5 minutes complete haemolysis of the erythrocytes has occured. The tubes are then centrifuged at 20,000 X g in an MSE 8 X 50ml rotor for 40 minutes at 4°C. The

supernatant is decanted and the red cell plasma membranes homogenised in 40ml buffer F and re-centrifuged as before. Membranes were subject to a total of 4 sedimentation/homogenisation cycles. The resulting washed membranes are pinkish in colour due to small amounts of trapped haemoglobin. Membranes were suspended in 250 mM sucrose, 10mM tris-HCl, 1mM EDTA to 1mg membrane protein ml^{-1} and stored at -50°C under nitrogen until required.

2.6. Preparation of sarcolemma from lean and obese mouse hind limb muscle tissue.

Sarcolemma was purified from muscle homogenate by the method of Cheng et al. (1977), preceded by a modified method of obtaining muscle homogenates.

Hind limb muscle tissue was obtained by killing 20 mice by cervical dislocation, removing the hind legs by cutting the pelvic joint, and stripping the skin away from the muscle with forceps. Muscle tissue was cut away from the leg bones and separated from the surrounding connective and adipose tissue as much as possible. However, it is not possible to completely separate all adipose tissue from muscle tissue, especially in tissue from obese mice.

Since adipocyte plasma membrane co-purifies with sarcolemma and is obtained in considerable better yield, it was initially necessary to remove the contaminating adipocytes as descirbed below.

Excised tissue was pooled in ice cold Krebs-HEPES buffer pH 7.4 at 4°C, as described in 'Preparation of adipocytes'. The mince was washed twice in this buffer. The washed mince was then transferred to 2 vol. of Krebs-HEPES buffer pH 7.4 at 37°C containing 1mg ml^{-1} collagenase and shaken for 30 minutes in a 37°C water bath at 120 cycles per minute. At the end of this period, the tissue suspension was centrifuged at 1,000 X g for 2 minutes. Muscle tissue, now completely free of adipose tissue, sedimented while liberated adipocytes and fat floated at the top of the tube. Adipocyte plasma membranes from burst adipocytes remained in the infranatant. The fat cake and infrana-

tant were discarded and the sediment washed 3 times in 250mM sucrose, 1mM tris-HCl pH 7.8 at 4°C and then homogenised in 4 vol. of this buffer in an Ultra-Turrax speed 5 for 10 sec. The homogenate was then filtered through nylon gauze and centrifuged at 2,000 X g for 10 minutes in an MSE 8 X 50 rotor. The sediment was suspended in 10 vol. (per initial muscle wt.) 0.5M LIBr, 0.05 mM EGTA, 10mM tris-HCl pH 8.5 at 4°C by 10 up and down strokes of a Jencons 50ml motor- driven homogeniser at 3,000rpm. This solution was then transferred to a 250ml glass breaker, covered with sealing film and magnetically stirred at 100rpm overnight, and then centrifuged at 2,500 X g for 15 minutes in an MSE 8 X 50ml rotor. The supernatant was then decanted and centrifuged in the same rotor at 150,000 X g for 30 minutes. The sediment was then suspended in 3 vol. (per initial muscle wt.) 0.6M KCl, 10mM tris-HCl pH 8.0 at 4°C by 20 up and down strokes of a tight fitting glass 50ml homogeniser of unknown brand available in the Department. The suspension was allowed to stand on ice for 20 minutes and then centrifuged at 5,000 X g for 15 minutes in an MSE 8 X 50ml rotor. The supernatant was then centrifuged at 150,000 X g for 30 minutes in the same rotor. The sediment was suspended in 6ml 250mM sucrose, 1mM tris-HCl pH 7.8 at 4°C and layered in top of 15ml 30% sucrose (± 0.1%, Zeiss refractometer) 1mM tris-HCl pH 7.8 at 4°C in a 25ml polycarbonate centrifuge tube. The gradient was centrifuged at 100,000 X g for 2 hours. The sarcolemma enriched fraction at the 250mM sucrose/30% sucrose interface was removed by pipette, diluted to 40ml with 250mM sucrose, 1mM EDTA, 10mM tris-HCl and centrifuged at 150,000 X g for 30 minutes. The membrane pellet was suspended to 1mg membrane protein ml^{-1} in the same buffer and stored at -50°C under nitorgen until required.

2.7. Preparation of whole brain synaptosomal membrane from lean and obese rats and mice.

The method employed was essentially that of Gray and Whittaker (1962), with some modifications.

The entire brains from 3 animals were removed from their crania, and homogenised in 40ml 0.32M sucrose, 10mM Histidine-HCl

pH 6.5 at 4°C (Buffer H). Histidine was chosen rather than tris-HCl, since a pH of 6.5 is too far removed from the pK of tris-HCl to be of any use as an effective buffer, and a pH of 6.5 is essential in the preparation of pure synaptosomes. Using a more effective buffer removed 'clumping' problems experienced by ourselves and other workers.

The homogenate was centrifuged at 800 X g for 10 minutes at 4°C. The supernatant was then re-centrifuged at 17,000 X g for 20 minutes in an 8 X 50 angle rotor in an MSE 21 centrifuge. The pellet was then re-homogenised in 50ml of buffer H. 6ml of this homogenate was then layered over a 2-step discontinuous Ficoll gradient. This consisted of 8ml 13% Ficoll in buffer H. and 8ml 7.5% Ficoll in buffer H. 3 tubes were prepared in this manner, and the gradients then centrifuged at 43,000 X g for 1 hour in a 3 X 25 swing-out rotor on an MSE Pegasus centrifuge, with the rotor acceleration at 15% of maximum. This procedure results in much improved separation for high loaded gradients.

The membrane fraction at the 7.5%/13% Ficoll interface was then removed, diluted to 40ml in buffer H and centrifuged at 17,000 X g for 15 minutes in an MSE 21 centrifuge.

The pellet is then taken up in 6ml of 0.32M sucrose, 1mM EDTA, 10mM Histidine-HCl, pH 6.5 at 4°C (Buffer I) and layered over a 2-step discontinuous gradient. This consisted of 8ml 12.5% Ficoll in buffer I and 8ml 8% Ficoll in buffer I. The gradient was then centrifuged at 43,000 X g for 1 hour in a 3X 25 swing-out rotor on an MSE Superspeed centrifuge.

The membrane fraction at the 8%/12.5% interface was removed, diluted to 40ml in buffer H and centrifuged at 50,000 X g for 15 minutes on an MSE 21 centrifuge. The membranes were then homogenised in buffer H to a membrane protein concentration of 1mg ml^{-1} and stored at -50°C under nitrogen until required.

Utilising a second gradient system, with altered gradient densities and EDTA present, resulted in considerably improved synaptosomal purity. Membrane yields are reduced using this technique, but purity is of overriding importance, since small

amounts of myelin considerably interfere with microviscosity studies on synaptosomes.

2.8. Preparation of salivary gland plasma membrane from lean and obese mice.

The 2 pairs of the submaxillary and parotid salivary glands were removed from 4 mice, and homogenised in 40ml 250mM sucrose, 1mM EDTA, 10mM tris-HCl pH 7.4 at 4°C (Buffer A). The homogenates were centrifuged at 1,000 X g for 4 minutes in an MSE minor centrifuge at 4°C. The supernatants were then centrifuged at 17,000 X g in an 8 X 50ml rotor for 20 minutes. The pellets were taken up in 50ml buffer A, 6ml of this solution was layered over 15ml 9% Ficoll in buffer A, and the gradients centrifuged at 60,000 X g in an MSE 3 X 25ml S.O. rotor for 45 minutes. The purified plasma membranes at the buffer A/9% Ficoll interface were aspirated from the gradient, diluted to 40ml with buffer A and centrifuged at 50,000 X g for 20 minutes in an MSE 8 X 50 rotor. The pellets were homogenised in buffer A to a membrane protein concentration of 1mg ml^{-1} and stored at -50°C under nitrogen until required.

In pilot experiments, 1% stepwise gradients from 7% - 12% Ficoll were employed to ascertain the isopycnic density of the bulk of the plasma membranes. 9% was found to be optimal for minimal mitochondrial contamination.

2.9. Preparation of hepatic mitochondrial inner membrane from lean and obese mice.

The method employed for this preparation was that of Chan et al. (1970). Ten lean and ten obese mice were killed by cervical dislocation and their livers removed and minced in 5 vol. 70mM sucrose, 220mM mannitol, 2mM HEPES pH 7.4 at 4°C containing 0.5mg ml^{-1} BSA (Buffer D). The minced tissue was washed twice in buffer D. 15g of the minced tissue was homogenised in 45ml buffer D by four up and down cycles of a Jencons 50ml homogeniser at 5,000rpm. The resulting homogenate was divided in 3 40ml centrifuge tubes and centrifuged at 660 X g for 10 minutes. The supernatants were then centrifuged at 7,000 X g in an MSE 8 X 50ml rotor for 15 minutes. The combined sediments were suspended

in 30ml buffer D and centrifuged for 15 minutes at 7,000 X g. The resulting mitochondrial pellet was suspended to a protein concentration of 100mg ml^{-1}, and stirred in a glass 20ml vial at low speed. A solution of 1.2% Digitonin was prepared in buffer D not containing BSA. An equal volume of this solution was added to the stirring mitochondrial suspension and stirred for 15 minutes. At the end of this period, the mitoplast suspension (mitochondria devoid of outer membrane) was diluted to 3 vol. with buffer D and homogenised in a glass homogeniser by hand and centrifuged at 9,500 X g for 10 minutes. The sediment was re-homogenised in half the previous volume of buffer D and centrifuged at 9,500 X g for a further 10 minutes. The sediment was then resuspended to 30-35mg ml^{-1} protein. A solution of 19mg ml^{-1} Lubrol WX in buffer D was added to the mitoplast suspension to give a final Lubrol concentration of 0.16mg per mg mitoplast protein. The final solution was well stirred and left to stand at 0°C for 15 minutes. At the end of this period, the membrane suspension was diluted 1:2 with buffer D and centrifuged for 1 hour at 140,000 X g. The pellet containing purified mitochondrial inner membrane was suspended to 10mg ml^{-1} protein (biuret) in buffer D not containing BSA and stored under nitrogen at -50°C.

2.10. Measurement of NADPH cytochrome C reductase activity in isolated liver microsomes from lean and obese mice.

Isolated microsomes, stored at 1mg microsomal protein per ml, were thawed in warm water and diluted 2 fold with 100mM NaCl, 20mM KCl, 1mM tris-HCl pH 7.4 at 20°C and left to stand on ice until required. Enzyme activity was assayed essentially by the method of Phillips and Langdon (1962) with some modifications. 100mM NaCl, 30mM KCl, 25mM HEPES pH 7.4 at 0°C was prepared and 2.66ml of this buffer was pipetted out into plastic 3ml cuvettes standing on ice, 2 cuvettes for each microsome preparation. The temperature of the buffer was raised by 1°C and the pH readjusted to 7.4, and more 2.66 aliquots taken. In this manner, the correct pH is maintained over the temperature range of the experiment. To each cuvette was added 100µl microsome suspension, 200µl 9.3mg ml^{-1} cytochrome C in distilled water, 20µl 9.9mg ml^{-1} KCN in 1mM tris-HCl pH 8.0, and mixed. Cuvettes were

assayed for cytochrome C reduction at the desired temperature by adding 20µl 5.3mg ml^{-1} NADPH in 1mM tris-HCl pH 8.0 at 4°C, and following the increase in optical density at 550nM on a pen recorder of a Pye-Unicam SP 600 spectrophotometer. The reference cell was retained in the oxidised state by omitting the NADPH. The rate of reduction of cytochrome C, proportional to enzyme activity, was expressed as OD units sec^{-1} and was plotted as a function of temperature.

2.11. Measurement of insulin-stimulated 2-deoxyglucose transport in adipose tissue slices.

Mice were killed by cervical dislocation and epididymal fat pads removed and placed on the chopping stage of a McIlwain tissue chopper. Slice thickness was set at 1mm. After completion of the chopping, the stage was rotated through 90° and the sample rechopped. Thus, pieces of adipose tissue were obtained of $1mm^2$ section. Chopped tissue was then dispersed in KREBS bicarbonate buffer, gassed with 95% O_2, 5% CO_2 for 1 hour, pH 7.4 at 37°C. 4-8 individual tissue pieces were then transferred to a 10ml flask containing 1ml KREBS bicarbonate buffer, 1% BSA, and incubated plus or minus 100µ units bovine insulin (BDH). At the end of this period, 20µl Krebs-bicarbonate buffer containing 2µ mol. 2-deoxy-D glucose and 1µCi 2-deoxy-D-1-^{3}H glucose (Amersham), specific activity 19.5Ci $mmol.^{-1}$ was added. The flasks were incubated at the desired temperature for 30 minutes. At the end of the incubation, the contents of the flasks were filtered through preweighed Whatman No. 1 filter circles, 1cm diameter. The filter circles were washed with 20ml 0.9% NaCl and left to dry at 37°C for 1 hour. The filter circles were then re-weighed and placed in a liquid scintillation vial. 10ml of scintillation fluid were added and the vials counted on a Phillips beta counter (PW 4501/01), gated for tritium. Extracellular 2-deoxy ^{3}H glucose was estimated with ^{3}H inulin, a non-transported sugar, and subtracted from all readings.

2.12. Measurement of insulin stimulated 2-deoxy-D-glucose transport into isolated adipocytes from lean and obese mice.

Adipocytes were isolated, as previously described. Care was

taken to ensure constant osmolarity of the buffer so that when Krebs-HEPES 4% BSA was replaced by Krebs-HEPES 1% BSA, the change in osmolarity was compensated by addition of the required volume of 9% NaCl to the buffer. This precaution, together with harvesting the cells by flotation rather than centrifugation considerably reduced cell breakage from obese mice adipose tissue. All obese mice used in the isolation of adipocytes were between 6-8 weeks of age. Animals used at 6 weeks of age gave very good cell yields. By 8 weeks, cell breakage is noticeable and by 10 weeks breakage is too severe to utilise isolated adipocytes for experimentation. 2-deoxyglucose transport into adipocytes was assessed by the method of Gammeltoft and Gliemann (1973).

Washed cells were diluted to between 2 X 10^5 and 4 X 10^5 cells ml^{-1}, with continual stirring. 200µl of this cell suspension was transferred to 5ml plastic incubation tubes at 35°C. Insulin stimulated glucose transport was assessed by the total uptake of ^{3}H-2-deoxyglucose in the presence and absence of insulin. This assay is based on the principal that deoxyglucose is transported and phosphorylated by the same process as glucose but cannot be further isomerised to fructose-1-phosphate (Wick et al., 1957). After incubation, the uptake was terminated by removing 200µl samples from the cell suspension and rapidly centrifuging (10,000 X g) the cells in plastic microfuge tubes (Beckman Instruments) to which 100µl silicone oil MS200 had been previously added. Silicone oil has a specific gravity intermediate between triglyceride and water and thus forms an intermediary layer above the incubation medium and below the compacted cell layer. Untransported deoxyglucose present in the extracellular water space and deoxyglucose entering the cell by simple diffusion was estimated in the presence of cytochalasin B (50µM) which completely inhibits the saturable (facilitated) transport system (Renner et al., 1972).

The microfuge tubes were then carefully sliced with a sharp knife in the middle of the silicone oil. The cell containing portion was placed in a scintillation vial with 10ml scintillation fluid, shaken and counted for tritium on a Philips beta counter (PW4501/01).

2.13. Adenylate cyclase activity assay.

Crude membrane fractions were obtained by differential centrifugation. 15-25µg protein of the 1,500 X g - 25,000 X g pellet was taken for adenylate cyclase assay. The total incubation volume of 0.2ml contained 0.5mM ATP, 5mM $MgCl_2$, 1.0mM EGTA, 1.0mM theophylline, 7.5mM phosphoenol pyruvate, 30mM tris-HCl pH 7.4 and 25U/ml pyruvate kinase, and was incubated for 10 minutes at 37°C in the presence of 0-1mM isoprenaline. After rapidly freezing to stop the reaction, cyclic 3':5' monophosphate (cAMP) was assayed by the receptor binding method of Brown et al. (1970), using bovine adrenal cortex 5,000 X g supernatant. The assay was linear from 1.25 to 10 pmol. All samples were assayed in triplicate.

2.14. Measurement of membrane cholesterol content.

Cholesterol content of isolated membranes was measured by the convenient and highly reproducable enzymatic procedure of Gamble et al. (1979). The membrane solution (containing between 50-500ng cholesterol) was added to a mixture containing detergents to solubilize membrane components, cholesterol oxidase, which generates H_2O_2, peroxidase which catalyses the reduction of H_2O_2 and the conversion of p-hydroxy phenyl acetic acid to yield a stable product of high relative fluorescence. For determination of esterified cholesterol, cholesterol ester hydrolase can also be included in the mixture. The assay solution is incubated for 30 minutes at 37°C, after which relative fluorescence is measured at 325nm excitation, 415nm emission. Samples containing 50-500ng cholesterol per 15µl ethanol were prepared to construct a standard curve. The composition of the assay mixture was as follows: 58µl 100mM potassium phosphate buffer pH 7.4 at 37°C (phosphate buffer), 14µl cholesterol oxidase (1U ml^{-1}) in phosphate buffer; 14µl horseradish peroxidase (10U ml^{-1}) in phosphate buffer, 14µl cholesterol ester hydrolase (0.1U ml^{-1}) in phosphate buffer, 7µl 0.5% triton X-100; 7µl 20mM sodium cholate; 21µl p-hydroxyphenylacetic acid (4mg ml^{-1}); 15µl sample or standard.

Fluorecence was measured in a 250µl microcuvette (American Instrument Co.) and unesterified cholesterol was estimated by substituting 14µl phosphate buffer for cholesterol ester hydrolase.

2.15. Extration of membrane lipids.

The procedure based on the technique of Bligh and Dyer (1959) as described by Veerkamp and Broekhuyse (1976) was adopted. All glassware used in lipid analysis was chromic acid washed and all solvents, obtained from Koch-Light, were distilled prior to use.

To 0.8ml membrane suspension, 3ml chloroform-methanol (1:2 $^{v}/_{v}$) was added. This mixture was homogenised in a motor driven teflon glass 12ml Jencons homogeniser at 5,000rpm. Usually 10 up and down strokes were sufficient to ensure complete extraction. The mixture was transferred to a pyrex 12ml glass centrefuge tube and centrifuged in an MSE minor centrifuge at 1,000 X g for 5 minutes. The supernatant was decanted off and the pellet re-extracted with 1ml chloroform and re-centrifuged as before. The chloroform washing was gently poured out of the tube, leaving behind the protein pellet lying on top of the chloroform layer. and combined with the first chloroform/methanol extraction.

To the combined washings, 1ml 100mM KCl was added and rotamixed for 2 minutes. The tube was then centrifuged for 1 minute at 1,000 X g in an MSE minor centrifuge. The upper phase was aspirated and the lower chloroform layer collected by means of a metal syringe, leaving behind material at the interface. The chloroform layer was evaporated in a 10ml round bottom flask under rotary vacuum evaporation. Residual water was removed by the addition of 1ml benzene-methanol (4:1 $^{v}/_{v}$) and re-evaporated. Lipids are taken up in 1ml chloroform-methanol (2:1 $^{v}/_{v}$) and transferred to a 12ml centrifuge tube. The tube was rotary evaporated under vacuum. Lipids were then dissolved in 200µl chloroform-methanol (2:1 $^{v}/_{v}$) and transferred to a 800µl brown glass sample vial. The solvent was evaporated off under vacuum by placing the vial inside a larger tube connected to the rotary evaporator. The dried lipids were stored under nitrogen at -50°C until required.

2.16. Thin layer chromatography of membrane lipids.

The conditions for successful chromatography of mammalian phospholipids are very critical and must be varied to suit an individual experiment. The basic method is described in detail by Veerkamp and Broekhuyse (1976) with some modifications. Silica gel H containing 3% magnesium hydroxycarbonate in deionised water was homogenised in a beaker by a rotating teflon pestle at 5,000rpm for 2 minutes to the correct consistency. 20 X 20cm glass plates were cleaned in chloroform methanol (1:1 $^{v}/_{v}$). The slurry was spread to a thickness of 0.6mm and air dried overnight. The plates were activated at 120°C for 1 hour and stored in a dry cupboard at room temperature until required.

Extracted lipid was dissolved in 20µl chloroform-methanol (2:1 $^{v}/_{v}$) and applied by syringe in a tight horizontal band, 2cm from the bottom of the plate. The end of the band was at least 2cm from the right hand edge of the plate. The applied lipid was then dried in a stream of nitrogen. Solvent systems were: 1st dimension (solvent front-parallel to lipid band), petroleum ether (40°-60°)-acetone (3:1 $^{v}/_{v}$); 2nd dimension (solvent front parallel to lipid band) chloroform-methanol-28% ammonia (65:25:5 by vol.); 3rd dimension (solvent front perpendicular to lipid band) chloroform-methanol-acetone-acetic acid-water (6:8:2:2:1 by vol.). After chromatography in the first solvent system the tlc plates were dried in a vacuum dessicator for 5 minutes. After the second solvent system, the plates were vacuum dessicated over 98% H_2SO_4 for 30 minutes. After the third solvent system, the plates were vacuum dessicated for 1 hour.

All chromatography tanks were paper lined and contained 10mg butylated hydroxytoluene (BHT) per 100ml solvent mixture and were equilibrated for 12 hours prior to chromatography. It was found that pre-development of the plates in petroleum ether-acetone allowed up to 2.5mg extracted lipid to be loaded onto the tlc plate without smearing of the resolved lipid spots. Also, this pre-treatment resulted in better separation of the phospholipid classes along with a higher degree of reproducability. This effect may result from a number of factors: for example,

moisture may be eluted from the plate which may give rise to better separation; also, the neutral lipids are eluted from the phospholipids, thus reducing the lipid load; and finally, the phospholipids may become more evenly distributed and adsorbed throughout the gel after pre-treatment in petroleum ether-acetone.

Because the chromatography time is fairly lengthy, BHT is present in the solvent systems to minimise oxidation of the lipids. The amino phospholipids are particularly sensitive to oxidation. On oxidation, PE turns bright yellow and even small amounts of oxidation products in PE are detectable. After chromatography and extraction of PE by this procedure, the final product was colourless.

2.17. Detection of resolved lipids on tlc plates.

Fluorescent probe studies of lipids encompass a wide range of techniques but common to all of these is that the lipid under study must be as pure as possible and must be free of fluorescent contaminants. Thus, after development of phospholipid classes by tlc, the lipid spots must be identified with a non destructive, easily removable marker before they can be extracted from the silica gel for fluorescence studies. A technique was developed which satisfied these criteria and which overcame the problems encountered using methods currently available in the literature (for a review see Veerkamp and Broekhuyse, 1976).

The technique employed utilises the fluorescent probe 1,6 diphenyl 2,3,5 hexatriene (DPH). Because of its hydrophobicity, it forms clustered domains when applied to silica gel. In this state, DPH is non-fluorescent, due to collisional quenching between adjacent excited and ground state molecules. This effect disappears when DPH is in free solution, (or associated with lipid adsorbed on the gel). Thus, when DPH was applied to tlc plates, any lipids present on the plate fluoresced under uv light. As DPH is also highly soluble in volatile, a polar solvent such as petroleum ether (40-60°C) it can be applied in the form of a spray to the plates and can be eluted from lipid spots by chromatographing in petroleum ether (40-60°C). Phospholipids

are immobile in this solvent and elution will be complete when the lipid spots cease to fluoresce.

10mg DPH was dissolved in a small volume of tetrahydrafuran (THF) (freshly distilled over $LiALH_4$) and made up to 100ml with petroleum ether (40-60°C). Chromatograms were sprayed with this reagent, care being taken to mask off the base of the plate to prevent contamination of the eluting solvent system 4. Only 1 light stroke of spray was required. Spots were visualised by viewing under long wave uv, lipid spots fluorescing violet. The spray reagent was stored in the dark at 4°C and was stable for at least 2 months.

DPH marker stain is removed after marking the positions of the spots by re-chromatographing the tlc plates in petroleum ether (40-60°C). However, since neutral lipids are soluble in petroleum ether they were first removed from the plate. Phospholipids and glycolipids are immobile in the petroleum ether system but DPH was rapidly eluted from the lipid spots. When all marked lipid spots ceased to fluoresce under uv, elution was complete. Respraying with DPH confirmed that the lipid spots had not moved during the elution of DPH stain with petroleum ether. It was sometimes necessary to use multiple development or 2-dimensional elution if the lipid spots were widespread on the plate. Petroleum ether also eluted BHT present on the plate from the previous solvent systems. Since the R_f value of BHT is greater than that of DPH in petroleum ether, this compound was also removed from the lipids.

DPH stained neutral lipid removed from the previous plate was extracted from the silica gel with chloroform-methanol (3:1 v/v), re-chromatographed on silica gel G and resolved by the method of Broekhuyse (1972). This involved running the tlc initially in petroleum ether (40-60°C) to the top of the plate, followed by petroleum ether (40-60°C)-diethyl ether-formic acid (80:20:2 by vol.) in the same direction to half-way up the plate. In this system, the DPH was separated from all lipid spots as shown in Table 2.1. The DPH was viewed under uv light before the solvent had dried, as DPH fluoresces in the presence of elut-

Table 2.1.

R_f values of neutral lipid and DPH on silica gel G tlc plates.[a]

Lipid	R_f
Monoacylglycerol	0.03
Diacylglycerol	0.08
Cholesterol	0.14
Free fatty acids	0.23
Triacylglycerol	0.35
DPH stain	0.52
Cholesterol esters	0.69
BHT	0.91
Hydrocarbons	0.98

[a]Solvent system A, petroleum ether (40-60°C); solvent system B, petroleum ether (40-60°C)-diethyl-formic acid (80:20:2 by vol.) run to half-way up the plate.

ing solvent system. After drying the plate, a narrow band of the plate was stained with DPH to ascertain the R_f values of the neutral lipid subclasses.

2.18. Extraction of lipids from tlc plates.

Elution of lipids from silica gel is essentially on the same lines as the Bligh and Dyer procedure for extraction of lipids from membranes, only with some modifications. The marked lipid spots were scraped off the plate onto filter paper and transferred to 12ml glass centrifuge tubes. To the silica gel 3ml chloroform-methanol (1:2 $^{v}/v$) were added. The tubes were then sonicated for 20 seconds in a bath sonicator (Megason). The tubes were then centrufuged at 1,000 X g for 10 minutes to sediment the silica gel in an MSE minor centrifuge at room temperature. The supernatants were then decanted into other 12ml centrifuge tubes. To these 1ml chloroform and 1.8ml 60mM KCl in CO_2 saturated deionized water was added. Tubes were then rotamixed for 1 minute and re-centrifuged for 1 minute. The upper phase was aspirated and the lower layer collected by syringe. (An aqueous extraction was necessary because magnesium hydroxycarbonate does not completely sediment with the silica gel; also, the magnesium hydroxycarbonate is slightly soluble in the eluting solvents. Any magnesium hydroxycarbonate remaining in the purified lipid interferes with fluorescence studies. Acididying the aqueous phase with CO_2 greatly increases the solubility of magnesium hydroxycarbonate in water (Merck Index). The lower phase was then filtered by suction through Millipore PTFE filters, 0.2μM pore size, to remove all traces of silica gel, which again seriously interferes with fluorescence studies. The filtrate was evaporated and dried with benzene-methanol 4:1, as described previously for extraction of membrane lipids. The extracted dry lipids are stored under nitrogen at -50°C until required.

2.19. Preparation of fatty acid methyl esters for CLC.

Dried lipids were taken up in 20μl chloroform-methanol (3:1 $^{v}/v$) previously dried for 12 hours over molecular sieve (Type 4A, BDH). Sodium methoxide was prepared fresh, just prior

to transmethylation of lipids. 230mg sodium metal was cut clean of any oxide and storage oil was removed in 3 changes of light petroleum ether. The sodium was then washed in dry methanol to rid the surface of sodium oxides. The clean, oxide-free sodium was then dissolved in 50ml dry methanol. 20µl of this sodium methoxide reagent was then added to the lipids in 20µl chloroform-methanol (2:1 $^{v}/_{v}$). The tubes were capped, well rotamixed and incubated for 15 minutes at room temperature. At the end of this period, 10µl dry acetyl chloride-methanol (2:48 $^{v}/_{v}$) was added, rotamixed and stored under nitrogen at +4°C until required. GLC of the fatty acid methyl esters was performed of a Pye Unicam Series 104 gas-liquid chromatograph at oven temperatures of 180°C and 225°C on a 9' X $^{1}/_{8}$" i.d. 5% silar (on diatomite) glass column by Dr. D.L. Corina.

2.20. Preparation of lyso phospholipids from lean and obese mice.

Phosphatidylcholine and phosphatidylethanolamine were obtained from lean and obese mice adipocyte plasma membranes as previously described.

Lipids were taken up in 0.8ml 10mM $CaCl_2$, 0.25mM $MgCl_2$, 5mM tris-HCl pH 8.9 at 25°C containing 1U ml^{-1} phospholipase A_2 from crotalus adamenteus. Lipids were shaken for 1 hour in a Griffin shaker, then sonicated for 1 minute in a Megason bath sonicator and re-shaken for 30 minutes.

Lipids were then extracted as described before by the Bligh and Dyer procedure. Lipids were then re-chromatographed on silica gel G in chloroform-methanol-acetic acid-water (90:40:12:2 by vol.) along with PC and PE standards. This solvent system does not separate the free fatty acids from native PE and consequently they were not removed for analysis. On staining the plate with DPH as previously described, both PC and PE had largely been converted to the lyso-derivatives.

The lyso-derivatives were removed from the plate and extracted from the silica gel as previously described. The lyso lipids were then transmethylated and the fatty acid methyl esters analysed by GLC.

No attempt was made to quantitate the percentage catalytic efficiency. However, as judged by the relative fluorescence intensities of the native phospholipids and the lyso derivatives at least 90% of the phospholipids had been converted to the lyso compounds. No material remained at the origin, as detected by charring the plate with H_2SO_4 after removal of the lyso compounds. This would suggest that the phospholipase had left the fatty acid on carbon atom 1 of the phospholipids intact.

The relative positions of the native phospholipids, lyso derivatives and free fatty acids are shown in table 2.2.

2.21. Preparation of samples for fluorescence studies.

All buffers used in these studies were filtered through 0.2μM pore size millipore filters. All sucrose solutions were dialysed against charcoal for 12 hours to remove fluorescent impurities.

Membrane solutions obtained as previously described were thawed and diluted to 100μg membrane protein ml^{-1} with 250mM sucrose, 1mM tris-HCl pH 7.4 at 4°C (Buffer G). 1μl 1mM DPH in freshly distilled THF was added per ml membrane suspension and immediately rotamixed. The membrane suspension was left to stand in the dark at room temperature for 1 hour or at 37°C for 20 minutes. At the end of this period, 0.1 vol. 1M NaCl, 50mM KCl, 10mM $CaCl_2$, 250mM HEPES pH 7.4 at 20°C (Buffer H) was added with mixing, giving a final membrane concentration of 100μg ml^{-1}. This was then incubated at 37°C for 15 minutes. (HEPES buffer was used because its pK is fairly temperature stable ($pK/t° = 0.008$). However, the presence of HEPES in the buffer prior to addition of DPH inhibited the entry of DPH into membranes by an unknown mechanism. Once DPH has equilibrated into membranes HEPES has no effect. The relative fluorescence intensity of a DPH labelled sample of adipocyte plasma membrane was stable over a 2 hour time period. The fluorescence polarisation, as a function of temperature, was plotted for a control sample of dimyristoyl phosphatidylcholine in the presence and absence of HEPES buffer. The values of P below and above the phase transition and the melting temperature (tm) of this lipid were in agreement with published data using either HEPES or tris buffer (Shinitzky and Barenholz, 1978).

Table 2.2. R_f values of phospholipids treated with phospholipase A_2.

Phospholipid	R_f value
Free fatty acids	0.75
Phosphatidylethanolamine *	0.72
Phosphatidylcholine *	0.55
Lyso phosphatidylethanolamine *	0.30
Lyso phosphatidylcholine	0.18

* standards of these compounds were also run for comparison.

In these studies a 200µl quartz cuvette was used which not only reduced the sample volume but also reduced light scatter parameters considerably.

Extracted membrane lipids and commercially prepared lipids were dried down in vacuo in 800µl dark glass vials. 200µl of the appropriate buffer was added and the tube then sonicated in a Megason bath sonicator for 20 sec. at 25°C. The lipid dispersion was then incubated at 37°C with 1µl ml^{-1} 1mM DPH in THF prior to fluorescence measurements. Probe uptake was complete after 15 min.

2.22. Measurement of fluorescence polarisation.

Fluorescent probe incorporated into biological membranes or liposomes is excited with vertically polarised light from a grating monochromator in an Aminco-Bowman spectrophotofluorimeter. The emitted light is analysed at 90° to the excitation beam. Polarisers were 1" quartz 'Polacoat' lenses, capable of being rotated manually through 90°. Excitation and emission slit width was 2.5 X 4.5mM. The sample was contained in an Aminco 200µl quartz cell enclosed by a flow-through water jacket connected to a heating unit. Cell temperature was monitored by means of a calibrated thermistor (Light Laboratories).

The common fluorescence polarisation can be measured by excitation with monochromatic light which is vertically polarised and the emission intensity detected through an analyser oriented parallel (I_{VV}) or perpendicular (I_{VH}) to the plane of polarisation of the excitation beam. A correction factor is introduced to compensate for monochromator polarisation artefacts. This is calculated by orientating the excitation polariser horizontally and measuring the fluorescence intensity with the emission analyser oriented parallel (I_{HH}) or perpendicularly (I_{HV}). Intensities due simply to light scattering in the absence of fluorophore are (L_{VV}, L_{VH}, L_{HH}, L_{HV}). The degree of polarisation, P is calculated from the equation:

$$P = \frac{\left(I_{VV} - I_{VH} \cdot I_{HH}/I_{HV}\right)}{\left(I_{VV} + I_{VH} \cdot I_{HH}/I_{HV}\right)}$$

P was either calculated at fixed temperature, in which case P was derived as the mean of n readings, or P was calculated over a range of temperatures, one reading at each temperature point. A plot of the correction factor ratio, $\frac{I_{HV}}{I_{HH}}$ against the temperature of measurement from 0-50°C yielded a scatter plot. These points were entered into a Hewlett-Packard calculator that had been programmed to solve different models of least squares fit. The 3 models were linear, parabolic and hyperbolic. A linear solution gave a regression coefficient nearest to ± 1.0.

A calculator programme was then constructed to deal with the following data input.

First, a least squares fit curve of the form $y = mx + c$ is generated from the following data

$$x = T°C, \qquad y = \frac{I_{HH} - L_{HH}}{I_{HV} + L_{HV}}$$

where T°C = cuvette temperature

I = fluorescent light intensity

L = light scattering intensity.

Light scattering parameters were kept constant at all values of T, since it was found that for membrane protein concentrations below 100µg ml^{-1} and lipid dispersions below 100µM light scattering intensities are independent of temperature for all orientations of polariser and analyser.

The generated curve has a negative slope (-m) and the correction at any given temperature can be obtained from the solution of $y = c - (x.m)$.

The value of P at any given temperature is, therefore:

$$P = \frac{(I_{VV} - L_{VV}) - ((I_{VH} - L_{VH}) \cdot y)}{(I_{VV} - L_{VV}) + ((I_{VH} - L_{VH}) \cdot y)}$$

For fixed temperature measurement with n reading, the mean value of the correction factor

$$\frac{\sum \frac{I_{HV} - L_{HV}}{I_{HH} - L_{HH}}}{n} = c$$

was calculated. This was then used to calculate P from

$$P = \frac{(I_{VV} - L_{VV} - ((I_{VH} - L_{VH}).C)}{(I_{VV} - L_{VV} + ((I_{VH} - L_{VH}).C)}$$

and hence the mean of n readings

$$\sum \frac{P}{n} = \bar{P}$$

The correction factor compensates for instrumentation artefacts. These arise because the distribution of transmitted dipoles through a polariseraround the axis of maximum transmittance is non-gaussian, due to partial polarisation of the excitation beam by the monochromator. Also, the cuvette, the sample under study and the emission monochromator further partially polarise the light beam. Orientating the excitation polariser 90° from the vertical creates a situation where the polarised intensities of the fluorophore cannot be measured in the photomultiplier axis. This is because the direction of propagation of a photon is always 90° to its vibrational dipole, therefore depolarisation of horizontally polarised photons by the fluorophore results in an angular displacement of the dipole to which the analyser is insensitive. Thus, in a system with no polarising artefacts, the polarised intensities measured with the analyser at 0° and 90° to the vertical will be equal. Polarised bias introduced by the instrumentation will result in an inequality of these two measurements. The ratio of these measurements will be proportional to the superimposed instrumentation artefacts on the polarisation measurements.

In order to evaluate the validity of correcting the value of P at t°C by the value of the correction factor determined for that temperature by regression analysis, the thermotropic properties of DPH were evaluated in a sample of dimyristoyl PC. DPH has well defined thermotropic properties when incorporated into this lipid (Shinitzky and Barenholz, 1978).

Arrhenius plots were constructed for DPH polarisation in Dimyristoyl PC, either by correcting the value of P by the correction factor obtained at each temperature, or by correcting P

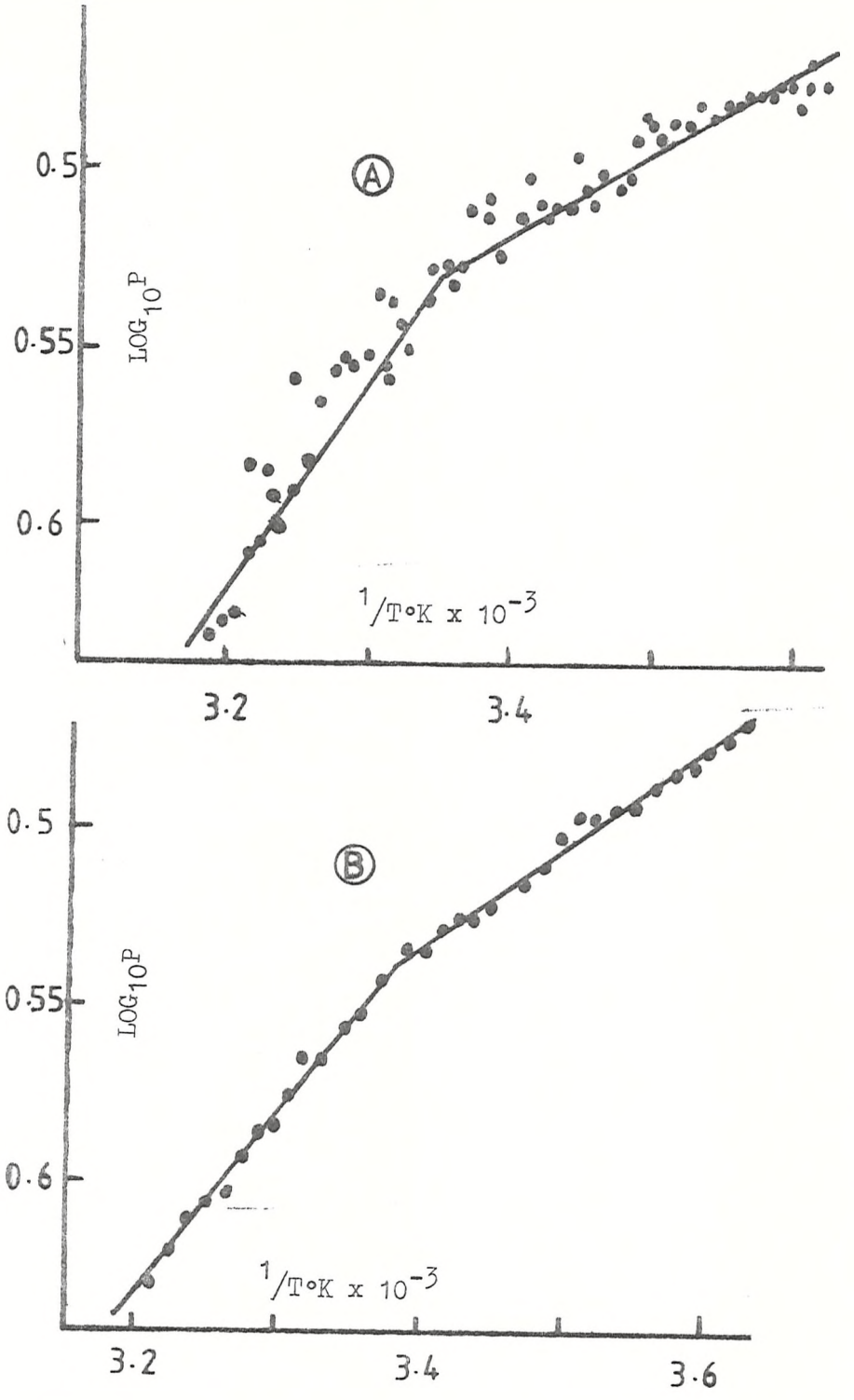

Fig 2.1. Arrhenius plots of fluorescence polarisation by DPH labelled native biological membranes by a) standard procedure, b) by the method described in section 2.22.

as described above. No difference was obtained in the value of P above, or below the melting temperature of this lipid, or the value of the melting temperature itself. Examples of Arrhenius plots obtained by the two methods for DPH labelled native biological membranes is shown in Fig 2.1. for comparison.

2.23. Synthesis and spectral properties of p-N,N,N trimethyl-phenylamine 6-phenyl hexa-1,3,5-triene ($TADPH^+$).

The synthesis of $TADPH^+$ was accomplished by the Wittig reaction (Wittig and Schöllkopf, 1960). 2gm cinnamyl bromide was dissolved in 25ml benzene. To this was added 2.6gm triphenyl phosphine, and the solution then stirred for 2hr under dry nitrogen at room temperature.

At the end of this period 5.5ml (1.8M) butyl lithium and 25 ml dry diethyl ether were added. The reaction mixture was then stirred for 1 hour, and 1.8gm dimethylamino cinnamaldehyde was then added. This mixture was heated under reflux for 2 hr. The reaction mixture was then cooled and acidified with 100mM HCl. The organic phase was separated and washed with water and was then dried over anhydrous Na_2SO_4. The solution was decanted and evaporated under reduced pressure to give a crystalline solid which was recrystallized from ethyl acetate-petroleum ether to yield 1.5gm of (E) (Fig 2.2). The identity of (E) was established using ^1H NMR spectroscopy (60M Hz, $CDCl_3$, TMS reference). The ^1H NMR spectrum of E exhibited two multiplets at 6.2-7.4ppm (15H), and a singlet at 2.9ppm (6H). The singlet resonance corresponded to the 6 methyl nitrogen protons, and the two multiplet resonances to the aromatic and unsaturated protons. This compound was then treated with excess of methyl iodide for 1 hour at 0°C. Evaporation of the solvent gave a solid which was recrystallized from chloroform to yield a green crystalline solid (M.P. 186°C - 188°C)

This compound was $TADPH^+$.

Spectral properties of TADPH

The excitation, emission, absorbance spectra of $TADPH^+$ in PC liposomes are shown in Fig 2.6. The excitation maxima is well separated from the emission maxima. $TADPH^+$ was

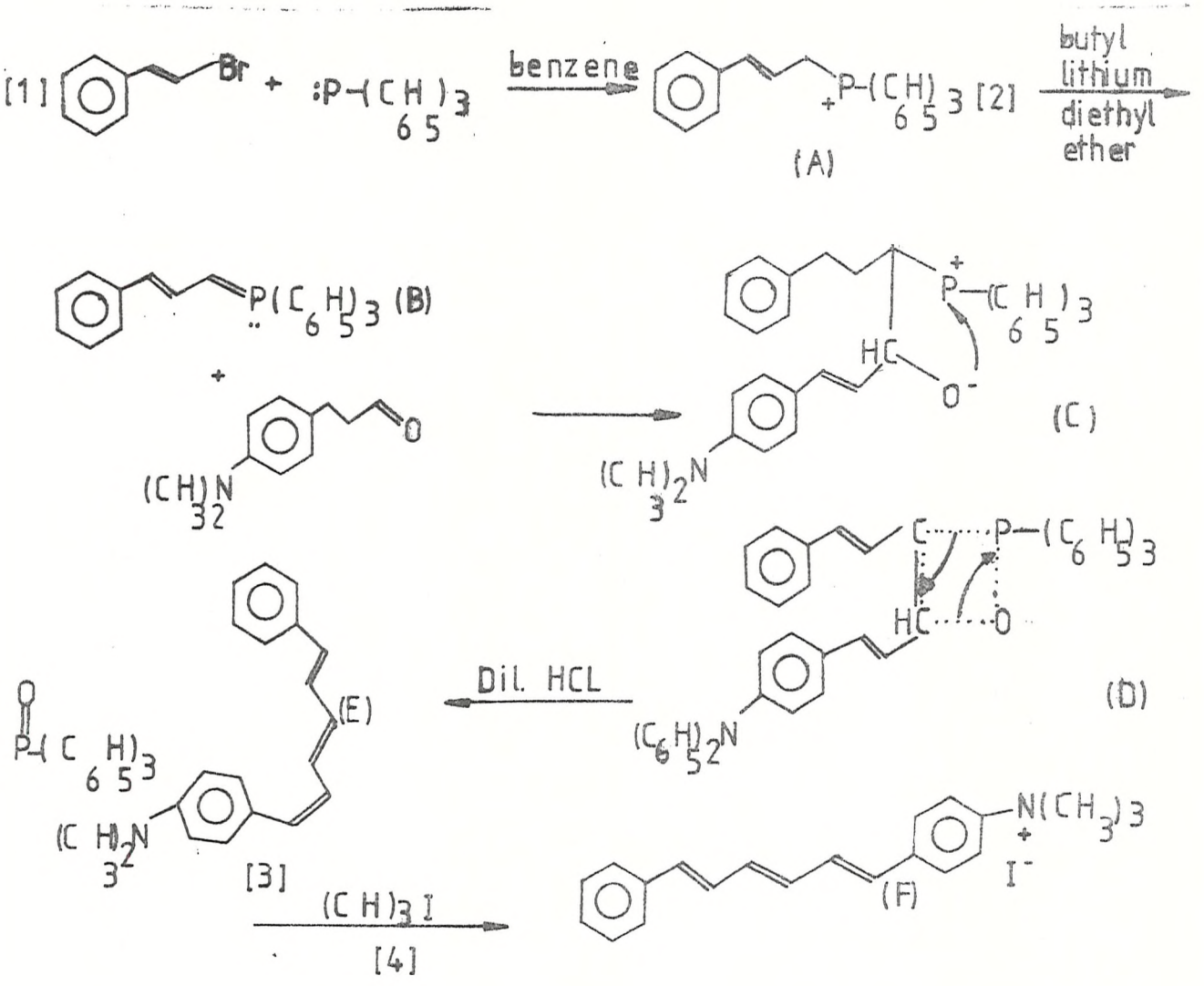

Fig 2.2. Route of synthesis of $TADPH^+$.

Reaction (1) Formation of quaternary phosphonium ion (A) from cinnamyl bromide.

Reaction (2) Formation of ylid (B) by treatment with strong base.

Reaction (3) Nucleophillic attack by ylid at carbonyl of dimethyl-amino cinnamaldehyde to form betaine intermediate (C). Reaction proceeds via cyclic four centre intermed-iate (D) to yield p-N,N, dimethylphenylamine-6-phenyl hexa-1,3,5-triene (E).

Reaction (4) p-amino group of (E) is quaternized by the reaction with methyl Iodide to yield $TADPH^+$ (F)

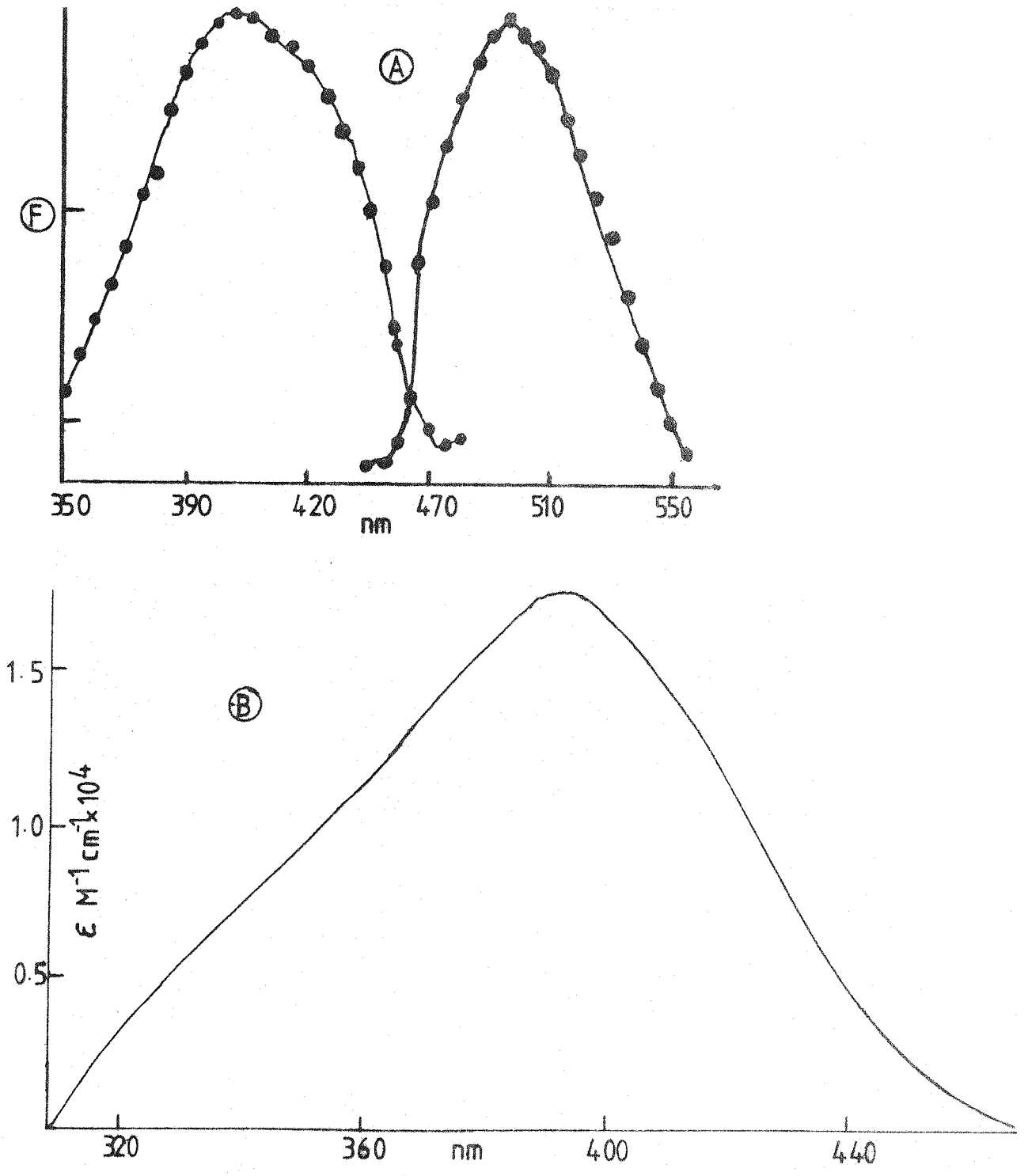

Fig 2.3. (A) Fluorescence excitation spectrum (emission recorded at 495nM), fluorescence emission spectrum (excitation at 408nm) of $TADPH^+$ in Dimyristoyl PE liposomes
(B) Absorbance spectrum of $TADPH^+$ in chloroform/methanol (1:)

incorporated into Dimyristoyl PC liposomes, and the polarisation measured form 0°C-40°C (Fig 2.5). The polarisation of this probe is sensitive to its environment, and monitors gel to liquid crystalline phase transitions, yielding a melting temperature for Dimyristoyl PC of 25°C, in agreement with published data. The fluorescence emission of this probe was highly stable over long periods, demonstrating that this probe did not photobleach.

Thus, $TADPH^+$ is a competant, novel fluorescent probe that may be of use in probing the environment of hydrophobic cations within the membrane matrix.

2.24. Synthesis and spectral properties of cholesta -5,7,9-triene-3-β-ol

Synthesis of this fluorescent sterol was by the method of Bergman and Stevens (1948). To 20g of 7-dehydrocholesterol acetate dissolved in 520ml chloroform was added 32.5g mercuric acetate in 520ml glacial acetic acid. The mixture was stirred for 18 hours in the dark. The mercurous acetate formed from the reaction was removed by filtration and washed with diethyl ether. The filtrate and ether washings were combined and concentrated in vacuo at a temperature below 45°C. The precipitate which formed was washed with acetic acid and methanol and the mother-liquors were reconcentrated until no further precipitate was obtained. The combined precipitates were dissolved in diethyl ether and the solvent evaporated until crystals began to form. 10ml of ethanol was added and the cholesta -5,7,9-triene-3-β-ol acetate was crystallized from ethanol-diethyl ether. This process was repeated until no impurities were detected by GLC.

About 2gm of the cholesta-5,7,9-triene-3-β-ol acetate were dissolved in 25ml diethyl ether. 200mg of anhydrous $LiAlH_4$ were added and the mixture refluxed under nitrogen for 20 min. On cooling, acetone was added dropwise until frothing ceased followed by 10-20ml distilled water and 2-3ml of 0.1M HCl. This mixture was extracted twice with 20ml diethyl ether:petroleum ether (40°-60°) 1:1 ($^v/_v$), the combined solvent layers retained and washed with distilled water before being dried over sodium

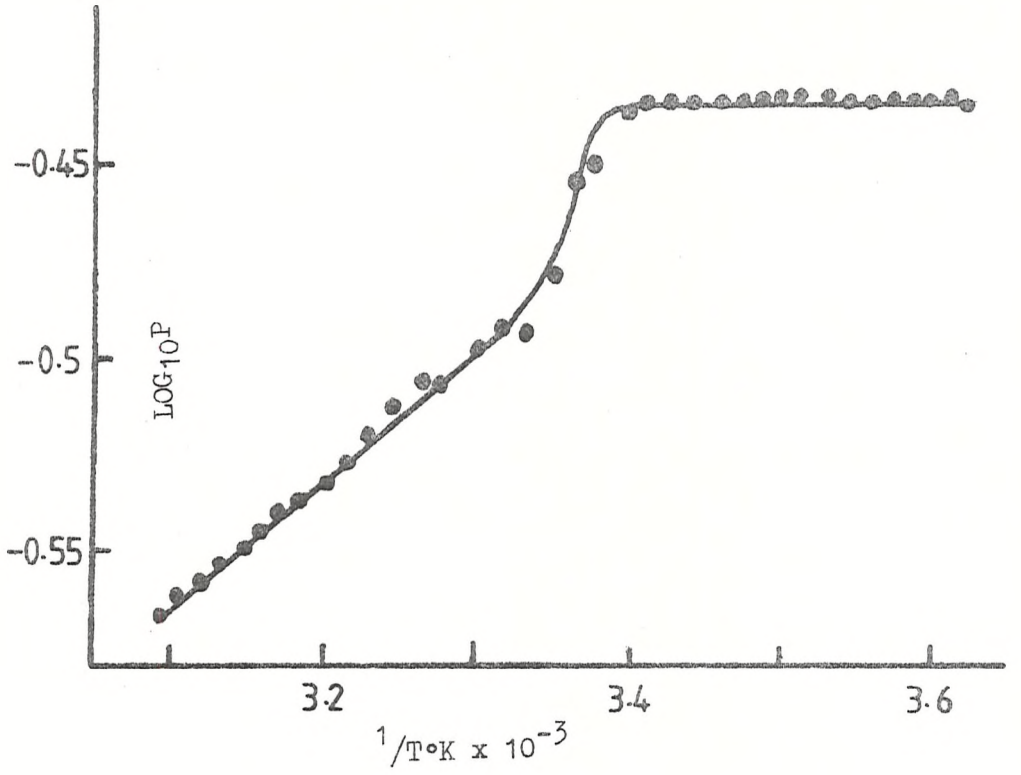

Fig 2.4. Arrhenius plot of fluorescence polarisation by $TADPH^{+}$ labelled dimyristoyl PC liposomes.

sulphate. After evaporation of the solvent, the fluorescent sterol was twice recrystallized from diethyl ether-methanol. The sterol gave a single spot on silica Gel G/10% $AgNO_3$ when chromatographed in chloroform/acetone/glacial acetic acid/ 194:4:6:1 (by vol) when visualized with U.V. light. The fluorescent sterol was stable for manymonths stored in liquid nitrogen, but is unstable in the presence of air. Experiments utilizing this fluorescent sterol have to be carried out quickly due to the unstability of this probe at room temperature.

Fig 2.5. shows the excitation, emission, absorbance and polarisation spectrum of this probe.

2.25. Synthesis of 1-acyl-2-docosahexaenoyl sn glycerophosphocholine (DHPC)

DHPC was synthesised from 1-acyl sn glycero-3-phosphocholine (lyso PC), and docosahexaenoic acid (DA) by the procedure of Warner and Benson (1977). 100mg of DA was dissolved in 3ml freshly distilled Tetrahydrafuran (THF). 70mg of carbonyldiimidazole was then added, and the reaction mixture stored for 45 min at room temperature under nitrogen. After this time, the solvent was removed under a stream of nitrogen, and the resulting fatty acid imidazolide was dissolved in 3ml freshly distilled dimethylsulphoxide (DMSO). To this mixture was added a solution of 100mg lyso PC in 6ml DMSO. After cooling the reaction mixture to 17°C, a 4ml aliquot of sodium methylsulphinylmethide solution (prepared from DMSO (7.2ml) and metallic sodium (4.4mmol))was slowly added by syringe to the rapidly stirring reactants. After 10 min, the reaction vessel was placed on an ice bath and the reaction was terminated by the rapid additon of 0.2M HCl at 4°C, until the pH of the solution was 1.0. The acidified reaction mixture was then made up to 40ml with water and extracted 3 times with 20ml chloroform-methanol (2:1 $^{v}/_{v}$). The chloroform extracts were pooled and washed twice with 20ml methanol-water (1:1 $^{v}/_{v}$) to remove most of the DMSO from the crude lipid extract. The organic layer was then evaporated under reduced pressure, remaining water and DMSO was removed under reduced pressure. The products were separeted by preparative layer chromatography on Silica Gel G (0.6mm). An authentic sample of PC was also included

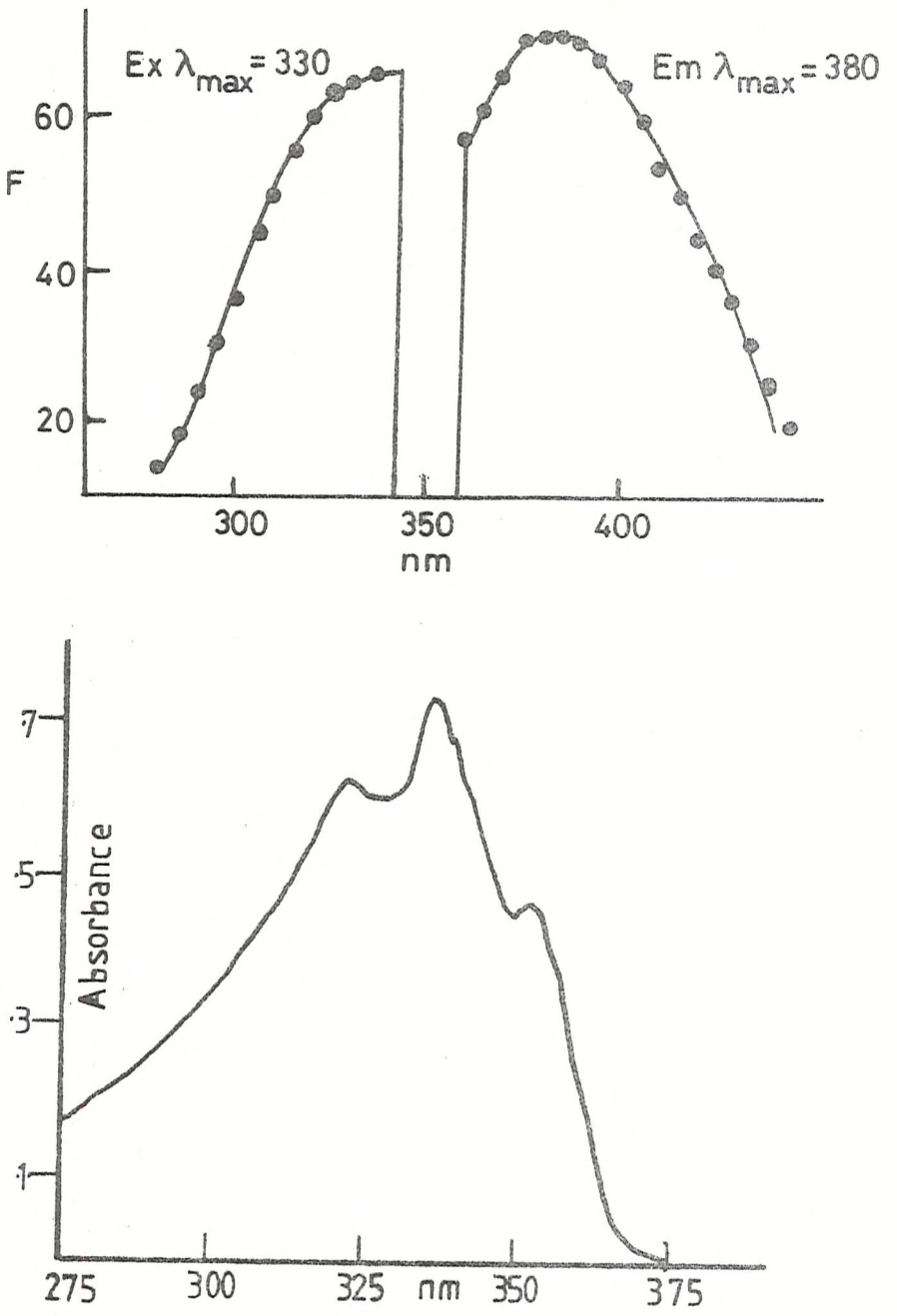

Fig 2.5. A. Fluorescence excitation spectrum (emission recorded at 380nm), fluorecence emission spectrum (excitat- at 330nm), and

B. absorption seectrum of 5.6×10^{-5}M cholesta-5,7,9-triene-3-β-ol in chloroform.

(Rogers, J. (1979) PhD thesis, University of Southampton).

on the plates. The chromatograms were developed in chloroform-methanol-water (65:25:4 by vol). After development of the plate with DPH spray reagent, the band co-migrating with PC was removed from the plate and eluted from the gel, to yield 35mg product. 60MHz ^{1}H NMR of this product in deuterated chloroform confirmed that the product was PC, and contained 35 more protons per molecule than the starting material, lyso PC.

2.26. Synthesis of 1-acyl, 2-docosahexaenoyl sn glycerophosphoethanolamine (DHPE) from DHPC.

The method employed was the transphosphatidylation reaction described by Smith et al. (1978). Phospholipase D (5mg) from cabbage (sigma) was dissolved in 10ml 40mM $CaCl_2$ in 10% ethanolamine acetate (pH 5.6 at 25°C). 20mg DHPC was dissolved in 10ml distilled ethanol-free chloroform and stirred with the aqueous enzyme solution for 48 hrs under nitrogen at room temperature.

At the end of this period, the reaction mixture was acidified with HCl to pH 1.0 and the lipid products extracted by the Bligh and Dyer proceedure in a final volume of 100ml chloroform. The chloroform layer was evaporated to dryness, and the products purified by preparative layer chromatography as described for the previous reaction. The solvent system employed was Chloroform/Methanol/28% Ammonia (90:50:11 by vol). After development 3 major bands were present under U.V. after spraying with DPH reagent. The top band (R_F 0.55) corresponded to an authentic PE sample run on the plate, the middle band (R_F 0.35) corresponded to authentic PC, and the bottom band (R_F 0.05) was phosphatidic acid (Smith et al., 1978). The band corresponding to PE was removed from the plate, and the product eluted. 100MHz ^{1}H NMR resulted in a spectra with a reduced number of protons at 3.8-3.0 ppm, consistant with the transformation of the choline headgroup to ethanolamine. The product was also ninhydrin positive. GLC analysis of the fatty acid composition of the product is shown in Table 2.2., as well as the Lyso PC starting material. The GLC spectrum of the fatty acid composition of the product confirmed that it was 1-acyl, 2-docosahexaenoyl PE.

