HOMEOSTATIC CONTROL OVER MEMBRANE LIPID COMPOSITION AND FUNCTION IN THE RAT LIVER

by

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SUMMARY

The present thesis is concerned with an examination of the concept of membrane homeostasis (Sabine, 1983), which implies that biological membranes tend to maintain a constant level of lipid fluidity in the face of potential exogenous and endogenous perturbations. Manipulations of dietary cholesterol and/or saturated (coconut oil) v/s unsaturated (sunflower seed oil) fatty acids have been used not only as a tool to study the relationship between membrane lipid composition, membrane lipid fluidity and membrane-bound enzymes of lipid metabolism but also to see whether these enzymes act co-ordinately for the maintenance of a membrane homeostasis under the above dietary conditions. The results presented can be summarized as follows:

(1) Dietary cholesterol produced significant alterations in both the cholesterol content and fatty acid composition of rat liver microsomal lipids. The major effect of dietary cholesterol was an accumulation of cholesterol in the microsomal membranes, mainly as cholesterol esters and significantly more so when cholesterol was fed in combination with sunflower seed oil. In terms of membrane fatty acids, dietary cholesterol lead to an increase in palmitoleic (16:1) and/or oleic (18:1) and linoleic (18:2) acids, with a concomitant decrease in palmitic (16:0) and/or stearic (18:0), arachidonic (20:4) and docosahexaenoic (22:6) acids.

(2) The dietary cholesterol-induced alterations in lipid composition

mentioned above were large enough to change thermotropic properties of microsomal membrane lipids, as determined by differential scanning calorimetry (DSC). Cholesterol enrichment of the liver microsomes caused by dietary cholesterol resulted in the lowering of phase transition temperature irrespective of the fatty acid supplement, but, the magnitude of the decrease was greater when cholesterol was fed in combination with sunflower seed oil. Furthermore, the influence of dietary cholesterol was predominantly on the phase transition of the bulk lipids of the membrane (designated T_1) rather than on higher melting point - lipid domains (designated T_2).

(3) Following cholesterol feeding, the fatty-acyl desaturase activities of rat liver microsomes were significantly modified. Consistent with the changes in the fatty acid profiles, the Δ^5 - desaturase activity was enhanced, whereas, Δ^6 - and Δ^9 -desaturase activities were inhibited in the liver microsomes of cholesterol-fed animals, which resulted in an overall decrease of membrane unsaturation (unsaturation index).

(4) Administration of 2% cholesterol in the rat diet affected the cholester metabolism of liver microsomes, and the type of fatty acid (saturated or unsaturated) supplement had a pronounced effect on this process. Following the feeding of sunflower seed oil, the cholesterol synthesizing capacity, as determined by the HMG-CoA reductase activity, was found to be decreased, whereas coconut oil feeding had no significant effect. All the cholesterol-supplemented diets suppressed this enzyme activity, but, the

suppression was greater when cholesterol was fed in combination with sunflower seed oil.

(5) Both the high fat diets, without cholesterol supplement, reduced the cholesterol 7α-hydroxylase activity in rat liver microsomes. Addition of 2% cholesterol, significantly increased cholesterol 7α-hydroxylase activity, irrespective of the fatty acid supplement.

(6) The rate of cholesterol esterification, as determined by the acyl CoA:cholesterol acyltransferase (ACAT) activity, was found to be elevated following the consumption of sunflower seed oil, whereas coconut oil feeding had no significant effect. Inclusion of 2% cholesterol in the reference diet resulted in a slight increase of ACAT activity in the liver microsomes, but the difference was not significant. When cholesterol was fed in combination with either sunflower seed oil or coconut oil, however the activity of microsomal ACAT was significantly elevated.

(7) Arrhenius plots of liver microsomal HMG-CoA reductase, cholesterol 7α-hydroxylase and ACAT activities from rats fed a reference diet exhibited a discontinuity at 28.8, 27.6 and 28.5°C respectively. Supplementation of the reference diet with 15% sunflower seed oil resulted in a lowering of the transition temperature (Tc) observed in the Arrhenius plot of HMG-CoA reductase, and an increase in activation energy (Ea_1) above the Tc. Addition of 2% cholesterol abolished the temperature

discontinuity in the Arrhenius plot of this enzyme regardless of the fatty acid supplement.

(8) No consistent relationship between the dietary fatty acids, membrane fatty acid composition and Arrhenius plot parameters of microsomal cholesterol 7α-hydroxylase was found. As with HMG-CoA reductase, addition of 2% cholesterol abolished the break point observed in the Arrhenius plot of cholesterol 7α-hydroxylase activity in liver microsomes of rats fed the respective low-cholesterol diets.

(9) Unlike HMG-CoA reductase and cholesterol 7α-hydroxylase, liver microsomal ACAT retained a temperature discontinuity in the Arrhenius plots even after cholesterol feeding. The Tc however, was lowered from 28.5°C in the reference diet group to 25.1 and 25.2°C in cholesterol plus sunflower seed oil and cholesterol plus coconut oil diet groups respectively. Addition of cholesterol to the reference diet had no significant effect either on Tc or activation energy values.

(10) The changes observed in the fatty acid composition of microsomal membranes, following cholesterol feeding, were reflected in the whole liver and plasma. The cholesterol content of whole liver also followed the same pattern as that of liver microsomes, but plasma cholesterol level was either unaltered or slightly elevated when 2% cholesterol was included in the diets.

From these observations it appears that liver microsomal membranes do

have a tendency to maintain a constant level of lipid fluidity, at least under the dietary conditions of the present investigations, and this is consistent with the proposed hypothesis of membrane homeostasis (Sabine, 1983). To compensate for the altered membrane fluidity caused by cholesterol enrichment following cholesterol feeding, the activities of membrane-bound enzymes of lipid metabolism are modified. The accumulation of cholesterol in the microsomal membranes suppresses its own synthesis by inhibiting HMG-CoA reductase activity. Secondly, removal of cholesterol from these membranes, either by its hydroxylation or esterification is accelerated following cholesterol feeding as indicated by elevated cholesterol 7α-hydroxylase and ACAT activities, but not enough to prevent cholesterol accumulation. On the other hand, activities of microsomal desaturase enzymes following cholesterol feeding are modified in such a manner that lead to the decrease of unsaturation index of the membrane phospholipids, which would have an opposite effect to that of increased cholesterol on the membrane lipid fluidity.

DSC results, in combination with those of Arrhenius plot parameters of the membrane-bound enzymes, suggest that the rate-limiting enzymes of cholesterol synthesis and metabolism, i.e. HMG-CoA reductase, cholesterol 7α-hydroxylase and ACAT might have a specific micro-environment ("boundary lipid") within the microsomal membranes, which has different physical properties when compared with that of the bulk lipids of these membranes.

DECLARATION

I declare that this thesis does not incorporate, without acknowledgement, any material previously submitted for a degree or diploma in any university, and to the best of my knowledge and belief it does not contain any material previously published or written by another person, except where due reference is made in the text.
I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

(Manohar Lal Garg)

Course - Ph.D.

Awarded -

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ABBREVIATIONS

ACAT	Acylcoenzyme A : Cholesterol Acyltransferase
$AgNO_3$	Silver nitrate
ATP	Adenosine triphosphate
BHT	Butylated Hydroxytoluene
CDL	Cardiolipin
CH	Cholesterol
C7H	Cholesterol 7α-hydroxylase
CoA	Coenzyme A
CO	Coconut oil
(CO + CH)	Coconut oil plus cholesterol diet
DMPC	Dimyristoylphosphatidylcholine
DPPC	Dipalmitoylphosphatidylcholine
DSC	Differential scanning calorimeter
DSPC	Distearoylphosphatidylcholine
EDTA	Ethylenediamine tetra-acetic acid
EFA	Essential fatty acids
ESR	Electron spin resonance
FAME	Fatty acid methyl esters
GLC	Gas liquid chromatography
HMG-CoA	β-Hydroxy-β-methylglutaryl coenzyme A
H_2SO_4	Sulphuric acid
KOH	Potassium hydroxide
L-TP	Lipid-transfer proteins
$MgCl_2$	Magnesium chloride
MUFA	Monounsaturated fatty acids
NADP	Nicotinamide adenine dinucleotide phosphate
NAD	Nicotinamide adenine dinucleotide

NaF	Sodium fluoride
NMR	Nuclear magnetic resonance
n mole	nanomole
nsPl-TP	Non specific phospholipid-transfer proteins
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL-TP	Phospholipid-transfer proteins
p mole	picomole
POPOP	1,4-Bis-[2-(5-phenyloxazolyl)]-benzene
PPO	2,5-Diphenyloxazole
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
REF	Reference diet
(REF + CH)	Reference plus cholesterol diet
S.D.	Standard deviation
SFA	Saturated fatty acids
SO	Sunflower seed oil diet
(SO + CH)	Sunflower seed oil plus cholesterol diet
SPH	Sphingomyelin
TADS	Thermal analysis data station
Tc	Transition temperature
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
UDP	Uridine diphosphate
U.I.	Unsaturation Index
UV	Ultraviolet

CHAPTER 1. PREFACE

Fluidity within the lipid bilayer is a central feature of the mosaic model of a biological membrane (Singer and Nicholson, 1972). The main chemical determinants of membrane lipid fluidity in their presumed order of significance are the cholesterol content and the degree of saturation/unsaturation of phospholipid acyl chains. Alterations in the cellular or sub-cellular lipid components including cholesterol content and/or phospholipid fatty acyl chains can be found related to some pathological processes, such as myocardial infarction and stress (Gudjarnason and Oskarsdottis, 1977), atherosclerosis (Sabine, 1977; Tahin et al, 1982), cardiac lesions (Tahin, 1982), diabetes (Huang et al, 1984) and carcinogenesis (Sabine, 1975; Chandrasekhara and Ananth Narayan, 1970; Perkins and Kummerow, 1976; Galeotti et al, 1984). The concept of membrane homeostasis (Sabine, 1983) implies that these changes in lipid composition and thus in the fluidity of cellular or sub-cellular membranes, may be a major event occurring under the influence of a variety of etiological factors. Then with a tendency to restore native fluidity, the membranes may exhibit properties distinctly different from normal membranes and thus the cell may start showing abnormal metabolic behaviour and loss of structural integrity. The enquiry described in this thesis is mainly concerned with this question of membrane homeostasis, i.e. whether or not a biological membrane does possess the tendency to maintain a certain constancy of the physico-chemical (fluidity) status of its lipids in the face of specific external stresses, namely in this instance, dietary cholesterol and/or fatty acids. Effects of such treatments on membrane lipid profiles, on their thermotropic behaviour and on the activity of

membrane-bound enzymes have been followed to examine the concept of membrane homeostasis (Sabine, 1983) and its implications. Some new developments of the hypothesis are also presented.

Part of the work described in this thesis has already been reported elsewhere:

ABSTRACTS

GARG, M.L. and SABINE, J.R. (1984)
Effect of dietary lipids (saturated v/s unsaturated) on hepatic microsomal composition and function.
Proc. Aust. Biochem. Soc., 16 : 11.

GARG, M.L., SNOSWELL, A.M. and SABINE, J.R. (1984)
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Intl. Union Biochem. Symp. "Microsomal Redox Systems", Bangalore, India.

GARG, M.L., SNOSWELL, A.M. and SABINE, J.R. (1984)
Fatty acid profiles of plasma and liver lipids from rats fed cholesterol-supplemented diets.
Proc. Nutr. Soc. Aust., 9 : 117.

GARG, M.L. and SABINE, J.R. (1985)
Dietary cholesterol modifies the fatty acid composition of hepatic microsomal membranes.

The Second International Congress on "Essential Fatty Acids and their Eicosanoids", London, England (U.K.).

GARG, M.L., SNOSWELL, A.M. and SABINE, J.R. (1985)
Membrane homeostasis and the effects of dietary cholesterol upon microsomal desaturase enzymes of rat liver.
13th International Congress of Biochemistry, Amsterdam, The Netherland.

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Dietary cholesterol modifies the fatty acid profiles of hepatic microsomal membrane.
"Regards sur la biochimie" (Edited by Societe de Chimie Biologie) 1985, No. 1, page 51.

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XIII International Congress of Nutrition, Brighton, U.K.

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Microsomal 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMGR) and cholesterol 7α-hydroxylase (C7H) in livers of rats fed high cholesterol diets.
7th International Symposium on Atherosclerosis, Melbourne, Australia.

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GARG, M.L., SABINE, J.R. and SNOSWELL, A.M. (1985)
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Biochem. Intl., 10 : 585-595.

GARG, M.L., SNOSWELL, A.M. and SABINE, J.R. (1985)
Dietary cholesterol modifies both cholesterol content and fatty acid profiles of hepatic microsomal membranes.
Nutr. Rep. Intl., 32 : 17-26.

GARG, M.L., SNOSWELL, A.M. and SABINE, J.R. (1985)
Effect of dietary cholesterol on cholesterol content and fatty acid profiles of rat liver and plasma.
Nutr. Rep. Intl., 32 : 117-128.

GARG, M.L. SNOSWELL, A.M. and SABINE, J.R. (1985)
Influence of dietary cholesterol upon desaturase enzymes of rat liver microsomes.
Progr. Lipid Res., (In Press).

GARG, M.L., McMURCHIE, E.J. and SABINE, J.R. (1985)
Membrane homeostasis: Thermotropic behaviour of microsomal membrane lipids isolated from livers of rat fed cholesterol-supplemented diets.
Biochem. Intl., (In Press).

CHAPTER 2. REVIEW OF LITERATURE.

This chapter is a comprehensive review of the information available until July, 1982, when this investigation was undertaken.

Initially a brief description of structure and composition of biological membranes will be given (Section 2.1.). The techniques involved in the modification of membrane lipids will then be covered with particular emphasis on the relationship between dietary lipids, membrane lipid composition and membrane functions (Sections 2.4. - 2.7.). The role of microsomal membranes in the overall regulation of fatty acid and cholesterol metabolism will be discussed but limited to a description of enzyme reactions involved (Section 2.8.).

A hypothesis, in which constancy of physico-chemical status of the membrane lipids under various external and internal contraints is proposed, has been discussed (Section 2.10.). Finally, in Section 2.11., the specific aims of this present study are presented.

2.1. Membrane Structure and Composition

Before proceeding with any description of membrane structure and composition, I must pause to acknowledge that the term membrane means different things to different people: "to the histologist it might be any boundary that can be visualized under a microscope; to the biochemist it might be a multimolecular complex, a fraction generally found at the bottom of a centrifuge tube; to the physiologist and particular to surgeon, it might be any enveloping or protective

tissue" (Sabine, 1977). My concern throughout this thesis is predominantly with the biochemist's membrane, a notion that expands the simple idea; membrane consists mainly of lipid and protein, with some carbohydrates in the form of glycoprotein or glycolipid.

Membranes form the boundary of the cells (plasma membranes) and organelles (such as mitochondria and nuclei) and constitute the interconnecting network within the cells (endoplasmic reticulum and golgi apparatus). Biological membranes exhibit a great diversity of physiological functions which correlate with a similar diversity in composition and structure. They are not merely inert hydrophobic barriers but are highly selective and permeable. Membranes are involved in the initiation, reception and transduction of endocrine stimuli, the transport of ions and metobolites and directly in the metabolism of the cell by means of many membrane-bound enzymes (Raison, 1973a; Cullis and de Kruijf, 1979).

Thus, the knowledge of composition and structure of biological membranes is fundamental to the understanding of their diverse functions. The relative proportions of protein and lipid vary considerably between membranes and often reflect their diverse functions so that the metabolically active, inner mitochondrial membrane is relatively rich in protein whereas the insulating myelin membrane is rich in lipid (Marsh, 1975; Thompson, 1980).

2.1.1. The lipids

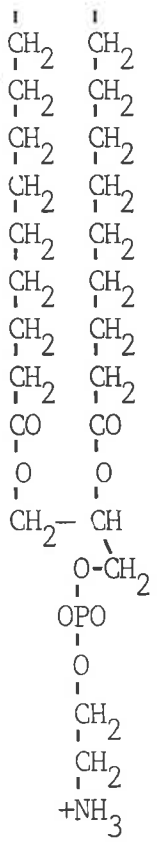
The lipid component of biological membranes consist largely of

different types of phospholipids and, depending upon the membrane, varying amounts of cholesterol and glycolipid (Van Deenen, 1965; Marsh, 1975; Thompson, 1980). Chemical structures of the major lipids of a membrane are shown in Figure 1. The main phospholipids in membranes are; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), sphingomyelin (SPH) and cardiolipin (CDL). The phospholipids are amphiphiles in that they contain in their structure regions which are polar and hydrophilic (e.g. choline, ethanolamine, inositol etc.) and regions which are non-polar and hydrophobic (acyl chains). Both of these regions are linked by a glycerol or sphingosine 'back bone'. The hydroxyl group of cholesterol is also hydrophilic but the remaining steroid ring is hydrophobic (Van Deenen, 1965; Marsh, 1975). The lipids are not readily miscible with water and this immiscibility of lipid and water is put to good use in the design of biological membranes as will be discussed in proceeding sections.

2.1.2. The proteins

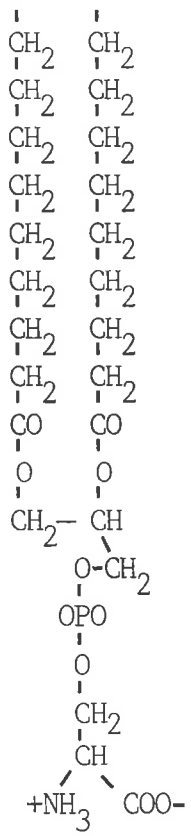
The protein molecules in membranes fall into two broad classes. Loosely attached to the surface of the membrane are the peripheral or the extrinsic proteins, which are bound to the membrane by electrostatic and hydrogen bond interactions (Marsh, 1975; Sandermann, 1978). These proteins can be washed off free from the remaining membrane using buffers of high ionic strength. The other class of membrane proteins are the integral or intrinsic proteins which are firmly embedded in the membrane and require detergent destruction of

FIGURE 1: The chemical formulae of the major lipids of membranes drawn to illustrate their approximate spacial appearance.



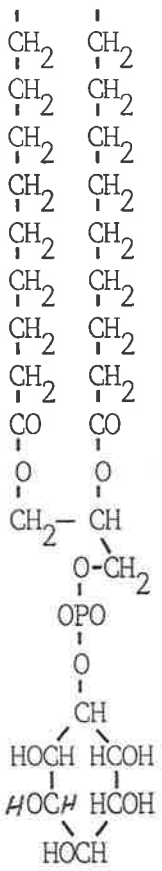
(PE)

Phosphatidyl-
ethanolamine



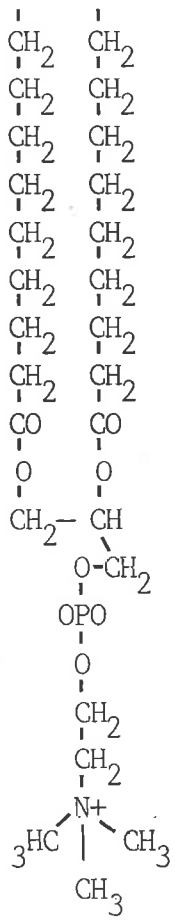
(PS)

Phosphatidyl-
serine



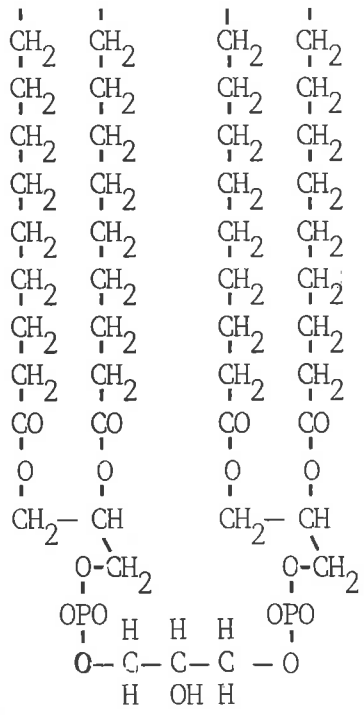
(PI)

Phosphatidyl-
inositol



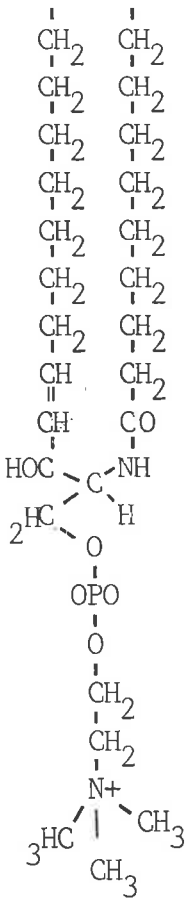
(PC)

Phosphatidyl-
choline



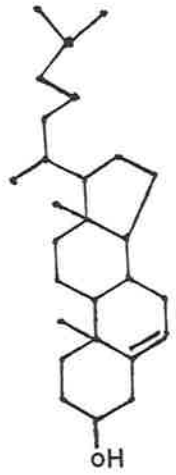
(CDL)

Cardio-
lipin



(SPH)

Sphingo-
myelin

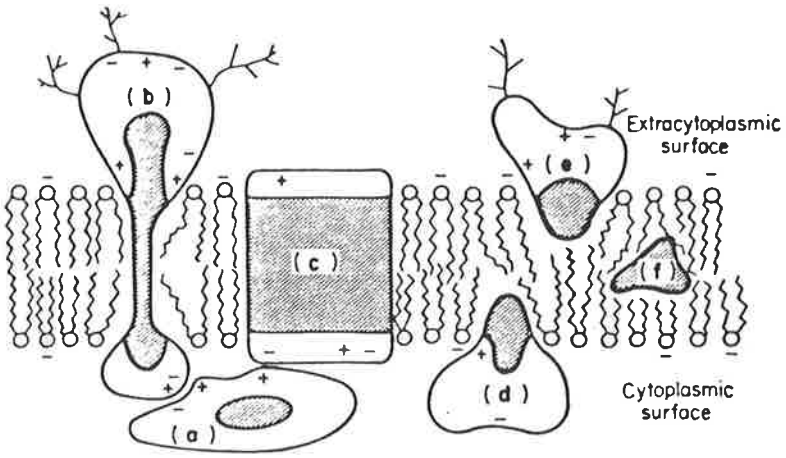


(CHOL)

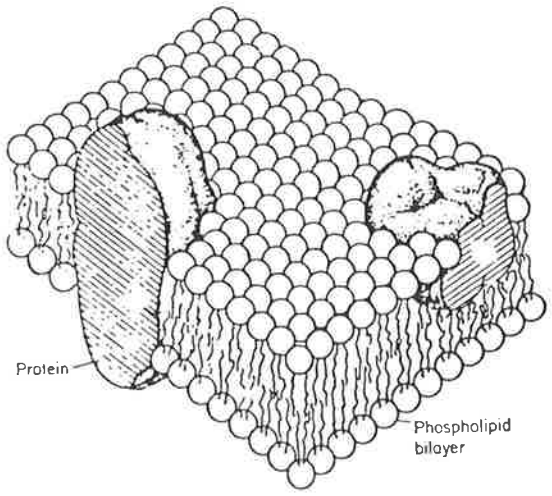
Cholesterol

FIGURE 2: (A) Possible arrangements of membrane proteins. Peripheral proteins (a) are hydrophilic and bind to the polar surfaces of other membrane proteins and lipids. Integral membrane proteins [(b)-(f)] interact with the lipid bilayer portion on the membrane. Hatched areas represent hydrophobic regions.
[From Houslay, M.D. and Stanley, K.K. (1981) Dynamics of Biological membranes. Wiley, Chichester.]

(B) The fluid-mosaic model of the membrane.
[from Singer, S.G. and Nicolson, G.L. (1972) Science, 175 : 720.]


(b)
(c)
(e)
(f)
(d)
(a)
Extracytoplasmic surface
Cytoplasmic surface

[A]


Protein
Phospholipid bilayer

[B]

2.3. Membrane Fluidity

The lipid molecules in a membrane are not rigid, their hydrocarbon chains flex and their degree of flexion may be greatest near the center of the bilayer and least adjacent to the polar heads (Hubbell and McConnell, 1977). Thus, the lipid component of a membrane is a two dimensional liquid in which molecules are restricted to their own monolayer. Various terms have been used to describe this physico-chemical character e.g. fluidity, microviscosity, order and many physical techniques such as NMR, ESR, DSC, wide angle x-ray diffraction and various spectroscopic methods have been used to measure such a parameter. All of these techniques have their accompanying advantages and disadvantages, the major disadvantage being that measurements can be made only on the bulk lipids of the membrane and that the answer obtained depends a great deal on the nature of particular probe used. Nevertheless, there may be a considerable advantage in using a standard physical measurement on all membranes and their lipid extracts.

At low temperatures, hydrated phospholipid bilayers exist as highly ordered gels with the C-C bonds of their fatty acyl chains in the rigid *trans* confirmation at an angle to the vertical plane which depend upon the extent of hydration. As the temperature is raised, the mobility of the fatty acyl chains gradually increases until, at a characteristic temperature, an abrupt thermal transition occurs concomitantly with an increase in heat absorption. The bilayer then exists in a highly disordered, liquid-crystalline state and the C-C

bonds have a partially *gauche* confirmation with an increase in torsional mobility (Marsh, 1975; Chapman, 1982). The temperature at which this reversible phase transition occurs is termed as the phase transition temperature (T_c). In natural membranes T_c, while basically a characteristic of the phospholipid acyl chains, is modified by the presence of cholesterol, polar head groups, presence of proteins and divalent cations (Thompson, 1980; Chapman, 1982). Any disturbance in one or more of these membrane constituents will influence the fluidity of these membranes.

2.4. Techniques for studying membrane lipids versus membrane function

Because the protein composition of most membranes is so complex, studies of how protein and lipid molecules interact to give a functionally competent membrane are difficult. Membrane proteins generally loose their functions when stripped of all lipid. It is assumed that membrane-associated functions require structural integrity of protein molecules, whereas phospholipids normally play a supportive role. Various approaches have been used to alter the lipid composition of membranes, both in vivo and in vitro, and their consequent effects on membrane functions have been postulated. Some of these are discussed below.

2.4.1. Phospholipase treatment

The classical way to demonstrate the lipid dependence of an enzyme has been the observation of a loss of activity on removal of lipid. Phospholipases are mild reagents and are very specific in their

action. Different phospholipases attack the phospholipid molecules at different sites as is shown in Figure 3. However, the loss of activity following such treatment is not sufficient to establish lipid dependency of an enzyme (Coleman, 1973). The inactivation may also be due to;

(i) specific denaturation, unconnected with lipids

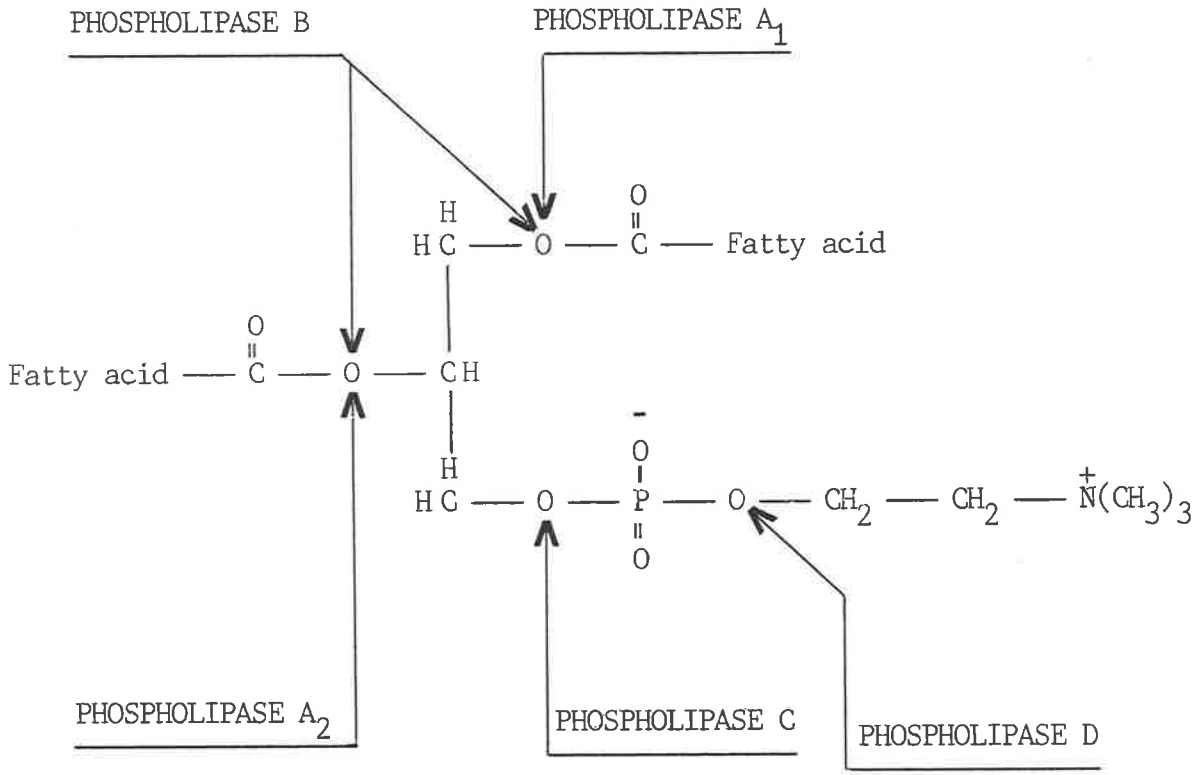
(ii) inhibitory action of the products of phospholipase action i.e. lysophospholipids and free fatty acids.