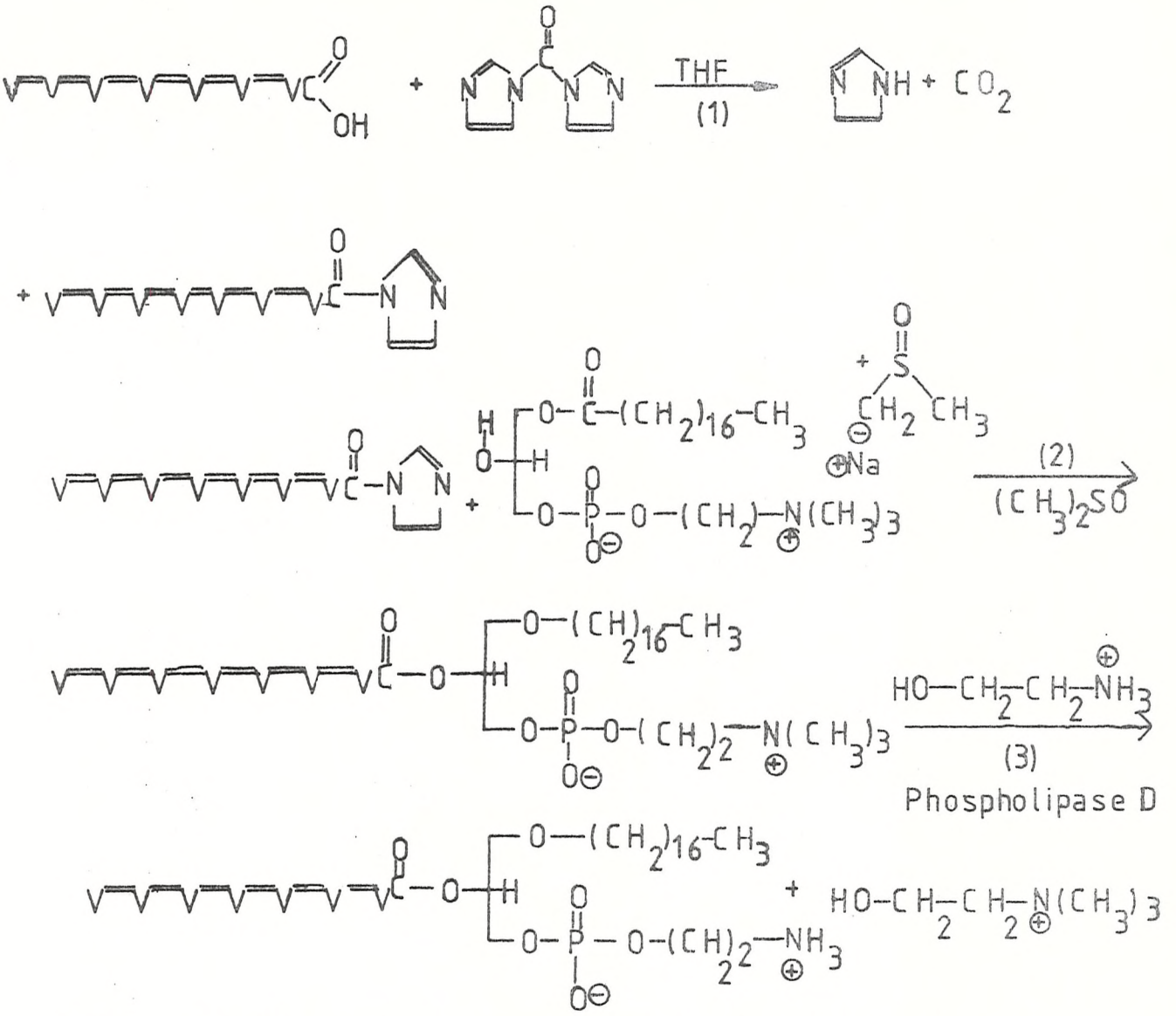

Fig 1. Reaction scheme for preparation of 2-docosahexaenoyl PE (22:6 PE).

Reaction 1 formation of docosahexaenoic acid imidazolide from carbonyldiimidazole in tetrahydrafuran (THF).

Reaction 2 Docosahexaenoyl inidazolide is reacted with 1-acyl sn glycerophosphocholine and sodium methylsulphinyl-methide in dimethylsulphoxide to yield 1-acyl 2-docosahexaenoyl sn gyycerophosphocholine.

Reaction 3 After purification of product from reaction 2, product is incubated in the presence of excess ethanolamine to yield 1-acyl 2-docosahexaenoul sn glycerophosphoethanolamine.

Table 2.2. GLC fatty acid spectrum of 1-acyl, 2-docosahexaenoyl sn glycerophosphoethanolamine (DPPE), synthesised from egg 1-acyl sn glycerophosphocholine (lyso PC) and docosahexaenoic acid.

Fatty Acid	DHPE	Lyso PC
16:0	12.3	28
18:0	21.4	42.3
18:1	1.4	5.0
18:2	3.0	7.2
18:3	2.5	6.0
20:4	4.8	11.5
22:6	54.6	ND

2.27. Quantitation of adipocyte cell suspensions using a Coulter counter.

500µl adipocyte cell suspension was fixed in 1ml of buffered 2% osmium tetroxide-collidine, for 24 hr at 37°C. The buffer was prepared as follows. 5.34ml syn-collidine was dissolved in 182ml water. 18ml of 1M HCl was then added. To 12.5ml of this buffer was added 25ml of a solution containing 1gm OsO_4 (BDH), and 12.5ml 300mM NaCl. The osmium-collidine buffer was stored in the dark at 4°C.

After fixation of the cells, cells were centrifuged on a bench MSE minor centrifuge for 1 min. The fixed cells were then washed with 5ml 0.9% NaCl, re-centrifuged and an aliquot transferred to a glass scintillation vial with 10ml 0.9% NaCl. Cells were counted by swirling the cell suspension while the 400µM probe of a Coulter ZB counter sampled 0.5ml of this cell suspension.

The counter settings were established from graphs of Coulter counts against the value of the particular setting under investigation. All graphs contained a region where coulter counts were independant of the setting value. The setting value was used at the mid-point of the plateau for counting OsO_4 fixed cell suspensions The conditions used for counting were as follows:

1. Probe pore size, 400µM
2. Chamber Volume, 500µl
3. Amplification, 1.0
4. Apperture Current, 0.088
5. Lower Threshold, 30
6. Upper Threshold, 00

A graph was constructed of coulter counts at the above setting against number of OsO_4 fixed cells. The counts were linear up to 1200 cells/500µL. Therefore, all cell samples were diluted to fall below 2,400 cells ml^{-1}.

2.28. Determination of amino phospholipid distribution in adipocyte plasma membrane using the derivatising reagent 2,4,6-trinitrobenzene sulphonic acid (TNBS).

2.28. (i). Derivatisation by TNBS of adipocyte plasma membranes.

The method employed was essentially that of Fontaine and Schroeder (1979) for fibroblasts, with some modifications.

Adipocytes were prepared as previously described, and washed thoroughly in Krebs-HEPES buffer containing 1% BSA. Just prior to incubation in the derivatising buffer, the cell suspension was lightly centrifuged, and about 10ml of the compacted cells were transferred to 100ml 1.5mM TNBS, 30mM NaCl, 1mM glucose, 1mM $CaCl_2$, 5mM KCl, 1% BSA and 120mM $NaHCO_3$, pH 8.5 at 25°C (Buffer J) for 20 and 35 min at 25°C. Incubations were performed in 250ml plastic beakers in a slowly shaking water bath. At the end of the incubation period, the cells were harvested by flotation, and the infranatant aspirated. Cells were washed 3 times in a stop buffer (170mM NaCl, 10mM Histidine, pH 7.0 at 25°C), in 40ml plastic centrifuge tubes, the cells being harvested after each washing with a light centrifugation.

Cell membranes were prepared from these cells as previously described, then pelleted and stored at -50°C until required.

In order to estimate the total amount of amino phospholipids available for derivatisation by TNBS on both sides of the plasma membrane, plasma membranes were prepared in the normal manner. 1mg plasma membrane protein was taken up in 5ml buffer by homogenisation, and transferred to a 10ml glass scintillation vial. The vial was then sonicated in a bath sonicator for 1 min at 25°C, and the contents transferred to a 10ml centrifuge tube. After 15 min, tubes were centrifuged for a total time of 20 min at 100,000 x g at 25°C. The pellets were immediately frozen at -50°C under nitrogen until required.

2.28. (ii). Preparation of standard derivatised phospholipids.

Standard derivatised phospholipids were prepared as follows. 500nM of either PE or PS was mixed with 500nM PC in chloroform/methanol (2:1 $^{v}/_{v}$). The lipid mixtures were evaporated to dryness in a 12ml glass centrifuge tube. To the lipid, 1.8ml Buffer J, not containing BSA, was added, and the tubes sonicated in a bath sonicator for 1 min at 25°C. After 35 min, 4ml chloroform/methanol (1:1 $^{v}/_{v}$) was added, and the tube well rotamixed. The tube was then centrifuged on an MSE bench centrifuge for 1 min at speed 6. The chloroform layer contained the derivatised phospholipids. The aqueous layer was completely removed, and the chloroform layer evaporated to dryness. Derivatised lipids were purified on 0.5mM silica gel G preparative chromatogrphy plates, by development in chloroform/methanol/water (60:25:4 by vol). Standard native phospholipids were also applied to the plate. After development, derivatised phospholipids appeared as yellow spots with increased R_F values compared to native lipids. The derivatisation procedure resulted in 100% conversion of the amino phospholipids to their trinitrophenyl (TNP) derivatives. The derivatised lipids were stored at -50°C until required.

2.28. (iii). Assessment of adipocyte viability after TNBS incubation.

Adipocyte viability was estimated after incubation with TNBS to ensure no cell damage had been caused by the reagent which may effect labelling of aminophospholipids. $2 \times 10^5 - 10^6$ cells in a total volume of 100µL were added to 2ml Krebs-HEPES buffer, pH 7.0 at 25°C containing 10µCi U-^{14}C glucose and 1mM glucose, in a screw-cap bottle. From the cap, a $1cm^2$ piece of blotting paper soaked in 7.5M KOH was suspended. The cap assembly was then located on the bottle after the addition of cells. Cells were incubated for 1 hour at 25°C with occasional swirling. At the end of this period, the blotting paper was removed and added to 1ml water in a scintillation vial. The pH was rapidly adjusted to 7.0 with acetic acid using pH papers, and 10ml scintillation fluid added. Vials were counted for ^{14}C in a Phillips liquid scintillation counter as described previously.

Approximate cell numbers in the aliquots was estimated by extracting total triacyl glycerol from the samples in propanol. This extract was then evaporated to dryness in small pre-weighed aluminium cups, and dried to constant weight. Cell number was estimated by dividing the total mass of triglyceride by the known mean volume of adipocytes calculated from a previous experiment.

2.28. (iv). Time course for derivatisation of phospholipids by TNBS.

Time courses for the derivatisation process were followed spectrophotometrically at 337nM, according to the method of Gordenky and Marinetti (1973) on various lipid mixtures. The optical density changes were quantitised by constructing a standard curve as follows: Various amounts of Dipalmitoyl PE (25-100nM) were dissolved in 1ml chloroform-methanol (1:1 $^{v}/_{v}$) containing 0.1ml 5% $NaHCO_3$ pH 8.6 for 2 hours at room temperature. At the end of this period, solvent was removed by evaporation with benzene-methanol (4:1 $^{v}/_{v}$) and the residue was sonicated for 1 min with 1ml water. Each tube was measured spectrophotometrically at 337nm, and a standard curve was constructed of OD 337nm against the molar concentration of phospholipid.

2.28. (v). Extraction and identification of derivatised phospholipids from native membranes.

Derivatised membranes were thawed and homogenised in 1.8ml 60mM KCl, and lipids extracted by the Bligh and Dyer procedure. Extracted lipids, derivatised standards and native phospholipids were chromatographed on silica gel H containing 3% magnesium hydroxycarbonate in chloroform-methanol-28% ammonia (65:25:5 by vol). After development, derivatised lipids were identified on the chromatograms as yellow spots, and other non-derivatised lipids were detected by DPH spray reagent as previously described. Spots of silica gel representing greater than a total of 50nmole phospholipid phosphorous were removed from the plate for phosphate analysis by the method of Fiske and Subbarow, (1925).

Derivatised lipids representing less then 50nmole phospholipid phosphorous were eluted from the gel by sonication of the gel in 10ml chloroform/methanol 2:1, and filtered through

a PTFE filter, (0.2μM pore size). The filter was evaporated to dryness under vacuum. The residue was taken up in 1ml ethanol, and the optical density measured at 337nm. A standard curve was prepared of TNP-PE in ethanol, the assay ranging from 10-100nm phospholipid. Spots containing less than 50nmoles of underivatised lipid were marked, and DPH eluted from the spots by chromatography in petroleum ether (40°C-60°C). Spots were removed from the plate and the lipids eluted, filtered and evaporated to dryness as described above. The lipids were then derivatised with TNBS in chloroform/methanol containing 0.1ml 5% $NaHCO_3$ pH 8.6 as described in (ii) The derivatised phospholipids were extracted by the Bligh and Dyer procedure, evaporated to dryness and taken up in 1 ml ethanol. Phospholipid concentration was estimated by measuring the optical density of the ethanol solution at 337nm.

2.29. Preparation of obese mice using goldthioglucose hypothalamic lesioning.

Goldthioglucose was injected subcutaneously at a dose of 1.2mg/g body weight in a saline vehicle. Mortality was about 20% and about 85% of the survivors became obese. Animals were utilised for experimentation that had attained a body weight in excess of 40g within 4 weeks of lesioning.

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CHAPTER 3.

PHYSICAL ASPECTS OF THE LIPIDS IN THE HEPATIC ENDOPLASMIC RETICULUM OF LEAN AND OBESE MICE HOUSED AT DIFFERENT ENVIRONMENTAL TEMPERATURES USING ENZYME AND FLUORESCENT PROBES

3.1. INTRODUCTION.

3.1.a. The endoplasmic reticulum (ER).

The nature of membrane biogenesis within the cell is poorly understood. However, it is now widely accepted that de novo membrane synthesis is confined to the endoplasmic reticulum, with extensive modification in the Golgi apparatus of both the protein and lipid components of the bilayer (Jamieson and Palade, 1967a, 1967b). Therefore, initial experiments to investigate the postulate of functionally altered membranes being involved in the aetiology of obesity were carried out on the microsomal fraction from liver. This fraction essentially contains a heterogeneous population of vesicles, derived from the endoplasmic reticulum (ER).

The ER of the liver cell is a complex membrane network envaginating all areas of the cytoplasm, on which are localized a large number of intrinsic and extrinsic enzyme systems. The ER is essentially concerned with the synthesis of membrane components, metabolic conversions and protein synthesis. Approximately 60% of the ER surface has ribosomes attached, engaged in protein synthesis. These areas are called rough ER while the remaining areas are ascribed as smooth ER.

The 'microsomal fraction' of a liver homogenate is the 100,000 X g sedimentable material contained in the post mitochondrial (10,000 X g) supernatant. In the fed animal, 30-40% of the ER is lost to the mitochondrial 10,000 X g pellet due to the presence of glycogen which tends to aggregate ER (Luck,1961).

Associated with the microsomal fraction are exogenous proteins. Washing of microsomes with 0.5M KCl or 0.15M Tris-HCl ph 8.0 removes 30-35% of the total protein, without loss of endoplasmic reticulum (Depierre and Dallner, 1976). However, these authors point out that it is not certain whether washing the microsomes in this fashion removes extrinsic proteins (e.g. ribosomal RNA) associated with the ER.

Rat liver microsomes are significantly contaminated with lyso-

somes and peroxisomes, (between 9-11% of the homogenate acid phosphatase is recovered with the microsomal fraction), mitochondria, plasma membrane and Golgi (Beaufay et al., 1974). These authors estimate that 20% of their total microsomal protein was due to the above contaminants. Unfortunately, further purification of the microsomal fraction results in greater loss of ER, and the resulting purified membranes may be less representative of the total cellular ER fraction.

These above considerations impose possible difficulties in the interpretation of data obtained from experiments with lean and obese ER fractions, since it is possible that subcellular fractions from obese animals may differ in their sedimentation characteristics. Evidence will be presented to show the ER fractions from obese animals are not dissimilar from that of lean in terms of protein/lipid/RNA ratios, although this does not necessarily mean that identical fractions are being harvested from the two livers. There is no simple solution to this problem, and in the following experiments we make the assumption that our microsomal fractions contain a representative sample of total organ ER, although at the same time, we are aware of the difficulties in making direct comparisons.

We have utilised an intrinsic ER enzyme as a reporter for its physical environment, and therefore information from this system should not be influenced by contaminating organelles.

The technique of depolarisation of fluorescence, first described by Weber, (1953) was used to assess both bulk microsomal membrane microviscosity and the viscosity of phospholipids extracted from both lean and obese mice. The fluorescent probe will not be selective in its membrane partitioning, and therefore will report the average microviscosity of all membrane types present. Similarly, extracted phospholipids will be derived from all membrane types present in the preparation.

Despite these drawbacks, the microsomal fraction provides an excellent area to begin research of this nature, as it is available in large amounts and is easily prepared.

Evidence is presented to show that the hepatic ER membrane from obese mice is in a more fluid state than that derived from lean, and that this phenomena is due, at least in part, to alterations in the fatty acyl moieties of the membrane phospholipids. Data is also presented suggesting altered metabolic regulation of membrane composition in ER membranes from obese mice.

3.1.b. Use of enzyme and fluorescent probes to study the physical properties and GLC to study the composition of microsomal membranes and extracted phospholipids.

(i). Enzymatic probes.

Considerations on the physical state of lipids within a biological membrane are often extrapolated from studies of the temperature dependence of membrane-bound enzymes (Lenaz, 1977). NADPH-cytochrome P-450 oxidoreductase, (EC 1.6.2.4) is a membrane bound enzyme found in mammalian ER. Its activity can be assessed by the addition of the artificial electron acceptor, cytochrome C. Mitochondrial cytochrome C reduction by NADPH can be prevented by the inclusion of KCN in the assay media, and therefore, under these conditions, the rate of cytochrome C reduction in a measure of ER NADPH-oxidase activity, since the binding and dissociation of oxidized and reduced cytochrome C is not rate limiting at the concentrations used in the assay (Philips and Langdon, 1962).

NADPH-cytochrome P-450 oxidoreductase has been characterised in mammalian hepatic microsomes, where it is especially abundant. The enzyme is a flavoprotein of M.W. 79,700 (Dignam and Strobel, 1977).

The cytochrome P-450 dependant mixed function oxidase system is responsible for hydroxylation of a wide variety of substrates, e.g. steroids, polycyclic hydrocarbons, fatty acids, carcinogens and toxic drugs (Orrenius and Ernster, 1974). The NADPH cytochrome P-450 reductase mediates the electron transfer between acceptor, NADPH and P-450 molecules. To date, no mobile electron carrier has been found between the reductase and cytochrome P-450, so it has been postulated that the two proteins

directly interact by lateral or rotational diffusion and collision (Dupple and Ullrich, 1976; Yang et al., 1977, 1978). This was later confirmed by Taniguchi et al. (1979), by kinetic studies with purified enzymes reconstituted in phosphatidylcholine vesicles.

A model for the organisation of the reductase and P-450 enzymes was first proposed by Stier and Sackman (1973), whereby the P-450 molecules surround the reductase, both enzymes being associated with annular lipid in a rigid quasicrystalline state. The stoichiometry of reductase and P-450 interaction has been assessed by Bosterling et al. (1979) using reconstituted enzymes in vesicles containing PC/PE/PA mixtures. They achieved a maximal activity with a stoichiometry of 1 molecule of reductase to 5 of P-450 in 1:2 $^{w}/w$ lipid. An important aspect of this work was that to achieve the correct (in vivo) stoichiometry in their reconstituted system coinciding with maximal activity, the presence of PE was essential in the lipid matrix. Thus, since microsomal enzymic oxygenation requires the presence of these two enzymes and specific phospholipids, the microsomal monooxygenase system provides a good model for the study of protein-protein and protein-lipid interactions.

Experiments were carried out to examine whether there were any changes in the form of the Arrhenius plots of reductase activity between lean and obese mice, as a method for probing changes in the lipid environment between enzymes from the two groups.

Changes in the form of Arrhenius plots are usually ascribed to changes in the physical environment of the enzyme (Warren et al., 1974; Kimelberg and Papahadjopoulos, 1974; Lee et al., 1974; Morrisett et al., 1975; Wunderlich et al., 1975; Houslay et al., 1976a, 1976b). The Arrhenius relationship is the equation which connects the rate constant (K) of a reaction with temperature (T).

$$K = Ae^{-E/RT} \qquad \text{(Eq. 1)}$$

where A is a constant, generally known as the frequency factor of reaction, (collision frequency).
E is the energy of activation for the reaction and
R is the gas constant.

In order to test the Arrhenius equation, we first take logarithms of both sides of Eq. 1

$$\ln K = \ln A - \frac{E}{RT} \qquad \text{(Eq. 2)}$$

It follows that, if the Arrhenius law applies, a plot of ln K against $^{1}/T$ will be linear, and the slope of this line will be $-^{E}/R$.

Taking common logarithms:-

$$\log_{10} K = \log_{10} A - {}^{E}/2.303\ RT \qquad \text{(Eq. 3)}$$

and the slope of a plot of $\log_{10}$ K against $^{1}/T$ is $-^{E}/2.303$ R or

$$^{E}/4.57 \qquad \text{(Eq. 4)}$$

and has the units of Kilocal $mol.^{-1}$.

Thus, as long as the energy of activation does not change, $\log_{10}$ K is proportional to $^{1}/T$.

In situations where a change in temperature leads to an abrupt change in the conformation of the reactive centre of a particapant in the reaction, then this may lead to a hindrance or shielding of the centre so that the fraction of collisions in which the energy is in excess of a particular value necessary to overcome local molecular repulsions is reduced.

With small molecules this sudden change does not take place. However with macro-molecules with secondary and tertiary structure this change does happen yielding biphasic or multiphasic Arrhenius plots.

This change can be due to sudden relaxation or formation of weak internal bonding forces (Van der Waal's) which alters the conformation of the reactive centre yielding a new energy of activation for that reaction, or can be due to associated molecules undergoing a conformational or phase change, which in turn alters the conformation of the reactive centre. Such is the situation for membrane bound enzymes. A phase change from liquid crystalline to gel phase of associated lipid molecules can physically constrain the enzymes under study, and the energy for activation concomitantly rises (Fourcans and Jain, 1974).

It is widely accepted that the amino acid sequence of polypeptides is highly conserved, and therefore a membrane bound enzyme from the same animal source has always the same primary structure. However, it is also accepted that animals are able to modify the composition of their membrane lipids as outlined in the introductory chapter. Therefore if the 'breakpoint' of an Arrhenius plot of a membrane bound enzyme can be shown to vary under certain situations, it is more likely to be indicative of a change in the nature of the membrane lipids rather than a change in the enzyme itself.

Interpretation of the significance of changes in activation energy, E_A between two similar, but not identical reactions, is difficult, because in multi-substrate reactions, especially those involving diffusional or rotational collisions within an ordered matrix, many factors can influence experimentally determined E_A.

Over the past 65 years, considerable theoretical effort has been made to predict the absolute magnitudes of the pre-exponential factor, A, and E_A for various reactions. Except for very simple gas phase reactions, attempts to quantitate E_A and A have not been possible. It is therefore sufficient to regard changes in E_A between two similar, but not identical, systems as reflecting a change in the organisation or assembly of the components participating in the reaction.

This change could be a change in the lipid fraction or the proteins themselves, or both. Protein conformation can be changed by phosphorylation/dephosphorylation reactions for example. It could also be changed by binding different lipid classes. Finally, the nature of the hydrocarbon chains of the phospholipid molecules can affect protein conformation. However whether the lipids themselves become more or less 'ordered' need not necessarily affect the change in activation energy in any particular direction.

As before, a higher activation energy of a system over another will be assigned as that system having a higher degree of 'order'. It should be remembered that this applies to the system as a whole, and the term 'order' may not have any real phys-

ical meaning.

(ii). Fluorescent Probes.

Our second approach to examining the thermotropic characteristics of the lipid components of microsomal membranes from lean and obese mice, was to use the technique of depolarisation of fluorescence of an incorporated fluorophore.

The Arrhenius kinetics of the depolarisation values give an insight into the degree of molecular motion, the degree of order and the temperature of onset into phase separations and transitions occurring within the membrane. A disadvantage with this system is that it is not possible to label native membranes with fluorophore, without first purifying them from glycogen. Glycogen causes a high degree of light scatter, and hence inaccurate fluorescence measurements. Fluorescence measurements were carried out on extracted lipids reconstituted into stable bilayer vesicles by the sonication procedure. Thus, the lipid components of these vesicles will no longer be ordered in the same manner as the native membrane, which again makes comparison between extracted membranes from lean and obese animals difficult. Information about specific membrane components such as phospholipids reconstituted into stable vesicles obtained from Arrhenius plots of fluorescence depolarisation is extremely useful in understanding where specific biochemical changes in membranes can affect the membrane as a whole and the proteins contained within it.

Because NADPH cytochrome P-450 reductase is thought to have annular lipid which is selected from the bulk lipid pool (Stier and Sackman, 1973; Bosterling et al., 1979), it was of interest to compare the information obtained from the enzyme study to Arrhenius plots obtained from fluorescence polarisation studies of membranes labelled with DPH, which samples the average bulk microviscosity of the membrane lipids (Shinitzky and Barenholz, 1978).

The two major phospholipid components of microsomal membranes are phosphatidylcholine (PC), and phosphatidylethanolamine (PE) which have been shown to be assymetrically distributed between

the outer (cytoplasmic) face and the inner (lumenal) face, respectively of the rat liver endoplasmic reticular membrane (Higgins and Dawson, 1977). The outer face contained 84% PC, 8% PE and the inner face 28% PC and 37% PE.

PC and PE were extracted and purified from lean and obese microsomes in order to establish microviscosity parameters for each lipid with a view to correlating changes in NADPH cytochrome P-450 reductase Arrhenius data between lean and obese animals with changes in PC and PE microviscosity. Further, correlating such changes may help to provide more evidence that this enzyme is associated with a specific lipid selected from the bulk lipid pool.

The terms membrane fluidity and membrane microviscosity are used interchangeably. Fluidity is simply reciprocal viscosity. Both these terms refer to intrinsic properties of a membrane system which regulate biophysical phenomena such as substrate permeability, membrane protein conformation, and the rotational and diffusional mobility of membrane proteins.

Since the degree of fluorescence polarisation is proportional to the microviscosity, changes in the value of P in a membrane system can be brought about by a number of membrane constituents. An increase in the cholesterol/phospholipid ratio will cause an increase in P (Hubbell and McConnell, 1971), and the degree of order in the system (Cogan et al., 1973). The two other major characteristics are the content of SM, which condenses the membrane and confers a high degree of rigidity on the membrane structure (Shinitzky and Barenholz, 1974) and the degree of unsaturation of the phospholipid acyl chains (Chapman and Wallach, 1968). Configuration (cis-trans) and position of the double bonds within the hydrocarbon chains strongly influence the physical properties of biological membranes. All these factors will influence fluorescence polarisation measurements, although detailed compositional analysis must be performed on a membrane system to assess the relative contribution of each of these factors.

It is more difficult to correlate compositional changes in membranes with changes in the observed flow activation energies calculated from Arrhenius plots of polarisation (or microviscosity) against absolute temperature. According to Shinitzky and Inbar, (1976) the flow activation energy, ΔE can be interpreted as the energy required to fuse the flowing segments to furnish a 'hole' which is large enough to maintain the flow. Thus, in ordinary liquids, ΔE can be interpreted as the kinetic energy required to overcome the intermolecular forces that oppose the flow, and thus will be numerically equal to the amount of energy required to associate a mole of 'flowing segment' from the bulk liquid (Kauzmann and Eyring, 1940).

Since intermolecular interactions between the flowing segment (in this case the segment containing the DPH) and the bulk liquid (the hydrocarbon core assembly of the phospholipids) tend to inhibit the flow process, ΔE will therefore increase with the number of degrees of freedom for intermolecular interactions. Thus, the less ordered a hydrocarbon assembly, the larger the segment to participate in the flow process, since intermolecular interactions become more diffuse.

Thus, it now becomes apparent that ΔE for fluorescence depolarisation studies bears no relationship to E_A from membrane enzyme probe studies, although both measure some degree of constraint on the system under study. While a reduced thermal mobility due to increased viscosity of the matrix a protein is imbedded in, will in all probability increase E_A, if the increased viscosity is due to increased order of the hydrocarbon matrix, ΔE will be reduced as measured by the fluorescence polarisation of a rotating hydrophobic fluorphore.

General rules about the contingency of ΔE on membrane molecular structure are difficult to establish from the literature. ΔE decreases with increasing cholesterol content in the system, which indicates an increase in order. The decrease in ΔE is concomitant with a marked decrease in both the unit flow volume and the average length of flowing segments (Shinitzky and Inbar, 1976). The interaction with the rigid backbone of cholesterol

with the phospholipid acyl chains thus condenses the system, and simultaneously reduces the average length of hydrocarbon segments which participate in the flow process.

The degree of unsaturation of fatty acyl groups esterified to phospholipids probably plays a large part in fixing the value of ΔE obtained from Arrhenius plots of fluorescence depolarisation studies on lipid mixtures, although little data is available from the literature concerning this parameter. Biological membranes contain a wide spectrum of fatty acyl species, not only differing in their degree of unsaturation, but also their carbon chain length. Pi bonds in naturally occurring fatty acids are always in the cis conformation (Hilditch and Williams, 1964). Polyenoic Pi bonds are always methylene bridged, that is they are separated by 1 carbon atom. Mammalian biological membranes contain large amounts of fatty acyl species containing 1, 2,3, 4 and 6 Pi bonds per fatty acyl molecule. Because the conformation of the methylene bridged Pi bonds distorts the shape of the hydrocarbon chain, polyenoic fatty acyl species have a larger molecular volume than saturated fatty acyl species (Chapter 5). Thus, according to Shinitzky and Inbar (1976), this phenomena should increase ΔE as the degrees of freedom for molecular interaction becomes more diffuse with increasing molecular volume.

(iii). <u>GLC analysis of fatty acids derived from membranes and phospholipids from lean and obese animals.</u>

Bulk microsomal membrane lipids were transesterified with sodium methoxide, and analysed by GLC. The resultant spectrum

is that of the fatty acids derived from both phospholipids and neutral lipids that comprise the bilayer.

To asses the overall degree of order that is likely to arise from these fatty acids comprising the hydrophobic core of the membrane, four parameters must be considered.

a). The fraction of totally saturated lipids. Totally saturated lipids confer a high degree of order on a membrane.

b). The number of pi bonds per unit number of fatty acids. The number of pi bonds markedly influences the degree of order within membranes (Chapter 1).

c). The average carbon chain length of the fatty acids.

d). The isomeric configuration of conjugated pi systems. Cis isomers in fatty acids give rise to considerable disorder compared to trans isomers.

From GLC data, changes in the number and distribution of pi bonds will give an indication of the degree of order within obese membranes compared to those of lean, and is useful in assessing the cause of changes observed in enzyme and fluorescence data.

The fatty acid spectrum of individual phospholipid classes, purified from the bulk ER, was obtained for comparison of compositional and microviscosity parameters. Changes in the viscosity of membranes of obese animals should be accompanied by changes in the fatty acid composition of the major membrane phospholipids.

GLC data for bulk microsomal membrane lipids from animals at different ages was assessed, in an attempt to ascertain whether the severity of insulin resistance that develops in growing animals was in any way connected with observed membrane changes.

Finally, the microsomal fraction from mice made obese with a single subcutaneous injection of gold thioglucose was analysed by GLC. Mice were sacrificed in the static phase of their obesity, in excess of 70g weight. Changes in the fatty acid composition of GTG-obese mice will be of use in assessing whether

membrane changes are specifically associated with the ob gene, or whether they are general changes occurring in the obese state.

3.2. RESULTS AND DISCUSSION.

3.2.a. Purity of membrane preparations.

Assessment of total ER in the whole liver homogenate and in the isolated microsomal fraction by measurement of NADPH cytochrome C reductase activity indicated a fairly constant recovery of ER over three microsomal preparations from lean animals, but a rather more variable recovery of ER from obese (Table 3.1.). Recovery was assessed by measuring the rate of reduction of cytochrome C per mg protein in the crude homogenate, and in the purified microsomal fraction. Thus, the percent recovery of ER can be calculated from the ratios of the total homogenate activity to the total microsomal activity of the NADPH cytochrome C reductase.

Despite variations in recovery of obese ER, phospholipid to protein and phospholipid to RNA ratios were very similar in both lean and obese microsomal fractions (Table 3.1.). Similar results were obtained on a repeat preparation. Thus, the purified microsomal fractions obtained are likely to be representative of total organ ER, although the drawbacks must be considered when making direct comparisons between microsomes derived from morphologically different tissues, such as liver from lean and obese animals. Contaminating organelles in the microsomal fraction will not interfere with enzyme Arrhenius plots, since the enzyme under study will only be present in ER membranes. Fluorescence and GLC data will be the average of all the membrane types present, and therefore must be viewed with some caution.

3.2.b. Environmental housing temperatures.

It is known that the rectal temperature of mice varies with their environmental temperature, and that the rectal temperature of obese mice falls faster in cold stress conditions (Trayhurn et al., 1976). Below 10°C housing temperature, obese mice cannot maintain homeothermia and death ensues. It has been postulated that obese mice poorly thermoregulate because of some thermogenic defect directly associated with their obesity (Bray and York, 1979; Trayhurn and James, 1978).

Fig 3.1. shows rectal temperatures of lean and obese mice

Table 3.1.

Phospholipid/protein/RNA phosphorous ratios in microsomal membranes purified from lean and obese mice livers. Animals were housed at 25°C.

		LEAN		OBESE	
Phospholipid:protein (μ moles mg^{-1})	a)	0.49		0.45	
	b)	0.44		0.41	
		0.47	(Mean)	0.43	
RNA phosphorous:protein (μ moles mg^{-1})	a)	0.33		0.32	
	b)	0.33		0.34	
		0.33	(Mean)	0.33	
RNA phosphorous:phospholipid (μ moles μ $mole^{-1}$)	a)	0.67		0.67	
	b)	0.75		0.83	
		0.71	(Mean)	0.75	
% Recovery of microsomes	a)	65		48	
	b)	62		57	
	c)	63		39	
		63.3	(Mean)	48.0	(Mean)

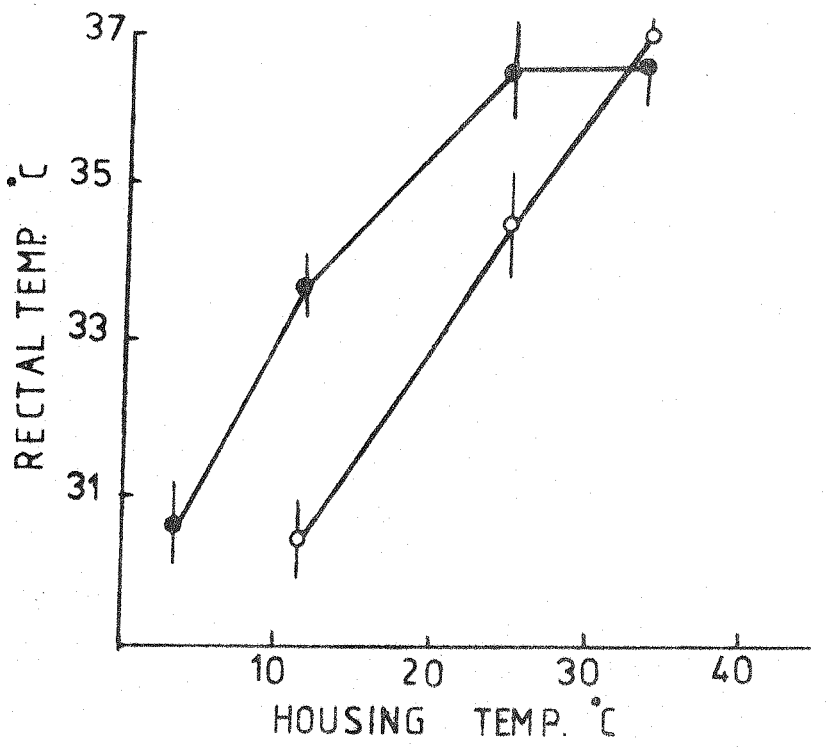


Fig 3.1.

Rectal temperature of lean (●) and obese (o) mice as a function of housing temperature.

at different environmental temperatures. Rectal temperatures are normalized in obese animals at 34°C. At housing temperatures of 12°C, the obese rectal temperature falls to around 30°C. Such changes in body temperature should be sufficiently large to perturb biochemical structures as cell membrane temperature has been shown to promote lipid cluster formation (Lee et al., 1974) and phase separation of membrane lipids into coexisting fluid and crystalline domains (Wunderlich et al., 1975), and to an alteration in the kinetics of membrane bound enzymes (Raison et al., 1971).

It was therefore of interest to find out whether mouse liver microsomal membranes adapted to changing body temperature to preserve their physical properties.

Arrhenius plots of enzyme activity and fluorescence polarisation by a marker probe in lean and obese microsomal membranes were analysed from animals housed at 34°C, 25°C and 12°C.

3.2.c. Intrinsic enzyme marker Arrhenius kinetics in lean and obese mice microsomal membranes.

Figs 3.2 and 3.3 show the Arrhenius plot for cytochrome C reduction at 550nM by hepatic microsomal NADPH cytochrome C reductase from lean and obese animals housed at 24°C and 12°C respectively. The curves are representative of 4 separate experiments on different batches of animals. (The mean values of the breakpoints and activation energies are summarised in Table 3.2). In all graphs presented in this thesis, lines through points are drawn free hand with an estimation of the breakpoint by inspection. Independant workers in this department drew similar lines, and agreed with the slopes of the lines and their intersections. Computer analysis of quadratic solutions was not deemed necessary if comparative graphs were self evidently different.

The data from these graphs, and the data from animals housed at 34°C and 4°C (plots not shown) is summarised in Table 3.2.

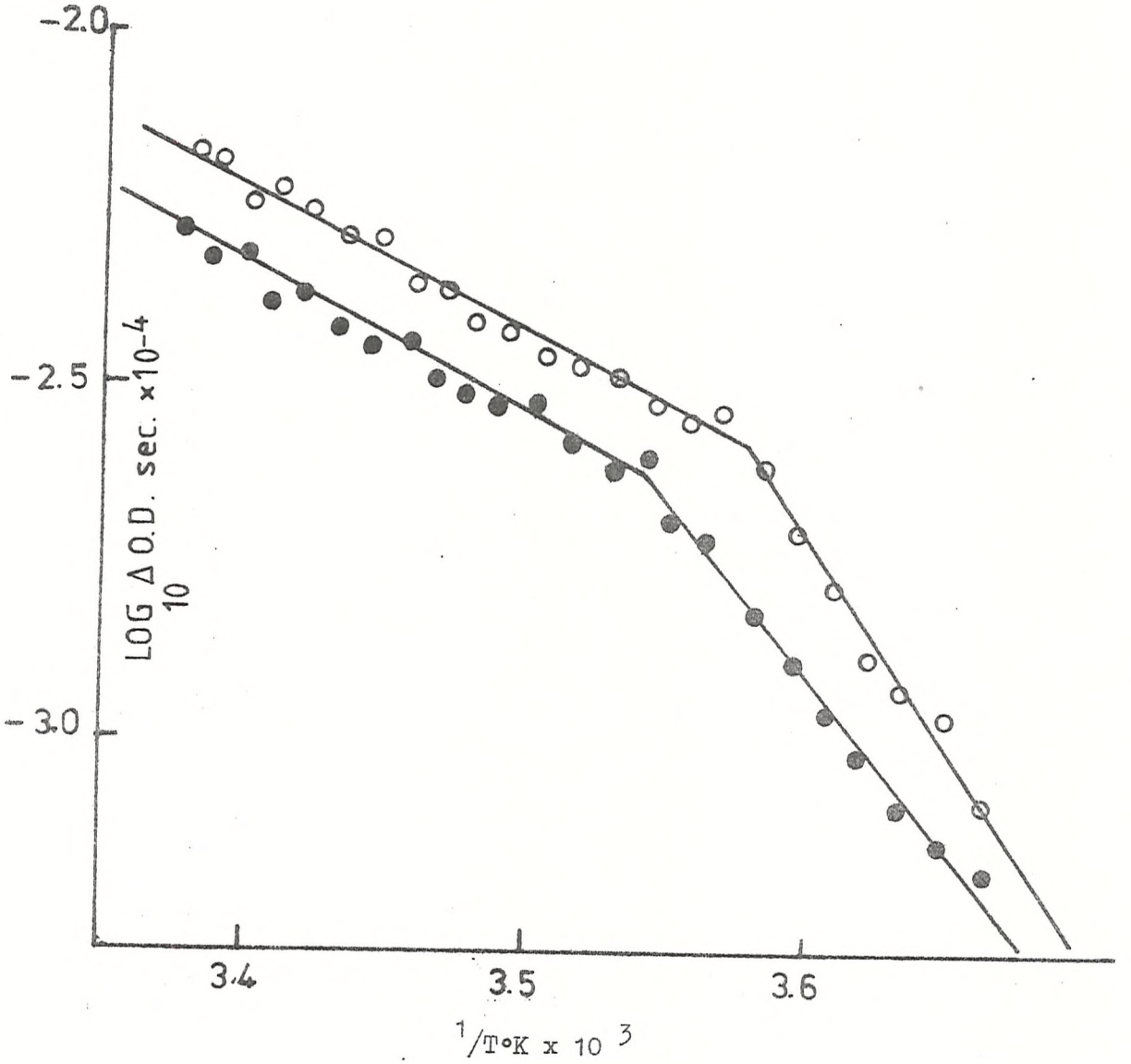


Fig 3.2.

Arrhenius plots of hepatic microsomal NADPH cytochrome P-450 reductase activity from lean (●) and obese (o) mice housed at 24°C.

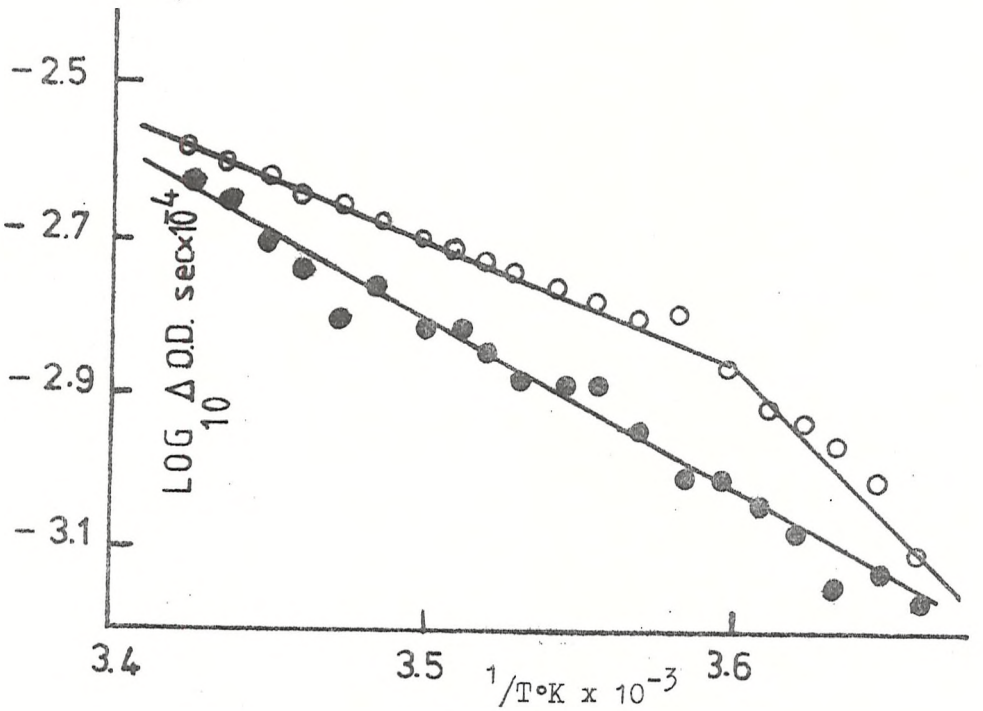


Fig 3.3.

Arrhenius plots of hepatic microsomal NADPH cytochrome P-450 reductase activity from lean (●) and obese (o) mice housed at 12°C.

Table 3.2.

Summary of data obtained from the Arrhenius plots of hepatic microsomal NADPH cytochrome P-450 reductase activity from lean and obese mice housed at various temperatures.

Phenotype	Housing temperature (°C)	Arrhenius Breakpoint (°C)	Activation energy above breakpoint (kcal. mol.$^{-1}$)	Activation energy below breakpoint (kcal. mol.$^{-1}$)
Lean ††	34	9.1 ± 0.1	7.1 ± 2.5	21.2 ± 1.2
Obese ††	34	7.5 ± 0.2	5.8 ± 2.3	27.5 ± 1.7
Lean †	24	9.5 ± 0.3	9.5 ± 0.4	21.3 ± 1.8
Obese †	24	6.5 ± 0.2	9.2 ± 1.2	27.9 ± 1.0
Lean *	12	-	9.2, 9.2	-
Obese *	12	5 - 5.2	6.7 - 6.9	17.8 - 20.3

† n = 4,, †† n = 3, * n = 2

The 'breakpoints' between lean and obese vary considerably at both housing temperature. This variability lends support to the concept that the enzyme is undergoing some physical constraint below the breakpoint imposed by the lipid matrix, rather then undergoing an internal organisational change independantly of its environment.

Breakpoints from Arrhenius plots of NADPH reductase activity from obese animals housed at 34°C and 24°C ('obese 34 and 24') were significantly lower than 'lean 34 and 24' indicating that the enzyme from 'obese 34 and 24' were sensing a phase separation occurring within the lipid matrix that was shifted down by 2-3°C, suggestive of a structural difference in the lipid matrix in obese preparations. Tentatively, this may suggest the presence of a structural component in a more fluid, or less ordered state in obese microsomal membranes. This may be an expected adaptation in obese membranes from animals housed at 25°C, since their apparent core temperature is 2.5°C lower than that of lean.

Housing the animals at 34°C for one week (after which apparent body temperatures are similar), resulted in a 1.6°C difference in breakpoint, as against 3°C at housing temperatures of 25°C, suggesting that the anomaly observed in obese microsomal membranes characterised by a change in the Arrhenius breakpoint is partially rectified by normalizing body temperature.

When animals were housed at 12°C the breakpoint in 'lean12' was reduced to below 0°C, and 'obese 12' shifted down to 5°C. Thus, by the same rationale, 'lean 12' microsomes now contain a component in a more fluid state than 'obese 12'. Both lean and obese rectal temperatures are subnormal at 12°C, and therefore this apparent fluidisation of some component in the ER membrane matrix from lean animals may reflect an attempt to maintain correct membrane function at reduced core temperature. Although the breakpoint of 'obese 12' was reduced to around 5°C, the reduction is not as dramatic as that in the lean.

In summary, lean breakpoints were not significantly changed at the two housing temperatures of 34°C and 24°C, when there was

no change in their apparent core temperature. However, housing at 12°C caused a reduced core temperature, and a downward shift in breakpoint, suggesting some change in the lipid matrix. This may be a physiological response to restore membrane microviscosity. In the obese membranes, where apparent core temperature was different at all 3 environmental temperatures, a small downward shift of breakpoint was observed as the environmental temperature was reduced. Thus, the regulation of some aspect of the lipid matrix in obese mouse ER is different to that of lean.

As previously discussed, interpretation of activation energy values should be approached with caution. Changes that occur in the shape of Arrhenius plots between two similar systems, are however, strongly indicative of different environmental conditions for the enzyme under study. In general, for membrane bound enzyme reactions, an increase in E_A is suggestive of a more constrained environment. Usually, this is translated to loosely describe changes in the hydrodynamic properties of lipids in the matrix, and the system is said to have become more ordered. For convenience, this term will be used.

From Table 3.2. it can be seen that the activation energies above the Arrhenius breakpoint are not significantly altered by housing animals at 34°C rather then 24°C. However, there is a suggestion from the 2 experiments performed at 12°C that the activation energy falls in obese 12 microsomes (6.8 v 9.2kcal $mol.^{-1}$). This would suggest that the enzyme activity is modulated by a component which is less ordered in 'obese12' membranes.

Below the breakpoint, activation energies were significantly higher in 'obese 24 and 34' than their lean counterparts, indicating that the enzyme is associated with a component with a higher degree of order in obese membranes. In 'obese 12' membranes, the activation energy was reduced to around 'lean 24 and 34' values, indicating that the component conferring the higher degrees of order in 'obese 24 and 34' was normalized in this situation.

To conclude, analysis of enzyme probe Arrhenius plots sug-

gests that the composition of the microsomal membranes of obese animals are different to those of lean. Tentatively, there may be a component that confers a higher degree of order, or more constraint, on the enzyme, and also there may be a component in a more fluid state, which may depress some lipid phase separation to which the enzyme is sensitive.

Housing the animals at 12°C, where the obese mouse rectal temperature is severely depressed, the component previously assigned to a 'higher degree of order' was normalized to that of 'lean 34 and 24'. The component in the lean assigned to a less fluid state became more fluid housing at 12°C, possibly as a result of a reduced core temperature in lean animals. It is clear from these results that there are differences in the membrane organisation in obese mice microsomes. Regulation of membrane composition in response to changing core temperature may be disturbed in the obese ER. Inability to correctly regulate membrane composition with changing core temperature may play a role in the poor thermoregulatory ability of obese animals. Equally, poor thermoregulation and inability to regulate membrane composition may be a failure of a process common to both.

3.2.d. Fluorescence polarisation of DPH labelled microsomal membranes from lean and obese mice.

Microsomal membrane lipids were labelled with DPH as described in 'Materials and Methods'. Arrhenius plots of fluorescence depolarisation with increasing temperature are shown for all three groups of animals housed at 34°C (Fig 3.4); 24°C (Fig 3.5); and 12°C (Fig 3.6), and data from these plots is summarised in Table 3.3. From Figs 3.4 and 3.5 the degree of polarisation is lower at around the animals body temperature, indicating that the bulk average microviscosity of obese membrane lipids is lower than that of lean. The flow activation energies were lower in obese membranes, and thus contained components in a more ordered state. Because of this, there was a gradual normalization of membrane microviscosity at reduced assay temperature.

To achieve the same microviscosity in obese membranes as

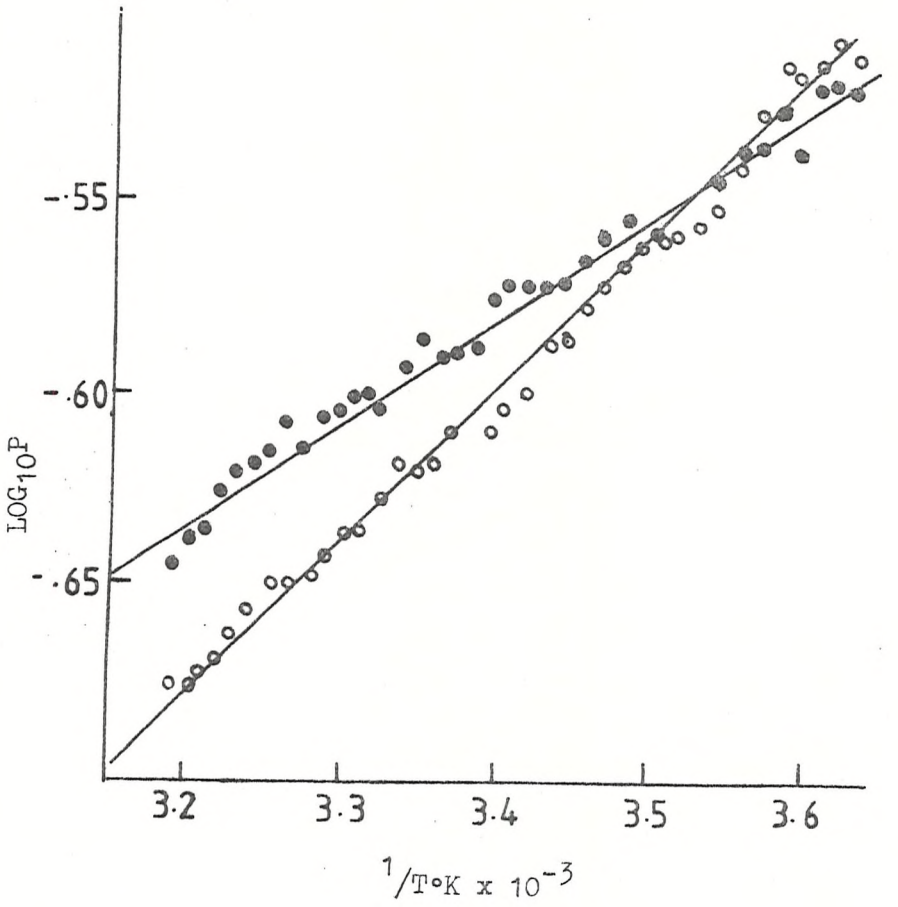


Fig 3.4.

Arrhenius plot of fluorescence depolarisation (P) by DPH in extracted hepatic microsomal lipids from lean (●) and obese (o) mice housed at 34°C.

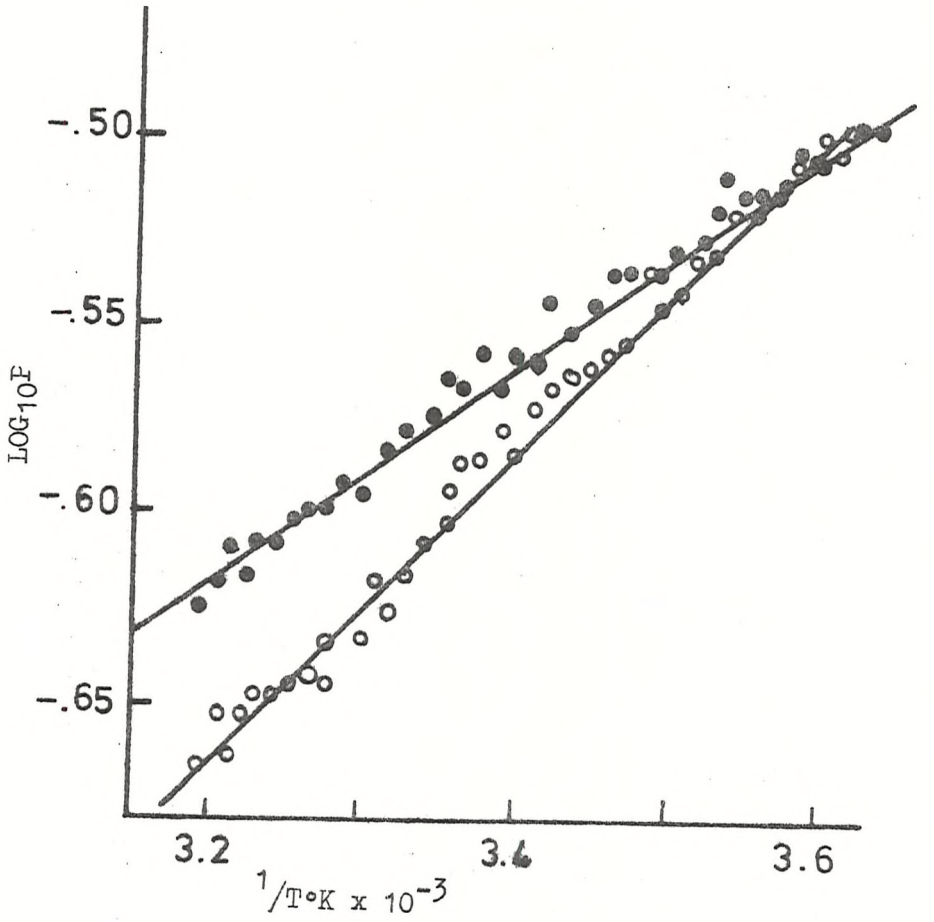


Fig 3.5.

Arrhenius plot of fluorescence depolarisation (P) by DPH in extracted hepatic microsomal lipids from lean (●) and obese (o) mice housed at 25°C.

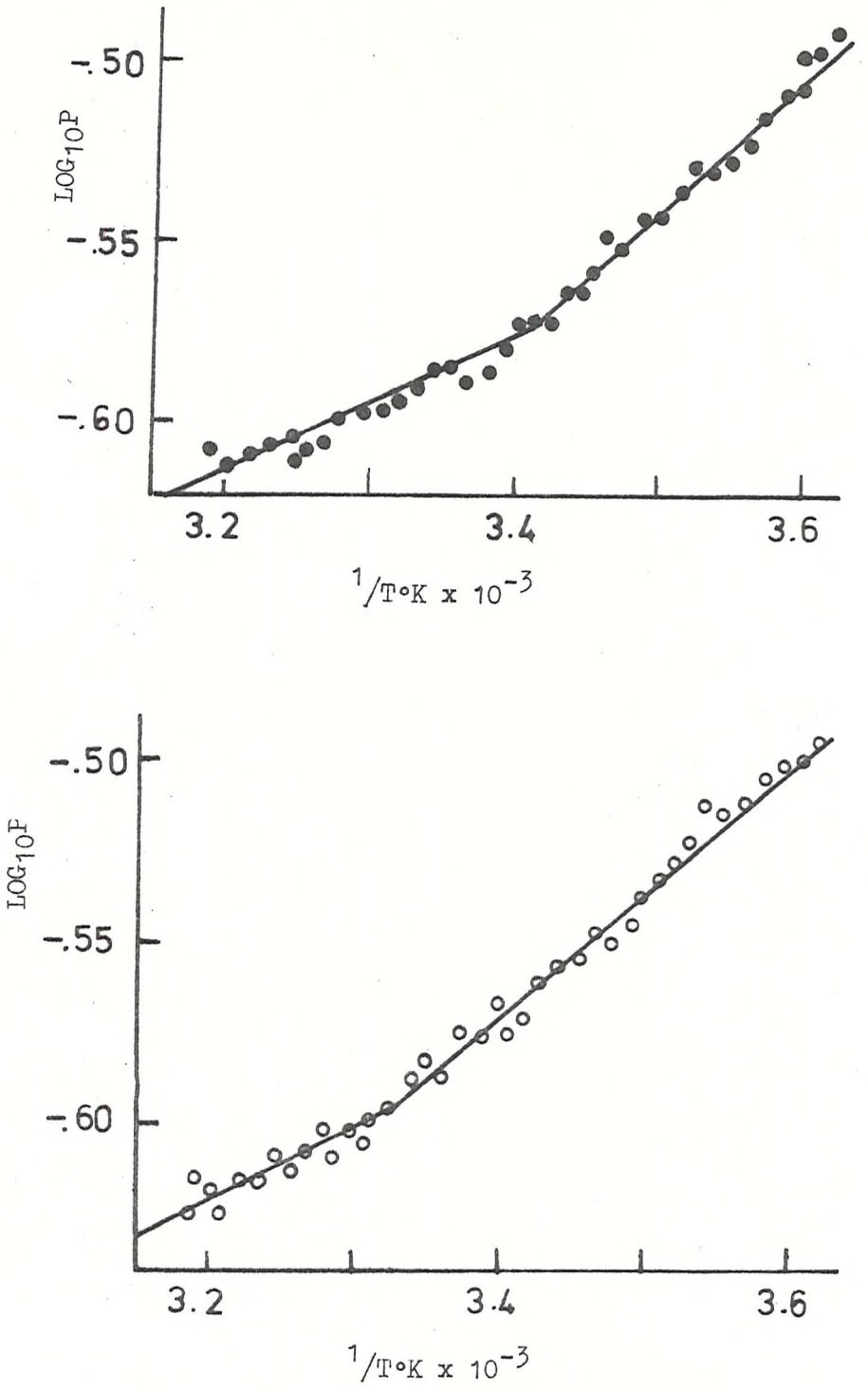


Fig 3.6.

Arrhenius plots of fluorescence polarisation by DPH in extracted hepatic microsomal lipids from lean (●) and obese (o) mice housed at 12°C.

Table 3.3.

Summary of data obtained from the Arrheius plots of fluorescence depolarisation by DPH in extracted microsomal lipid vesicles from lean and obese mice housed at different temperatures.

Phenotype	Housing Temperature °C	Arrhenius Breakpoint °C	Polarisation at 37°C	Fluidity Ratio $\oint R_{37}$	Activation energy above breakpoint (kcal. mol.$^{-1}$)	Activation energy below breakpoint (kcal. mol.$^{-1}$)
Lean	34	-	0.237	1.211	-	16.9
Obese	34	-	0.215		-	11.44
Lean	25	-	0.245	1.232	-	15.9
Obese	25	-	0.221		-	11.44
Lean	12	20.3	0.247	1.045	23.9	12.6
Obese	12	27.8	0.242		23.9	12.6

that of lean assayed at 37°C, membranes from obese would have to be assayed at 28.5°C - a difference of 11°C. In fluidity terms, obese membranes are fluidised by around 22% over lean when housed at room temperature.

Housing at 12°C produced Arrhenius plots with breakpoints at 20.3°C in lean and 27.8°C in obese. Gross membrane fluidity was decreased in obese membranes to lean values. Flow activation energies above and below breakpoints were identical.

This result is somewhat surprising, since the core temperatures of obese mice are as low as is possible to sustain life, since housing at 10°C, death ensues. Therefore under these conditions a fluidisation of obese membranes might be expected if membrane physical properties are to be preserved. Since this was not the case, this observation implies a changed regulation of membrane composition in obese membranes.

The biphasic nature of these 12°C Arrhenius plots suggests the breakpoints mark the end of a broad phase transition. Because the activation energies below the breakpoints (12.6 kcal $mol.^{-1}$) are nearer to those obtained from the monophasic Arrhenius plots in membranes from lean and obese animals housed at 34°C and 24°C (mean value = 13.92 kcal $mol.^{-1}$), it is likely that the end of this broad phase transition was previously above 40°C in membranes from animals housed above 12°C. The breakpoint in obese 12°C microsomes is higher than that of lean, suggesting that there may be at least one component in a less fluid state in 12°C microsomes.

However, it is possible that the appearance of this breakpoint is an artefact caused by selective partitioning of the fluorescent probe into different lipid domains above and below the break temperature. Further experimentation is necessary to evaluate the significance, if any, of this phenomenon.

Thusfar, our results with enzyme and fluorescent membrane probes suggest structural and regulatory anomalies in the lipid composition of obese microsomal membranes. At housing temperatures above 24°C, membranes from obese animals are in a more

fluid state, but at the same time probably contains at least one component in a less fluid state. Housing at 12°C normalises obese microsomal membrane bulk fluidity, although a second component may be maintained in a less fluid state than in lean microsomes.

Again, we must conclude that the observed regulatory changes in the membranes from obese animals are multifactorial with respect to the membrane components.

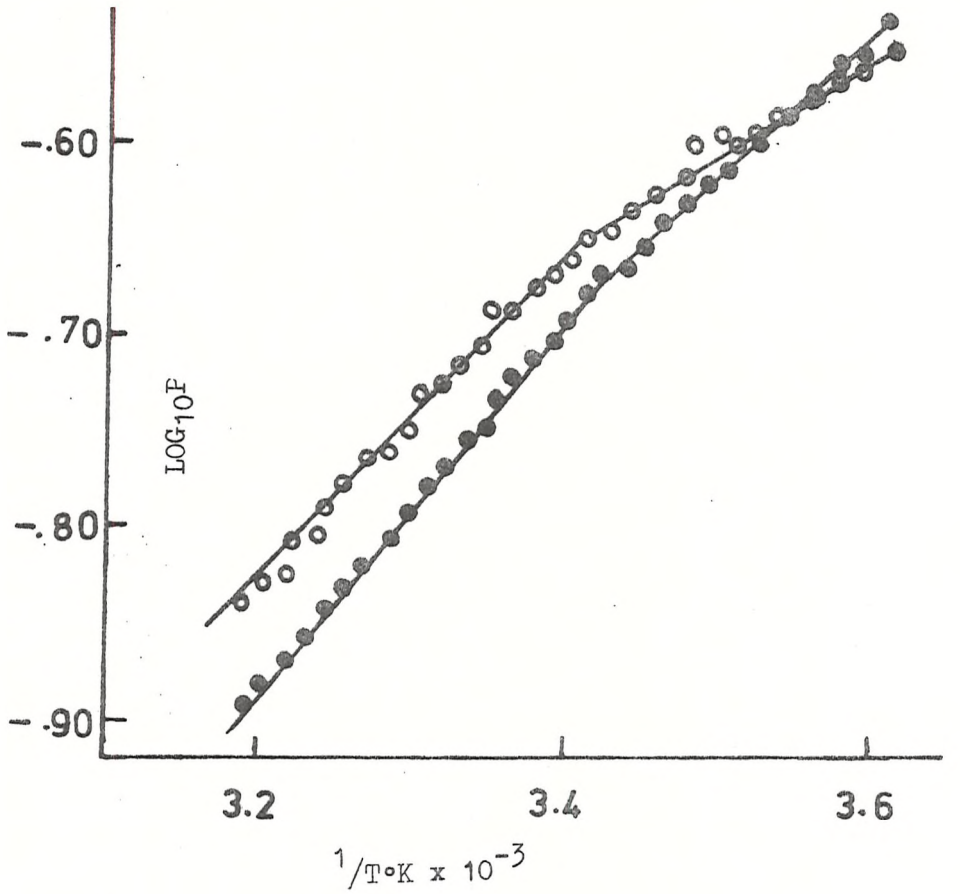
3.2.e. Fluorescence polarisation studies on extracted PC and PE from lean and obese microsomal membranes.

PC and PE were extracted from the bulk membrane lipids by tlc for fluorescence depolarisation and fatty acid compositional studies.

Fluorescent labelled vesicles were prepared by sonication (Chapter 1). PC forms stable bilayers while PE forms stable hexagonal phase (H_{II}) multilayers under these conditions (Cullis and De Kruijff, 1979). Figs 3.7 and 3.8 show Arrhenius plots of fluorescent phospholipid vesicles.

Lean PC, at an assay temperature of 37°C is more fluid than that of obese PC. With decreasing temperature, the curves converge, due to lower flow activation energies in the lean above and below the breakpoints. Thus PC from obese ER is less fluid at physiological temperatures than that of lean, and the acyl chains in obese PC are more disordered with respect to their molecular structure. As the assay temperature is reduced, so the acyl chains from lean PC become more condensed. As DPH partitions equally well into fluid and quasi-crystalling domains, within the matrix, so the rotational mobility of the probe is reduced, decreasing the measured bulk microviscosity.