(iii) direct inhibitory action of the reagents or their contaminants e.g. proteases (Coleman, 1973).

2.4.2. Reconstitution

An increasing number of membrane-bound enzymes have been shown to require lipids for their correct functioning. When lipids from these enzymes were carefully removed, they lost their activity. By reconstituting the enzyme by lipid supplementation, the activity was restored (Triggle, 1970; Rothfield and Romeo, 1971). Sandermann (1978) has summarized a list of enzymes that have an absolute requirement for lipid for full activity. It is interesting to note that prominent amongst these enzymes are those involved in electron transport, lipid metabolism, transport of metabolites and ions and information transmission. In some cases, the lipid requirement have proved to be very specific e.g. PG for phosphotransferases (Kundig and Roseman, 1971), CDL for malate dehydrogenase (Tobari, 1964) and PC for β-hydroxybutyrate dehydrogenase (Jurtshuk et al, 1961) etc.; in other cases the specificity has been quite wide and it appears that

FIGURE 3: Sites of attack of phospholipases on phosphatidylcholine.


PHOSPHOLIPASE B
PHOSPHOLIPASE A1
Fatty acid
Fatty acid
PHOSPHOLIPASE A2
PHOSPHOLIPASE C
PHOSPHOLIPASE D

almost any phospholipid, and in some cases, even non-ionic detergents, glycerides or fatty acids will substitute and lead to the enzyme reactivation (Sandermann, 1978).

2.4.3. Lipid-transfer proteins

The observation that cytosol preparations from various sources mediate the exchange of lipids may be explained by the presence of lipid-transfer proteins in these extracts (Kader, 1977). Wirtz and Zilversmit (1968) discovered that an exchange of phospholipid, in vitro, occured between mitochondria and microsomes of rat liver, and postulated that this intermembrane exchange is catalyzed by a particular category of proteins named phospholipid-transfer proteins (PL-TP). The successful isolation of highly purified monospecific transfer proteins from beef liver (Kamp et al, 1973) or rat liver (Lamb et al, 1976; Poorthuis et al, 1980), and partially purified protein from sheep lung (Robinson et al, 1978) and Caster bean (Yamada et al, 1980) provided the first demonstration of the presence and activity of these proteins. No protein strictly specific for PI has been isolated so far. For PE exchange, several authors have found such activity in soluble proteins prepared from various tissues, including those from plants (Boussangeet al, 1980). The isolation of protein able to transfer PE led to the discovery of non-specific proteins mediating the movement of PC, PI, SPH and cholesterol in addition to PE in rat liver. As far as I know, no protein able to catalyze a transfer of CDL has been isolated. Also no protein, exhibiting a specificity for molecular species of particular phospholipids (comprising saturated or unsaturated acyl chains) has

been isolated, although the presence of such proteins in rat liver cytosol has been postulated (Schulze et al, 1977). No evidence is yet available that these transfer proteins are specific for certain natural membranes.

Kader et al (1982) have explained a number of important aspects of PL-TP for membrane research. The lipid-transfer proteins have been frequently used to modify the phospholipid composition of the outer monolayer of natural membranes, and then to examine the effect of this change on membrane properties (Bergelson and Barsukov, 1977; Barsukov et al, 1978). Crain and Zilversmit (1981) have shown that activity of glucose-6-phosphatase is modified when lipid composition of microsomal membranes is manipulated by non-specific phospholipid-transfer proteins.

2.4.4. External stress

Fluidity in the lipid bilayer is a central feature of the mosaic model of a biological membrane, and the maintenance of this fluidity at an appropriate level is essential for life processes (Singer and Nicholson, 1972). Membrane fluidity is a function of many variables, including the nature of membrane lipids (type of phospholipids, fatty acid chain length and degree of unsaturation, head group and cholesterol content), temperature, water content and presence of ions (especially divalent cations). Various external stresses e.g. nutritional (Spector et al, 1980; Innis and Clandinin, 1981 b;McMurchie et al, 1983; Clandinin et al, 1983), environmental (McMurchie et al, 1973; Lyons and Raison, 1970), hormonal (Emilsson

and Gudbjarnson, 1981), pharmacological (Nhamburo et al, 1982) and physiological (McMurchie and Raison, 1975; Raison and Lyons, 1971; Schatta et al, 1977) have been demonstrated to alter one or more of these fluidity parameters and in turn to modify membrane function.

2.5. Dietary lipids: A useful tool to modulate membrane lipid composition, structure and function

Studies on the relation between lipid structure and membrane function have depended on the correlation between a measured enzyme of physiological function and the molecular ordering of fluidity of membrane lipids in response to changes induced by some external variables. The most widely used variables altering membrane lipid fluidity, in vivo, are dietary fatty acids and temperature. The lipid composition of bacteria and plants may be altered by changing their growth temperature. With animals, it is more difficult to alter membrane lipid composition by changing growth temperature. Thus, at least under physiological conditions, dietary fatty acids and cholesterol are by far the most common variable used to alter membrane lipids in animals. The next part of this chapter therefore deals with the changes in membrane lipid composition, structure and function induced by dietary lipids.

2.6. Dietary lipids affect membrane lipid composition

Membrane lipid composition can be extensively modified in vivo both by direct incorporation from the diet and by modifications of intrinsic mechanisms such as desaturation and chain elongation reactions. Many

of the earlier studies, aimed at investigating the response of membrane fatty acid composition to variations in dietary fat, were concerned with the state of essential fatty acid deficiency (Guarnieri and Johnson, 1970; Holman, 1978). A fat-free diet or a diet containing only saturated fatty acids is often still used as one extreme point of reference. Later, interest developed in the effect of feeding PUFA much in excess of the minimum dietary requirement for health, partly due to the suggestion that this could lower blood cholesterol level, with beneficial effects against heart disease (Vergroesen and Gottenbos, 1975).

Membrane lipids can be divided into two major constituents; acyl lipids (predominantly phospholipids) and sterols (mainly cholesterol). The effect of dietary lipids on each of these membrane components will be discussed in turn.

2.6.1. Acyl lipids

A diet rich in PUFA has been shown to increase the degree of unsaturation of membrane phospholipids in rat liver and heart (Innis and Clandinin, 1981 a, b; Tahin et al, 1981; Spector et al, 1980), sheep liver, kidney and heart (McMurchie and Raison, 1979), rabbit intestine (Field and Salome, 1982) and human blood (Farquhai and Ahrens, 1963). The membranes studied were: endoplasmic reticulum (Hammer and Wills, 1978; Tahin et al, 1981; Spector et al, 1980), plasma membranes (King et al, 1977; Im et al, 1979), mitochondrial membranes (Haeffner and Privett, 1975; McMurchie and Raison, 1979; Innis and Clandinin, 1981 a, b) and erythrocyte membranes (Farquhar

and Ahrens, 1963; Popp-Snijders et al, 1984). Dietary cholesterol also affects the phospholipid composition of biological membranes (Ramirez et al, 1984). Cooper et al (1980) reported an increase in plasma and red cell membrane phospholipids, when dogs were fed a cholesterol-supplemented diet. However, no attention has yet been given to the effects of dietary cholesterol upon membrane fatty acid composition.

2.6.2. Sterols

Cholesterol, being a component of membrane lipids is also known to influence the fluidity of biological and artificial membranes (Vanderkooi et al, 1974; Naito, 1978). Cholesterol content of biomembranes can be altered experimentally in some tissues by dietary means (Cooper et al, 1980; Garcia-Gonzalez et al, 1984). Mitropoulos and Venkatsan (1977) reported that supplementation of the diet with cholesterol produces a significant increase in the cholesterol content of rat liver microsomes. The nature of the fat fed with the cholesterol seems to be critical for the determination of cholesterol content of membranes. Field and Salome (1982) have demonstrated that both saturated and unsaturated fats fed with cholesterol raised the cholesterol content of rabbit intestinal microsomes to the same extent. However, significantly higher amounts of cholesterol esters are accumulated when cholesterol is fed with unsaturated fat (Field and Salome, 1982). On the other hand, others (Spector et al, 1980; Brenneman et al, 1977) have reported that the nature of fat (saturated versus unsaturated) in the diet has no effect on either free or esterified cholesterol content of rat liver or Ehrlich ascites

microsomal membranes.

2.7. Membrane functions affected by dietary lipids

The type of modifications in the lipid bilayer structure and composition, discussed above, can account for the altered membrane functional properties such as, the transport systems and membrane-bound enzyme activities.

2.7.1. Membrane transport processes

An essential role of biomembranes is to allow movement of all compounds necessary for the normal function of a cell, across the membranal barrier. These compounds include a vast array of sugars, amino acids, steroids, fatty acids, anions and cations to mention a few. There are at least three general mechanisms by which metabolites (solutes) can pass through biomembranes.

A few metabolites of low molecular weight such as water, carbon dioxide, oxygen and anaesthetic molecules can penetrate membranes by simple diffusion. The rate of simple diffusion of a substance is determined by its solubility in the membrane lipids (Collander, 1954), by its diffusion coefficient in the membrane (Stein, 1967; Stein, 1981) and by the difference in its concentration between the outside and the inside of the cell (Stein, 1981). Although simple diffusion was believed to be an important transport mechanism in cells, current information suggests that this process is very limited.

Facilitated diffusion is somewhat similar to simple diffusion in that a concentration gradient is required and the process does not involve an expenditure of energy. A distinguishing feature of facilitated diffusion is the presence of a saturation effect i.e. a tendency to reach a maximum rate of flow through the membrane as the concentration of the diffusing substance is increased. Secondly, a mobile carrier, often a protein, combines with the material to be transported that speeds up the rate of diffusion. Compounds such as α-aminoisobutyric acid, methotrexate, choline, glutamate etc. are transported across the membrane by facilitated diffusion (Kaduce et al, 1977; Burns et al, 1979; Hyman and Spector, 1982; Balcar et al, 1980).

Active transport is one of the most important properties of all plasma membranes which is very similar to facilitated diffusion with the critical exception that the metabolite or solute moves across the membrane against a concentration gradient and this requires energy input. Use of inhibitors such as azide or iodoacetate that markedly decrease the production of energy in the cell, greatly inhibits active transport (Hokin, 1972). Neither passive diffusion nor facilitated diffusion would be affected by the use of these inhibitors.

Cholesterol, fatty acids and chemical nature of the polar head groups of a membrane have been shown to affect its permeability (Van Deenen et al, 1972; Deuticke, 1977).Rates of permeation of electrolytes and non-electrolytes have been found to be maximum at, or near, the transition temperature (T_c) and decrease in both the gel and fluid phases below and above this temperature (T_c) (Blok et al, 1976). Since the order-disorder transition of lipids is accompanied by a

decrease in the area per molecule, it was postulated that pores developed in the bilayer at the T_c; the number and life time of these pores is dependent upon the fatty acyl chain length of these lipids (Blok et al, 1976). de Gier et al (1968), using liposomes made of various synthetic lecithin species have demonstrated that a decrease in chain length causes a higher permeability of the lipid bilayer. Also, an increase in the number of cis-double bonds can lower the permeability barrier (McElhaney et al, 1970; de Gier et al, 1968).

Further, cholesterol is also known to increase the tightness of the packing of cell membranes (Chapman, 1973). The permeability is increased when the cholesterol content of the membrane is reduced while permeability decreases when membrane cholesterol increases (Bruckdorfer et al, 1969; de Kruyff et al, 1972).

7.2.2. Membrane-bound enzyme properties

In addition to determining the permeability properties of cells and cellular compartments, membranes also provide a suitable micro-environment for many membrane-bound enzyme systems. Several enzymes have been shown to have a specific lipid requirement for optimal activity (Charnock et al, 1973; Eytan and Racker, 1977; Farias et al, 1975; Sandermann, 1978) and some of these require the membrane to be in a fluid state (Kimelberg and Papahadjopoulos, 1972). Phospholipids have been directly implicated both in supporting the activity of the enzymes and restoring their maximum activities (Hallinan, 1974; Brenneman et al, 1977).

Of the many membrane-bound enzymes studied in relation to the changes in dietary fatty acids, the ATPases and adenylate cyclose have been most frequently used. Several other enzymes located on different cellular and sub-cellular membranes: e.g. 5'-nucleotidase (Merisko et al, 1981; Brivio-Haugland et al, 1976) in plasma membrane, acetylcholinesterase (Galo et al, 1981; Farias, 1980) in erythrocyte membrane, succinic dehydrogenase (McMurchie and Raison, 1979) in mitochondrial membranes, desaturases (Pugh and Kates, 1977; Pugh et al, 1980; Mahfouz et al, 1980), enzymes of cholesterol synthesis and metabolism (Sabine and James, 1976; Mitropoulos and Venkateson, 1977; Spector et al, 1980; Field and Salome, 1982), drug metabolizing enzymes (Hammer and Willis, 1979; Lamber and Wills, 1977 a, b; Dupple and Ulbrich, 1976) and UDP-glucuronyl transferase (Spector et al, 1980) in microsomal membranes, have been shown to be affected by dietary lipids.

The correlation of Arrhenius plot discontinuities in enzyme activity with a physical parameter, has been a useful approach to ascertain the relationship between membrane physical properties and enzyme activities. For Ca^{2+}-ATPase such a positive correlation has been found (Hesketh et al, 1976; Hidalgo et al, 1976) while others have suggested that the correlation is with a conformational change (Dean and Suarez, 1981) as well as with the state of aggregation of the enzyme in the membrane (Hidalgo et al, 1978; Hoffman et al, 1979). Cullis and de Kruijff (1979) have demonstrated that the temperature at which the phase transition of the membrane lipids occur is not necessarily the same as the breakpoint in the Arrhenius plot and depends on the position of the probe in the lipid matrix.

Spin label and fluorescent probe experiments have also revealed that some membrane-bound enzymes are surrounded by a coat of lipid which has been immobilized by lipid-protein interactions. This 'boundary lipid' has a different transition temperature from that of the bulk lipid and appears to be essential for the attainment of maximal activity of the enzyme (Overath and Träuble, 1973; Stier and Sackmann, 1973). However, the absolute specificity of this boundary lipid is questionable since a variety of phospholipids and indeed detergents can replace the initial coat (Warren et al, 1974, 1975; Dean and Tanford, 1977). Also there is strong evidence for a rapid exchange between boundary lipid and bulk lipid (Cullis and Grathwohl, 1977). However, the physical state of the lipid matrix or lipid coat or both, does influence the functioning of membrane-bound enzymes.

2.8. Microsomal membranes and lipid metabolism

As discussed above, many of the enzymes of fatty acid metabolism (desaturases and elongases) and of cholesterol synthesis and metabolism (HMG-CoA reductase, ACAT, C7H) are located on the endoplasmic reticulum. Therefore, microsomal membranes may play and important role in the overall regulation of lipid metabolism.

2.8.1. Metabolism of fatty acids

It is quite clear from the preceeding sections that fatty acids play a dynamic role in the structure and function of biological membranes. Certain fatty acids (unsaturated only) are essential to the animals, yet can be synthesized in the plants. Animals cannot introduce a

double bond beyond 9th carbon atom whereas plants can only introduce beyond 9th carbon atom from the carboxylic group of the fatty acid (Gurr and James, 1971). The first double bond in both animals and plants is always at 9th carbon atom from the carboxyl end e.g. oleic acid;

$$^{18}CH_3\text{-}^{17}CH_2\text{-}^{16}\_^{15}\_^{14}\_^{13}\_^{12}\_^{11}CH_2\text{-}^{10}CH = {}^{9}CH\text{-}^{8}CH_2\text{-}^{7}\_^{6}\_^{5}\_^{4}\_^{3}\_^{2}CH_2\text{-}^{1}COOH$$

Desaturation in plants ← | → Desaturation in animals

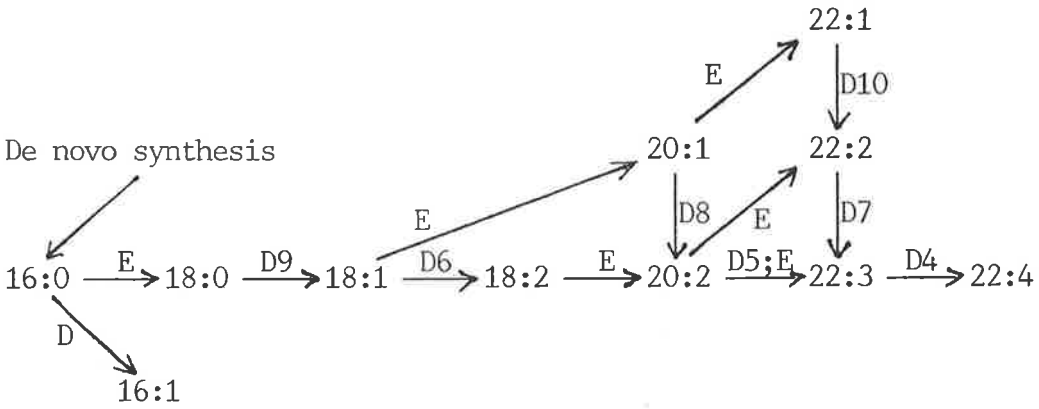
Consequently, from a metabolic, physiological and pathological point of view, the fatty acids have to be distributed in 3 main families, named ω9-, ω6- and ω3- families (Figure 4), which cannot be interconverted. The major pathways of PUFA synthesis appears to be the alternate desaturation and chain elongation (Bernert and Sprecher, 1975) as is shown by the horizontal sequences in Figure 4. All human, vegetal or animal cells have fatty acids of all these series in different proportions. The saturated (SFA) and monounsaturated (MUFA) fatty acids from ω9-family are the major fatty acid species of adipose tissue (storage lipid) while PUFA from ω6- and ω3- families are largely found in the membrane phospholipids (structural lipids).

2.8.1.1. Desaturation of fatty acids

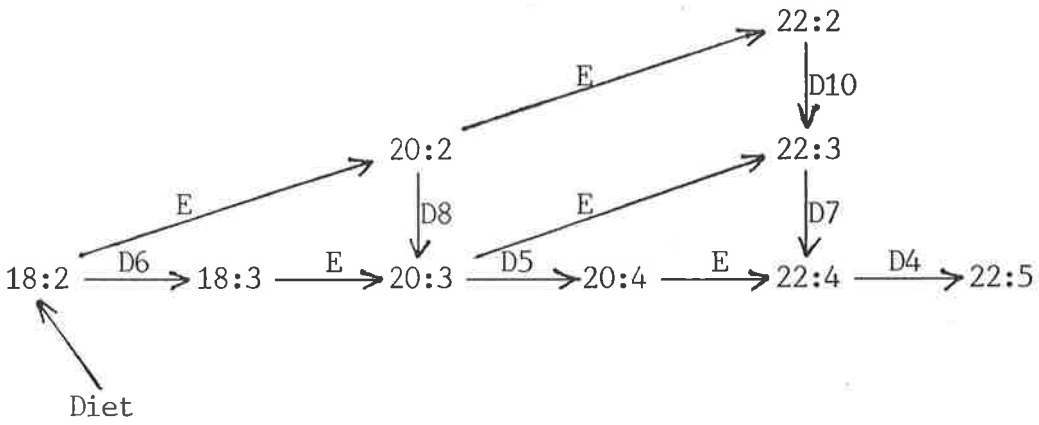
The relative availability of saturated and unsaturated fatty acids for membrane phospholipid synthesis is determined by the activity of membrane-bound desaturases. These enzymes catalyze the stereospecific removal of two hydrogen atoms from a pre-formed fatty acid CoA

FIGURE 4: Families of long chain fatty acids in animals;
DX = Desaturation on carbon x from the carboxyl group
E = Elongation.

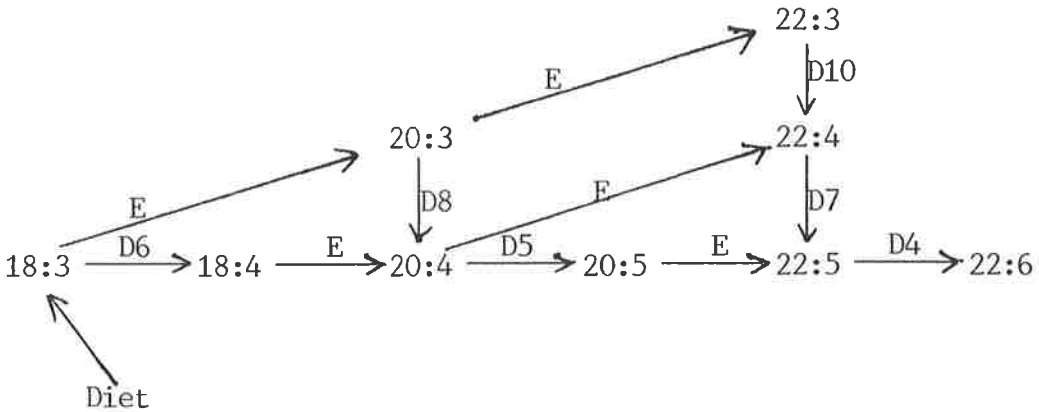
w9 FAMILY



w6 FAMILY



w3 FAMILY

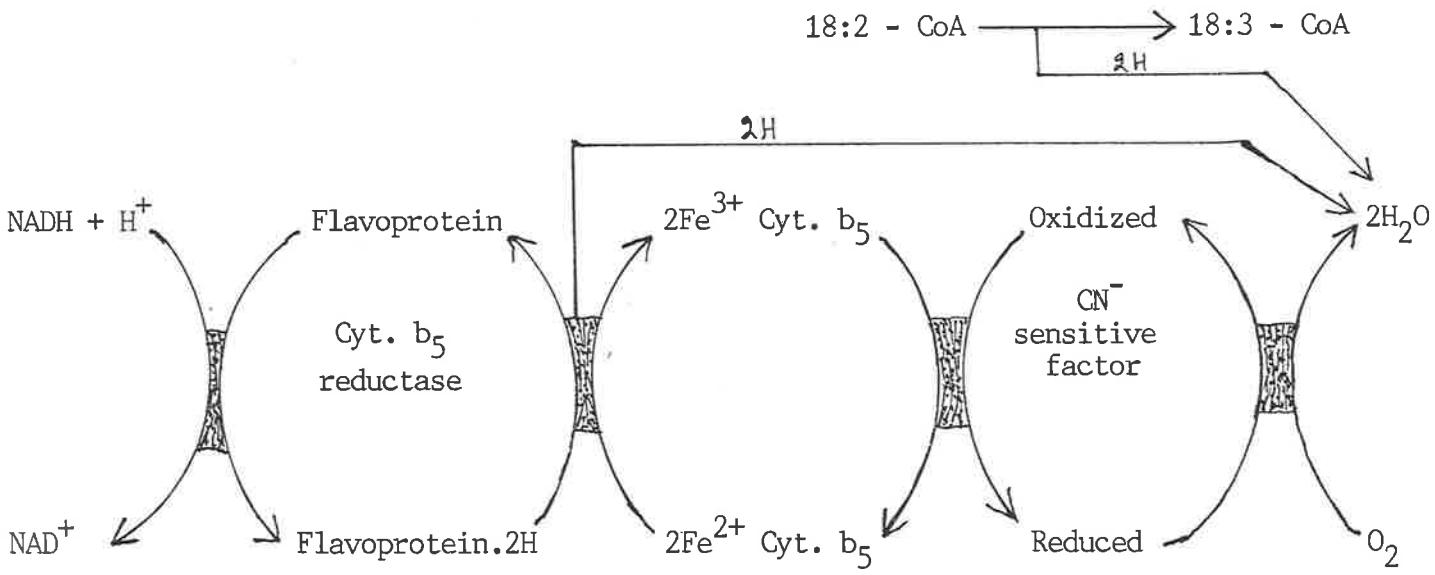


derivative of either exogenous or endogenous origin, which results in the formation of a cis-double bond (Gurr and James, 1971; Jeffcoat, 1979). The enzymes are prefixed according to the position on the fatty acid from which they remove hydrogen atoms e.g. Δ^9-, Δ^6-, Δ^5- and Δ^4-. Acyl CoA is not always the required precursor for desaturation as 20:3 lecithin has been shown to be directly desaturated to 20:4 lecithin (Pugh and Kates, 1977, 1979, 1984).

The desaturase enzymes are tightly bound to the endoplasmic reticulum as intrinsic proteins (Rogers and Strittmatter, 1973, 1974; Strittmatter and Rogers, 1975; Holloway and Holloway, 1975, 1977). They require molecular oxygen, and electron donor (NADH or NADPH) and a short electron transport chain consisting of NADH- cytochrome c reductase and cytochrome b5 (Figure 5). The mechanism of desturation is thought to be similar for all the desaturases (Oshino, 1972; Oshino and Sato, 1972; Brenner, 1971) as is shown in Figure 5. The desaturases are difficult to purify and characterize because they require microsomal lipids for activity (Jones et al, 1969). Partial purification has been achieved (Strittmatter et al, 1974) and evidence has been obtained that the terminal cyanide (CN^-) sensitive factor is the desaturase (Figure 5) (Brenner, 1974). Fatty acyl desaturase activity can play an important role in the maintenance of appropriate membrane fluidity by regulating saturation/unsaturation of membrane phospholipids. It has been shown that Escherichia coli changes the fatty acid composition of its membrane lipids as its growth temperature is altered (Marr and Ingraham, 1967). With increased growth temperature, there is a greater tendancy to incorporate longer and more saturated fatty acids into phospholipids (Sinenski, 1971). Thus it has been hypothesised that changes in the fatty acid composition of phospholipids produce membranes whose lipids have a relatively constant fluidity at the growth temperature - a process dubbed "Homeoviscous adaption" (Sinenski, 1974).

The desaturase activities have been shown to be influenced by various nutritional and hormonal factors (Brenner, 1981). Food deprivation decreases the activity of Δ^9- desaturase enzyme and induction of Δ^9- desaturase is most responsive to carbohydrates refeeding (Brenner et al, 1968; de Gomez-Dumm et al, 1970). Dietary PUFA markedly inhibit Δ^9- and Δ^6- desaturase activities in various tissues of rat (Mahfouz

FIGURE 5: Postulated mechanism of desaturation using linoleic (18:2) acid as an example.
[From Brenner, R.R. (1971) lipids, 6 : 567].


18:2 - CoA
18:3 - CoA
2H
2H
NADH + H+
Flavoprotein
2Fe3+ Cyt. b5
Oxidized
2H2O
Cyt. b5
reductase
CN-
sensitive
factor
NAD+
Flavoprotein.2H
2Fe2+ Cyt. b5
Reduced
O2

et al, 1980; Cook, 1981; Shimp et al, 1982). Different hormones affect the desaturase activities of rat liver microsome differently. Insulin increases Δ^9-, Δ^6- and Δ^5- desaturation whereas all other metabolism hormones: glucagon, epinephrine, corticoids and thyroxine decrease all the three desaturase enzyme activites (de Gomez-Dumm et al, 1975, 1976; Enser, 1979; Brenner, 1981).

2.8.1.2. Chain elongation

Fatty acids up to and including palmitic acid (16 carbon atoms) can be synthesized by the condensation of acetyl CoA and malonyl CoA units by the cytosolic enzyme complex known as fatty acid synthetase. Palmitic (16:0) acid is then further elongated by the action of mitochondrial or microsomal membrane-bound elongases. In contrast to the relatively large amount of work carried out on individual desaturases, little work has been directed to the study of elongation of fatty acids. Partial purification of elongases has been achieved by Bernert and Sprecher (1977).

Starvation (Donaldson et al, 1970) and the administration of hypolipidaemic drugs (Landriscina et al, 1977) have been shown to depress the activity of microsomal chain elongation. Elongase activity is largely unaffected by the action of thyroxine, but is stimulated by triiodothyronine (Faas et al, 1972; Landriscina et al, 1976; Ginoni et al, 1978). These results clearly indicate that the chain elongation is under a different set of controls than that which influences fatty acid synthetase and desaturation.

2.8.2 Metabolism of cholesterol

During the last 200 years cholesterol, probably more than any other organic molecule, has drawn the attention of the scientists all over the world. This is understandable in view of the correlation between abnormal levels of plasma cholesterol and the occurrence of various serious diseases in man (Sabine, 1977). Plasma and tissue cholesterol levels can be generally regarded as the nett result of several different but interrelated processes, as shown in Figure 6. The largest contribution to total body synthesis of cholesterol in rat is made by the liver, with the intestine being the second major site, while other tissues are also capable of some degree of synthesis (Dietschy and Siperstein, 1967).

The principal metabolic products of cholesterol in mammals are bile acids, cholesterol esters and steroid hormones. The conversion of cholesterol to these end productions is shown in Figure 7. The following discussion will be limited to the major regulatory enzymes of cholesterol synthesis and metabolism. Details of each of these pathways can be found elsewhere (Goad, 1975; Sabine, 1977).

2.8.2.1. Biosynthesis of cholesterol

Cholesterol is a derivative of perhydrocyclopentanophenanthrene, with 27 carbon atoms, containing one hydroxyl group in the 3β position and a branched aliphatic side chain, as follows;

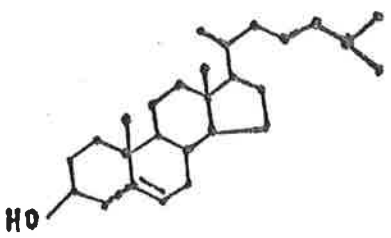
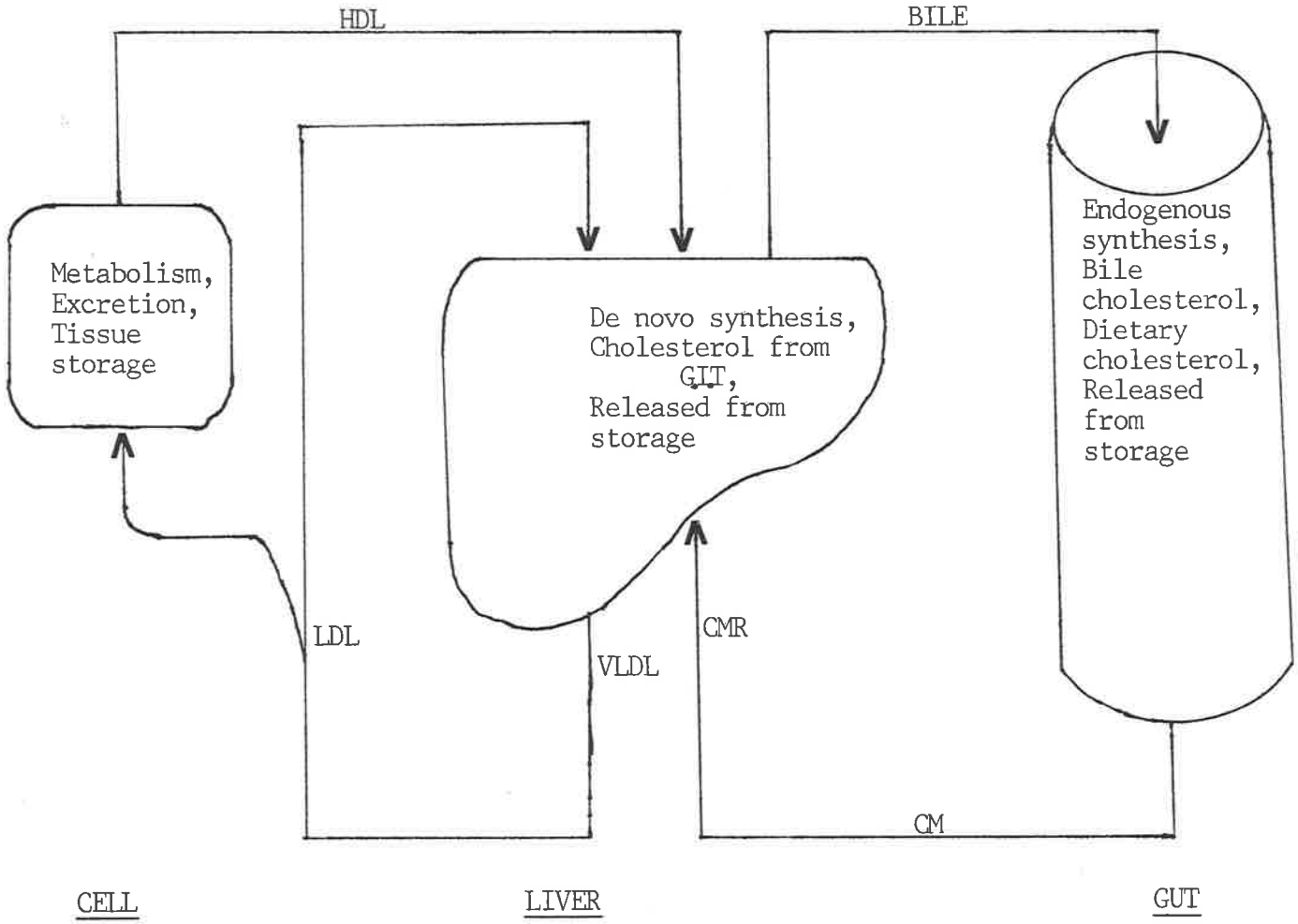


FIGURE 6: Overview of cholesterol transport. Cholesterol from gut (diet, bile, endogenous production and release from storage) reaches liver as part of chylomicron (CM) remnant (i.e. CMR). Liver cholesterol is mainly excreted in bile or as a component of very-low-density lipoprotein (VLDL); through lipolysis and other intravascular processes, VLDL is converted to low-density lipoprotein (LDL). LDL may be taken up by peripheral cells or by liver. High-density lipoprotein (HDL) is formed in the gut, in liver and intravascularly and probably functions in reversed transport of cholesterol from cell to liver. UC, unesterified cholesterol; CE, cholesterol ester.


HDL
BILE
Metabolism,
Excretion,
Tissue
storage
De novo synthesis,
Cholesterol from
GIT,
Released from
storage
Endogenous
synthesis,
Bile
cholesterol,
Dietary
cholesterol,
Released
from
storage
LDL
VLDL
CMR
CM
CELL
LIVER
GUT

Cholesterol is synthesized from acetyl CoA units, which is a multienzymic process involving at least 27 enzymes localised both in the cytosol and on the membranes of the endoplasmic reticulum. Altogether 18 molecules of acetyl CoA are involved to form one molecule of cholesterol, during which 18 molecules of ATP and 20 molecules of NADPH are also consumed (Sabine, 1977). The formation of hydroxymethylglutaryl-coenzyme A (HMG-CoA), an intermediate of cholesterol biosynthetic pathway, involves the condensation of 3 acetyl CoA units, catalyzed by acetoacetyl CoA thiolase and HMG-CoA synthase, enzymes located in the cytosol (Lane et al, 1973). HMG-CoA is converted to mevalonic acid by the enzyme HMG-CoA reductase, which is the first membrane - bound enzyme in cholesterol biosynthetic pathway. Mevalonic acid is then converted to cholesterol by a number of steps including phosphorylation, decarboxylation, isomerisation and reduction. A more thorough discussion of these reactions can be found in several reviews (Goad, 1970, 1975; Sabine, 1977).

The conversion of HMG-CoA to mevalonic acid by HMG-CoA reductase is considered to be the rate-limiting step in the biosynthesis of cholesterol (Rodwell et al, 1976). The reductase is tightly bound to the endoplasmic reticulum (Bucher et al, 1960) with its active site situated on the cytosolic surface of the endoplasmic reticulum (Kleinsek et al, 1981; Phillips and Ness, 1984).

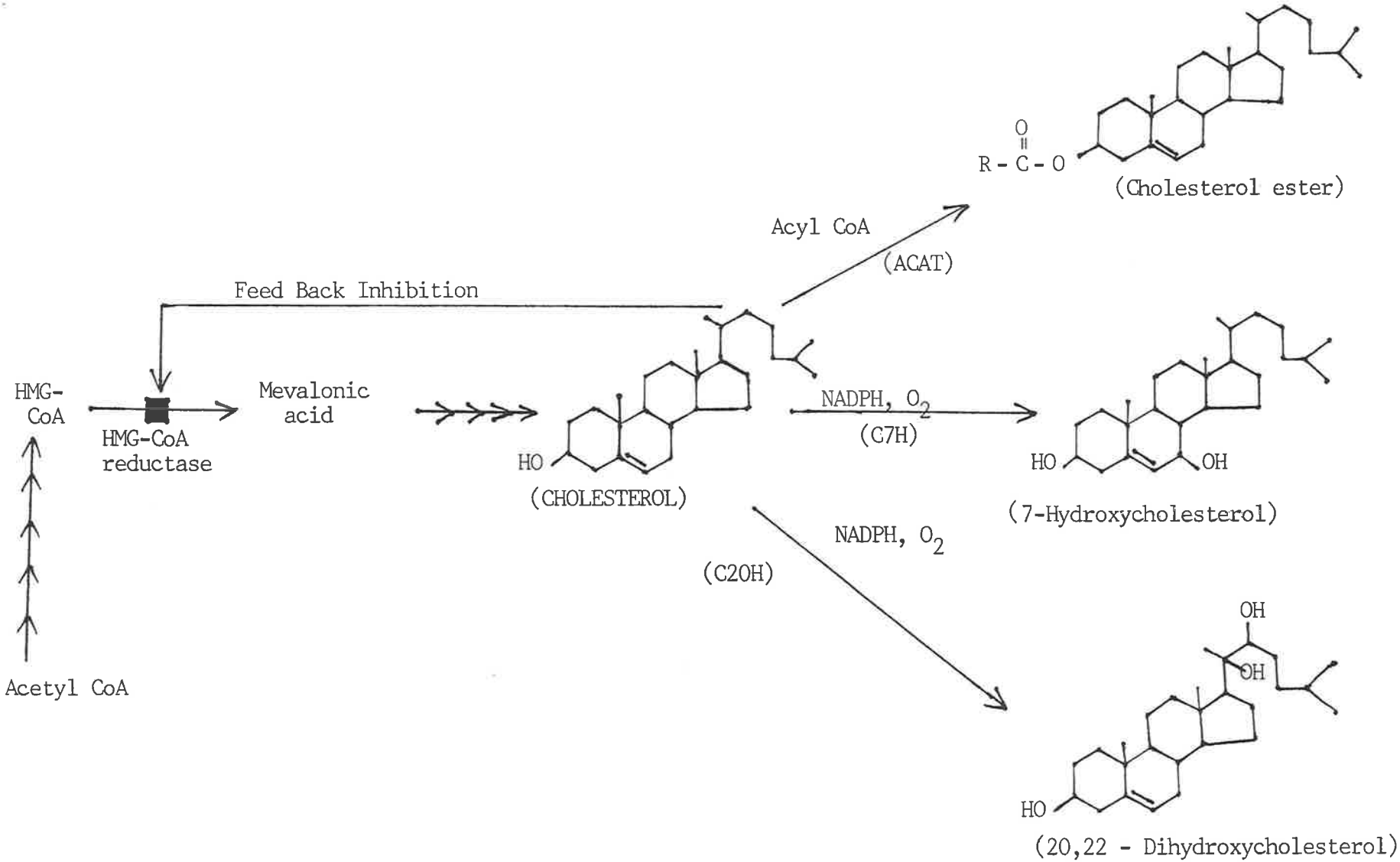
HMG-CoA reductase activity in liver is inhibited when rats are fed a cholesterol-rich diet (Rodwell et al, 1976). The exact molecular mechanism of feedback regulation of cholesterol biosynthesis is not known. Synthesis and degradation of HMG-CoA reductase have been

suggested to be a major factor in this regulation (Rodwell et al, 1976; Shapiro and Rodwell, 1971; Chen at al, 1982). In addition, phosphorylation - dephosphorylation of HMG-CoA reductase is thought to play a role in the short term regulation (Gibson and Ingebritsen, 1978; Arebalo et al, 1981). A direct effect of microsomal cholesterol level on HMG-CoA reductase activity has also been reported (Mitropoulos and Venkatesan, 1977). The enzyme activity is strongly inhibited when rat liver microsomes are enriched with cholesterol by incubation with serum (Venkatesan and Mitropoulos, 1982; Mitropoulos et al, 1981).

2.8.2.2. Metabolism of cholesterol to bile acids

The synthesis of bile acids from cholesterol take place entirely in the liver. In most mammals cholic acid and chenodeoxycholic acid are the major products, which are then conjugated with either taurine or glycine, prior to their secretion into the bile. The first step in the synthesis of bile acids is hydroxylation of cholesterol molecule at the 7α-position to form 7α-hydroxycholesterol (Figure 7) and this is considered to be the rate-limiting step in this process (Myant and Mitropoulos, 1977). This reaction is catalyzed by microsomal cholesterol 7α-hydroxylase (Shefer et al, 1968; Danielsson et al, 1967). In vitro, the enzyme requires molecular oxygen and NADPH as co-factor. The active site of the enzyme has very specific structural requirements e.g. for the side chain of cholesterol (Boyd et al, 1973). The enzyme can use microsomal-bound cholesterol as a substrate, in vitro (Van Cantfort and Gichen, 1975).

FIGURE 7: Metabolism of cholesterol to cholesterol esters, bile acids and steroids.


HMG-
CoA
HMG-CoA
reductase
Mevalonic
acid
Feed Back Inhibition
Acetyl CoA
HO
(CHOLESTEROL)
Acyl CoA
(ACAT)
R - C - O
O
(Cholesterol ester)
NADPH, O2
(C7H)
HO
OH
(7-Hydroxycholesterol)
NADPH, O2
(C20H)
OH
OH
HO
(20,22 - Dihydroxycholesterol)

The location of the control of bile acid synthesis at the cholesterol 7α-hydroxylase level has several important implications. Firstly, it ensures that there is no build up of the intermediates to the bile acids from cholesterol. Secondly, the accumulation of cholesterol in the liver might also inhibit its own synthesis by suppressing HMG-CoA reductase activity. On the other hand, too much cholesterol will enhance the rate of hydroxylation of cholesterol so as to affect the removal of the excess (Myant and Mitropoulos, 1977). Feeding cholesterol to intact animals results in decreased activity of HMG-CoA reductase, in increased activity of cholesterol 7α-hydroxylase and also in increased activity of cholesterol acyltransferase (Balasubramaniam et al, 1978). Consequently, such a condition is associated with an increased cellular concentration of cholesterol esters, and increased rate of bile acid biosynthesis and a decreased rate of hepatic cholesterogenesis (Nervi et al, 1975; Wilson, 1964). On the whole, the control acting over cholesterol biosynthesis and its metabolism to bile acids and cholesterol esters are very much inter-connected, with all the three regulatory enzymes located on the same membranes (Mitropoulos et al, 1978).

2.8.2.3 Metabolism of cholesterol to steroid hormones

The adrenal gland, the ovary and the testis convert a significant amount of cholesterol to steroid hormones. The amount of cholesterol metabolized to steroid hormones may not be very large relative to other processes but is of vital importance. The common precursor for all the steroid hormones is pregnenolone, which is synthesized from cholesterol and is known to involve three enzyme, two hydroxylases and

a lyase (Gower, 1975). Cholesterol molecule is initially hydroxylated at the 20α- and then at 22β -position to form 20α,22β - dihydroxycholesterol (Figure 7). These reations are catalyzed by mixed function oxidases and require molecular oxygen NADPH and the participation of cytochrome P450 (Gower, 1975). The formation of 20α,22β - dihydroxycholesterol is believed to be the rate-limiting step in the synthesis of pregnenolone and of the steroids from cholesterol.

The cholesterol pool for the synthesis of prenenolone is derived from synthesis in-situ in these tissues and does not equilibrate with circulating pools (Gower, 1975). Therefore, in relation to metabolism of cholesterol in the liver, steroidogenesis is quantitatively insignificant, although it is critically important for the physiological well-being of the animal.

2.8.2.4 Metabolism of cholesterol esters

Under normal conditions, in most mammals, esterified cholesterol represents about 20% of the total liver cholesterol, while the proportion in the serum is much higher, ranging from 60% to 80% (Goodman, 1965; Field et al, 1960). In the microsomes of rat liver, however more than 90% of total membrane cholesterol is unesterified (Baqir and Booth, 1977; Mitropoulos and Venkatesan, 1977).

Esterification of cholesterol in the rat liver is catalyzed by acyl CoA: cholesterol acyltransferase (ACAT) enzyme (Figure 7), which is tightly bound to the microsomal membranes and is mainly recovered in

the rough endoplasmic reticulum (Balasubramaniam et al, 1978). Free fatty acids are not incorporated into cholesterol esters unless ATP and CoA are present in the reaction medium (Brenneman et al, 1977); otherwise they are inhibitory to the enzyme.

Cholesterol esters have two functions in animals and man. They serve as intracellular storage forms of cholesterol and to transport cholesterol through the blood plasma. The ACAT thus protects against unesterified cholesterol accumulation (Nilsson, 1975) and also esterifies the cholesterol released intracellularly during the catabolism of plasma lipoproteins taken up from the extracellular fluid (Goldstein et al, 1974; Rothblat et al, 1976).

Cholesterol can also be esterified in the plasma by the enzyme which differs from liver acyltransferase in that the fatty acyl donor is the number 2 fatty acid of a lecithin molecule, and hence is named lecithin: cholesterol acyltransferase (LCAT). LCAT is found only in the plasma, although it originates from the liver and is possibly removed from the circulation by this organ (Nordby et al, 1976). The role of LCAT is apparently to esterify some of the free cholesterol present in the lipoprotein particles and hence increase their capacity to take up more free cholesterol from their surroundings (Myant, 1971).

The hydrolysis of cholesterol esters in the liver is catalyzed by a cholesterol esterase. The bulk of this activity is found in the cytosol, with only about 1% to 32% associated with the microsomal fraction (Goodman, 1969). In addition, two other esterases have been

reported to be present in the rat liver homogenates (Nilsson, 1976). Not all tissues are capable of synthesis and hydrolysis of cholesterol esters (d'Hollander et al, 1976). The liver and adrenal gland are capable of both synthesis and hydrolysis, whereas other tissues are capable only of esterification (e.g. lung) or hydrolysis (e.g. adipose tissue) or neither (e.g. heart).

Since ACAT catalyses cholesterol esterification in the arterial intima, it has been implicated in the development of atherosclerosis (Brecher and Chobanian, 1974; Hashimoto and Dayton, 1977). Atherosclerosis is characterized by lipid accumulation in the arterial intima, and cholesterol esters are the main lipid material that accumulates. It is generally believed that the cholesterol esters which initially accumulate are synthesized intracellularly. The cholesterol moiety is probably derived from cholesterol esters in plasma lipoproteins, but the accumulation of cholesterol esters inside the cells requires an initial hydrolysis followed by subsequent re-esterification (Brown and Goldstein, 1975). On the other hand, one can speculate that cholesterol ester formation may be protective mechanism, preventing undue accumulation of the unesterified form, which actually may be the "toxic" material as suggested by Spector et al (1979).

Both free and esterified cholesterol also constitute a significant proportion of total lipids in the membranes as has been discussed previously in this chapter. They affect the activity of membrane-bound enzymes and hence influence metabolic functions indirectly (Kimelberg and Papahadjopoulos, 1974). In relation to this

last effect, it has been observed that dietary feedback suppression of microsomal membrane-bound HMG-CoA reductase is paralleled by an increase in the microsomal cholesterol ester content (Rodwell et al, 1976); Mitropoulos and Venkatesan, 1977).

2.9. Summary of literature review

Membranes consist of protein and lipid. The protein component is responsible for specific membrane functions whereas the lipid component provides the basic matrix of the membrane and serves as a permeable barrier. The activity of membrane-bound proteins (enzymes) is dependent on the lipid composition of the membrane and a perturbation in any one or more of the lipid profiles (cholesterol content, fatty acyl chains and polar head groups) can lead to altered membrane properties and function. Both exogenous (mainly from diet) and endogenous (de-novo synthesized) lipids participate in the genesis of the membrane but the relative contribution by these exogenous and endogenous sources is not known.

Further, many of the enzymes of lipid synthesis and metabolism are bound to the membranes of endoplasmic reticulum. Thus there is ample evidence to establish that the molecules (fatty acids and cholesterol), which give membranes their special functions are, themselves, dependent on membranes for their synthesis and metabolism. It seems important to know the factors that determine the lipid composition of membranes.

The overall pathways of cholesterol and fatty acid synthesis from

acetate, metabolism of cholesterol to bile acids, steroid hormones and cholesterol esters and of fatty acid metabolism appear to be well established. Therefore, at present greater effort is being directed towards the questions of how these pathways are regulated and what mechanisms are involved. Given that membrane-bound HMG-CoA reductase, cholesterol 7α-hydroxylase, acylcoenzyme A: cholesterol-acyltransferase are important enzymes for cholesterol metabolism and similarly desaturases are important for fatty acid metabolism, the critical questions are, how their activities are regulated at membrane level and what are the physiological effectors involved.

2.10. Working hypothesis

The concept of lipid homeostasis of cellular membranes (Sabine, 1983) implies that the physico-chemical status, sometimes referred to as fluidity or microviscosity, of the lipids of cellular and subcellular membranes is maintained relatively constant in the face of various stresses e.g. metabolic, nutritional, hormonal, physiological, environmental. It is in the cell's best interests to maintain this constancy in the face of various internal and external constraints. During this study several aspects of this hypothesis and its consequences will be examined by feeding various lipid-supplemented diets to the rats followed by measuring liver microsomal lipid composition, microsomal lipid fluidity and microsomal-bound enzyme activities.

Alterations in the membrane lipid fluidity can be induced experimentally by varying the temperature, cholesterol content and

nature and composition of membrane phospholipids (Farias et al, 1975; Feo et al, 1976; Malkiewicz-Wasowicz et al, 1977). Furthermore, these manipulations of the membranes also result in profound changes in the activities of several bound enzymes, thus indicating a role of membrane lipids and membrane fluidity in the control of their activities.

While most of these observations were made under non-physiological conditions, it is nevertheless quite possible that the same mechanism of enzyme regulation may be operative under physiological conditions e.g. when certain lipids are included in the diet (Farias et al, 1975). This concept of enzyme regulation and its significance in the control of cellular metabolim is now gaining wide recognition (Innis and Clandinin, 1981 b; Jenke et al, 1982).

A classical approach to ascertain the relationship between membrane physical properties and enzyme activities has been to correlate the Arrhenius Plot discontinuities with those of a physical parameter. Such a positive correlation has been found in some cases where occurrence of a phase transition determined by a physico-chemical method coincides with the temperature break in the Arrhenius Plot of a membrane-bound enzyme (Raison, 1973b; Fox, 1975; Hidalgo et al, 1976).

Many of the enzymes of lipid metabolism e.g. desaturases, HMG-CoA reductase, ACAT, cholesterol 7α-hydroxylase are exclusively located on the membranes of endoplasmic reticulum. Therefore, it is necessary to determine the relationship between microsomal lipids and the overall

regulation fatty acid and cholesterol metabolism. In the present study, a variety of high lipid diets will be used as a tool to investigate this relationship.

2.11 Research Objectives

The purpose of the work presented in this thesis is to examine and to extend the above hypothesis (Sabine, 1983) along the following lines of questions:

(1) are the changes in dietary cholesterol level followed by the alterations in liver microsomal lipid composition and has quality and quantity of fat-fed with or without cholesterol any significant effect on this process?

(2) If so, how do these changes in microsomal lipid composition affect microsomal lipid fluidity?

(3) Are these changes in microsomal lipid composition and fluidity accompanied by alterations in the activities of microsomal-bound enzyme activities and their Arrhenius Plot characteristics.

(4) Are the changes in liver microsomal lipid composition reflected in the whole liver and/or plasma?

CHAPTER 3. MATERIALS AND GENERAL METHODS

3.1. Materials

3.1.1. Animals

Male buffalo strain (Wistar origin) rats weighing between 200-300g, bred and raised at the Waite Institute were used throughout the course of this investigation. These animals were housed in a windowless, well ventilated room with the lights off at 0700 hr and on again at 1900 hr and the temperature of the room maintained at 21°C ± 2°C. Food (M and V cubes) and tapwater were offered ad libitum unless stated, otherwise.

3.1.2. Radiochemicals

The following radiochemicals were purchased from Amersham International Plc. (Buckinghamshire, England): [1 - ^{14}C] stearic acid (57 mCi/mmol), [1 - ^{14}C] linoleic acid (58 mCi/mmol), [2 - ^{14}C] eicosa-8, 11, 14 - trienoic acid (56 mCi/mmol) and [3 - ^{14}C] 3-hydroxy-3-methylglutaryl-coenzyme A. [1 - ^{14}C] Palmitoyl-coenzyme A (54.6 mCi/mmol) and [7 - ^{3}H(n)] cholesterol (13 Ci/mmol) were obtained from New England Nuclear, Boston, Mass., U.S.A. All the radiochemicals were more than 98% pure and were used without further purification.

3.1.3. Biochemicals

Unlabelled cholesterol, cholesterol esters, HMG-CoA, unlabelled fatty acids, glucose-6-phosphate, $NADP^+$, glucose-6-phosphate dehydrogenase, glutathione, NADH, CoASH, Triolein, Tween 80, BSA, dithiothreitol, dithioerythritol, ATP, histidine, Tris-chloride, nicotinamide, Palmitoyl-CoA, EDTA, phospholipids and FAME for TLC and GLC and silica gel H were obtained from Sigma Chemical Co. (St. Louis, MO). Some of the FAME were supplied by Nu-Chek Prepn. Inc. Silica gel G from Merck, Dermstadt, Germany and bulk cholesterol from Ajax Chemicals, Sydney, Australia.

3.1.4. Others

Coconut oil and sunflower seed oil were purchased from Vegetable Oils Pty. Ltd. (Vic., Australia). Rat food (M and V cubes) was supplied by Milling Industries Pty. Ltd. (S.A., Australia). The composition of this diet is given in Table 1.

3.2. General methods

3.2.1. Preparation of experimental diets

Crushed M and V cubes (about 1 - 2 mm^3 in Size) were used as the basic (Reference or control) diet in all the experiments, which contained 3% total fat derived from a variety of sources. Experimental diets were prepared by supplementing the reference diet with one of the following: 20g cholesterol/Kg diet; 150ml sunflower seed oil/Kg diet;

Table 1. Composition of the basal diet*.

Minimum Crude Protein (%)	21.0
Minimum Crude Fat (%)	3.0
Maximum Crude Fibre (%)	7.0
Maximum Added Salt (%)	1.0
Vitamin A	10,000 I.U./kg
Vitamin D3	2,500 I.U./kg
Vitamin E	20 I.U./kg
Vitamin K	1.1 mg/kg
Vitamin B_{12}	10 µg/kg

* Information supplied by the manufacturer "Milling Industries Pty. Ltd., Adelaide, South Australia.

Ingredients: Wheat/Barley/Oats/Groats/Sorghum/Triticale/Wheat Offal, Soyabean Meal/Peanut Meal/Sunflower Meal/Cottenseed Meal/Meat Meal/Lucerne Meal/Lupins/Peas, Salt, Calcium, Phosphate, Trace Minerals and Vitamins.

20g cholesterol plus 150 ml sunflower seed oil/Kg diet; 150 ml coconut oil/Kg diet; 20g cholesterol plus 150 ml coconut oil. For the high fat - high cholesterol diets, 20g cholesterol was dissolved in 150 ml of respective oil before mixing it with 1 Kg of crushed cubes. The low fat - high cholesterol diet was prepared by mixing cholesterol with crushed cubes. To ensure adequate mixing of the added cholesterol, the mixture was moistened with a little chloroform which was subsequently removed by drying overnight at 40°C. The reference diet was treated in a similar way with chloroform only. After preparation of the diets, total lipids were extracted and their fatty acid compositions were determined by GLC as explained in section 3.2.5.6.

3.2.2. Blood and Liver Collection

Unless noted otherwise, the animals were fed the experimental diets for a period of 28 days. All the rats were killed between 0800 and 1000 hr using a guillotine. Following decapitation, the blood was collected in a heparinized tube with the aid of a heparinized funnel. The livers were quickly excised, rinsed in ice cold saline, extrahepatic tissue if any, removed, blotted dry, weighed and suspended in 0.25M sucrose - 0.01M tris-chloride buffer (pH 7.4). The livers were always processed immediately after collection without further storage. Blood was centrifuged at 1200 x g for 15 minutes to get the plasma.