With PE vesicles labelled with DPH, polarisation curves shown in Fig 3.7 were obtained. Obese PE vesicles were far more fluid at physiological temperatures. The assay temperature would have to be reduced by some 20°C to obtain equivalent fluidity in lean PE vesicles. The activation energies above



Lean

ΔE above break = 4.49 kcal mol^{-1}

ΔE below break = 7.55 kcal mol^{-1}

Breakpoint = 20.3°C

Obese

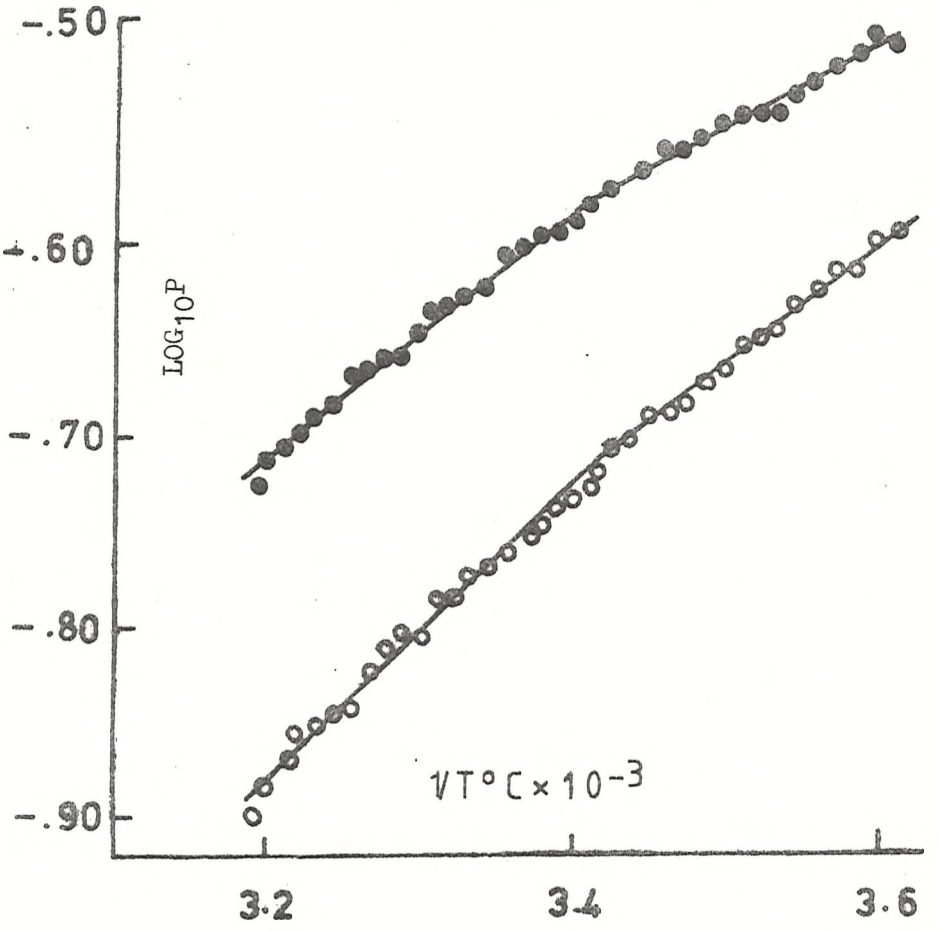
ΔE above break = 5.39 kcal mol^{-1}

ΔE below break = 8.99 kcal mol^{-1}

Breakpoint = 20.3 °C

Fig 3.7.

Arrhenius plot of fluorescence depolarisation (P) by DPH in extracted hepatic microsomal PC vesicles from lean (●) and obese (o) mice housed at 25°C.



Lean

ΔE above break = 8.47 kcal mol^{-1}

ΔE below break = 10.37 kcal mol^{-1}

Breakpoint = 20.3°C

Obese

ΔE above break = 5.82 kcal mol^{-1}

ΔE below break = 7.67 kcal mol^{-1}

Breakpoint = 20.3°C

Fig 3.8.

Arrhenius plot of fluorescence depolarisation (P) by DPH in extracted hepatic microsomal PE vesicles from lean (●) and obese (o) mice housed at 25°C.

and below the breakpoints were lower than those of lean, suggesting that the acyl chains are more ordered in obese ER compared with those of lean.

Since the phospholipid fraction of the ER membrane is composed of approximately 54% PC and 25% PE (Depierre and Dallner, 1976) the fluidity and order parameters obtained from the Arrhenius plots of these two parameters should by and large dictate these properties in the whole membrane. By considering the relative contributions of each lipid species, it is possible to explain the results of the polarisation Arrhenius plots in terms of the major phospholipid species. Assuming the compositional results of Depierre and Dallner, (1976) for rat hepatic ER, then if the membrane contained no other phospholipids, and the same neutral lipid content, than the mean contributions of the two lipid classes to the bulk polarisation at an assay temperature of 37°C would be 0.159 for lean and 0.152 for obese. In other words the net effect would be a more fluid membrane in obese ER. Although this is consistent with the observed situation, the possible contribution of the remaining 25% of phospholipids, PS and SM, and also membrane neutral lipids (especially cholesterol) to the change in fluidity cannot be ignored, and requires further systematic investigation.

The work of Bosterling et al. (1979), has demonstrated that the presence of PE is essential in vesicles containing reconstituted NADPH cytochrome P-450 oxidoreductase enzyme systems, for full expression of enzyme activity. It thus would appear likely that the enzyme is associated with PE. Our results are consistent with this concept, since the Arrhenius plots of 'obese 25' enzyme activity suggest association with a more fluid lipid. PE, therefore is a good possibility. Since the major microsomal phospholipid is PC, it may well be this lipid that determines the activation energy of the enzyme reaction, since 'obese 25' Arrhenius activation energies suggest association with a more ordered component.

Higgins and Dawson (1977) have demonstrated that the rat liver ER is assymetric with respect to the localisation of phos-

pholipid species. Their findings indicate that PE is largely confined to the luminal monolayer, and that the cytoplasmic monolayer comprised mainly of PC. This raises the possibility that the obese microsomal membrane may have altered bilayer fluidity assymetry, the luminal monolayer being more fluid than that of lean, and in contrast the cytosolic monolayer may be less fluid. This concept may help to rationalize the Arrhenius data for NADPH cytochrome C reductase activity, since the enzyme system is likely to interact with both halves of the bilayer.

3.2.f. GLC analysis of microsomal membrane fatty acyl methyl esters from lean and obese mice.

Table 3.4 shows the fatty acid spectrum from the total microsomal lipid extract. The major components are shown with standard errors, and represent the accumulated data from 6 observations. Initially, experiments were performed to determine if the fatty acid changes in obese liver microsomes were dependant of the severity of the developing hyperglycaemic syndrome. GLC data was obtained from animals aged from 6 weeks to 6 months.

As no age dependant differences could be discerned in the fatty acid composition, the data is presented as the accumulated means and standard errors. It can be concluded that the observed changes in Table 3.4 are age independant.

The major changes in fatty acid composition of microsomes in obese compared to lean mice were a reduction of 16:0, an increase in 18:0, and 18:1, a decrease in 18:2 and an increase in 22:6 and 24:1. Overall, the total lipid extract is less saturated with more pi bonds $mol.^{-1}$ lipid. This is consistent with both the increased fluidity that was measured by fluorescence depolarisation by DPH, and the lower Arrhenius breakpoint for NADPH-cytochrome C reductase.

Thus, the fluidisation of obese microsomal membranes is due, at least in part, to increased desaturation of the phospholipid fatty acyl moieties.

Table 3.4.

GLC fatty acid methyl ester spectrum of total microsomal membrane lipids from lean and obese mice housed at 25°C.

FATTY ACID	% OF TOTAL			
	LEAN		OBESE	
14:0	0.2		0.5	
14:1	0.4		0.3	
16:0	26.9	± 1.44	16.6	± 0.98
16:1	3.7		3.5	
18:0	17.6	± 0.73	20.6	± 1.2
18:1	13.1	± 0.44	15.8	± 1.8
18:2	12.5	± 0.5	10.0	± 0.2
18:3	2.0		1.2	
20:0	ND		0.22	
20:2	1.6		1.2	
20:3	3.0	± 0.04	3.4	± 0.2
20:4	8.4	± 0.7	8.4	± 0.6
22:1	0.3		1.4	
22:4	0.6		0.6	
22:6	8.9	± 0.9	12.6	± 1.4
24:1	0.7		2.7	

3.2.g. GLC analysis of microsomal membrane PC and PE fatty acyl methyl esters from lean and obese mice.

Table 3.5 shows the fatty acid spectrum of PC from lean and obese mice hepatic ER. From the table, there appear to be no major differences, except that the obese PC molecules are more saturated and contain less pi bonds $mol.^{-1}$ than lean (Table 3.7), which is consistent with the fluorescence depolarisation data.

Table 3.6 shows the fatty acid spectrum of PE from lean and obese hepatic ER. There is a marked reduction of 16:0 and 18:0, and a large increase in 20:4 and 22:6. Consequently, there is a considerable reduction (over 40%) in the content of saturated fatty acids, with nearly a threefold increase in the number of pi bonds $mol.^{-1}$ (Table 3.7). The mean carbon chain length is increased by 1 carbon atom in the obese PE molecules.

Thus, the marked fluidisation of PE vesicles from obese ER can be accounted for by an increase in 20:4 and 22:6, at the expense of 16:0 and 18:0.

3.2.h. GLC analysis of fatty acyl methyl esters from microsomal membranes from mice made obese by injection of gold thioglucose.

Gold thioglucose-obese mice (GTG) were prepared as described in 'Materials and Methods'. Animals were in the static phase of their obesity, and were housed at 25°C. Total microsomal membrane fatty acid methyl esters were analysed by GLC. A similar fatty acid spectrum was obtained as that for ob/ob mice (Table 3.8) the major changes being a decline in 16:0, with an increase in 22:6. The total phospholipid extract contained a lower percentage of saturated fatty acids, and an increased number of pi bonds $mol.^{-1}$ to both lean and ob/ob mice.

Thus, changes in fatty acid saturation parameters were obtained in two separate models of obesity, genetic autosomal recessive, and chemically induced hypothalamic. This alteration of membrane composition may, therefore, be a general feature of the obese state.

Table 3.5.

GLC fatty acid methyl ester spectrum of phosphatidylcholine extracted from total microsomal membranes from lean and obese mice housed at 25°C.

FATTY ACID	% OF TOTAL	
	LEAN	OBESE
14:0	1.2	1.1
14:1	0.4	0.5
16:0	24.2	23.5
16:1	3.3	3.3
18:0	12.6	14.9
18:1	13.1	13
18:2	12.6	7.7
18:3	1.1	1.5
20:0	0.72	1.26
20:2	ND	1.0
20:3	2.7	1.4
20:4	10.5	7.4
22:2	1.32	0.4
22:4	0.7	1.7
22:6	12.1	13.8
24:1	3.4	5.8

Table 3.6.

GLC fatty acid methyl ester spectrum of phosphatidylethanolamine extracted from total microsomal membranes from lean and obese mice housed at 25°C.

FATTY ACID	% OF TOTAL	
	LEAN	OBESE
14:0	0.9	0.4
14:1	0.9	0.3
16:0	34.3	16.3
16:1	ND	2.8
18:0	25.0	19.4
18:1	14.8	13.1
18:2	6.8	5.6
18:3	2.4	1.8
20:0	2.1	ND
20:2	ND	ND
20:3	2.7	1.4
20:4	3.7	14.2
22:2	1.1	0.8
22:4	1.8	0.9
22:6	1.9	22.0
24:1	1.5	ND

Table 3.7.

Summary of data from GLC analysis of the fatty acyl methyl esters from lean and obese hepatic microsomal lipids, and extracted PC and PE.

	% saturated fatty acids		pi bonds 100 molecules^{-1}		Mean carbon atom chain length.	
Phenotype	L	O	L	O	L	O
Total extract	44.7	37.9	150.8	171.5	18.1	18.6
PC	38.7	40.8	175.5	167.3	18.4	18.3
PE	62.3	36.1	80.6	230.2	17.7	18.7

Table 3.8.

GLC fatty acid methyl ester spectrum from total microsomal lipid extract from GTG obese mice. The total fatty acids are 39.5% saturated, and contain 188.2 pi bonds per 100 lipid molecules. Lean values are those shown in table 3.4.

FATTY ACID	% OF TOTAL	
	GTG OBESE	LEAN
14:0	0.3	0.2
16:0	20.0	26.9
16:1	0.4	3.7
18:0	19.2	17.6
18:1	18.5	13.1
18:2	9.6	12.5
18:3	1.1	2.0
20:3	3.2	3.0
20:4	9.7	8.4
22:4	1.1	0.6
22:6	15.0	8.9
24:1	2.0	0.7

3.2.i. Interpretation of flow activation energy changes in fluorescence depolarisation studies in membranes and liposomes from lean and obese mice.

As has been outlined in section 3.1.(ii) the interpretation of the value of ΔE is not always easy. In membrane lipid extracts it is particularly difficult since the full composition of the individual lipid classes is not known. In phospholipid vesicles of a single class, theoretically it is only the fatty acyl chains that are determinant in the value of ΔE. Therefore comparison of GLC data and ΔE values for each lipid class should help to establish a stronger link between ΔE and molecular structure. Efforts in this direction are hampered by lack of knowledge of the isomeric heterogeneity of each unsaturated fatty acid class. There is much evidence that mammalian fatty acids are predominantly cis, however (Hilditch and Williams, 1964).

The total lipid extract from obese membranes was more fluid at physiological assay temperatures. Due to a decreased ΔE, fluidity was normalized at low assay temperatures. Extracted PE showed an increase fluidity and also a decreased ΔE, suggesting the characteristics of the bulk membrane may be strongly influenced by PE. Since the larger changes were a reduction in 16:0 with an increase in 22:6 in both whole lipid extracts and PE, then this may account for the enhanced fluidity. The configuration of all cis 22:6 is such that it adopts a relatively rigid three dimensional structure (Chapter 5).

It may be this aspect of this lipid that may give rise to a higher degree of order in obese membranes and PE vesicles. At the same time the large volume and poor packing ability of this lipid serve to increase fluidity. This will be dealt with in greater detail in Chapter 5.

The cause of the increase in ΔE in PC from obese microsomal membranes is obscure, perhaps a decrease in 18:2 or 20:4 might serve to decrease the order of PC vesicles from obese mice.

3.2.j. Findings of Rouer et al. (1980).

Recently Rouer et al., (1980) studied the Arrhenius data of an intrinsic microsomal enzyme, ethoxycoumarin-O-diethylase in microsomal membranes from lean ob/ob and db/db mice. Breakpoints in ob/ob and db/db membranes were reduced to below zero, suggesting that the enzymes from these two mutant strains were sensing a more fluid environment. Our results with NADPH cytochrome C are in agreement with those obtained by Rouer et al. for ethoxycoumarin-O-diethylase.

These workers also obtained subzero breakpoints in Arrhenius plots of DPH fluorescence depolarisation in microsomal membranes from mutant mice, but obtained a breakpoint at 22°C for lean membranes. They detected no difference in membrane microviscosity at body temperature between any of the groups. Our results are not consistent with this observation, but the possibility that glycogen and extrinsic proteins may be masking fluidity differences in their membranes cannot be ruled out.

The GLC data obtained by Rouer et al. (1980) demonstrate that microsomal membranes from obese animals contain altered proportions of fatty acids. Although their data and ours show many points of agreement (for example, reduced 16:0, 18:2 and increased 18:0, 18:1), no data was presented for 22:6 or the more minor fatty acid components. However, these authors noted an increase in 20:4, not obtained by ourselves.

3.3. CONCLUSIONS.

Studies using an enzymic marker and fluorescent probe to assess changes in the microsomal membranes from obese mice suggest that there are compositional changes in these membranes. Furthermore, housing the animals at different environmental temperatures caused compositional changes within the hepatic ER. These changes are not mirrored by similar changes in those from obese mice suggesting that a regulatory defect in the control of membrane composition may be present. This may be related in some way to the obese animals poor thermoregulatory ability, compared to lean animals.

Fluorescence polarisation studies on membrane lipids demonstrate that obese membranes are more fluid than lean. Gross membrane fluidity measurements also demonstrate that there are differential changes in membrane composition between lean and obese under different housing conditions.

Fluorescence measurements on isolated phospholipids from lean and obese ER shows that ER from obese membranes is considerably more fluid than that of lean, while PC in obese ER is less fluid. It is likely that PE is the dominant factor in causing an increased gross membrane fluidity in obese ER.

GLC data demonstrates that the major cause of the increased fluidity in PE from obese ER is the presence of 22:6. The presence of this lipid in greater amounts in membranes from lean mice made obese by hypothalamic lesioning suggests that the observed fatty acid changes in microsomal membranes may be a general feature of the obese condition.

Thus, obese mice microsomal membranes are structurally altered compared to those from lean mice. PE and PC are assymetrically distributed in the ER, and thus the obese ER membranes may have a more viscous cytoplasmic monolayer, while having a less viscous luminal monolayer compared to lean controls. This factor may strongly influence the biochemistry of intrinsic and extrinsic membranes proteins, and may help to

partially explain the many metabolic anomalies associated with membrane bound proteins in the obese state.

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CHAPTER 4.

COMPOSITIONAL AND ENZYMATIC STUDIES ON THE ADIPOCYTE PLASMA MEMBRANE FROM LEAN AND OBESE MICE

4.1. INTRODUCTION.

4.1.a. Function of the adipocyte.

By far the most obvious anatomical change in the obese state is that of the white adipose tissue, since it is this that is the site of lipid storage in mammalian and avian species. Not surprisingly, much interest has been shown in probing the nature of the regulation of fat metabolism in this tissue in the search for metabolic changes that give rise to the obese condition.

The fat cell (adipocyte) has been a popular model for investigating the action of hormones on cellular metabolism, since its ease of preparation, durability and its high sensitivity to various hormones make it ideal for in vitro study. One disadvantage of this cell, however, is the very wide range of size that it can attain. Comparison of metabolic parameters between two populations of cells of different mean diameter is difficult, since metabolic data can be'normalised' by expressing results in terms of cell number, surface area or intracellular water space.

In recent years certain aspects of adipose tissue metabolism in the obese condition have been elucidated, and are now widely accepted. The adipocyte is concerned with fatty acid uptake, storage and release, also it is capable of de novo fatty acid synthesis from glucose. In the fed animal, the adipose tissue provides a reserve of fats to provide the animal with energy for periods of starvation, or for periods of strenuous exercise. During normal periods, in the mature animal, the tissue is in the steady state whereby the rate of lipolysis is equivalent to the rate of re-esterification, and esterification of newly synthesized fatty acids. Through mechanisms unknown, the adipose tissue mass is accurately controlled over long periods, such that the adipose tissue mass is adequate for moderate starvation, but at the same time not too extensive to impair the animals mobility. In the obese condition, when this is not the case, a number of metabolic parameters in the adipose tissue are altered. Principally, these changes are brought about by changes in the response of adipocytes to plasma hormones.

4.1.b. Hormonal control of fat metabolism.

Triacylglycerol accumulation in adipocytes is ultimately regulated by the supply of fatty acids from dietary fat and glucose, and the demand for fatty acids by the tissues. Insulin enahnces anabolic processes that lead to triacylglycerol (TG) formation in the adipocyte in the presence of dietary substrate. The catecholamines, glucagon and growth hormone promote TG catabolism to fatty acids which are released from the cells. These hormones are responsible for maintaining the steady-state levels of plasma metabolites in the fed, starved and stressed condition.

The relative sensitivity of adipose tissue to the above mentioned hormones is modified by other plasma hormones, particularly thyroid hormone and the glucocorticoids. The long-term control of adipose tissue mass in the fed state is likely to be due to the ability of adipocytes to respond to subtle changes in the relative concentrations of these hormones in the plasma. Fig 4.0. illustrates the control of TG metabolism in adipose tissue by insulin and hormones acting via the adenylate cyclase. Insulin promotes TG accumulation by modulating a variety of intracellular events. Insulin increases the transport of glucose into the adipocyte by increasing the number of transporters in the plasma membrane (Cushman and Wardzala, 1980). Since transport is rate limiting for glucose utilization, intracellular substrates for TG synthesis are elevated (i.e. glucose -1-phosphate, NADPH and fatty acids). Insulin may also exert a direct enhancement of esterification. Lipolysis is decreased by insulin, although the mechanism for this action is not known, but there is some evidence that insulin may reduce intracellular cAMP. The role of cAMP in the control of lipolysis is well documented (Kuo and Greengard, 1969). Intracellular cAMP may be reduced by the increase in activity of the phosphodiesterase enzymes following exposure of adipose tissue to insulin (Loten and Sneyd, 1970). Insulin also increases the activity of lipoprotein lipase in adipose tissue. This serves to promote the entry of fatty acids into adipocytes for esterification.

The role of catabolic hormones in adipose tissue metabolism

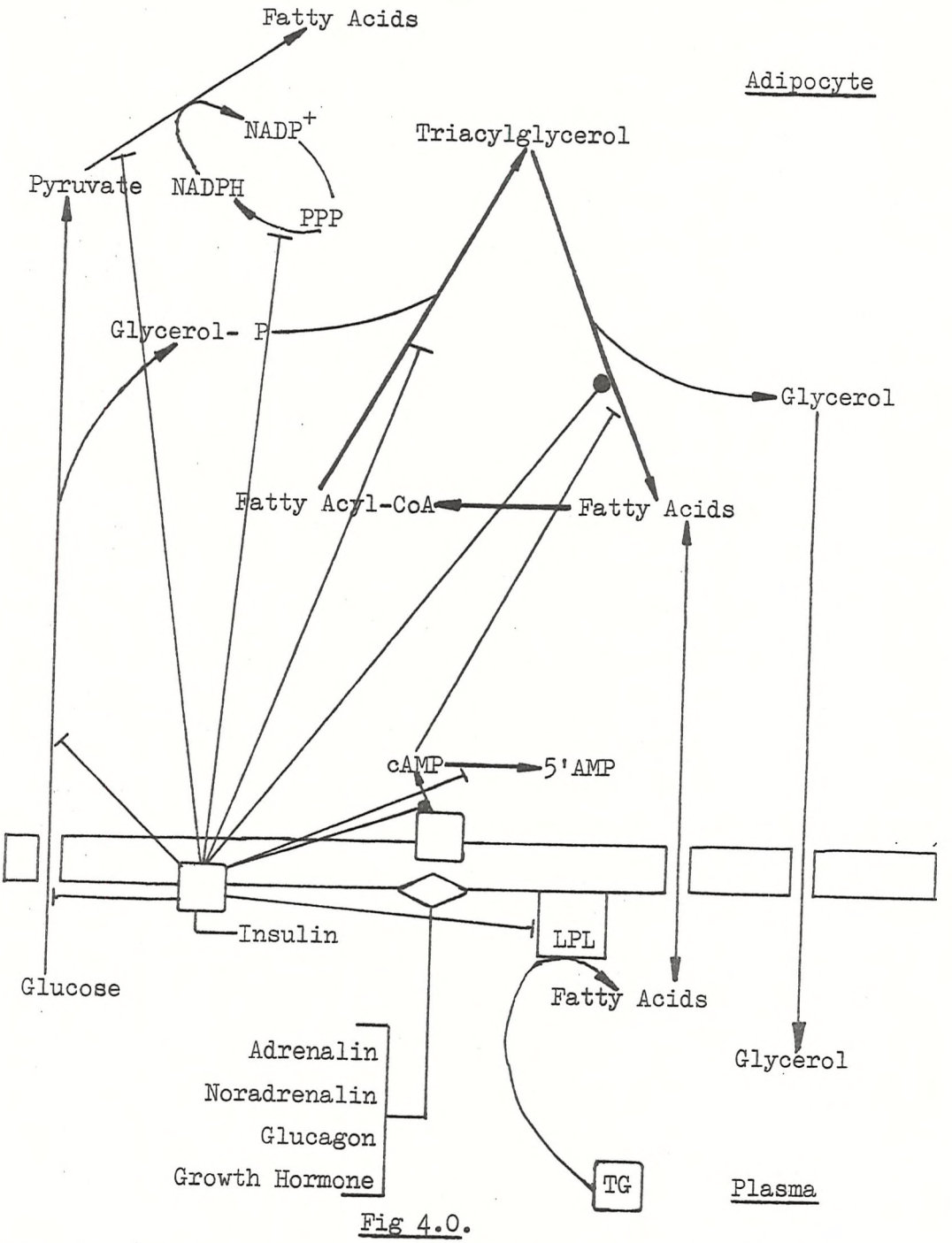


Fig 4.0.

Hormonal control of triacylglycerol/fatty acid cycle and glucose metabolsim in adipose tissue.

LPL - Lipoprotein Lipase

PPP - Pentose Phosphate pathway.

—| Stimulation

—● Inhibition

are better understood. The catecholamines, glucagon and growth hormone all stimulate the adenylate cyclase directly via their receptors. The resulting elevation of intracellular cAMP activates a protein kinase which phosphorylates an inactive lipase, converting this enzyme into its active form (Corbin et al., 1970). TG metabolism, therefore, is regulated primarily by the relative antagonism between insulin and hormones activating adenylate cyclase.

Thyroid hormones modulate catecholamine action in a variety of tissues including adipocytes (Malbon et al., 1978). The affinity of β receptors for agonists is dependant on thyroid status (Malbon, 1980), increasing plasma T_3 concentrations producing an increase in binding affinity for isoprenaline. Thyroid hormone also modulates the regulation of agonist binding to β receptors by GTP, the rightward shift in the dose-response curve to adrenaline caused by the presence of guanine nucleotides is abolished in hypo and hyperthyroid states (Malbon, 1980). Thyroid hormones may also modulate the α to β receptor ratio in adipose tissue (Rosenquist, 1972), α receptor occupancy antagonises β receptor occupancy in adipose tissue. The effect of thyroid hormones on modulating the coupling efficiency between ligand-receptor complex and adenylate cyclase has been established, and is discussed by Malbon (1980). Although little is known about the nature of this action of the thyroid hormone, nevertheless Malbon (1980) has shown that guanine nucleotides are not involved in the altered coupling efficiency caused by changed serum T_3 levels.

The glucocorticoid hormones, corticosterone and hydrocortisone promote fatty acid release from adipocytes in the presence of glucose. Although little is known about the specific mechanisms by which the hormones act, protein synthesis is an obligatory step in the action of the glucocorticoids (Fain and Czech, 1975). Glucocorticoids specifically inhibit the membrane transport of glucose, thereby reducing the availability of glycerol-1-phosphate for re-esterification of fatty acids (Czech and Fain, 1971). Glucocorticoids also alter the sensitivity of fat cells to lipolytic enzymes. Since glucocorticoids do not alter the

steady state levels of cAMP (Fain et al., 1971), Fain and Czech (1975) suggest that the glucocorticoids enhance the potency of cAMP to activate lipolysis by some unknown mechanism.

4.1.c. Hormonal control of fat metabolism in obesity.

Whatever the locus of increased metabolic efficiency observed in obese animals compared to lean, hypertrophy of adipose tissue in obese animals must result from an alteration in the relative rates between lipogenesis and lipolysis in the whole animal during the dynamic phase of weight gain.

Under normal growth conditions of an animal, net TG accumulation is strictly controlled in adipose tissue. It is at this stage of development that adipose tissue is at its most sensitive to hormonal status. Net TG accumulation in adipose tissue from obese animals, however, occurs also under conditions where adipocytes display a marked blunted response to circulating hormones (Bray and York, 1979). Whatever the reasons for this blunted hormonal response in adipose tissue from obese animals, it is likely that this factor coupled with the actual hormonal status of the obese animal contributes largely to increased TG accumulation in the adipose tissue.

The poor hormonal response of adipose tissue in obese animals is not primary to the aetiology of obesity, since tissue transplant studies reveal that adipose tissue from either phenotype adopts the same characteristics as host tissue (Hausberger, 1959; Meade et al., 1978). However, as Bray and York (1979) point out, hormone resistance may be a general feature of many tissues in the obese condition. Clarification of the possible cellular locus of this resistance is therefore necessary in order to understand more clearly the biochemical changes occurring in the whole animal that ultimately give rise to an increased adipose tissue mass. The general features of hormone resistance displayed by adipose tissue in the ob/ob mouse have been discussed in the introduction (Chapter I).

Dehaye et al., (1978) have demonstrated the likely possibility of reduced coupling efficiency between ligand-receptor

complex and adenylate cyclase in ob/ob mice adipocyte plasma membranes compared to lean controls. Also, from perturbation studies with ethanol on guanyl nucleotide coupling to adenylate cyclase, these authors postulate an altered lipid composition in membranes from obese mice compared to lean. The data of Dehaye et al., (1978) suggests for the first time that the observed blunted response of lipolysis to catacholamines in obese adipose tissue may be a result of reduced receptor-cyclase coupling efficiency due to an altered lipid environment.

The early events that trigger responses in target cells to circulating hormones occurs on the cell plasma membrane. In Chapter 3 it was established that gross perturbations occur in hepatic microsomal membranes from obese mice when compared to lean control membranes. Experiments were therefore designed to characterise adipocyte plasma membranes from lean and obese mice in order to establish whether similar perturbations occur in plasma membranes as in microsomal membranes, and if so, whether altered plasma membrane composition contributes to the altered responsiveness of adipose tissue to hormonal stimulation.

4.1.d. <u>The effect of membrane lipid composition on basal and hormone stimulated adenylate cyclase activity</u>.

In order to evaluate the possible effect of altered membrane composition on the poor hormonal stimulation of adenylate cyclase by catabolic hormones observed in the obese condition, it is worthwhile reviewing the literature on the effect of lipids on the basal and hormone stimulated activity of adenylate cyclase.

Although data between experimental groups is not always consistent, considering the wide variety of animal sources from which data has been obtained, and also variations in experimental conditions used, it becomes clear that a feature of this enzyme system is its high degree of sensitivity to both the fluidity and lipid composition of its environment in the cell plasma membrane.

Early studies with phospholipase treatments of membrane preparations resulted in changes in basal adenylate cyclase

activity, hormone stimulation, or both (Pohl et al., 1971; Rabalcava and Rodbell, 1973). In several studies, readdition of phospholipids resulted in restoration of basal activity or hormone stimulation to an extent that was dependant on the nature of the polar headgroup (Pohl et al., 1971; Levey, 1971; Rethy et al., 1971). These workers established that phospholipids were essential in the coupling process, and also to normal cyclase function, and that phospholipids were preferentially associated with the hormone-receptor-cyclase complex.

Membrane perturbing agents have been shown to have a variety of effects on the adenylate cyclase system. Gorman and Bitensky (1970), first showed that short chain alcohols could selectively activate the glucagon-responsive adenylate cyclase in liver plasma membrane, complimentary to the findings of Dehaye et al., (1978), who observed a similar effect of ethanol on the β-adrenergic stimulated adenylate cyclase. Dipple and Houslay (1978) demonstrated that benzyl alcohol activates both the glucagon stimulated and uncoupled fluoride stimulated adenylate cyclase in rat liver plasma membrane. At higher concentrations, this agent inhibited both activites, although the concentration required for onset of inhibition was quite different. Longer chain phenolic alcohols did not inhibit the uncoupled unit until higher concentrations were reached, when the coupled unit also became inhibited.

Since the aryl ring of benzyl alcohol only interacts with phospholipid headgroups, these authors postulated that the lipid annulus of the catalytic unit determines the activity of the uncoupled unit, a process overridden when coupling occurs to the glucagon receptor. Englehard et al., (1978) performed a series of experiments using mouse cells in culture. Cells were supplemented with fatty acids and various phospholipid headgroup derivatives. These workers then correlated changes in plasma membrane adenylate cyclase activity with changes in bulk cell phospholipid composition. Since the bulk of membrane lipids contained in a 48,000 X g pellet are microsomal and mitochondrial in origin, difficulties arise in interpretation of their results in terms of plasma membrane perturbations by lipid supplementation.

Increasing membrane PE/PC ratio by incubating cells with ethanolamine increased basal adenylate cyclase activity, but reduced NaF, PGE and PGE + GTP-induced stimulation over basal. Increasing membrane unsaturation led to further increases in basal activity in both choline and ethanolamine supplemented cells. Increasing the content of unsaturated fatty acids in cells with choline supplementation potentiated the PGE, stimulation of adenylate cyclase, while conversely, when cells were supplemented with ethanolamine there was a fall in the simulatory effects of PGE.

Supplementing cells with choline and unsaturated fatty acids produced a 35% increase in the K_m for ATP of the adenylate cyclase, whereas ethanolamine and unsaturated fatty acid supplementation decreased the K_m by 225%. These results suggest that PC and PE interact differently with the adenylate cyclase, and that unsaturated fatty acids modulate basal and hormone stimulated adenylate cyclase activity.

The polyene antibiotic, filipin has been shown to alter adenylate cyclase activity in frog erythrocytes (Limbird and Lefkowitz, 1975) and in pigeon erythrocytes (Puchwein et al., 1974). Filipin is an agent known to bind cholesterol in membranes, with high affinity.

Filipin was found, in both systems, to uncouple the adenylate cyclase from the β adrenergic receptor occupied with agonist. No change in the [^{3}H]-dihydroalprenalol or [^{3}H]isoproterenol specific binding was noted. Using perylene to probe the hydrophobic interface, and aminonapthylsulphonic acid to probe the hydrophobic/hydrophilic interface, Puchwein et al. (1974) concluded that the filipin-induced uncoupling effect took place in the hydrophobic region of the membrane. Cholesterol may thus be exerting a regulatory effect on adenylate cyclase, either directly, or more likely, by indirectly interfering with protein-lipid interactions.

More recently, Sinensky et al. (1979) have examined the wide range of cholesterol incorporated into mutated hamster overy cells, defective in the regulation of plasma membrane cholesterol content.

Sinensky et al. (1979) found that decreasing membrane cholesterol content decreased basal adenylate cyclase activity, while increasing membrane order and cholesterol content increases basal activity. From parallel ESR studies, Sinensky et al. (1979) concluded that this phenomenom was due to acyl chain ordering by cholesterol rather than to a direct cholesterol/adenylate cyclase interaction. Increased acyl chain ordering also led to a decreased NaF and PGE-stimulation, although the maximal potency of these agents was unaffected by acyl chain ordering.

Finally, Gidwitz et al. (1980) have compared adenylate cyclase activities between normal and transformed chicken embryo fibroblasts. These two cell lines have different membrane composition. They found that transformed cells had both a higher PE/PC ratio and also contained more unsaturation in the acyl chains than normal cells. A lower basal adenylate cyclase activity was also observed in transformed cells. Supplementing both cell lines with exogenous lipids demonstrated that increasing the PE/PC ratio or the degree of unsaturation reduced basal activity of the adenylate cyclase.

These authors postulate from their studies on transformed cells that lipid composition may be modulating the effectiveness of the regulatory subunit to activate the cyclase enzyme. In view of this evidence for the effect of membrane composition on the regulation of adenylate cyclase activity, if similar changes occur in the adipocyte plasma membranes from obese mice as reported for microsomes in Chapter 3, then this altered lipid composition may provide an explanation for the altered characteristics of adenylate cyclase, observed in adipocyte plasma membranes from obese animals by Dehaye et al. (1978).

Since thyroid hormones exert an effect on the coupling efficiency between hormone-receptor complex and adenylate cyclase, then altered membrane composition may also interfere with this locus of thyroid hormone action, as apparent resistance to thyroid hormones of the lipolytic system in ob/ob mice has been observed (Otto et al., 1976).

4.1.e. Effects of membrane lipid composition on insulin action.

There have been few studies reported on the effects of lipids on insulin stimulation of hexose transport. Recently, Amatruda and Finch (1978), have evaluated the effect of temperature on insulin-stimulated glucose transport and insulin binding. These studies demonstrate that the glucose transporter is sensitive to its lipid environment, since a thermal transition for gluocse uptake into isolated adipocytes occurred at a temperature where a phase separation occurred in the plasma membrane, as detected by ESR. Also, in the presence of insulin, thermal transitions for hexose uptake occurred at 37°C, such that a 1°C change in temperature altered hexose transport by 30%. Such large thermally induced changes are likely to be mediated by protein-lipid interactions, although no definate proof of this was apparent from their results.

Amatruda and Finch (1979) also found that basal glucose transport was unaffected by incubation temperature. Insulin stimulated hexose transport was markedly increased at higher incubation temperatures. This was not mediated by increased insulin binding at high temperatures. Indeed, insulin binding at 37°C is only 50% of that observed at 27°C, due to decreased numbers of binding sites. Binding affinity of the receptors to insulin at 25°C is also greater than at 37°C. Thus, the efficiency of hormone-receptor complexes to activate transporter units must be considerably greater at physiological temperatures than at reduced temperatures. Again, these changes in activating efficiency of the insulin-receptor complex may be a result of membrane viscosity changes, although evidence is scanty.

More recently, Pilch et al. (1980) demonstrated that fluidising membranes by incorporation of cis unsaturated C_{18} fatty acids stimulated glucose transport in isolated plasma membranes to the same degree as prior incubation of the cells with insulin before membrane isolation. The effects of fatty acid fluidisation and insulin treatment were not additive. The authors conclude that fluidisation of annular lipid surrounding the glucose transport protein may be involved in the mechanism of insulin

action. This somewhat surprising result, although both interesting and controversial in the light of the findings by Cushman and Wardzala (1980), necessarily must be viewed with some caution, since the incorporation of 0.5 mol. ratio of fatty acid to phospholipid into membranes is highly unphysiological, and may seriously disrupt membrane architecture.

In this chapter some properties of adipocyte plasma membranes of lean and obese mice are examined. Adipocyte plasma membranes were isolated from lean and obese mice to investigate any compositional differences between the two groups that may give rise to altered membrane physical characteristics, as measured with fluorescent probes. Changes that occur in membranes from obese animals were correlated with changes in hormone stimulated adenylate cyclase activity and insulin stimulated hexose transport into isolated cells. Housing animals at 34°C produces normalisation of core temperature to that of lean mice (Chapter 3), and as it was of interest to examine the above mentioned parameters in animals housed at 34°C. Starvation causes a significant reduction of plasma insulin and glucose levels in obese mice (Le Marchand et al., 1977). Therefore, starving obese animals should help to determine if compositional changes in membranes from obese animals may be secondary to hyperinsulinaemia/hyperglycaemia.

4.2. Results.

PHYSICAL STUDIES ON MEMBRANES AND MEMBRANE COMPONENTS.

4.2.a. Fluidity studies using fluorescence polarisation by DPH labelled adipocyte plasma membranes from lean and obese mice housed at different environmental temperatures.

Adipocyte plasma membranes from lean and obese animals housed at different environmental temperatures were labelled with DPH, and the fluorescence polarisation measured from 4°C to 40°C. Fig 4.1 shows the Arrhenius plot from membranes of animals housed at 25°C, and Table 4.1 summarises the results.

From Table 4.1 it can be seen that obese mouse adipocyte plasma membranes were significantly more fluid (P decreased) than those of lean.

The Arrhenius break temperature was also reduced in obese membranes, indicative also of a membrane component in a more fluid state. The flow activation energy was considerably reduced below the breakpoint in obese membranes, suggestive of a component in a more rigid or ordered state in obese membranes.

Above the breakpoint, the flow activation energy was little changed between obese and lean control adipocyte plasma membranes.

Similar results were obtained from lean and obese animals housed at 12°C (Table 4.1) and for lean animals housed at 4°C. Since the body temperature of lean mice housed at 4°C is similar to that of obese mice housed at 25°C, then changes in obese adipocyte plasma membrane fluidity are unlikely to be simply an adaption to a lower core temperature.

The data from Arrhenius plots of fluorescence depolarisation by DPH labelled adipocyte plasma membranes from lean and obese mice housed at 34°C is shown in Fig 4.2. The data from these plots is summarised in Table 4.1. This data demonstrates that the obese mouse adipocyte plasma membranes mormalise to lean values, when the mice are housed at 34°C. Housing temperature made little difference to lean adipocyte plasma membrane fluidity.

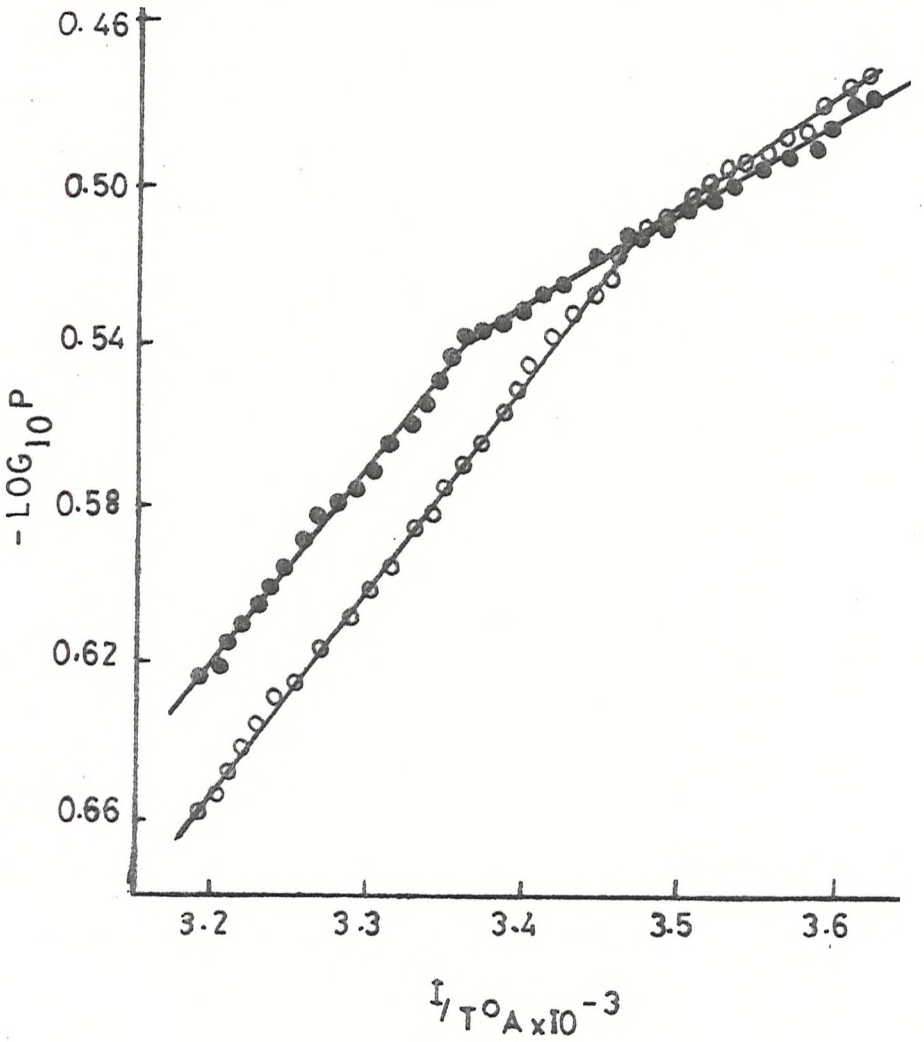


Fig 4.1.

Arrhenius plot of fluorescence depolarisation by DPH labelled adipocyte plasma membranes from lean (●) and obese (o) mice housed at 25°C (room temperature).

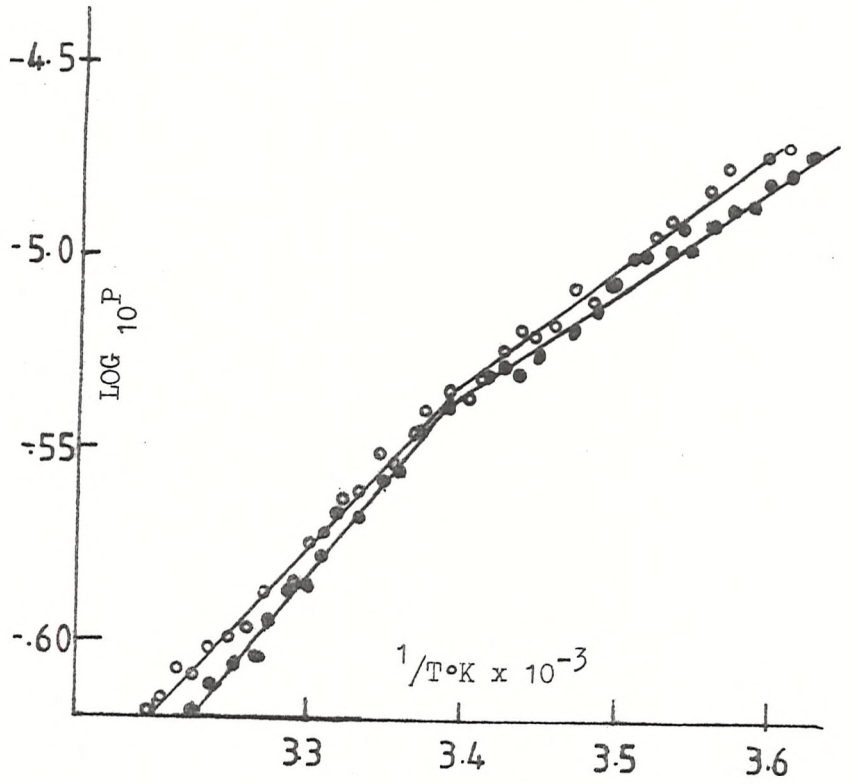


Fig 4.2. Arrhenius plots of fluorescence polarisation by DPH labelled adipocyte plasma membranes from lean (●) and obese (o) mice housed at 34°C.

TABLE 4.1.

Combined data from fluorescence polarisation by DPH labelled adipocyte plasma membranes from lean and obese mice housed at various environmental temperatures.

Phenotype	Housing Temp.	Breakpoint °C	Signif. P	Polarisation at 37°C	Signif. P	E above $_{1}$ Kcal mol^{-1}	Signif. P	E below $_{1}$ Kcal mol^{-1}	Signif. P	ΦR_{37}
n = 3 LEAN	34	23.6 ± 0.51	NS	0.255 ± 0.013	NS	9.33 ± 0.21	NS	18.52 ± 1.82	NS	0.91
n = 3 OBESE	34	23.5 ± 0.12		0.254 ± 0.001		9.78 ± 1.1		15.63 ± 0.66		
n = 4 LEAN	25	25.05 ± 0.26	S**	0.249 ± 0.001	S**	8.02 ± 0.45	NS	20.36 ± 0.93	S*	1.24
n = 4 OBESE	25	17.6 ± 0.7		0.224 ± 0.003		8.32 ± 0.27		13.22 ± 1.50		
n = 3 LEAN	12	23.4 ± 0.32	S**	0.253 ± 0.005	S*	8.21 ± 0.30	NS	20.3 ± 0.50	S*	1.24
n = 3 OBESE	12	17.49 ± 0.22		0.228 ± 0		10.5 ± 1.14		15.87 ± 0.14		
n = 1 LEAN	4	23.3		0.258		9.5		19.5		

S* = (P = 0.01), S** = (P = 0.001).

4.2.b. Fluidity studies using fluorescence polarisation by DPH labelled liposomes of adipocyte bulk plasma membrane lipids from lean and obese mice housed at different environmental temperatures.

Adipocyte plasma membranes were prepared from lean and obese animals housed at 25°C and 34°C. Total membrane lipids were extracted, and liposome suspensions prepared as described in Chapter 2. The liposomes were labelled with DPH and the fluorescence polarisation measured. The Arrhenius plots generated from this experiment are shown in Figs 4.3 and 4.4. The data from these plots are summarised in Table 4.2.

From Table 4.2 it can be seen that when animals are housed at 25°C, adipocyte plasma membrane lipids are more fluid than lean controls. The flow activation energy above and below the breakpoint was lower than lean controls, indicative of a component in a more ordered state in obese membranes.

Housing the animals at 34°C did not alter the fluidity or flow activation energy above the breakpoint in lean. However, the breakpoint was shifted below zero. This suggests that at least one component of the membrane lipids in lean adipocyte plasma membranes is altered at this housing temperature, although this compositional alteration is not large enough to perturb the fluidity of either liposomes or native membranes.

Housing at 34°C produced a large decrease in adipocyte plasma membrane lipid fluidity, such that the membrane lipids were less fluid than lean when housed at 34°C. Again, this breakpoint was reduced to below zero in obese membrane lipids.

These results are quantitatively similar to those obtained with native membranes, since similar fluidity ratios were obtained, although the values of P are lower in liposomes compared to membranes. Also, the values of the Arrhenius breakpoints were similar in liposomes derived from adipocyte plasma membranes from animals housed at 25°C. This suggests that the phase separations observed at the breakpoint are to some degree dependant on protein-

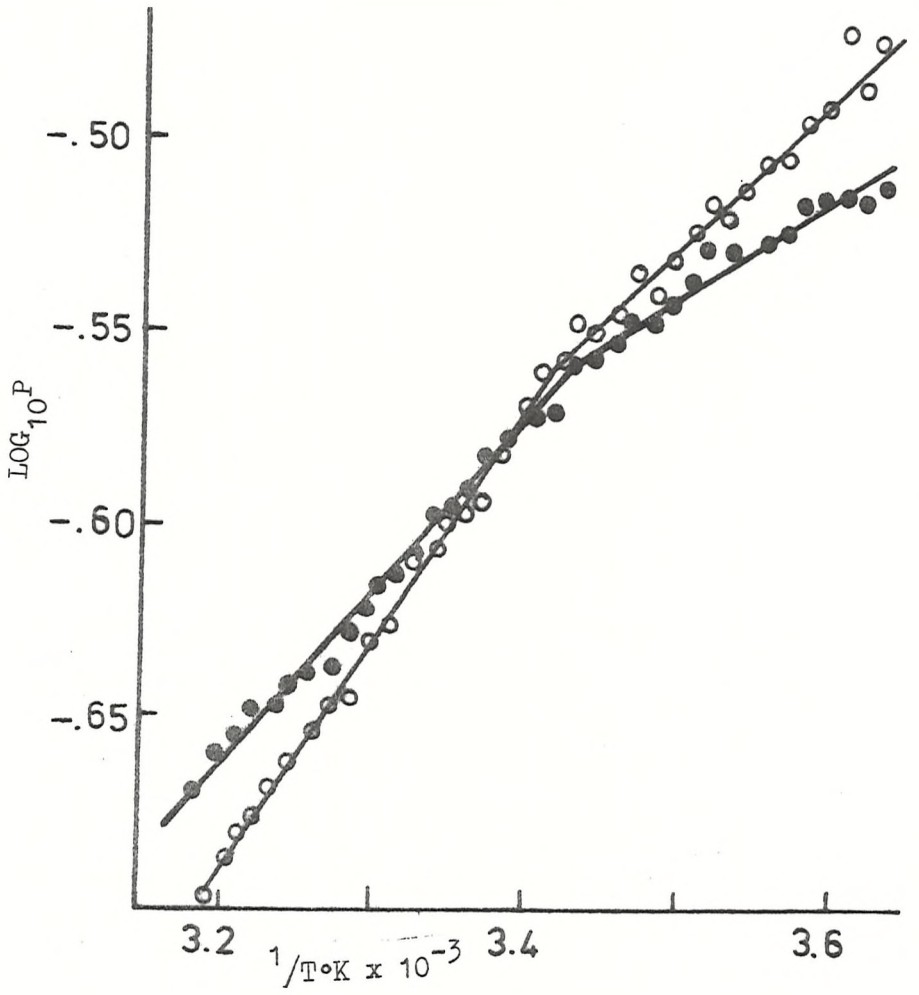


Fig 4.3.

Arrhenius plots of fluorescence polarisation by DPH labelled adipocyte plasma membrane extracted total lipid vesicles from lean (•) and obese (o) mice housed at room temperature (25°C).

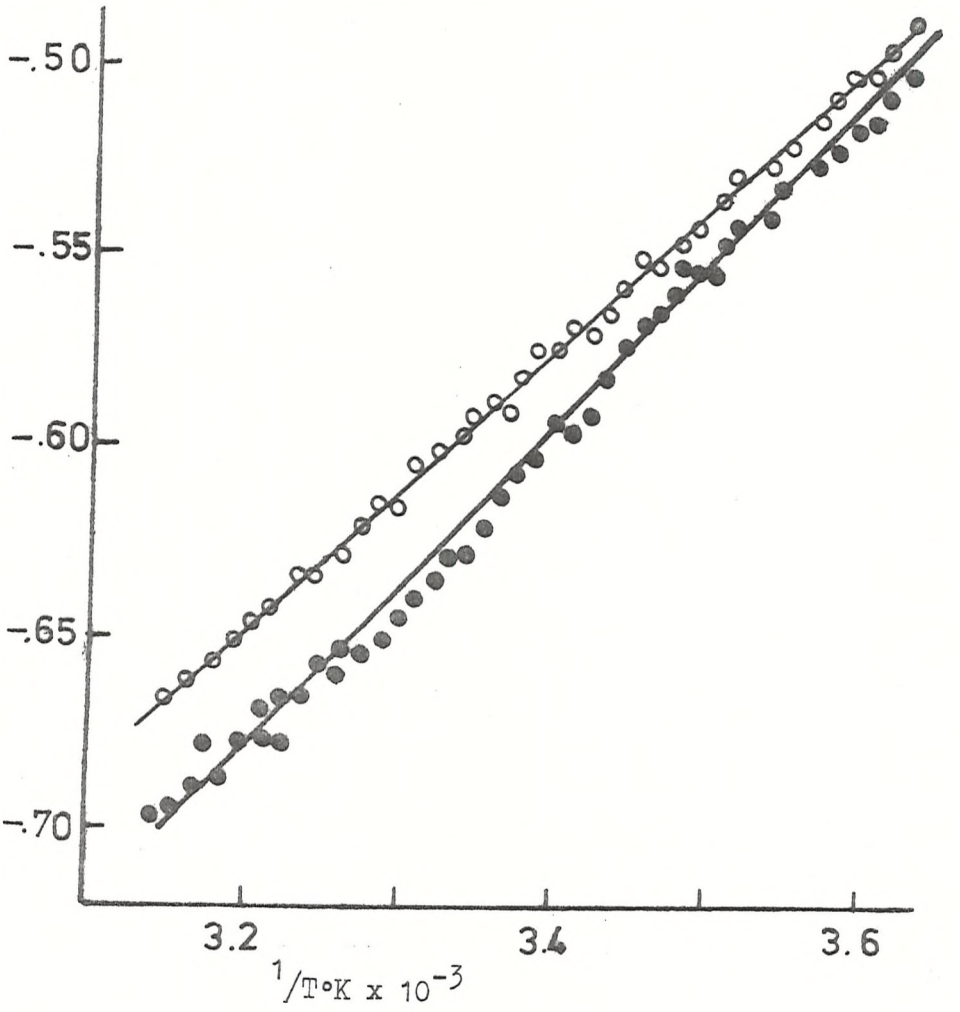


Fig 4.4.

Arrhenius plots of fluorescence polarisation by DPH labelled adipocyte plasma membrane extracted total lipid vesicles from lean (●) and obese (o) mice housed at 34°C.

TABLE 4.2.

Combined data from Arrhenius plots of fluorescence polarisation by DPH labelled adipocyte plasma membrane total lipid extracts from lean and obese mice housed at 34°C and 25°C.

Phenotype	Housing Temp. °C	Breakpoint °C	Polarisation 37°C	ΔE above Breakpoint (Kcal mol^{-1})	ΔE below Breakpoint (Kcal mol^{-1})	Fluidity Ratio R_{37}
LEAN	34	<4	0.224	12.3	-	0.917
OBESE	34	<4	0.234	10.3	-	
LEAN	25	18.6	0.232	9.88	17.7	1.311
OBESE	25	18.8	0.201	7.7	11.9	

Results are mean values for combined membranes obtained from 10 lean and 5 obese mice.

lipid interactions, or to membrane asymmetry. However, membrane proteins do not appear to be involved in the apparent increase in membrane fluidity observed in animals housed below 25°C.

4.2.c. Fluidity studies using fluorescence polarisation by DPH labelled liposomes of adipocyte bulk plasma membrane phospholipids from lean and obese mice housed at different environmental temperatures.

The major neutral lipid present in membrane lipid extracts is cholesterol, which exerts an ordering effect on membrane phospholipid acyl chains (Chapter 5). Neutral lipids were removed from extracted membrane lipids by TLC and liposomes made from the remaining phospholipid fraction.

Polarisation studies on phospholipid liposomes from adipocyte plasma membrane of lean and obese mice were performed to assess the contribution of cholesterol (and other minor neutral lipids) to the observed changes in lipid fluidity in adipocyte plasma membranes from obese animals compared to lean. The Arrhenius plots obtained from these studies are shown in Figs 4.5 and 4.6. The data from these plots is summarised in Table 4.3.

Fluidity ratios, activation energies and breakpoints were similar to those observed in total membrane lipid liposomes. The values of P were lower, since the 'ordering' effect of cholesterol was absent in liposomes containing only the phospholipid fraction.

These results demonstrate that the changes in fluidity observed in membranes from obese animals reside in the phospholipid fraction. When obese animals are housed at 34°C, their membrane phospholipids become less fluid than lean controls.

4.2.d. Fluidity studies on individual phospholipid classes from adipocyte plasma membranes of lean and obese mice housed at 25°C.

Individual phospholipid classes were prepared from lean and

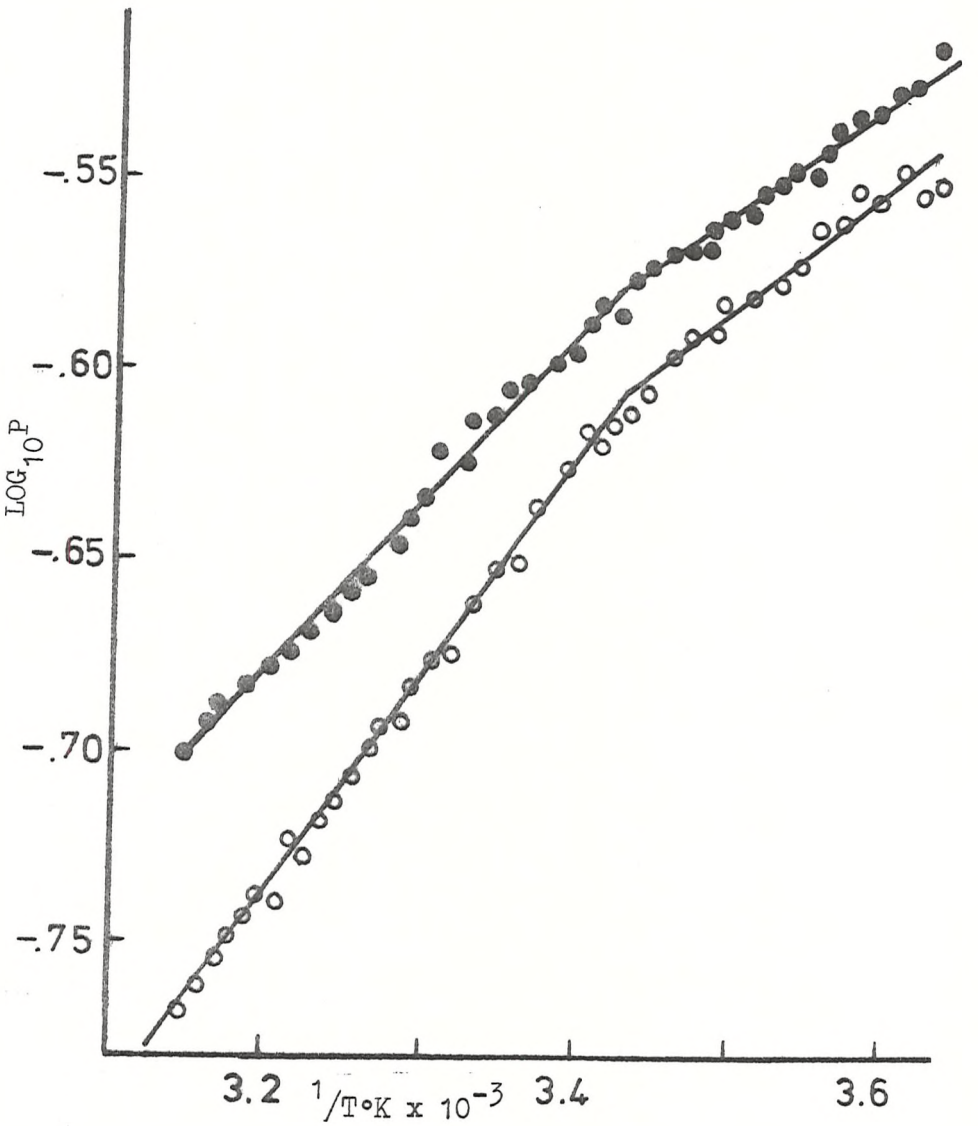


Fig 4.5.

Arrhenius plots of fluorescence polarisation by DPH labelled adipocyte plasma membrane extracted phospholipid vesicles from lean (●) and obese (o) mice housed at 25°C.

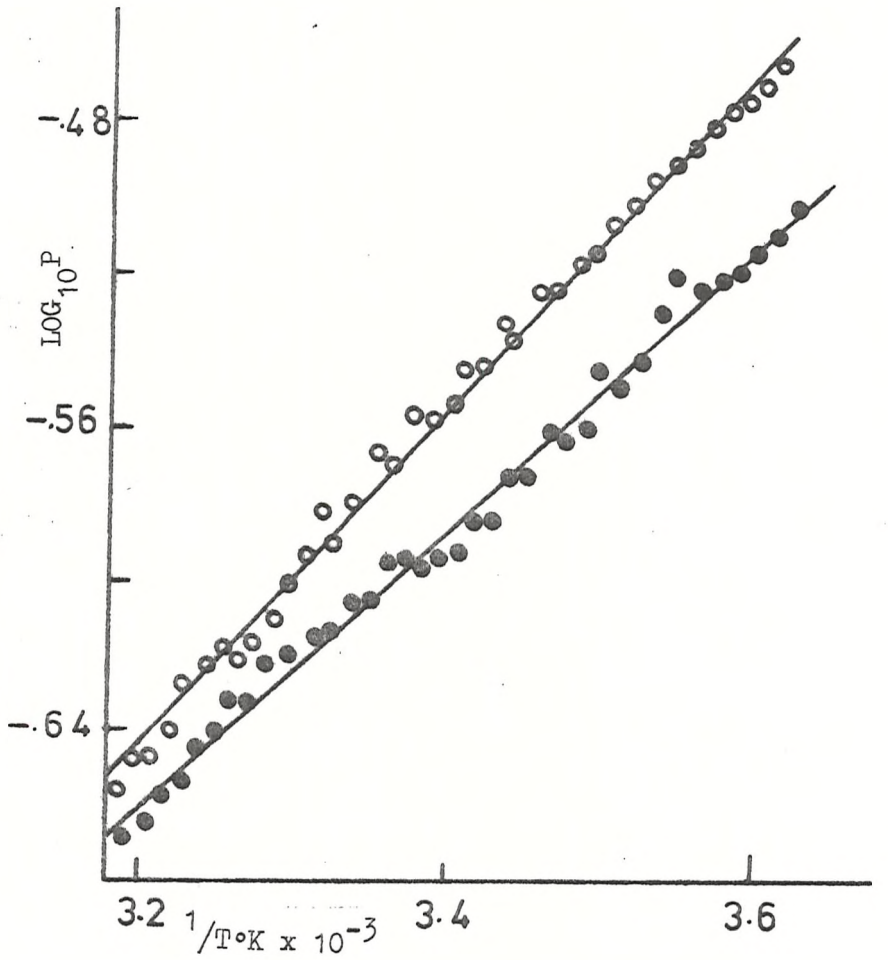


Fig 4.6.

Arrhenius plots of fluorescence polarisation by DPH labelled adipocyte plasma membrane extracted phospholipid vesicles from lean (•) and obese (o) mice housed at 34°C.

TABLE 4.3.

Combined data from Arrhenius plots of fluorescence polarisation by DPH labelled adipocyte plasma membrane phospholipid vesicles from lean and obese mice housed at 34°C and 25°C.

Phenotype	Housing Temp. °C	Breakpoint °C	Polarisation 37°C	ΔE above Breakpoint (Kcal mol^{-1})	ΔE below Breakpoint (Kcal mol^{-1})	Fluidity Ratio Φ R_{37}
LEAN	34	-	0.214	10.75	-	0.88
OBESE	34	-	0.229	12.58	-	
LEAN	25	19.0	0.215	10.77	17.16	1.24
OBESE	25	18.1	0.191	8.13	14.92	

Results are mean values for combined membranes obtained from 10 lean and 5 obese mice.

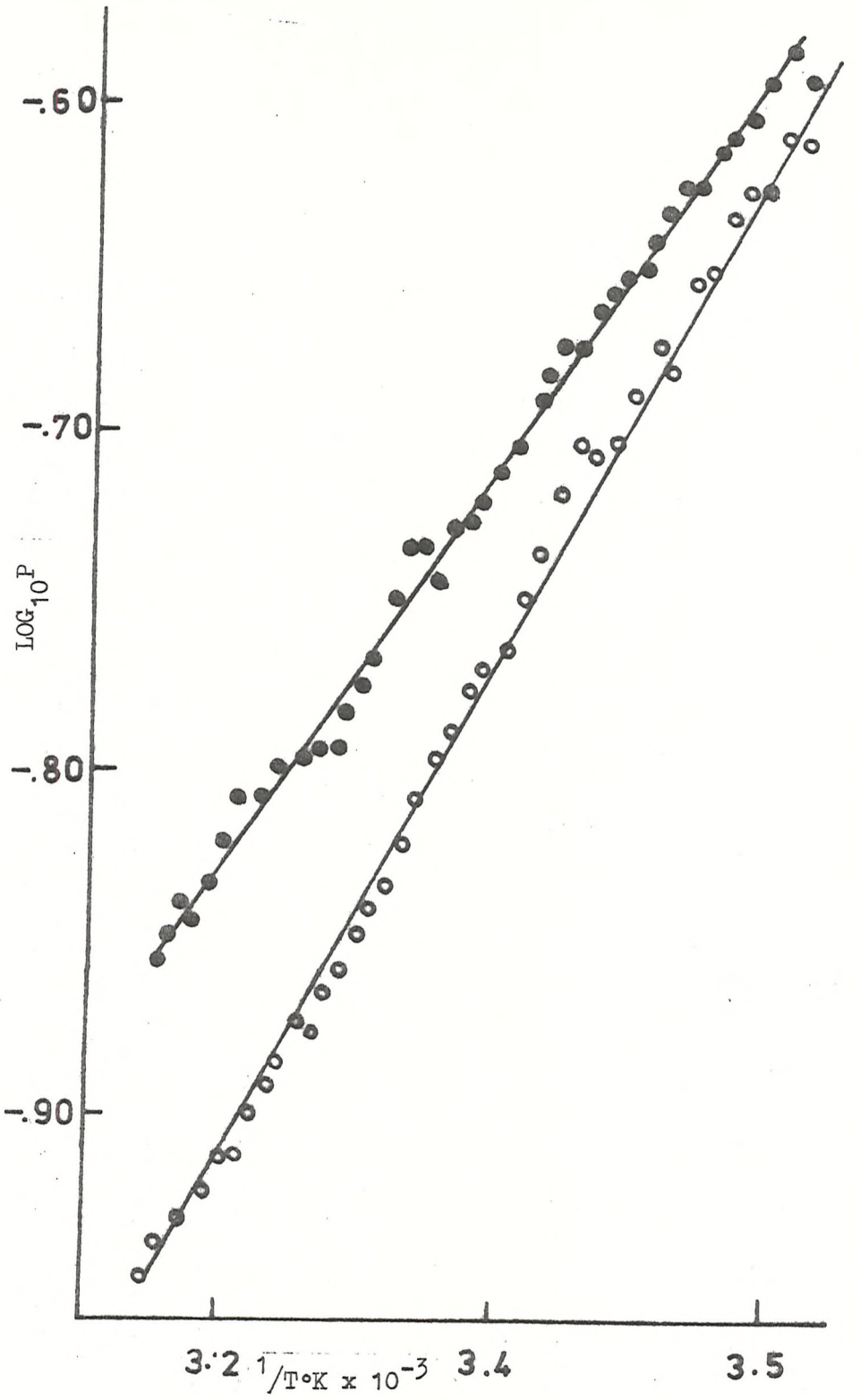


Fig 4.7.