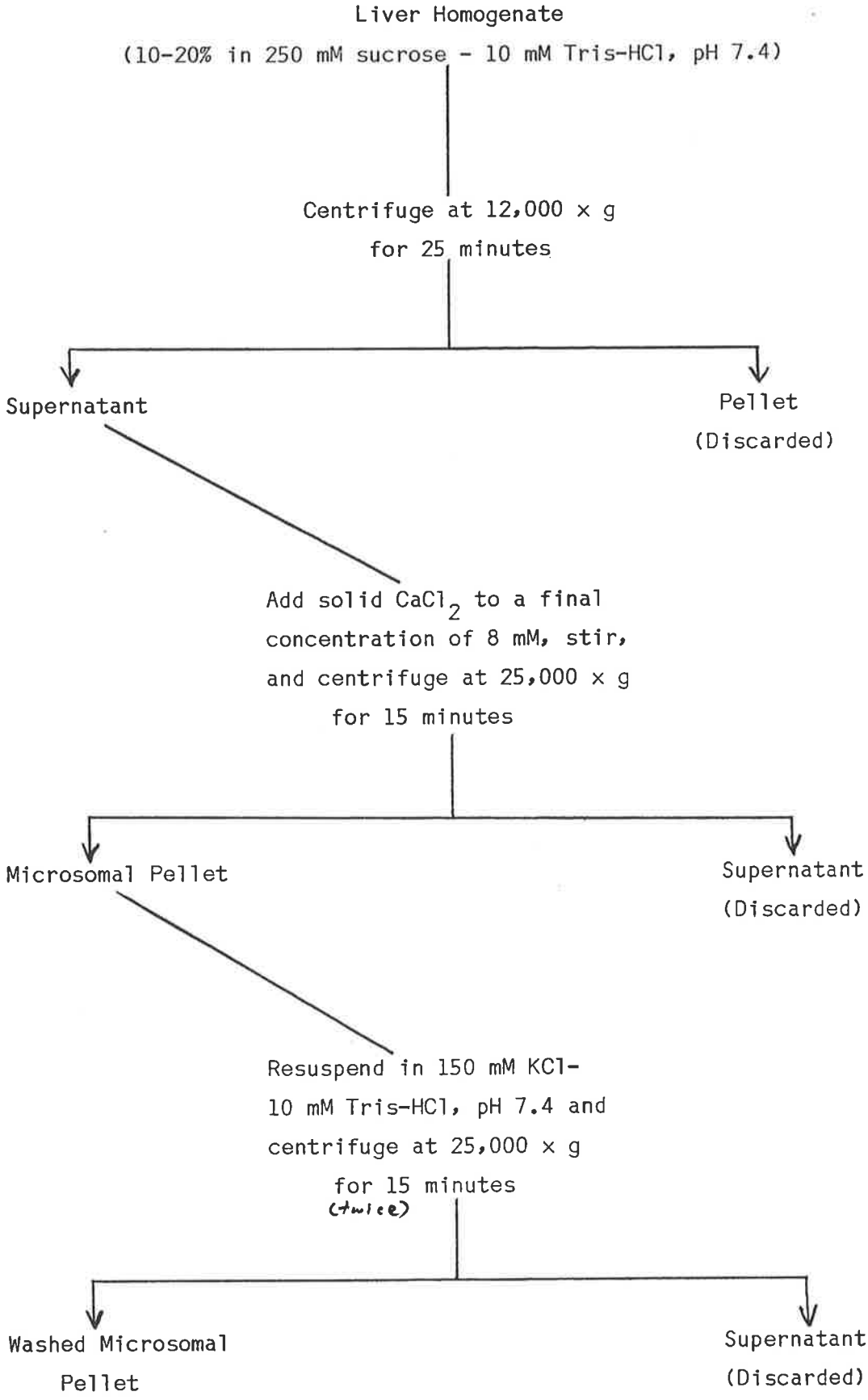
3.2.3. Preparation of hepatic microsomes

Microsomes from livers were isolated essentially by the method of Schenkman and Cinti (1978). Livers were homogenized by mincing with scissors, followed by five passes in a Potter-Elvehjem tissue grinder equipped with a motor driven teflon pestle. The homogenate was centrifuged at 12,000 x g for 25 minutes to obtain the post-mitochondrial supernatant. Addition of solid calcium chloride (8 mm final concentration) to the post-mitochondrial supernatant allowed complete sedimentation of the microsomes at 25,000 x g in 15 minutes. The pellet was then washed twice by resuspending in an equal volume of 0.15 M KCl - 10 mM tris-chloride buffer (pH 7.4) and was resedimented at 25,000 x g for 15 minutes. The resultant pinkish pellet of microsomes was dispersed in the appropriate buffer for enzyme assay and hence referred to as freshly-prepared microsomal suspension. Enzyme activities and microsomal membrane constituents including cholesterol and phospholipids, are not affected by this calcium aggregation method (Schenkman and Cinti, 1978; Kamath and Narayan, 1972). The centrifugation scheme followed for the sedimentation of microsomes is given in Figure 8.

3.2.4. Lipid extraction

Plasma, liver and liver microsomes were homogenized in 20 volumes of chloroform: methanol (2:1, v/v) mixture containing 0.005% butylated hydroxytoluene (BHT) as an antioxidant. Total lipids were extracted by the method of Folch et al (1957). After shaking for 30 minutes, 0.88% NaCl was added to get the separation of two distinct layers.

FIGURE 8: Flow diagram of procedure for the sedimentation of microsomes. All steps are carried out at 0-5°C.


Liver Homogenate
(10-20% in 250 mM sucrose - 10 mM Tris-HCl, pH 7.4)
Centrifuge at 12,000 x g
for 25 minutes
Supernatant
Pellet
(Discarded)
Add solid $CaCl_2$ to a final
concentration of 8 mM, stir,
and centrifuge at 25,000 x g
for 15 minutes
Microsomal Pellet
Supernatant
(Discarded)
Resuspend in 150 mM KCl-
10 mM Tris-HCl, pH 7.4 and
centrifuge at 25,000 x g
for 15 minutes
(twice)
Washed Microsomal
Pellet
Supernatant
(Discarded)

The upper layer of methanol and water containing soluble impurities was discarded. The bottom layer of chloroform containing the lipids was decanted and stored under nitrogen at -20°C in dark until further use.

3.2.5. Analysis of lipids

3.2.5.1. Fractionation of total lipids

The fractionation of total lipids into polar and non-polar lipids was done by the solvent partition method of Nichols (1964). According to this method, total lipids were dissolved in a mixture of petroleum ether (60° - 80°): 95% methanol in water (1:1, v/v), pre-equilibrated with each other for 24 hours. After shaking thoroughly, the contents were allowed to stand and the two layers were separated. The methanol-water layer contained mainly the polar lipids and the petroleum ether layer was enriched in non-polar lipids.

3.2.5.2. Separation of lipid fractions into classes

Both polar and non-polar lipids were subjected to thin layer chromatography (TLC) for further fractionation and purification. For all TLC, 20 x 20 cm glass plates were coated with 0.25mm thick layer of silica gel using a Desaga adjustable applicator (Model S-II). Some of the plates were prepared by spreading the silica gel slurry on TLC plates and then tapping it on the surface of a smooth table. This also resulted in a uniform distribution of the gel particles on TLC plates. Plates were allowed to air-dry before being activated at

110°C for 3 hours. Plates were then cooled to room temperature and stored in a dessicator and could be used without re-activation for 4 days. Spotting of standards and extracts was preformed using a microlitre syringe (S.G.E. Melbourne, Australia).

Plates were developed in glass chromatography tanks sealed with ground glass covers, and with solvent saturated chromatographic paper lining the sides of the tank to ensure a vapour-saturated atmosphere. The solvent run in all cases was approximately 15cm.

3.2.5.2.1. TLC of phopspholipids

TLC plates were coated with silica gel H for the separation of phospholipid classes. The polar lipids (mainly phospholipids) were concentrated and applied to TLC plates which were developed in chloroform : methanol : acetic acid : water (25 : 15 : 4 : 2, v/v/v/v) according to the method of Skipski et al (1964). The plates were dried under nitrogen and bands were visualized by keeping the plate in an iodine chamber. Phospholipid classes were identified by co-chromatography with authentic standards and by comparison of their Rf-values.

3.2.5.2.2. TLC of neutral lipids

TLC plates were coated with silica gel G for the fractionation of neutral lipids into free cholesterol, free fatty acids, triacylglycerols and cholesterol esters. The plates were developed in petroleum ether (60° - 80°) : diethyl ether : acetic acid (80 : 20 :

1, v/v/v). Lipid bands were visualized and identified as explained in section 3.2.5.2.1.

3.2.5.3. Determination of phospholipids

Phospholipid fractions were eluted and estimated according to the method of Raheja et al (1973). Silica gel spots containing the lipids were scrapped off the plates and were shaken with chloroform : methanol (1 : 1, v/v). After addition of water, the chloroform layer was decanted which contained the phospholipid fraction. The recovery of different phospholipid fractions were more than 98% by these methods (Raheja et al, 1973). The chloroform was evaporated under nitrogen and the residue redissolved in 0.4 ml chloroform. To this was added 0.1 ml of chromogenic solution, which is a modification of the spray reagent described by Vaskovsky and Kostetsky (1968):

16g of ammonium molybdate was dissolved in 120 ml of water to give Solution I. 40 ml of concentrated HCl and 10 ml of mercury were shaken with 80 ml of Solution I for 30 minutes which gave, after filtration, Solution II. 200 ml of concentrated H_2SO_4 was carefully added to the remainder of Solution I. To the resultant solution was added Solution II to give Solution III. 45 ml of methanol, 5 ml of chloroform and 20 ml of water was added to 25 ml of Solution III to give the chromogenic solution, which was stable for at least 3 months when stored at 5°C.

After adding chromogenic solution, the samples were placed in a boiling water-bath for 1 - 1.5 minutes. After cooling to room

temperature, 5 ml of chloroform was added and shaken gently. The Prussian blue colour in the chloroform layer was read at 710 nm against a blank. A calibration curve (1 - 10 µg phosphorus) was prepared using standard dipalmitoyl phosphatidylcholine (DPPC). Total phospholipids were quantified in a similar manner in the aliquots of total lipid extracts.

3.2.5.4. Determination of cholesterol and cholesterol esters

All the iodine was allowed to sublime before the elution of cholesterol and cholesterol esters (section 3.2.5.2.2.) which were then estimated individually by the method of Stadtman (1957). To the lipid residue in a test-tube 5 ml of chloroform was added. 2 ml of cold acetic anyldride : sulphuric acid (4 : 1, v/v) reagent was added and mixed thoroughly. The tubes were placed in a water-bath maintained at 16 - 18°C and the colour was allowed to develop for 15 minutes in dark. The absorbance was measured at 625 nm against a blank containing chloroform and the reagent only. A standard curve was also prepared by taking cholesterol in the range of 25 to 125 µg.

3.2.5.5. Transesterification of lipid classes

For the preparation of fatty acid methyl esters TLC spots were first visualized under U.V. light at 245 nm, after spraying with 0.2% 2.7-dichlorofluoroscein in ethanol. Spots containing the appropriate lipid fractions were scrapped off and methyl esters were prepared without elution from the gel (Henderson et al, 1982). This method avoids losses incurred in elution of lipids from gel and is

quantitative. The lipid fractions in the gel were heated at 70°C for 3 hours with 5% H_2SO_4 in anhydrous methanol. Fatty acyl methyl esters (FAME), thus formed, were eluted with petroleum ether (40° - 60°) after cooling the samples to room temperature. Dichlorofluorescein and silica gel remained in the aqueous layer. The solvent was subsequently removed under nitrogen and the FAME redissolved in a small volume of chloroform.

3.2.5.6. GLC of fatty acid methyl esters

FAME, prepared as explained above, were analysed with a gas chromatograph (Hewlett Packard, Model 5840A) equipped with a 25 LC2/BP1 glass capillary column (Alltech Assoc.) and a flame ionization detector (FID). The operating conditions were : carrier gas hydrogen at a flow rate of 20 ml/minute and split ratio of 60:1; detector and injector temperatures were maintained at 250°C and oven temperature was programmed to rise from 175° to 230°C at a rate of 5°C per minute. Identification of individual components was made by comparison of their retention times with authentic standards. Peak areas were measured automatically with a GC terminal (Hewlett Packard, Model, 5840A) with built-in integrating capacity.

3.2.6. Protein determination

Protein concentration was determined by the method of Hartee (1972), after precipitating with 10% trichloracetic acid (TCA). BSA was used as standard protein. The liver microsomal protein in rats fed reference diet was 22.3 $\pm$ 4.8 mg (mean $\pm$ S.D.)/g wet weight of liver

which is comparable with the published values (Gregory and Booth, 1975).

3.2.7. Statistical methods

Unless stated, otherwise, the results are shown as mean ± S.D. and the experimental groups were compared for significance using student's t-test.

CHAPTER 4. DIETARY CHOLESTEROL - INDUCED ALTERATIONS IN MICROSOMAL LIPID COMPOSITION OF THE RAT LIVER.

The fatty acyl chains of the phospholipids and the cholesterol content are important determinants of the lipid fluidity of biological membranes, which in turn affect several functional properties of these membranes. Fatty acid composition of membrane lipids can be manipulated by changing the nature of dietary fatty acid intake (Kurata and Privett, 1980a,b; Innis and Clandinin, 1981a, Tahin et al, 1981). These changes are now being recognized as functionally important as the specific role of subcellular membrane lipids in modulating membrane function becomes more clear (Clandinin, 1978; Chapman, 1975). Cholesterol content, another major determinant of the membrane lipid fluidity, can also be manipulated by changing the cholesterol content of the diet (Mitropoulos and Venkatesan, 1977). However, little or no information is available as to the effects of exogenous cholesterol on membrane lipid constituents other than cholesterol (e.g. polar head groups and fatty acid profiles). Therefore, before any discussion of the effect of dietary cholesterol on membrane function, it is important to know the effect of such treatment on membrane lipid composition, including cholesterol content and phospholipid composition and fatty acid profiles.

In the present exercise, microsomal membranes have been analysed for their lipid profiles; mainly because the enzymes that are of most interest in the present investigation are bound to these membranes. Rats were fed the cholesterol-supplemented diets (explained in section 3.2.1.) for a period of 28 days. Microsomes from the livers of these treated rats were isolated and analyzed for lipid composition,

including fatty acid profiles of different lipid fractions as previously explained (sections 3.2.3. - 3.2.5.).

4.1. Fatty acid composition of lipid-supplemented diets

The fatty acid composition of various lipid-supplemented diets is given in table 2. The sunflower seed oil (SO) diet contained 57.6% linoleic acid (18:2) whereas coconut oil (CO) diet contained only 5.6%. On the other hand, the CO diet was greatly enriched in saturated fatty acids (86.8%) versus 17.9% in the SO diet (Table 2). P/S ratios, therefore, of both SO (3.274) and CO (0.065) diets departed greatly from that of the reference (1.014) diet. The addition of 2% cholesterol into the reference (REF) or SO or CO diet had no effect on the relative percentage of fatty acids in these diets.

4.2. Effect of dietary lipid treatments on general condition, body weights and liver weights

After the 28 days experimental period, all of the animals appeared healthy. The rats on coconut oil (CO) diet did not show any signs of essential fatty acid deficiency. There was no significant difference in the mean relative weight gain of the rats on different diets (Table 3). The animals fed 15% sunflower seed oil plus 2% cholesterol (SO + CH) diet, however tended to gain weight faster than all other groups. All the dietary groups studied had similar liver weights except for the group fed (SO + CH) diet, which had significantly higher liver weights when compared to the respective low cholesterol (SO) diet

Table 2. Fatty acid composition of lipid-supplemented diets

Fatty acid[1]	Reference diet[2]	Sunflower seed oil diet[2]	Coconut Oil diet[2]
	% (w/w)		
8:0	-	-	7.1
10:0	-	-	6.1
12:0	-	-	42.9
14:0	1.9	T[3]	16.8
16:0	25.3	11.8	11.4
16.1(ω7)	1.6	T	T
18:0	9.2	6.1	3.5
18:1(ω9)	22.9	24.5	7.6
18:2(ω6)	35.6	57.6	5.6
18:3(ω3)	1.3	T	T
20:1(ω9)	2.2	T	T
Saturated	36.4	17.9	86.8
Monounsaturated	26.7	24.5	7.6
Polyunsaturated	36.9	57.6	5.6
P/S ratio	1.014	3.274	0.065
U.I.	101.8	139.7	18.8

1. Fatty acids are designated as the number of carbon atoms followed by the number of double bonds together with the designated fatty acid series for particular unsaturated fatty acids.

2. Addition of 2% (w/w) cholesterol to these diets had no effect on their fatty acid compositions.

3. T represents trace amounts (less than 0.1%).

group (Table 3).

4.3. Microsomal lipid composition after dietary lipid treatments

The alterations in the lipid composition of rat liver microsomes, produced by dietary fatty acids and/or cholesterol, are of considerable interest. Table 4 contains the data on cholesterol content of liver microsomes from rats fed the various experimental diets. 15% sunflower seed oil (SO) diet elevated the cholesterol content of liver microsomes, whereas 15% coconut oil (CO) diet had no significant effect when compared with a low fat, reference (REF) diet. This increase was confined mainly to the ester fraction which was found to be 2.5 fold higher than in microsomes from animals fed CO or REF diet (Table 4). As expected, all three high-cholesterol diets also raised the cholesterol content of liver microsomes. This increase in cholesterol content followed the order : sunflower seed oil plus cholesterol (SO + CH) > coconut oil plus cholesterol (CO + CH) > reference plus cholesterol (REF +CH) diets (Table 4). Although both the free and the esterified cholesterol contents were elevated, the increase was greater in the ester fraction. Feeding cholesterol with sunflower seed oil (SO + CH) resulted in the greatest accumulation (6 times more than SO diet) of cholesterol esters followed by cholesterol plus coconut oil (5 times more than CO diet) and reference plus cholesterol (2 times more than REF diet) diets (Table 4).

All experimental animals, except for those on the SO diet, had similar values for microsomal total phospholipid content (Table 5). The group

Table 3. Effect of dietary fatty acids and/or cholesterol on body and liver weights

	Cholesterol (2%,w/w)	Reference diet	Sunflower seed oil diet	Coconut oil diet
Body wt. (g)	-	315 ± 9	314 ± 8	323 ± 7
	+	319 ± 23	331 ± 24	318 ± 22
Liver wt. (g)	-	12.6 ± 0.4	12.4 ± 0.4	13.5 ± 0.3
	+	13.4 ± 0.9	14.9 ± 1.1**	13.5 ± 1.0

Data are presented as mean ± S.D. of n = 4 - 5 rats from each dietary group.

**: Significantly different from the corresponding low cholesterol diet-fed animals, $P < 0.01$.

Table 4. Effect of dietary fatty acids and/or cholesterol on cholesterol content of rat liver microsomes

Diet	Cholesterol (2%, w/w)	Microsomal cholesterol		
		Total	Free	Esterified
		(μg/mg microsomal protein)		
Reference diet	-	27.8 ± 1.1	23.6 ± 1.0	4.3 ± 0.2
	+	39.2 ± 1.3***a	29.3 ± 1.5***a	9.9 ± 0.7***a
Sunflower seed oil diet	-	36.0 ± 1.4***b	25.2 ± 2.0	10.8 ± 1.0***b
	+	97.2 ± 1.7***a	33.2 ± 3.0***a	64.0 ± 3.0***a
Coconut oil diet	-	26.1 ± 1.1	21.9 ± 1.9	4.2 ± 0.3
	+	55.6 ± 1.2***a	34.7 ± 1.1***a	20.9 ± 1.0***a

Data are presented as mean ± S.D. of liver microsomal preparations (n = 4 - 5) from each dietary group.

***a : Significantly different from the corresponding low cholesterol diet-fed animals, $P < 0.001$.

***b : Significantly different from the reference diet-fed animals, $P < 0.001$.

on SO diet had significantly less phospholipids when compared to that on REF diet. This loss, however was eliminated when cholesterol was also included in the diet (Table 5). There were no appreciable differences in the relative percentages of the major phospholipid fractions in the various microsomal membranes. In all cases, phosphatidylcholine (PC) accounted for more than 60% of total phospholipids, with phosphatidylethanolamine (PE) being the second major phospholipid (about 25%) in these membrane preparations (Table 5).

4.4. Fatty acid profiles of liver microsomal lipids from rats fed high lipid diets

Fatty acid profiles of total lipids from liver microsomes of rats fed various diets is given in Table 6. Medium chain fatty acid (8:0 - 14:0) from coconut oil (CO) diet was not incorporated into the microsomal lipids despite a large (72%) dietary load. Sunflower seed oil (SO) diet feeding lowered the 16:0, 20:4 and 22:6 fatty acid content, with a concomitant rise in 18:2 fatty acid, while on the other hand coconut oil (CO) diet lowered the 18:2 and 20:4 content with an accompanying increase in 22:6 content (Table 6). Therefore, sunflower seed oil feeding increased, whereas coconut oil feeding decreased, the ω6/ω3 fatty acid ratios in the microsomal membranes as compared to that in REF diet-fed animals (Table 6). The inclusion of 2% cholesterol into the otherwise low cholesterol diets resulted in several distinct alterations in the fatty acid composition of the liver microsomes. Exogenous cholesterol led to the accumulation of 18:1, with an accompanying depletion of 18:0, in the microsomal

Table 5. Effect of dietary fatty acids and/or cholesterol on phospholipid content of rat liver microsomes[1]

Phospholipid Fraction	REF	(REF + CH)	SO	(SO + CH)	CO	(CO + CH)
Total (µg/mg protein)	512 ± 7	491 ± 12	425 ± 10***	499 ± 11	520 ± 5	528 ± 12
	(% of total phospholipid)					
Phosphatidylcholine	64.3 ± 3.6	66.3 ± 2.9	63.2 ± 4.4	62.5 ± 2.1	67.8 ± 5.1	63.4 ± 3.3
Phosphatidylethanolamine	26.1 ± 1.4	24.1 ± 1.6	25.5 ± 0.9	27.0 ± 1.9	22.1 ± 1.8	25.0 ± 1.1
Phosphatidylserine + Phosphatidylinositol	7.6 ± 1.1	6.9 ± 0.6	7.9 ± 0.7	8.1 ± 1.3	6.7 ± 1.3	7.9 ± 0.9
Sphingomyelin	2.0 ± 0.3	2.7 ± 0.5	3.4 ± 1.1	2.4 ± 0.1	3.4 ± 0.4	3.7 ± 1.0

Data are presented as mean ± S.D. for liver microsomal preparations (n = 4 - 5) from each dietary group.

1. REF, Reference diet; (REF + CH), Reference diet plus 2% cholesterol diet; SO, 15% Sunflower seed oil diet; (SO + CH), 15% Sunflower seed oil plus 2% cholesterol diet; CO, 15% coconut oil diet; (CO + CH), 15% Coconut oil plus
2% cholesterol diet.

*** : Significantly different from the reference diet-fed animals, P <0.001.

membranes irrespective of the type and amount of fat-fed (Table 6). Amongst the polyunsaturated fatty acids (PUFA), the arachidonic (20:4) acid content of these membranes was lowered, with a simultaneous increase of linoleic (18:2) acid. In addition, dietary cholesterol reduced the proportion of docosahexaenoic (22:6) acid in the liver microsomes, leading to an increased ω6/ω3 fatty acid ratios (Table 6). The unsaturation index of microsomal membrane was also decreased following the feeding of 2% cholesterol in the diet. Similar changes in fatty acid composition were observed when cholesterol was fed either without any added fat or with saturated or unsaturated fat (Table 6).

Microsomal total lipids were further fractionated into neutral lipids and phospholipds and their fatty acid profiles were compared. Table 7 shows that some of the medium chain fatty acids (12:0 and 14:0) which were detected only in trace amounts in the total lipids, constitute a considerable proportion of neutral lipids. On the other hand, in the phospholipid fraction, these acids are completely absent (Table 8). In the neutral lipids, dietary cholesterol reduced the percentage of saturated (16:0 and/or 18:0) and 20:4 with an increase of monounsaturated (16:1 and/or 18:1) and in some cases of diunsaturated (18:2) fatty acids (Table 7). The unsaturation index (U.I.) of neutral lipids was increased by the SO diet and decreased by the CO diet, compared with that of REF diet, but was not influenced by dietary cholesterol (Table 7). The fatty acid composition of phospholipids was similar to that of the microsomal total lipids, exhibiting a decrease in 18:0, 20:4 and 22:6 with an accompanying increase in 18:1 and 18:2. By contrast with the total neutral lipids the unsaturation index (U.I.) of the

Table 6. Effect of dietary cholesterol on fatty acid composition of liver microsomal total lipids

Fatty acid	REF	(REF + CH)	SO	(SO + CH)	(CO)	(CO + CH)
			% (w/w)			
14:0	-	-	-	-	1.5 ± 0.0	3.2 ± 0.0***
16:0	23.7 ± 0.4	23.8 ± 0.3	15.4 ± 0.2	14.4 ± 0.2	21.4 ± 0.3	21.5 ± 0.4
16:1 (ω7)	-	-	-	-	-	2.1 ± 0.2
18:0	19.3 ± 0.1	15.1 ± 0.2**	18.6 ± 0.7	15.5 ± 0.1**	22.3 ± 0.2	15.6 ± 0.3***
18:1 (ω9)	10.6 ± 0.1	15.2 ± 0.5**	11.1 ± 0.0	16.2 ± 0.3***	10.8 ± 0.1	17.8 ± 1.0***
18:2 (ω6)	18.6 ± 0.1	23.6 ± 0.3***	32.4 ± 0.6	39.6 ± 0.7**	17.8 ± 0.3	22.4 ± 0.5**
20:4 (ω6)	23.0 ± 0.8	18.9 ± 0.2**	18.9 ± 0.1	14.0 ± 0.2***	19.5 ± 0.2	13.4 ± 0.1***
22:6 (ω3)	4.8 ± 0.1	3.5 ± 0.2**	3.4 ± 0.2	T	6.6 ± 0.0	3.9 ± 0.1***
Saturated	43.0	38.9	34.0	29.9	45.2	40.3
Monounsaturated	10.6	15.2	11.1	16.2	10.8	19.9
Polyunsaturated	46.4	45.9	54.9	53.6	43.9	39.8
ω6/ω3	8.67	12.14	15.09	-	5.65	9.18
U.I.	168.6	159.0	171.9	151.6	164.0	141.7

Data are presented as the mean ± S.D. for liver microsomal preparations (n = 4 - 5) from each dietary group.

Values with a superscript are significantly different from their respective low cholesterol diet-fed animals : * , $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 7. Effect of dietary cholesterol on fatty acid composition of liver microsomal neutral lipids

Fatty acid	REF	(REF + CH)	SO	(SO + CH)	CO	(CO + CH)
			% (w/w)			
12:0	-	-	-	-	2.1 ± 0.0	3.1 ± 0.0***
14:0	-	-	-	-	5.1 ± 0.0	7.1 ± 0.1***
16:0	30.9 ± 0.6	28.0 ± 0.5	18.8 ± 0.5	13.7 ± 0.0***	30.2 ± 1.3	26.1 ± 0.9
16:1 (ω7)	3.7 ± 0.0	6.1 ± 0.1***	-	T	2.4 ± 0.0	4.9 ± 0.0***
18:0	10.1 ± 0.1	5.3 ± 0.1***	4.8 ± 0.0	3.8 ± 0.0***	10.7 ± 0.7	5.5 ± 0.2***
18:1 (ω9)	23.6 ± 0.2	28.9 ± 0.3**	20.5 ± 0.3	27.0 ± 1.0**	25.0 ± 0.6	31.4 ± 0.5**
18:2 (ω6)	22.6 ± 0.3	26.4 ± 0.4*	50.8 ± 2.1	52.1 ± 1.9	19.2 ± 0.1	21.8 ± 0.3*
20:4 (ω6)	9.0 ± 0.1	5.0 ± 0.0***	4.8 ± 0.0	3.3 ± 0.1***	7.8 ± 0.1	4.2 ± 0.0***
Saturated	41.0	33.3	23.6	17.5	48.3	41.8
Monounsaturated	27.3	35.0	20.5	27.0	27.4	36.3
Polyunsaturated	31.6	31.4	55.6	55.5	27.0	26.0
U.I.	108.5	107.8	141.3	144.4	97.0	96.7

Data are presented as the mean ± S.D. microsomal preparations (N = 4 - 5) from each dietary group.

Values with a superscript are significantly different from their respective low cholesterol diet-fed animals : *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 8. Effect of dietary cholesterol on fatty acid composition of liver microsomal phospholipids

Fatty acid	REF	(REF + CH)	SO	(SO + CH)	CO	(CO + CH)
			% (w/w)			
16:0	22.4 ± 0.3	23.8 ± 0.4	14.7 ± 0.2	15.4 ± 0.3	19.8 ± 0.2	19.4 ± 0.2
18:0	21.7 ± 0.2	18.1 ± 0.0**	28.6 ± 0.3	24.7 ± 0.1**	27.0 ± 0.4	23.4 ± 0.3*
18:1 (ω9)	7.9 ± 0.0	11.2 ± 0.1***	5.8 ± 0.1	8.5 ± 0.0***	6.6 ± 0.0	11.6 ± 0.0***
18:2 (ω6)	18.1 ± 0.1	22.5 ± 0.2***	21.6 ± 0.2	28.5 ± 0.2***	18.1 ± 0.1	23.0 ± 0.3**
20:4 (ω6)	24.9 ± 0.3	21.3 ± 0.5*	25.4 ± 0.3	20.5 ± 0.4**	21.8 ± 0.3	19.6 ± 0.1*
22.6 (ω3)	4.8 ± 0.1	3.6 ± 0.3**	3.7 ± 0.0	2.5 ± 0.1***	6.6 ± 0.1	4.8 ± 0.2**
Saturated	44.1	41.9	43.3	40.1	46.8	42.8
Monounsaturated	7.9	11.2	5.8	8.5	6.6	9.6
Polyunsaturated	47.8	47.2	50.7	51.5	46.5	47.4
ω6/ω3	8.96	12.17	12.70	19.60	6.04	8.89
U.I.	176.3	163.0	172.8	162.5	169.6	162.8

Data are presentd as the mean ± S.D. for liver microsomal preparations (n = 4 - 5) from each dietary group.

Values with a superscript are significantly different from their respective low cholesterol diet-fed animals : *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

phospholipids was decreased by cholesterol feeding and was essentially unaltered by the type and amount of fat-fed (Table 8).

Neutral lipids were in turn further fractionated into triacylglycerols and cholesterol esters and similarly total phospholipids were fractionated into phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine plus phosphatidylinositol (PS +PI) fractions. The fatty acid composition of these individual fractions was determined and compared. Tables 9 and 10 show that the changes observed in the individual fractions followed the same pattern as observed in the total neutral lipids or total phospholipids. Quantitatively, however, the largest differences in the fatty acid composition were associated with the cholesterol ester fraction (Table 9).