Arrhenius plots of fluorescence polarisation by DPH labelled sonicated phosphatidylcholine vesicles from adipocyte plasma membranes from lean (•) and obese (o) mice housed at 25°C.

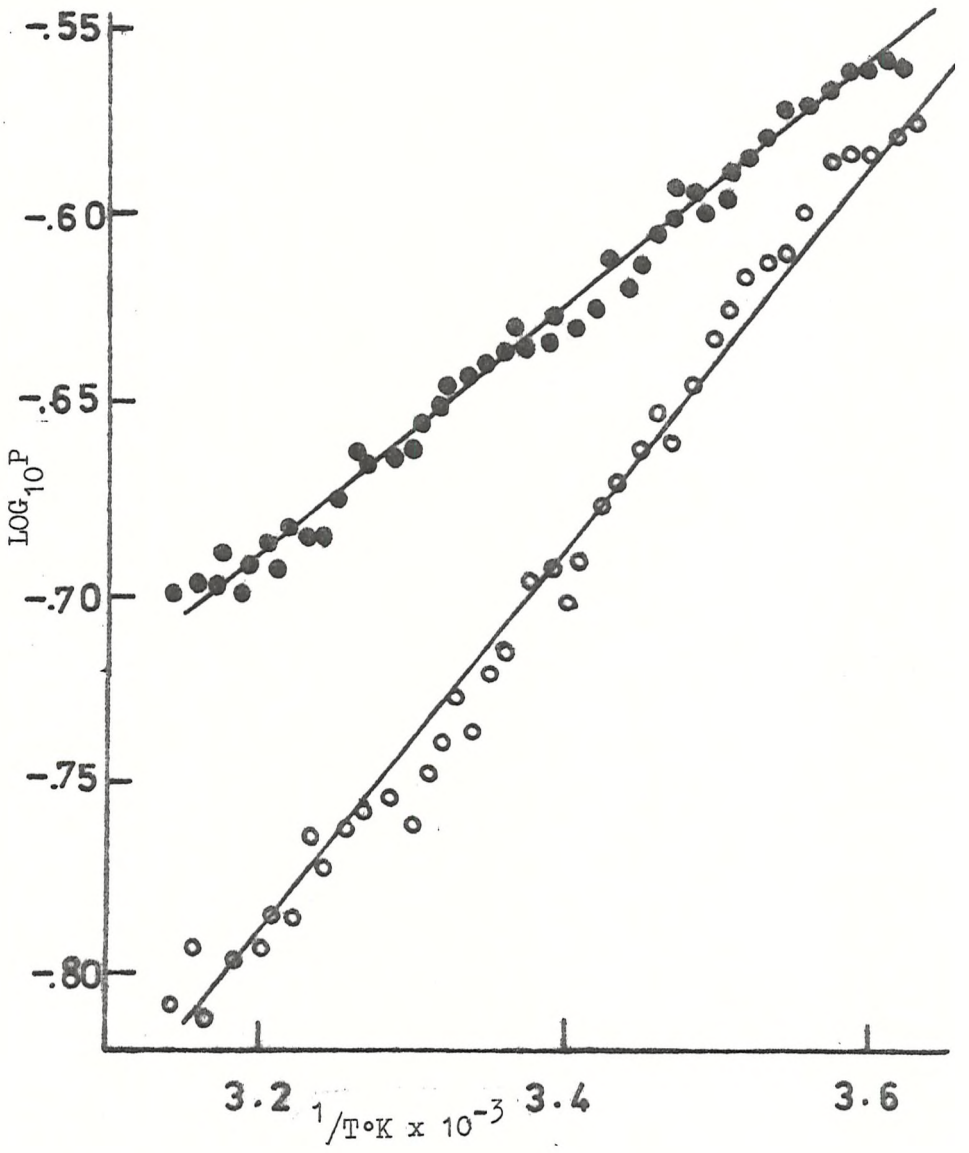


Fig 4.8.

Arrhenius plots of fluorescence polarisation by DPH labelled sonicated phosphatidylethanolamine vesicle from adipocyte plasma membranes from lean (•) and obese (o) mice housed at 25°C.

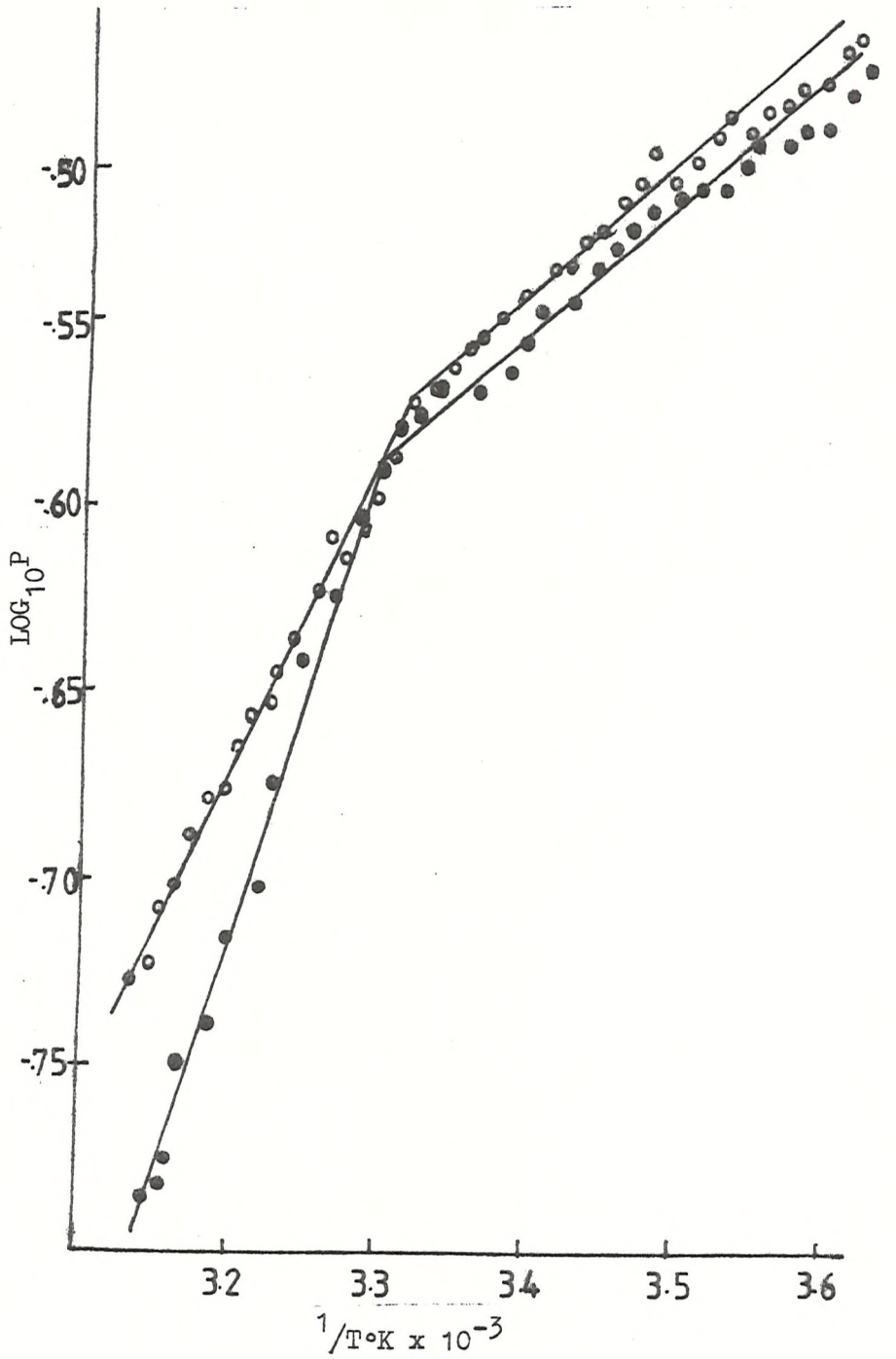


Fig 4.9.

Arrhenius plots of fluorescence polarisation by DPH labelled sonicated sphyngomyelin vesicles from adipocyte plasma membranes from lean (•) and obese (o) mice housed at 25°C.

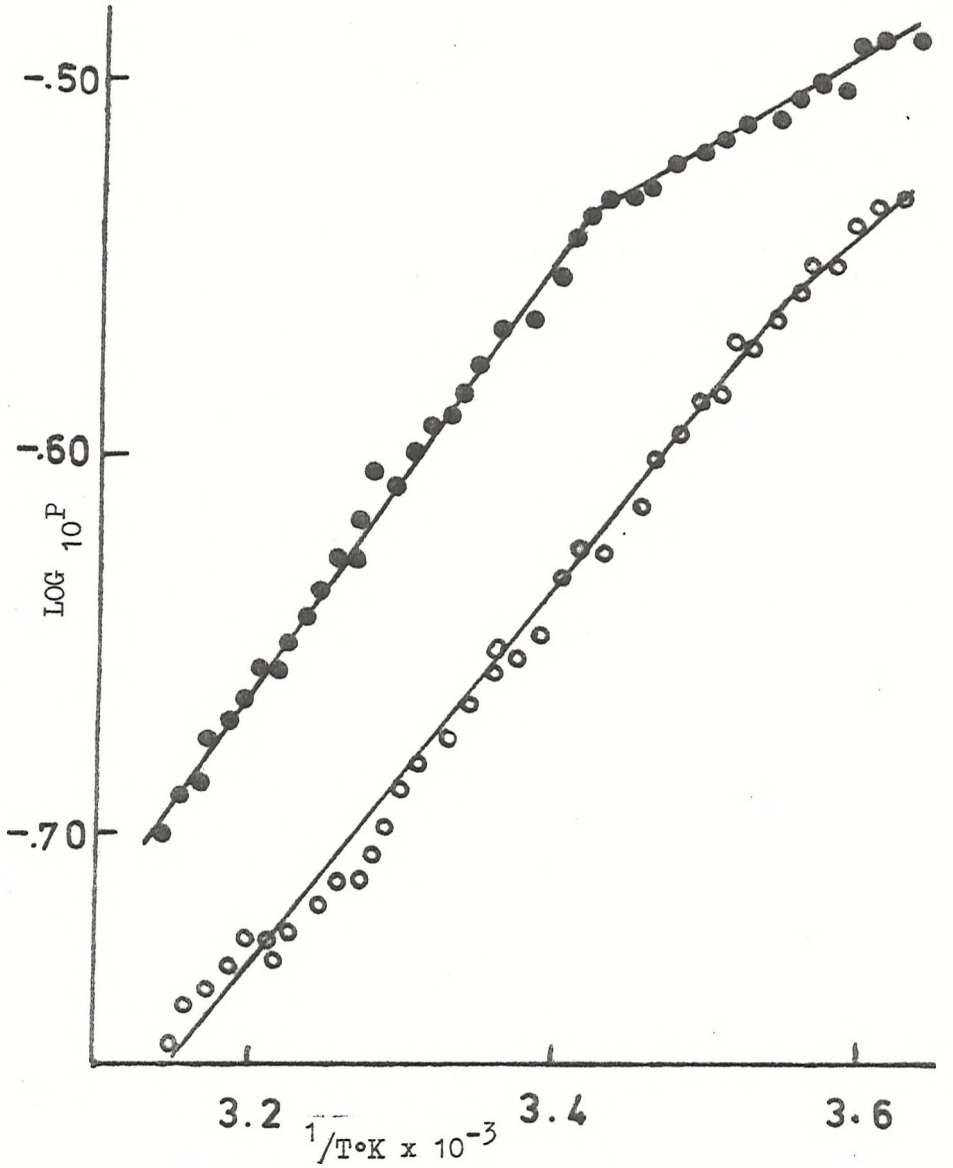


Fig 4.10.

Arrhenius plots of fluorescence polarisation by DPH labelled sonicated phosphatidylserine vesicles from adipocyte plasma membranes from lean (•) and obese (o) mice housed at 25°C.

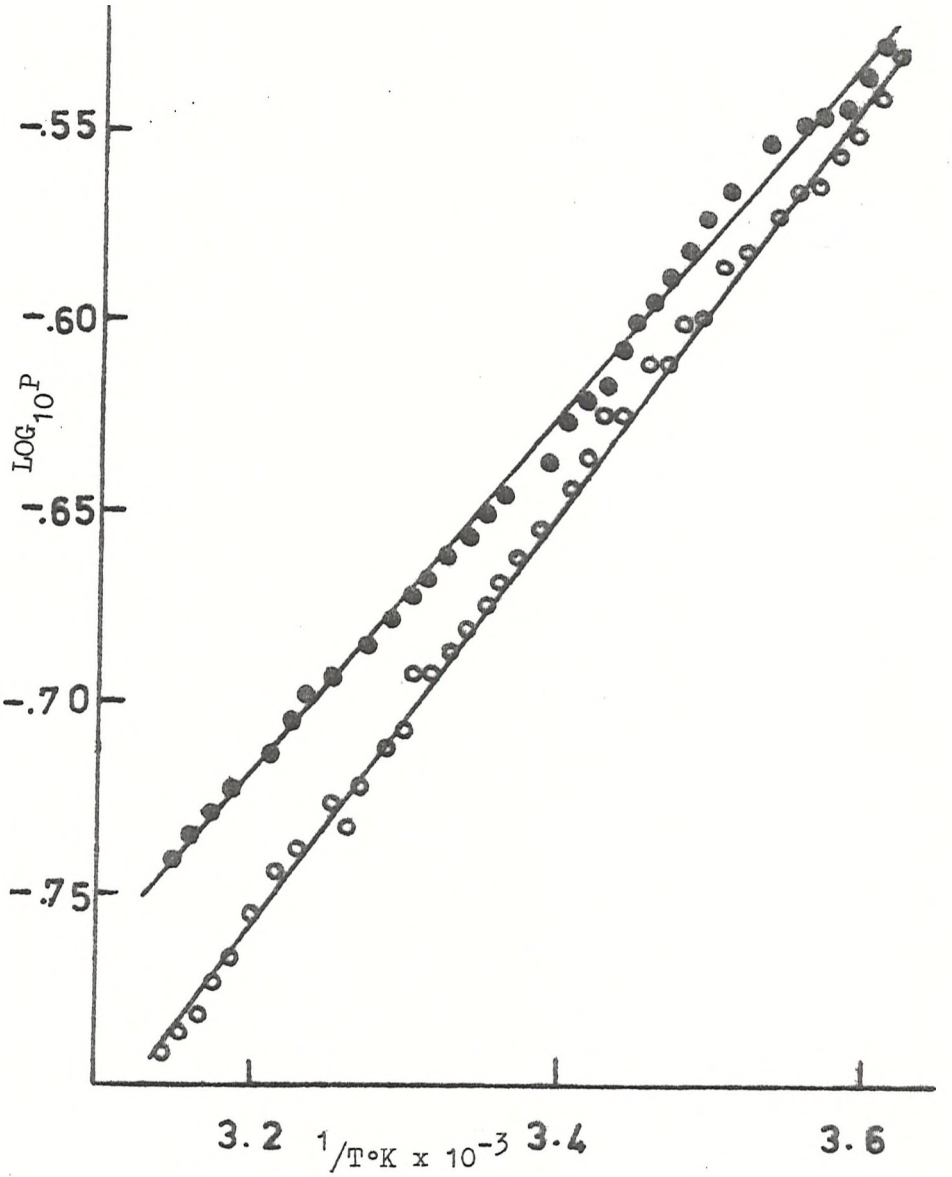


Fig 4.11.

Arrhenius plots of fluorescence polarisation by DPH labelled sonicated phosphatidylinositol vesicles from adipocyte plasma membranes from lean (•) and obese (o) mice housed at 25°C.

obese mouse adipocyte plasma membranes, as described in Chapter 2. Sonicated DPH labelled liposomes were then prepared, and the fluorescence polarisation measured for each class. Arrhenius plots of this data are shown in Figs 4.7 to 4.11. Data from these plots is summarised in Tables 4.4 and 4.5.

PC, PE, PS and PI were all more fluid from obese membranes compared to lean controls. SM, however was less fluid. The largest fluidity ratios were observed in PE and PS, with smaller changes in PI and PC.

In general, within each class of phospholipid, the more fluid component displayed the lower activation energy, suggesting that increased fluidity was accompanied by insertion of a more rigid or more ordered component.

Breakpoints were only observed in SM and PS, indicating that these components may be involved in phase separations observed in total phospholipids, total lipids and adipocyte plasma membranes. Little difference in the values of these breakpoints between SM or PS from lean and obese animals was observed. From these studies, it would appear that PE is the phospholipid which largely contributes to the observed increased fluidity of adipocyte plasma membranes from obese animals, as was observed in Chapter 3 for hepatic microsomal membranes.

ANALYSIS OF MEMBRANE COMPOSITION.

4.2.e. Measurement of Protein, Cholesterol and Phospholipid composition of lean and obese mouse adipocyte plasma membranes.

Physical studies indicated that changes in the content of protein or cholesterol in adipocyte plasma membranes from obese mice was unlikely to be the cause of the observed fluidity increase in obese adipocyte plasma membranes. This was confirmed by measuring the total cholesterol and phospholipid to protein ratios in membrane preparations from the two groups of animals (Table 4.6). No change was observed in either total cholesterol or total protein with respect to membrane phospholipid content.

TABLE 4.4.

Summary of data from Arrhenius plots of fluorescence polarisation by DPH labelled sonicated PC and PE vesicles from lean and obese mouse adipocyte plasma membranes.

Phospholipid	Phenotype	Breakpoint °C	Polarisation	ΔE above Breakpoint kcal mol^{-1}	ΔE below Breakpoint kcal mol^{-1}	Fluidity Ratio R_{37}
Phosphatidyl-Choline	LEAN	<4	0.136	7.88	-	1.1
	OBESE	<4	0.128	6.34	-	
Phosphatidyl-ethanolamine	LEAN	<4	0.209	14.39	-	1.50
	OBESE	<4	0.168	9.05	-	

Results are mean values for combined membranes phospholipids from 10 lean and 5 obese mice.

TABLE 4.5.

Summary of data from fluorescence polarisation by DPH labelled sonicated SM, PS and PI vesicles from lean and obese mice adipocyte plasma membranes.

Phospholipid	Phenotype	Breakpoint °C	Polarisation 37°C	ΔE above Breakpoint °C	ΔE below Breakpoint °C	Fluidity Ratio ΦR_{37}
Sphyngomyelin	LEAN	30.0	0.204	3.46	12.79	0.83
	OBESE	29.1	0.225	5.33	12.3	
Phosphatidyl Serine	LEAN	19.4	0.224	8.1	19.3	1.35
	OBESE	9.5	0.190	9.25	12.8	
Phosphatidyl Inositol	LEAN	<4	0.197	10.0	-	1.18
	OBESE	<4	0.179	8.47	-	

Results are mean values for combined membrane phospholipids from 10 lean and 5 obese mice.

TABLE 4.6.

Compositional analysis of adipocyte plasma membranes from lean and obese mice housed at 25°C.

	LEAN	OBESE
Cholesterol (μmol./mg protein)	0.42	0.43
Total phospholipid (μmol./mg protein)	1.20	1.20
Phospholipid Composition (% Total)		
Phosphatidylcholine	40.1	39.5
Phosphatidylethanolamine	27.1	27.5
Sphyngomyelin	16.1	16.5
Phosphatidylserine	10.1	10.0
Phosphatidylinositol.	6.5	6.6

Results are means of duplicate determinations on pooled membrane samples derived from 10 lean and 5 obese mice.

Total cholesterol is the sum of both free cholesterol and cholesterol esters. The latter was very low in adipocyte plasma membranes from both groups of animals (<5%). Since it is not possible to accurately determine such small amounts, total cholesterol was measured.

The remainder of the neutral lipid fractions were examined qualitatively by TLC as described in Chapter 2. Chromatograms from the two groups of animals, and visualised with DPH spray reagent. All neutral lipid subclasses described in Chapter 2 were present in both lean and obese lipid extract. Also, identical fluorescence intensity was observed between neutral lipid subclasses from membranes derived from each group of animals. It was concluded that there was no major differences between the neutral lipid fractions of adipocyte plasma membranes from lean and obese mice. Since the content of neutral lipids in membranes other than cholesterol is low, it is unlikely that small changes in the neutral lipid fraction not appar ent with such a simple, qualitative technique, contribute significantly to changes in membrane fluidity between the two groups of animals.

No change was observed between phospholipid subclass composition betweeen the two groups of animals.

It was concluded that there are no major compositional changes in the protein, phospholipid or neutral lipid fractions of obese mouse adipocyte plasma membranes that can account for the altered physical properties of these membranes.

4.2.f. GLC analysis of fatty acids derived from adipocyte bulk plasma membrane lipids from lean and obese mice housed at 25°C.

Fatty acid methyl esters were prepared from bulk lipid extracts from adipocyte plasma membranes from lean and obese animals, by transmethylation with sodium methoxide. The GLC spectra obtained are shown in Table 4.7 and the data is summarised in Table 4.10.

TABLE 4.7.

GLC spectra of fatty acid methyl esters derived from the total lipid extract from lean and obese mice adipocyte plasma membranes. Animals were housed at 25°C.

	LEAN	OBESE
12:0	1.5	2.9
14:0	4.9	5.0
16:0	21.0	16.5
16:1	2.8	2.9
18:0	23.0	17.0
18:1	8.6	14.3
18:2	14.9	9.3
18:3	2.4	1.0
20:1	0.3	ND
20:2	3.6	3.9
20:4	6.8	8.1
22:2	0.9	1.5
22:4	1.3	ND
22:6	7.7	18.0

Results are mean values from membranes derived from 10 lean and 5 obese animals.

From Table 4.7 similar changes are observed between lean and obese membrane fatty acids as were observed in microsomal fatty acids (Chapter 3). An increase in 22:6 with a concomitant decrease in 16:0 and 18:0 was observed, along with an increase in 18:1 and decrease in 18:2 in obese membranes. Thus, the observed fluidisation of obese membranes is a result of increased 22:6 and 18:1. The presence of increased 22:6 may be responsible for the observed decrease in flow activation energy in obese membranes (Chapter 5).

4.2.g. GLC analysis of fatty acids derived from adipocyte plasma membrane individual phospholipid classes from lean and obese mice housed at 25°C.

Fatty acids from phospholipids isolated from lean and obese adipocyte plasma membranes were transmethylated with sodium methoxide and analysed by GLC. The fatty acyl methyl ester spectra for each phospholipid class is shown in Tables 4.8 and 4.9. The data from these tables is summarised in Table 4.10.

Since 70% of the total adipocyte plasma membrane phospholipids are PE and PC, then it is within these groups that any gross alterations in membrane fluidity would most likely be reflected. From Table 4.8, obese PC contains more 18:1, 20:4 and 22:6, and less 14:0. The net effect of these alterations is shown in Table 4.10. Obese PC has a slightly reduced saturated fatty acid content, with a 17% increase in the number of double bonds per lipid molecule. This is in agreement with fluorescence studies on this lipid, since obese PC was found to be fluidised compared to lean.

PE, however, showed striking changes between the two groups. All saturated fatty acid classes were reduced, while all the major unsaturated species were elevated. The major change was substitution of 22:6 for 16:0 and 18:0. The net affect of these changes (Table 4.10) was a 53% decrease in saturated fatty acids, with an 87% increase in the number of double bonds per lipid molecule.

This observation agrees well with the large fluidity change observed in PE liposome by fluorescence studies.

TABLE 4.8.

GLC spectra of fatty acid methyl esters from PC and PE isolated from adipocyte plasma membranes from lean and obese mice housed at 25°C.

	Phosphatidylcholine		Phosphatidylethanolamine	
Fatty Acid	Lean	Obese	Lean	Obese
12:0	1.3	T	1.3	1.3
14:0	5.6	T	6.5	0.7
16:0	21.6	23.1	26.5	14.4
16:1	3.1	2.2	0.2	3.0
18:0	15.4	17.2	18.9	9.2
18:1	10.4	18.7	8.2	10.7
18:2	22.9	18.1	8.5	12.4
18:3	2.8	ND	3.1	2.9
20:1	ND	ND	1.2	T
20:2	3.3	3.2	1.5	T
20:4	5.0	6.8	13.1	18.1
22:2	2.2	1.2	0.2	T
22:4	3.2	ND	0.2	ND
22:6	3.2	9.5	10.6	28.3

Results are mean values from membrane phospholipids derived from 10 lean and 5 obese animals.

TABLE 4.9.

GLC spectra of fatty acid methyl esters from SM, PS and PI isolated from adipocyte plasma membranes from lean and obese mice housed at 25°C.

Fatty Acid	Sphingomyelin		Phosphatidyl-serine		Phosphatidyl-inositol	
Phenotype	LEAN	OBESE	LEAN	OBESE	LEAN	OBESE
12:0	-	-	2.5	1.4	3.9	3.1
(Sphingosine 14:0)	(50)*	(50)*	-	-	-	-
14:0	ND	1.9	3.6	2.6	7.0	2.6
16:0	6.7	19.0	32.5	30.4	18.9	23.7
16:1	1.9	2.4	3.3	7.4	3.7	2.9
18:0	15.3	7.4	20.7	10.3	33.5	23.7
18:1	6.7	10.0	14.1	20.2	7.4	13.3
18:2	8.6	ND	5.2	5.0	2.0	5.2
18:3	ND	ND	ND	ND	6.2	6.5
20:2	ND	ND	4.6	3.2	ND	ND
20:4	7.2	4.8	8.6	9.0	9.7	11.2
22:6	ND	ND	5.3	10.5	7.8	7.8
24:0	3.8	4.8	ND	ND	ND	ND

* Assumed from the known structure of SM.

Results are mean values from membrane phospholipids isolated from 10 lean and 5 obese animals.

TABLE 4.10.

Summary of GLC data for lipid extracts and phospholipids from lean and obese mouse adipocyte plasma membranes. Percent change in saturation and number of double bonds is compared between values obtained for 'Total lipid extract' and the mean values obtained for these parameters by summing the relative contributions of each phospholipid class ascribed as 'Total phospholipid fraction'.

	% Saturated Fatty Acids		Pi bonds per 100 mol.	
Source	LEAN	OBESE	LEAN	OBESE
Lipid Extract	50.7	41.4	136	190
PC	43.9	40.3	131	153
PE	54.4	25.6	154.9	289.4
SM	75.8	83.1	54.6	31.6
PS	59.3	44.7	101.6	143.0
PI	65.3	53.1	122.6	143.4

	'Total Lipid Extract'	'Total phospholipid fraction'
% decrease in saturated fatty acid content of obese membranes.	82%	78%
% increase in Pi bonds per 100 fatty acids of obese membranes.	140%	140%

The GLC fatty acid spectrum of SM was also in agreement with fluidity studies, since obese SM showed an increase in total saturated fatty acid content (Table 4.10), elevated by about 10%, and a decrease in double bonds per lipid molecule of 42%. The major point of interest is that 16:0 was largely substituted for 18:0 in obese SM. A reduction of 18:2 and 20:4 were the major contributors to the decreased fluidity of obese SM liposomes.

Obese mouse PS and PI both showed a decreased unsaturated fatty acid content and an increased number of double bonds per lipid molecule (Table 4.10). Again, these observations are consistent with the observed fluidity changes in obese mouse adipocyte plasma membrane PS and PI. Obese mouse membrane PS was fluidised by increased 18:1 and 22:6, with a concomitant fall in 18:0. Obese mouse membrane PI was fluidised by an increase in 18:1 and 20:4. Like obese mouse membrane PS, a fall in 18:0 content was observed. However, the overall fluidisation was less in PI than PS (Table 4.5) due to an increase in 16:0 content.

By comparison of Tables 4.4 and 4.5 with Table 4.10, the observed fluidity ratios between the phospholipids from lean and obese mouse adipocyte plasma membranes are in good agreement with both the degree of saturated species and the number of double bonds per lipid molecule within each phospholipid class.

Since the majority of the bulk fatty acid species reside on phospholipids within a biological membrane, then the summed relative contribution of each phospholipid to the total percentage unsaturated fatty acids and the number of double bonds per lipid molecule should be in the same ratios between lean and obese as those obtained for the total lipid extract. This manipulation provides a cross check that phospholipid recovery and transmethylation efficiency were similar between the two groups. Table 4.10 demonstrates that this was indeed the case.

These results demonstrate that PE is the major phospholipid species altered in obese adipocyte plasma membranes compared to lean controls. In this lipid, 22:6 is substituted for 16:0 and 18:0. It is this change in the fatty acid composition of obese

mouse adipocyte plasma membrane PE that accounts for the gross fluidisation observed in PE from obese membranes compared to lean controls.

ASPECTS OF MEMBRANE FUNCTION.

4.2.h. Adenylate cyclase stimulation by isoprenaline in adipose tissue membrane for lean and obese mice.

Adenylate cyclase activity was measured in crude adipose tissue plasma membrane preparations from lean and obese mice housed at 25°C and 34°C for 1 week. Since obese mouse adipocyte plasma membrane fluidity is normalized by housing at 34°C, it was of interest to determine the possible effects of this observation on the established defect in the adenylate cyclase system in obese mouse adipocyte plasma membranes, discussed in the introduction.

The activity of adenylate cyclase from lean and obese animals housed at 25°C and 34°C was measured under basal, NaF stimulated and Isoprenaline stimulated conditions. The dose-response curves for adenylate cyclase to isoprenaline are shown in Fig 4.12. The data from these experiments is summarised in Table 4.11. The reduced basal and β-agonist stimulated activity of adenylate cyclase in membranes from obese mice housed at 25°C was confirmed in this study.

Basal adenylate cyclase activity was not substantially altered in membranes from either group of animals when housed at 34°C

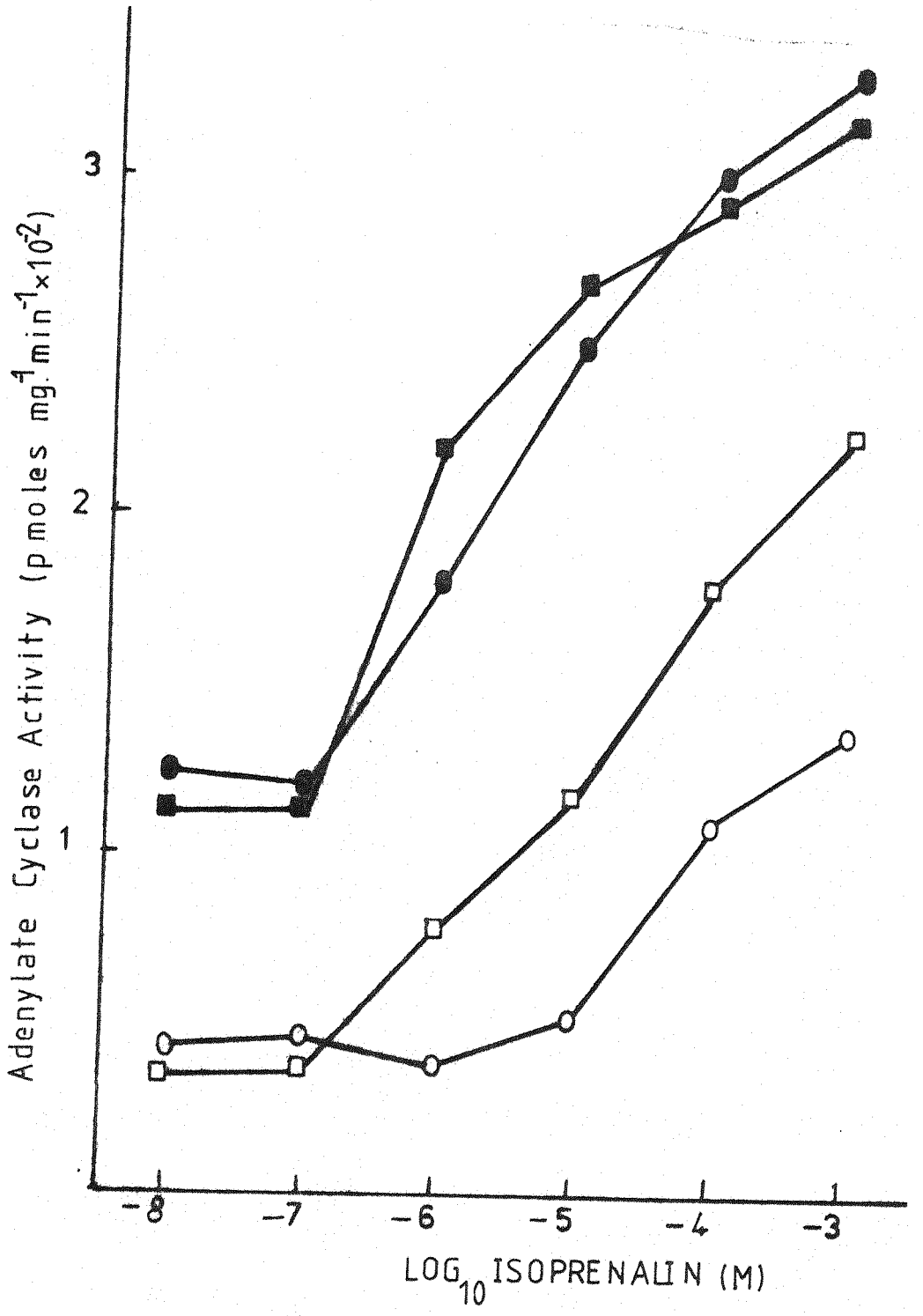


Fig 4.12.

Dose response curve adenylate cyclase activity in crude membrane preparations from adipose tissue of lean (●■) and obese (○□) mice housed at 25°C (○●) and 34°C (□■).

TABLE 4.11.

Phenotype - Housing temp (°C)	Basal Activity *	Max. β Adrenergic Stimulated Activity *	NaF Stimulated Activity *	NaF Stimulation %	Max. β Adrenergic Stimulation %	(β Agonist) for Threshold Response (M)	(β Agonist) for ½ max. Response (M)	Basal Activity as % NaF Activity	% Recruitment of NaF Activity by (β Agonst)
L - 25	1.22	3.35	8.70	713	275	3.16×10^{-7}	2.5×10^{-6}	14.0	38.5
L - 34	1.13	3.15	7.50	663	278	3.16×10^{-7}	2.5×10^{-6}	15.1	42.0
0 - 25	0.42	1.35	5.84	1390	321	3.16×10^{-5}	3.98×10^{-5}	7.2	23.1
0 - 34	0.35	2.25	4.38	1250	643	3.16×10^{-7}	1.78×10^{-5}	8.0	51.4

* Activities are pmol. mg^{-1} min^{-1} $\times 10^{-2}$.

Brackets denote concentration.

compared to housing at 25°C. Since basal activity was reduced to 34% of lean values in obese mouse adipocyte plasma membranes, this suggests that the catalytic efficiency of the enzyme in obese membranes is reduced in the basal state. Further, as NaF-stimulated activities suggest a reduction of 33% in the number of catalytic units per mg protein in membranes from obese animals, then it follows that to attain the relative basal activities recorded in Table 4.11, obese adenylate cyclase units must only be operating at 50% efficiency compared to lean.

The dose-response curves of obese mouse adipocyte plasma membrane adenylate cyclase to isoprenaline was shifted to the right compared to lean controls when animals were housed at 25°C. This data agrees well with the findings of Dehaye et al.,(1978). The agonist concentration necessary for both a threshold and half-maximal response was elevated in membranes from obese mice compared to lean controls. Maximal concentrations of isoprenaline produced a smaller stimulation over basal activity in obese membrane compared to lean.

Housing at 34°C produced substantial changes in these parameters in obese mouse membranes, but no change was observed in lean membranes. A normalisation was observed in the threshold response of adenylate cyclase to isoprenaline in obese adipocyte plasma membranes from animals housed at 34°C. To achieve half maximal activation by isoprenaline of the adenylate cyclase in obese mice adipocyte plasma membranes, 17 times the concentration of isoprenaline is nesessary compared to lean controls. Housing at 34°C reduces this to 7 times the isoprenaline concentration required for half-maximal activation of the cyclase of obese membranes over that of lean.

Maximal isoprenaline stimulation of adenylate cyclase was improved in obese plasma membranes by housing at 34°C, from 40% of that of lean for animals housed to 25°C, to 71% of that of lean for animals housed at 34°C. Since basal activity was unaffected by housing temperature and the NaF-stimulated activity was reduced by a similar degree in lean and obese animals, these

results show that the efficiency of the hormone-receptor complex to activate adenylate cyclase was improved in obese membranes from animals housed at 34°C. When the maximal agonist stimulated activity is expressed in relation to the NaF-stimulated activity, the obese agonist-receptor complex was able to recruit only 23.1% of the total adenylate cyclase activity compared to 38.5% in lean. When the animals were housed at 34°C, obese adipocyte plasma membrane agonist-receptor complex was then able to recruit 51.4% of NaF-stimulated activity. Taken together, these results suggest that membrane fluidity affects either the number of β receptors and/or the coupling efficiency of the hormone-receptor-adenylate cyclase complex.

4.2.i. Insulin stimulated hexose transport into adipocytes isolated from lean and obese mice housed at different environmental temperatures.

2 groups of 5-7 week old lean and obese mice were housed at 25°C or acclimatised to 34°C for 8-10 days. Basal and insulin stimulated ^{3}H 2-deoxy D-glucose (DOG) uptake was assessed in isolated adipocytes as described in methods. Prior experiments were performed to evaluate the time course of uptake for 0.13mM DOG into lean and obese adipocytes. Uptake was linear in both lean and obese cells for at least 15 minutes. An assay duration of 10 minutes was therefore chosen. Corrected data was expressed as total hexose uptake per min. per million cells, as a function of medium insulin concentration. Typical experimental plots are shown in Figs 4.13 and 4.14. The data from these plots and similar experiments is summarised in Table 4.12. Basal transport of DOG was found to be 2-fold increased into adipocytes from obese animals compared to lean, and was not affected by housing temperature in both groups.

The dose-response curve to insulin was shifted to the right in cells from obese animals housed at 25°C, the insulin ooncentration required for half-maximal stimulation being increased four-fold over that of lean. At concentrations of insulin that produced maximal DOG transport rates, the ability of insulin to elevate the relative number of transporter sites was similar

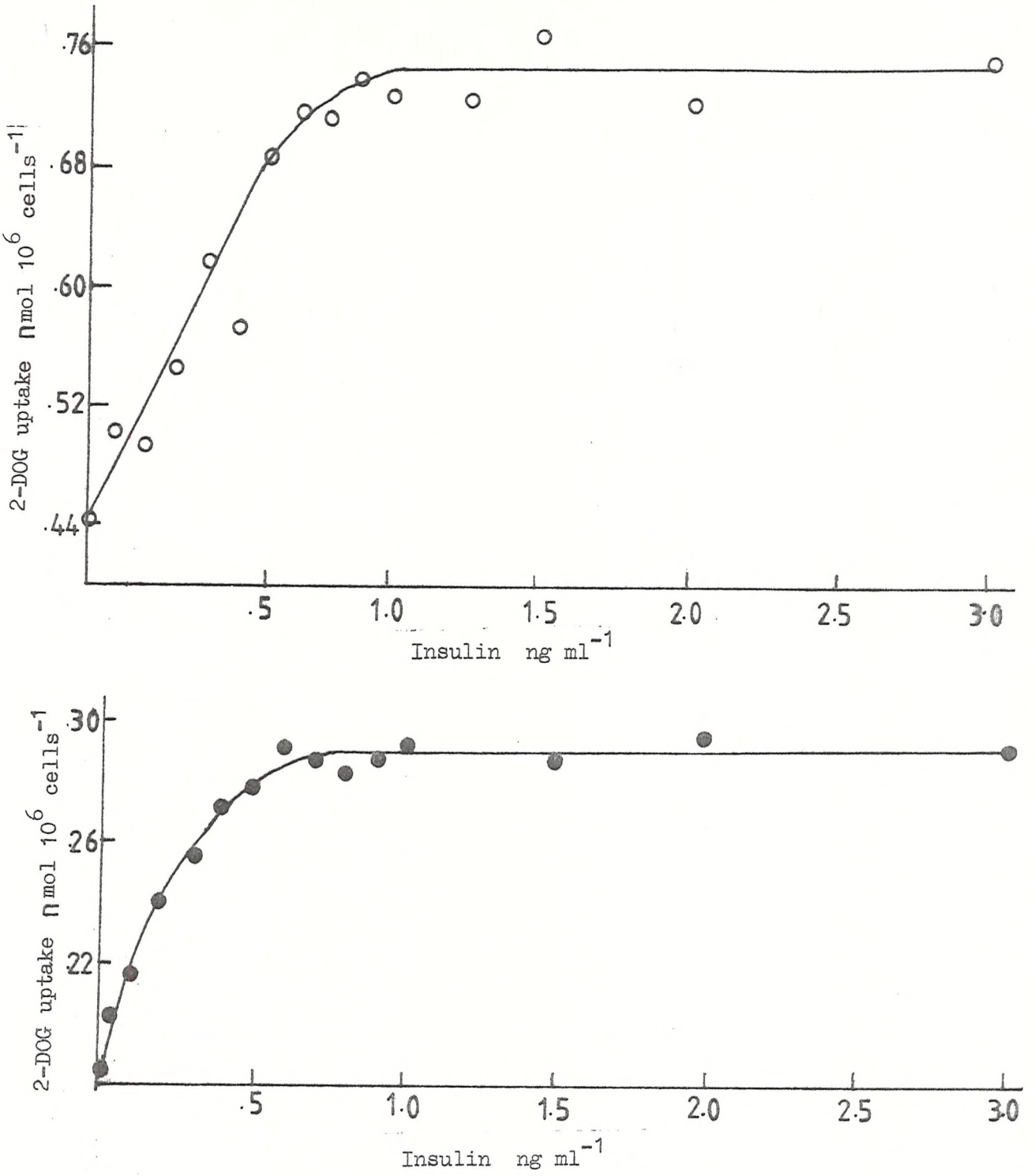


Fig 4.14.

Insulin dose-response curves for 125μM DOG uptake into isolated epididymal adipocytes from lean (•) and obese (o) mice housed at 34°C. DOG transport was assayed at 35°C.

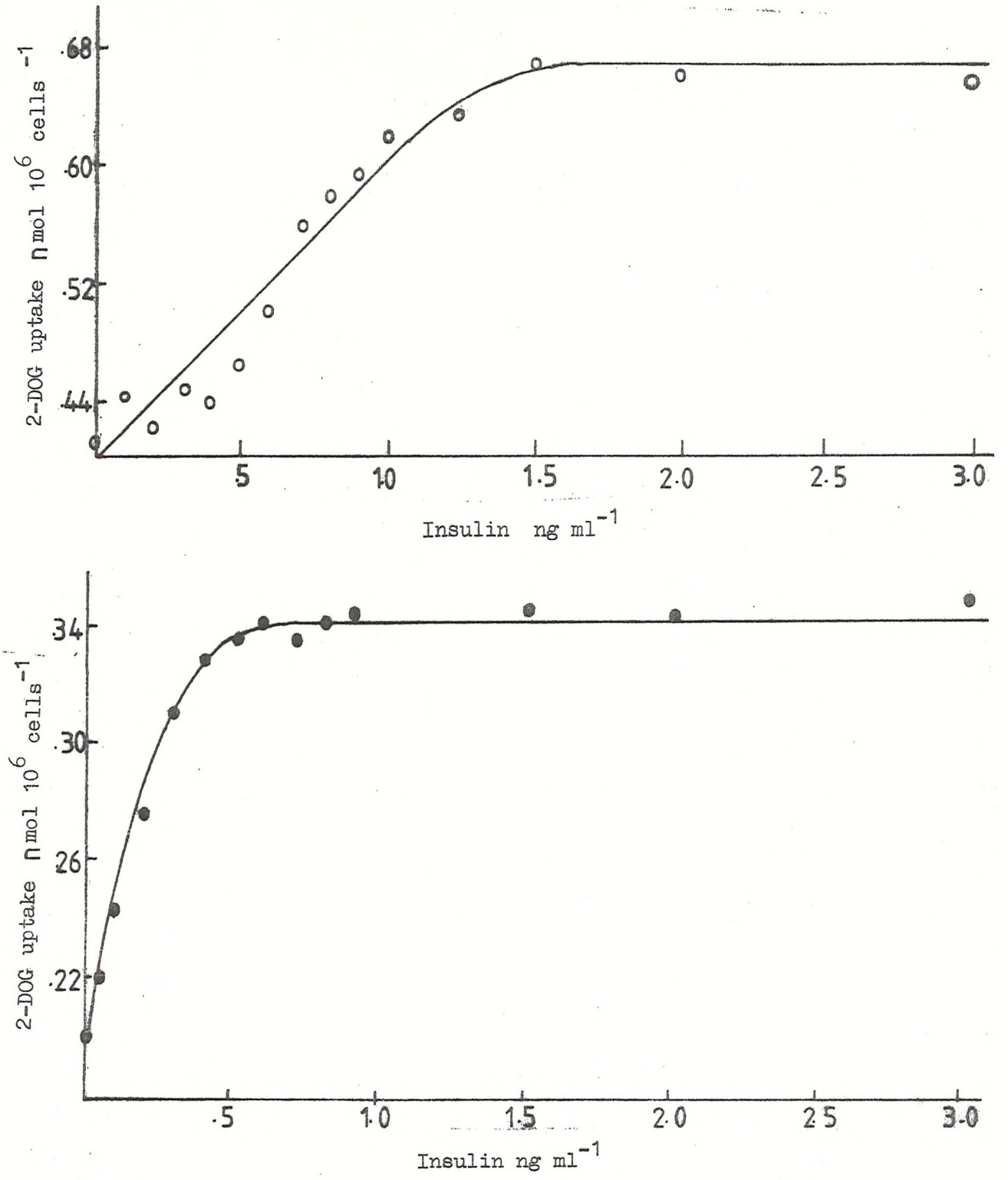


Fig 4.13.

Insulin dose-response curves for 125μM DOG uptake into isolated epididymal adipocytes from lean (•) and obese (o) mice housed at room temperature. DOG transport was assayed at 35°C.

TABLE 4.12.

Summary of data obtained from dose-response curves for insulin stimulated DOG transport into adipocytes and insulin binding to isolated membranes. Both experiments were carried out on lean and obese animals housed at 25°C and 34°C.

	Lean 25°C	Obese 25°C	Lean 34°C	Obese 34°C
$\frac{1}{2}$ max. stimulation * (ng insulin ml^{-1})	0.15, 0.2, 0.21 (3 exp.)	0.7, 0.7 (2 exp.)	0.22 (1 exp.)	0.4, 0.26 (2 exp.)
Mean $\frac{1}{2}$ max. stimulation (ng insulin ml^{-1})	0.19	0.7	-	0.33
Maximal stimulation (Fold)	1.7, 1.68 1.7	1.75, 1.60	1.54	1.66, 1.71
Mean maximal stimulation (Fold)	1.7	1.68	-	1.69
Specific Insulin bound (f mol. mg^{-1})	1.93	0.76	1.90	1.85
Non-specific Insulin bound (f mol. mg^{-1})	3.95	5.41	5.20	6.20
Specific as % total	33%	12%	27%	23%

Results are values obtained from tissue derived from 10 lean and 5 obese mice.

* No. of experiments performed in brackets.

between cells from lean and obese animals housed at 25°C and 34°C. The maximal fold stimulation of DOG transport was around 1.6.

Housing the two groups of animals at 34°C produced a leftward shift in DOG uptake dose-response curve of obese adipocytes to insulin. No significant change was observed in the dose-response curve of DOG uptake to insulin in cells from lean animals housed at 34°C.

This data suggests that obese adipocytes loose their insulin resistance when housed at 34°C. Freychet et al. (1972) have demonstrated that the rightward-shifted dose-response curve of DOG uptake to insulin is due primarily to a decreased number, but not affinity, of plasma membrane insulin receptors in obese mouse adipocytes. Insulin receptor number was therefore assessed by the total binding capacity of purified plasma membranes from lean and obese mice incubated in the presence of 0.15mg ml^{-1} ^{125}I insulin. The results of this experiment are presented in Table 4.12. Insulin binding was reduced in plasma membranes from obese mice housed at 25°C to only 40% of the level observed in membranes prepared from lean mice. After housing at 34°C the binding to membranes of obese mice increased to that observed in the lean group. This result suggests that the normalised dose-response curve of DOG transport to insulin in cells from obese animals housed at 34°C is due to an increase in the number of plasma membrane insulin receptors.

Plasma glucose was also measured in lean and obese mice housed at 25°C and 34°C (Table 4.13). Plasma glucose was increased 2.5 fold in obese mice plasma compared to lean when housed at 25°C. When animals were housed at 34°C, plasma glucose was reduced in both groups. However, the reduction in obese mouse plasma glucose was dramatic compared to lean. Plasma glucose in obese mice was now only 1.5 fold in excess of lean values.

Plasma immuno-reactive insulin (IRI) was also measured (Table 4.13). Plasma IRI was over 6 fold higher in plasma

TABLE 4.13.

Plasma Immunoreactive insulin and plasma glucose concentrations in lean and obese mice housed at 25°C and 34°C.

	Housing Temp °C	Lean	Obese
Plasma Insulin μUml^{-1}	25	119	734
	34	114	161
Plasma Glucose (mM)	25	11.5	28.4
	34	8.8	12.8

Results are derived from duplicate determinations on pooled blood samples from 4 animals.

from obese animals. Housing at 34°C resulted in a large reduction of obese plasma IRI, with little change in lean plasma IRI. Obese mouse plasma IRI was only 1.4 fold elevated over lean controls when housed at 34°C.

Housing obese animals at 34°C produced changes in adipocyte membrane fluidity, hexose transport into adipocytes, hormone stimulated adenylate cyclase activity, insulin receptor number

in adipocyte plasma membranes and plasma IRI and glucose concentrations. All of these factors may be inter-dependant.

4.2.j. Insulin-stimulated hexose transport into adipose tissue in vitro from lean and obese mice. The effects of assay temperature.

As adipocyte plasma membrane fluidity was altered in cells from obese mice, the possible effects of this observation on the temperature-dependance of hexose transport was examined. In this experiment, hexose transport was assessed into tissue pieces rather than isolated cells. Results were expressed as stimulation of basal hexose transport by 100μU ml^{-1} insulin.

Fig 4.15 shows the results of insulin stimulation of ^{3}H-DOG uptake into adipose tissue pieces from lean and obese animals housed at 25°C over the temperature range 33°C-44°C. Between 33-36°C, little difference was observed between lean and obese tissue with respect to insulin stimulated DOG uptake. This is in agreement with previous results with isolated adipocytes, in both experiments, stimulation was around 1.5 fold. At 37°C, an inhibitory action of insulin was actually observed in obese tissue, with little change in the response of lean tissue, although between 37-38°C, a significant reduction in insulin stimulation was observed. The apparent suppression of DOG transport by insulin was maintained in obese tissue to 39.5°C. At the higher incubation temperature of 43°C, insulin was without effect in obese tissue, while at 44.5°C, a significant stimulatory effect of insulin returned.

These results demonstrate differential responses of insulin stimulated hexose transport to temperature into adipose tissue in vitro between lean and obese animals, this phenomena may be a function of altered plasma membrane composition of adipocytes from obese mice compared to lean controls.

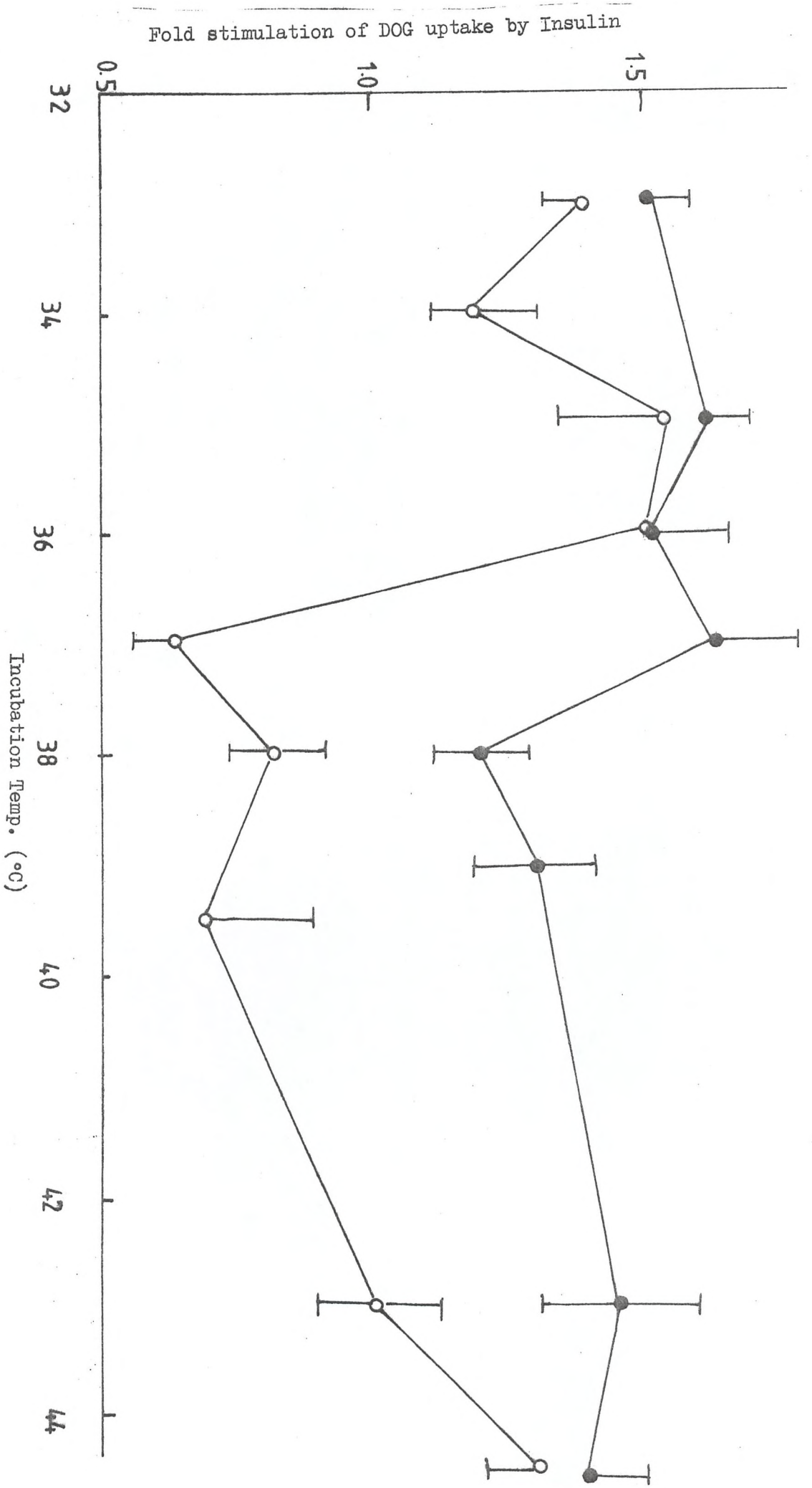
4.2.k. Effect of starvation on adipocyte plasma membrane fluidity in obese mice housed at 25°C.

Starvation has been shown to reduce plasma IRI and glucose concentration in obese mice, and to improve insulin stimulated

Fig 4.15.

Stimulation of ^{3}H-DOG transport by Insulin into adipose tissue pieces from lean and obese mice as a function of incubation temperature.

Results are expressed as means ± S.E. from 4 different experiments. Each experiment utilized samples of tissue pooled from 4 mice.


Fold stimulation of DOG uptake by Insulin
0.5
1.0
1.5
32
34
36
38
40
42
44
Incubation Temp. (°C)

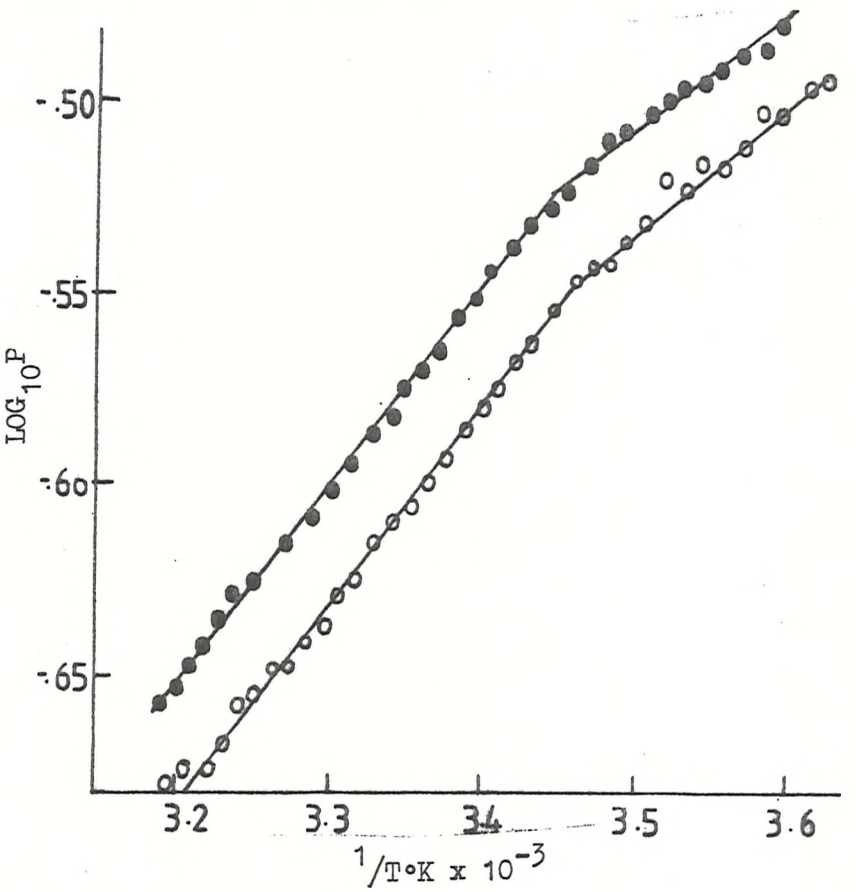


Fig 4.16. Arrhenius plots of fluorescence polarisation by DPH labelled adipocyte plasma membranes from obese mice fed ad libitum (•) and starved obese mice (o).

TABLE 4.14.

Summary of data from Arrhenius plots of fluorescence depolarisation by DPH labelled adipocyte plasma membranes from obese mice fed ad lib. and starved 2 weeks prior to preparation of membranes.

Feeding Regimen	Polarisation 37°C	ΔE above Breakpoint (Kcal mol^{-1})	ΔE below Breakpoint (Kcal mol^{-1})	Breakpoint °C	Fluidity Ratio $\frac{\Phi}{\Phi} R_{37}$
Ad Libitum.	0.224	8.32	13.22	17.6	1.08
Starved 2 weeks.	0.215	8.8	13.5	16.0	

Results are derived from pooled membrane samples from 4 mice in each group.

glucose transport into isolated adipocytes (Introduction to this chapter). It was therefore of interest to examine the effect of a prolonged starve on obese mouse adipocyte plasma membrane fluidity.

Arrhenius plots from fluorescence studies on adipocyte plasma membranes from obese mice starved for 2 weeks and for control obese mice fed ad lib. are shown in fig 4.16. The data from these plots is summarised in Table 4.14.

Starvation increases the fluidity of obese mouse adipocyte plasma membranes (Table 4.14). No major change in flow activation energy was observed, although there was a slight reduction in the break temperature, consistent with the observed fluidisation of membranes from starved animals.

The results from this experiment suggest that the decrease in fluidity of obese mouse adipocyte plasma membranes observed when the animals were housed at 34°C, may not be secondary to reduced plasma IRI or glucose concentrations, since during starvation these parameters are also reduced.

4.3. Discussion.

4.3.a. Compositional changes in adipocyte plasma membranes from obese animals.

Experiments reported in this chapter on the composition of adipocyte plasma membranes, clearly demonstrate differences between the membranes of lean and obese animals. As with microsomal membranes, adipocyte plasma membranes of obese mice are more fluid than those of lean mice. No differences were detected in the proportions of protein, cholesterol or phospholipid classes between the two groups, fluidity differences residing completely with the phospholipid fraction.

Fluidity measurements on individual phospholipid classes revealed that PE was very much more fluid from obese mice membranes compared to lean. Smaller changes were observed in PC, PS and PI. SM, however, was less fluid from obese membranes. The increase in fluidity observed in PE accounts for the large difference in fluidity observed in native adipocyte membranes in obese mice.

If mouse adipocyte membrane phospholipids are orientated asymmetrically, as they are in mouse transformed fibroblasts (Fontaine and Schroeder, 1979), with the amino containing phospholipids comprising the inner half of the bilayer (PE and PS) and the choline containing phospholipids comprising the outer half (PC and SM), then as was postulated for microsomes, this will result in asymmetric membrane viscosity, with the inner monolayer being considerably fluidised in obese adipocyte plasma membranes compared to lean. The outer monolayer may have similar viscosities between the two groups, since PC and SM displayed opposing fluidity changes in obese membranes. PE from obese animals was fluidised because 16:0 and 18:0 were replaced by 20:4 and 22:6. As highly unsaturated PE has a low affinity for cholesterol (Demel et al., 1972) and the transbilayer distribuation of cholesterol is likely to be dependent on its relative affinity for each monolayer (Demel et al., 1977), the possibility arises that the asymmetric distribution of cholesterol may be different in adipocyte plasma membranes from obese animals. The net effect of

this altered cholesterol asymmetry alone would be to increase the order of the outer monolayer and decrease that of the inner monolayer in membranes from obese animals compared to lean.

Housing animals at 34°C normalised obese plasma membrane fluidity. This normalisation occurred in the phospholipid fraction of the membrane. Since P values, activation energies and the breakpoints of the Arrhenius plots derived from lean and obese mouse membranes housed at 34°C were identical, it is highly likely that the fatty acyl moieties of the phospholipids were similar between the two groups. Indeed this has recently been confirmed by French and York (unpublished observations).

4.3.b. Hormone stimulated adenylate cyclase activity in crude membrane fractions from lean and obese mice.

Adipose tissue from genetically obese (ob/ob) mice has been found to be relatively insensitive to the lipolytic actions of catecholamines (Enser, 1970; Yen et al, 1972). The maximum rise in intracellular cAMP in fat cells of obese mice due to catecholamine-stimulation in the presence of theophylline is impaired (Dehaye et al., 1977), as is the stimulation of adenylate cyclase in fat cell membrane preparations by β agonists (Laudat and Pairault, 1975; Dehaye et al., 1975). Shepherd et al. (1977) concluded from their experiments that the coupling between the hormone-receptor complex and the adenylate cyclase may be defective on obese mouse adipocytes. Studies by Dehaye et al. (1978) provided evidence for this hypothesis by observing the effects of the membrane perturbing agent ethanol on GTP stimulation of adenylate cyclase in membranes from lean and obese animals, and these authors suggested that the lipid matrix of adipocyte plasma membranes may be different in some way.

Results presented in this chapter on hormone stimulated adenylate cyclase activity in plasma membranes from lean and obese animals are consistent with the results obtained by Dehaye et al. (1978), demonstrating an impaired basal, NaF and hormone stimulated activity of adenylate cyclase from obese animals compared to lean. Also, concomitant with this observation is the altered phospholipid composition observed in membranes from obese animals. The large

increase in the degree of unsaturation of PE may be responsible for the decreased hormone responsiveness of obese adipose tissue plasma membrane, as this observation is consistent with the data of Englehard et al., (1978) who also obtained reduced hormone stimulated adenylate cyclase activity in membranes enriched with unsaturated PE (4.1.d). These authors also found that increasing PE unsaturation gave rise to increased basal activities in their membranes. Basal activity of adenylate cyclase in obese mouse membranes is reduced over that of lean. This may possibly be due to a decreased number of cyclase units in obese mouse membranes, as shown by reduced activity in the presence of NaF. Another factor may be a decreased inner monolayer cholesterol content of obese mouse membranes, due to a lower affinity of cholesterol to long-chain polyunsaturated fatty acids, as Sinensky et al. (1979) found that basal activity of the enzyme decreased with decreasing cholesterol content of hamster ovarian membranes. At first sight, these observations may seem at variance with the observation of Englehard et al. (1978) who observed an increased basal adenylate cyclase activity with increased unsaturated PE fatty acid content. However, unsaturation was increased with the insertion of 18:2 in their experiments. This lipid interacts well with cholesterol (Chapter 5), and therefore the cholesterol content of the inner monolayer may increase after incorporation of 18:2 PE, this increasing basal adenylate cyclase activity.

Maximum β adrenergic stimulation of adenylate cyclase activity (when expressed as percentage of basal activity) is similar in membranes derived from lean and obese animals. This observation is not consistent with previous work (e.g. Dehaye et al., 1978) which uniformly demonstrates a reduction in this parameter in membranes from obese animals compared to lean. However, a repeat experiment performed more recently on mice from the Southampton colony demonstrated a blunting of the percentage maximum β adrenergic stimulation of adenylate cyclase in membranes from obese mice compared to lean (Steer, York and Poat, unpublished observations). The reasons for this discrepancy are unclear at present.