4.5. Discussion

The results presented in this chapter demonstrated that dietary cholesterol produces remarkable alterations not only in the cholesterol content but also in the fatty acid composition of microsomal membranes of rat liver. The results also clearly show that the quality of the fat consumed by the animals has a pronounced effect on the enrichment of microsomes with cholesterol. The accumulation of cholesterol in the liver microsomes following unsaturated fat-feeding may be a consequence of higher influx of intestinal cholesterol into the hepatocytes. Absorbed cholesterol from the intestine is transported predominantly in the ester form (Dietschy and Wilson, 1970a,b) and this esterification is catalyzed by the enzyme

Table 9. Effect of dietary cholesterol on fatty acid composition of liver microsomal neutral lipid fractions

Fraction	Fatty acid	REF	(REF + CH)	SO	(SO + CH)	CO	(CO + CH)
Triacylglycerols	<16:0	-	-	-	-	10.5 ± 0.3	13.5 ± 0.1**
	16:0	44.5 ± 0.7	34.5 ± 0.8***	22.2 ± 0.5	19.0 ± 0.2*	37.1 ± 0.3	28.2 ± 0.3***
	16:1 (ω7)	5.3 ± 0.1	7.4 ± 0.4**	-	-	3.0 ± 0.0	3.9 ± 0.1**
	18:0	4.2 ± 0.0	2.4 ± 0.2**	2.1 ± 0.1	2.3 ± 0.2	3.7 ± 0.0	2.5 ± 0.0***
	18:1 (ω9)	30.6 ± 0.5	33.5 ± 0.2*	23.2 ± 0.3	27.0 ± 0.3**	30.4 ± 0.5	33.9 ± 0.6*
	18:2 (ω6)	15.3 ± 0.1	22.4 ± 0.4***	48.7 ± 0.8	51.6 ± 1.1	15.3 ± 0.2	17.6 ± 0.1*
	U.I.	66.5	89.3	120.6	130.2	64.0	73.0
Cholesterol Esters	16:0	54.0 ± 2.3	44.0 ± 1.8*	25.5 ± 1.1	18.0 ± 0.6***	50.5 ± 2.8	45.8 ± 1.7*
	16:1 (ω7)	1.1 ± 0.0	3.3 ± 0.1***	1.2 ± 0.4	2.4 ± 0.0*	2.1 ± 0.0	4.5 ± 0.1***
	18:0	19.1 ± 1.4	10.3 ± 0.3***	16.3 ± 0.3	5.9 ± 0.2***	21.4 ± 1.2	13.8 ± 0.7***
	18:1 (ω9)	10.9 ± 1.0	21.1 ± 1.3***	20.2 ± 0.1	35.6 ± 1.9***	12.8 ± 0.6	22.5 ± 1.0***
	18:2 (ω6)	8.8 ± 1.0	17.9 ± 0.6***	18.8 ± 0.8	36.3 ± 1.9***	7.6 ± 0.3	13.4 ± 1.0***
	20:4 (ω6)	6.1 ± 0.2	3.4 ± 0.1***	8.0 ± 0.7	1.8 ± 0.2***	5.6 ± 0.1	2.0 ± 0.3***
	U.I.	54.0	72.8	91.0	117.8	52.5	61.8

Values with a superscript are significantly different from their corresponding low cholesterol diet-fed animals : *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 10. Effect of dietary cholesterol on fatty acid composition of liver microsomal phospholipid fractions.

Fraction[1]	Fatty acid	REF	(REF + CH)	SO	(SO + CH)	CO	(CO + CH)
PC	16:0	32.6 ± 1.2	29.8 ± 1.7	20.1 ± 0.2	23.0 ± 1.1	26.5 ± 0.8	27.9 ± 1.2
	18:0	30.1 ± 0.5	22.8 ± 1.3***	35.4 ± 0.6	32.7 ± 0.3*	36.3 ± 0.3	29.7 ± 0.3***
	18:1 (ω9)	8.9 ± 0.2	12.6 ± 0.1***	6.1 ± 0.1	8.6 ± 0.1***	7.3 ± 0.1	11.0 ± 0.0***
	18:2 (ω6)	15.7 ± 0.2	20.4 ± 0.1***	21.6 ± 0.2	24.9 ± 0.4**	16.0 ± 0.4	22.2 ± 0.3***
	20:4 (ω6)	12.5 ± 0.5	12.0 ± 0.2	16.7 ± 0.3	10.7 ± 0.1***	11.2 ± 0.1	9.2 ± 0.0***
PE	16:0	35.3 ± 1.2	34.8 ± 0.4	23.7 ± 0.3	21.6 ± 0.5*	34.3 ± 0.5	33.8 ± 0.3
	18:0	34.0 ± 1.3	26.7 ± 0.3***	42.1 ± 1.2	39.3 ± 1.7	37.5 ± 0.8	34.2 ± 0.2*
	18:1 (ω9)	7.3 ± 0.2	12.8 ± 0.2***	8.2 ± 0.0	12.2 ± 0.2***	6.1 ± 0.0	10.2 ± 0.3***
	18:2 (ω6)	8.8 ± 0.1	12.6 ± 0.2***	13.0 ± 0.1	16.4 ± 0.1***	9.6 ± 0.2	12.1 ± 0.1***
	20:4 (ω6)	13.0 ± 0.1	12.2 ± 0.2*	13.0 ± 0.1	10.7 ± 0.1***	11.6 ± 0.2	9.7 ± 0.0**
PS + PI	16:0	4.6 ± 0.6	4.4 ± 0.8	8.9 ± 1.0	8.1 ± 0.6	8.8 ± 0.2	7.9 ± 0.3
	18:0	70.0 ± 2.3	71.1 ± 2.1	61.9 ± 1.8	62.3 ± 2.3	66.4 ± 1.2	66.5 ± 1.9
	18:1 (ω9)	9.8 ± 0.2	13.0 ± 0.5*	7.0 ± 0.2	10.8 ± 0.3**	10.7 ± 0.2	14.4 ± 0.4**
	18:2 (ω6)	0.8 ± 0.2	1.4 ± 0.4	2.9 ± 0.1	4.6 ± 0.1**	T	1.9 ± 0.1***
	20:4 (ω6)	14.8 ± 0.8	10.1 ± 0.2***	19.3 ± 0.3	14.2 ± 0.2***	14.1 ± 0.5	9.3 ± 0.7***

1. PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PS + PI, Phosphatidylserine + Phosphatidylinositol.

Values with a superscript are significantly different from their corresponding low cholesterol diet-fed animals : *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

acylcoenzyme A cholesterol acyltransferase (ACAT) in the gut wall (Haugen and Norum, 1976). Unsaturated fat-feeding has been shown previously to increase ACAT activity in the intestinal microsomes (Field and Salome, 1982). Thus, the conditions for cholesterol transport are more favourable when unsaturated fat rather than saturated fat is fed, which would likely result in an increased concentration of cholesterol in the liver. Alternatively, the higher concentration of cholesterol in the microsomes as a result of unsaturated fat-feeding may be due to an increased ability of the microsomal membranes to accommodate cholesterol, because of the altered fatty acid composition of their phospholipids (Reiber, 1978).

The reason for the significant decrease in total phospholipid content of liver microsomes fed unsaturated fat diet is not clear. However, the relative percentage of none of the major phospholipid fractions is altered. Further, dietary cholesterol has no appreciable effect on the phospholipid composition of these membranes. Contradictory reports are available in the literature on the effect of dietary cholesterol on membrane phospholipid content. Cooper et al (1980) have shown that dietary cholesterol increases the phospholipid content of plasma lipoprotein and red cell membrane lipids. More recently microsomal membrane phospholipids have been reported to be lowered by the administration of cholesterol in the chick (Ramirez et al, 1984).

The medium chain fatty acids (8:0 - 14:0) from the saturated fat diet are not incorporated into microsomal phosphoolipids, but are detected in considerable quantities in neutral lipids. The chain elongation mechanism of liver microsomes appears to be effective enough to

prevent medium chain fatty acid build-up inspite of a large dietary load. These results are consistent with the observations of Mayorek and Jacobs (1983) who demonstrated that only fatty acids longer than myristic (14:0) acid can be incorporated into phospholipids of cultured rat hepatocytes. The medium chain fatty acids (8:0 - 12:0) are specific substrates for diacylglycerol transferase, which leads to the formation of triacylglycerols, a major fraction of neutral lipids (Mayorek and Jacobs, 1983).

Excess dietary cholesterol increases the percentage of 16:1 and/or 18:1 with an accompanying depletion of 16:0 and/or18:0 in the lipids of liver microsomal membranes. These results are consistent with an enchancement of Δ^9-desaturase activity in the rat liver microsomes. On the other hand, a decrease in 20:4 content with concomitant increase in 18:2 suggests an inhibition of Δ^5- and/or Δ^6-desaturase activity in the liver microsomes of cholesterol-treated animals. Similar alterations of fatty acid composition in response to a diet containing an elevated cholesterol content have been reported in the lipids of dog platelets (Pitas et al, 1979). In contrast to these results, other workers (Bochenek and Rodgers, 1978; Morin et al, 1962), with respect to liver lipids, have shown that dietary cholesterol produces a decrease in the percentages of both 18:2 and 20:4, thus suggesting that this decrease is due to an increased utilization of 20:4 for more cholesterol ester and phospholipid synthesis and their secretion as very low density lipoprotein from the liver into the plasma. In the present exercise, however, the decrease in 20:4 is followed by an increase in 18:2, suggesting that 18:2 was readily available for the synthesis of 20:4 to overcome any increased

demand of utilization. Therefore, accumulation of 18:2 in the liver microsomes is suggestive evidence for a possible block in the synthesis of 20:4 from 18:2.

Unsaturated fat diet lowered and saturated fat elevated the docosohexaenoic (22:6, ω3) acid level of liver microsomes when compared with that of a low fat, reference diet. The 22:6 (ω3) was not present in any of the diets, and thus, it must have been derived from the desaturation and elongation of 18:3 (ω3), which was present however only in trace amounts in the high fat diets. Increased level of 22:6 (ω3) following saturated fat feeding suggests that 18:3 (ω3) is a preferred substrate for Δ^6- desaturation when 18:2 (ω6) is available in relatively low amounts. These results are consistent with the observations of Brenner (1974) who demonstrated that there is a competition between 18:2 (ω6) and 18:3 (ω3) for Δ^6- desaturation and 18:3(ω3) has a higher affinity for Δ^6-desaturase than 18:2 (ω6). Irrespective of the quality and quantity of fat fed, excess dietary cholesterol reduces the proportion of 22:6 (ω3) in liver microsomes. These results suggest that dietary cholesterol inhibits the desaturation of both ω6 (18:2) and ω3 (18:3) fatty acids in the liver microsomal membranes.

The changes in fatty acid composition mentioned above are more or less distributed in all the major lipid fractions, however the greatest differences are associated with the cholesterol ester fraction. Regardless of the lipid fraction studied, the results demonstrate that the unsaturation index (U.I.) of phospholipids is independent whereas that of neutral lipids is highly dependent on the dietary fatty acid

intake. On the other hand dietary cholesterol has an opposite effect i.e. the U.I. of phospholipids is entirely dependent on, whereas that of neutral lipids is essentially unaltered by the administration of cholesterol in the diet. These results suggest that under the conditions of dietary fatty acid treatment, neutral lipids act as a buffer for the maintenance of membrane phospholipid saturation/unsaturation in order to preserve a constancy of membrane lipid fluidity. When the animals are treated with high cholesterol diets, the cholesterol content of the membranes is increased, which is expected to change the fluidity of these membranes. In order to restore the native fluidity, the phospholipid saturation/unsaturation has to be altered. These results are in line with the proposed concept of lipid homeostasis (Sabine, 1983) discussed earlier in section 2.10. The fatty acid composition of microsomal membranes is altered in order to minimize the changes in membrane fluidity caused by dietary cholesterol.

The microsomal lipids that have been studied in the present chapter, constitute the micro-environment of several microsomal-bound enzymes. The relationship of the changes in these lipids with some of the membrane-bound enzymes is presented in the following chapters.

CHAPTER 5. THERMOTROPIC BEHAVIOUR OF LIVER MICROSOMAL MEMBRANE LIPIDS FOLLOWING DIETARY LIPID MANIPULATIONS

A number of physical techniques have shown that pure phospholipids undergo reversible thermotropic phase transitions involving changes in the molecular ordering of the fatty acyl chains (Chapman et al, 1967). For mixtures of membrane phopholipids the transition temperature, while basically a characteristic of the phospholipid acyl chains, is also dependent on their polar headgroups, cholesterol content, amount of water and divalent cations. For biological membranes, the phase transition of the lipids has been shown to be influenced by membrane proteins in a manner, which depends on whether the interaction is primarily with the hydrophobic region of the bilayer or in the vicinity of the polar headgroups (Oldfied and Chapman, 1972; Quinn, 1981; Chapman, 1975; Melchior and Steim, 1976). The presence of cholesterol at sufficient relative concentration has the effect of removing the phase transition (Ladbrooke et al, 1968a). Those biomembranes which contain large amounts of cholesterol e.g. myelin membranes, as well as their total lipid extracts, do not show any phase transition (Ladbrooke et al, 1968b). Other biomembranes, such as mitochondria and microsomes which contain little cholesterol, however, show transitions similar to those observed with simple lipid systems (Steim et al, 1969; Blazyk and Steim, 1972).

Phase transitions of aqueous lameller dispersions of phospholipids are readily detectable by differential scanning calorimetry (Chapman et al, 1974; Bach and Chapman, 1980). Such transitions involve a highly co-operative reversible change in the fatty acids between a fluidus,

liquid-crystalline phase and a solidus, crystalline or cogel phase (Chapman et al, 1967; Quinn, 1981). Thermal techniques have been applied to detect phase transitions associated with membranes and membrane lipids from a wide variety of organisms (Steim et al, 1969; Ashe and Steim, 1971; Baldassare et al, 1976). Using differential scanning calorimetry (DSC), Blazyk and Steim (1972) published a preliminary report on microsomal membranes showing only one lipid phase transition centred around 0°C. With more sensitive DSC instrumentation, however Bach et al (1976) were able to show that the microsomal membranes have a second phase transition also, in the region between 18° - 40°C. Recent reports have confirmed that lipids of mammalian membranes, including those of microsomal membranes, do indeed undergo phase transitions at temperatures well above 0°C, although these transitions are of relatively low enthalpy (Bach et al, 1977; Bach et al, 1978; Livingstone and Schachter, 1980). The phase transitions occuring between 18° - 40°C may be responsible in part for the discontinuities that have been reported for the Arrhenius plots of numerous membrane-associated enzyme systems, generally at temperatures near 25°C (Brasitus and Schachter, 1980; Sipat and Sabine, 1981).

The enzymes of lipid metabolism (e.g. HMG-CoA reductase, cholesterol 7α-hydroxylase, ACAT and desaturases) that are of interest in the present investigation are bound to the membranes of endoplasmic reticulum. It is quite clear from Chapter 4 that the cholesterol content and fatty acid profiles of liver microsomes can be extensively modified by dietary lipid treatment. These changes in lipid composition of microsomal membranes might be expected to alter the physical properties of the membrane lipids. The objective of the

present exercise was to elucidate the nature of alterations in the physical properties of microsomal membrane lipids produced by dietary fatty acids and/or cholesterol. For this purpose, the lipids, isolated from liver microsomes of rats fed various high lipid diets (explained in section 3.8.1) were subjected to DSC.

5.1. Differential Scanning Calorimetry of microsomal lipids

DSC was performed on aqueous buffer dispersions of lipids isolated from rat liver microsomes. Lipid extracts from liver microsomes of rats fed the experimental diets were concentrated by evaporating the solvent under a nitrogen stream at 40°C and then approximately 40 mg of isolated lipid was added to each 75-µl stainless-steel pan (Perkin-Elmer, Norwalk, CT, USA). These lipid samples also contained about 40-50 µg of antioxidant (BHT). Cooling and heating scans of aqueous buffer dispersions of dimyristylphosphatidylcholine (DMPC) or dipalimitoylphosphatidylcholine (DPPC) in the presence of BHT (at a concentration of 0.1% of dry weight of the phospholipid) have been previously shown to have no effect on the transition temperature or enthalpy of these phospholipids (McMurchie et al, 1983a). The solvent from the pans containing the lipids was evaporated by vacuum desiccation for at least 24 hours and samples were then hydrated by adding approximately 50 µl (i.e. over 100% hydration) of liposome buffer comprising 50 mM Tris / 2 mM EDTA / 15% (v/v) ethylene glycol (pH 7.2). Ethylene glycol at this concentration has no considerable effect on the phase transition behaviour of aqueous dispersions of distearylphosphatidylcholine (DSPC), when examined by DSC (Mabrey and Sturtevant, 1976). Pans were hermetically sealed using a Perkin-Elmer

sealing press and left to equilibrate (without sonication) at 4°C, at least overnight.

Thermal scans were performed using a Perkin-Elmer Differential Scanning Calorimeter (Model DSC-2B) and analyzed using a dedicated Perkin-Elmer Thermal Analysis Data Station (TADS). The instrument was first calibrated with respect to both temperature and enthalpy measurements using water, indium (Perkin-Elmer) and aqueous dispersions of DMPC and DPPC. Scans were made at a rate of 5°C per min. against an empty sealed reference pan. All the samples were scanned twice in the cooling mode and at least once in the heating mode, which was initiated above the ice-water phase transition for the observation of the thermal behaviour of microsomal membrane lipids. The phase transition of the lipid dispersions has been defined as that temperature at which a significant departure from the baseline is first evident in the cooling mode or when the scan reunites with the baseline in the heating mode.

5.2. Influence of dietary fatty acids and/or cholesterol on the thermal behaviour of rat liver microsomal lipids

Representative DSC thermograms obtained in the cooling mode for aqueous buffer dispersions of liver microsomal membrane lipid from rats fed various lipid-supplemented diets are shown in Figure 9. For each of the samples, two exothermic transitions were detected, the first designated T_1 occuring between -10 to 5°C and the second designated T_2, occuring between 21 to 25°C. In some of the samples, a third, very small transition designated T_3, was also observed at a

FIGURE 9: Representative differential scanning calorimetric scans of aqueous buffer (pH 7.2) dispersions of liver microsomal membrane lipids isolated from rats fed either a reference (REF) diet, sunflower seed oil (SO) diet or coconut oil (CO) diet supplemented with or without cholesterol (CH). Scans were made in the cooling mode at a rate of 5 C deg/min. Arrows indicate the temperature at which a significant departure from the baseline was observed.

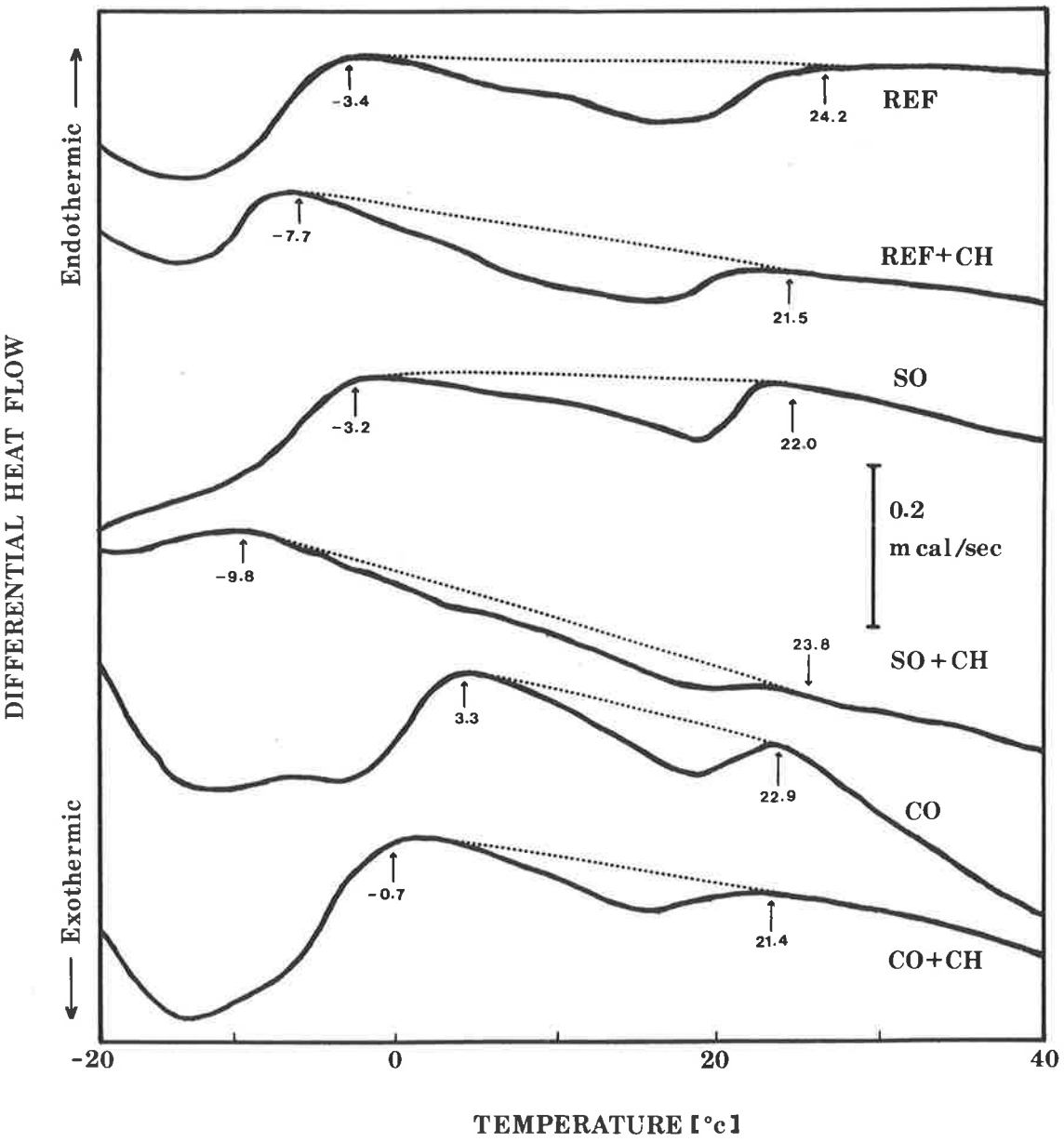
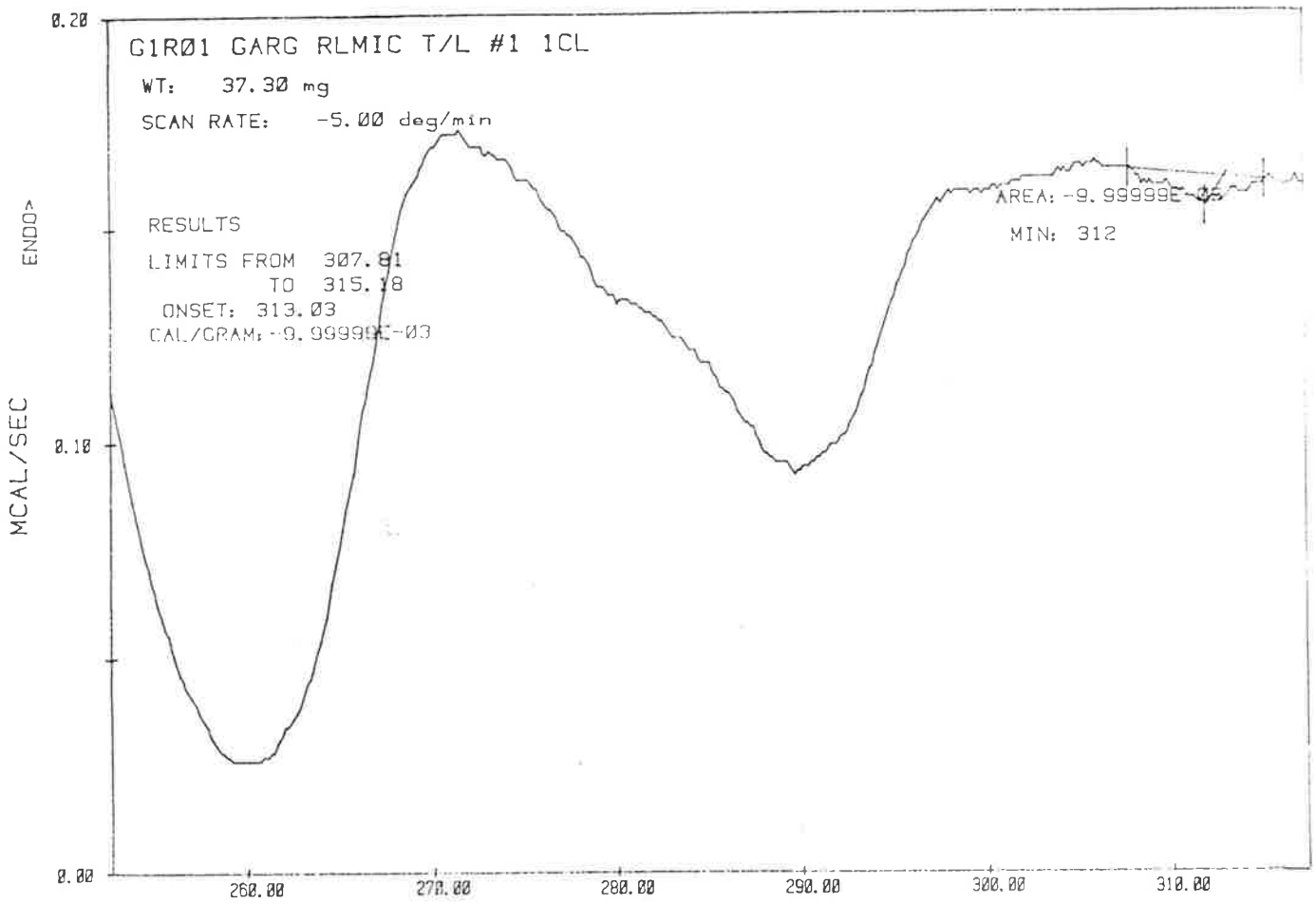

DIFFERENTIAL HEAT FLOW
Endothermic
Exothermic
-3.4
24.2
REF
-7.7
REF+CH
21.5
SO
-3.2
22.0
0.2
m cal/sec
-9.8
23.8
SO+CH
3.3
22.9
CO
-0.7
21.4
CO+CH
-20
0
20
40
TEMPERATURE [°c]

FIGURE 10: Differential scanning calorimetric scan of aqueous buffer (pH 7.2) dispersions of liver microsomal membrane lipids isolated from rats fed a reference (REF) diet - scan blown up to show the occurence of a third phase transition (designated T_3).


G1RØ1 GARG RLMIC T/L #1 1CL
WT: 37.3Ø mg
SCAN RATE: -5.ØØ deg/min
RESULTS
LIMITS FROM 3Ø7.81
TO 315.18
ONSET: 313.Ø3
CAL/GRAM: -9.9999E-Ø3
AREA: -9.99999E-Ø5
MIN: 312
ENDO>
MCAL/SEC
Ø.2Ø
Ø.1Ø
Ø.ØØ
26Ø.ØØ
27Ø.ØØ
28Ø.ØØ
29Ø.ØØ
3ØØ.ØØ
31Ø.ØØ

temperature near 37°C (Figure 10). For all samples, the phase transitions T_1 and T_2 were repeatedly observed in both cooling and heating scans but the values of the respective transition temperatures were slightly different due presumably to the change in scanning mode (McMurchie et al, 1983a).

Table 11 describes the effect of dietary fatty acids and/or cholesterol on the phase transition temperatures designated T_1, T_2 and T_3. The various lipid-supplemented diets had their major effect on the lipid phase transition designated T_1. This transition occured at 3.3°C in the coconut oil (CO) diet group whereas in the reference (REF) and sunflower seed oil (SO) diet groups, it was detected at -3.4 and -3.2°C respectively (Table 11). Inclusion of 2% cholesterol into the REF diet resulted in a 4.3 centigrade degree lowering of the temperature of the phase transition T_1. Similar results were obtained when cholesterol (CH) was fed with CO or SO lipid supplements, however, the magnitude of the decrease was greater in the (SO + CH) group (6.6 centigrade degree) than in the (CO + CH) diet group (4.0 centigrade degree). The phase transition T_2, was not greatly affected by any of the experimental diets (Table 11). The phase transition T_3, when detected was observed in the first cooling scan and was absent in subsequent scans.

Enthalpy values of the phase transitions designated T_1 and T_2 for all the experimental groups are given in Table 12. The enthalpy values for transition T_1 (ΔH_1), where measurable, were far greater than those of transition T_2 (ΔH_2), irrespective of the nature of dietary supplement. In the REF, CO and SO dietary groups, the addition of 2%

Table 11. Transition temperatures for liver microsomal lipids from rats fed various lipid-supplemented diets.

Exothermic Phase Transition	2% Cholesterol	Transition Temperature (° C)		
		REF[a]	SO[b]	CO[c]
T_1	-	-3.4	-3.2	+3.3
	+	-7.7	-9.8	-0.7
T_2	-	24.2	22.0	22.9
	+	21.5	23.8	21.4
T_3	-	S.T.[d]	N.D.[e]	N.D.
	+	S.T.	N.D.	S.T.

The values represent liver microsomal preparations from n = 4 - 5 rats in each dietary group. The phase transition temperatures for successive scans in the cooling mode differed by less than 0.3 centigrade degrees for the phase transitions designated as T_1 and T_2.

a. Reference diet

b. Sunflower seed oil diet (15%, w/w)

c. Coconut oil diet (15%, w/w)

d. Small transition detected

e. Transition not detected

cholesterol decreased the enthalpy values for the phase transition T_1 (ΔH_1), and in addition had a tendency to lower ΔH_2 values (Table 12). The enthalpy values for the T_3 transition (ΔH_3), when detected, were very small as compared to ΔH_1 or ΔH_2 values for the same group (data not shown).

5.3. Discussion

The results presented in this chapter demonstrate that both dietary fatty acids and cholesterol affect the thermotropic properties of rat liver microsomal lipids, at least, as determined by DSC. Both the transition temperature and enthalpy values of the lipid phase transitions are affected by dietary lipid supplementation. Presumably, these changes recorded with microsomal total lipids essentially represent the events occurring in the microsomal membrane phospholipids as these have been reported to consitute more than 90% of the microsomal total lipids (Kamath and Narayan, 1972).

For REF diet group, the temperatures at which phase transitions T_1 and T_2 occur, are similar to those reported by Bach et al (1977) for lipids extracted from liver microsomes of rats fed a normal, low fat diet. Addition of 2% cholesterol lowered the phase transition T_1, irrespective of the dietary fatty acid supplement, however, the magnitude of the decrease was greater when cholesterol was fed in combination with the sunflower seed oil. It is evident from Chapter 4 that dietary cholesterol increases the level of cholesterol in rat liver microsomal membranes and that this increase, which is particularly evident in the cholesterol ester fraction, is greatest

Table 12. Enthalpy values for liver microsomal lipids from rats fed various lipid - supplemented diets.

Exothermic[a] Enthalpy Values	2% Cholesterol	REF[b]	SO[c]	CO[d]
ΔH_1	-	-0.93	N.M.[e]	-1.10
	+	-0.52	N.M.	-0.79
ΔH_2	-	-0.33	-0.22	-0.14
	+	-0.28	-0.18	-0.11
ΔH_3	-	-9.99x10	N.M.	N.M.
	+	N.M.	N.M.	N.M.

a. The enthalpy values are expressed in cal/g dry weight of lipid and represent liver microsomal preparations from n = 4 - 5 rats in each dietary group

b. Reference diet

c. Sunflower seed oil diet (15%, w/w)

d. Coconut oil diet (15%, w/w)

e. Not able to be measured

when unsaturated fatty acids and cholesterol are combined together in the diet. Therefore the results presented here are consistent with those of Hinz and Sturtevant (1972) who demonstrated that cholesterol causes a progressive lowering of the transition temperature between the gel and liquid-crystalline phase, when added up to a level of 33 mole % in pure phospholipids. In the present study, the mole % of cholesterol in the microsomal membranes was found to be 9.5, 13.3, 13.8, 27.5, 8.8 and 17.0 for the REF, (REF + CH), SO, (SO + CH), CO and (CO + CH) diet group respectively (these values were derived from cholesterol/protein and phospholipid/protein values given in Chapter 4). In addition to this direct effect, dietary cholesterol also results in a decreased unsaturation index (U.I.) for liver microsomal membranes (c.f. Chapter 4), with the later parameter causing a possible rise in the transition temperature as suggested by Chapman (1975). The actual phase transition temperature of rat liver microsomal lipids as a function of dietary cholesterol would therefore seem to be a result of a balance between the transition lowering effect of increased membrane cholesterol and the decreased U.I. of membrane fatty acids as a result of dietary cholesterol supplement. These results are in agreement with the concept of membrane homeostasis (Sabine, 1983) which implies that biological membranes tend to maintain a constant level of lipid fluidity in the face of potential exogenous and endogenous perturbations. To minimize the changes in microsomal membrane fluidity produced by dietary cholesterol, compensatory alterations occur in the membrane fatty acid profiles. In addition to the lowering of phase transition T_1, dietary cholesterol also lowers the phase transition T_2, when fed with REF or saturated fat (CO) diets, however this transition occured rather at a

higher temperature when cholesterol (CH) was fed with unsaturated fat (SO). The reason for this decrease in T_2 following (SO + CH) diet feeding is not clear.

The T_3 transition was not observed in all samples, nor was it observed with repeated scans. With aqueous buffer dispersion of egg-yolk phosphatidylethanolamine, Hardman (1982) has shown that its thermal behaviour exhibits a transition between fluid lameller (L_α) and reverse hexagonal (H_{II}) liquid-crystalline phases, which is dependent on the pH of the liposome buffer. McMurchie et al (1983a) have shown that in liver mitochondrial lipids, the transition T_1 and T_2 are independent, whereas T_3 is dependent, on the pH of liposome buffer used. In the present study, microsomal membrane lipids exhibited a T_3 transition at about the same temperature (near 37°C) as previously reported in the liver mitochondrial lipids (McMurchie et al, 1983a). Therefore, it is likely that the transition T_3 represents a phase change between fluid lameller (Lα) and reverse hexagonal (H_{II}) lipid-crystalline phases.

The enthalpy values for transition T_1 (ΔH_1) and T_2 (ΔH_2), would suggest that a much smaller proportion of lipids in the microsomal membranes participate in transition T_2 in comparison to transition T_1. For REF diet group, ΔH_1 is about 3-times greater in comparison with ΔH_2 value while for CO diet group ΔH_1 is about 8-times greater than the ΔH_2 value. These results indicate that the phase transition T_2 may represent the formation of the solidus phase of some higher-melting point lipids. On further cooling, the bulk of the lipids, which were not involved for transition T_2, would then undergo

solidus formation at a lower temperature (phase transition T_1) as previously suggested by McMurchie et al (1983a) for the liver mitochondrial lipids. Dietary cholesterol lowers ΔH_1 and has a tendency to lower ΔH_2 values regardless of the nature of fatty acid supplement. Hinz and Sturtevant (1972) reported similar results using aqueous dispersions of dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) and suggested that each molecule of added cholesterol removes 2 molecules of lipid from the hexagonal phase which undergoes the co-operative transition to liquid-crystal phase upon heating.

In view of the above discussion, it can be concluded that at physiological temperature, rat liver microsomal lipids are in fluidus state. As the results presented are obtained from the isolated lipids of microsomal membranes, it rules out the possibility that the phase transitions observed arise from denaturation of proteins or some consequence of lipid-protein interactions. The observation of two clearly separate transitions indicates that some microsomal lipids can undergo a phase change independently of other membrane lipids. Altered fatty acid composition of microsomal membranes may have a compensatory role, to minimize the changes in membrane fluidity produced by dietary cholesterol. This would support the concept of membrane homeostasis (Sabine, 1983) which stimulated this investigation.

CHAPTER 6. MICROSOMAL FATTY ACYL DESATURASE (EC 1.14.99.5) ACTIVITIES IN LIVERS OF RATS FED CHOLESTEROL-SUPPLEMENTED DIETS.

It is evident from Chapter 4 that dietary cholesterol evokes changes in the cholesterol content and fatty acid composition of liver microsomes that in turn undoubtedly alter the fluidity of membrane lipids (c.f. Chapter 5). The major effect of dietary cholesterol observed in these experiments was an accumulation of 18:1 and 18:2 fatty acids with accompanying depletion of 16:0 and/or 18:0, 20:4 and 22:6 fatty acids in the microsomal membranes, suggesting that exogenous cholesterol accelerates the conversion of 18:0 to 18:1 and inhibits the synthesis of 20:4 from 18:2. Other workers, however (Bochenek and Rodgers, 1978; Morin et al, 1962), with respect to the whole liver, have demonstrated that the decrease in 20:4 after cholesterol feeding is due to its increased utilization for the synthesis of cholesterol ester and phospholipid, and their secretion into the plasma as very low density lipoproteins.

The fatty acyl desaturating enzymes which regulate tissue fatty acid composition, are located primarily in the microsomal membranes (Stoffel, 1962; Holloway et al, 1962). Therefore, in order to obtain a better understanding of the mechanism(s) whereby dietary cholesterol brings about changes in the fatty acid composition of microsomal membranes, presumably for the maintenance of lipid homeostasis, the effect of added cholesterol in the diet on microsomal desaturase activities was investigated.

6.1. Desaturase measurements

Rats were fed the experimental diets for a period of 28 days and microsomes were isolated from the livers of treated animals, as previously described in Section 3.2.3. Microsomes were resuspended in 0.15M KCl - 0.25M sucrose solution for the measurement of desaturase activities.

Desaturation of fatty acids by liver microsomes was measured by estimation of the percentage conversion of [1-^{14}C] stearic acid to oleic acid for Δ^9-desaturase, [1-^{14}C] linoleic acid to γ-linolenic acid for Δ^6-desaturase and [2-^{14}C] 8, 11, 14 - eicosatrienoic acid to arachidonic acid for Δ^5-desaturase, according to the procedure of de Gomez-Dumm et al (1983). The desaturase reaction medium contained 5 µmol ATP, 0.1 µmol CoA, 1.25 µmol NADH, 5 µmol $MgCl_2$, 2.25 µmol glutathione, 62.5 µmol NaF, 0.5 µmol nicotinamide, 62.5 µmol phosphate buffer (pH 7.0) and 200 nmol of [1-^{14}C] stearic acid for Δ^9-desaturase or 200 nmol of [1-^{14}C] linoleic acid for Δ^6-desaturase or 100 nmol of [2-^{14}C] 8, 11, 14 - eicosatrienoic acid for Δ^5-desaturase. The incubations were carried out with 5 mg of microsomal protein in a shaking water bath at 37°C for 10 minutes in a total volume of 1.5 ml. The reaction was stopped by adding 2 ml of 10% KOH in methanol. The lipids were saponified by heating for 2 hours at 85°C, acidified with 1 ml of 8N HCl, the fatty acids extracted with petroleum ether (40° - 70°) and methylated by heating for 1 hour with methanolic 5% H_2SO_4 at 70°C. Fatty acid methyl esters were extracted with petroleum ether (40° - 70°) and separated by chromatography on thin layer

chromatographic (TLC) plates, impregnated with 5% $AgNO_3$ on silica gel G.

The argentation TLC plates were prepared by mixing 40 g of silica gel G with 2 g $AgNO_3$ dissolved in 100 ml of distilled water. 2, 7 - Dichlorofluoroscein (0.2 ml of 0.2% methanol solution) was added to the slurry and spread over 4-5 glass plates (20 x 20 cm). This resulted in a uniform distribution of fluoroscein.

Carrier methyl esters of 18:0, 18:1, 18:2, γ18:3, 20:3 and 20:4 were spotted along with the labelled samples. Plates were developed in petroleum ether : diethyl ether (9:1, v/v) for the separation of saturated fatty acids from monoenes, petroleum ether : diethyl ether (4:1, v/v) for the separation of dienes from trienes and petroleum ether : diethyl ether (3:2, v/v) for the separation of trienes from tetraenes. The spots were visualized under ultraviolet light, scrapped off the plates directly into scintillation vials and counted with 10 ml of scintillation fluor (5 g PPO, 0.1 g POPOP/litre of toluene) using a liquid scintillation spectrometer. Percentage conversion was calculated by dividing the counts in the product by the total counts in the substrate plus the product, multiplied by 100.

6.2. Δ^9 - Desaturation activity

The results from the in vitro measurements of Δ^9-desaturase activity in liver microsomes from rats fed various high-lipid diets are presented in figure 11. The maintenance of rats on high cholesterol diets resulted in a significant elevation of Δ^9-desaturase activity

when compared to the animals fed the respective low cholesterol diets. This effect was most pronounced when the rats were supplemented with cholesterol without any added fat (Figure 11). Both the high fat (SO and CO) diets produced a reduction in the conversion of 18:0 to 18:1, but nevertheless the addition of cholesterol to these diets increased the Δ^9-desaturation. High unsaturated fat (SO) diet significantly decreased Δ^9-desaturation when compared with the high saturated fat (CO) diet (Figure 11).

6.3. Δ^6-Desaturation activity

The effect of dietary cholesterol on the desaturation of linoleic (18:2) acid to γ-linolenic (18:3) acid is shown in Figure 12. Feeding cholesterol-supplemented diets to the rats inhibited Δ^6-desaturase activity in the liver microsomes. The inhibition was most pronounced when cholesterol was fed without any added fat (Figure 12). Both the high fat diets (without added cholesterol) lowered Δ^6-desaturation, the coconut oil diet more than sunflower seed oil diet, when compared with the reference diet (Figure 12).

6.4. Δ^5-desaturation activity

Excess dietary cholesterol reduced the conversion of 8, 11, 14-eicosatrienoic (20:3) acid to arachidonic (20:4) acid (Figure 13). Like Δ^9- and Δ^6-desaturase activities, Δ^5-desaturation was also influenced to a greater extent when no fat was added to the high cholesterol diet. As with Δ^6-desaturase, both the high fat diets inhibited Δ^5-desaturation, the coconut oil diet more than sunflower

FIGURE 11: Δ^9 - desaturase activity in the liver microsomes of rats fed either a reference (REF) diet, sunflower seed oil (SO) diet or Coconut Oil (CO) diet supplemented with or without cholesterol (CH).

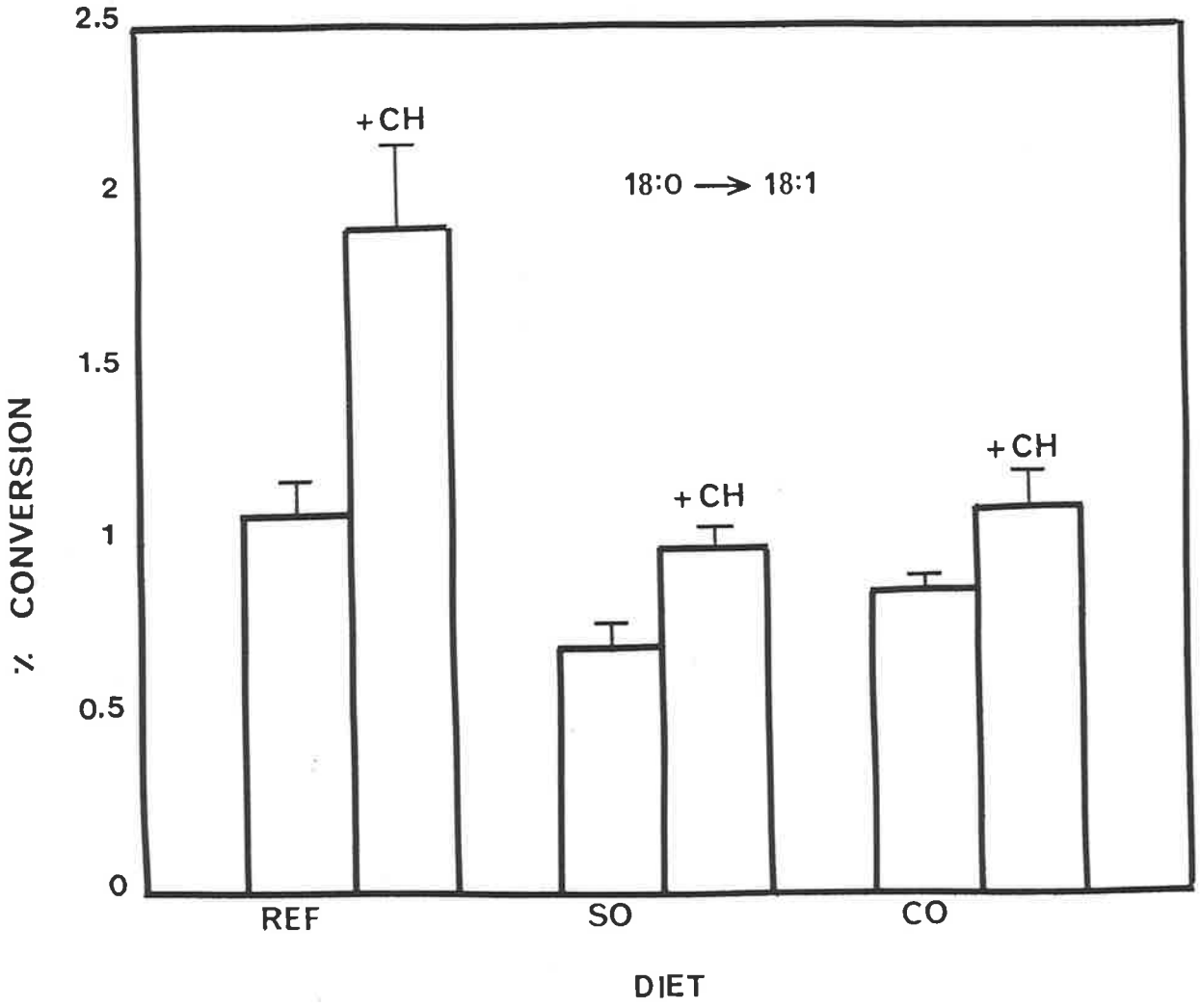
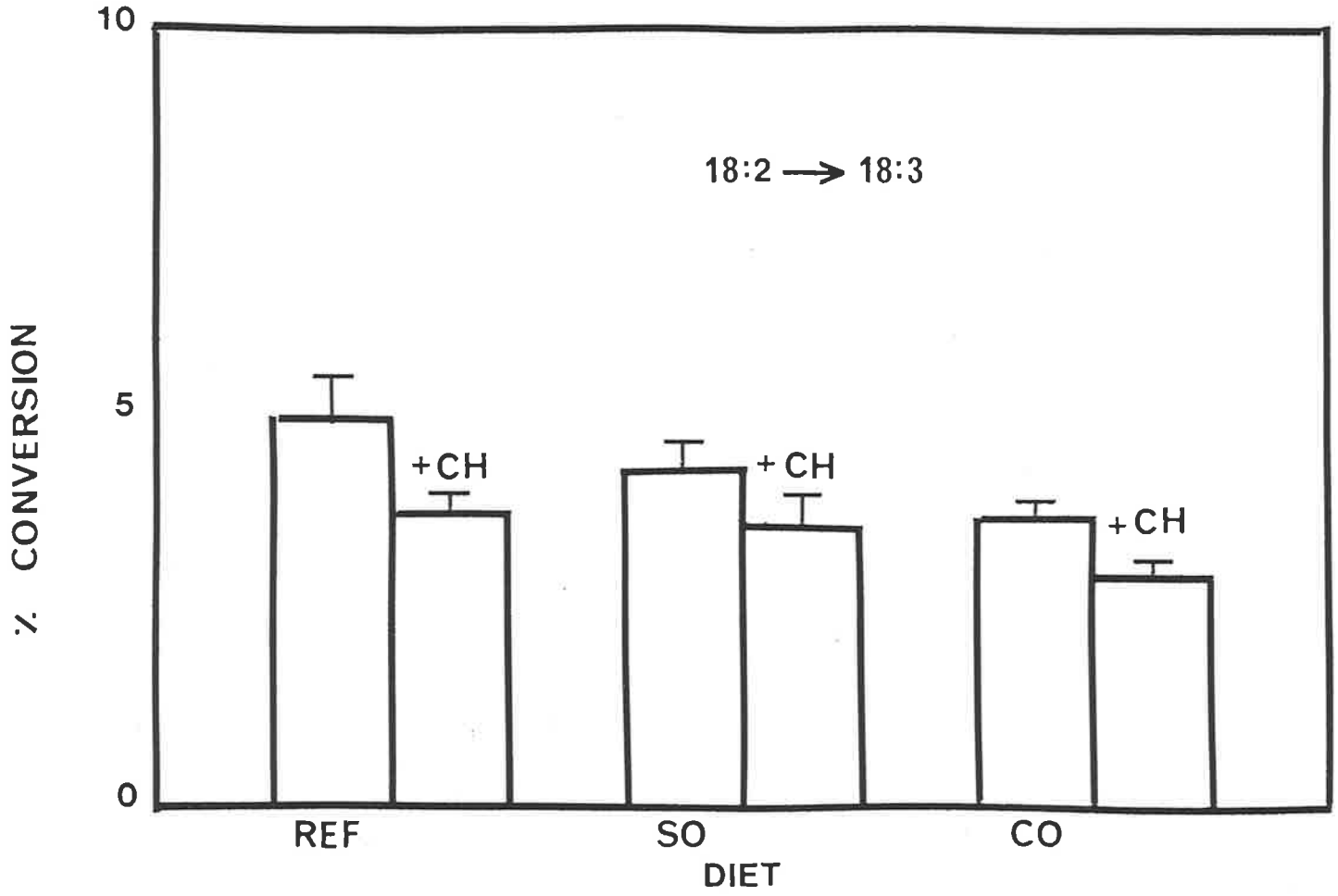

2.5
2
1.5
1
0.5
0
% CONVERSION
+CH
18:0 → 18:1
+CH
+CH
REF
SO
CO
DIET

FIGURE 12: Δ^6 - desaturase activity in the liver microsomes of rats fed either a reference (REF) diet, sunflower seed oil (SO) diet or coconut oil (CO) diet supplemented with or without cholesterol(CH).


10
5
0
% CONVERSION
18:2 → 18:3
+CH
+CH
+CH
REF
SO
CO
DIET

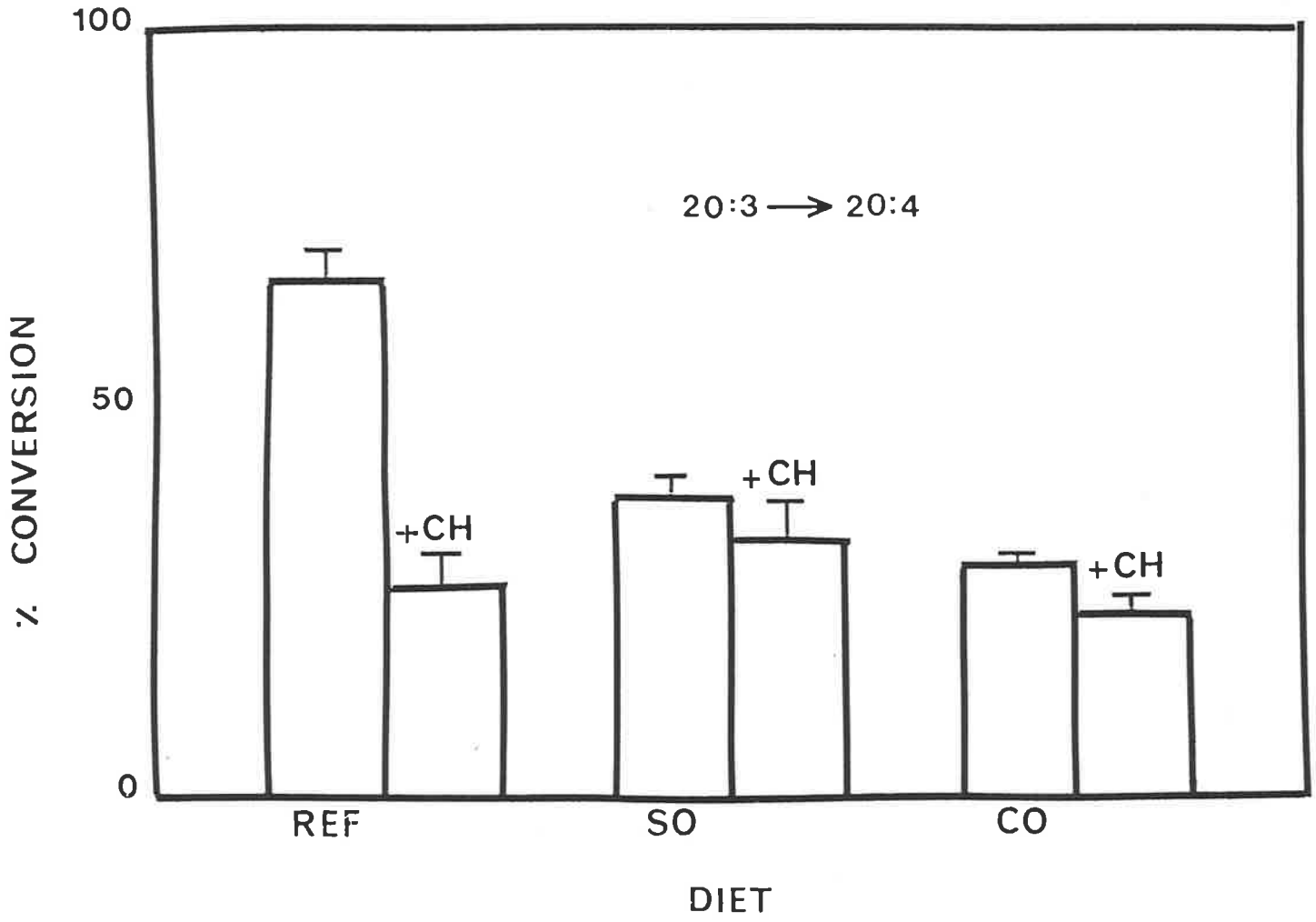
seed oil diet, when compared with the reference diet (Figure 13). Addition of 2% cholesterol to the coconut oil diet further reduced the Δ^5-desaturase activity in the liver microsomes. When cholesterol was supplemented in combination with sunflower seed oil, the Δ^5-desaturase activity in the microsomal preparations was inhibited, but was not significantly different from the respective low cholesterol (SO) diet fed animals (Figure 13).

6.5. Discussion

The present experiments were concerned with an examination of the fatty acid desaturating enzymes since these may regulate the fatty acyl composition of cellular and subcellular membranes as well as that of the whole tissue. The results presented in this chapter demonstrated that dietary cholesterol produces marked alterations in Δ^9-, Δ^6- and Δ^5- desaturase activities of rat liver microsomes. Excess dietary cholesterol accelerates the conversion of 18:0 to 18:1 by activating the Δ^9-desaturase enzyme and slows down the synthesis of 20:4 from 18:2 by inhibiting Δ^6- and Δ^5- desaturase activities. These changes in desaturase are consistent with the previously observed alterations in the fatty acid composition caused by dietary cholesterol (Chapter 4).

The data presented also clearly show that the changes observed in desaturase activities are more pronounced when cholesterol was fed without the addition of any exogenous fat. Both the high fat (SO and CO) diets, i.e. without the addition of exogenous cholesterol, lowered Δ^9-, Δ^6- and Δ^5- desaturation, but the inclusion of cholesterol in

FIGURE 13: Δ^5 - desaturase in the liver microsomes of rats fed either a reference (REF) diet, sunflower seed oil (SO) diet or coconut oil (CO) diet supplemented with or without cholesterol (CH).


100
50
0
% CONVERSION
20:3 → 20:4
+CH
+CH
+CH
REF
SO
CO
DIET

these diets further modified the enzyme activities. Within the high fat groups Δ^9-desaturase activity in liver microsomes from rats on SO diet was lower than those fed CO diet. This difference may be attributed to the high content of 18:2 in the SO diet, as suggested previously by Jeffcoat and James (1977). However, Pugh and Kates (1984) recently demonstrated that a diet rich in 18:2 (corn oil) increases Δ^9-desaturase activity in comparison to a diet poor in 18:2 (coconut oil). On the other hand Δ^6- and Δ^5- desaturase activities were found to be elevated in animals fed SO diet compared to the CO diet. Similar results on Δ^6- and Δ^5- desaturase activities have been previously reported (Pugh and Kates, 1984). Irrespective of the amount and type of fat fed in the diet, the Δ^9-desaturation was enhanced and the Δ^6- and Δ^5- desaturation activities were inhibited in cholesterol-fed animals. These results are consistent with the observed increase in 18:1 and 18:2 with accompanying depletion of 18:2. 20:4 and 22:6 in the liver microsomes of rats fed 2% cholesterol.

It is also evident from Chapter 4 that the SO diet lowered and the CO diet elevated the 22:6 (ω3) level of liver microsomes as compared to the REF diet group. 22:6 (ω3) is not present in any of the diets fed to the rats, so it must have been derived from the desaturation and elongation of 18:3 (ω3) which was present in very small amounts in all the diets. These results suggest that 18:3 (ω3) is a preferred substrate for Δ^6-desaturation when 18:2 (ω6) is available in relatively low amounts. Further, when cholesterol was added to the diets, 22:6 (ω3) in the liver microsomes was significantly lowered as compared to those fed respective low-cholesterol diets (Chapter 4).

This indicates that dietary cholesterol inhibits the desaturation of 18:3 (ω3) as well, irrespective of the quantity and quality of fat fed. Therefore, in brief, dietary cholesterol increases the percentage of monounsaturated fatty acids and reduces the percentage of polyunsaturated (20:4 and 22:6) fatty acids, overall resulting in a decrease of U.I., which in turn modifies the thermal behaviour of the microsomal membrane lipids (Chapter 5). The major effect of dietary cholesterol on thermal behaviour, as shown in Chapter 5, was a decrease in the phase transition temperature of the bulk lipids of the microsomal membranes as well as a decrease in the enthalpy values for this transition (Chapter 5). These changes, observed in the transition temperature and enthalpy values following the feeding of 2% cholesterol are consistent with the enrichment of microsomal membranes with cholesterol, whereas altered fatty acid composition (decrease in U.I.) following cholesterol feeding is expected to have the opposite effect. These results suggest that dietary cholesterol leads first to the accumulation of cholesterol in the microsomal membrane, which in turn changes the fluidity of these membranes. This altered fluidity then modifies microsomal-bound desaturase activities, so as to produce a decrease in the membrane unsaturation index, which, in its turn has a tendency to restore the native fluidity. Nevertheless, irrespective of the precise mechanism(s) by which desaturase activities are modified, the results presented in this chapter further confirm our previous observations that the decrease in 20:4 of liver microsomes, apparently, caused by dietary cholesterol, is due, at least in part, to a decreased synthesis of 20:4 from 18:2. Also these results are consistent with the proposed hypothesis (Sabine, 1983) of membrane homeostasis. Increased cholesterol content of microsomal membrane,

following cholesterol feeding, produces changes in the microsomal-bound desaturase activities, in such a manner that tends to nullify the effect of accumulated cholesterol on membrane fluidity, for the maintenance of lipid homeostasis.