The concentrations of isoprenaline required to produce threshold and half-maximal response are increased in obese mouse membranes compared to lean. This could be due to the decreased coupling efficiency of the hormone-receptor complex and adenylate cyclase postulated by Dehaye et al.,(1978), or to a change in the binding kinetics of the hormone to the receptor, or possibly both. Scatchard analysis of the β antagonist ^{3}H Dihydroalprenalol (DALP) binding to lean and obese mouse adipocyte plasma membranes by Steer, Poat and York (unpublished) suggest that the maximal binding capacity for DALP is reduced in obese membranes to 70% of that of lean, with little change in the apparent K_D for the ligand. This observation is unlikely to fully account for the 100-fold increase in isoprenaline concentration necessary to promote a threshold response observed in obese membranes compared to lean, and therefore it seems likely that this phenomena may be due to a combination of factors.

Housing animals at 34°C produced striking changes in the response of adenylate cyclase to isoprenaline in obese preparations, suggesting an improvement in hormone-receptor adenylate cyclase coupling efficiency. Threshold response in both lean and obese preparations of adenylate cyclase to isoprenaline was identical, although the dose-response curve was still shifted to the right in obese preparations. This can be accounted for by the observation of Steer, Poat and York (unpublished) who have observed a reduced binding capacity for ^{3}H DALP for obese membranes compared to lean when animals were housed at 34°C. This would suggest that decreased receptor number is not a large contributory factor to the observed poor hormonal stimulation of adenylate cyclase in membranes from obese mice, and that the major locus of adenylate cyclase hormonal stimulation resistance in this tissue lies in hormone receptor cyclase coupling in the membrane matrix.

Starvation of obese animals has been shown to reduce plasma insulin and glucose concentrations (Laudat and Pairault, 1975). These authors have also demonstrated that the dose-response of adenylate cyclase for adrenaline is not altered in membranes from obese mice after a period of starvation, and therefore the blunted hormonal stimulation of adenylate cyclse to β agonists is not

secondary to the diabetic state of obese mice. Starvation was shown to potentiate the increased fluidity observed in membranes from obese animals. Thus, normalisation of obese mouse adipocyte plasma membrane fluidity to lean control values when animals are housed at 34°C is also unlikely to be secondary to the observed improvement in the diabetic condition of obese mice housed at this temperature.

These experiments provide further evidence that the physical state of membrane lipids can influence hormone-receptor adenylate cyclase coupling, and that the altered lipid composition of obese mouse adipocyte plasma membranes may be at least partially responsible for the poor elevation of intracellular cAMP concentration observed in the presence of β agonists, in adipose tissue from obese mice.

4.3.c. Insulin stimulated hexose transport into adipocytes from lean and obese mice.

In 5-7 week old ob/ob mice, maximal response of DOG uptake into isolated adipocytes was similar to that observed in lean control cells. This finding is at variance with the findings of Czech et al. (1977) who observed only a small stimulation of 3-O-methylglucose uptake by insulin into lean adipocytes, and very large stimulation in insulin stimulated 3-O-methylglucose uptake into cells from obese animals. By 12 weeks of age, a complete reversal of this phenomena occurred, obese cells becoming refractory to the effects of insulin, and lean cells becoming their most sensitive. We have tried to prepare cells from obese animals aged 12 weeks. The extent of cell lysis during the preparation is too great to yield interpretable results.

Insulin stimulation of DOG uptake into adipose tissue pieces from 12 week old lean and obese animals demonstrated that, below an assay temperature of 37°C, insulin stimulated DOG uptake was similar between the two groups of animals. As the core temperature of obese animals is below 37°C at housing temperatures of 25°C, then no evidence is apparent from the data presented in this chapter to support the findings of Czech et al. (1977) that

hexose transport in cells from obese mice becomes refractory to the effects of insulin at 12 weeks of age. However, as Czech et al. (1977) were using an incubation temperature of 37°C, then it is possible that their apparent lack of insulin stimulation was caused by a higher than physiological temperature. These results on insulin stimulated hexose transport into lean and obese adipocytes are consistent with the findings of Christophe et al. (1961) and Chlouverakis and White (1969) for glucose utilization by fat pads in lean and obese mice. These authors reported a similar percentage stimulation by insulin of glucose utilization for fat pads from both phenotypes. As transport is rate limiting for gluocse utilization (Olefsky, 1977) it is likely that insulin is able to increase the numbers of plasma membrane transporters in obese cells to the same degree as lean.

A rightward shift in the dose-response curve of DOG transport to insulin, and a reduced binding capacity for insulin per mg membrane protein, strongly suggest that the number of insulin receptors per unit area of cell surface is reduced in cells from obese animals compared to lean, as has been observed by Freychet et al. (1972). Despite a receptor concentration per unit area of plasma membrane of approximately 30% compared to lean controls, nevertheless, these receptors are able to activate proportionally the same maximal numbers of transporter units per unit area of plasma membrane in obese cells compared to lean. This is consistent with the concept that full receptor occupancy is not necessary for full expression of insulin action.

Housing animals at 34°C produced a rightward shift in the dose-response curve of hexose uptake to insulin in cells from obese mice, with no change observed in lean cells. The insulin binding capacity of membranes isolated from these cells was increased to lean values. This is of interest, because Freychet et al.,(1972) found that chronic food restriction, which normalized body weight and drastically reduced plasma insulin and glucose concentrations, did not appear to have any effect on the apparent low insulin receptor concentration in ob/ob mouse fat cell membranes. Tentatively, this may suggest that membrane

fluidity may be regulating plasma insulin receptor concentration. This concept is supported by the observation of Amatruda and Finch (1979) that reducing the incubation temperature form 37°C to 27°C resulted in a 50% increase in the apparent number of receptors in plasma membranes.

It must be stressed that the binding data obtained in this study of insulin to plasma membranes is entirely preliminary as only a single concentration of labelled insulin was used. Although this does give some indication of the total binding capacity of membranes, as the affinity of receptors to insulin is not affected in the obese state (Freychet et al. 1972), nevertheless multi-point Scatchard plots must be obtained to ascertain the correct relative numbers of receptors on plasma membranes.

Hormone stimulation of hexose transport was abolished in adipose tissue pieces from obese mice housed at 25°C at incubation temperatures of 37°C and above, but was relatively temperature insensitive in lean tissue. An explanation for such an event with the available data is not possible. However this phenomena may in some way be connected with the observations of Amatruda and Finch (1979) that a hormone mediated thermal transition occurs at 37°C. The altered lipid composition of obese mouse plasma membranes may in some way interfere with this process. The suggestions of Cushman and Wardzala (1980) that insulin may stimulate hexose transport by fusing submembranous transporters with the plasma membrane may also provide an explanation. It may be that the compositional differences observed in obese adipocyte membranes may inhibit the fusion process above 37°C. Whatever the reason for this observation, it is not physiologically important when obese animals core temperature is below 37°C. However when housed at 34°C, core temperature rises to 37°C (Chapter 3). In this case, adipose tissue plasma membrane may become refractory to insulin stimulation. It may be this event that brings about normalisation of membrane fluidity, in order to return insulin sensitivity to the tissue when obese animals are housed at 34°C.

4.3.d. Conclusions.

Adipocyte plasma membranes from obese mice are fluidised by containing highly unsaturated PE. When gross membrane fluidity is normalised to lean values by housing at 34°C, the poor hormonal responsiveness of adenylate cyclase in obese membranes improves. Housing animals at 34°C also produces a normalisation of the dose-response curve of hexose transport to insulin, by increasing the membrane receptor concentration. These observation suggest that the altered membrane composition in the obese state contributes significantly to the poor hormonal responsiveness of adipose tissue in the obese state.

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CHAPTER 5.

INVESTIGATIONS INTO ASPECTS OF THE MOLECULAR ORGANISATION OF THE ADIPOCYTE PLASMA MEMBRANE FROM LEAN AND OBESE MICE

5.1. Introduction.

5.1.a. Phospholipid Asymmetry in membranes.

In Chapter 4, it was established that the major phospholipid components of ob/ob mouse adipocyte plasma membrane are altered in comparison to lean controls. In order to evaluate the possible significance of these findings to the altered properties of these membranes in the ob/ob mouse, it is necessary to measure the phospholipid composition of each half of the bilayer, so that the distribution of each phospholipid class within the bilayer can be predicted.

The orientation of proteins and carbohydrates within the membrane is maintained throughout the lifetime of these components. The orientation of these components within the bilayer is critical to their function. The polar nature of proteins and carbohydrates at the cell surface prevent their reorientation within the membrane, since the energy of activation for the dehydration process necessary for polypeptides to diffuse through the hydrocarbon core of the membrane is very large. Lipids, however, exhibit a much smaller activation energy for transbilayer movement. However, the transbilayer movement of phospholipids (flip-flop) is relatively slow, with half times in the order of days (De Kruyff and Wirtz, 1977) for stable liposomes. In contrast, in biological membranes this process is much faster, e.g. in bacterial membranes the half life of flip-flop of phospholipids is in the order of minutes. The presence of proteins within the bilayer is thought to be responsible for the shorter half life of flip-flop. For example, the presence of the transmembrane protein, glycophorin, in PC liposomes, increased the rate of flip-flop of lyso PC by 100 fold (Van Zoelen et al., 1976). Thus, it may be expected that phospholipids would be randomly distributed between the two halves of the bilayer. However, as the membrane comprises phospholipids with different headgroups, it might be expected that flip-flop times for different phospholipids may be different.

Coupled with this, is the possibility that specific phospholipids may have a greater affinity for proteins components selectively located on one side of the bilayer, or alternatively, they

may have a preference for the overall geometry of the particular bilayer half. The flip-flop process may then give rise to a dynamic segregation of phospholipids species within the membrane. Much evidence exists to suggest this is indeed the case. Phospholipid asymmetry studies have been performed using a number of different techniques. The most convenient, and most widely used technique employs reagents that can react with the amino function of phospholipid head groups. Both PE and PS are able to react with such reagents. Negative reactivity of cell membranes to such chemical reagents need not necessarily imply the absence of the amino phospholipids in a monolayer. Hence, careful control experiments have to be performed to establish that all the amino phospholipids are available for derivatisation with the chemical reagent, providing they are exposed to it. Since the early work of Maddy (1964) phospholipid assymetry has been evaluated in a number of different cell types. Maddy (1964) first demonstrated that the amino phospholipids in erythrocytes were not available for derivatisation by 4 acetamido-4' -isothiocyanostilbene-2,2'-disulphonate.

Trinitrobenzenesulphonic acid (TNBS) has been used as a non-permeant chemical derivatising reagent. Phospholipids exposed to this reagent are converted to their trinitrophenol derivatives (TNP-phospholipids). Assays with TNBS and amino phospholipids have demonstrated that the derivatisation process goes to completion (Marinetti and Love, 1976).

Using TNBS to label amino phospholipids, it has been shown that only 12-18% of the total PE, and none of the total PS was labelled in intact human platelets by this reagent (Schick et al., 1976). In mouse LM cells TNBS reacted with only 4% of the total PE and 5% of the total PS (Fontaine and Schroeder, 1979). These authors also demonstrated that 10-15% of the total PE and 20% of the total PS was labelled in isolated murine synaptosomes exposed to TNBS (Fontaine and Schroeder, 1980). Rothman and Kennedy, (1977) demonstrated that 33% of the total PE of Bacillus megaterium membrane was labelled with TNBS. From these and other studies using derivatising reagents, phospholipase enzymes and immunological techniques it has become apparent that a high degree of phospholipid assymetry is a general feature of many membranes.

In many membranes, the amino phospholipids PS and PE occupy the inner monolayer, and the choline containing phospholipids (PC and SM) comprise the outer monolayer.

TNBS reagent has been used to determine the phospholipid distribution in mouse adipocyte plasma membranes, and also to determine if any difference in phospholipid assymetry exists between adipocyte plasma membranes from lean and obese mice.

5.1.b. Use of p-N,N,N trimethylphenylamine 6-phenyl hexa-1,3,5-triene ($TADPH^+$) to investigate plasma membrane inner monolayer fluidity.

Deuticke (1968) first observed that lipophilic anions and cations produced different alterations of shape in erythrocytes. Cations caused the cells to assume a 'cup-like' appearance, whereas anions produced a characteristic 'crenated' appearance. Sheetz et al. (1976) have interpreted this observation in terms of their bilayer couple hypothesis. Essentially, this hypothesis predicts that if the two halves of a membrane bilayer differ in their protein and lipid composition, then each half can respond differently (and independantly) to membrane perturbations. A wide range of drugs, mostly negatively charged, are known to cause erythrocytes to crenate, and similarly positively charged drugs all cause erythrocytes to assume a 'cup'-shape. It has been proposed that all of these drugs bind with their hydrophobic regions in the bilayer hydrocarbon region, and their ionic headgroups at the surface. However, due to the extensive net negative charge of the inner half of the erythrocyte bilayer, due to the presence of PS, cationic drugs preferentially partitioned into and anionic drugs out of, the inner monolayer. If the adipocyte plasma membrane also contains a negatively charged inner monolayer, then positively charged fluorescent probes might preferentially partition to the inner surface, to give selective information concerning this region. $TADPH^+$ was equilibrated into adipocyte plasma membranes by initial sonication, and incubation for 2 hr in the dark under nitrogen at room temperature. Fluorescence polarisation was measured to determine if any difference in inner monolayer fluidity could be detected between lean and obese mouse adipocyte plasma membranes.

The success of this technique depends on demonstrating that the fluorescent probe is located in the inner monolayer. Liposomes were therefore prepared of reconstituted inner and outer monolayers, and labelled with $TADPH^+$ in order to provide evidence that $TADPH^+$ was monitoring the inner monolayer environment.

5.1.c. Fatty Acid localisation on phospholipids.

The major phospholipids of mammalian cell membranes contain 2 fatty acid moieties. The exception to this is SM, where the 1-position of the glycerophospholipids is replaced by the hydrocarbon chain (14 carbon atoms) of sphyngosine. In recent years, it has become apparent that the acyl moieties of the glycerophospholipids are subject to considerable variation, and that phospholipids from the same membrane systems can show an asymmetric distribution of fatty acid classes between the 1 and 2 carbon atom positions of the glycerophospholipids. Miller et al., (1976) demonstrated that phospholipids isolated from goldfish intestinal microsomes contained a much higher percentage of unsaturated fatty acids at carbon atom 2 of PC and PE than that found at carbon atom 1. The degree of unsaturation asymmetry between carbon atom 1 and 2 was more marked in PE than PC. These authors also demonstrated that both carbon atoms 1 and 2 of PE and PC became more unsaturated by housing the goldfish at 6°C rather than 15°C. Housing at 6°C produced an increase in 22:6 and 20:4 at carbon atom 2 of PE, and also the appearance of 22:6 at carbon atom 1 in this phospholipid. The changes in PC were less marked at carbon atom 2 with only an increase in 20:4, but the similar appearance of 22:6 at position 1 of PC was also observed in 6°C acclimated goldfish. Thus, positional asymmetry is regulated differently between phospholipids of the microsomal membrane in goldfish in response to changing environmental temperature, and the major regulated fatty acid in this species is 22:6, which is substituted for the unsaturated species 16:0 and 18:0 in both PE and PC.

Hazel (1979) has performed similar studies on the trout liver phospholipid fraction. The major regulated fatty acid was again 22:6 at the expense of 18:0 on cold acclimation. However, the carbon 2 position of PC was the acceptor site for 22:6. Both PE and PC demonstrated a high degree of unsaturation assymetry between

the 1 and 2 positions. Virtually all of the polyunsaturated fatty acids were esterified at the 2 position, while the saturated and monoenoic fatty acids were located at position 1.

Sun and Horrocks (1968) demonstrated that mouse brain PE and PC contained a highly asymmetric distribution of fatty acids. Virtually all of the saturated fatty acids were located at carbon atom 1. The positional assymetry of the fatty acids was more marked in PE compared to PC. Thus, positional as metry of the fatty acids can be varied by specific changes within phospholipids, which appear to be independant of other phospholipid classes within the same membrane. In view of these considerations, the positional location of fatty acids in mouse adipocyte plasma membrane was assessed, in order to determine where the changes occurring within phospholipid classes from ob/ob mouse adipocyte plasms membranes had taken place.

5.1.d. Function of sterols in membranes.

5.1.d(i).

Cholesterol is the major sterol found in mammalian cell membranes. It is especially abundant in cell plasma membarnes, where its proportion varies between 0.3-1.3 mole per mole phospholipid. Cholesterol interacts strongly with other membrane components such as phospholipids and proteins. Cholesterol-phospholipid interaction has been extensively studied , but the exact nature of the interactions are not completely understood. X-ray and neutron defraction studies have shown that cholesterol is largely immersed in the hydrocarbon core of the bilayer with the methyl chain terminus near the centre of the bilayer, and its β-hydroxyl group in the vicinity of the acyl carbonyl group of the phospholipid. Brockerhoff (1974) proposed that the sterol hydroxyl group and the phospholipid carbonyl group may be associated in a 'hydrogen belt' with water, however, as yet no direct evidence for hydrogen bonding between the two groups has been found, although conversion of the 3β-hydroxy to 3α hydroxy group to form 3-α-epicholesterol abolishes cholesterol-phospholipid interaction (Demel et al., 1972). Thus, an orientated polar interaction is essential.

Cholesterol also interacts with phospholipid acyl chains by hydrophobic interaction. In the liquid crystalline phase, cholesterol reduces the mean molecular area of phospholipids and considerably reduces molecular motion (Demel and De Kruyff, 1976). The degree of this ordering effect depends on acyl chain length, and the degree of unsaturation as would be expected from a predicted hydrophobic interaction with phospholipids. A planar sterol structure is required for interaction, since the non-planar coprostanol does not interact with phospholipids. Androstenol (lacks 8-carbon atom side chain) also does not interact with phospholipids. Thus the unique structuring of cholesterol allows stable interaction with membrane phospholipids, increasing the degree of order within bilayer structures.

Although direct interaction of cholesterol with membrane proteins is difficult to demonstrate, cholesterol has been shown to bind to myelin proteolipid apoprotein with high affinity (London et al., 1974), and to be excluded from the lipid annulus of the Ca^{++}-ATPase (Warren et al., 1975). However, cholesterol certainly modulates the activity of membrane proteins, e.g. adenylate cyclase (see Chapter 4). Whether this effect of cholesterol on proteins is direct or indirect in each case is difficult to demonstrate.

Apart from ordering membrane lipids and regulating the activity of some membrane proteins, the incorporation of cholesterol confers properties on the membrane as a whole. For example, cholesterol incorporation into liposomes of several phospholipids reduces the permeability of these phospholipids to water (Blok et al., 1977) and small organic molecules such as glycerol and glucose (de Gier et al., 1969) and to cations (Vanderkooi and Martonosi, 1971). The reduced permeability has been attributed to increased packing and decreased mobility of the fatty acyl chains which result from the interaction of cholesterol with the phospholipids. The degree of cholesterol content has been shown to influence permeability in biological membranes. The permeability of all the above molecules is reduced when the cholesterol content is increased in Mycoplasma laidlawii membranes (De

Kruyff et al., 1972). Also a reduction in Na^+ transfer was demonstrated in guinea pig erythrocytes after dietary elevation of membrane cholesterol (Kroes and Ostwald, 1971). Thus, possibly a major role of cholesterol in plasma membranes of mammalian cells is perhaps to minimise the leakage of electrolytes and substrate molecules across the cell membrane.

Another important aspect of cholesterol incorporation into biomembranes is the effect of the sterol on lateral diffusion of membrane bound proteins. Using the technique of fluorescence recovery after photobleaching, Vaz et al. (1979) demonstrated that the mobility of the amphipathic apolipoprotein labelled with fluoresceine in egg PC bilayers was reduced 10 fold when the liposomes contained 50% cholesterol. Within the lipid matrix of plasma membranes there are postulated to be modulating proteins that collision couple with effector proteins (Rimon et al., 1978) the steady state activity of which may be governed by the ratio of coupled to uncoupled effector proteins. As the steady state concentration of the coupled species is likely to be governed by the rate constants for the coupling and the decoupling process, then in theory the activity of the effector protein could be a function of the diffusion coefficients of the two proteins within the membrane matrix, if the coupling process was rate limiting. Cholesterol, therefore, may have an important function in biological membranes in regulating diffusion controlled processes.

5.1.e. Cholesterol-phospholipid interaction and the organisation of cholesterol within biological membranes.

Rogers et al. (1979) have investigated the organisation of cholesterol within PC liposomes. From their studies with a fluorescent cholesterol analogue, they concluded that the sterol-lipid system was ordered in such a manner as to prevent sideways overlap of probe molecules, an event that would normally quench the probe fluorescence. As the cholesterol/lipid molar ratio of 1:1 demonstrated little quenching of incorporated probe, it was postulated that the most likely model for the organisation of this system was regions of sterol molecules arranged in rows, with rows of phospholipid running parallel to the rows of sterol. Studies by Demel et al. (1972) demonstrated little difference in the

permeability properties of liposomes of cholesterol and a number of different fatty acid positional isomers of PC. Thus, the acyl chains of PC do not appear to be preferentially orientated toward cholesterol.

The fatty acid composition of PC can markedly influence the permeability of the liposomes. Cholesterol incorporation drastically reduces the permeability of these liposomes, liposomes permeability being an indicator of the degree of condensation of the acyl chains by cholesterol (Demel et al., 1972). The ability of cholesterol to condense PC liposomes is high when the phospholipid contains one saturated and one unsaturated species of any kind (18:1, 18:2, 18:3 or 20:4). However,1-palmitoyl,2-docosahexaenoyl PC was not condensed at all by cholesterol incorporation. When liposomes contained PE with two unsaturated fatty acids of any kind except 18:1, the ability of incorporated cholesterol to condense these liposomes was poor (Dioleiyl PC, however is effectively condensed by cholesterol). Decreased permeability of liposomes has been shown to closely correlate with the Van der Waals interaction forces between cholesterol and the acyl chains, as measured by chain mobility in the presence of cholesterol by a wide range of techniques (Demel et al., 1972). Therefore, it can be concluded that cholesterol/phospholipid interaction can be modulated by the fatty acid compositions of phospholipids. It is not known whether the degree of interaction modifies cholesterol organisation within bilayers.

As well as differential interaction between different PC species, cholesterol also demonstrates preferential interaction with different phospholipid classes. Egg PC has a lower affinity for cholesterol than bovine brain SM, as detected by Differential Scanning Calorimatory (DSC) (Demel et al., 1977) and by liposome partition studies by Lange et al., (1979). Both SM and PC were found to have a higher affinity for PE by DSC (Demel et al., 1977). In a later study by Van Dijck (1979), the affinity of PS for cholesterol was found to be intermediary between SM and PC. In equimolar mixtures of PE and PC, Cullis et al. (1978) established that cholesterol preferentially associates with PE, using ^{31}P NMR

and DSC. The variable affinities of cholesterol for different phospholipids first led Demel et al. (1977) to speculate that the asymmetric distribution of phospholipids across the erythrocyte plasma membrane bilayer might result in an unequal distribution of cholesterol between the two bilayer halves.

This concept is extended further in this chapter to include the hypothesis that membranes containing polyunsaturated fatty acids with a low affinity for cholesterol may also influence cholesterol compartmentalisation within the plane, and across the bilayer.

The techniques used to study cholesterol/phosphlipid interaction mentioned above become increasingly difficult to employ as the complexity of the lipid mixture under study increases. It was therefore decided to develop a technique utilizing a fluorescent probe to investigate the interaction with cholesterol of complex lipid mixtures. The validity of the method was assessed by measurement of phospholipid systems already measured for cholesterol affintiy by classical methods. After establishing the composition of each monolayer of adipocyte plasma membranes from lean and obese mice, liposomes were reconstituted from extracted lipids of native membranes, and the cholesterol affinity for each monolayer was assessed, in order to estimate the effect of the altered fatty acid composition observed in obese mouse membranes on cholesterol interaction with phospholipids.

5.1.f. The use of fluorescent probes to monitor cholesterol/phospholipid interaction.

(i) Use of Cholesta-5,7,9 triene-3-β-ol (CTO)

The comparison of the properties of this fluorescent sterol to those of cholesterol have been assessed by Rogers (1979). Comparison of the physical properties of the fluorescent sterol with cholesterol incorporated into PC bilayers were studied using chlorophyll a fluorescence and stopped-flow measurements of liposome permeability to compare the interactions of the fluorescent sterol with PC to that of native cholesterol. No difference could be detected between either sterol, and it was concluded that the lipid interactions of the CTO probe were indistinguishable from that of cholesterol.

Aqueous dispersions of CTO probe yield highly quenched fluorescence above the critical micelle concentration. The absorbtion spectra is also very different compared to those seen in organic solvent. The altered fluorescence and absorbtion properties can be attributed to micelle formation in which the probe experiences concentration quenching. Rogers et al. (1979) demonstrated that similar quenching could also occur in lipid bilayers using perylene as a fluorescent quencher. Resonance energy transfer between probe and perylene demonstrated that the planar moieties of the fluorophors could overlap in the bilayer, quenching the fluorescence of the probe. Since sterol nuclei are relatively planar, and that juxtaposition of the β-faces of the molecules could result in 'sideways' overlap of the triene systems. Rogers et al. (1979) postulated that sideways overlap between probe molecules could occur in membranes and give rise to non fluorescent dimers or aggregates. Fluorescence quenching could further take place by resonance energy transfer between monomers and dimers. If chlorophyll a is used as a model for collisional quenching in PC bilayers, then fluorescence quenching due to deactivating collisions between excited and ground state chlorophyll a molecules takes place (Beddard et al., 1976) and the fluorescence intensity at any concentration of chlorophyll a would follow a Stern-Volmer relation of the type;

$$\frac{N_o}{N} = 1 + \frac{C}{C^*}$$

where: N_o is the intensity in the absence of quenching,

N is the observed fluorescence,

C is the molar ratio of the fluorophore, and

C^* is the molar ratio at which the fluoresence yield has fallen by half.

The Stern-Volmer relationship describes the dynamic quenching process for collisions between fluorophores that are randomly distributed in a matirx with no preferred orientation preventing overlap of the molecular orbitals undergoing energy transfer. The quenching process for the incorporation of CTO probe does not follow the Stern-Volmer relationship. Indeed the Stern-Volmer equation predicts that at a 1:1 lipid to probe molar ratio, the fluorescence intensity would be a factor of 24 smaller than at 500:1 lipid to

to probe molar ratio, whereas it is in fact only 3 times smaller (Rogers et al., 1979).

This observation, supported by fluorescent lifetime measurements, demonstrates that cholesterol is ordered within PC liposomes so as to prevent sideways overlap of the triene systems. This ordering of PC/ cholesterol mixture is likely to arise at least in part from the strong interaction between cholesterol and phospholipid. If indeed this is the case, then it would be expected that the weaker the interaction between cholesterol and phospholipid, the closer to a Stern-Volmer prediction of the quenching process would occur experimentally, as was observed for chlorophyll a which does not appear to interact preferentially with, or be orientated by, phospholipids to any observable degree. Experiments were therefore designed to investigate the fluorescence quenching properties of the probe in lipid mixtures known to have varying affinities for cholesterol.

Measurements of CTO concentration dependant quenching as a method for probing cholesterol affinity has one disadvantage. It is possible that more than one quenching process may be operative. For example, resonance energy transfer can occur between excited probe monomers and ground state probe dimers over distances of between 10-100Å. (Badley, 1976) as well as collisional energy transfer. Therefore, in different lipid mixtures with the same affinity for cholesterol, there may possibly be different types of quenching mechanisms operative. Therefore fluorescence intensity measurements at different CTO probe concentrations may not necessarily reflect the interaction of CTO probe with phospholipid liposomes. Therefore a second method for probing cholesterol interaction with phospholipid liposomes by fluorescent sterols was performed, using the technique of fluorescence polarisation.

The degree of fluorescence polarisation (P) of the CTO probe is sensitive to the viscosity of its environment (Rogers et al., 1979). Therefore the P values of CTO probe incorporated into phospholipid liposomes should be dependant on cholesterol incorporation, as cholesterol is known to increase the viscosity of phospholipid liposomes (Shinitzky and Inbar, 1976) by the ordering effect

of cholesterol on the acyl chains. This ordering effect is reduced in liposomes of phospholipids of low cholesterol affinity, as shown by permeability studies (Demel et al., 1977). Therefore the ability of increasing cholesterol content in CTO labelled liposomes to alter the polarisation by the CTO probe may be a useful measure of cholesterol interaction with phospholipids.

Another factor influencing polarisation by CTO probe in phospholipid liposomes, is depolarisation of fluorescence by the Förster effect. Förster (1948) first described the process of depolarisation of fluorescence by energy transfer between excited and ground state molecules. If energy transfer occurs freely between randomly distributed molecules in the lipid bilayer, then the depolarisation effect will be a function of fluorophore concentration. This is indeed the case with randomly distributed chlorophyll a as fluorescent probe, and experimentally determined values for concentrations of chlorophyll a that produce complete depolarisation of fluorescence agree well with predicted values from equations describing this process (Rogers et al., 1979). Using CTO as probe, however, Rogers et al. (1979) found that even at very high probe concentrations, the fluorescence of this probe was still highly polarised. These authors attributed this phenomenom to the organisation of cholesterol in PC bilayers considerably reducing Förster energy transfer effects. Thus the net effect of increasing cholesterol concentration on the fluorescence polarisation of CTO labelled liposomes may be the result of 2 opposing effects:-

(a) the increase in P caused by increased microviscosity, and
(b) decreased polarisation caused by increased Förster energy transfer.

To minimise the influence of (b), CTO concentration was kept to the minimum required for reasonable fluorescence intensity measurements, and was kept at constant concentration over the range of cholesterol/PC used in the fluorescence polarisation titration. At the probe concentration chosen, Förster energy transfer effects are negligible in PC liposomes (Rogers et al., 1979).

This may not be the case for phospholipid mixtures with a very low affinity for cholesterol. However, in such a situation

increasing cholesterol concentration in CTO labelled liposomes should have less effect on probe polarisation than in liposomes with a higher affinity for cholesterol (if this technique is going to be of any use). Under these conditions, it might be expected that Förster energy transfer could occur more freely between probe molecules in a less ordered environment, thus reducing probe polarisation, and this effect may be dependant on cholesterol/phospholipid molar ratio. Thus, in liposomes of reduced affinity for cholesterol, the degree of probe polarisation may be reduced by one or both these effects. As both effects are additive, a reduced increase in probe polarisation by cholesterol in one phospholipid mixture compared to another should be the result of a reduced cholesterol/phospholipid affinity.

It was hoped that the combined results of CTO probe fluorescence and fluorescence polarisation would validate the use of fluorescent probes as a tool for probing the interaction of cholesterol with phospholipid mixtures.

(ii) Use of 1,6, Diphenyl hexa 1,3,5 triene (DPH) to assess cholesterol/phospholipid affinity.

DPH polarisation is extremely sensitive to the viscosity of its environment (Chapter 1). Therefore the effect of increasing cholesterol content on polarisation by DPH labelled liposomes was investigated. The dependance on the value of P obtained by increasing cholesterol content of different phospholipid mixtures was compared to results obtained using CTO as fluorescent probe, to validate the use to DPH in these studies.

For DPH, the value of P is related to the microviscosity of the hydrocarbon matrix; using the formula of Shinitzky and Barenholz (1978)

$$\eta = \frac{2p}{0.46 - P}$$

An'interaction index' can be calculated from titration slopes of polarisation versus cholesterol:phospholipid molar ratio, and is suggested to be defined as:-

$$i37° = \frac{x_1 - x_2}{\eta_1 - \eta_2} \quad \text{mol poise}^{-1}$$

where (η_1, η_2) are the microviscosity of the liposomes at cholesterol: phospholipid molar ratios (x_1, x_2) respectively, and $x_1 > x_2$.

The calculated value for i_{37} describes the number of moles of cholesterol required to raise the viscosity of 1 mole of phospholipid by 1 poise at 37°C, and is suggested to be a measure of the affinity of cholesterol for phospholipid acyl chains.

5.1.g. Evaluation of the value of the flow activation energy obtained from Arrhenius plots of fluorescence polarisation in terms of molecular structure.

In Chapters 3 and 4, it was suggested that membranes derived from obese mice contained components that were more 'ordered', based on evaluation of the flow activation energy parameters of fluorescence polarisation experiments. This concept, based on absolute reaction rate theory applied to flow processes by Kauzmann and Eyring (1940) was validated for DPH polarisation by Shinitzky and Inbar (1976). These authors observed the effect of cholesterol on ΔE for various liposomes, and showed a decrease in ΔE with increasing cholesterol content. This is in accordance with the concept of a reduction of both the unit flow volume and the average length of the segments participating in the flow process within the hydrocarbon matrix as cholesterol concentration is increased. These thermodynamic conclusions have been supported by a variety of other techniques, for example cholesterol has been shown to reduce the moleculr volume of phospholipids by monolayer film compression techniques (Gains, 1966). However, interpretation of differences in ΔE between different phospholipids and phospholipid mixtures is considerably more diffecult, and even more so in heterogenous lipid mixtures. Adipocyte plasma membranes from lean and obese mice, for example, display different flow activation energies, and yet have similar cholesterol content. Although this observation could be due to any number of factors, the large difference in the unsaturation of PE between membranes from lean and obese animals may be involved. To investigate the possible dependance of ΔE on fatty acid composition of phospholipids, Arrhenius plots of DPH polarisation of a variety of different PE liposomes has been examined.

5.2. Results.

5.2.a. Phospholipid assymetry of adipocyte plasma membranes from lean and obese mice.

5.2.a. i) Effect of 2,4,6-trinitrobenzenesulphonic acid (TNBS) on adipocyte viability.

Glucose utilization as a measure of cell viability was assessed by the conversion of U-^{14}C glucose into $^{14}CO_2$ in isolated adipocytes from lean and obese mice. The effect of preincubation with TNBS buffer for 35 minutes on $^{14}CO_2$ production is shown in Table 5.1. The results suggest that although there was a reduction in glucose utilization of about 30% in the two groups treated with TNBS, nevertheless the cells remained viable. Visual inspection of cells at the end of the incubation with TNBS buffer suggested that TNBS treatment did not enhance cell breakage in cells from either lean or obese mice. Membranes from broken cells were removed in the washing procedure after derivitization, and therefore would not affect results. A degree of cell lysis was observed in cells from obese anaimals, but not lean, after incubation for 1 hour with U-^{14}C glucose, but again there appeared to be no difference in the degree of lysis in cells from obese animals that had been treated with or without TNBS.

These results are consistant with the observation of Fontain and Schroeder (1979) who observed a 20% decrease in activity of mouse LM cell Na^+/K^+-ATPsse activity after incubation with TNBS. Thus cell viability, as measured by glucose transport and oxidation, is not seriously impaired by treatment of lean and obese mouse adipocytes with TNBS.

5.2.a. ii) Reaction times for the aminophospholipids with TNBS.

PE and PS comprise 28% and 10% respectively of the total phospholipid fraction of adipocyte plasma membranes. Because these phospholipids do not form bilayer structures in the presence of Ca^{++} (Cullis and De Kruyff, 1979) they were mixed individually or together with PC such that the amino phospholipid:PC ratio in the resulting bilayer vesicles was as the *in vivo* situation. Reaction times were investigated (fig 5.1) for TNBS derivitisation under

Table 5.1. Rates of glucose conversion by adipocytes isolated from lean and obese mice after preincubation in buffer ± 1.5mM TNBS.

	D- U-^{14}C glucose conversion (pmole/10^6 cells/hr) to $^{14}CO_2$	
	Lean	Obese
Control buffer	25.1	20.3
1.5mM TNBS buffer	18.1	13.8
% reduction in Glucose utilization by TNBS.	27.9	32.0

Results are values obtained from pooled tissue from 10 lean and 5 obese mice.

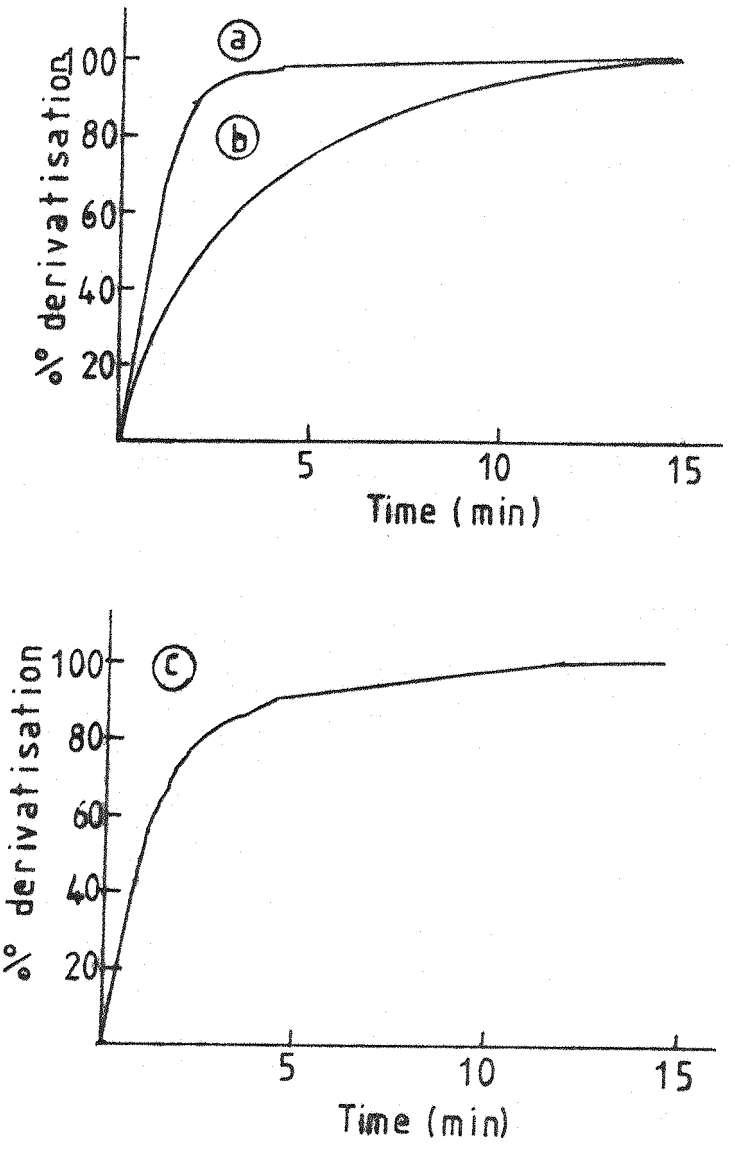

Fig 5.1. (a) Time course of derivatisation of 50nmoles PE in liposomes of PE and PC (50:34 mole/mole).

(b) Time course of derivatisation of 50nmoles PS in liposomes of PS and PC (5:20 mole/mole).

(c) Time course of derivatisation of 50nmoles of (PE + PS) in liposomes of PE, PS and PC (3:1:1 mole/mole/mole).

these hypothetical situations i.e. when PE only or PS only or PE/PS mixtures were located in the outer monolayer of native membranes exposed to the reagent.

It can be concluded that PE is entirely derivitised in 4 minutes and PS by 12 minutes under conditions where each was the sole amino phospholipid occupying the outer monolayer exposed to the reagent. The derivatisation of each phospholipid species is independant of the presence of the other, as a mixture of the two amino phospholipids yielded a time-course of derivatisation that approximated to a theoretical hyperbola obtained from the mean relative optical density contributions of each lipid class.

Thus, in a model membrane system, TNBS is able to completely derivatise the amino phospholipids in 12 min, at 25°C.

5.2.a. iii) Chromatography of adipocyte plasma membranes exposed to TNBS from intact cells; and from membranes associated with TNBS buffer.

Lipids were extracted from adipocyte plasma membranes prepared from intact adipocytes that had been exposed to TNBS and also from isolated plasma membranes that had been subsequently sonicated with TNBS buffer, and re-sedimented after the addition of a stop-reagent. Extracted lipids from the two groups of membranes from lean and obese mice were chromatographed as described in Materials and Methods along with commercially prepared standards of PE and PS, and also TNP-derivatives of these lipids prepared as previously described.

After development of the chromatogram, TNP-derivatives of the phospholipids, co-migrating with the standard TNP phospholipids were identified as yellow spots on the chromatogram. Very small ($\simeq$1nmole) amounts of TNP phospholipids could be detected visually. Other non-derivatised phospholipids were detected by DPH spray reagent, and the relative proportions of all phospholipids were determined as described in Materials and Methods. The results of these determinations are reported in Table 5.2.

When plasma membranes were sonicated into TNBS buffer, 100% of membrane PS and about 93% membrane PE was available for deriv-

Table 5.2. Phospholipid composition of adipocyte plasma membranes from lean and obese mice after exposure of adipocytes to TNBS buffer and isolated plasma membranes sonicated in TNBS buffer.

Phospholipid		PS	PI	SM	PC	TNP-PS	PE	TNP-PE
R_F		0.04	0.17	0.30	0.39	0.52	0.55	0.87
Lipid extract from plasma membranes from Adipocytes exposed to TNBS buffer	Lean*	9.5	7.0	16.0	38.0	ND	28.6	0.9
	Obese*	10.2	6.8	15.0	41.0	ND	26.2	0.8
Lipid extract from Adipocyte plasma membranes sonicated in TNBS buffer	Lean*	ND	6.8	15.5	41.7	8.0	2.0	26.0
	Obese*	ND	7.2	15.5	37	10.5	1.8	28.0

TNBS was 1.5mM, and results represent mean values for 2 determinations after 20 and 35 mins. exposure to TNBS. Results are means of 2 determinations from Adipocyte plasma membranes derived from 20 lean and 10 obese mice.

ND - not detectable.

* - Percentage of total phospholipid fraction.

TNP - Trinitrophenyl derivative of phospholipid.

atisation by TNBS. These results are in agreement with Fontaine and Schroeder (1979) who suggest that residual PE unavailable for derivatisation by TNBS may be tightly associated with membrane proteins. Plasma membranes from adipocytes exposed to TNBS buffer, however, demonstrated that in plasma membranes from both phenotypes PS was unavailable for derivatisation by TNBS, and most (97%) of PE was also unavailiable for derivatisation. These results suggest that the amino phospholipids occupy the inner half of the bilayer in adipocyte plasma membranes from lean and obese mice.

5.2.b. Analysis of the positional location of fatty acyl groups within PC and PE derived from adipocyte plasma membranes from lean and obese mice.

Phospholipase A2 was utilized to remove the fatty acyl moiety at carbon atom 2 of the glycerophopholipid under study. The 1-acyl lysophospholipid was then separated by TLC, and the fatty acid remaining at postion 1 was then trans-esterified and analysed by GLC. Tables 5.3 and 5.4 tabulate the results of this experiment. The composition of the 2-acyl moieties was calculated from the difference in composition of the acyl moieties of the phospholipid and the lysophospholipid, and these results are also included in the tables.

In both phospholipids, from both phenotypes, the fatty acids on the 1 position of the glycerol skeleton were more saturated than those at the 2 position, in agreement with published data, (see Miller et al., 1976). PC from adipocyte plasma membrane was more saturated at the 1 carbon position of the glycerocholine, and little difference was observed in the fatty acid spectrum at this position between PC from lean and obese mouse plasma membrane (Table 5.3). The fatty acids at the 2 carbon position, however, displayed a marked difference between the two phenotypes. Saturated fatty acid content was reduced, 18:1 and LCPUFA were increased (Table 5.3). Thus, the 2 carbon position of obese mouse adipocyte plasma membrane PC was more unsaturated than that of lean (Table 5.5). Similar, although more exaggerated, changes were observed in PE (Table 5.4). Again, fatty acids at carbon atom position 1 were little changed between the two phenotypes. How-

Table 5.3. Fatty acid composition of 1 acyl and 2 acyl lyso PC from lean and obese mouse adipocyte plasma membrane.

	Fatty Acid Composition (% total)					
	LEAN			OBESE		
	PC	1-LYSO PC	2-LYSO PC*	PC	1-LYSO PC	2-LYSO PC*
12:0	1.3	1.3	1.3	T	1.9	0
14:0	5.6	1.2	10	T	1.5	0
16:0	21.6	33	10.2	23.1	36.7	9.5
16:1	3.1	4.5	1.7	2.2	6.6	0
18:0	15.4	21.3	9.5	17.2	25.7	8.7
18:1	10.4	13.6	7.2	18.7	15.6	21.8
18:2	22.9	5.4	40.4	18.1	5.0	31.2
18:3	2.8	2.8	2.8	ND	ND	ND
20:2	3.3	1.7	4.9	3.2	1.0	5.4
20:4	5.0	2.1	7.9	6.8	2.9	10.7
22:2	2.2	1.3	3.1	1.2	0.7	1.7
22:4	3.2	0.9	5.5	ND	1.0	0
22:6	3.2	ND	6.4	9.5	1.4	17.6

* Calculated by subtraction of 1 acyl fatty acids from total phospholipid fatty acids.

Data represents lipids obtained from pooled tissue from 10 lean and 5 obese mice.

Table 5.4. Fatty acid composition of 1 acyl and 2 acyl PE from lean and obese mouse adipocyte plasma membrane.

	Fatty Acid Composition (% total)					
	LEAN			OBESE		
	PE	1-LYSO PE	2-LYSO PE*	PE	1-LYSO PE	2-LYSO PE*
12:0	1.3	1.1	1.4	1.3	0.7	1.65
14:0	6.5	0.8	10.96	0.7	1.3	0.2
16:0	26.50	33.5	17.5	14.4	23.7	4.4
16:1	0.2	6.3	0	3	4.6	1.2
18:0	18.9	24.8	11.7	9.2	26.6	0
18:1	8.2	19.6	0	10.7	18.3	2.6
18:2	8.5	3.6	12.1	12.4	8.8	13.9
18:3	3.1	0.5	5.1	2.9	5.9	0
20:0	1.2	1.0	1.3	T	1.1	0
20:2	1.5	1.7	1.3	T	1.1	0
20:4	13,1	3.7	20.4	18.1	2.9	28.9
22:2	0.2	1.0	0	T	1.6	0
22:4	0.2	1.6	0	ND	1.0	0
22:6	10.6	0.85	18.4	28.3	2.4	47.2

* Calculated by subtraction of 1 acyl fatty acids from total phospholipid fatty acids.

Data represents lipids obtained from pooled tissue from 10 lean and 5 obese mice.

ever, carbon atom 2 position fatty acids were altered to quite a considerable degree in PE from obese mouse membranes (Table 5.4). Saturated fatty acids were almost totally absent from position 2, and an impressive increase in 22:6 was observed.

Thus, the phospholipids from mouse adipocyte display marked positional assymetry in the distribution of unsaturated carbon bonds (Table 5.5).

The fluidisation observed in phospholipids from adipocyte plasma membranes of obese animals is achieved by the esterification of LCPUFA's at carbon atom 2 of the phospholipids, in place of saturated fatty acids (Table 5.5), 20:4 was increased at carbon atom 2 similarly in both PC and PE. However, the relative increase in fluidity observed for PE compared to PC in lipids from ob/ob mice arises from the increase in esterification of 22:6 at carbon atom 2 of PE.

5.2.c. Use of p N,N,N trimethylphenylamine 6 phenyl hexa-1,3,5-triene ($TADPH^+$) to probe adipocyte plasma membrane fluidity.

As the inner monolayer of a membrane is more negatively charged than the outer monolayer then assuming that the bilayer couple model of Sheetz and Singer (1974) applies to $TADPH^+$, as it does to other cationic drugs, $TADPH^+$ should partition into the inner monolayer.

$TADPH^+$ was incorporated into adipocytic plasma membrane isolated from lean and obese mice as previously described (Chapter 2). The fluorescence polarisation by probe labelled membranes was measured from 0°C-40°C, and the Arrhenius plots from this study are shown in Fig 5.2.

$TADPH^+$incorporation probed an environment that was considerably more fluid in obese membranes compared to lean controls. Although in practise the location of $TADPH^+$ is difficult to prove, no phase separation was apparent using $TADPH^+$ as probe, whereas phase separations were apparent in native membranes from lean and obese mice labelled with DPH (Chapter 4). $TADPH^+$ is sensitive to

Table 5.5. Summary of data obtained from GLC analysis of the positional location of fatty acids from PC and PE derived from lean and obese mouse adipocyte plasma membranes.

Phenotype	Phospholipid	% Saturated fatty acids	Pi bonds per 100 mol	% total Phospholipid LCPUFA at each position
Lean	PC	43.9	131	-
Obese	PC	40.3	153	-
Lean	1-ACYL LYSO PC	57	55.3	3
Obese	1-ACYL LYSO PC	63.9	56.8	5.3
Lean	2-ACYL LYSO PC*	31	206.1	19.8
Obese	2-ACYL LYSO PC*	18.1	247	28.3
Lean	PE	54.4	154.9	-
Obese	PE	25.6	289.4	-
Lean	1-ACYL LYSO PE	61.2	66.3	6.15
Obese	1-ACYL LYSO PE	53.4	93.6	6.3
Lean	2-ACYL LYSO PE*	42.3	234	38.8
Obese	2-ACYL LYSO PE*	6.3	430	76.1

* Calculated by subtraction of 1-acyl fatty acids from total phospholipid fatty acids.

Data represents lipids obtained from pooled tissue from 10 lean and 5 obese mice.

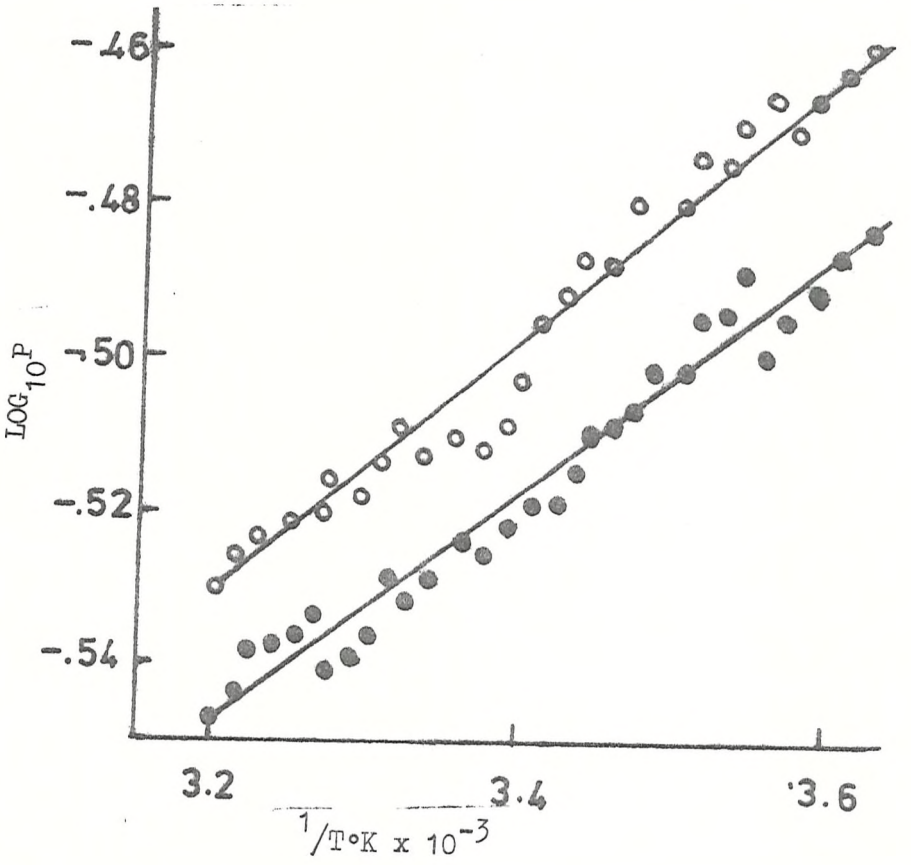

Fig 5.2. Arrhenius plots of depolarisation of fluorescence by $TADPH^+$ labelled adipocyte plasma membranes from lean (●) and obese (o) mice.
Membranes were derived from adipose tissue for 8 lean and 4 obese mice.

the gel to liquid crystalline phase transition of dimyristoyl PC (Chapter 2) and also to phase separations observed in SM/PC liposomes (Fig 5.3). These observations suggest that $TADPH^+$ is not located in regions containing lipids that undergo thermal phase separations in native adipocyte membranes. As phase separations were absent in lipid mixtures comprising the inner monolayer (section 5.2.d.) from lean and obese mice adipocyte plasma membranes, it is likely that $TADPH^+$ is probing the inner monolayer when incorporated into native membranes.

Flow activation energies for $TADPH^+$ and DPH probing of native membranes are different, and therefore the response of these two probes to changes in the viscosity of their environment are different. Because of this, fluidity ratio determinations are not comparable between polarisation measurements obtained with the two probes.

One solution to this problem is to convert polarisation differences (measured with the same probe) between two membrane systems into the temperature span that represents the polarisation difference. This can directly be interpolated from the Arrhenius polarisation plots, and is the temperature at which the more fluid membrane has the same viscosity as the less fluid membrane has at 37°C.

$TADPH^+$ probing of adipocyte membranes demonstrates that the obese mouse membranes would have to be cooled from 37°C to 25.5°C (a difference of 11.5°C) to achieve the same fluidity as that observed in lean membranes at 37°C. However, with DPH probing of adipocyte plasma membranes, a temperature difference of 5.3°C is observed (Chapter 4). This suggests that $TADPH^+$ is located in a lipid region more fluid than DPH. This observation is consistent with a fluidized inner monolayer of obese mouse adipocyte plasma membranes compared to lean controls in the native membrane. $TADPH^+$ equilibrated into liposomes of reconstituted outer and inner monolayers from lean and obese mouse adipocyte plasma membrane lipids * demonstrated that this probe was capable of detecting fluidity differences in complex lipid mixtures (Table 5.6) in a similar manner to DPH. The fluidity difference observed in reconstiuted

* This is explained more fully in section 5.2.d.

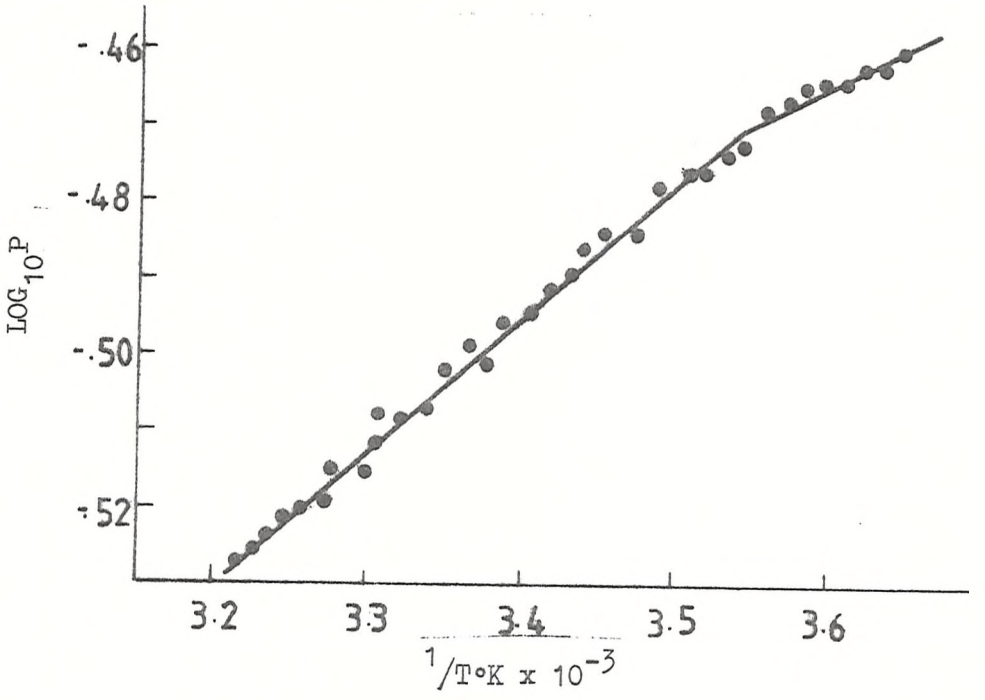

Fig 5.3. Arrhenius plots of depolarisation of fluorescence by $TADPH^{+}$ labelled liposomes of Egg PC and Bovine Brain SM (2:1 mole/mole)

Table 5.6. Summary of data from fluorescence polarisation studies on liposomes and membrane vesicles using $TADPH^+$ as fluorescent probe.

System Under Study	Source	P_{37}	ΔE above Breakpoint kcal mol^{-1}	ΔE below Breakpoint kcal mol^{-1}	Breakpoint °C
Adipocyte *					
Plasma	Lean	0.298	6.2	-	-
Membrane	Obese	0.283	6.6	-	-
Adipocyte Plasma					
Membrane *	Lean	0.301	-	-	-
PC:SM:C (2:1:1.1)	Obese	0.299			
Adipocyte Plasma					
Membrane *	Lean	0.290	-	-	-
PE:PS:PC:C (3:1:1:1.8)	Obese	0.279			
Liposomes	Egg/				
PC/SM (2:1)	Bovine	0.291	5.90	10.0	9.1
	Brain				

* Membranes and lipid extracts were derived from 8 lean and 4 obese mice.

inner monolayer liposomes from lean and obese mice was strikingly similar to that observed in native membranes.

This study provides further evidence for the altered fluidity assymetry of obese mouse adipocyte plasma membranes compared to lean controls.

5.2.d. Fluidity studies on reconstituted lipid vesicles comprising the inner and outer monolayers of adipocyte plasma membranes from lean and obese mice.

For these studies the molar ratio of cholesterol to phospholipid of each reconstituted monolayer was assumed to be identical between lean and obese membranes and also an equal symetrical distribution between outer and inner monolayer was assumed. Reconstituted liposome of outer monolayer phospholipids comprised PC:SM:Cholesterol in molar ratios 2:1:1.1 and inner monolayer liposomes, PE:PC:PS:Cholesterol, 3:1:1:1,8. These molar ratios were calculated on the basis of asymmetry studies performed in this chapter, with the assumption that the inner monolayer phospholipid content was 50% of the total phospholipid content of the membrane, and that PC was present as the only other phospholipid species in the inner monolayer with PE and PS. Cholesterol was added to a total Cholesterol to phospholipid molar ratio of 0.36, (established for the native membrane in Chapter 4), in both inner and outer monolayer model systems, as its relative distribution is unknown at the present. As the location of PI is unknown, this phospholipid was not included in these studies. The reconstituted liposomes were labelled with DPH, and the fluorescence polarisation measured from 2°C-40°C. The results of this study are shown in Figs 5.4 and 5.5, and the results are summarised in Tables 5.7 and 5.8. Experiments with reconstituted outer monolayer liposomes (Fig 5.4) demonstrated that lipid mixtures from obese mouse membranes were more fluid than those of lean, although the fluidity ratio was substantially lower than for whole membrane lipid mixtures. The influence of both SM and Cholesterol on PC fluidity was also investigated (Table 5.7). Mixing SM with PC reduced the fluidity ratio, as expected as SM was found to be more viscous from obese mouse membranes. The presence of cholesterol in PC mixtures did not produce the same reduction in fluidity ratio observed with SM.

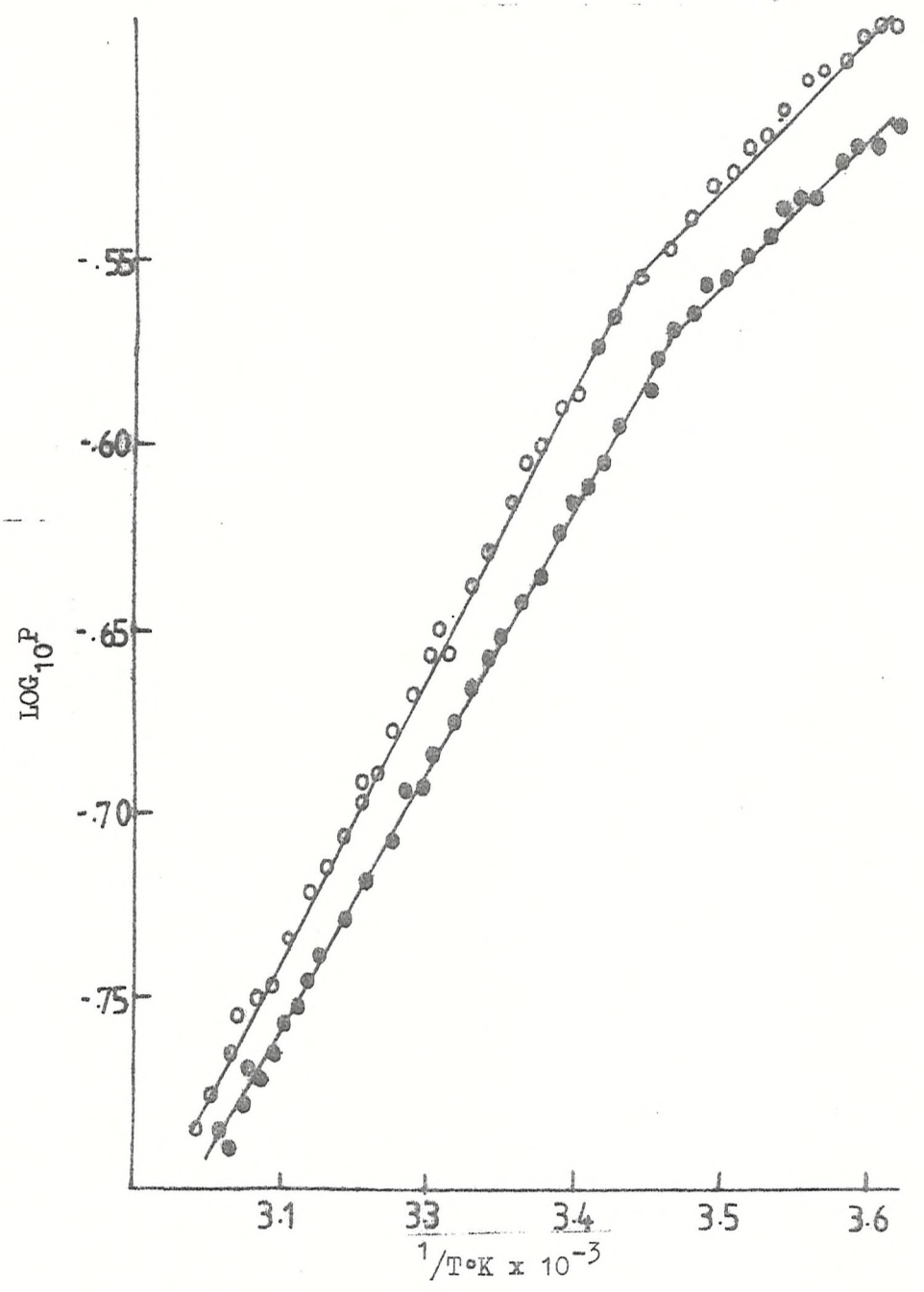

Fig 5.4. Arrhenius plots of fluorescence depolarisation by DPH labelled liposomes from reconstituted outer monolayer lipids from adipocyte plasma membranes from lean (●) and obese (o) mice.
Data represents lipids derived from 10 lean and 5 obese mice.

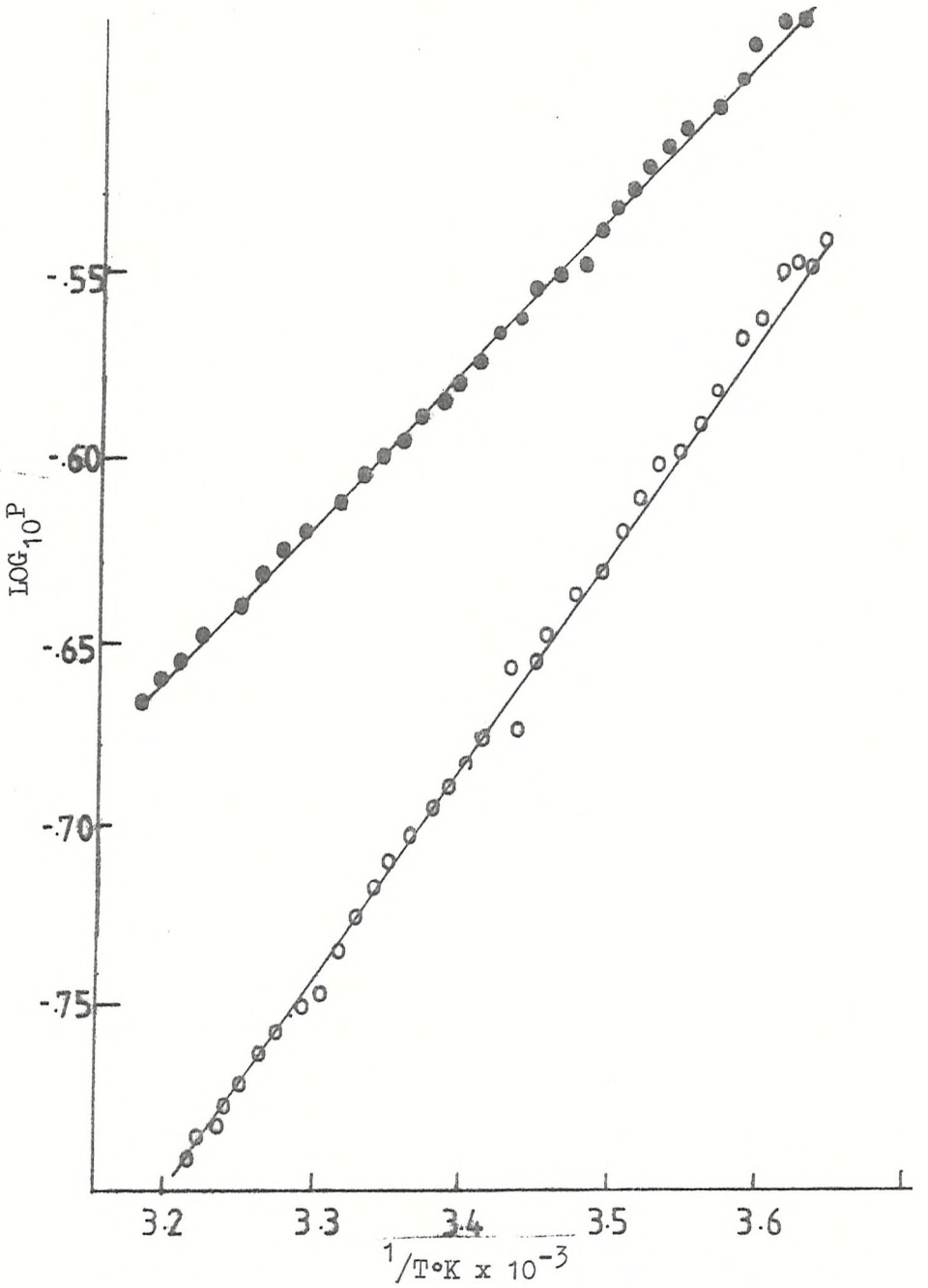

Fig 5.5. Arrhenius plots of fluorescence depolarisation by DPH labelled liposomes from reconstituted inner monolayer lipids from adipocyte plasma membranes from lean (●) and obese (o) mice.
Data represents lipids derived from 10 lean and 5 obese mice.