CHAPTER 7. REGULATION OF THE MEMBRANE-BOUND ENZYMES OF CHOLESTEROL SYNTHESIS AND METABOLISM BY DIETARY CHOLESTEROL AND/OR FATTY ACIDS

The liver plays a dynamic role in the metabolism and turnover of both exogenous and endogenous cholesterol (Hotta and Chaikoff, 1955). Hepatic cholesterol, including that reaching the liver from plasma and that synthesized de novo, is converted into bile acids, or is esterified, or is secreted into the plasma as lipoproteins. Cholesterol biosynthesis and its conversion to bile acids are known to be regulated to a major extent by the enzymes, HMG-CoA reductase and cholesterol 7α-hydroxylase respectively. Alternatively, cholesterol can be esterified with a long chain fatty acid by the enzyme, acylcoenzyme A : cholesterol acyltransferase (ACAT). ACAT, like HMG-CoA reductase and cholesterol 7α-hydroxylase, is confined to the microsomal membranes of rat liver (Chesterton, 1968; Stokke and Norum, 1970). Thus microsomal membranes may play an important role in the overall metabolism and turnover of cholesterol in the liver.

The location of the control of cholesterol synthesis and metabolism on the same membranes has several important implications. Firstly, the accumulation of cholesterol in the microsomal membranes might inhibit its own synthesis by suppressing HMG-CoA reductase activity. Secondly, too much cholesterol in these membranes might enhance the rate of hydroxylation of cholesterol so as to effect the removal of the excess. On the other hand, enrichment of microsomal membrane with cholesterol might increase cholesterol esterification so as to transport more of cholesterol as very low density lipoproteins. On

the whole then the controls acting over cholesterol biosynthesis and metabolism to bile acids and cholesterol esters seem to be very much inter-connected, with all the three enzymes (i.e. HMG-CoA reductase, cholesterol 7α-hydroxylase and ACAT) localized on the same membranes.

It is evident from Chapter 4 that dietary cholesterol leads to the accumulation of cholesterol in the microsomal membranes, mainly as cholesterol esters and significantly more when the cholesterol is fed along with PUFA. These changes in the lipid composition of liver microsomal membranes following cholesterol feeding are associated with profound alterations in the thermal behaviour of microsomal lipids (Chapter 6). Therefore, in order to see whether the three enzymes of cholesterol metabolism act co-ordinately for the maintenance of a cholesterol homeostasis under conditions (dietary cholesterol and/or fatty acids) known to alter the metabolism of cholesterol in the liver, the activities of these enzymes, together with their Arrhenius parameters, were determined. A hypothesis linking these effects has been presented earlier from this laboratory (Sabine, 1983). He proposed that there is normally a certain constancy in the "fluidity" of the membrane lipids and it is in the cell's best interest to maintain this constancy in the face of various external and internal constraints.

7.1. HMG-CoA reductase (EC 1.1.1.34)

3-Hydroxy-3-methylglutaryl Coenzyme A reductase (EC 1.1.1.34) is the rate-limiting enzyme of cholesterol biosynthetic pathway (Dempsey, 1974; Rodwell et al, 1976). Given the importance of cholesterol

synthesis in many serious diseases (Sabine, 1977), it is clearly important that detailed work should be done with the specific objective of clarifying our knowledge of the physiological and intracellular mechanisms by which the catalytic capacity of the enzyme is regulated. Two hypotheses have gained importance to explain the molecular mechanism(s) controlling the activity of HMG-CoA reductase. Several workers (Ingebritsen et al, 1978; Nordstrom et al, 1977; Arebalo et al, 1981) have shown that the enzyme activity is reversibly modified by protein-mediated phosphorylation/dephosphorylation reactions. This phenomenon, however, does not provide a satisfactory explanation when all of the available data are critically examined (Ness, 1983). The second hypothesis for molecular control, namely 'the activity of HMG-CoA reductase is regulated by the fluidity of its supporting microsomal membrane' first proposed from this laboratory (Sabine and James, 1976) and later confirmed by others (Mitropoulos and Venkatesan, 1977), has been implicated as a possible general intracellular mechanism common to many regulatory situations. The present experiments were thus concerned with attempts to explore further this possibility.

HMG-CoA reductase activity and its Arrhenius behaviour, in the liver microsomes of rats fed cholesterol-supplemented diets with or without high levels of either saturated (coconut oil) or unsaturated (sunflower seed oil) fat were determined. Experimental diets and preparations of liver microsomes have been explained in Sections 3.2.1. and 3.2.3. respectively.

7.1.1. Assay for HMG-CoA reductase

HMG-CoA reductase activity was measured essentially as described by Shapiro et al (1974). Microsomal protein (125 µg) was pre-incubated for 5 minutes at 37°C in 50 µl of 0.25 M sucrose - 0.005 M potassium phosphate - 0.001 M EDTA (pH 6.8) buffer containing 0.01 M dithioerythritol. Then 30 µl of 0.02 M EDTA-KOH (pH 6.8) containing 5.0 µmol of glucose-6-phosphate, 0.5 µmol of $NADP^+$ and 1 unit of glucose-6-phosphate dehydrogenase was added. After another 5 minutes, 20 µl of 1 mM DL-[3-^{14}C] HMG-CoA (400 dpm/µmol) was added and the incubations were continued for another 30 minutes. The reaction was stopped with 25 µl of 4N HCl containing 4 µmol of [5-^{3}H] mevalonic acid (5000 dpm/µmol) as an internal standard. The mixture was further incubated for at least 15 minutes at 37°C to permit mevalonic acid to lactonize and then centrifuged for 5 minutes at 2000 g to sediment denatured proteins. 100 µl of this protein-free supernatant was directly applied to the activated silica gel G, TLC plate. This plate was then developed in a solvent system comprising benzene-acetone (1:1, v/v) and air-dried. The region (Rf = 0.5-0.8) containing the mevalonolactone was scraped off directly into a scintillation vial and counted with 10 ml of scintillation fluor (5 g PPO and 0.1 g POPOP/litre of toluene). Raw ^{14}C data were corrected for recovery by use of ^{3}H mevalonic acid internal standard data. Enzyme activity was expressed as picomoles mevalonic acid synthesized per minute per milligram of microsomal protein. The effect of substrate concentration and microsomal protein as well as of incubation time were studied for HMG-CoA reductase reaction to obtain the optimum conditions.

7.1.2. Determination of Arrhenius plot parameters

The Arrhenius plots were obtained by measuring the enzyme activity at different temperatures and then plotting logarithm of the enzyme activity against the reciprocal of the absolute temperature. The lines were fitted by the linear regression analysis. The coefficient of determination (r^2) and the residual sum of squares was calculated for all possible combinations of points fitted to two straight lines from the upper to the lower temperature extremes. A change in slope was considered to occur at the first minimum for the sum of the residual sum of squares of two straight lines. The difference in slope about a designated point of slope change, was tested for statistical significance and was adopted as actual point of slope change if the level of significance was $P < 0.005$ (McMurchie and Raison, 1979). The temperature at which the change in slope occurred was termed the transition temperature (Tc). The magnitude of activation energy (Ea) of the enzyme reaction was then calculated from the slope of the Arrhenius plot which has a negative value of (Ea/2.303R); R being the Gas Constant having a value of 1.98 Cal/mole/°K.

7.1.3. Assay conditions for HMG-CoA reductase

The rate of reduction using [3-^{14}C] HMG-CoA as a substrate, was found to be linear with time, over at least 60 minutes incubation time (Figure 14). The rate of ^{14}C-mevalonic acid formation was also proportional to the microsomal protein concentration upto 500 µg protein/incubation (Figure 15). For convenience, so that a large

number of assays could be carried out in a single run, a 30 minute incubation time and 125 µg microsomal protein per incubation were employed in most of the subsequent experiments. The incubations were carried out with 1 mM HMG-CoA as a substrate, however, similar results were obtained when HMG-CoA concentration was varied between 1.0 and 2.0 mM as shown in Figure 16.

The direct application of the deproteinized samples to TLC plates eliminated the ether extraction step and raised the recovery of mevalonic acid to above 90%. Since, as much as 80% of the deproteinized reaction mixture was used for TLC, the sensitivity was correspondingly increased.

7.1.4. HMG-CoA reductase activity in liver microsomes of rats fed lipid-supplemented diets

The activity of microsomal HMG-CoA reductase from livers of rats fed low- and high-cholesterol diets, with and without high levels of either saturated (coconut oil, CO) or unsaturated (sunflower seed oil, SO) fat, is presented in Table 13. HMG-CoA reductase activity was significantly reduced in microsomal preparations from rats fed SO diet than that in the preparations from rats fed either on REF or CO diet. Addition of 2% cholesterol in all the three diets depressed the enzyme activity, irrespective of the quality and quantity of fat-fed. The level of suppression of HMG-CoA reductase was lowest when cholesterol was added to the SO diet (Table 13).

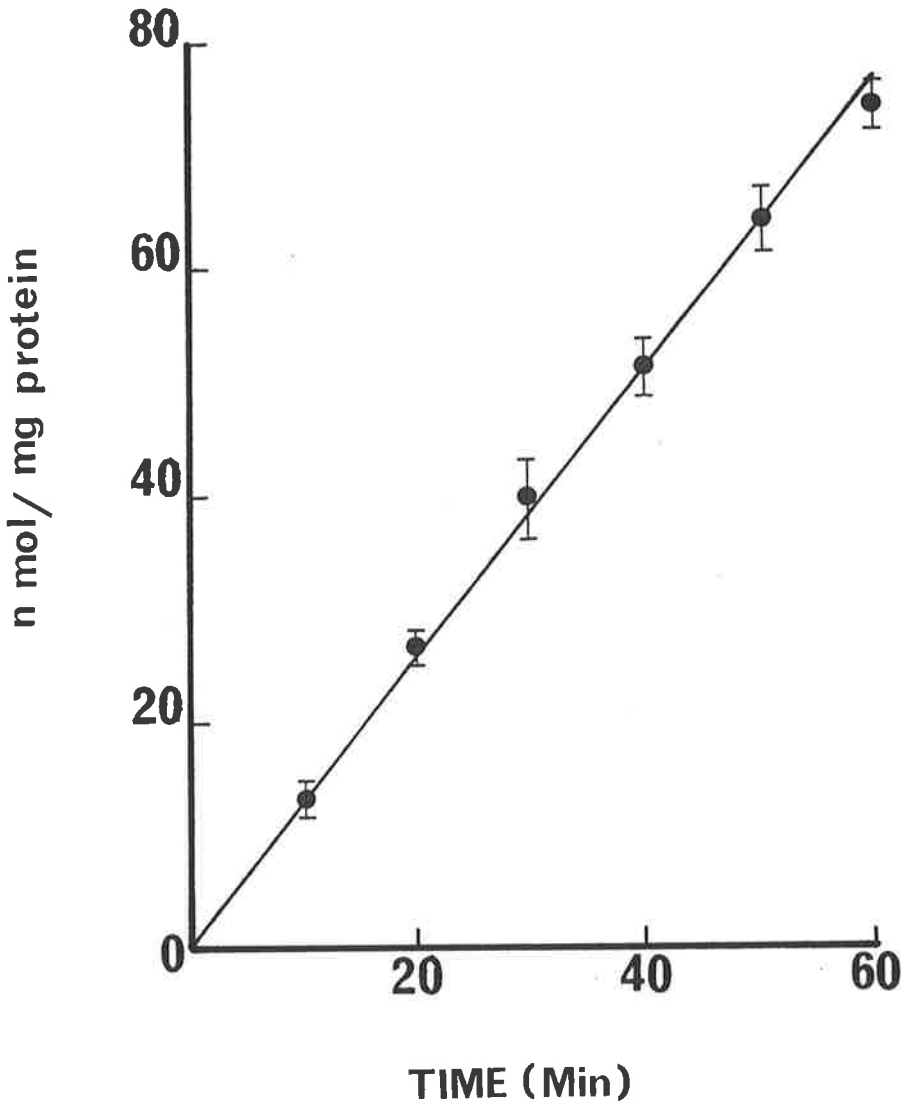


Figure 14

Time course of HMG-CoA reductase activity. Freshly prepared microsomal reductase was assayed for activity as described in section 7.1.1. except that the time of incubation was varied as shown. The assay was carried out in duplicates and the vertical bars indicate the S.D.

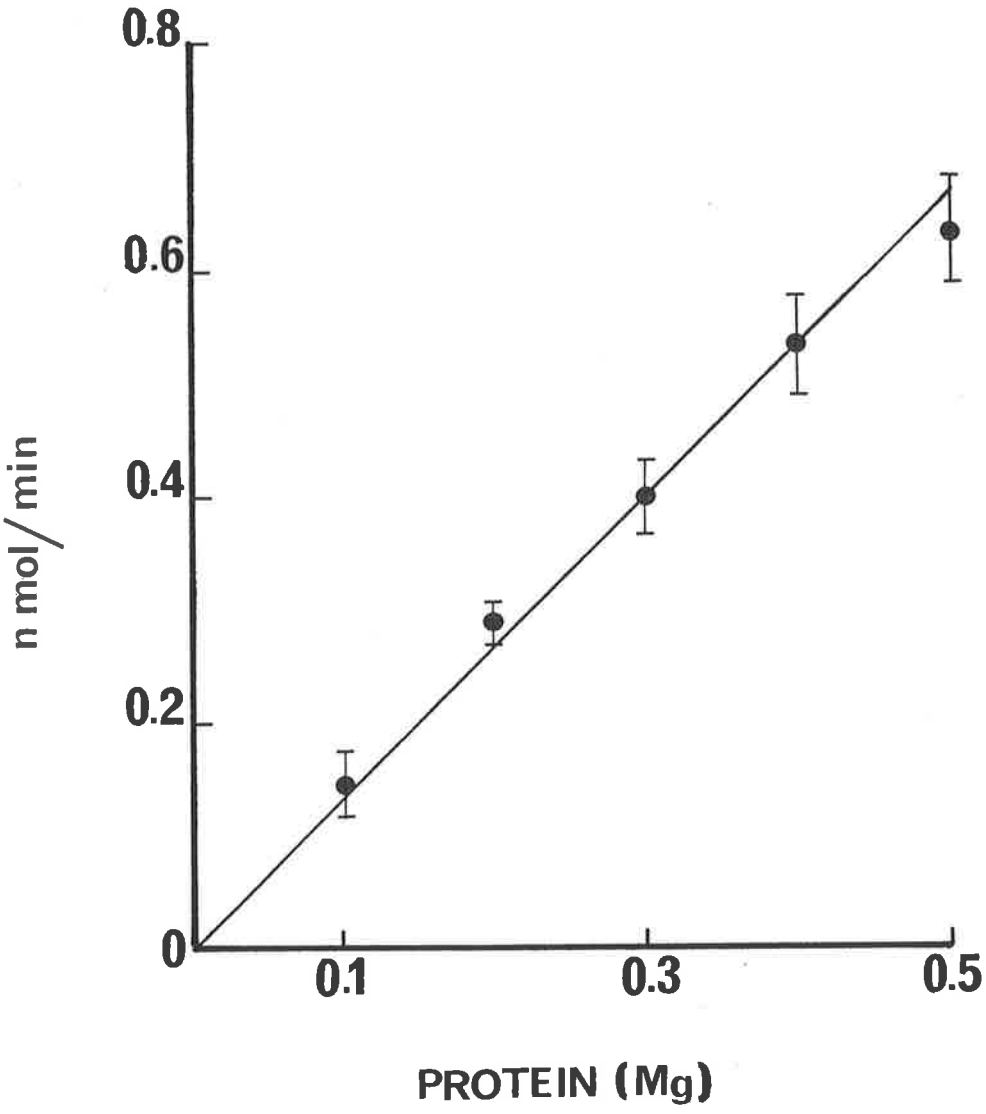


Figure 15

Effect of varying the protein concentration on the activity of microsomal HMG-CoA reductase. The enzyme was assayed for activity as described in section 7.1.1. except that the protein concentration was varied as shown. The assay was carried out in duplicates and the vertical bars indicate the S.D.

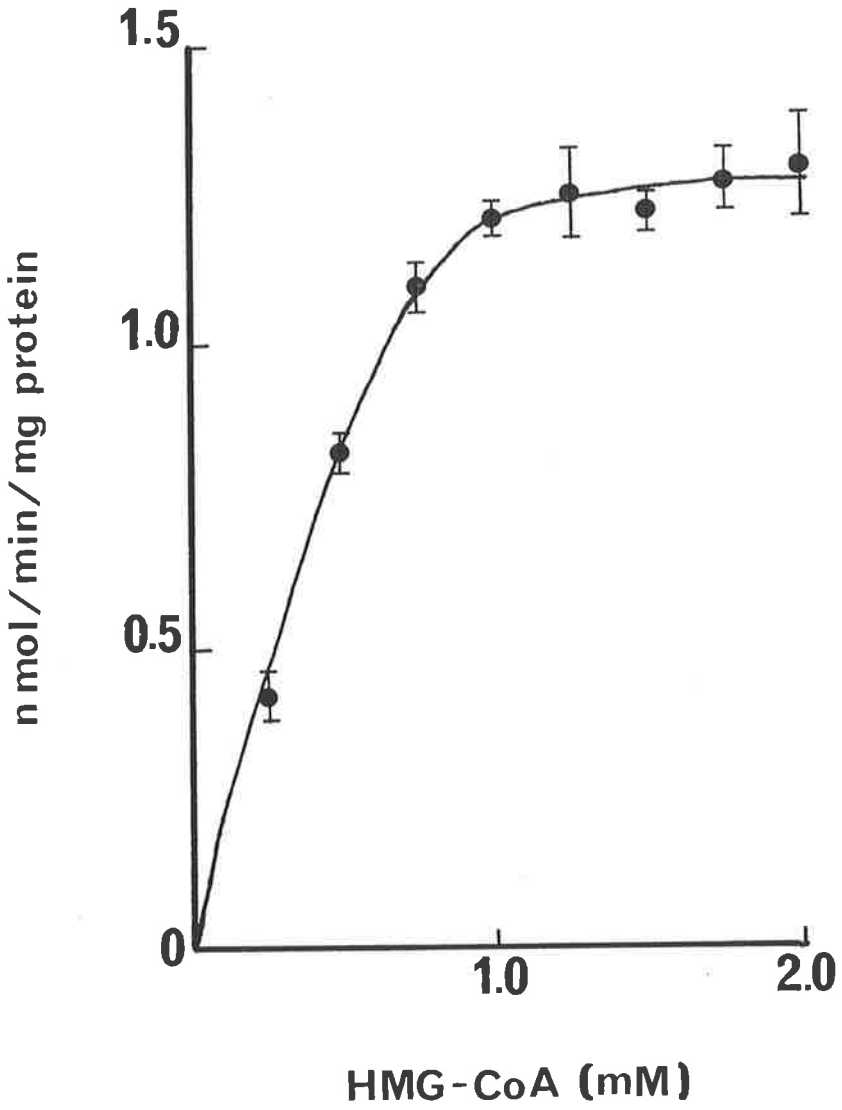


Figure 16

Substrate saturation curve for microsomal HMG-CoA reductase. Freshly prepared microsomal reductase was assayed for activity as described in section 7.1.1. except that the initial concentration of HMG-CoA was varied as shown. The assay was carried out in duplicates and the vertical bars indicate the S.D.

Table 13. HMG-CoA reductase activity in liver microsomes of rats fed various lipid - supplemented diets

2% Cholesterol	Reference diet	Sunflower seed oil diet	Coconut oil diet
	p mol mevalonate formed / mg protein / minute		
-	1120 ± 126	860 ± 49[a]	1068 ± 99
+	588 ± 88[b]	426 ± 52[b,c]	702 ± 86[b]

The experimental conditions are described in section 7.1 and the enzyme assay in section 7.1.1.
The values are the mean ± S.D. of triplicate measurements in liver microsomal preparations from rats (n = 4 - 5) in each dietary group.

a. Significantly different from the reference diet or coconut oil diet-fed animals; $P < 0.0$

b. Significantly different from the respective low cholesterol diet-fed animals; $P < 0.001$

c. Significantly different from the cholesterol plus coconut oil diet-fed animals; $P < 0.05$

7.1.5. Arrhenius plots of microsomal HMG-CoA reductase prepared from rats fed various experimental diets

Arrhenius plots of liver microsomal HMG-CoA reductase activity from rats fed either a low cholesterol - low fat (REF) diet, high cholesterol-low fat (REF + CH)diet, low cholesterol - high fat (SO and CO) diets or high cholesterol - high fat (SO + CH and CO + CH) diets for 4 weeks are shown in Figures 17-19. HMG-CoA reductase activity from rats fed REF diet was biphasic between 15-37°C, with critical temperature (Tc) at about 28.8°C. The mean value for activation energy in the temperature region above Tc (Ea_1) was 10.8 K Cal/mole and that for below the Tc (Ea_2) was 23.2. (Table 14).

Supplementation of the REF diet with 15% sunflower seed oil resulted in lowering of Tc in comparison to the REF diet and the diet supplemented with 15% coconut oil. Activation energy above Tc (Ea_1) was significantly increased and below Tc (Ea_2) was significantly decreased in SO diet group than that in REF or CO diet groups (Table 14).

The presence of 2% cholesterol in either REF or CO or SO diet resulted in the Arrhenius plots of HMG-CoA reductase activity having a constant activation energy (Ea) i.e. elimination of Tc between 15 - 37°C. Within the high cholesterol groups, microsomal preparations from rats fed cholesterol plus coconut oil (CO + CH) exhibited a higher Ea and rats fed cholesterol plus sunflower seed oil (SO + CH) exhibited a lower Ea, than those fed REF diet supplemented with 2% cholesterol (REF + CH) without any added fat (Table 14).

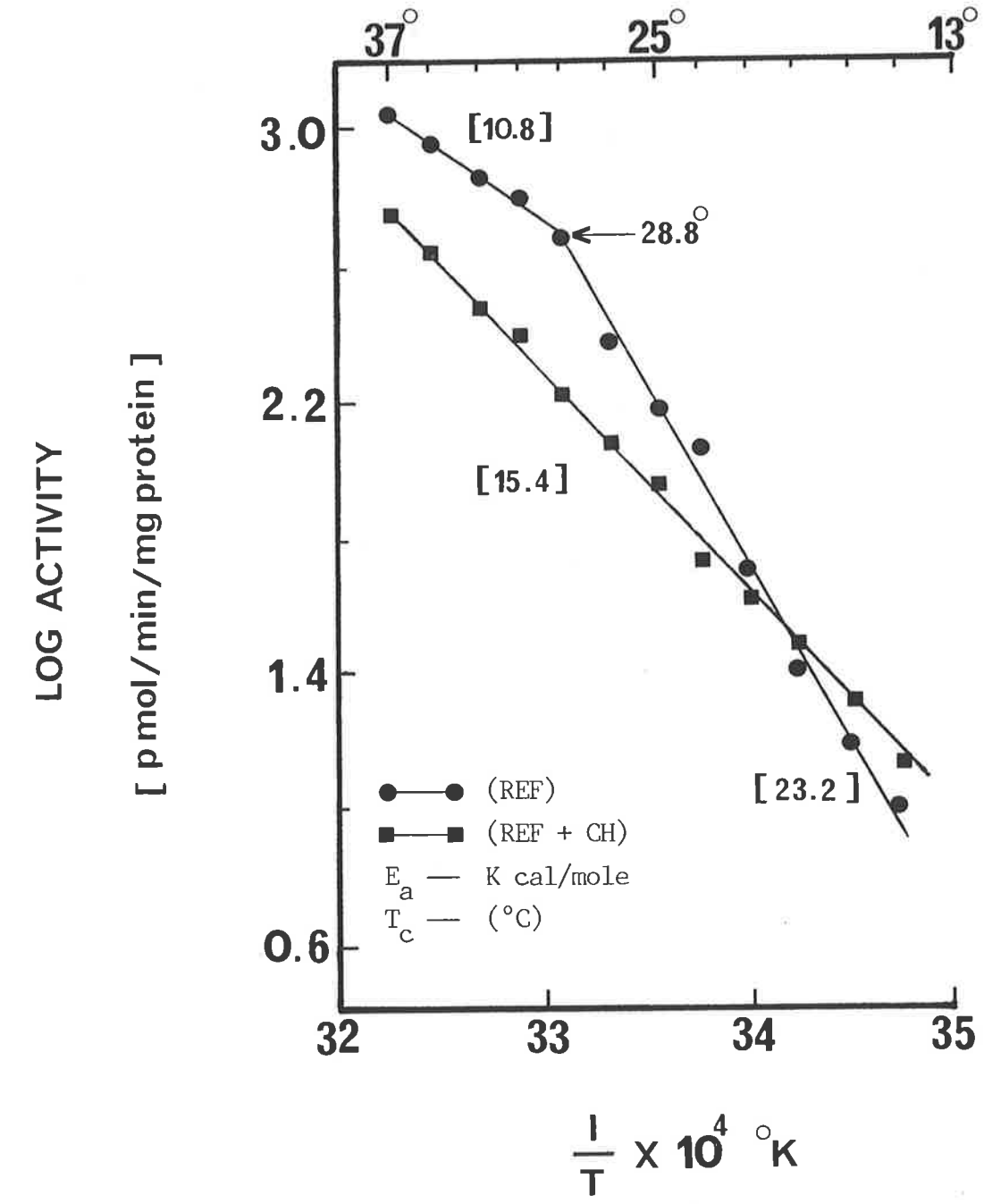


Figure 17

Arrhenius plots of liver microsomal HMG-coA reductase from rats fed a reference (REF) or 2% cholesterol (REF + CH) diet. The activation energy (E_a) values are shown in brackets and arrows indicate the transition temperatures (T_c).

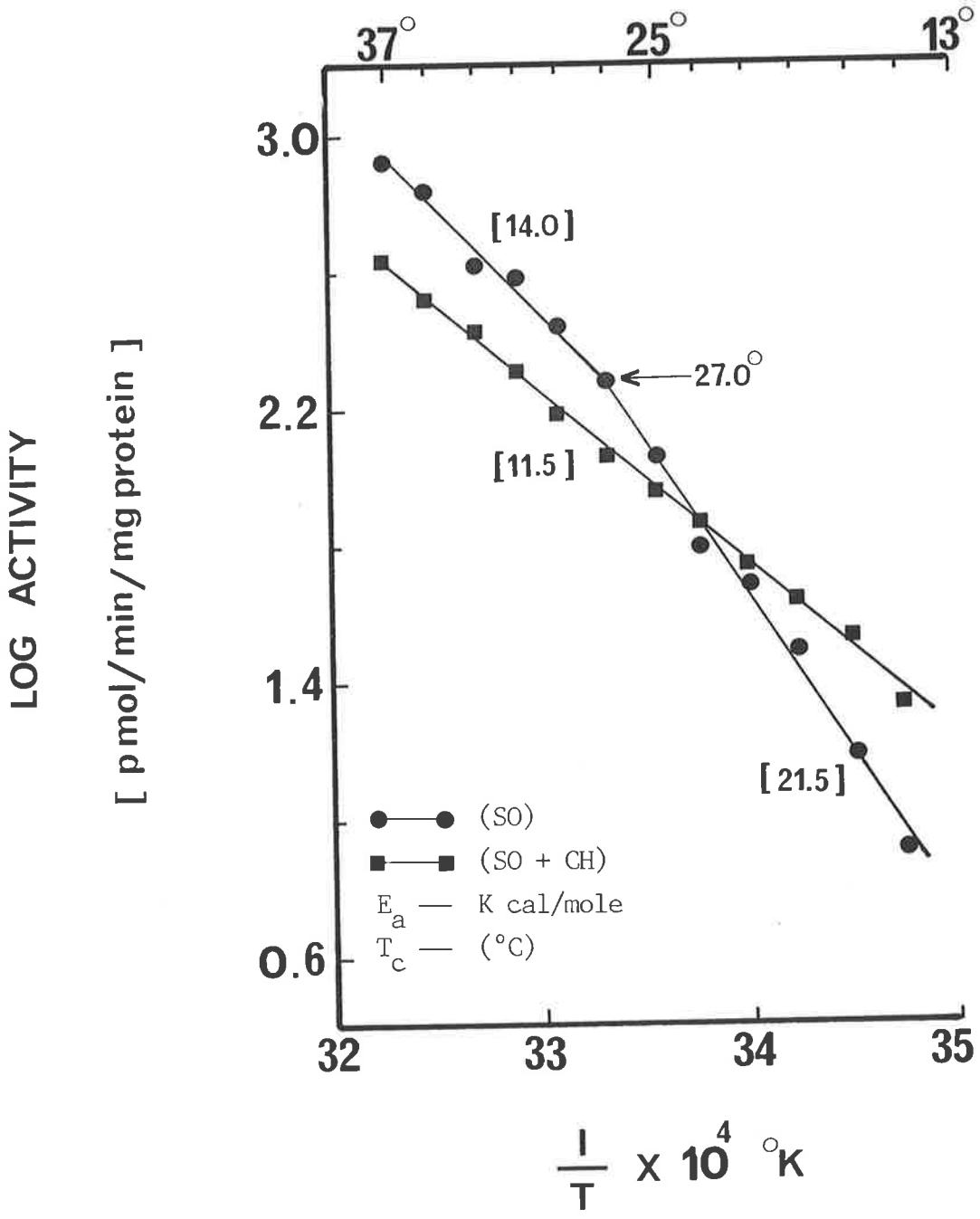


Figure 18

Arrhenius plots of liver microsomal HMG-coA reductase from rats fed a 15% sunflower seed oil (SO) or 15% sunflower seed oil plus 2% cholesterol (SO + CH) diet. The activation energy (E_a) values are shown in brackets and arrows indicate the transition temperatures (T_c).