Table 5.7. Summary of data from Arrhenius plots of depolarisation of fluorescence by DPH labelled sonicated liposomes of various lipid mixtures derived from adipocyte plasma membranes from lean and obese mice housed at 25°C.

Phenotype	LEAN				OBESE				
Lipid Mixture (Molar ratio)	Break-point °C	P_{37}	E above breakpoint kcal mol^{-1}	E below Breakpoint kcal mol^{-1}	Break-point °C	P_{37}	ΔE above Breakpoint kcal mol^{-1}	ΔE below Breakpoint kcal mol^{-1}	Fluidity Ratio $\frac{\Phi}{\Phi} R_{37}$
PC -	-	0.136	7.88	-	-	0.129	6.34	-	1.10
SM -	30.0	0.204	3.46	12.79	29.1	0.225	5.33	12.3	0.83
PC + C 3 : 1.1	-	0.174	7.12	-	-	0.159	6.05	-	1.15
PC + SM 2 : 1	9.5	0.165	8.08	12.04	12.7	0.159	6.44	10.54	1.06
*PC + SM + C 2 : 1 : 1.1	15.6	0.190	6.58	11.16	18.1	0.183	6.07	13.30	1.07

* Lipid mixture for model for outer monolayer of adipocyte plasma membrane.

Data represents lipids obtained from pooled adipose tissue from 10 lean and 5 obese mice.

Table 5.8. Summary of data from Arrhenius plots of depolarisation of fluorescence by DPH labelled sonicated liposomes derived from adipocyte plasma membranes from lean and obese mice housed at 25°C.

Phenotype	LEAN				OBESE				
Lipid Mixture (Molar Ratio)	Break-point °C	P_{37}	ΔE above Breakpoint kcal mol^{-1}	ΔE below Breakpoint kcal mol^{-1}	Break-point °C	P_{37}	ΔE above Breakpoint kcal mol^{-1}	ΔE below Breakpoint kcal mol^{-1}	Fluidity Ratio ΦR_{37}
PE	-	0.209	14.39	-	-	0.168	9.05	-	1.45
PS	19.4	0.224	8.1	9.25	19.3	0.190	9.6	12.8	1.35
PC	-	0.136	7.88	-	-	0.129	6.34	-	1.10
*PE:PC:PS:C 3: 1: 1:1.8	-	0.2	10.8	-	-	0.165	7.9	-	1.

* Lipid mixture for model 0.224 for inner monolayer of adipocyte plasma membrane 169

Data represents lipids obtained from pooled adipose tissue from 10 lean and 5 obese mice.

An anomaly observed in this experiment was that the breakpoint in outer monolayer model lipid mixtures was lower in liposomes from lean compared to those from obese mice (Table 5.7), whereas in native membranes, and lipid extracts, the reverse is true. The explanation for this paradox is not clear at present. Thus, this experiment suggests that the outer monolayer is more fluid in adipocyte plasma membranes from obese animals, but is less fluid than the total membrane lipid extract.

Liposomes modelling the inner monolayer of adipocyte plasma membranes from obese mice were considerably fluidized compared to those from lean (Fig 5.5). The fluidity ratio was greater than that observed for whole membrane lipid extracts (Table 5.8), and the flow activation energy was reduced. These observation support the concept that the inner monolayer is more fluid in adipocyte plasma membranes from obese mice compared to lean controls. The reduced flow activation energy is a factor that appears to be associated with more fluid phospholipids, and is likely to be associated either with a decreased saturated fatty acid content or an increased LCPUFA content of phospholipids.

5.2.e. The use of cholesta-5,7,9-triene-3-β-ol (CTO) to determine the incorporation of cholesterol into liposomes of varying phospholipid composition.

Liposomes containing commercially prepared phospholipids of different classes were assessed for their ability to form ordered mixtures with cholesterol. CTO probe was used as a fluorescent cholesterol anlaogue, and titrations were performed of fluorescence intensity against molar ratio probe:phospholipid (0.1-0.6 mole mole^{-1}). At 0.6 mole mole^{-1} CTO, the optical density of the liposomes suspension did not exceed 0.3. (Inner filter and self absorbtion effects become significant in decreasing the fluorescence yield at an OD>0.375, (Rogers, 1979)). For all phospholipids under study except PE, the fluorescence yield was proportional to probe concentration (Fig 5.6) over the range of probe concentrations studied. The difference in slopes of the plots (Table 5.9) suggest that CTO probe is interacting in a different manner with phospholipids of different classes. The slope of the fluorescence titration for PE (Fig 5.6) is biphasic. The slope of this titra-

tion is recorded in Table 5.9 as that between 0.2-0.6 molar ratio cholesterol to PE. Assuming that a decreasing rate of increase of fluorescence intensity between liposomes of different phospholipid classes is caused by an increasing concentration quenching effect, then the slopes of these plots may reflect the ability of CTO and phospholipids to form ordered arrays so as to prevent the sideways overlap of triene system of the probe. The ordering ability of the phospholipids and phospholipid mixtures is therefore SM>PS>PE/PS/PC>PC>>PE (Table 5.9). This series compares favourably to the affinity of cholesterol for the phospholipids, SM>PS>PC>>PE observed by Demel et al. (1977) using DSC techniques.

That the slopes of these plots were a reflection of the degree of concentration quenching of CTO probe within the liposomes matrix, and not a function of differing quenching mechanisms, was supported by polarisation studies performed in the same manner as the previous investigation. The concentration of CTO probe was kept constant, and the cholesterol content of different phospholipid liposomes was varied between 0-0.8 mole per mole phospholipid. At each titration point, the polarisation by the CTO probe was measured at 37°C. The results of these studies are shown in Fig 5.7. Each experiment was repeated twice and the mean values of the slopes of these plots were determined. This value represents the relative increase in polarisation produced by incorporation of a unit amount of cholesterol (ΔP). These results are summarised in Table 5.10. ΔP was found to decrease in liposome classes in the same order as that found for fluorescence intensity studies, i.e. PC/SM>PE/PS/PC>PS>>PE. Cholesterol/polarisation titrations were also measured for liposomes containing Dilauryl PE/PS/PC and 1-acyl, 2-docosahexaenoyl PE/PS/PC (Fig 5.7). P for these studies (Table 5.10) was found to decrease for different PE species in the order Hen Egg PE> Dilauryl PE>>1-acyl, 2-docosahexaenoyl PE.

The increase in polarisation of the probe caused by increasing cholesterol concentration is the result of increasing the viscosity of the liposomes by the addition of cholesterol within

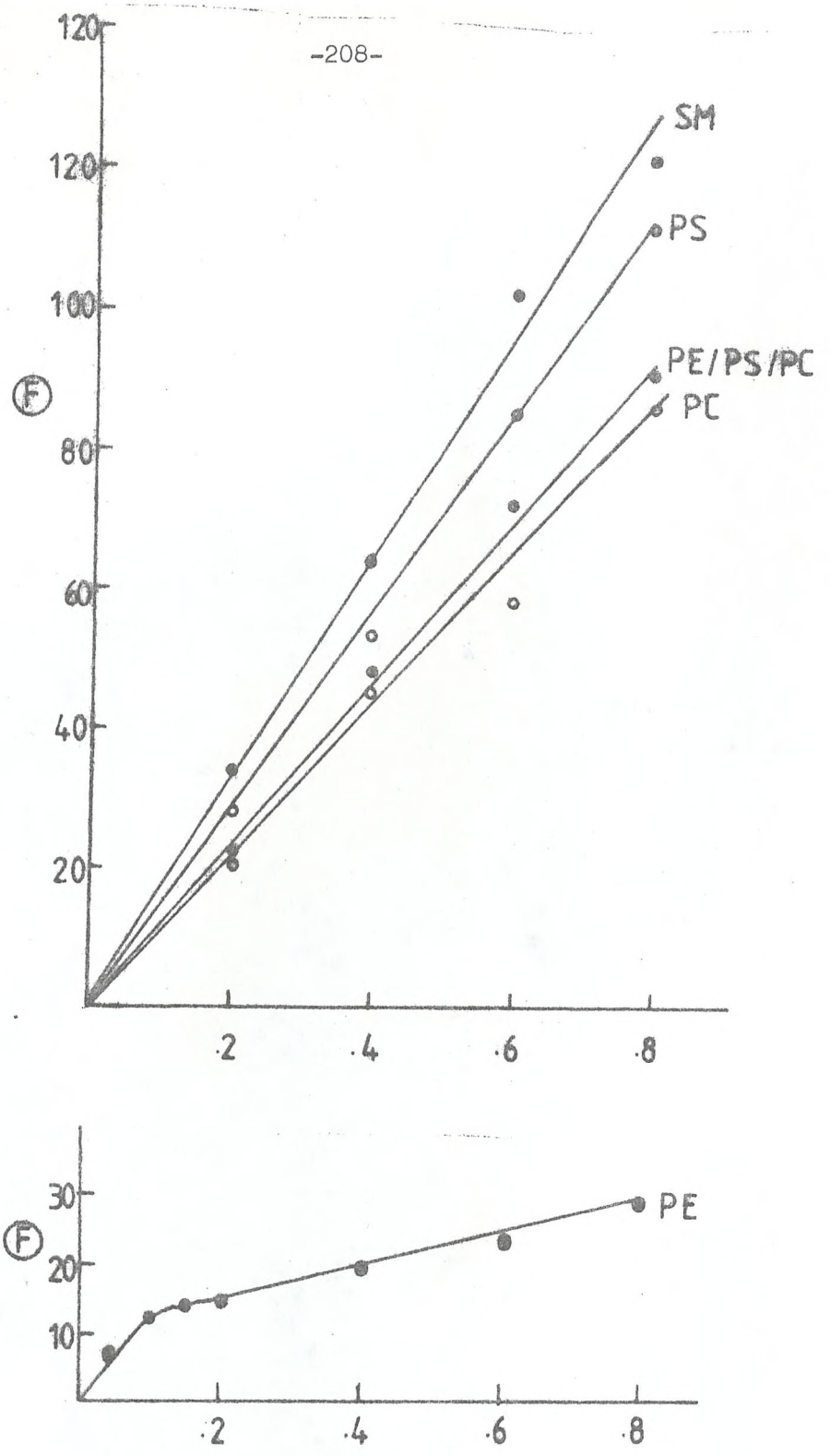

Fig 5.6. Plots of fluorescence intensity against concentration of the fluorescent cholesterol analogue, Cholesta-5,7,9-triene-3-β-ol in phospholipid liposomes at 37°C.

Abscissae are molar ratio cholesterol to phospholipid

Table 5.9. Titration slopes of fluorescence intensity as a function of the molar increase in Cholesta-5,7,9-triene-3-β-ol in various phospholipid liposomes at 37°C.

Phospholipid	Molar Ratio	Source	Slope of fluorescence titration
PC	-	Hen Egg	1.08
PS	-	Bovine Brain	1.36
PE	-	Hen Egg	0.24
SM	-	Bovine Brain	1.53
PE/PS/PC	3:1:1	Hen Egg/Bovine Brain/Hen Egg	1.15

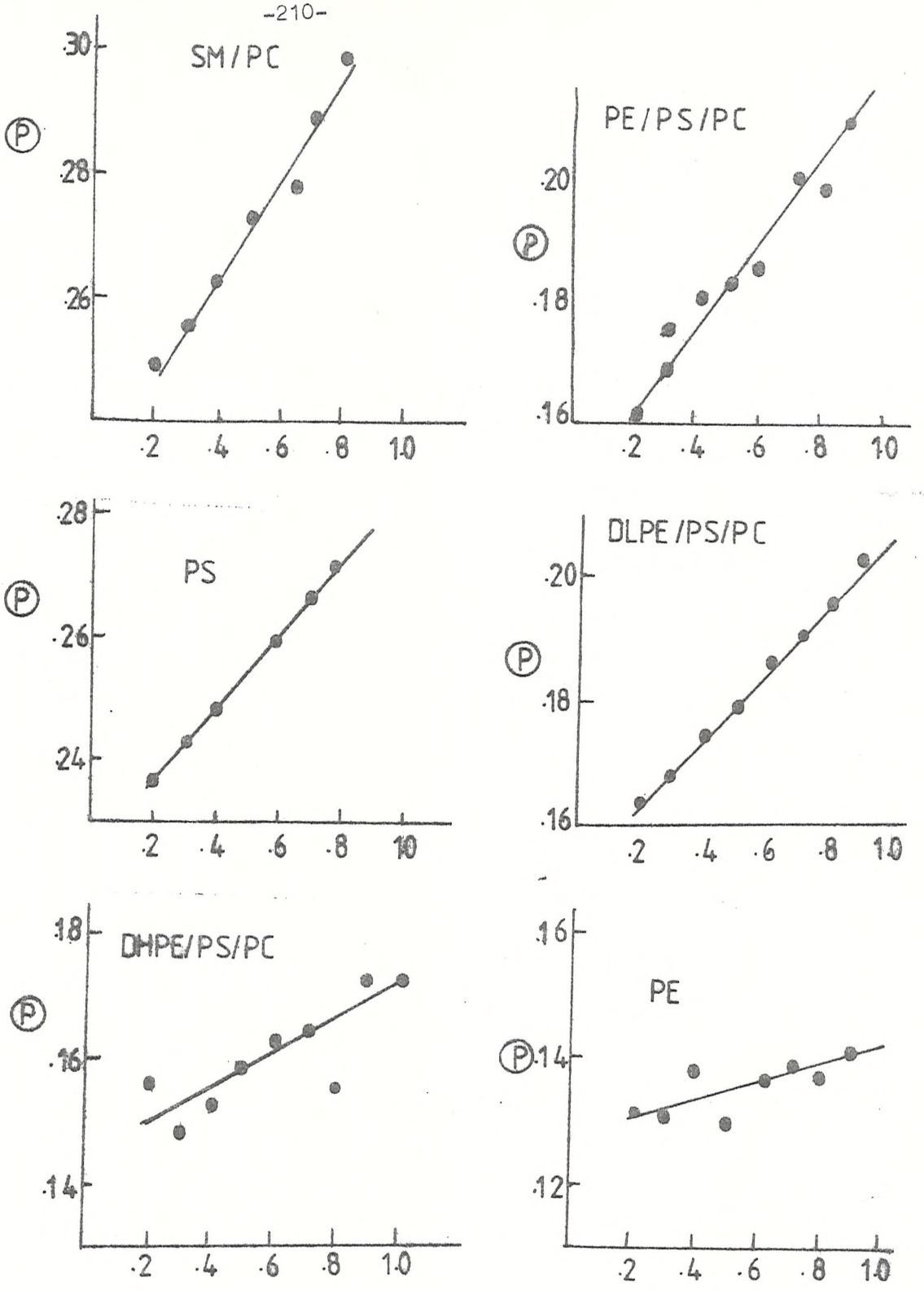

Fig 5.7. Plots of fluorescence polarisation of Cholesta-5,7,9-triene-3-β-ol labelled liposomes against increasing cholesterol concentration at 37°C.

Abscissae are molar ratio cholesterol to phospholipid

Table 5.10. The effect of increasing cholesterol incorporation on the fluorescence polarisation of Cholesta-5,7,9-triene-3-β-ol labelled phospholipid liposomes at 37°C.

Lipid	Molar Ratio	Source	Buffer	Slope (ΔP)
PC/SM	2 : 1	Hen Egg/Bovine Brain	A	0.083
PE	-	Hen Egg	B	0.016
PS	-	Bovine Brain		0.059
PE/PS/PC	3 : 1 :1	Hen Egg/Bovine Brain/Hen Egg	B	0.068
DLPE/PS/PC	"	Synthetic/Bovine Brain/Hen Egg	B	0.053
DHPE/PS/PC	"	"	B	0.028

Buffer A - 100mM NaCl, 20mM KCl, 1mM $CaCl_2$, 10mM Tris-HCl pH 7.4

Buffer B - 100mM KCl, 20mM NaCl, 100μM $CaCl_2$, 10mM Tris-HCl pH 7.4

DLPE - dilauryl PE

DHPE - 1-acyl, 2-docosahexaenoyl PE

any one phospholipid mixture. A smaller value of ΔP will tend to indicate that cholesterol has less influence on the acyl chain mobility of the phospholipids in one liposome type compared to another. If this reduced ability of cholesterol to increase the degree of order of the acyl chains is related to reduced cholesterol/phospholipid ordering within the liposomes, then depolarising influences of Förster energy transfer may also influence the value of ΔP.

The results of this study serve to suggest that fluorescent techniques may be useful in probing the organisation of cholesterol/phospholipid mixtures, simply by observing the relative changes of fluorescent properties of an incorporated probe with increasing cholesterol/phospholipid ratio. As the fluorescent probe used here is an analogue of cholesterol, it is hoped that fluorescent measurement gives direct information about the environment of cholesterol, and hence its association with phospholipid acyl chains in the liposome matrix. The ability of different phospholipids to cause changes in fluorescence properties of incorporated probe closely parallel affinity sequences of cholesterol for phospholipids, measured by DSC (Demel et al. 1977), and therefore this technique may be very useful in probing affinities of cholesterol for complex lipid mixtures.

5.2.f. <u>The interaction of cholesterol with various phospholipids and phospholipids derived from lean and obese mouse adipocyte plasma membranes measured by DPH polarisation.</u>

The validity of the use of fluorescent probes in elucidating cholesterol-phospholipid interaction has been described. Further supportive evidence for this technique is described using DPH as viscosity probe. The ability of cholesterol to increase the viscosity of liposomes can be sensitively probed by DPH polarisation. The possible use of DPH was evaluated on a series of commercially prepared phospholipids for comparison with data obtained using CTO probe, and DPH was also used to assess the interaction of cholesterol with liposomes of various phospholipid mixtures obtained from lean and obese mice.

Commercially available phospholipids were assessed for their ability to interact with varying concentrations of cholesterol

in sonicated liposomes as described in Materials and Methods using DPH as a viscosity probe. The results of this study are shown in Figs 5.10 and 5.11, and the interaction indices of these phospholipids (Table 5.11) suggests that the affinity for cholesterol of these phospholipids is SM>PS>PC>>PE, in close agreement with published data for studies employing differential scanning calorimetry to probe phospholipid-cholesterol interaction (Demel et al., 1972).

Phospholipids from commercial sources were combined to form liposomes that represented model systems for examining the cholesterol interaction with native adipocyte membrane inner and outer monolayers. From the previous study investigating the amino phospholipid distribution in lean and obese mouse adipocyte plasma membranes, the outer monolayer was represented by PC/SM (2:1 mole/mole) and the inner monolayer by PE/PS/PC (3:1:1 mole/mole/mole/). The buffers used in this study approximated to the ionic composition of the extracellular fluid for PC/SM mixtures and the cytosolic ionic composition for PE/PS/PC mixtures (Table 5.11). When liposome models for the inner and outer monolayers of adipocyte plasma membranes were prepared from commercially available phospholipids, there was no apparent difference in their interaction with cholesterol. Substituting dilauryl PE for egg PE in PE/PS/PC inner monolayer liposomes had little effect on the interaction of these liposomes with cholesterol. However, when 2-docosahexaenoyl PE (Synthesis described in Materials and Methods) was substituted for Egg PE, the interaction of cholesterol with these liposomes was reduced, suggesting that 22:6 interacts less well with cholesterol than either 18:1 (major fatty acid on 2 position of Egg PE) or 12:0 (on position 2 of dilauryl PE). Liposomes containing only 2-Docosahexaenoyl PE were found to exhibit a very low interaction with cholesterol (Table 5.11).

These results suggest that the presence of 22:6 on PE can affect the cholesterol interaction with the monolayer as a whole. When 22:6 PC was substituted for Egg PC to model outer monolayers, an increase in the interaction index was observed (Table 5.11). This suggests that 22:6 is capable of modifying the cholesterol affinity for the outer monolayer. The reduction in cholesterol affinity for model outer monolayers, by incorporation of 22:6 PC was not as great as incorporation of 22:6 PE in model inner monolayer lipid mixtures; despite a greater molar ratio of PC to

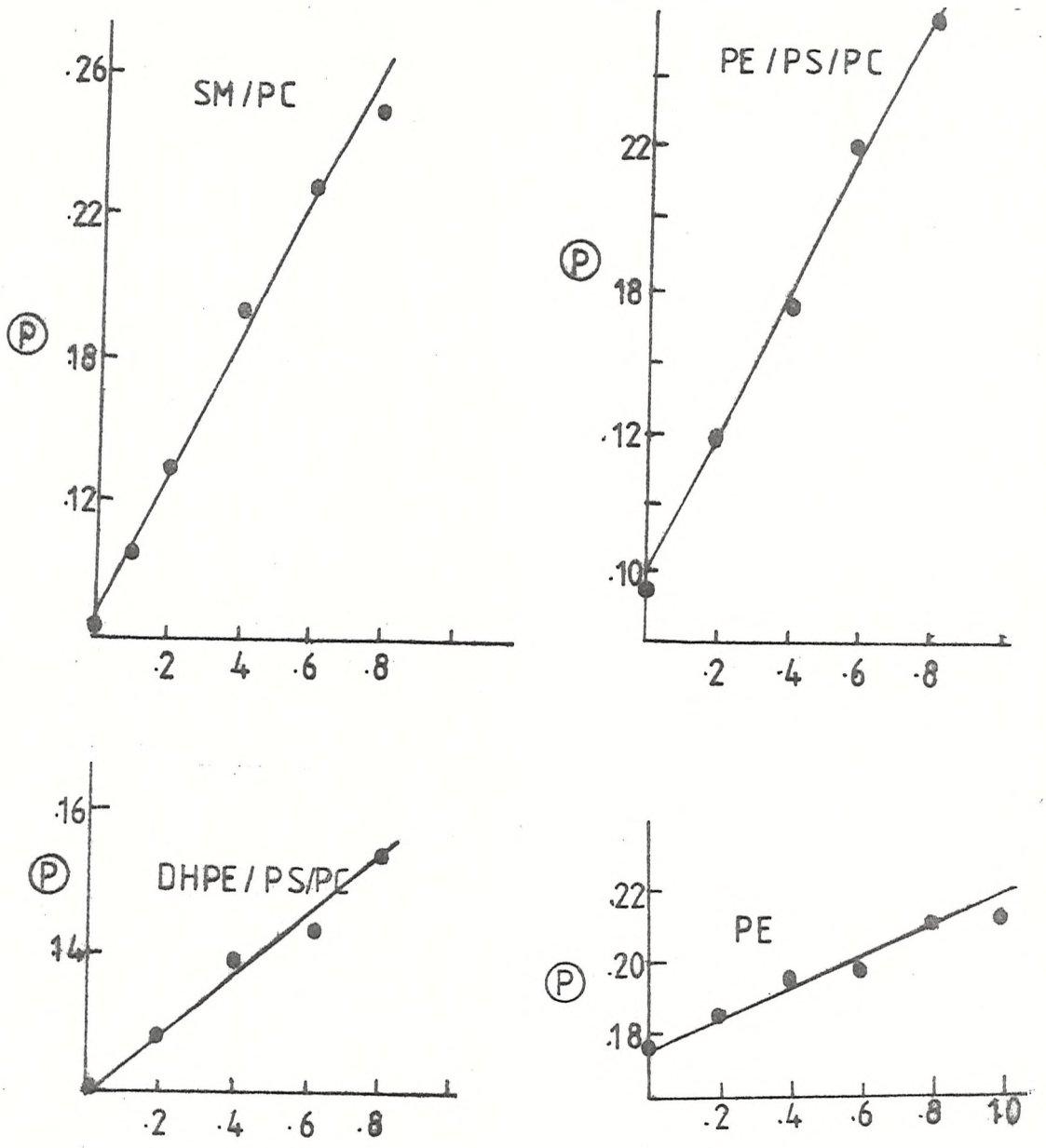

Fig 5.10. Plots of fluorescence polarisation of DPH labelled phospholipid liposomes against increasing cholesterol concentration at 37°C.

Abscissae are molar ratio cholesterol to phospholipid

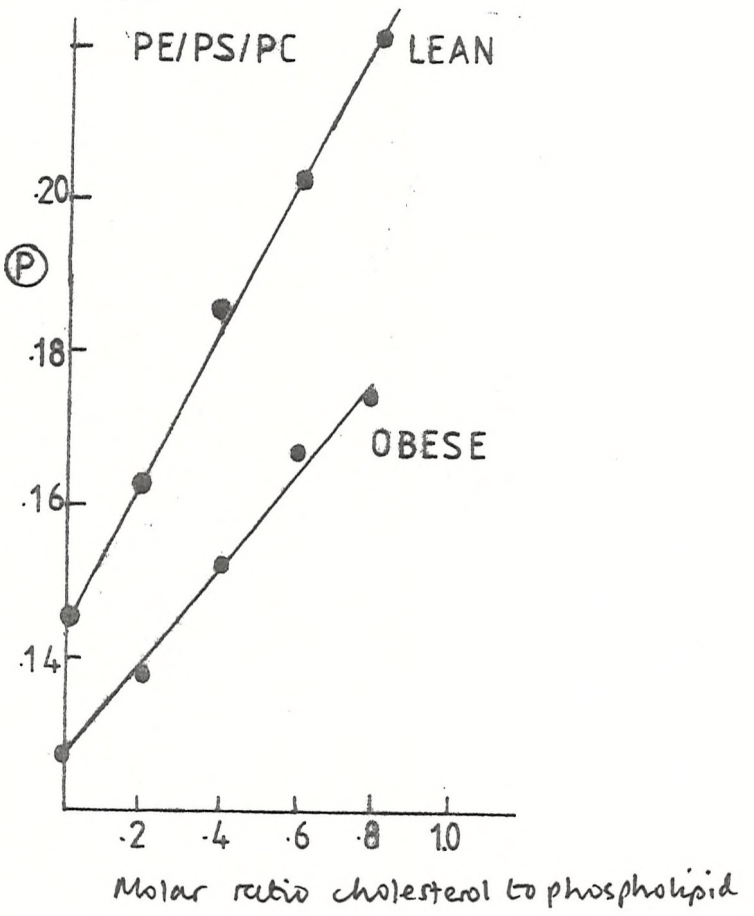

Fig 5.11. Plots of fluorescence polarisation of DPH labelled phospholipid liposomes against increasing cholesterol concentration at 37°C.

Table 5.11. Cholesterol interaction indices (i_{37}) for various phospholipids and phospholipid mixtures at 37°C measured by DPH fluorescence polarisation.

Lipid	Molar ratio	Source	Buffer	i_{37}
SM	-	Bovine Brain	A	0.24
PC	-	Hen Egg	A	0.50
PC/SM	2:1	Hen Egg/Bovine Brain	A	0.45
2-(22:6) PC/SM	2:1	Synthetic/Bovine Brain	A	0.65
PS	-	Bovine Brain	C	0.31
PE	-	Hen Egg	B	2.5
PI	-	Soya Bean	C	1.1
2-(22:6) PE	-	Synthetic	B	3.5
PE/PS/PC	3:1:1	Hen Egg/Bov. Br./Hen Egg	B	0.46
1,2 (12:0) PE/PS/PC	"	Synth./Bov. Br./Hen Egg	B	0.45
2 (22:6) PE/PS/PC	"	"	B	1.89

Results are mean values of 2 experiments.

Buffer A - 100mM NaCl, 20mM KCl, 1mM $CaCl_2$, 10mM Tris pH 7.4 at 37°.

Buffer B - 100mM KCl, 20mM NaCl, 100μM $CaCl_2$, 10mM Tris pH 7.4 at 37°.

Buffer C - 100mM KCl, 20mM NaCl, 1mM EDTA, 10mM Tris pH 7.4 AT 37°.

total phospholipid than PE to total phospholipid (Table 5.11). This observation may be due to PE having a lower affinity for cholesterol than PC, and suggests that 2-docosahexaenoyl PE has the lowest affinity for cholesterol of any of the naturally occurring phospholipids.

Table 5.12. Cholesterol interaction indices (i_{37}) for phospholipid mixtures from lean and obese mouse adipocyte plasma membranes measured by DPH fluorescence depolarisation at 37°C.

Lipid	Molar Ratio	Source	Buffer	i_{37}
PC/SM	2:1	Lean mice	A	0.51
PC/SM	2:1	Obese mice	A	0.52
PE/PS/PC	3:1:1	Lean mice	B	0.68
PE/PS/PC	3:1:1	Obese mice	B	1.57

Results are values from 1 experiment.

Phospholipids were prepared from pooled adipocytes from 20 lean and 10 obese mice.

Buffers are those described in Table 5.11.

Liposomes prepared from phospholipids isolated from lean and obese mouse adipocyte plasma membranes were reconstituted to model the inner (PE/PS/PC, 3:1:1) and outer (PC/SM, 2:1) monolayer of the native membrane, as determined in section 6.1, and the cholesterol interaction index (i_{37}) was measured by DPH polarisation. The results from this study are shown in Table 5.12. PC/SM mixtures from both lean and obese mice adipocyte plasma membranes displayed a higher i_{37} than Hen Egg/Bovine Brain SM mixtures, but were not different from each other. Lean and obese mouse PE/PS/PC mixtures, however, displayed a marked difference. Obese mouse lipid mixtures requiring 2.3 times as much cholesterol to raise the viscosity of these liposomes by 1 Poise as do those from lean mice adipocytes.

Thus, the inner monolayer of obese mouse adipocytes has a lower affinity for its endogenous cholesterol than does the inner monolayer of lean mouse adipocyte plasma membrane. The outer monolayers, however, appear to have an equal affinity for endogenous cholesterol from lean or obese mouse adipocyte plasma membrane.

5.2.g. Investigations into the influence of the fatty acyl composition of PE on the flow activation energy of DPH labelled liposomes.

In order to elucidate some general rules relating the value of the flow activation energy (ΔE) to molecular structure, Arrhenius plots of fluorescence polarisation by DPH labelled liposomes were plotted for a number of different PE species. ΔE was obtained from Arrhenius plots ot these results (Table 5.13).

Low ΔE values were obtained for dilauryl PE (DLPE), mouse brain synaptosomal PE and docosahexaenoyl PE (DHPE), indicating a high degree of order within these systems. Mixing equimolar proportions of DLPE and DHPE gave ΔE an intermediary value between ΔE for each component, suggesting that a value of ΔE in lipid mixtues may be an averaged value for each component. Dipalimtoyl PE (DPPE) undergoes a gel to liquid crystalline phase transition at 41°C, therefore this lipid exists in the gel phase over the assay temperature range. Equimolar mixtures of DPPE and DHPE

Table 5.12. Flow activation energies (ΔE) of various PE liposomes labelled with DPH.

Phospholipid	Source	Flow Activation Energy (kcal mol^{-1})
PE	Hen Egg	13.5
DLPE *	Synthetic (Sigma)	3.70
PE	E Coli	12.8
PE	Soya Bean	13.2
PE	Lean mouse Adipocyte	9.05
PE	Obese mouse synaptosome	3.48
DHPE	Synthetic	5.23
DHPE/DLPE (1:1)*	Synthetic/synthetic	4.58
DPPE/DHPE (1:1)	Synthetic/synthetic	12.9

DLPE - 1,2,dilauryl PE

DPPE - 1,2,dipalmitoyl PE

DHPE - 1 acyl, 2 docosahexaenoyl PE

Temperature ranges were 2°C-40°C except * which was 12°C-40°C.

increased ΔE from that obtained with DHPE. As gel phase lipid is highly ordered, this observation indicates that general rules concerning the relationship of ΔE to molecular structure cannot be applied when gel phase lipid exists within liposome systems. However, as only SM is the only phospholipid that contains some gel phase lipid over the normal assay range, this factor may not be important in interpretation of data using mammlian biological membrane lipids. E Coli PE, Soya bean PE and Hen Egg PE all had a relatively high ΔE. All these PE species contain mainly 1-saturated 2-unsaturated fatty acyl species.

Therefore the substitution of docosahexanoic acid for oleic acid (main position 2 fatty acid on E Coli PE and Soya bean PE) results in a marked change in ΔE. As this species is increased in obese mouse membranes it is likely that this fatty acid contributes to the consistant changes in ΔE observed in phospholipid liposomes, lipid extract liposomes and membrane vesicles from obese mice compared to those from lean controls.

5.3. Discussion

5.3 . Organisation of the lipids of adipocyte plasma membrane.

Studies with the non-permeant amino group derivatising reagent TNBS, revealed that in the amino phospholipids, adipocyte plasma membranes from both lean and obese mice are unavailable for derivatisation. Parallel control experiments support the concept that these phospholipids are located in the inner monolayer of the phospholipid bilayer. As the localisation of PI is unknown, the approximate phospholipid distribution in the adipocyte plasma membrane can be calculated assuming equal PI in each bilayer half (Fig 5.12). Thus, the adipocyte plasma membrane phospholipids are asymmetrically oriented vectorially in the adipocyte membrane, this asymmetry of the amino phospholipids is in close agreement with several other reports using TNBS as derivatising reagent. Shick et al.,(1976) evaluated the lipid sidedness of human platelets, and showed that only 12-18% of the total PE was accessible to TNBS and that PS was not labelled. In a murine cell line, TNBS reacted with only 4% of PE and 5% of the PS. (Fontaine and Schroeder, 1979). Asymmetry has also been investigated in isolated sealed synaptosome vesicles from mouse brain by Fontaine et al., 1980. These authors demonstrated that 10-15% PE and 20% PS were available for reaction with TNBS, and therefore the majority of the amino phospholipids occupy the inner monolayer of right-side-out synaptosomal vesicles.

Although the factors causing the generation of trans-bilayer phospholipid asymmetry are unclear at the present, Fontaine et al. (1980) have demonstrated that neither plasma lipoprotein phospholipid exchange processes or sequential head group methylation of selective monolayer PE molecules are necessary events in the generation of transmembrane phospholipid asymmetry. As 'flip-flop' times for phospholipids in membranes are comparatively short compared to the lifetime of the membrane, then the maintainance of a phospholipid asymmetry must require that either phospholipids interact preferentially with asymmetric membrane proteins, or that the presence of certain species of phospholipids in membrane regions is thermodynamically favourable. Evidence exists for both postulates. Many membrane proteins interact specifically with certain

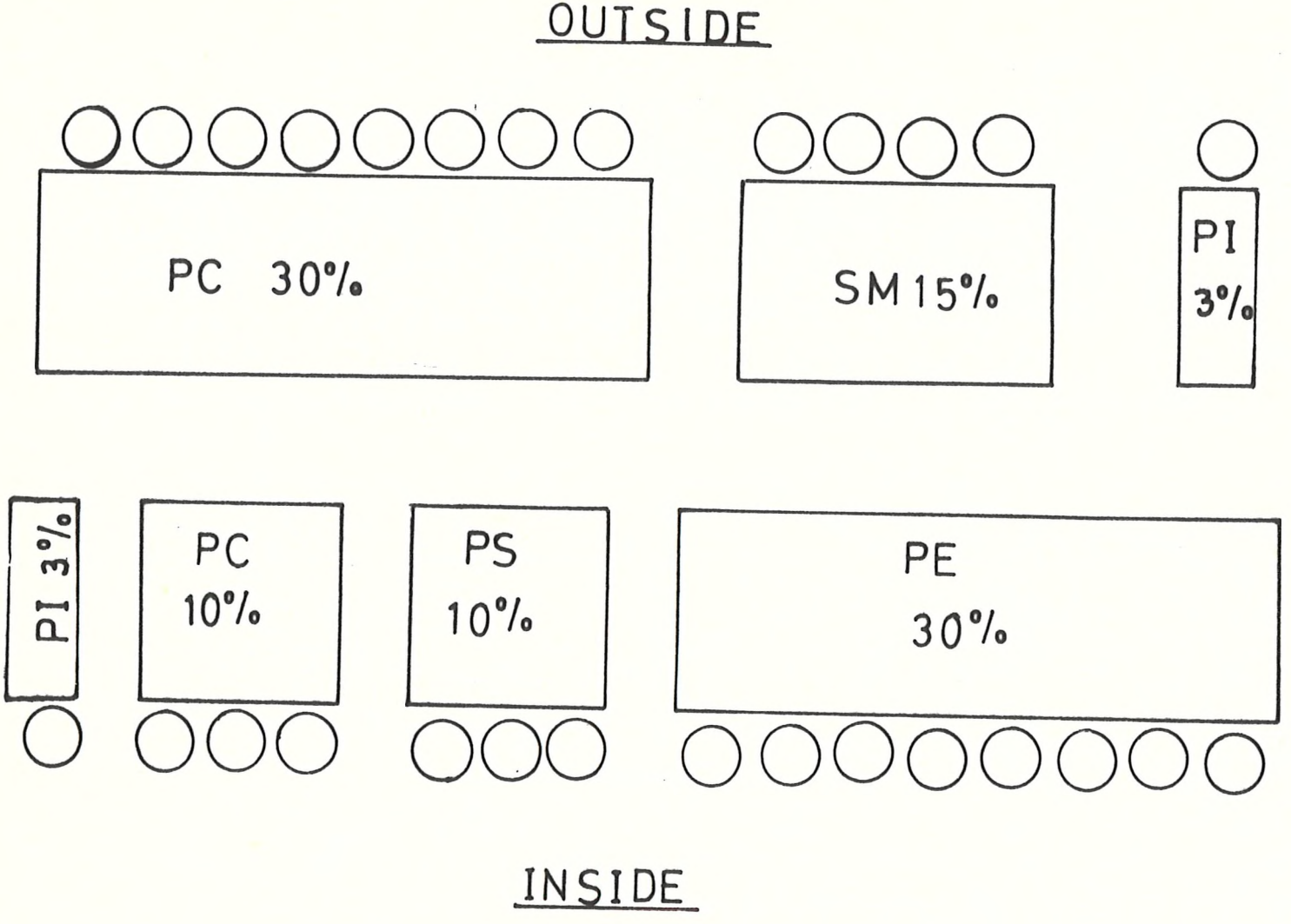

Fig 5.12. Phospholipid assymetric distribution across the plasma membrane bilayer of mice adipocytes.

phospholipid classes. (This has been discussed in Chapter 3) and it has been proposed that the phospholipid assymetry of erythrocyte plasma membrane is maintained by the cytosolic submembrane microfillamentous protein, Spectrin (Haest et al., 1977). De Kruyff and Baken (1978) provided evidence that the negatively charged phospholipid, phosphatidic acid is rapidly translocated to the inner half of vesicles of PE, with the exchange of PC from inner to outer monolayer. Negatively charged phospholipids have been suggested to preferentially reside in the inner monolayer due to their smaller molecular volume (De Kruyff and Baken, 1978).

The composition of the fatty acyl chains themselves may also result in phospholipid asymmetry. It has been demonstrated that a greater percentage of unsaturated PE acyl groups were present in the inside monolayer of both murine LM cell plasma membrane (Fontaine and Schroeder, 1979) and murine synaptosomal membranes (Fontaine et al., 1980). This may arise as a result of the observed preferential orientation of certain fatty acyl/head group combinations for different bilayer halves (Yeagle et al., 1976; De Kruyff and Baken, 1978). As PE and PS contain more unsaturated fatty acids than PC and SM, this may provide some of the driving force for the observed assymetry of these phospholipids. In conclusion, phospholipid assymetry in membranes may be a result of both the interaction between the fatty acyl chains and the phospholipid headgroups, and also from the preferential interaction of different phospholipid classes with oriented proteins. However, the lack of any observable difference in phospholipid assymetry between lean and obese mouse adipocyte plasma membrane suggests that gross changes in the fatty acid composition of phospholipids observed in ob/ob mouse membranes does not alter the transbilayer phospholipid composition.

Studies with the inner monolayer fluorescent probe $TADPH^{+}$, support the concept that the inner monolayer of membranes from obese animals is more fluid than the lean controls. Although this evidence by itself is not conclusive, other experiments have been initiated to probe the viscosity characteristics of the outer monolayer of native membranes, and thus it is hoped that a high

degree of experimental consistency will validate the concept of differing monolayer fluidities apparent from membrane aminophospholipid labelling experiments.

Having established that the gross membrane architecture does not appear to be altered in adipocyte membrane from obese animals compared to lean controls, more specific identification of the gross fatty acid compositional changes observed in Chapter 4 between the phospholipids isolated from plasma membranes from lean and obese mice revealed that carbon atom 2 of the glycerophospholipid backbone contained variable quantities of long chain polyunsaturated fatty acids 20:4 and 22:6 (LCPUFA). Variable LCPUFA content was observed between similar phospholipid classes from each phenotype, and also between different phospholipid classes from the same phenotype, in agreement with results from phospholipids from goldfish (Miller et al., 1976) and trout (Hazel, 1979). These authors also found that 22:6 was the major regulated LCPUFA on cold acclimation of these species, and carbon atom 2 was the major acceptor site for LCPUFA. In goldfish intestinal microsomes, PE was the major regulated phospholipid on cold adaption, whereas in trout liver, PC was the major regulated phospholipid. In the development of obesity in the ob/ob mouse, it is PE that undergoes the largest increase in LCPUFA compositional change in the adipocyte plasma membrane. These observations suggest that the insertion of LCPUFA's at carbon atom 2 of phospholipids may have an important regulatory function on membrane related processes.

5.3.2. Phospholipid fatty acid composition and cholesterol interaction.

Fluorescent probes were used to investigate the interaction between cholesterol and phospholipids within the membrane bilayer. Studies reported in this chapter strongly support the use of these probes to investigate the interaction of cholesterol with complex lipid mixtures. The variability of cholesterol to interact with different lipid mixtures reported here are in agreement with DSC studies and further supports the concept of Demel et al., (1972) that membranes may contain regions of variable cholesterol content.

Using DPH as a probe for cholesterol-phospholipid interaction,

it was apparent that there was no apparent difference in the interaction between cholesterol in model inner and outer monolayers of the adipocyte plasma membrane. This observation suggests that the low affinity of pure PE for cholesterol is overcome when it is mixed with other phospholipids to comprise the inner monolayer of adipocytes and other similarly asymmetric membranes. However, Demel et al. (1977), using differential scanning calorimetry presented evidence that cholesterol preferentially associated with phospholipids other than PE in PE lipid mixtures. DSC studies, however, do not provide specific information about the cholesterol interaction with a lipid mixture as a whole. Thus, the transbilayer asymmetric distribution of cholesterol proposed by Demel et al. (1977) to result from altered interaction between cholesterol and different phospholipid classes within the monolayers is not supported by our observations. A number of studies have investigated the effect of fatty acid composition on cholesterol-phospholipid interaction. Demel et al. (1972) suggest from small molecule permeability studies into phospholipid liposomes, that cholesterol is unable to interact with liposomes of 1-palimtoyl, 2-docosahexaenoyl PC. In contrast, all other naturally occurring lecithins examined by these workers interacted well with cholesterol,was measured by reduction in permeability.

A comprehensive study by Gosh and Tinoco (1972) of the condensing effect of cholesterol on 1-palimtoyl and 1-steroyl substituted lecithins, with naturally occurring mono, di, tri and tetraenoic unsaturated species of carbon atom 2 of the phospholipid gave similar results using monolayer pressure-area studies to the permeability studies of Demel et al. (1972). All the natural lecithins were well condensed by cholesterol, although the condensing effect of cholesterol on both 1-palimtoyl and 1-stearoyl PC was reduced when the 2 position was occupied by arachidonic acid (20:4) instead of mono, di or trienoic acids.

The studies of Demel et al. (1977) suggesting that 22:6 is different from other naturally occurring fatty acids in its ability to interact with cholesterol is extended further here. 22:6 is present in animal phospholipid species, and its presence is found to be altered both by temperature acclimation and also in the

obese condition. Its presence in certain phospholipids within the bilayer may result in non-random distributions of cholesterol within monolayers, postulated by Demel et al. (1977), and may result in an assymetric distribution of cholesterol between the two bilayer halves.

In order to assess the effect of the presence of 22:6 (docosahexaenoic acid) on phospholipid cholesterol interaction, 1-acyl 2-docosahexaenoyl PC and PE were synthesised. Using these lipids in monolayer models of inner and outer halves of the plasma membrane demonstrated that the presence of 22:6 in PC/SM mixtures reduced the affinity of this lipid mixture to cholesterol. However, the presence of 22:6 in PE/PS/PC liposomes resulted in a drastic reduction of the affinity of these liposomes to cholesterol. When phospholipids from lean and obese mice were reconstituted to model the outer and inner bilayer halves of the adipocyte plasma membrane, little difference in the affintiy of lean and obese mouse membrane outer monolayer mixtures with cholesterol could be demonstrated. However, lipid mixtures from obese mouse inner monolayer had a much reduced affinity for cholesterol compared to lean controls. It is suggested that the presence of increased 22:6 at the 2 carbon position of PE in obese mouse adipocyte plasma membranes is responsible for this observation. In vivo, the reduced affinity for cholesterol in the inner bilayer half may result in a more random distribution of cholesterol in the plane of the inner monolayer in obese mouse membranes compared to lean, and also a different transbilayer asymmetry may result between the two phenotypes.

5.3.3. Rationale for the relationship between fatty acid unsaturation and cholesterol interaction.

It is becoming clear that in many membranes, fatty acyl groups are not randomly located within bilayers, but are preferentially located within certain phospholipid classes and on specific carbon atoms of the glycerophosphoryl moieties (see introduction for references). As the phospholipid classes themselves are segregated within the bilayer, it can be concluded that membranes contain a high degree of molecular organisation and 3

dimensional segregation. Membrane organisation exists in a dynamic equilibrium, and this equilibrium position can be locally modified by the presence of proteins or cholesterol for specific metabolic purposes. The biological membrane is also highly adaptable, the composition and content of each phospholipid component can be varied by the cell in order to modify either the overall function of the membrane or to modify the activity of specific target protein components within the cell membrane. There is little doubt that this process is highly influenced by the fatty acid species esterified to the phospholipids, although little attention has been given to the question of why such a wide range of fatty acids are esterified to phospholipids.

Over recent years, it has been established that in most animal membranes, the majority of phospholipids present must be in the liquid crystalline phase to maintain correct membrane function. (Chapter 1). In order to achieve this, the presence of at least one unsaturated fatty acid must be present, as disaturated phospholipids are in the gel phase over the temperature range experienced by animals. The presence of saturated species on phospholipids may also be necessary, for phospholipid interactions with proteins (Lee, 1976) and also for the regulation of membrane fluidity. Indeed, the two most common phospholipid types found in many animal membranes are either 1-palimtoyl, 2-oleoyl phospholipid or 1-stearoyl, 2-oleoyl phospholipid (Hilditch, 1956). Saturated fatty acids at the carbon-1 position may be important for the interaction of phospholipids with cholesterol when they contain di, tri or tetra enoic fatty acids at their 2 carbon position (Demel et al., 1977).

The presence of a cis unsaturated bond in fatty acids of phospholipids gives rise to a 'kink' in the hydrocarbon chain, which expands the mean molecular area of phospholipids. The angle between the limbs of the hydrocarbon chain form an angle of about 110° for oleic acid (Fig 5.13.a) when all the sigma carbon bonds were fully extended (trans configuration) (cis Δ^9 18:1 (trans)). Above the gel to liquid crystalline phase transition, the molecule undergoes rotation about sigma carbon bonds, and the molecule can rearrange, with, for example, a sigma bond in the gauche configuration at Δ^{11}, thus cis Δ^9 (Δ^{11} gauche) 18:1 (trans) (Fig 5.13.b) can be

stabilised in this configuration by hydrophobic interaction with cholesterol, reducing the mean molecular area occupied by phospholipid and cholesterol in the absence of condensation. It is unlikely that cholesterol can interact with cis Δ^9 18:1 (trans) (Fig 5.13.a) as cholesterol does not interact with 9 carbon acyl phospholipids (Joos and Demel, 1969). Thus the presence of 18:1 in phospholipids renders them liquid crystalline over a large temperature range and it also allows the phospholipid to be effectively condensed by cholesterol. Indeed, 1-stearoyl, 2-oleoyl PC/cholesterol mixtures occupy the smallest mean molecular area of any naturally occurring phospholipid (Table 5.14). This phenomena reduces the permeability of phospholipid bilayers by 80% (Table 5.14), and thus 18:1/cholesterol content of membranes can effectively regulate permeability of membranes to small molecules.

The introduction of a second cis pi bond into oleic acid to form cis $\Delta^{9,12}$ 18:2 (trans) (linolenic acid) (Fig 5.13.c) expands the mean molecular area of phospholipids still further (Table 5.14) with a concommitant doubling of small molecule permeability. Cholesterol is able to effectively condense 18:2 containing phospholipids, such that a reduction in permeability similar to that with 18:1 phospholipids, is observed, although the percentage reduction in molecular area by cholesterol is not as great (Table 5.14). Assuming that the molecule must be stabilised by cholesterol such that its mean molecular volume is reduced, then shielding of the bulky pi orbitals of the double bond system from the surface of the molecule interacting with cholesterol must take place. It is suggested that this occurs by rotation of the methylene bridge carbon atom between the two cis pi bonds, such that the pi bond axis are out-of-plane and spiralled. Thus, all that is required for a planar surface to be generated for interaction with cholesterol on the lipid molecule is the formation of α sigma gauch bonds at Δ^{14}, and it is the resulting structure that has the lowest molecular volume.

Cholesterol/fatty acid hydrophobic Van der Waal's interaction is not permanent, and these intermediate structures are continually dissociating and reforming. In a heterogenous mixture of lipids,

the degree of interaction with any one lipid class will depend on the affinity of cholesterol for the favourable interacting surface of each lipid, and also the activation energy of the rearrangement process necessary to generate the correct conformation to occur within the lipid molecule. If these factors are reduced as the number of cis pi bonds increases then the presence of 18:2 in particular classes of phospholipid in certain areas within the membrane may result in local reductions of cholesterol. 18:2 may also serve to increase permeability of the membrane, and also to increase the fluidity of certain phospholipids relative to each other.

Elongation and desaturation of 18:2 results in the production of cis $\Delta^{5,8,11,14}$, 20:4(trans) (arachidonic acid) (Fig 5.13.e). Despite an increase in the number of pi bonds, the ability of cholesterol to condense phospholipids containing 20:4 is relatively unchanged compared to 18:2 (Table 5.14) although cholesterol is not able to modify the permeability of 20:4 containing phospholipids as effectively as 18:1 and 18:2 (Table 5.14), possibly because the mean molecular area of this lipid is larger than either 18:1 or 18:2.

In order to explain the ability of cholesterol to interact with this lipid, the CPK® model of arachidonic acid is presented in Fig 5.14. As with 18:2, if all the methylene bridge sigma bonds are rotated clockwise, then a linear, compact structure results generating a surface for interaction with cholesterol, and a low molecular volume. Arachidonic acid, therefore may confer similar properties on a phospholipid as described for 18:2, except that these properties may be more extreme with 20:4, as it is more fluid at given temperatures (more highly expanded). Because the E_A may be higher for the rearrangement process, cholesterol may interact with 18:1 and 18:2 in preference to 20:4 in mixtures of these lipids. The presence of a cis Δ^5 pi bond in 20:4 provides support for the concept that cholesterol interaction with 18:1, 18:2 and 18:3 is not due solely to the presence of the saturated C-8 hydrocarbon segment between the carbonyl ester carbon atom and cis Δ^9. Thus the presence of 20:4 in membranes may allow a fine adjustment of membrane fluidity and in addition it may

alter the permeability of the membrane through its possible effects on cholesterol segregation. Another role of membrane associated 20:4, is for precursors of prostaglandin synthesis. Membrane bound phospholipase A_2 can quickly mobilize 20:4. Therefore 20:4 stored in membranes is a reservoir of precursors for these important metabolic regulators.

The W-3 series of fatty acids found in membranes arise from the parent 18:3 fatty acid, although the presence of this fatty acid in mammalian membranes is low. The properties of this lipid differ little, and are intermediate between 18:2 and 20:4, and therefore need not be discussed. The major product of desaturation and elongation of 18:3 is cis $\Delta^{4,7,10,13,16,19}$ 22:6 (docosahexaenoic acid). This lipid when present in phospholipid liposomes is hardly condensed by cholesterol (Table 5.14) and the permeability of these lipsomes to small molecules is not affected by cholesterol. It was established in this chapter that cholesterol had a very low affinity for phospholipids containing 22:6, and that the increase in 22:6 in obese mouse adipocyte PE reduced the affinity of the inner monolayer phospholipids to cholesterol compared to inner monolayer lipids of lean control animals.

The CPK® model of 22:6 in Fig 5.14 is presented to suggest a reason for this property. The presence of six methylene bridged pi bonds converts the molecule into a helix, with the pi bonds spiralling around its axis. This structure cannot rearrange, and is structurally rigid compared to all other naturally occurring fatty acids in the lipid-crystalline phase. Therefore cholesterol cannot condense this structure, nor can it interact with it (Fig 5.14) as the helical structure of this molecule prevents any hydrophobic interaction with planar molecules like cholesterol. Thus, 22:6 is a special lipid, having a highly ordered secondary structure, a large molecular volume, with poor packing ability and interaction with cholesterol. 22:6 may also interact poorly with non-specific hydrophobic regions of membrane proteins, but there may be membrane proteins with special binding sites for this uniquely structured lipid. All of these factors suggest that 22:6 may be involved in the regulation of membrane related processes.

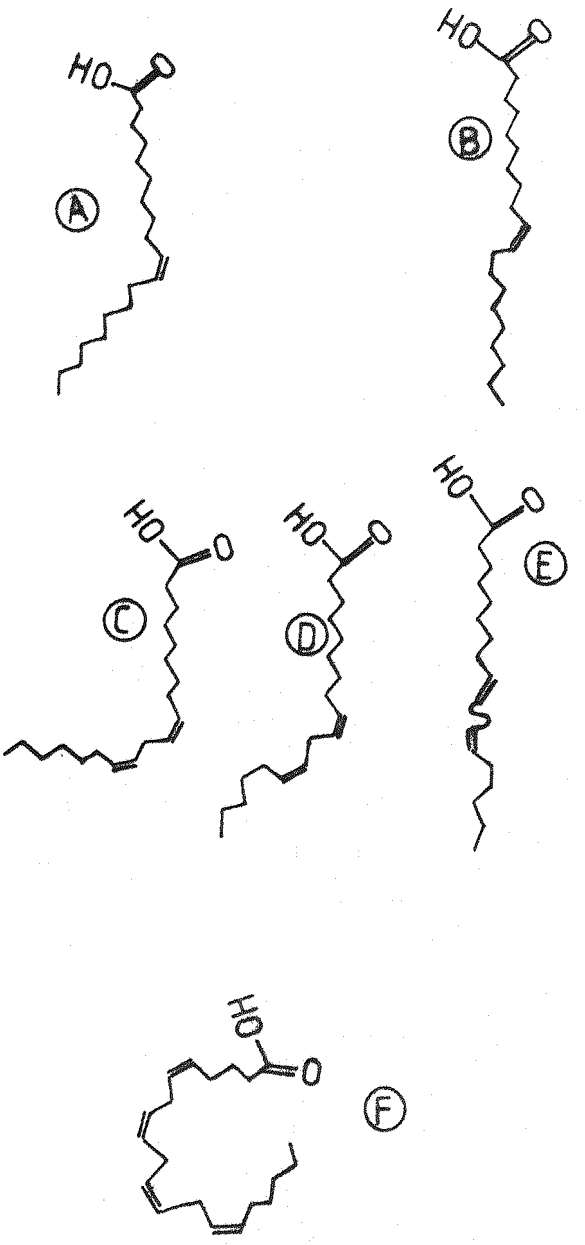

Fig 5.13. Isomeric configurations of fatty acyl groups a) cis Δ^{9} oleic acid (trans) b) cis Δ^{9} (Δ^{11}gauch) oleic acid (trans) c) cis $\Delta^{9,12}$ linoleic acid (trans) d) cis $\Delta^{9,12}$ (Δ^{11} gauche) linoleic acid (trans) e) cis $\Delta^{9,12}$ (Δ^{14} gauche) linoleic acid with rotated Pi methylene bridged sigma bonds. f) cis $\Delta^{5,8,11,14}$ arachidonic acid (trans). Isomeric configurations in brackets refer to sigma carbon-carbon bonds.

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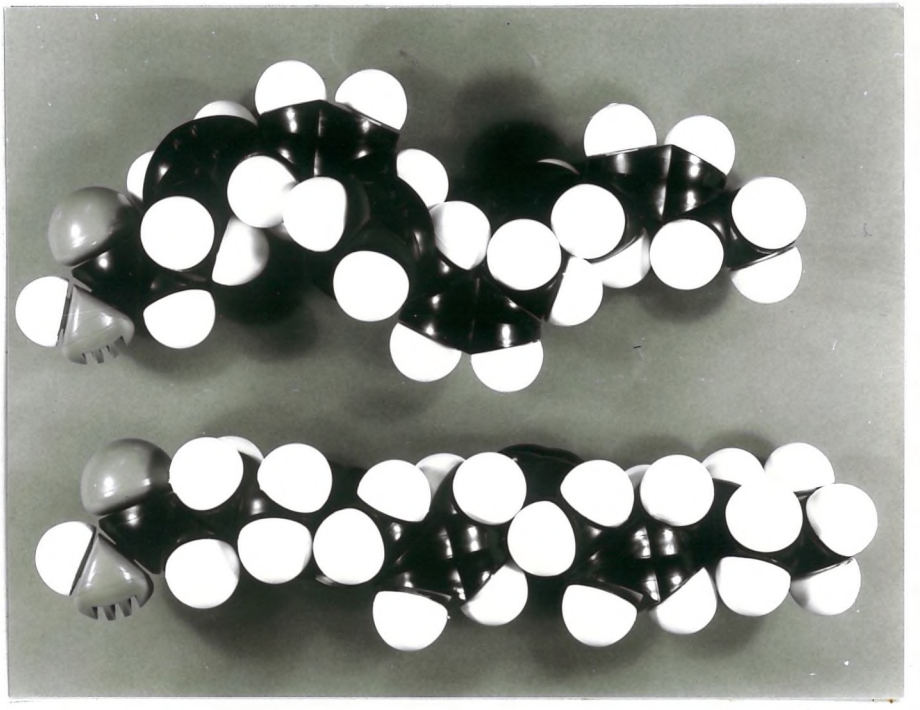

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(i)

(ii)

Fig 5.14. CPK® models of A) 1-steroyl, 2-docosahexaenoyl PE interacting with cholesterol at the 2-docosahexaenoyl position and B) docosahexaenoic acid (i) and arachidonic acid with clockwise rotated Pi methylene bridged sigma bonds(ii).

Table 5.14. (A) The area per molecule of mixed synthetic pure PC and mixed synthetic PC-cholesterol films, molar ratio 1:1 at a pressure of 12 dynes cm^{-1} and 22°C.

(B) The relative amount of glucose released from liposomes of mixed synthetic pure PC and mixed synthetic PC-cholesterol mixtures after 1 hour at 40°C.

Phospholipid	(A) Area/molecule (A^{o2}) (12 dynes cm^{-1}, 22°C)	% change	(B) % max. glucose release (1hr, 40°C)	% change
18:0/18:1	13.7	21.2	24.5	80.4
18:0/18:1/Cholesterol	10.8		4.8	
16:0/18/2	14.9	18.1	50.7	82
16:0/18:2/Cholesterol	12.2		9.33	
16:0/18:3	14.9	16.8	59.2	67.6
16:0/18:3/Cholesterol	12.4		19.2	
16:0/20:4	15.1	16.6	57.3	66
16:0/20:4/Cholesterol	12.6		19.2	
16:0/22:6	15.4	7.1	72	0
16:0/22:6/Cholesterol	14.3		72	

All data from Demel et al., (1972)

5.3.4. Role of 1-acyl 2 docosahexaenoyl PE and cholesterol distribution in the regulation of membrane processes.

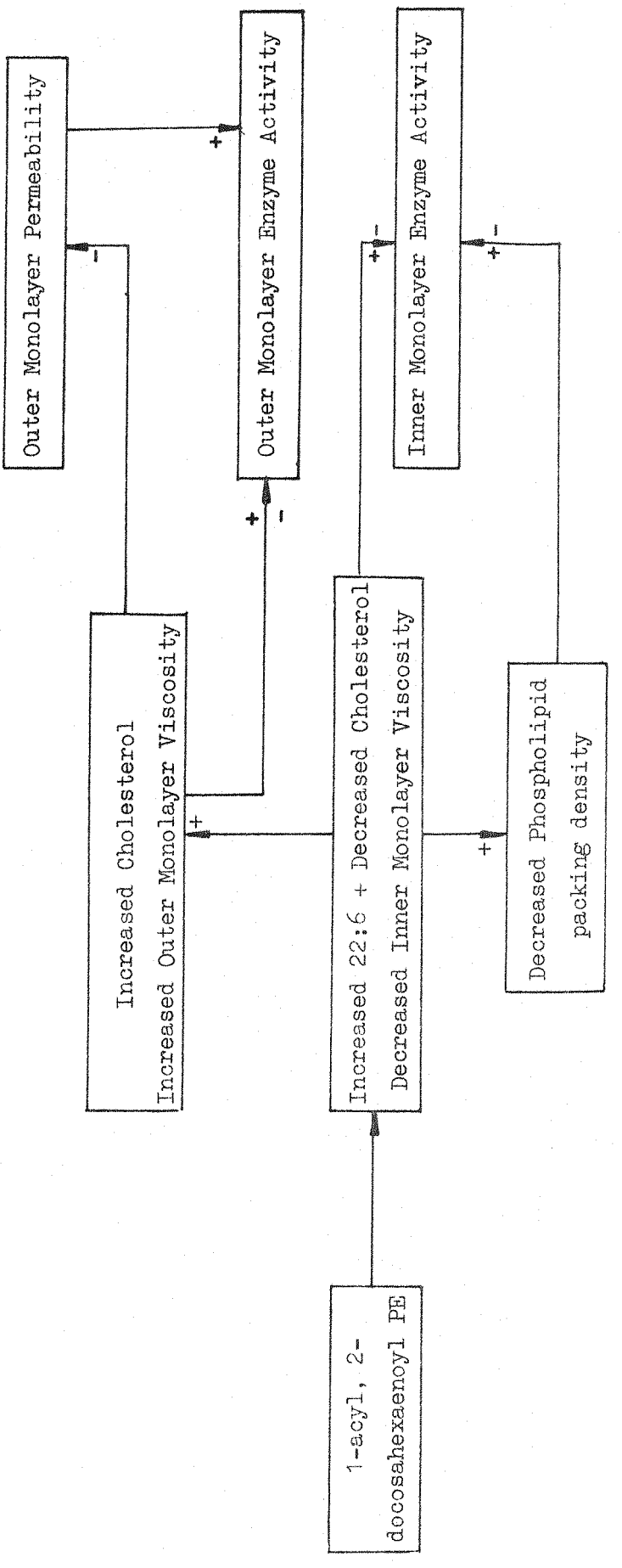
PE has only a small zwitterionic head group. For this reason the headgroups can be tangentially to the plane of the bilayer (Phillips et al., 1972) so that PE can pack closer with other PE molecules than can other phospholipids such as PC, whose bulky headgroups increase the molecular area of these molecules. Therefore the packing ability of PE monolayers is more sensitive to changes in fatty acid composition of the side chains compared to PC. Increasing inner monolayer 22:6 may alter the architecture of the phospholipid matrix to an extent that may affect the activity of membrane bound enzymes. Their activity may also be influenced by the increased fluidity of membranes high in 22:6.

As the half time of flip-flop of cholesterol between the two bilayer halves is short (Backer and Davidowicz, 1979), it is not unreasonable to assume that the equilibrium distribution of cholesterol between the two bilayer halves will be in the ratio of the relative partition coefficients for cholesterol and lipid matrix. Increasing inner monolayer 22:6 content may cause a repartitioning of cholesterol such that a net transfer of cholesterol from the inner to the outer half may occur. This is illustrated in a flow diagram (Fig 5.15). This event may in itself cause a further increase in fluidity of the inner monolayer, with a concomitant decrease in its fluidity, and a condension of the outer monolayer. This factor may influence the activity of outer monolayer enzymes, and also reduce the permeability of the outer monolayer to small molecules and ions. These factors may significantly contribute to the altered membrane processes observed in the ob/ob mouse.

5.3.5. Flow Activation Energy of 22:6 phospholipids.

Throughout chapters 3 and 4, the interpretation of the flow activation energy (ΔE) results obtained from native membranes, extracted lipids and phospholipids labelled with DPH assumed that lipids from ob/ob mouse membranes contained a more 'ordered' component. Experiments with PE liposomes demonstrate that the presence of 22:6 reduces ΔE to values observed in PE from ob/ob mice. These results can now be rationalised in terms of the structure

Fig 5.15. Flow diagram for postulated events occurring after incorporation of 1-acyl, 2-docosahexaenoyl PE into the inner monolayer of a cell plasma membrane.



of 22:6. The rigid secondary structure of this lipid results in a reduced number of degrees of freedom for molecular interaction with other acyl groups, and therefore reduces the volume of a flowing segment labelled with DPH.

5.3. . Conclusions.

The results presented in this chapter identify the phospholipid 1-acyl, 2-docosahexaenoyl PE as the major phospholipid contributing toward the fluidisation of obese mouse adipocyte plasma membranes. This phospholipid is present in the inner monolayer of the plasma membrane. Evidence has been presented that the inner monolayer is more fluid in membranes from obese mice compared to lean controls. The presence of 22:6 PE in the inner monolayer results in an altered cholesterol affinity for the monolayer as a whole. This factor may result in the inner plasma membrane monolayer from ob/ob mice adipocytes containing regions of random phospholipid/cholesterol mixtures or perhaps domains of homogenous lipid pools. These factors may cause an increased partitioning of cholesterol into the outer half of the plasma membrane.

The rationale for the poor interaction of 22:6 with cholesterol has been interpreted in this thesis from space-fitting models constructed for each lipid class. The presence of more than four methylene bridged pi bonds causes a high degree of steric hinderance within the molecule. This factor causes 22:6 to adopt a rigid helical structure. This structure is unique in naturally occurring fatty acids, preventing interaction with cholesterol and decreasing the packing ability of phospholipids. Phospholipids containing this fatty acid may be involved in the regulation of membrane-bound enzyme activity. Evidence has been presented in Chapter 4 that the adenylate cyclase/hormone receptor coupling efficiency may be regulated by the presence of 22:6 PE. Recently, Emilsson and Gudbjarnason (1981) have observed that chronic exposure of rat heart to noradrenaline increases PE docosahexaenoic acid by 25%, further indicating a regulatory role for this lipid in the expression of catecholamine resistenace.

The possible increased cholesterol content of this outer monolayer may result in reduced ionic permeability of membranes containing 22:6 PE in the inner monolayer, possibly resulting in reduced ionic pumping, altered outer monolayer enzyme activity, and reduced lateral mobility of membrane proteins.

The postulated reduced lateral mobility of enzymes in the outer monolayer of membranes from obese mice may help to explain the impaired hormonal stimulation by β adrenergic receptors of the adenylate cyclase in adipocytes from these animals. If lateral diffusion of the β receptor within the plane of the membrane is a rate limiting step in the activation process of adenylate cyclase, then an increase in outer monolayer viscosity would be expected to decrease the number of β receptor/adenylate cyclase coupled species.

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CHAPTER 6.

INVESTIGATIONS INTO THE PHYSICAL PROPERTIES AND COMPOSITION OF VARIOUS CELL MEMBRANES FROM LEAN, OBESE, HYPERTHYROID AND STARVED ANIMALS.

6.1. Introduction.

6.1.a. Fluidity changes in various membranes from ob/ob and lean mice.

In chapters 3 and 4 it was established that there are gross compositional pertubations in the hepatic ER and adipocyte plasma membrane of obese mice. The fluidisation of PE, with the insertion of LCPUFA's at position 2, result in a fluidised inner monolayer of the adipocyte plasma membrane. This perturbation may give rise to a redistribution of cholesterol between the two halves of the bilayer. These factors are likely to produce altered lipid-protein interactions, and may be responsible for the poor coupling efficiency between the β receptor and the adenylate cyclase enzyme observed in obese mouse adipocyte plasma membrane.

This information warrants the expansion of this investigation to other tissue membranes of lean and obese mice, to establish whether metabolic abnormalitiies observed in other tissues of ob/ob mice can be related to compositional changes within cell membranes.

i) Brain synaptosomes.

The brains of ob/ob mice have a reduced mass, reduced neuronal size in most areas of the brain (Bereiter and Jeanrenaud, 1979), a decrease in the total number of brain cells (Van der Kroon and Speijers, 1979) and an increase in hypothalamic catecholamine content (Lorden et al., 1976). All these observations are consistent with congenital hypothyroidism (Van der Kroon and Speijers, 1979). Hypothyroidism has been shown to influence the coupling efficiency of the β receptor-adenylate cyclase system by an as yet unidentified plasma membrane perturbation (Malbon, 1980). Experiments were therefore carried out to examine if obese mouse brain synaptosomal membranes have an altered lipid composition compared to lean controls, that may have contributed in utero to impaired brain development.

ii) Pancreas.

Hyperinsulinaemia is a factor associated with many different types of obesity. The metabolic events leading to the hyperins-

ulin secretion are unclear. Studies by York and Bray (1972) demonstrated that Ventro-medial hypothalamic (VMH) lesioned rats that normally develop obese hyperinsulinaemic syndrome did not do so if β cell function was destroyed pre-operatively. This suggests that hyperinsulinaemia contributes significantly to the maintainance of the obese state in VMH lesioned animals. Bray et al. (1981) have conclusively demonstrated that the hypersecretion of the VMH lesioned rat is mediated via the autonomic nervous system (ANS). The ANS input to the pancreas comprises the cholinergic parasympathetic and the adrenergic sympathetic, both of which modulate α, β and Δ cell secretions (Samols et al., 1981). The β cell insulin output is stimulated by Acetelycholine and inhibited by catacholamines (both circulating and from the sympathetic ANS).

The exaggerated insulin secretion by ob/ob mice β cells may therefore be mediated by an altered response of the β cell to the ANS, or more specifically to a reduced suppression of insulin secretion by catecholamine resistance of the β cells. If the impaired coupling efficiency of hormone-receptors and adenylate cyclase exists in the β cell plasma membrane, as it does in the adipocyte plasma membrane, then β cell plasma membrane lipids may be involved in this process. A preliminary study was therefore carried out to exaimine the fluidity of isolated pancreatic cell plasma membranes, although as the ratio of β cells to total cells is low in whole pancreatic cells digests, the interpretation of data from this study in terms of β cell function is difficult.

iii) Muscle.

Muscle is the largest organ of the mammal. In the ob/ob mouse a dramatic reduction in insulin sensitivity and responsiveness has been observed in vitro (Genuth et al., 1971). A defect has been observed in muscle of ob/ob mice insulin receptor number and basal glucose utilization (Le Marchand-Brustel, 1978). Chan and Dehaye (1981) also found that adrenaline did not stimulate glucose transport into muscle from db/db mice, while significantly stimulating glucose entry into control mice muscle. These authors found that insulin sensitivity of muscle was not

altered in young db/db mice, and suggest that all factors distal to glucose transport and phosphorylation are not impaired in young db/db mice. They further suggest that the impaired glucose utilization of young db/db mouse muscle resides solely at the glucose transport level, i.e. at the sarcolemmal membrane. Although direct comparisons cannot be made between the db/db and ob/ob mouse muscle, nevertheless many metabolic defects are common to both. It was therefore of interest to examine the fluidity of lean and ob/ob mouse sarcolemmal membrane, to ascertain if an altered sarcolemmal lipid composition could be involved in the altered basal and catecholamine stimulated hexose transporter systems observed in the db/db mouse muscle.

iv) Salivary Gland.

Salivary gland has a high degree of ANS input. Evidence has been presented by Bray et al. (1981) that VMH lesioning reduces sympathetic activity of the ANS, and this was connected with atrophy of the salivary gland. Salivary gland is also atrophied in ob/ob mice compared to lean controls, despite their increased demand for saliva, due to their hyperphagia. Again, the possibility must be considered that neurotransmitter resistance of secretory cells in the salivary gland could account for these observations in the absence of any defect in sympathetic tone. Salivary gland may also be a model system for brown fat tissue, as both have a high ANS supply to enable these tissues to respond immediately to environmental stimuli. Brown fat, like salivary gland, is atrophied in ob/ob mice, despite an increased demand on thermogenic tissue in these hypometabolic animals. Therefore, salivary gland plasma membrane fluidity was examined in order to assess the likelyhood of membrane composition being involved in the possible resistance to catecholamine neurotransmitters postulated for these two tissues.

v) Liver.

Decreased insulin and glucagon binding has been observed in hepatic plasma membranes from ob/ob mice. Of interest is that inhibition of fatty acid synthesis by vasopressin is absent in ob/ob mice, despite a normal vasopressin inhibition of glycogenolysis (Hems and Ma, 1976). Although the mechanism for

this process is unknown, ion fluxes, possibly across the plasma membrane, may be involved (York and Bray, 1979). Hepatic plasma membrane was examined from lean and obese mice, and the fluidity of these membranes was assessed. Plasma membranes were also prepared from animals housed at 34°C in order to ascertain whether housing temperature affected hepatic plasma membrane composition, as was observed for adipocyte plasma membrane in the obese animal.

vi) Erythrocyte.

The obese mouse is hypercholesterolaemic (York and Bray, 1979). The erythrocyte is anucleate, and unable to control its plasma membrane lipid composition, and therefore the obese mouse erythrocyte plasma membrane may contain more partitioned cholesterol than those from lean animals, which may decrease the fluidity of erythrocyte plasma membranes. This hypothesis was evaluated by measuring the fluidity of purified erythrocytes obtained from both phenotypes. Diabetes is known to result in a more viscous erythrocyte plasma membrane (Baba et al., 1979) and therefore the erythrocyte plasma membrane phospholipid extract fluidity of lean and obese mice was also assessed, to examine if the diabetic syndrome of ob/ob mice affected the erythrocyte membrane phospholipids.

vii) Mitochondria.

Mitochondria isolated from ob/ob mouse livers have more respiratory chain enzymes, and also have an increased respiratory chain activity than lean controls (Katyare and Howland, 1977). Mitochondria are known to alter their degree of respiratory efficiency in response to thyroid status. Hulbert, (1976) has proposed that this is at least partially achieved by the alteration of the inner membrane phospholipid composition, and this author has provided impressive correlation between thyroid status and the degree of unsaturation of the inner mitochondrial membrane. Mitochondrial respiratory efficiency declines with decreasing plasma thyroid hormone concentration, and the inner membrane phospholipids become more saturated. The increased respiratory efficiency observed in ob/ob mouse hepatic mitochondria demonstrates that the proposed functional hypothyroidism of ob/ob

animals (Joosten and Van der Kroon, 1974) does not operate at the mitochondrial membrane level. However, if the hypothesis that mitochondrial inner membrane fluidity can be related to mitochondrial efficiency in the lean and ob/ob mouse, then inner mitochondrial membrane lipids from ob/ob mice should contain relatively unsaturated phospholipids compared to lean controls. Inner mitochondrial membrane lipids were extracted for fluidity studies to investigate this hypothesis from ob/ob and lean control mice.

6.1.b. Other animal models of obesity.

Thusfar, it has been established that there is a good case for the altered physical state of the plasma membrane observed in the adipocyte of ob/ob mice contributing to the poor hormonal response of the cell. In this chapter, this concept is extended to other cell types in the ob/ob mouse, and also to the genetically obese (fa/fa) rat, first described by Zucker and Zucker (1961). Demonstration that membrane changes occur in other models of obesity will establish that such changes are involved in either the expression or as a result of the obese condition, and not simply a manifestation of the ob gene. Adipocyte plasma membranes and brain synaptosomal membranes from the fa/fa and lean control Zucker rats were studied for comparison with results obtained with the same membranes from ob/ob and lean control mice.

6.1.c. Plasma membrane and metabolic efficiency of cells.

Obesity is a disease characterised by excessive storage of energy, and in several animal models has been shown to be the result of an increased metabolic efficiency. The mechanisms whereby animals can regulate their metabolic efficiency are obscure, but the role of hormones and target cell sensitivity or maximal response to these hormones may play an important role. If the cell plasma membrane composition can alter the cellular responsiveness to hormones involved in regulating metabolic efficiency, then similar changes in plasma membrane composition observed in the ob/ob mouse may be observed under varying metabolic conditions characterised by an increase in metabolic efficiency, e.g. both starvation and obesity. Giacobino and Chemlar (1977) demonstrated that the adipocyte plasma membrane, the mitochondrial membrane

and the microsomal membrane of rat adipocytes, demonstrated drastic changes in fatty acid composition when the rats were fasted. In all three membranes these was an increase in the degree of unsaturation of the fatty acids with substitution of LCPUFA for 16:0 when isolated from fasted animals. The fatty acid spectrum from adipocyte plasma membranes from fasted rats was strikingly similar to the results presented in Chapter 4 for obese mouse adipocyte plasma membrane. It should be noted that the uncellular prokaryote Tetrehymena pyroformis also increases its membrane fatty acid unsaturation considerably when added to nutrient-free media (Nozawa et al., 1980), suggesting that the observed membrane changes may be a significant general feature of adaption by organisms to starvation. The effects of starvation on lean (Ob/?) mouse adipocyte plasma membrane fluidity was therefore examined, to ascertain if starvation could mimic changes in membrane fluidity observed in obese littermates.

Metabolic rate of animals is known to be under the control of thyroid hormones (Garrow, 1968). Hyperthyroidism results in elevated basal metabolism and weight loss. Conversely, hypothyroidism results in weight gain and a fall in metabolic rate (Garrow, 1978). Thyroid hormones may be involved in the decreased metabolic rate observed in starvation, as studies by Palmblad et al. (1977) have demonstrated a 50% fall in serum triiodothyromine (T_3) and two fold increase in serum reverse triiodothyromine (rT_3) in starving humans. Plasma T_3 concentration is elevated under long-term cold-stress conditions when heat production is of paramount importance. This is achieved by an acceleration of metabolic rate (Jansky, 1973). There also has been some evidence that the obese mouse may be functionally hypothyroid (Joosten and Van Kroon, 1974) as these authors demonstrated many similarities between the metabolism of the ob/ob mouse and hypothyroid animals. Furthermore, ob/ob mice do not survive cold stress to the same degree as thin littermates (Trayhurn et al., 1976). Thus, it would appear that thyroid hormones may be involved in the regulation of metabolic efficiency such that energy and thermic homeostatis can be achieved.

An important site of action of thyroid hormones is there modulation of catecholamine action in a variety of tissues including heart (Wildenthal, 1974), liver (Malbon et al., 1978a) and adipose tissue (Malbon et al., 1978b). The degree of potentiation of catecholamine-induced lipolysis is correlated to thyroid status (Reckless et al., 1976)in this tissue. Thyroid hormones have been shown to improve the impaired catecholamine stimulated lipolysis observed in adipose tissue from ob/ob mice (Otto et al., 1978) and hypothyroid rats (Malbon et al., 1978b). Malbon et al. (1978b) have demonstrated that intracellular hormone stimulated cAMP concentration of rat adipose tissue is directly correlated with thyroid status.

The possible loci of action of thyroid hormone at the cell plasma membrane level may therefore be:-

a) Modulation of the number, or affinity of α (inhibitory) or β (excitatory) adrenergic receptors.
b) Modulation of adenylate cyclase activity by an increase in the number of units or affinity for its substrates.
c) Modulation of either or both of the phosphodiesterase enzymes (cytosolic high K_m or membrane bound low K_m).
d) Modulation of the efficiency of coupling between β adrenergic receptors and adenylate cyclase units.

Malbon (1980) demonstrated that thyroid status has no effect on the number of β receptors, but does alter the affinity of the receptor for β agonists in rat fat cells, increasing thyroid status producing an increase in binding affinity for β agonists. This phenomena may be important in vivo for setting the threshold of lipolysis to catecholamine stimulation. It does not have any bearing of the blunted maximal response of adipose tissue to catecholamines in hypothyroidism or the elevated maximal response observed in hyperthyroid states. The modulation of α-receptor activity by thyroid hormone is controversial at the moment (Grill and Rosenquist, 1975; Reckless et al., 1976).

Adenylate cyclase activity has been found to be independant of thyroid status. Caldwell and Fain, (1971) demonstrated similar activities in basal cyclase activities in euthyroid and hyperthy-

roid rat adipocytes. Armstrong and Stouffer (1974) demonstrated similar basal activities in euthyroid and hypothyroid rat adipocyte ghosts. Furthermore, Malbon et al. (1978b) demonstrated no change in the maximal sodium fluoride stimulated cyclase activity in hypothyroid rats compared to euthyroid controls.

Substrate availability for adenylate cyclase has been discounted by Caldwell and Fain (1971). They postulated that accumulation of cAMP in fat cells from T_3 treated rats could be due to increased availability of ATP to the cyclase. However, T_3 treated rat adipocytes contained less ATP than did cells from euthyroid controls. This would suggest that ATP availability is not rate limiting.

Neither the membrane-bound (Malbon et al., 1978b) or the cytosolic (Caldwell and Fain, 1971) phosphodiesterase activities are altered by thyroid status. Thus, it is clear from the above considerations that the major aspect of the regulation of adenylate cyclase by thyroid status has yet to be elucidated.

The role of thyroid hormone in modulating the coupling efficiency between hormone-receptor complex and cyclase has recently been discussed by Malbon (1980). In the absence of any other mechanism for the observed contingency of hormone stimulated adenylate cyclase activity on thyroid status, this author suggests that a protein or lipid component within the membrane is responsible for this observation.

In view of the observed effects of membrane fluidity on hormone-stimulated adenylate cyclase activity described in Chapter 4, and the similarity between hormone-stimulated lipolysis in obesity and hypothyroidism, an experiment was performed to elucidate the effect of hypothyroidism on adipocyte plasma membrane fluidity.

6.2. Results.

6.2.a. Fluidity studies on lean and obese mouse hepatic plasma membranes from animals housed at 25°C and 34°C.

Hepatic homogenates contain organelles from a heterogeneous population of cells, comprising hepatocytes, Kupffer cells, bile canaliculi, sinusoidal endothelial cells and fat storage cells. The plasma membrane fraction will contain membranes derived from all of these cell types. Although livers from obese animals are unlikely to contain these cells in the same proportions as livers from lean animals, preliminary investigations were carried out on entire livers.

Membrane purity was assessed by measurement of total cardiolipin content (inner mitochondrial membrane phospholipid marker) for mitochondrial contamination, and NADH-cytochrome c reductase (in the presence of KCN) activity was used as an enzymic marker for ER. From these criteria, lean and obese mouse hepatic plasma membranes contained 12-15% contamination by these organelles (data not shown). As purity was similar between the two groups, further subfractionation was not attempted.

Hepatic plasma membranes were prepared from lean and obese animals housed at 25°C and 34°C for 1 week. Fluorescence polarisation by DPH-labelled membranes was measured from 2°C-40°C. The Arrhenius plots derived from measurements of membranes from animals housed at 25°C are shown in Fig 6.1. Table 6.1 summarises the results.

Obese mouse hepatic plasma membranes are more fluid than those of lean at both housing temperatures. No breakpoint was detected in plasma membranes from obese mice. As the flow activation energy was close to that observed in lean membranes above the breakpoint, then it is likely that the breakpoint is below 2°C in Arrhenius plots from obese mice plasma membranes, consistant with the more fluid membrane. Housing at 34°C produced a rise in the breaktemperature in obese membranes with little change in that of lean, indicating that at least one component became less fluid in plasma membranes from obese animals housed at 34°C. The fluidity of both lean and obese membranes was

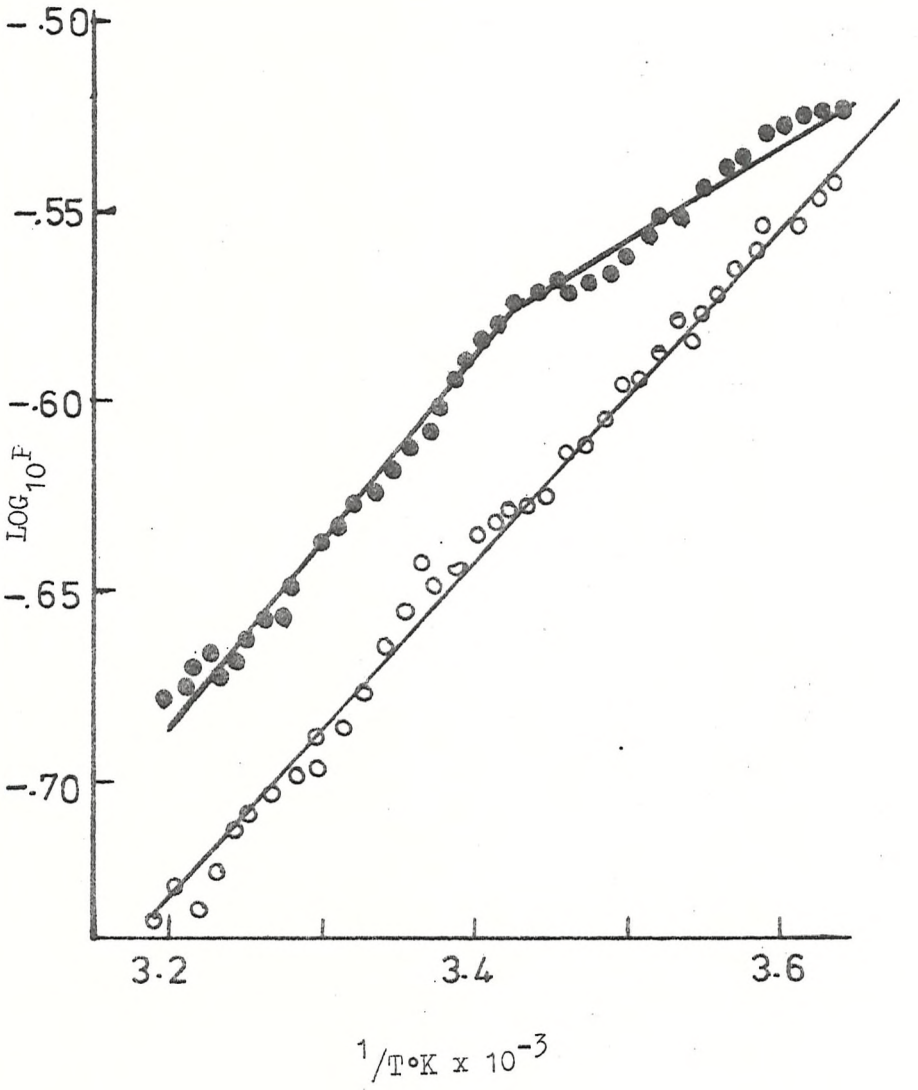


Fig. 6.1. Arrhenius plots of fluorescence depolarisation by DPH labelled liver plasma membranes from lean (●) and obese (o) mice housed at 25°C.
Date represents values for membranes derived from 4 animals in each group.

Table 6.1. Summary of data from Arrhenius plots of fluoresecence depolarisation by DPH labelled hepatocyte plasma membranes from lean and obese mice housed at 25°C and 34°C.

Phenotype Housing Temp °C	Breakpoint °C	Polarisation 37°C	ΔE above Breakpoint kcal mol^{-1}	ΔE below Breakpoint kcal mol^{-1}	Fluidity Ratio $\frac{\phi}{\phi}$ R_{37}
LEAN					
	18.8	0.212	9.15	18.8	
25					
					1.20
OBESE					
	<2	0.191	10.35	-	
25					
LEAN					
	19.0	0.214	7.63	16.6	
34					
					1.18
OBESE					
	12.7	0.195	7.69	13.20	
34					

Data represents values obtained from pooled hepatocyte plasma membranes from 4 lean and 4 obese mice.

slightly reduced by housing at 34°C, although it is not possible to assess whether this is significant on one preparation. However, the flow activation energies above the breakpoint of the DPH labelled plasma membranes were reduced from both groups when housed at 34°C, consistent with the incorporation of a more ordered component in both groups of membranes from animals housed at 34°C.

These results demonstrate that hepatic plasma membranes are more fluid from obese mice compared to lean controls, and that housing at 34°C does not have the same fluidising effect on hepatic plasma membranes from obese mice as adipocyte plasma membranes, although at least one component of membranes from both phenotypes becomes more ordered when animals are housed at 34°C.

6.2.b. Fluidity studies on lean and obese mouse hind limb muscle sarcolemmal membrane lipid extract.

Pure sarcolemmal membranes were prepared as described in Materials and Methods from 20 lean and 20 obese mice. Yields of sarcolemmal membrane were extremely low ($\hat{=}$ 750µg membrane protein). Because of this, it was necessary to check an enzyme marker for plasma membrane against values published in the literature. Na^+/K^+-ATPase activities in these membranes was found to be higher in two different preparations than those reported in the literature for rat sarcolemmal membrane (data not shown). It was concluded that the final membrane suspension from muscle consisted of highly purified sarcolemmal vesicles. Unfortunately, the paucity of material precluded further characterisation of sarcolemma. A lipid extract was labelled with DPH and the fluorescence polarisation measured from 2°C-40°C. The Arrhenius plots from this experiment are shown in fig 6.2, and the data is summarised in Table 6.2.

Obese mouse sarcolemmal membrane was more fluid than that of lean mice and was characterised by the reduction in breakpoint temperature. Flow activation energies were similar between lean and obese mouse membranes both above and below the breakpoint. The results of this study indicate an altered lipid composition of the sarcolemmal membrane of obese animals.

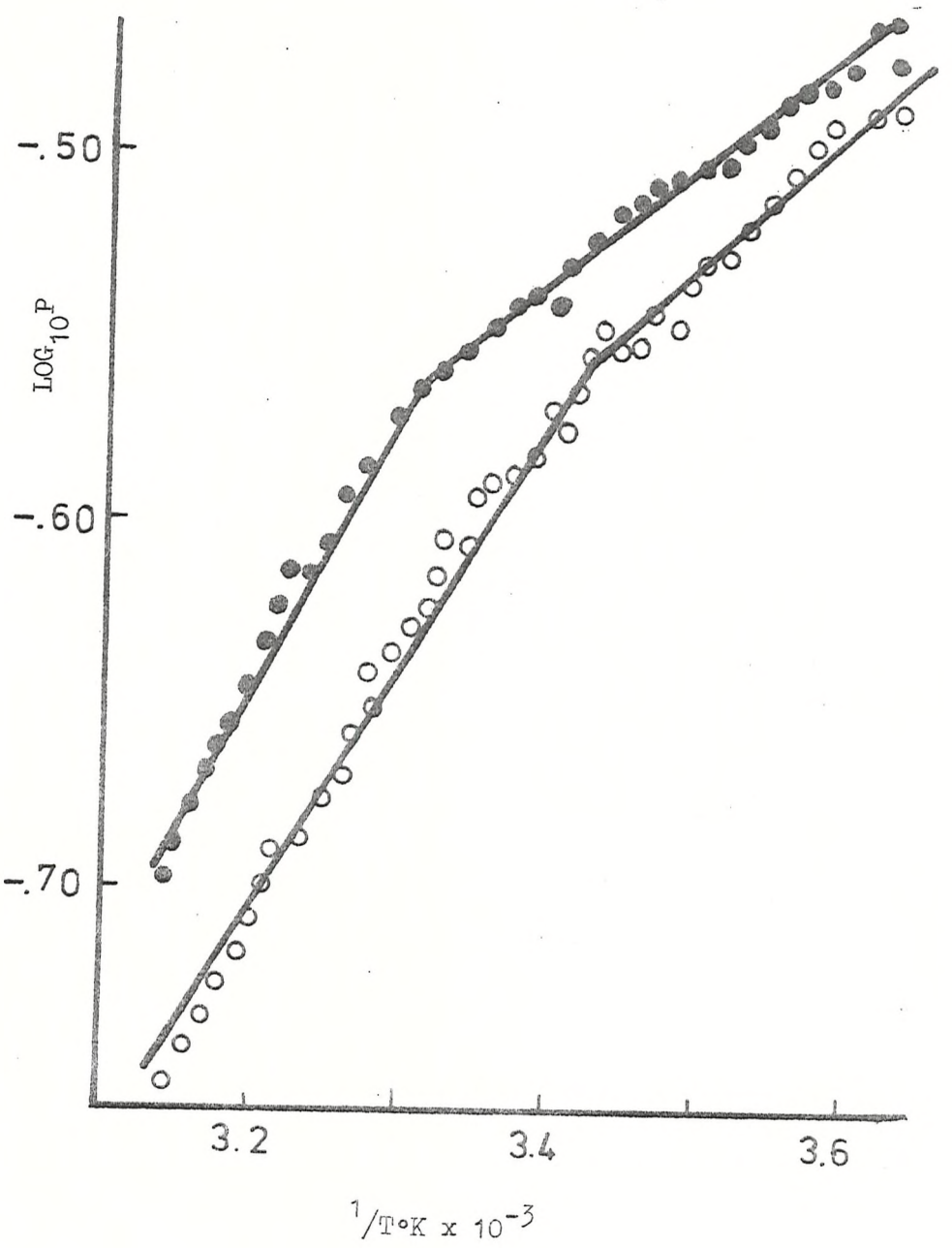


Fig 6.2. Arrhenius plots of fluorescence depolarisation by DPH labelled liposomes derived from extracted sarcolemmal lipids from lean (●) and obese (o) mice.
Data represents values for membranes derived from 20 lean and 20 obese mice.

Table 6.2. Summary of data from Arrhenius plots of fluorescence depolarisation by DPH labelled membrane vesicles from lean and obese mouse hind limb sarcolemma.

Phenotype	P_{37}	Breakpoint °C	ΔE above break-point kcal mol^{-1}	ΔE below break-point kcal mol^{-1}	Fluidity Ratio R_{37}
Lean	0.237	29.1	6.1	14.4	1.31
Obese	0.206	19.4	6.94	12.93	

Data represents values for membranes derived from 20 lean and 20 obese mice.

6.2.c. Fluidity studies on the inner mitochondrial membrane and its individual phospholipids from lean and obese mouse liver.

Mitoplast vesicles were prepared from the 17,000 X g mitochondrial fraction of lean and obese mouse liver homogenates, as described in Materials and Methods. PE, PC and CL were obtained from lipid extracts of the mitoplasts by TLC. Fluorescence polarisation of DPH labelled liposomes derived from lipid extracts was measured from 4°C to 40°C, and the Arrhenius plots from this experiment are shown in fig 6.3 and the results are summarised in Table 6.3.

Obese mouse inner mitochondrial membrane was more fluid than lean controls, and a reduction in the Arrhenius breakpoint was observed, with little change in the flow activation energy. The fluidity of the three major phospholipids isolated from obese mouse inner mitochondrial membrane were studied by DPH polarisation (Table 6.3). Each phospholipid class was more fluid from obese mouse mitochondrial inner membrane compared to lean controls. The minor phospholipids, SM and PI were not recovered for analysis. Thus, the mitochondrial inner membrane in obese animals contains phospholipids that have more fluid acyl chains than do lean controls. Cardiolipin is the most fluidised phospholipid measured, and it is this phospholipid that is found to be tightly and specifically bound to the respiratory chain enzymes, NADH dehydrogenase (Heron et al., 1977) and cytochrome oxidase (Awasthi et al., 1970).

6.2.d. Fluidity studies on erythrocyte plasma membranes and membrane phospholipids from lean and obese mice.

Erythrocytes contain no internal organelles, and therefore the plasma membrane is prepared pure, with only haemoglobin as a major contaminant. This protein is water-soluble, and the haem moiety does not fluoresce at 430nM, it does not interfere with fluorescence studies with DPH. Membranes were prepared, and fluorescence depolarisation by DPH-labelled membrane vesicles was measured from 2°C-40°C. The results from this study are shown in Table 6.4, and summarised in Table 6.2. Erythrocyte plasma

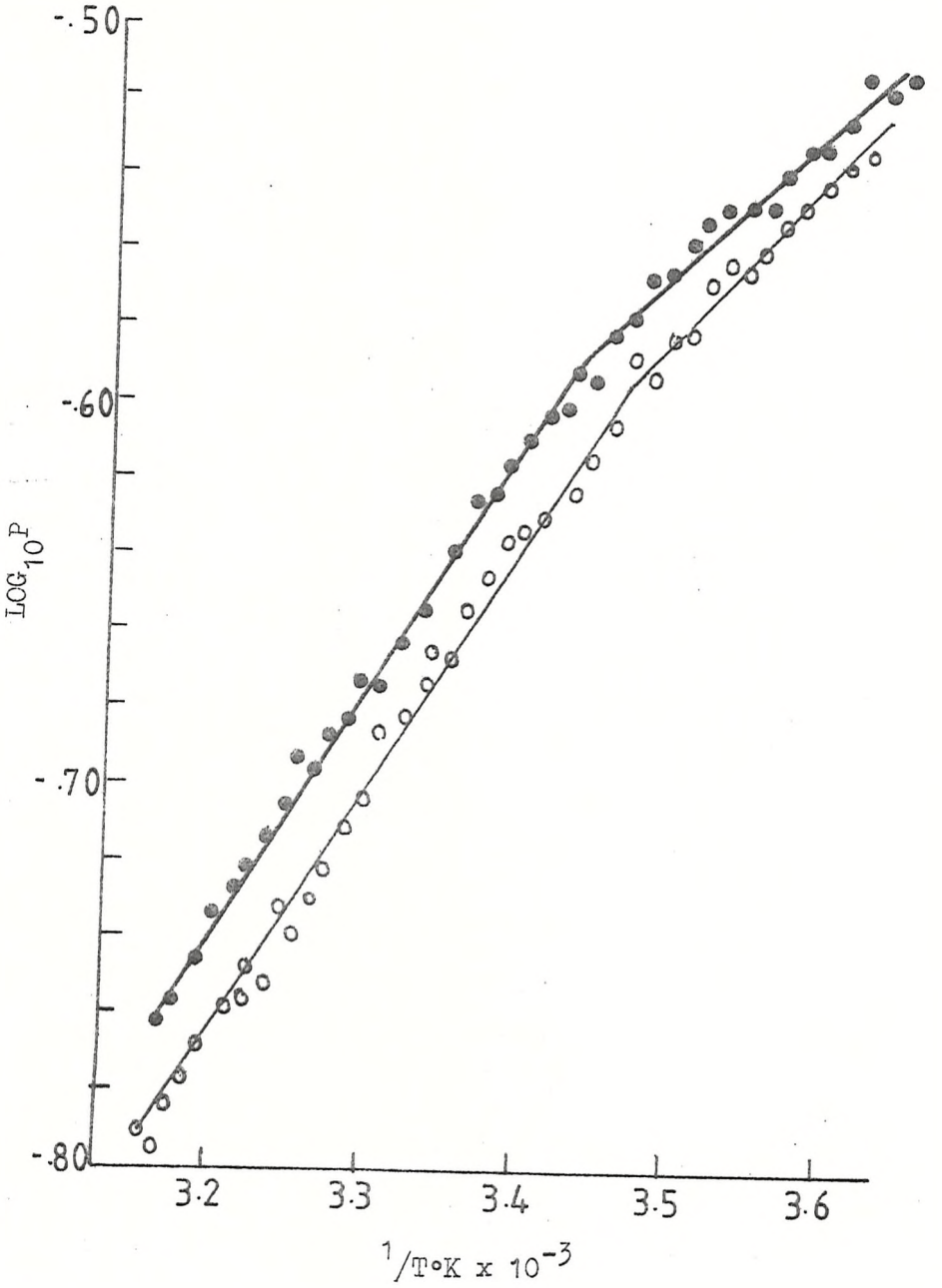


Fig 6.3. Arrhenius plots of fluorescence depolarisation by DPH labelled liposomes derived from lean (●) and obese (o) mouse liver mitochondrial inner membrane.
Data represents values for membranes derived from 20 lean and 20 obese mice.

Table 6.3. Summary of data from fluorescence polarisation by DPH labelled hepatic inner mitochondrial membranes and extracted phospholipids from lean and obese mice.

	Phenotype	P_{37} (mean) ± SEM (n = 5)	Breakpoint °C	ΔE above Breakpoint kcal mol^{-1}	ΔE below Breakpoint kcal mol^{-1}	Fluidity Ratio ΦR_{37}
Native Inner Membrane	Lean	0.197	19.8	7.04	12.38	1.12
	Obese	0.178	15.4	7.44	10.62	
PC***	Lean	0.154 ± 0.002	-	-	-	1.21
	Obese	0.135 ± 0.002	-	-	-	
PE***	Lean	0.191 ± 0.003	-	-	-	1.25
	Obese	0.167 ± 0.002	-	-	-	
CL***	Lean	0.195 ± 0.004	-	-	-	1.34
	Obese	0.163 ± 0.002				

Data represents values for membranes and phospholipids derived from 4 animals in each group. Polarisation values at 37°C represent mean of 5 observations ± SEM. Students t for each phospholipid group *** $p < 0.001$.

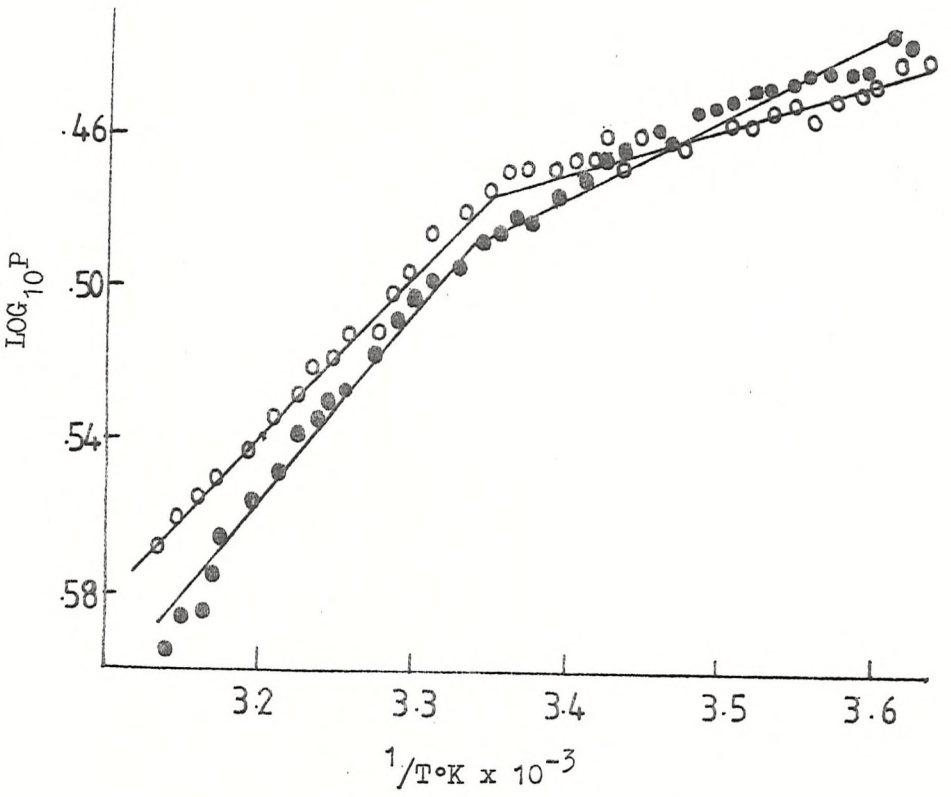


Fig 6.4. Arrhenius plots of fluorescence depolarisation by DPH labelled erythrocyte plasma membranes from lean (●) and obese (o) mice.
Data represents values for membranes derived from 4 lean and 4 obese mice.

Table 6.4. Summary of data from fluorescence polarisation by DPH labelled erythrocyte plasma membranes, extracted total lipids and phospholipids from lean and obese mice.

	Phenotype	P_{37} ± SEM (n=5)	Breakpoint °C	ΔE above Breakpoint kcal mol^{-1}	ΔE below Breakpoint kcal mol^{-1}	Fluidity Ratio $R_{37°C}$
Native plasma membrane	Lean	0.286	26.0	10.4	38.1	0.89
	Obese	0.299	28.2	9.28	22.2	
Total Phospholipid extract	Lean	0.200	32.3	3.1	10.6	1.09
	Obese	0.210	29.6	2.8	12.1	
PC (NS)	Lean	0.165 ± 0.003	-	-	-	1.0
	Obese	0.164 ± 0.003	-	-	-	
PE***	Lean	0.209 ± 0.003	-	-	-	1.36
	Obese	0.175 ± 0.005	-	-	-	

Data represents values for membranes, lipid extract and phospholipids derived from 4 animals in each group. Student's t for each phospholipid group *** $p < 0.001$, NS - not significant.

membranes from obese animals are less fluid than lean controls. The break temperature of the Arrhenius plot was increased in comparison to lean controls. Also, the flow activation energy of obese preparations was considerably reduced below the breakpoint. All these factors suggest that the erythrocyte plasma membrane from obese animals is more ordered compared to lean controls. However, when the total phospholipid extract of lean and obese mouse erythrocyte plasma membranes was analysed, it was found that phospholipid vesicles were more fluid from obese mouse erythrocyte membranes compared to lean controls. Fluorescence depolarisation by DPH labelled vesicles of individual phospholipid classes (PE and PC) recovered from the total lipid extract by TLC, revealed that PE was more fluid from obese mouse erythrocyte plasma membranes compared to lean controls, whereas PC was not altered between the two groups.

The increase in fluidity of the phospholipid extract from obese mouse erythrocyte membranes is thus probably due to a more fluid state of PE. The increase in fluidity was not accompanied by a reduction in the Arrhenius breakpoint, and the flow activation energy was unaltered.

The disparity of a decrease in fluidity of native membrane but an increase in fluidity of extracted phospholipids in the ob/ob mouse may possibly result from an increase in cholesterol levels associated with obese mouse erythrocyte membranes. Obese mice are hypercholesterolaemic (Salmon and Hems, 1973), and therefore more cholesterol may partition into erythrocyte plasma membranes. Erythrocytes are enucleate and therefore unable to regulate their membrane composition.

6.2.e. Fluidity studies on uncharacterised plasma membrane lipid fractions from lean and obese mouse pancreatic cells and salivary gland.

A collagenase digest of pancreas yields a heterogeneous population of islet, acinar and epithelial cells, all of which sediment at very low centrifugal force. Salivary gland homogenates contain organelles derived from epithelial cells (basket cells) serous and mucous cells. 17,000 X g supernatants of

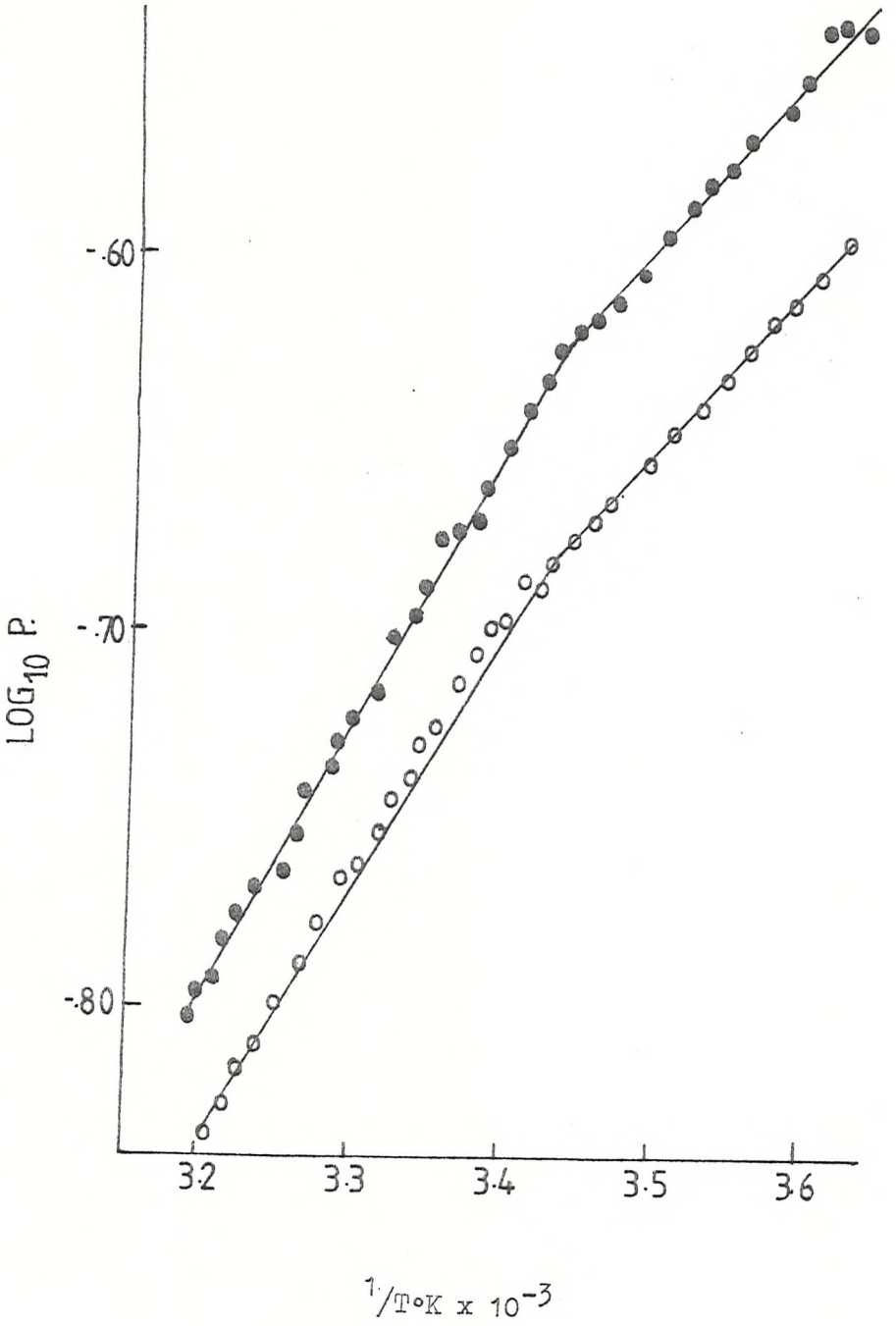


Fig 6.5. Arrhenius plots of fluorescence depolarisation by DPH labelled pancreatic plasma membranes from lean (●) and obese (o) mice housed at 25°C.
Data represents values for membranes derived from 4 animals in each group.

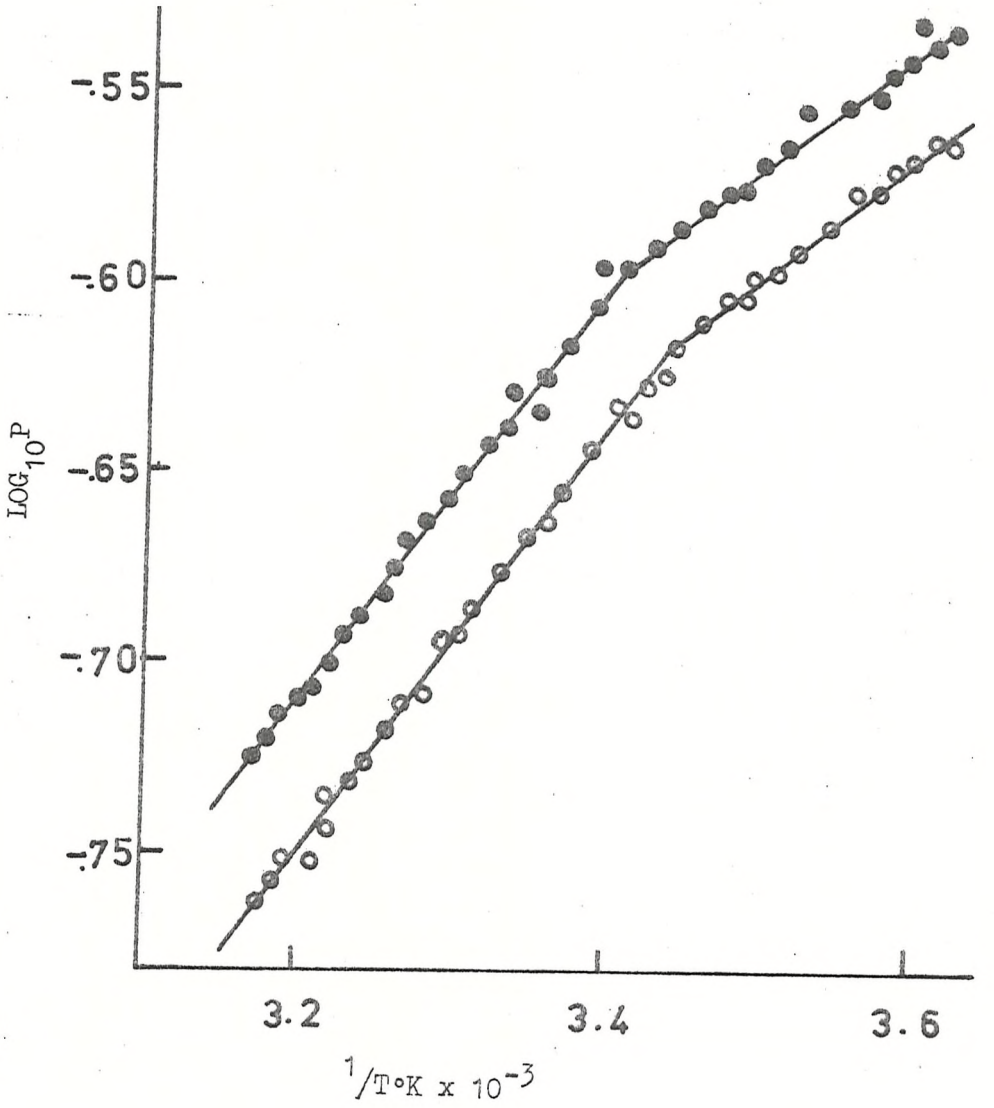


Fig 6.6. Arrhenius plots of fluorescence depolarisation by DPH labelled liposomes derived from extracted salivary gland cell plasma membranes from lean (●) and obese (o) mice. Data represents values for lipids derived from salivary gland cell membranes from 4 animals in each group.

Table 6.5. Summary of data from Arrhenius plots of depolarisation of fluorescence by DPH labelled liposomes from lipid extracts of salivary gland and pancreatic cell plasma membranes from lean and obese mice.

Membrane	Phenotype	$P_{37°}$	Breakpoint	ΔE above Breakpoint kcal mol^{-1}	ΔE below Breakpoint kcal mol^{-1}	Fluidity Ratio ϕR_{37}
Salivary Gland plasma membrane lipid extract	Lean	0.200	20.3	8.78	15.3	1.15
	Obese	0.184	17.7	8.39	14.5	
Pancreatic Cell plasma membrane lipid extract	Lean	0.167	17.7	6.40	10.1	1.16
	Obese	0.152	18.1	6.80	10.4	

Data represents values for membranes from 4 animals in each group.

homogenates from lean and obese mouse pancreatic cells and salivary gland yielded an isopycnic plasma membrane fraction with 9% Ficoll/ 8.6% sucrose. No attempt was made to characterise the resulting membrane fractions in terms of yield or purity, the absence of cardiolipin in the TLC analysis of extracted lipids suggests that there was no major mitochondrial contamination.

Lipid extracts from lean and obese mouse salivary gland and pancreatic cell plasma membranes were labelled with DPH, and Arrhenius plots of fluorescence depolarisation studies are shown in figs 6.5 and 6.6. The results are summarised in Table 6.4.

Both salivary gland and pancreatic cell plasma membranes were more fluid from obese mice compared to lean controls, although Arrhenius breakpoints and activation energies were similar between the two groups of membranes. As membranes used in this study were derived from a heterogeneous population of cell types which may not necessarily be in the same ratios between lean and obese, interpretation of this data is made somewhat more difficult. However, the magnitudes of the fluidity changes in lipids from the two tissues from obese mice were similar to those observed for other homogeneous plasma membranes, and therefore it is possible that all cell types in each tissue from obese mice have more fluid plasma membranes.

6.2.f. Fluidity studies on brain synaptosomal plasma membranes and phospholipids isolated from lean and obese mice, and the GLC analysis of the fatty acid composition of these phospholipids.

Purified nerve terminal synaptic membrane vesicles (synaptosomes) were prepared from lean and obese mice as described in Materials and Methods. An extra gradient purification step in the preparation of these vesicles removed the major part of the mitochondrial and myelin contamination present after preparation by the normal procedure. TLC analysis of the membrane fraction demonstrated a very low cardiolipin content, suggesting the minimal of mitochondrial contamination. Myelin has a very high microviscosity, due to a high cholesterol content (Rumsby, 1978) and therefore the

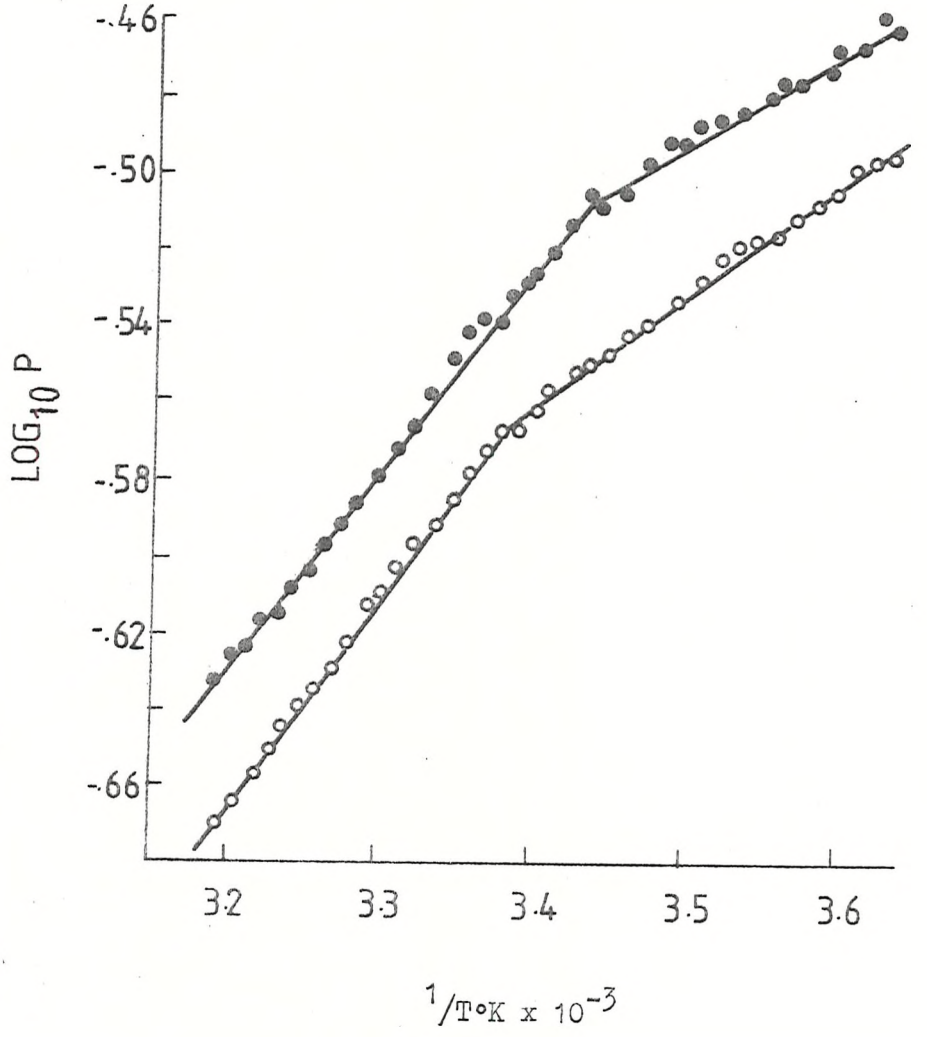


Fig 6.7. Arrhenius plots of depolarisation of fluorescence by DPH labelled brain synaptosomal membrane vesicles from lean (•) and obese (o) mice.
Data represents values for membranes derived from 4 animals in each group.

greater the purity of a synaptosomal fraction, the lower will be its viscosity. The microviscosity of lean and obese samples of synaptosomal membranes was reduced after additional purification, by a small but significant degree (data not shown). Also, after additional purification, mitochondria and myelin fractions were clearly visible on the discontinuous gradients.

Enzymic marker assays are currently underway to assess the exact degree of purity of membranes prepared using the modified purification procedure.

The fluorescence polarisation by DPH labelled synaptic vesicles was measured, and the Arrhenius plots from this study are presented in Fig 6.7. The results are summarised in Table 6.6. Obese mouse synaptosomal membranes are more fluid than lean controls, although the breakpoint was higher than that of lean. The flow activation energy below the breakpoint was lower in obese membranes, although little change was apparent in the flow activation energy above the breakpoint in synaptosomal membranes of obese mice.

PC and PE were extracted from lean and obese mouse synaptosomal membranes and the Arrhenius plots of fluorescence depolarisation by DPH labelled liposomes are shown in Fig 6.8 for PC liposomes and Fig 6.9 for PE liposomes. Samples of these lipids were analysed for fatty acid content (Table 6.7).

Extracted PC liposomes from obese mouse synaptosomal membranes were less fluid than those from lean controls. From the GLC data, obese mouse PC contained a higher percentage saturated fatty acids, and also a smaller average number of double bonds per fatty acid molecule. This observation accounts well for the decreased fluidity of obese mouse PC. PE from obese mouse synaptosomal membranes was considerably fluidized compared to lean controls. Indeed, the fluidity ratio of obese to lean PE was the highest recorded in these studies. GLC analysis of the fatty acid composition of lean and obese mouse synaptosomal PE revealed that both 22:6 and 20:4 were increased from obese mouse synapto-

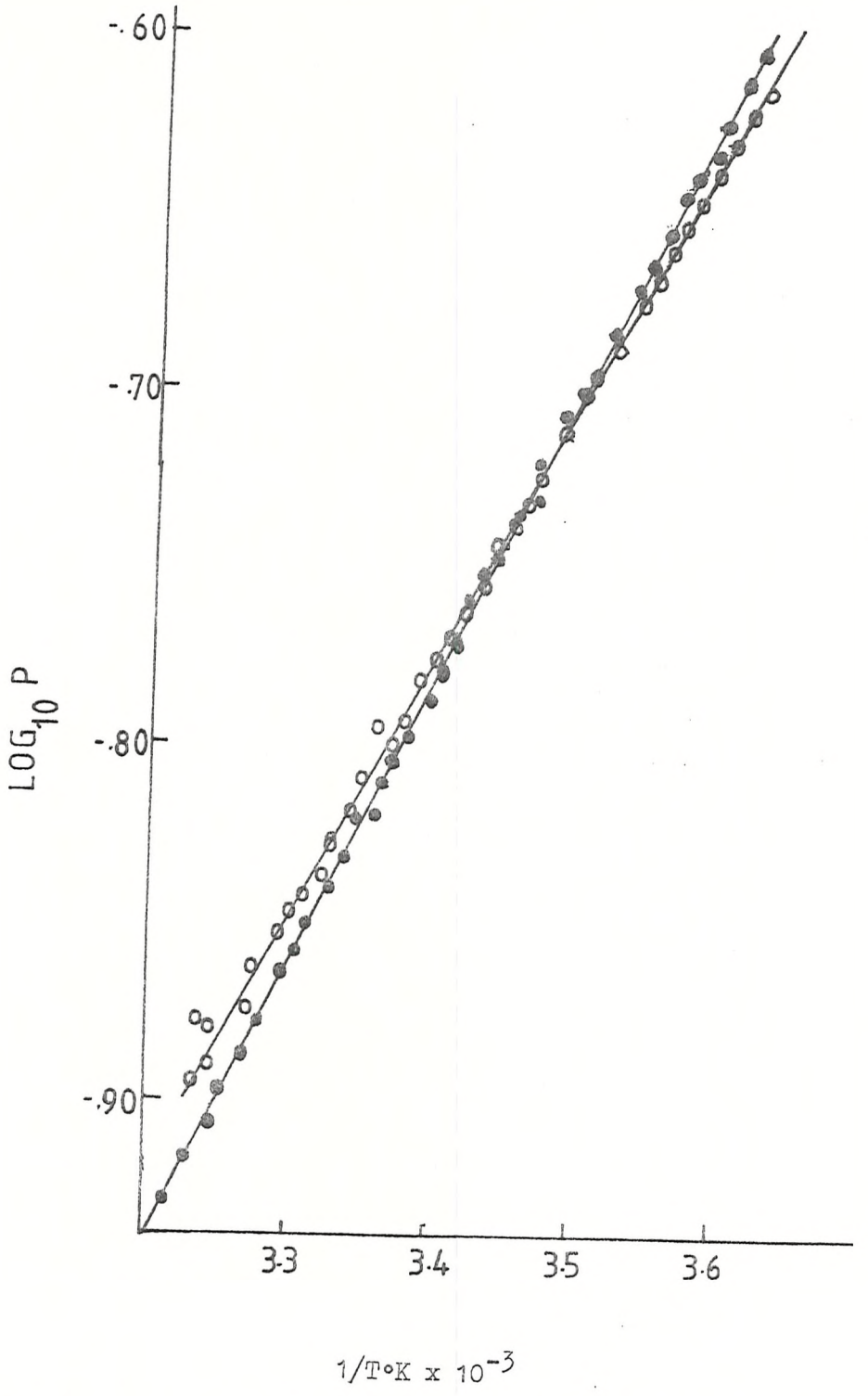


Fig 6.8. Arrhenius plots of depolarisation of fluorescence by DPH labelled brain synaptosomal PC liposomes from lean (●) and obese (o) mice.
Data represents values for membranes derived from 4 animals in each group.

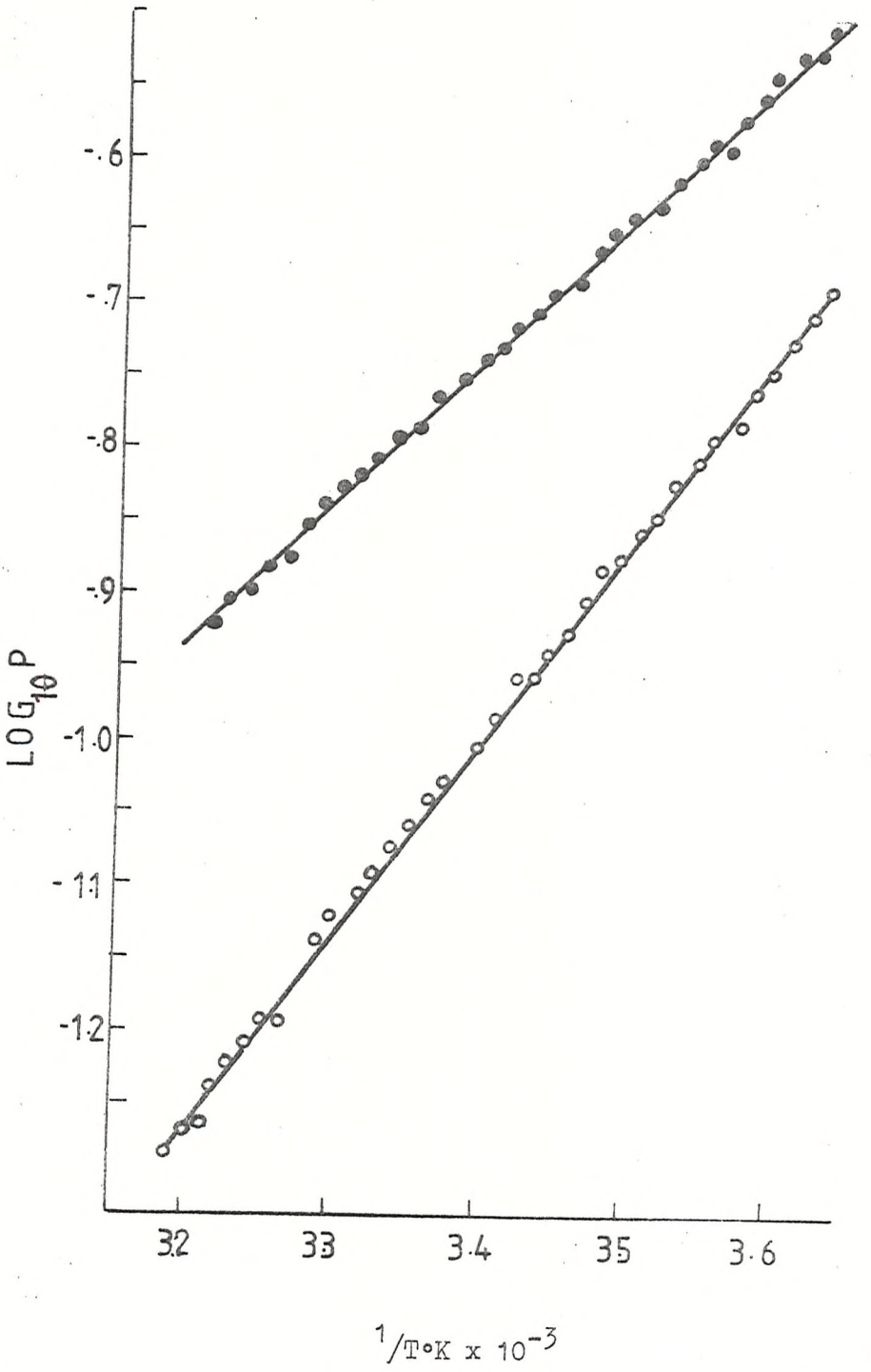


Fig 6.9. Arrhenius plots of depolarisation of fluorescence by DPH labelled brain synaptosomal PE liposomes from lean (●) and obese (o) mice.
Data represents values for membranes derived from 4 animals in each group.

Table 6.6. Summary of data from Arrhenius plots of depolarisation of fluorescence by DPH labelled membrane vesicles and phospholipid liposomes derived from lean and obese mouse brain synaptosomes.

System under study	Phenotype	$P_{37°}$	Breakpoint °C	ΔE above Breakpoint kcal mol^{-1}	ΔE below Breakpoint kcal mol^{-1}	Fluidity Ratio ΦR_{37}
Native membranes	Lean	0.241	18.6	8.72	19.7	1.18
	Obese	0.222	23.3	8.2	16.0	
PC	Lean	0.118	-	5.72	-	0.92
	Obese	0.125	-	6.2	-	
PE	Lean	0.124	-	4.72	-	2.56
	Obese	0.058	-	3.48	-	

Data represents values obtained for membranes and phospholipids from 4 lean and 4 obese animals.

Table 6.7. GLC spectra of fatty acids from PE and PE derived from lean and obese mouse brain synaptosomes.

	Phospholipid fatty acids (% total)			
	PC		PE	
Fatty Acid	Lean	Obese	Lean	Obese
16:0	32.9	41.7	16.9	8.2
16:1	ND	ND	2.7	T
18:0	9.4	12.2	17.6	7.9
18.1	23.3	19.6	23.6	13.2
18:2	2.9	7.2	3.5	1.0
18:3	1.5	2.6	2.7	T
20:0	ND	ND	2.7	ND
20:1	14.9	5.5	1.1	6.4
20:3	3.1	3.8	9.6	1.0
20:4	8.6	3.7	6.1	26.5
22:3	ND	ND	4.7	ND
22:6	3.4	5.0	8.8	25.3
24:1	ND	ND	T	6.0
% saturated fatty acids	42.3	53.9	37.2	16.1
pi bonds per 100 molecules	112.6	103.5	162.6	288.4

Data represents values for membranes derived from 4 animals in each group.

somal membranes, while 16:0, 18:0 and 18:1 were decreased. The net effect of these substitutions was to decrease the total percentage of saturated fatty acid in obese mouse synaptosomal PE to less than 50% of that present in lean, and also to increase the average number of Pi bonds per lipid molecule by 1.8 fold. Again, these observations agree well with fluorescence data.

The flow activation energies of the more fluid PE species was lower than the flow activation energy of the less fluid PE, consistent with the conceptof theLCPUFA's being more ordered than other fatty acids in the liquid-crystalline phase.

Thus, mouse brain synaptosomal membranes display similar characteristics to the adipocyte plasma membrane. As synaptosomal membrane bilayers have assymetric phospholipid distribution, with PE on the inside and PC on the outisde (Fontaine et al., 1980), this is likely to result in a transbilayer fluidity assymetry in obese mouse brain synaptosomal membranes.

6.2.g. Fluidity studies on adipocyte plasma membranes, and brain synaptosomal membranes from the genetically obese Zucker rat (fa/fa) and lean control rats (Fa/?).

Adipocyte plasma membranes from genetically obese Zucker rats and their lean littermates were prepared by the normal method. Membranes were labelled with DPH, and the fluorescence polarisation measured between 4°C-40°C. The Arrhenius plots from this experiment are shown in Fig 6.10 and the data is summarised in Table 6.8. Rat brain synaptosomes were also prepared in an identical fashion to mouse brain synaptosomes. These membranes were then labelled with DPH, and the fluorescence polarisation measured from 0°C-40°C (Fig 6.11). PC and PE were isolated by TLC from a lipid extract of the synaptosomal membranes. Samples were taken of these lipids for GLC analysis, and the results are shown in Table 6.9.

Adipocyte and synaptosomal plasma membranes from obese rats were more fluid than lean controls, with lower Arrhenius breakpoints. Flow activation energies were similar between membranes from lean and obese animals, although the flow activation energies tended

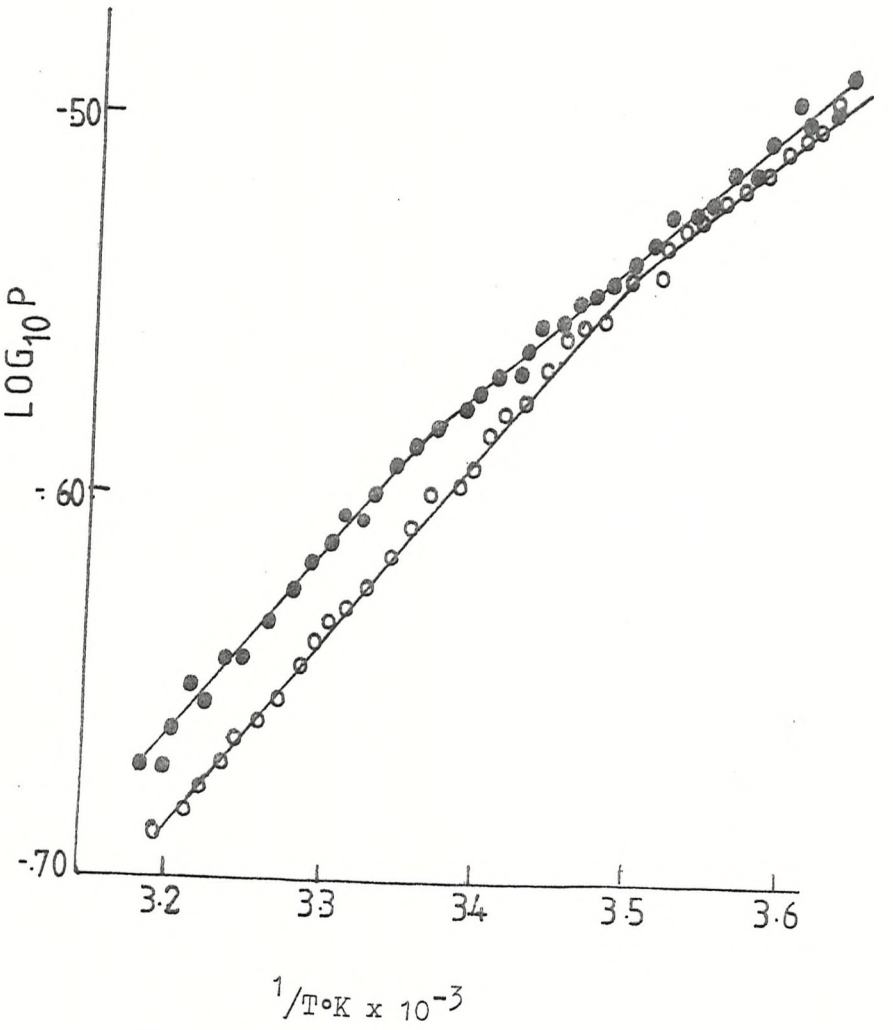


Fig 6.10. Arrhenius plot of depolarisation of fluorescence by DPH lablelled adipocyte plasma membranes from lean (●) and obese (o) Zucker genetically obese rats. Data represents values for membranes derived from 2 animals in each group.

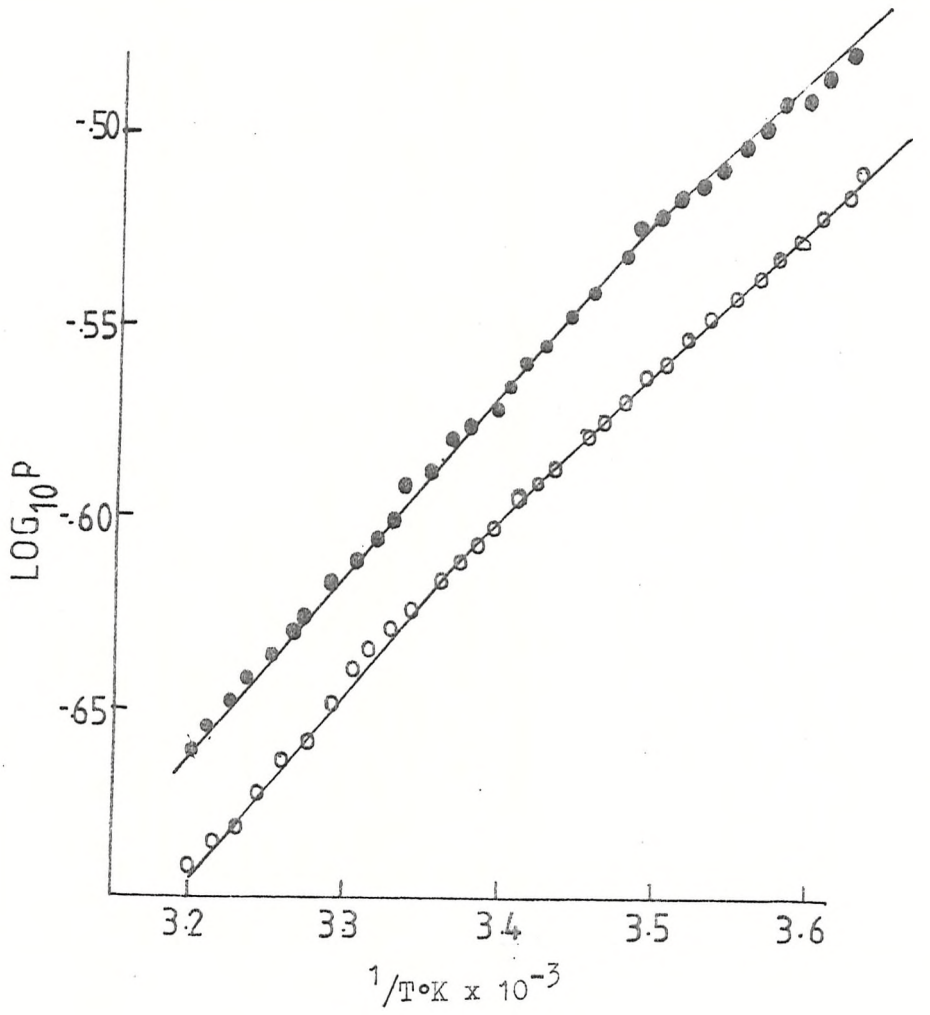


Fig 6.11. Arrhenius plot of depolarisation of fluorescence by DPH labelled brain synaptosomal membranes from lean (●) and obese (o) Zucker genetically obese rats. Data represents values for membranes derived from 4 animals in each group.

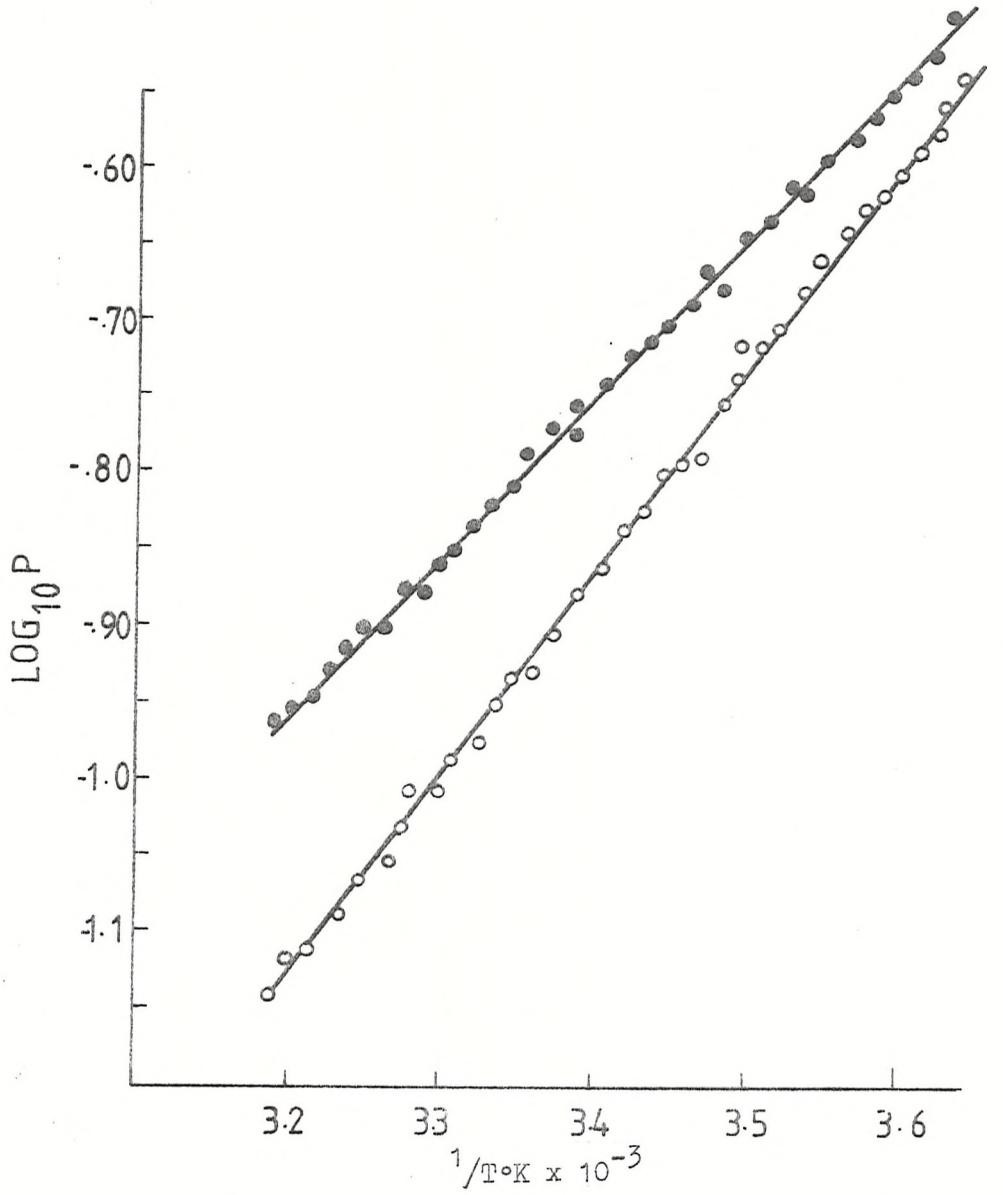


Fig. 6.12. Arrhenius plot of depolarisation of fluorescence by DPH labelled brain synaptosomal PE liposomes from lean (●) and obese (o) Zucker rats.
Data represents values for membranes derived from 4 animals in each group.

Table 6.8. Summary of data from fluorescence studies of membrane vesicles and phospholipid liposomes derived from membranes of lean and obese Zucker rats.

System under study	Phenotype	P_{37}	Breakpoint	ΔE above Breakpoint kcal mol^{-1}	ΔE below Breakpoint kcal mol^{-1}	Fluidity Ratio ϕR_{37}
Adipocyte* plasma membrane	Lean	0.223	23.7	9.8	12.5	1.14
	Obese	0.208	13.5	9.5	12.3	
Brain ± synaptosomal membranes	Lean	0.251	25.8	8.5	20.2	1.13
	Obese	0.237	19.3	8.0	17.3	
Synaptosomal PE ±	Lean	0.116	-	4.35	-	1.60
	Obese	0.080	-	3.5	-	
PC ±	Lean	0.169	-	-	-	1.0
	Obese	0.168	-	-	-	

Data represents values for membranes derived from *2 and ±4 animals in each group.

Table 6.9. GLC spectra of fatty acids derived from PC and PE derived from lean and obese rat brain synaptosomes.

	Phospholipid fatty acids (% total)			
	PC		PE	
Fatty Acid	Lean	Obese	Lean	Obese
16:0	21.9	27.5	14.4	12.4
16:1	ND	ND	1.7	1.8
18:0	41.0	34.0	17.0	11.6
18:1	10.1	12.0	18.9	16.6
18:2	11.2	9.2	2.0	2.0
18:3	1.1	2.6	2.0	0.9
20:1	10.7	9.3	2.3	5.8
20:3	ND	ND	3.9	0.8
20:4	3.9	5.4	12.7	18.4
22:3	T	T	3.9	4.8
22:4	ND	T	3.7	ND
22:5	ND	ND	T	T
22:6	T	T	12.9	22.0
24:1	ND	ND	4.7	2.4
% unsaturated fatty acids	62.2	61.5	31.4	24
Pi bonds per 100 molecules	62.2	69.5	199.3	255.7

Data represents values for membranes derived from 4 animals in each group.

to be lower above the breakpoint, consistent with observations on obese mouse membranes. Fluidity ratios were similar to those obtained for lean and obese mouse membranes.

PE and PC were extracted from lean and obese rat synaptosomal membranes, and the data from the Arrhenius plots of fluorescence depolarisation by DPH labelled liposomes is summarised in Table 6.8. The Arrhenius plot from measurements on PE liposomes is shown in Fig 6.12. Samples of these lipids were analysed by GLC for fatty acid content (Table 6.9). Phospholipids from synaptosomal membranes from lean and obese rat demonstrated similar changes to those observed from mice, although the differences were less exaggerated. No difference could be detected in the fluidity at 37°C between lean and obese rat synaptosomal PC and this observation is supported by the GLC data for those phospholipids, since little difference was discernable in the percentage unsaturated fatty acid content, although the number of double bonds per molecule was slightly increased in obese rat synaptosomal PC. Extracted PE, however, was considerably more fluid from obese rat synaptosomes compared to lean controls, and demonstrated a reduced flow activation energy. The GLC data for this phospholipid demonstrate increased 22:6 and 20:4, and reduced 16:0 and 18:0 in obese rat PE compared to lean.

The results from this study demonstrate that increased membrane fluidity, and fluidised PE is a general feature of at least two animal models of genetic obesity, and therefore altered membrane fluidity assymetry may be a general feature of the obese condition, and may therfore be instumental in the wide range of cell membrane anomalies observed in mammalian obesity.

6.2.h. <u>The effect of starvation and hypothyroidism on mouse adipocyte plasma membrane fluidity.</u>

Plasma membranes were prepared from lean mice that had been starved for 48 hrs, and from animals made hypothyroid with 0.03% PTU in their diet for 10 days. The ability of this dietary regimen to induce hypothyroidism has been assessed by Shimomura et al. (1981). Membranes were labelled with DPH, and the fluorescence polarisation measured from 4°C-40°C. The Arrhenius plots from

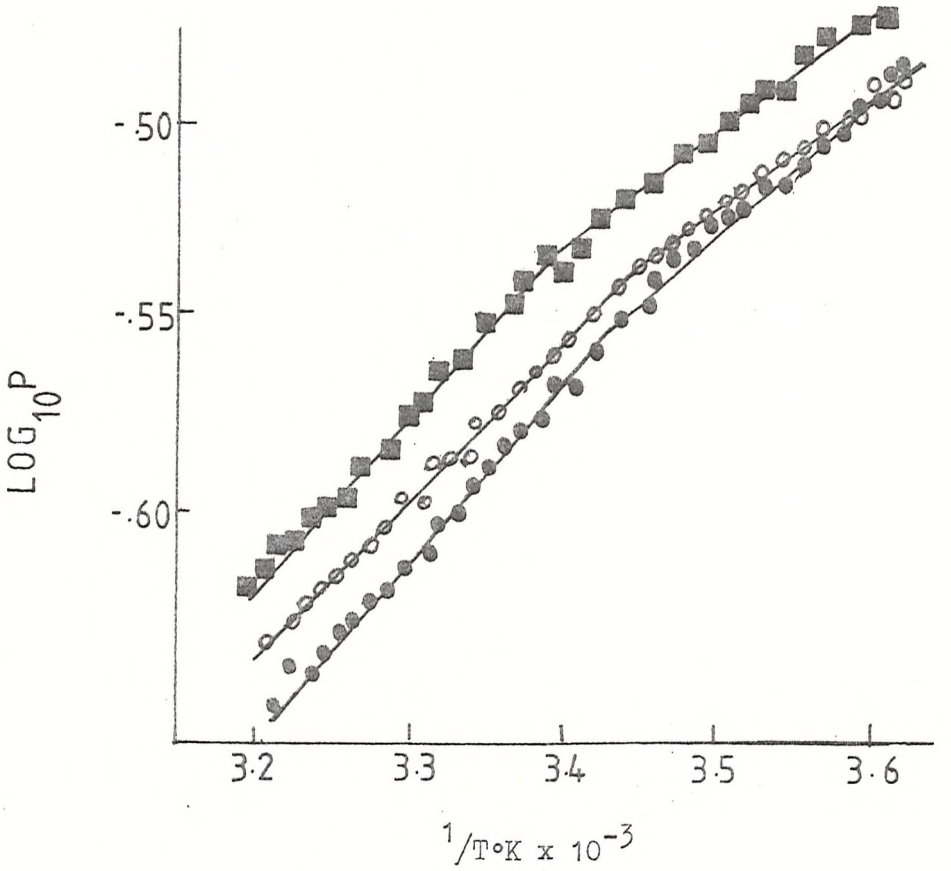


Fig 6.13. Arrhenius plots of fluorescence polarisation by DPH labelled membranes from control (■), starved (●) and hypothyroid (o) mice.
Data represents values obtained from membranes of 4 mice in each group.

Table 6.10. Summary of data from Arrhenius plots from fluorescence depolarisation by DPh labelled adipocyte plasma membranes from control, starved and hypothyroid lean mice.

Treatment	Phenotype	P_{37}	Breakpoint	E above Breakpoint kcal mol^{-1}	E below Breakpoint kcal mol^{-1}	Fluidity Ratio R_{37}	
Ad Libitum feeding Euthyroid	Lean *	0.249 ± 0.001	25.1 ± 0.26	8.02 ± 0.45	20.36 ± 0.93		
						1.23	
Starved Euthyroid	Lean	0.225	19.8	10.12	12.93		1.13
Ad Libitum feeding Hypothyroid	Lean	0.235	17.3	11.5	15.8		

* Values are mean ± SEM

Data represents values for membranes derived from 4 animals in each group.

these studies are shown in Fig 6.13 and the data from these plots is summarised in Table 6.10.

Starving mice and PTU-induced hypothyroidism in mice induces a fluidisation of the adipocyte plasma membrane, although the effect of starvation was apparently greater than the effects of hypothyroidism. A reduction in the Arrhenius breakpoint was also observed after starvation and PTU induced hypothyroidism, hypothyroidism inducing a greater change than starvation. Flow activation energies below the breakpoint were reduced by hypothyroidism and starvation.

Thus, all of these changes observed in the form of the Arrhenius plots of fluorescence depolarisation by DPH after induction of hypothyroidism and starvation in lean animals are similar to changes that occur in the Arrhenius plots from adipocyte plasma membranes isolated from obese mouse membranes.

6.3. Discussion

6.3.1. Studies on brain synaptosomal membranes from lean and obese mice.

Brain synaptosomal membranes from ob/ob mice are more fluid than those from lean controls. Fluidity studies on isolated phospholipids from the membranes also demonstrated a large increase in both fluidity and LCPUFA incorporation suggesting that the change in membrane fluidity results from the changed phospholipid composition. The fluidity of PE was much increased, and that of PC unchanged, in phospholipids isolated from membranes from ob/ob mice compared to lean controls. Fontaine et al. (1980) have calculated that pure rat brain synaptosomal membranes are comprised of about 44% PC and 38% PE. Furthermore, these authors demonstrated that only 10-15% of the PE resided in the outer monolayer of these membranes. Assuming mouse brain synaptosomal membranes are similarly assymetric with respect to the localisation of the major phospholipid components PC and PE, then clearly a massive fluidity assymetry exists in membranes from obese animals, much greater than that observed for adipocytes. A large increase in 22:6 was observed in synaptosomal PE from obese mice. As the inner synaptosomal monolayer contains a large amount of PE, the cholesterol affinity for this monolayer is likely to be low, and thus there may be an increase in outer monolayer cholesterol in obese mouse synaptosomes (Chapter 5) compared to lean controls, assuming equal cholesterol content of these membranes. Thus, the permeability to ions and small molecules may be decreased in ob/ob mouse synaptosomes, and thus ion pumping may be reduced in these membranes. Indirect support for this concept has been provided by Sun and Sun (1971). These workers studied the lipid composition and activity of the Na^+/K^+ ATPase in isolated synaptosomes from normal and essential fatty acid deficient mice. The amount of 22:6 was substantially reduced in PE from synaptosomal membranes of EFA-deficient animals, and the activity of the Na^+/K^+ ATPase was significantly elevated. The reduced unsaturation of EFA deficient mice synaptosomes is unlikely to elevate the specific activity of Na^+/K^+ ATPase units, as Kimmelberg and Papahadjopoulos (1974) have observed that the activity of solublised ATPase was increased

when unsaturated fatty acids were added to the preparation. Although Sun and Sun, (1974) did not compare cholesterol content of EFA and control mice synaptosomal membranes, it is possible that the increase in activity of the ATPase results in an increase in the permeability of Na^+ and K^+, possibly caused by a net transfer of cholesterol from the outer to the inner monolayer, when inner monolayer 22:6 was reduced by EFA deficiency.

Reduced ionic pumping in brain synaptosomes of ob/ob mice is also supported by the observation that Na^+/K^+-ATP ase activity is low in other tissues of ob/ob mice (York et al., 1978; Lin et al., 1978), and that increased neurotransmitter levels have been observed in hypothalamus, telencephalon and brain stem of ob/ob mice (Lorden et al., 1976). The reuptake of transmitter from the synaptic cleft into the pre synapse is the major control of synaptic activity, the reuptake process is a Na^+/K^+-ATPase-dependant process (Iwatsuka et al., 1970). Thus a low activity of this enzyme in ob/ob mouse synaptosomes may contribute to the altered hypothalamic control of pituitary function observed in the ob/ob mouse (Chapter 1).

Increased synaptosomal fluidity in ob/ob mice may impair the β receptor/adenylate cyclase coupling efficiency in these membranes as it possibly does in adipocytes. Although the role of cAMP in the functioning of neuronal activity is unclear at present, nevertheless the presence of large levels of adenylate cyclase in synaptosomes and the wide range of agents that stimulate this enzyme via receptor molecules suggest a functional significance for this nucleotide in brain (Daly, 1977). Thus, poor receptor/adenylate cyclase coupling efficiency in brain synaptosomes of ob/ob mice may also contribute to their altered pituitary function.

Impaired cAMP production in response to neurotransmitter secretion in the hypothalamus of ob/ob mice may also contribute to the postulated changes occurring in this region of the brain in ob/ob mice (Bray and York, 1979) such as impaired satiety centre function, thermoregulatory function and fertility control. All three processes are disturbed in the ob/ob mouse (Chapter 1).

6.3.2. Pancreatic cell plasma membrane in lean and ob/ob mice.

Bray and York (1979) have suggested that the autonomic nervous system (ANS) is involved in the expression of ventromedial hypothalamic (VMH) obesity (autonomic hypothesis). This has been extended by Bray et al. (1981) to conclude that sympathetic tone is reduced in both the β cell and salivary gland in VMH obese rats, and this factor largely contributes to both the hyperinsulinaemia and reduced salivary gland size in these animals. The ob/ob mice is also hyperinsulinaemic and has reduced salivary gland size, and therefore reduced sympathetic tone may contribute to the hyperinsulinaemic syndrome of the ob/ob mouse. Sympathetic stimulation of the β cell of ob/ob mice may be further blunted if the increased fluidity of pancreatic cell plasma membranes observed in this chapter leads to impaired stimulation of adenylate cyclase by catecholamines. This postulate is based on the possibility that the β cell membrane is disturbed in the same manner as in the adipocyte plasma membrane. Thus, it is possible that the change in membrane fluidity could be fundamental to the increase in insulin secretion in the ob/ob mouse.

Noradrenaline from the sympathetic nerve terminals (Campfield and Smith, 1980) and adrenaline from the adrenal medulla (Beard and Porte, 1980) inhibit insulin release in response to glucose and amino acids, either by direct inhibition of insulin output from secretory granules within the β cell, or by inhibition of the glucose signal for insulin secretion, or probably through both mechanisms (Fig 6.14.a). The parasympathetic nervous system, via acetyl choline, has been shown by Campfield and Smith (1980) to stimulate insulin secretion. Again this effect may be either direct or indirect (Fig. 6.14.a). Further the acetylcholine stimulation itself is under inhibitory control by sympathetic ANS, although the inhibitory influence of the parasympathetic ANS on the sympathetic ANS inhibition of insulin release is weak (Campfield and Smith, 1980). Thus in the β cell of ob/ob mice (Fig 6.14b) poor receptor-cyclase coupling would result in a reduction in the adrenalin/noradrenalin

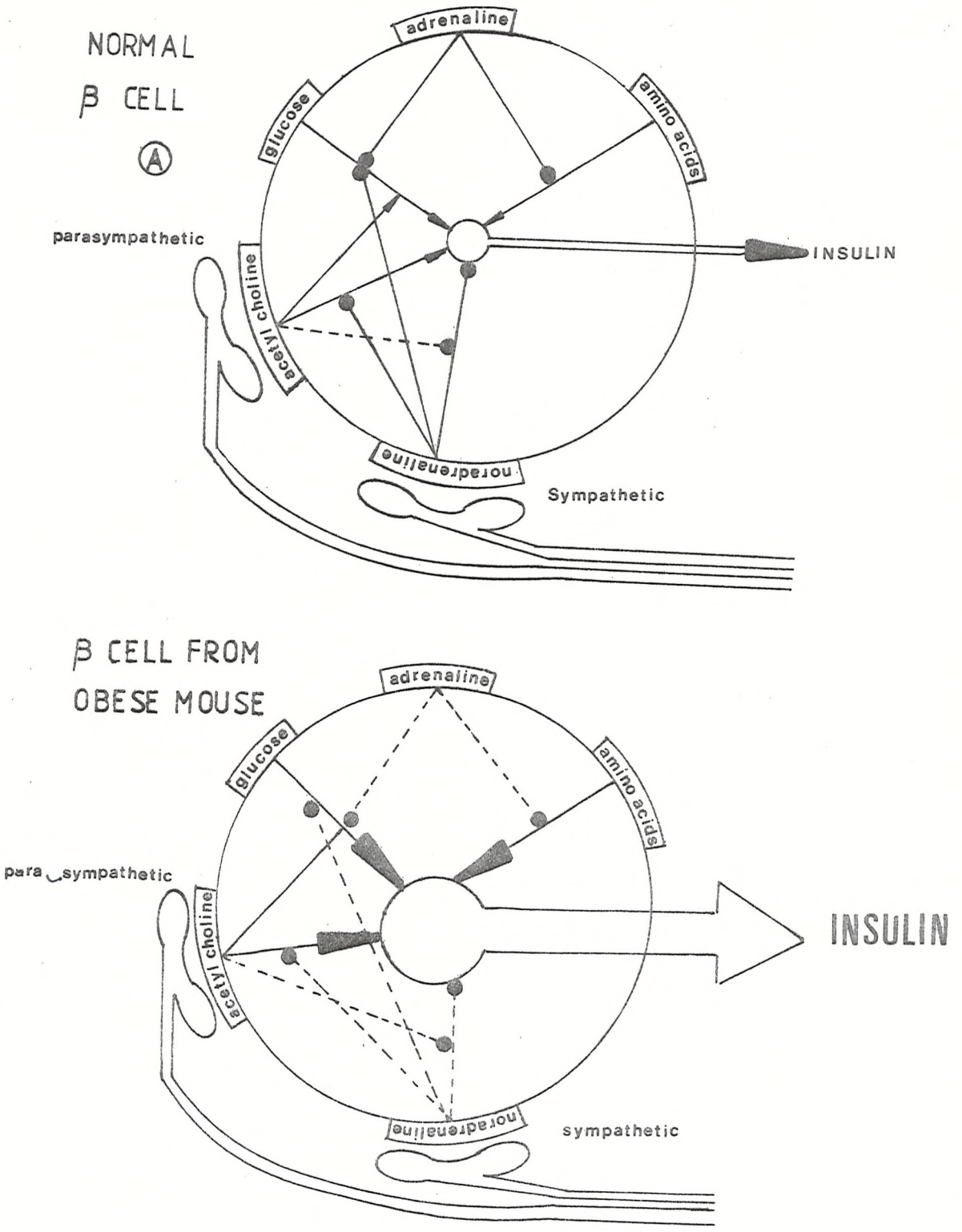


Fig 6.14. Model for β cell hyperinsulin secretion due to impaired sympathetic tone, and impaired β receptor/adenylate cyclase coupling efficiency.

Normal Stimulation

Exaggerated Stimulation

Normal Inhibition

Weak Inhibition

effects of insulin secretion in response to glucose, amino acids, and parasympathetic stimulation, and hyperinsulinaemia would result. The lipid fluidity of the β cell plasma membrane may therefore contribute to the hyperinsulinaemic syndrome of ob/ob mice.

6.3.3. Muscle cell plasma membrane fluidity in lean and ob/ob mice.

Sarcolemmal membrane lipid extracts from ob/ob mice demonstrate an increased fluidity compared to lean controls. This observation may help to explain the results of Chan and Dehaye (1981) who observed that adrenalin did not significantly stimulate glucose transport into muscle from db/db mice, whereas a significant stimulation was obtained for lean controls. The role of catecholamines in the regulation of fed state metabolism is underrated at the present time. Rowe et al., 1981, have demonstrated that plasma noradrenaline levels rise in a dose-dependant fashion to plasma insulin content independantly of blood glucose concentrations. This elevation in catecholamines is due to an increase in sympathetic activity. The plasma catecholamine concentration remains elevated after cessation of insulin secretion in the post-absorbative phase of feeding. Thus, the impaired catecholamine stimulated basal uptake of glucose into muscle of db/db mice observed by Chan and Dehaye (1981) may contribute to the impaired glucose clearance in the animal. Thus, impaired glucose clearance in the db/db and ob/ob mouse may result from the observed increased fluidity of the sarcolemmal membrane reducing the coupling efficiency between β receptor and adenylate cyclase.

In the ob/ob mouse, the muscle tissue is insulin resistant and has a reduced basal glucose uptake. The decreased basal glucose uptake is not secondary to hyperinsulinaemia or hyperglycaemia as Cuendot et al.,(1976) have demonstrated that reduced basal uptake of 2-deoxy-glucose in soleus muscle of ob/ob mice is observed by 4 weeks of age, (when the animals are still normoglycaemic) and is not improved after starvation or streptozotocin

treatment. Both these reduce blood insulin and glucose in ob/ob mice. In Chapter 4 it was established that glucose transport may possibly be impaired into adipocytes in ob/ob mice due to altered membrane characteristics. The increased fluidity of sarcolemmal membrane may contribute to this phenomenon.

6.3.4. Salivary Gland plasma membrane fluidity in lean and ob/ob mice.

The reduced size of salivary glands in ob/ob mice suggests reduced sympathetic stimulation of this tissue by the ANS. However, the observation that the plasma membrane of cells from this tissue are more fluid from ob/ob mice than from lean controls raises the possibility that ob/ob mouse salivary gland may not be as responsive to catecholamine neurotransmitters as lean controls, and this itself may cause atrophy. Salivary gland may be a model for brown adipose tissue (BAT), in that both tissues are heavily supplied by ANS, and both tissues are atrophied in ob/ob mice, dispite an increased demand for both saliva and thermogenesis. The quantity of BAT in ob/ob mice is actually three times greater than lean controls, but the protein content is only 0.66 that of lean controls (Girardier and Seydoux, 1981) hence the metablic mass is reduced, although the triacylglycerol content is markedly elevated.

BAT occurs at multiple sites in mammals, is highly innervated by the ANS, and is unique in its ability to sustain heat production on stimulation by the ANS for long periods of time. Its role in thermogenic homeostasis in rodents in cold stress is well established (Lindberg, 1976). As sympathetic activity is increased in response to insulin (Rowe et al., 1981), the thermic output of BAT may be expected to increase following insulin release, and this may be involved in the elevated thermic output of animals following a meal (Garrow, 1978). Dietary induced thermogenisis (DIT) (long term thermogenic response to elevated food uptake) also involves chronic stimulation of the sympathetic nervous system (by mechanisms unknown), to activate BAT thermogenesis (Rothwell and Stock, 1979). Thus, BAT may be useful to an animal as a thermic organ and as a regulated energy shunt. BAT activation results in a decrease in the efficiency

of energy utilisation, and therefore accelerates the oxidation of energy substrates. This ability of BAT first led Himms-Hagen (1979) to speculate that BAT may be involved in the maintainance of energy balance in mammals, and that obesity in ob/ob mouse may result from a malfunction in this tissue. This concept has been supported by Girardier and Seydoux (1981) who found that BAT was not activated by electrical stimulation in ob/ob mice to anything like that observed in lean control animals. This observation suggests that the impaired response of BAT to electrical stimulation may contribute to the impaired thermoregulatory thermogenesis and the impaired response to catecholamines in these animals (Trayhurn and James, 1978).

The observations of Girardier and Seydoux (1981) suggest that BAT in ob/ob mice is functionally disconnected from the ANS. This is not due to a decrease in the number or affinity of β receptors (Girardier and Seydoux, 1981), therefore, a post receptor defect seems likely. Although BAT plasma membrane was not isolated, the observation that salivary gland plasma membrane was more fluid from ob/ob mice points to a potential plasma membrane defect in BAT,possibly at the level of neurotransmitter resistance.

The acute response to noradrenaline has been studied with isolated brown adipocytes. Within 1 s of noradrenaline binding, a partial depolarisation of the plasma membrane occurs (Seydoux and Girardier, 1978), and intracellular cAMP levels rise to a maximum after 30-180 sec (Pattersson and Vallin, 1976). Eight different protein kinases are stimulated by cAMP (Knight and Skala, 1977) in BAT, the role of these proteins is not understood, however the induction of lipolysis observed in catecholamine binding in this tissue is almost certainly controlled by proteinkinase, as it is in other tissues (Newsholme and Start, 1973).

The nature of the regulation of mitochondrial function in this tissue by cAMP and the extent to which the lipolytic and respiratory responses of this tissue are interdependant is unknown. However, it is clear that a plasma membrane defect reducing the coupling efficiency between β receptors and adenylate cyclase may be involved in the poor response of this tissue to stimulation by the ANS.

6.3.5. Mitochondrial function in the ob/ob mouse.

Liver mitochondria from ob/ob mice are capable of a higher rate of ATP synthesis than lean controls (Katyare and Howland, 1978). However, ADP/O ratios and the degree of respiratory control was identical at each phosphorylation site in mitochondria from the two phenotypes. The increase in oxidative phosphorylation rates observed in mitochondria from ob/ob mice was largely due to an increase in respiratory chain enzymes, although the ratio of the oxidation rates in mitochondria from obese and lean mice was always greater then the respective cytochrome content ratio of their mitochondria (Katyare and Howland, 1978). In vivo, the ATP/ADP translocator in the inner mitochondrial membrane may be the rate limiting step in state 3 respiration (Pfaff et al., 1969), and therefore an increase in activity of this enzyme may account for this observation. Thus, the increase in fluidity observed in the inner mitochondrial membrane from ob/ob mice does not appear to influence the efficiency of either phosphorylation or respiratory control under the experimental conditions used by Katyare and Howland (1978) compared to lean control. However, the activity of the ADP/ATP translocator is very sensitive to the physical state of its environment (Pfaff et al., 1969), the activity of the enzyme doubling over a 5°C temperature increase. Thus, the increase in fluidity of the ob/ob mouse inner mitochondrial membrane may be influencing the activity of the ADP/ATP exchange process, such that state 3 respiration rates may be greater in mitochondria from ob/ob mice.

The degree of unsaturation of mitochondrial membrane lipids is known to vary with both thyroid (Hulbert, 1978) and nutritional status (Giacobino and Chmelar, 1977). Starvation and hyperthyroidism increasing the total percentage unsaturation of the fatty acids of mitochondrial membranes. Thyroid hormones increase both the specific and total activity of mitochondrial respiratory chain enzymes (Hulbert, 1978). Thus, membrane lipid fluidity may be an adaptation to provide a greater ATP synthesising capacity under conditions of accelerated metabolism in hyperthyroidism, and possibly to sustain the increased lipid synthesis of obese mouse livers. In starvation, perhaps it is simply an adaptation to econ-

omise on mitochondrial protein.

Similar fluidity changes have been observed for BAT mitochondrial membranes by French and York (unpublished observations) as have been reported in this chapter for hepatic mitochondria. The contribution of BAT to non-shivering thermogenesis is high (Foster and Frydman, 1979), and its role in themoregulatory thermongenesis has been discussed by James and Trayhurn (1981). The lower expenditure of food energy on thermoregulatory thermogensis in ob/ob mouse determined by James and Trayhurn (1981) is thought to lead to increased metabolic efficiency in these animals. As the activation of BAT by the sympathetic nervous system is severely blunted in ob/ob mice housed at 25°C (Girardier and Seydoux, 1981) the possible contribution of a fluidized inner mitochondrial membrane to the poor response of BAT in ob/ob mice to noradrenaline release by the ANS should be considered.

Mitochondrial inner membrane dehydrogenases donate electrons to molecular oxygen via the respiratory chain enzymes. Since the respiratory chain is composed of resistive electron carriers, then induction of net directional electron transport requires that potential energy be dissipated through each resistive element in the chain. Electron potential energy is transduced into kinetic vectorial proton translocation out of the mitochondria. If the conductance of the membrane to protons is low, energy is conserved as electrostatic charge separation (membrane potential, $\Delta\mu H^+$) across the membrane. Since this charge is not dissipated a steady state is reached. The generated membrane potential inhibits electron transport, as it is sufficient to drive protons back through the proton translocators at a rate which equals the rate of respiration driven proton extrusion, and thus, no net electron transport occurs. $\Delta\mu H^+$ can be dissipated by proton translocators that are coupled to ATP synthesis. Thus, in the absence of any other dissipation mechanism, electron transport is said to be tightly coupled to ATP synthesis. In practise, the membrane is slightly leaky to protons, thus reducing $\Delta\mu H^+$. The energy given to ion-pair separation during genesis of the membrane potential is dissipated as heat on charge neutralisation.

Electrons flow down the respiratory chain as $\Delta\mu H^+$ is not now at equilibrium with the redox potential of the electron donator/acceptor couple. In the absence of ATP synthesis, the mitochondria are in state 4 respiration. Clearly, the value of $\Delta\mu H^+$ * determined by the proton conductance leakage of the membrane, determines respiration rate, and thus $\Delta\mu H^+$ electron transport in the respiratory chain becomes rate-limiting. Reducing $\Delta\mu H^+$ further has no effect on respiration rate, and the mitochondria is said to be 'uncoupled' when all respiratory control is lost.

BAT mitochondria differ from mitochondria from other tissues in that they possess a symport anion uniporter within the membrane matrix, and OH^- ions can be extruded from the mitochondrial matrix, thus neutralising H^+ (Nicholls, 1979). $\Delta\mu H^+$ is reduced, respiratory control is lost, and electron transport results in heat generation. In the presence of substrate and oxygen, BAT mitochondria are capable of producing large quantities of heat. BAT adipocytes are tightly packed with mitochondria and triacylglycerol and the tissue is also well inervated with sympathetic nerve endings. The mechanism by which the activity of the anion uniporter is controlled, such that under non thermogenic conditions, BAT mitochondria are subject to respiratory control, is unclear. The primary event in activation of BAT is the binding of noradrenaline to the brown adipocyte plasma membrane receptor. The intracellular cAMP level is elevated (section 6.3.5) increasing lipolysis and NADH concentration. The plasma membrane is depolarised, resulting in an increase of cytosolic Ca^{++} (Nedergaard, 1980). It has been suggested that there are two events that contribute to the conduction of mitochondrial thermogenesis. The anion uniporter of BAT mitochondria have been shown to be regulated by a 32,000 M.W. protein (Heaton et al., 1978). This protein, associated with the mitochondrial inner membrane, inhibits the anion uniporter when it binds purine nucleotides at the outer surface of the inner membrane. The amount of purine nucleotide binding, and the proportion of the 32,000 mw protein correlates well with the thermogenic capacity of the tissue (Desautels et al., 1978). On exposure to cold there is an immediate increase in GDP binding in mitochondria from BAT through an unmasking of

* exerts respiratory control over the mitochondria. At a certain value of $\Delta\mu H^+$

existing binding sites. This is followed after 24 hrs by an increase in the 32,000 protein.

Hogan and Himms-Hagan (1980) have demonstrated that GDP binding of BAT mitochondria from obese mice was only 30% of that in lean controls, although no change was observed in the amount of 32,000 M.W. protein in their BAT mitochondrial inner membranes. When the mice were acutely cold exposed at 4°C, lean mice mitochondria increased GDP binding whereas obese mice did not, and subsequently died. These changes mirror the poor thermogenic ability of BAT from ob/ob mice tissue and its failure to 'switch on' on cold exposure (Girardier and Seydoux, 1981).

Thus this work raises the possibility that a level of control is operating on the prevention of the uncoupling process in BAT mitochondria from ob/ob mice. This inability to 'switch on' thermogenesis in BAT from ob/ob mice is not due to a lack of thermogenic potential by this tissue, as when the animals were adapted to less severe cold (14°C), BAT from ob/ob mice gradually increases its thermogenic capacity and an increase in the level of GDP binding in their BAT mitochondria was observed (Hogan and Himms-Hagan, 1980).

The relationship of the purine nucleotide binding protein to the anion uniporter is unknown, except that purine nucleotide binding regulates the effective proton conductance of the inner mitochondrial membrane (Nicholls, 1979). The number of anion uniporters in BAT mitochondria may not significantly alter respiration rate per se. For example, Nicholls (1979) has demonstrated that in cold adapted guinea pig BAT mitochondria, the effective proton conductance of the inner membrane is considerably in excess of that required to merely avoid respiratory control and that 50% inhibition of membrane conductance had no effect on respiration, although $\Delta\mu H^+$ doubled. The stoichiometry of inhibitory protein and anion uniporter is unknown. Thus, it is impossible to predict whether or not the reduction in GDP binding in BAT mitochondria from ob/ob mice is directly coupled to a possible reduced anion uniporter concentration (and even if it is whether this will effect the degree of uncoupling) or

whether it is reduced simply because the tissue is in a refractory state, due to a metabolic block. In other words, reduced GDP binding protein and the unmasking event on cold exposure need not represent an inability to reduce $\Delta\mu H^+$ to a level necessary to escape respiratory control in mitochondria from ob/ob mice.

It is possible to speculate that an increased requirement for this inhibitory 32,000 M.W. protein in active, uncoupled mitochondria is necessary in order to exert maximum control over mitochondria in this state. However, the purine nucleotide content of the matrix is unlikely to vary (Nicholls, 1979). A possible role for the purine nucleotide binding protein is that it regulates mitochondrial respiration under conditions of varying pH (Nicholls, 1979). This author has shown that the affinity of the regulatory protein for GDP is highly pH dependant. In highly active thermogenic BAT, the effective proton conductance of the membrane and the ATP-ase content of the membrane are low (Nicholls, 1979). Thus, the ability of these mitochondria to synthesise ATP may fall due to $\Delta\mu H^+$ being reduced to a level thermodymanically unfavourable for ATP synthesis. Substrate level phosphorylation does not contribute significantly to ATP synthesis under these conditions (Nicholls and Bernson, 1977). Thermogenic BAT has a high ATP requirement for substrate activation, for normal metabolic requirements of the cell, and also for the high Na^+/K^+ ATP-ase activity required to maintain the increased plasma membrane depolarisation of BAT adipocytes. Thus, as intracellular phosphate levels rise due to ATP hydrolysis, so the pH falls inhibiting the anion uniporter, raising $\Delta\mu H^+$ to a value where ATP synthesis can proceed.

Thus, it is possible that the increase in GDP binding protein in mitochondria is a function of the necessity for greater control of $\Delta\mu H^+$ in highly active uncoupled mitochondria. Reduced GDP binding in the mitochondria of ob/ob mice BAT may represent a deliberate de-repression of anion uniporter activity, in order to increase the thermogenic activity of this refractory tissue (Girardier and Seydoux, 1981), metabolically blocked at some stage. Expression of new binding sites for GDP in mitochondria of lean

mice cold exposed, may be a secondary event to thermogenic induction. Because thermogenesis is not induced in tissue from ob/ob mice exposed to cold, the GDP binding sites need not be increased.

These considerations pose the important question of what mitochondrial components are responsible for the poor thermogenic activity of BAT in ob/ob mice. The activity of BAT can be modulated by starvation, BAT activity decreasing in lean and increasing in ob/ob mice, such that the levels of activity are comparable (Girardier and Seydoux, 1981). Thus, the mechanisms for alteration of BAT activity in ob/ob mice are intact, however the setting of these mechanisms are inappropriate to the animals requirement of the tissue. Thus, whatever the nature of this regulatory component, it would be expected to be changed between the two phenotypes. Also it should change in response to starvation, and other conditions where the activity of BAT changes, such as cold adaption, (Hogan and Himms-Hagan, 1980) and thyroid status, (Ricquier et al., 1976). As ob/ob mice have a fluidised inner mitochondrial membrane compared to lean controls, it is possible that regulation of BAT mitochondria through such variable nutritional, hormonal and environmental influences may be mediated by a lipid component of the mitochondrial inner membrane.

As well as fluidity differences in the mitochondrial inner membrane of ob/ob mice compared to lean controls, Giacobino and Chmelar (1977) have demonstrated that fatty acid unsaturation in the phospholipids of mitochondria in starved lean rats is decreased. Hulbert (1976) has demonstrated that mitochondrial fatty acid unsaturation is highly dependant on thyroid status. Ricquier et al. (1975) have demonstrated that cold adaption leads to large changes in the total phospholipid content, the ratio of phospholipid classes and the fatty acid composition in BAT mitochondria.

Thus, the phospholipids themselves may be regulating the activity of the anion uniporter. This suggestion has some support from the observation that fatty acids are powerful uncouplers of respiratory control in BAT, but much less so in liver mitochondria (Heaton and Nicholls, 1976), although both liver and BAT

mitochondria bind fatty acids with the same affinity (Nicholls, 1979). Interestingly, this uncoupling of BAT by high concentrations of fatty acids occurs by a mechanism independant of the purine nucleotide sensitive pathway (Nicholls, 1979). Fatty acids in vivo generated by stimulation of triacylglycerol lipase activity, however, are not physiological uncouplers (Bleber et al., 1975).

It is postulated here that a phospholipid moiety may be associated with the anion uniporter, such that the physical state of the lipid exerts control over its OH^- conductance. The alteration of the fatty acid classes on the regulatory phospholipid may be involved in the altered response of BAT under various nutritional, hormonal and environmental conditions.

It is interesting to speculate further on the possible role of a lipid moiety in the regulation of the uniporter. Could it function as a thermal switch? The flux through a metabolic pathway is usually under control by a negative feedback process operating from the final end product of the pathway. In this case, the end product of BAT mitochondrial respiration is heat. For sufficient heat flow from the brown adipocyte to the blood plasma to occur in order to effect the extraordinary heat output of BAT under thermogenic conditions, the mitochondrial membranes may encounter temperatures several degrees above normal physiological temperatures. In order to prevent thermal denaturation of mitochondrial components, it may be necessary to prevent excessive heat production. This could occur by either increasing GDP binding, or perhaps the regulatory lipid postulated here might reach a critical fluidity and inhibit the anion uniporter conductance. If the fluidity of this hypothetical regulatory lipid is altered, then the temperature at which the onset of inhibition of the uniporter may change. This may help to explain why the activity of BAT mitochondria varies with the phospholipid composition of the mitochondrial membrane.

BAT in ob/ob mice is not activated by ANS stimulation, (Girardier and Seydoux, 1981) or cold stress (Hogan and Himms-Hagen, 1981). The fluidity of the mitochondrial inner membrane

is vastly increased compared to lean controls (French and York, unpublished). Therefore, these mitochondria may 'switch off' at a temperature little different from physiological, resulting in blunted thermogenesis. Thus ob/ob mouse mitochondria may exhibit a high degree of respiratory control. Also they may have a lower mitochondrial GDP binding protein content in order to try to overcome respiratory control. With a 'faulty' lipid thermostat, it is clear cold exposure may not effect a thermogenic response.

Although this hypothesis may explain the observed adaptions of BAT activity in normal animals, another mechanism must also prevent the thermogenic response in ob/ob mice, during chronic cold stressed conditions, their body temperature falls, and yet their BAT apparently does not switch on. This could be due to a very much depressed switching temperature, or a failure of some other mechanism. It has been suggested that a plasma membrane defect may inhibit the cAMP accumulation and possibly the membrane depolarisation effects of noradrenalin, hence reducing thermogenesis in the ob/ob mouse BAT. Recently, Thurlby and Trayhurn (1980) have observed that the sympathetic nervous vasodilatory reflex in BAT was much reduced in the ob/ob mouse. This could also inhibit thermogenesis by decreasing oxygen supply to this tissue.

6.3.6. Fluidity changes in the erythrocyte in ob/ob mice.

The ob/ob mouse erythrocyte plasma membrane is less fluid than that of lean controls. As the phospholipid fraction was more fluid from ob/ob mice, it can be assumed that either more protein or cholesterol was present in the plasma membrane of ob/ob mice. As ob/ob mice are hypercholesterolaemic, and erythrocytes are anucleate and are therefore unable to regulate their membrane composition, it seems likely that the observed decrease in fluidity was due to a higher membrane cholesterol content. PE isolated from ob/ob mice membrane was more fluid than PE from lean control membranes, conversely, PC was less fluid. As the erythrocyte plasma membrane has PC and PE assymetrically organised within the bilayer (Chapter 5) a different fluidity assymetry must exist between the membranes from the two phenotypes.

Baba et al. (1979) observed a decreased erythrocyte fluidity in human diabetics, which may possibly be due to increased cholesterol content or reduced phospholipid fluidity. This remains to be elucidated.

6.3.7. Fluidity changes in liver plasma membrane in lean and obese mice.

Liver plasma membranes from ob/ob mice are more fluid than lean controls, and this was not corrected by housing at 34°C. Liver plasma membranes from ob/ob mice bind less insulin and glucagon than lean controls, but this may be due to down regulation mechanisms in the presence of increased hormone concentration (Bray and York, 1979).

Studies by French and York (unpublished) have demonstrated that the observed fluidity changes occurring in the liver plasma membrane from ob/ob mice resides in the phospholipid fraction. These workers have observed that the fatty acid composition of PE is similar from the two phenotypes. However, PC and SM were fluidised from ob/ob mice membranes compared to those from lean controls. Fatty acid analysis revealed that the fluidisation of obese mouse PC and SM resulted from an increase in 22:6 incorporation. If the hepatic plasma membrane is assymetrically oriented in a similar manner to other membranes, then the outer half of the bilayer will be considerably more fluid in ob/ob mouse hepatic plasma membranes compared to lean controls.

Compared to most metabolically important tissues in the ob/ob mouse, the liver does not appear to have many gross funcional defects. Two exceptions to this are first, an impaired response of hepatic protein turnover rate of ob/ob mice to dietary manipulation (Miller et al., 1979) which may be secondary to the persistant hyperinsulinaemia in the ob/ob mouse. Secondly, livers from ob/ob mice display a lack of inhibition of fatty acid synthesis by vasopressin, despite a normal vasopressin inhibition of glycogenolysis (Hems and Ma, 1976). This factor may well result from altered membrane composition, as vasopressin is thought to act via phosphatidyl inositol metabolism. Phosphatidylinositol modulates intracellular calcium ion fluxes (Kirk and Michell, 1981).

As the ob/ob mouse hepatic plasma membrane fluidity did not normalise to lean values on housing the animal at 34°C it is clear that liver plasma membrane metabolism differs from that of the adipocyte, and suggests that changes in plasma membrane fluidity are not just a manifestation of generalised elevation in desaturase enzyme activities, but specific biochemical adaptions specialised to each cell surface.

6.3.8. Changes in membrane fludity in response to starvation and hypothyroidism.

Giacobino and Chmelar (1977) have demonstrated that adipocyte plasma, microsomal and mitochondrial membranes all fluidise in response to starvation. This was confirmed, at least in the plasma membrane, in both lean and ob/ob mice. Thus, starvation is capable of eliciting the same responses in the plasma membrane as obesity. As basal metabolic rate falls both in starvation and obesity (Garrow, 1978), fluidisation of membranes may be involved in energy conservation at the cellular level by mechanisms discussed in this chapter.

Hypothyroidism also results in a fluidisation of the plasma membrane in lean mice. This observation is consistent with the observations of Malbon (1980) that hypothyroidism in rats results in a reduced adenylate cyclase coupling efficiency in adipocyte plasma membrane. Indeed, the altered component in the membrane matrix proposed by Malbon (1980) to account for these observations may well be a fluidised lipid.

The fall in metabolic rate observed in hypothyroid states (Garrow, 1978) may thus be mediated by the same mechanism, at the cell plasma membrane level, as starvation and obesity. Indeed, the observations of Palmblad et al. (1977) suggest that the observed fluidisation of the adipocyte membrane may in fact be secondary to hypothyroidism in starvation, since in this condition a 50% fall in serum T_3 was observed in starving humans.

The effects of thyroid status on mitochondrial membranes, however are different in both starvation and obesity. The data of Hulbert et al. (1976) for rat, clearly demonstrates that increas-

ing plasma T_3 from hypothyroid to hyperthyroid states linearly increases the degree of hepatic mitochondrial membrane unsaturation in rats over a spectacularly wide range of fluidity. Conversely, in starvation, T_3 levels are reduced (Palmblad et al., 1977), however the rat adipocyte mitochondrial membranes become more unsaturated. These points serve to illustrate the complexity of metabolic adjustment to different hormonal, nutritional and pathological states in animals.

6.3.9. Other animal models of obesity.

In Chapter 4, it was observed that the GTG-obese mouse hepatic microsomes contained more LCPUFA than lean non-obese animals, and this was interpreted to suggest that the observed fluidisation of the ob/ob mouse microsomal fraction was a consequence of obesity and not a manifestation particular to the ob gene. This concept is further extended to include studies on the obese rat (fa/fa). Both adipocyte plasma membrane and synaptosomal membranes were more fluid in this animal model of obesity. Indeed, the changes in phospholipid composition of synaptosomal membranes in the fa/fa rat mirrored those observed for the ob/ob mouse, only the changes were not as acute as for the ob/ob mouse, which is consistent with the milder degree of obesity observed in the fa/fa rat (Bray and York, 1979). The similar changes in the fluidity of PE and PC in rat synaptosomal membranes suggest that the cholesterol segregation hypothesis discussed in Chapter 5 may also operate in the fa/fa rat, as Fontaine et al., 1980 have observed a high degree of PE/PC assymetry in rat synaptosomes. Thus, membrane fluidity changes observed in ob/ob mice apply to other models of obesity and may therefore be an intergral part of the expression of the obese syndrome.

6.3.10. Conclusions.

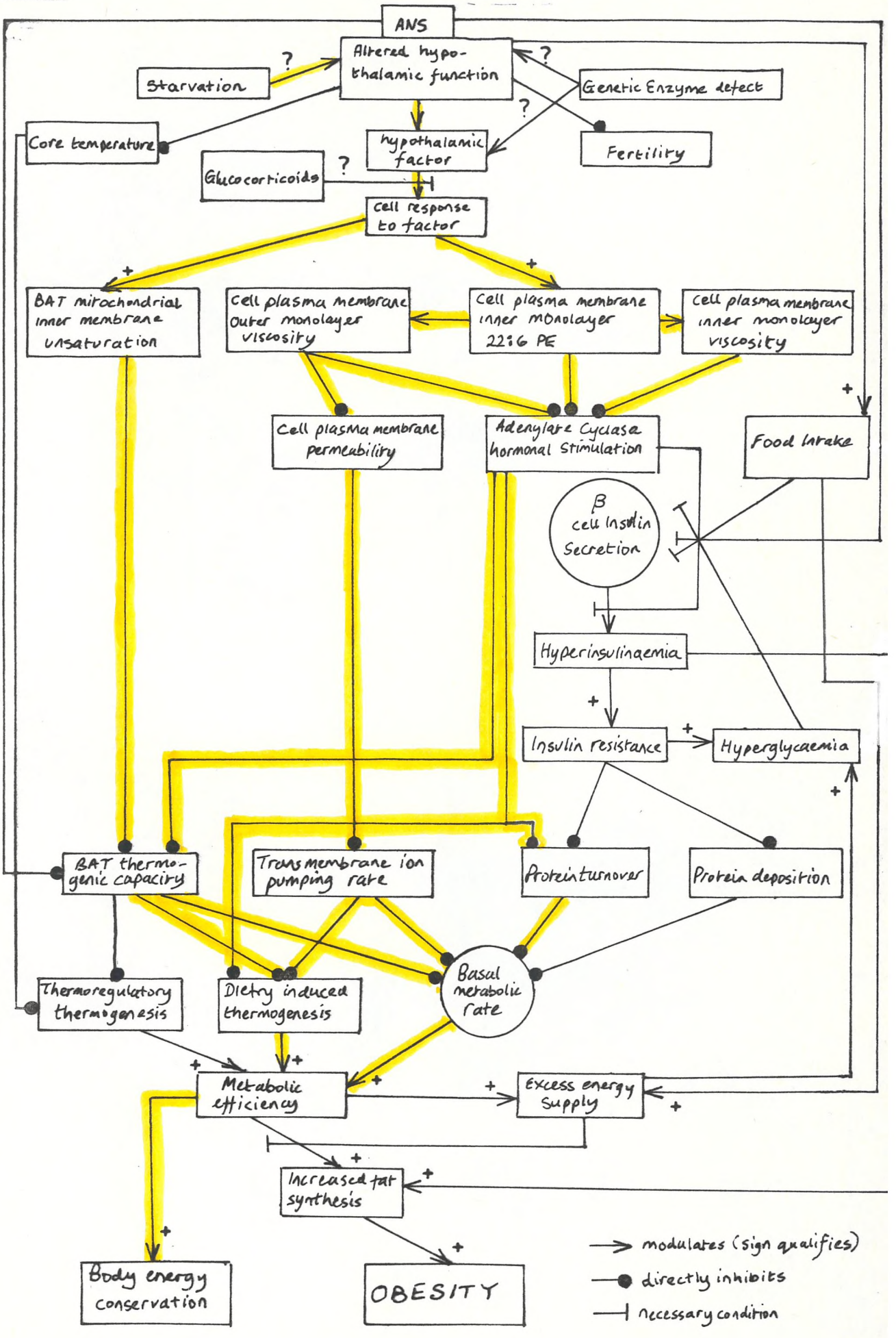
The results in this chapter serve to illustrate the wide range of membrane changes that occur in animals in response to pathological, hormonal, nutritional and environmental conditions. Furthermore, results presented in this Chapter support the concept that membrane metabolism is altered in the obese condition, and that many of the gross metabolic lesions observed in the ob/ob mouse can be rationalised by considering the metabolic impact of altered membrane related processes and the metabolic interdependance of each tissue. A flow chart is presented in Fig 6.13. to illustrate this point. The details of this chart are considerably speculative, and doubtless will require extensive modification in the light of future work. The enzyme defect in the ob/ob mouse is postulated to either interfere with the hypothalamic control of a circulating factor controlling membrane metabolism or to alter the responsiveness of the target cells to this factor.

The change in membrane composition may give rise to reduced cell membrane permeability, and reduced hormonal responsiveness is postulated to enhance insulin secretion from the β cells of the pancreas, resulting in hyperinsulinaemia; this syndrome results in insulin resistance of the tissues. This factor coupled with reduced glucose uptake and utilisation by the muscle promotes hyperglyceamia, and also impaired protein metabolism. Impaired hormonal stimulation of adenylated cyclase by catecholamines, and also impaired mitochondrial function in BAT results in a reduced thermogenic capacity of this tissue. The reduced energy requirement due to impaired thermogenic pathways, transmembrane ion pumping and protein turnover of tissues from the ob/ob mouse results in increased metabolic efficiency of the whole animal. When the animal is fed ad libitum, energy utilisation is less than total energy intake. The positive energy balance created by this situation gives rise to energy storage as fat. Some of the mechanisms described on the flow diagram (6.13) for the expression of the obese syndrome are postulated to be involved in the normal biochemical adaption of the tissues to starvation conditions. Some of these factors may be mediated by thyroid hormone status. Finally, the reduced responsiveness of BAT

and the tissues to catecholamine stimulation may directly result in impaired cold induced thermogenesis in the ob/ob mouse.

It is concluded that the cell plasma membrane phospholipids have an important regulatory role in adjusting the cellular responsiveness to circulating hormones, and may ultimately influence energy balance in the whole animal.

Fig 6.13. Flow diagram to illustrate some of the biochemical events occurring within the tissues of the ob/ob mouse that may give rise to the obese hyperglycaemic syndrome. Metabolic events that also occur in the normal adaption of the tissues to starvation conditions are enhanced in yellow.


ANS
Altered hypothalamic function
Starvation
Genetic Enzyme defect
Core temperature
Fertility
hypothalamic factor
Glucocorticoids
Cell response to factor
BAT mitochondrial inner membrane unsaturation
Cell plasma membrane outer monolayer viscosity
Cell plasma membrane inner monolayer 22:6 PE
Cell plasma membrane inner monolayer viscosity
Cell plasma membrane permeability
Adenylate Cyclase hormonal Stimulation
Food Intake
β cell Insulin Secretion
Hyperinsulinaemia
Insulin resistance
Hyperglycaemia
BAT thermogenic capacity
Transmembrane ion pumping rate
Protein turnover
Protein deposition
Basal metabolic rate
Thermoregulatory thermogenesis
Dietry induced thermogenesis
Metabolic efficiency
Excess energy supply
Increased fat synthesis
Body energy conservation
OBESITY
modulates (sign qualifies)
directly inhibits
necessary condition

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Chapter 7.

Final Discussion

The central aim of this project was to undertake a comparative study of cell membranes from genetically obese and lean control mice. Compositional changes were observed in various cell membranes from ob/ob mice, and an attempt was made to correlate this finding with the altered behaviour of certain membrane related metabolic processes occurring in the ob/ob mouse.

The results presented in this thesis strongly support a causal link between membrane composition and impaired metabolic events occurring within cell membranes in the ob/ob mouse. From this tenet, a hypothesis has been developed that suggests that biochemical alterations in the cell membrane phospholipids cause many of the membrane related changes that are characteristic of the ob/ob mouse. Similar membrane changes have also been observed in the genetically obese rat and hence may be a general feature of the obese condition. Membrane compositional alterations also have been shown to occur in mice under different hormonal, nutritinal and environmental conditions. The possibility therefore, arises that the cell membrane composition may be strictly controlled in order to regulate processes involved in energy and thermoregulatory homeostasis in animals. Thus, starvation induces compositional changes in mammalian cell membranes that metabolically adjust the tissues for efficient energy utilisation. If this metabolic adjustment for starvation conditions occurs when the animal has a normal dietary intake, the dietary energy in excess of that required for heat production, tissue maintainance and normal growth can only be stored, and is deposited largely as fat.

Identifying the metabolic process responsible for the alterations in membrane phospholipid composition is clearly vital to the understanding of obesity, partly because it is an obvious area for pharmacological manipulation, but mainly as it facilitates studies which eventually will lead to the identification of the primary defect in this form of genetic obesity.

It has also come to light in the course of this project that docosahexaenoic acid metabolism has an extremely important role in the biochemistry of mammalian biomembranes. Indeed, it is suggested that this rather unique fatty acid regulates the efficiency of energy utilisation in individual cells, and may also be involved in governing the response of membrane bound enzymes to metabolic regulators such as hormones.

Docosahexaenoic acid is the end product in the desaturation and elongation of the essential dietary component, linolenic acid, and thus the results presented in this thesis identify an important metabolic role for this compound. Hitherto, the reasons for the requirement of linolenic acid by mammals has been obscure, because deficiency experiments have only been performed in laboratory animals that are well fed and kept at a constant environmental temperature. It is suggested here that docosahexaenoic acid metabolism contributes to the biochemical changes that occur in mammals in response to changing nutritional and environmental conditions. Obesity, then may result in a disturbance of linolenic acid metabolism, such that the biochemistry of the tissues is not properly adjusted for the animals nutritional status.

Of particular concern is the possible underestimation of the nutritional requirements of this fatty acid for starving people. It has been proposed in this thesis that the increase in efficiency of energy utilization observed in starving animals may be mediated by an increase in cell plasma membrane docosahexaenyl phospholipids. Although the catabolism of docosahexaenoic acid is slow, nevertheless a gradual decline in membrane content must occur during starvation. When this happens, the body tissues energy conserving mechanisms themselves may become less efficient. This observation may be of particular importance if, prior to starvation, dietary intake of linolenic acid was low. Thus, essential fatty acid deficiency during chronic starvation may actually accelerate weight loss. This point serves to illustrate the importance of understanding the biochemical mechanisms that control energy balance.