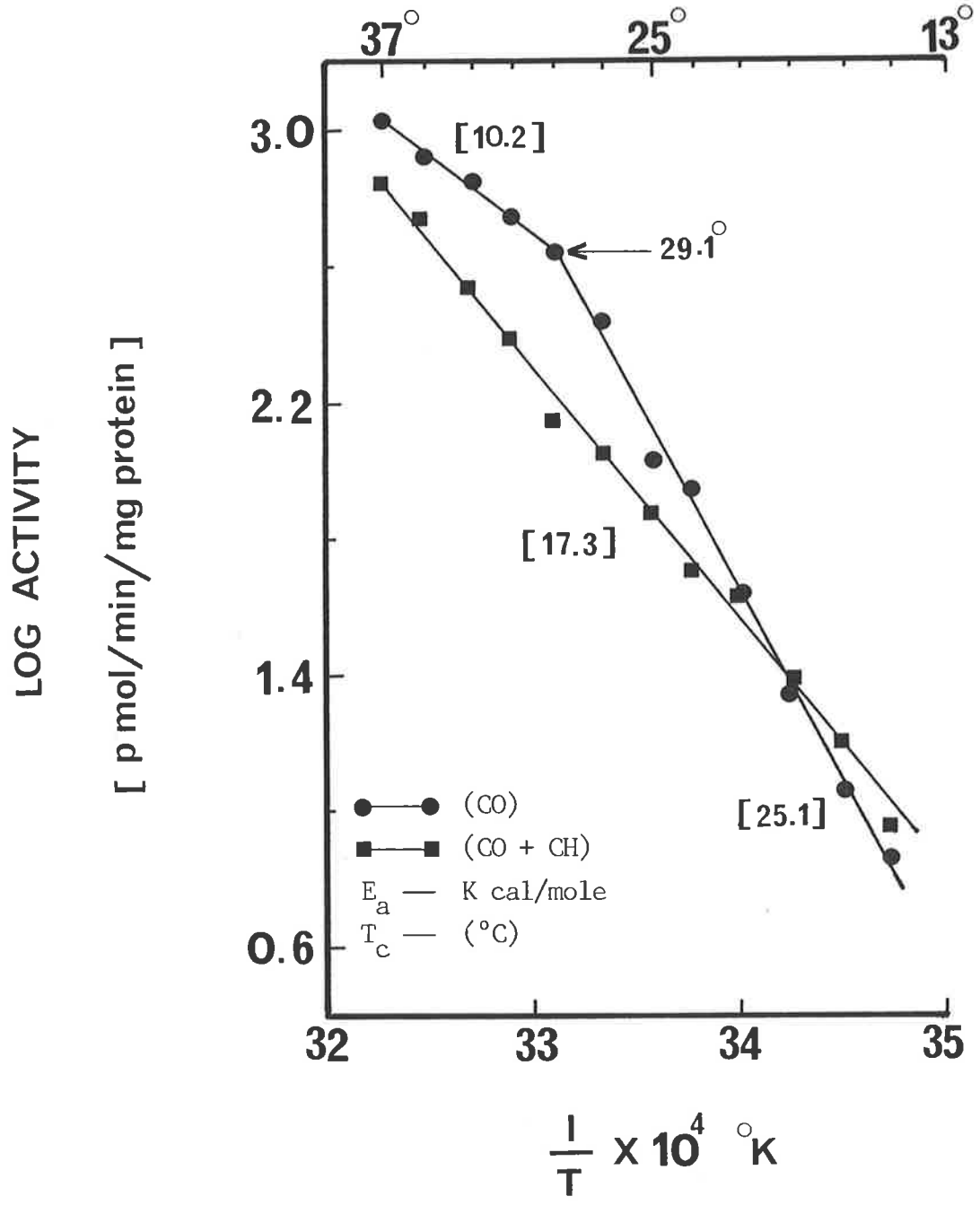


Figure 19

Arrhenius plots of liver microsomal HMG-coA reductase from rats fed a 15% coconut oil (CO) or 15% coconut oil plus 2% cholesterol (CO +CH) diet. The activation energy (E_a) values are shown in brackets and arrows indicate the transition temperatures (T_c).

Table 14. Arrhenius plot characteristics of HMG-CoA reductase from liver microsomes of rats fed various lipid-supplemented diets.

Parameter	2% Cholesterol	REF	SO	CO
T_c	-	28.8	27.0	29.1
	+	-	-	-
Ea_1	-	10.8 ± 0.8	14.0 ± 0.5[a*]	10.2 ± 0.2
	+	15.4 ± 0.4	11.5 ± 0.2[b**]	17.3 ± 0.3[b*]
Ea_2	-	23.2 ± 1.1	21.5 ± 0.6	25.1 ± 0.3
	+	-	-	-

Arrhenius plot parameters were determined as described in section 7.1.2. T_c represent the transition temperature in degree centigrades. Ea_1 represents activation energy above the T_c and Ea_2 represents activation energy below the T_c and are expressed in Kcals / mole.

a. Significantly different from reference diet - fed animals : $P < 0.05$.

b. Significantly different from reference diet plus 2% cholesterol - fed animals : *, $P < 0.05$; **, $P < 0.01$.

7.1.6. Discussion

The results presented in this section demonstrate that HMG-CoA reductase activity in liver microsomal preparations depends a great deal on the dietary lipid intake. Consumption of unsaturated fat for 4 weeks decreased the HMG-CoA reductase activity whereas saturated fat feeding had no appreciable effect. These results are in agreement with the observations of Mitropoulos et al (1980), while Bochenek and Rodgers (1978) have reported that both saturated and unsaturated fat feeding lowers the HMG-CoA reductase activity in the rat liver microsomes. As was previously discussed in Chapter 4, dietary unsaturated fat feeding does two things, increases unsaturation of microsomal membranes and increases cholesterol content of these membranes. The lowering of the transition temperature (Tc) in the Arrhenius plot of HMG-CoA reductase followed by the feeding of unsaturated fat is consistent with the increased membrane unsaturation, however, the increase in activation energy above the Tc (Ea_1) is opposite to what is expected from the consequence of increased unsaturation on membrane fluidity. On the other hand, enrichment of microsomal membranes with cholesterol as a result of unsaturated fat consumption, would be expected to suppress the activity of HMG-CoA reductase by feed back mechanism. Also a decrease in transition temperature (Tc), an increase in activation energy above the Tc (Ea_1) and a decrease in the activation energy below the Tc (Ea_2) of the enzyme would also, be anticipated, as previously indicated by Mitropoulos et al (1980). Such an elevation in the microsomal cholesterol content following dietary unsaturated fat feeding has been actually observed in the present study (Chapter 4).

Therefore, the increased cholesterol content of liver microsomes may be the primary event influencing the regulation of cholesterol biosynthesis under the conditions of dietary unsaturated fat.

The increase in cholesterol content of microsomes following SO diet feeding is localised in the ester fractions of cholesterol (Chapter 4). These results suggest that it is the accumulation of cholesterol esters in the microsomal membranes which is critical for the regulation of HMG-CoA reductase actvitiy. This is in contrast to the observations of Mitropoulos and Venkatesan (1977) who demonstrated that the real 'culprit' for the regulation of HMG-CoA reductase activity is the accumulation of free cholesterol in the microsomal membrane. Furthermore, in the present study, feeding 2% cholesterol to rats raised the cholesterol content of liver microsomes, mainly as cholesterol esters, and significantly more when cholesterol was fed with unsaturated fat (Chapter 4). Both saturated and unsaturated fat feeding with cholesterol elevated the free cholesterol content of microsomes to the same extent, however more cholesterol esters accumulated when cholesterol was fed with unsaturated rather than saturated fat (Chapter 4). The greater inhibition of HMG-CoA reductase activity following cholesterol feeding with unsaturated fat is consistent with the greater accumulation of cholesterol esters in the microsomal membranes. These results further suggest that the accumulation of cholesterol esters and not of free cholesterol in the membranes is critical for the regulation of HMG-CoA reductase activity under the dietary conditions of the present study. These results get some further support from the recent observations of Van Heudsen and Wirtz (1984) who, using non-specific lipid transfer protein or serum

for microsomal cholesterol enrichment, in vitro, have shown that the microsomal pool of non-esterified cholesterol has no direct effect on HMG-CoA reductase activity. In this respect, it is interesting to note that Morris hepatomas failed to display feedback control of HMG-CoA reductase activity by dietary cholesterol treatment because they failed to accumulate cholesterol esters in their livers, whereas livers from the host animals accumulated cholesterol esters and exhibited inhibitions of HMG-CoA reductase (Horton and Sabine, 1973; Harry et al, 1971).

Inclusion of 2% cholesterol in the rat diets abolished the break point observed in the Arrhenius plot of HMG-CoA reductase activity seen with the respective low-cholesterol diets, resulting in a constant activation energy between 15-37°C. Mitropoulos and Venkatesan (1977) have suggested that the presence of cholesterol at high concentration following cholesterol feeding imparts an "intermediate fluid" state i.e. the presence of cholesterol increases the activation energy above Tc (observed in the Arrhenius plot of HMG-CoA reductase from rats fed low-cholesterol diet) and decreases the activation energy below Tc, ultimately resulting in a constant activation energy. The results with (REF + CH) and (CO + CH) diets are in line with this concept. However (SO + CH) diet exhibited somewhat different results. The activation energy after (SO + CH) feeding was decreased both above and below the Tc, observed in the Arrhenius plot of HMG-CoA reductase in microsomal preparations from livers of rats fed SO diet without any added cholesterol. This means that the fatty acyl chains of liver microsomal membranes from rats fed (SO + CH) diet are more mobile both above and below the transition temperature (Tc) than those from rats

fed SO diet without added cholesterol. This may well be the reason why HMG-CoA reductase activity, in liver microsomal preparations from rats fed (SO + CH) diet, is considerably higher even at 15°C (lowest temperature at which the enzyme activity is measured) despite the fact that microsomal preparations from rats fed this diet exhibited the lowest enzyme activity at 37°C of all other experimental groups. The thermotropic behaviour of microsomal membrane lipids obtained by differential scanning calorimetry (DSC) revealed two transitions, located between -10 to 5°C and 20 to 25°C (Chapter 5). The second transition between 20 & 25°C may be in part responsible for the break observed in the Arrhenius plots of HMG-CoA reductase activity. Administration of 2% cholesterol in the diets resulted in the abolition of break point observed in the Arrhenius plot. However thermal scans of microsomal lipids still exhibited the two phase transitions, in spite of the cholesterol enrichment caused by dietary cholesterol. These results suggest that HMG-CoA reductase might have a specific micro-environment (boundary lipid) which has different physical properties than those of the bulk lipids of microsomal membranes.

Using mixtures of synthetic phospholipids and cholesterol, Ladbrooke et al (1968a) have shown that cholesterol, up to 50 mole % concentration, lowers the transition temperature between the gel and liquid crystalline phases. Above 50 mole % cholesterol concentration, no transition is observed (Ladbrooke et al, 1968a). In the present study, the cholesterol concentration in the microsomal membranes remained below 50 mole % even after dietary cholesterol treatments. Consequently, two clear phase transitions were observed in the DSC scans of these membrane lipids. Srikantaih et al (1977) have reported

that the cholesterol concentration in purified HMG-CoA reductase decrease from 0.59 nmol to 0.39 nmol/nmol of the enzyme, i.e. more than a 30% decrease, when 2% cholestyramine was fed in the rat diet. In another study Mitropoulos and Venkatesan (1977) showed that 5% cholestyramine feeding to rats resulted in only 10% decrease in the cholesterol content of liver microsomes. Although the percentage of cholestyramine in the diet and length of feeding time were different in the two studies, it appears that the cholesterol concentration in the immediate environment (boundary lipid) of HMG-CoA reductase is affected more than that of microsomal membranes in which HMG-CoA reductase is localised. Thus it is quite likely that in the present study, feeding 2% cholesterol, increased the cholesterol concentration in the 'boundary lipid' of HMG-CoA reductase, to more than 50 mole % which then led to the abolition of the break temperatures in the Arrhenius plot of the enzyme. On the other hand, microsomal cholesterol concentration remained below 50 mole % even after dietary cholesterol treatments and consequently two phase transitions were seen in the DSC scans of microsomal membrane lipids (Chapter 5). Such a concept of "cholesterol pool" similar to "boundary lipid" has been previously proposed for HMG-CoA reductase and cholesterol 7α-hydroxylase present in the microsomal membranes (Mitropoulos and Venkatesan, 1977). However, it is difficult to establish a strict relationship between the activity of HMG-CoA reductase and cholesterol concentration in endoplasmic reticulum, since no method is yet available to prepare a pure population of such membranes (Mitropoulos, 1983). The pre-incubation of rat liver microsomal fraction has been shown to modulate the activity of HMG-CoA reductase (Venkatesan and Mitropoulos, 1982). This is suggestive evidence that the pre-incubation may result in the transfer of microsomal cholesterol to endoplasmic-reticulum vesicles and thus change the HMG-CoA reductase activity (Venkatesan and Mitropoulos, 1982).

In brief then, the results presented here demonstrate that dietary cholesterol alters the activity of rat liver microsomal HMG-CoA reductase and that the type of fat fed has a profound effect on this process. The results on Arrhenius plots, combined with those of DSC (Chapter 5), suggest that HMG-CoA reductase is surrounded by "boundary

lipids" which appear to be affected differently than those of the bulk lipids of microsomal membranes by dietary cholesterol treatment. Much more work will be necessary in order to understand the molecular mechanisms associated with the observed changes in Arrhenius plots of HMG-CoA reductase upon cholesterol feeding. Firstly, it will be essential to purify the enzyme to establish the occurrence or otherwise of a change in the physical state of the enzyme microenvironment (boundary lipid). Secondly, reconsitution studies of HMG-CoA reductase may throw some light on the role of the phosholipids and cholesterol that are associated with the enzyme (Heller and Shrewbury, 1976) in the functioning of its catalytic site. An Arrhenius plot study of the purified enzyme from cholesterol fed animals would further indicate whether membrane association is involved in determining the linear nature of the plot.

7.2. Cholesterol 7α-hydroxylase (EC 1.14.13.17)

In any consideration of the regulation of cholesterol biosynthesis, its further fate cannot be ignored. This is becomming more critical now that various end products of cholesterol, such as bile acids and steroids, have been shown to exert some control over its biosynthesis (Gaylor, 1974). The conversion of cholesterol to bile acids in the liver is not only of significance in the digestion and absorption of dietary lipids, but also is the main route by which exchangeable plasma and tissue cholesterol is excreted (Sabine, 1977).

The first step in the conversion of cholesterol to bile acids is the hydroxylation of cholesterol molecule at the 7α-position, in a

reaction catalyzed by cholesterol 7α-hydroxylase. The mechanism of hydroxylation and the regulation of enzyme activity have been well reviewed by Myant and Mitropoulos (1977). Cholesterol 7α-hydroxylase is generally regarded as the rate-limiting enzyme in the sequence of bile acid synthesis from cholesterol. Thus, the conditions, such as a high cholesterol diet, fasting, cholestyramine feeding and diurnal rhythm, which are known to alter the rate of bile synthesis, also alter the activity of the enzyme (Sabine, 1977; Myant and Mitropoulos, 1977).

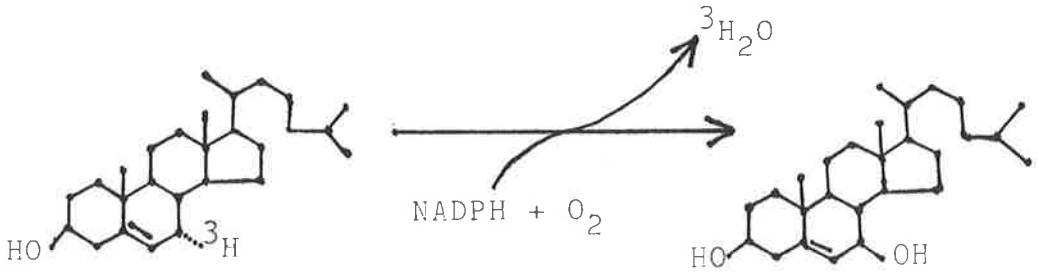
Interest in cholesterol 7α-hydroxylase in the present work is due both to its association with the membranes of microsomal fraction, a property that it has in common with HMG-CoA reductase, and to its co-ordinated role with the reductase in the maintenance of cholesterol homeostasis. The objective of this exercise was to determine whether cholesterol 7α-hydroxylase was prone to regulation by dietary lipid-induced alterations in the physical state of its membrane lipids, as revealed by DSC as well as by Arrhenius plots of the enzyme activity.

7.2.1. Assay method for cholesterol 7α-hydroxylase

Cholesterol 7α-hydroxylase activity has been usually determined by incubating [^{14}C] cholesterol in the presence of microsomal membrane and the required co-factors. 7α-Hydroxycholesterol formed at the end of the enzymic reaction is then extracted, purified by thin layer chromatography and assayed for radioactivity. The enzyme activity can be expressed as the percentage of labelled 7α-hydroxycholesterol

formed from [^{14}C] cholesterol (Shefer et al, 1968; Boyd et al, 1973). Alternatively, the mass of 7α-hydroxycholesterol has been measured, either by acetylation technique (Mitropoulos and Balasubramaniam, 1972) or by a method based on combined gas chromatography - mass spectrometry (Bjorkhem and Danielsson, 1975). As a large number of manipulations are involved in the extraction, isolation and derivation of 7α-hydroxycholesterol, these methods are impractical for carrying out a large number of enzyme assays in a single run.

Van Cantfort et al (1975) have described an assay enabling a quick and accurate determination of the activity of cholesterol 7α-hydroxylase. This method is based on the stereospecific hydroxylation at the 7α-position of cholesterol molecule, thus releasing water into the medium. [7α-^{3}H] cholesterol is used as a substrate and cholesterol 7α-hydroxylase activity can be evaluated by counting the tritiated water released into the incubation medium, as shown below:



Irrespective of the method employed, there are some general problems in the assay of microsomal cholesterol 7α-hydroxylase (Myant and Mitropoulos, 1977) which include : the water insoluble nature of the cholesterol used as a substrate; the presence of an endogenous pool of cholesterol accessible to the enzyme; susceptibility of cholesterol to non-enzymic auto-oxidation to various derivatives including

7α-hydroxycholesterol; and further utilization of 7α-hydroxycholesterol by other microsomal enzymes under certain conditions.

Bearing these problems in mind and so as to enable a large number of assays to be carried out in a single run, the method of Van Cantfort et al (1975) was adopted in the present study. Rats were fed the experimental diets (explained in Section 3.2.1.) for 28 days. Microsomes from the livers of these animals were prepared according to the method described in Section 3.2.3. using a buffer (pH 7.3) containing 250 mM sucrose, 2.5 mM EDTA and 30 mM nicotinamide. The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.3).

The final incubation mixture contained microsomal suspension, about 1.5 - 2.0 mg protein, 4 mM $MgCl_2$, 1 mM $NADP^+$, 10 mM glucose-6-phosphate, 1 I.U. of glucose-6-phosphate dehydrogenase, 20 mM cysteamine and 300 μM (0.5 μCi) of [7(n) - ^{3}H] cholesterol in a final volume of 1 ml. A detergent (Tween 80) in a concentration of 1 mg/assay was used to solubilise the [7(n) - ^{3}H] cholesterol in the incubation medium. After 30 minutes at 37°C in a shaking water-bath, the reaction was stopped with 0.2 ml of 50% TCA.

[^{3}H] water was isolated by distillation under reduced pressure according to the method described by Hutton et al (1966). An aliquot of the thawed [^{3}H] water was counted in 10 ml of Bray's scintillation fluor containing PPO (5 g), POPOP (0.1 g), naphthalene (80 g) in one litre of toluene, dioxane and ethanol (3.5 : 3.5 : 2.0 , v/v/v).

7.2.2. Enzyme activity

Under the conditions explained above, the tritiated water released into the incubation medium reflects the amount of 7α-hydroxycholesterol formed and hence, determines accurately the cholesterol 7α-hydroxylase activity. Assuming that stoichiometry was achieved and there were no complicating isotopic effects (Johnson et al, 1976) enzyme activity was expressed as p moles of cholesterol hydroxylated per minute per mg microsomal protein. In addition it was assumed that the tritiated water released was mainly, if not entirely, due to the action of cholesterol 7α-hydroxylase and contribution by other microsomal enzymes, such as cholesterol 7β-hydroxylase and non-enzymic auto-oxidation was negligible.

The participation of endogenous cholesterol was taken into account while calculating the enzyme activity as suggested by Van Cantfort and Gielen (1975). They proposed that 70% of the microsomal-bound cholesterol is directly accessible to cholesterol 7α-hydroxylase and this endogenous cholesterol makes a single pool with the exogenously added substrate. They further demonstrated that cholesterol esters do not participate in the cholesterol 7α-hydroxylation reaction.

According to the manufacturer's specifications (New England Nuclear, Boston, Mass., U.S.A.) approximately 70 - 90% of the tritium in [7(n) - ^{3}H] cholesterol resides at C-7, with most of the remainder at C-4. Since the ratio of 7α to 7β tritium is not known, no correction could be made and thus the observed cholesterol 7α-hydroxylase activity is not absolute. However, as the same batch of [7(n) - ^{3}H] cholesterol

was used in the same assay run, the results obtained should be comparable, as the correction factor would be the same.

7.2.3. Assay Conditions for microsomal cholesterol 7α-hydroxylase

On using a saturating level of [7(n) - ^{3}H] cholesterol, the enzyme activity was found to be linear with time over at least 60 minutes incubation period (Figure 20). The rate of cholesterol hydroxylation was also proportional to the microsomal protein concentration upto 2 mg per incubation (Figure 21). Liver microsomal cholesterol 7α-hydroxylase appears to be saturated at 300 μM concentration of exogenous cholesterol (Figure 22). These results are in agreement with the observations of Van Cantfort et al (1975). Therefore, in most of the subsequent assays, a 30 minute incubation time was employed and the microsomal protein concentration varied from 1.5 - 2.0 mg/incubation.

7.2.4. Cholesterol 7α-hydroxylase activity after feeding the rats various lipid-supplemented diets

The addition of either 15% coconut oil or 15% sunflower seed oil to the reference diet resulted in a lowering of enzyme activity (Table 15). Inclusion of 2% cholesterol into these diets enhanced the rate of cholesterol hydroxylation approximately 2 fold, irrespective of the amount and type of the fat-fed in the diet. However, microsomal preparations from rats fed sunflower seed oil plus cholesterol exhibited significantly lower cholesterol 7α-hydroxylase activity than in the microsomal preparations from those fed either coconut oil plus

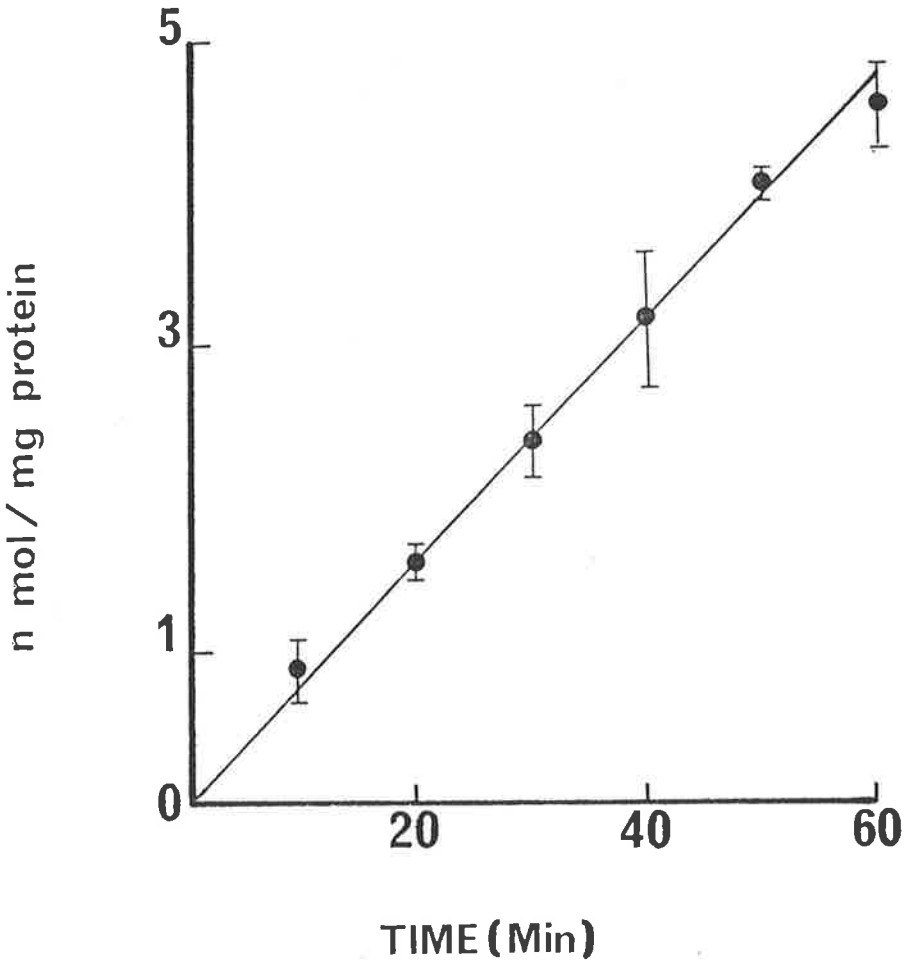


Figure 20

Time course of the activity of microsomal cholesterol 7α - hydroxylase. The freshly prepared microsomal ~~enzyme~~ preparation was assayed as described in section 7.2.1. except that the time of incubation was varied as shown. Each point on the curve is the mean ± S.D. of duplicate measurements.

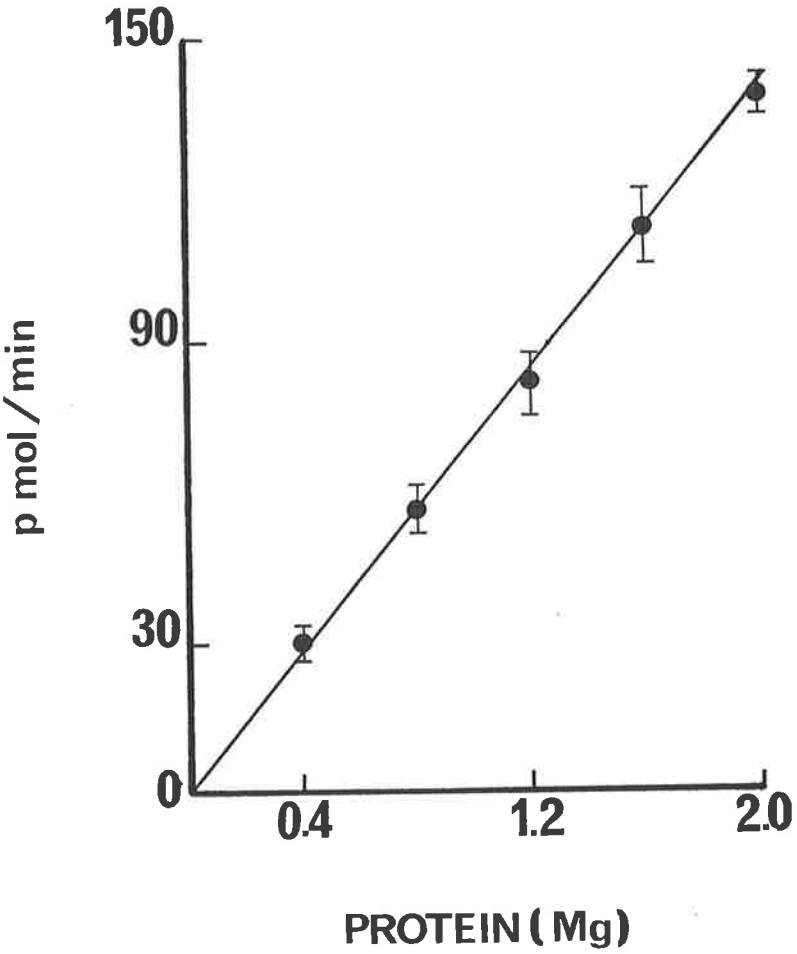


Figure 21

Effect of varying the microsomal protein concentration on the activity of cholesterol 7α - hydroxylase. Freshly prepared ~~enzyme was~~ microsomes were assayed as described in section 7.2.1. except that the microsomal protein concentration was varied as shown. Each point on the curve represents the mean ± S.D. of duplicate measurements.

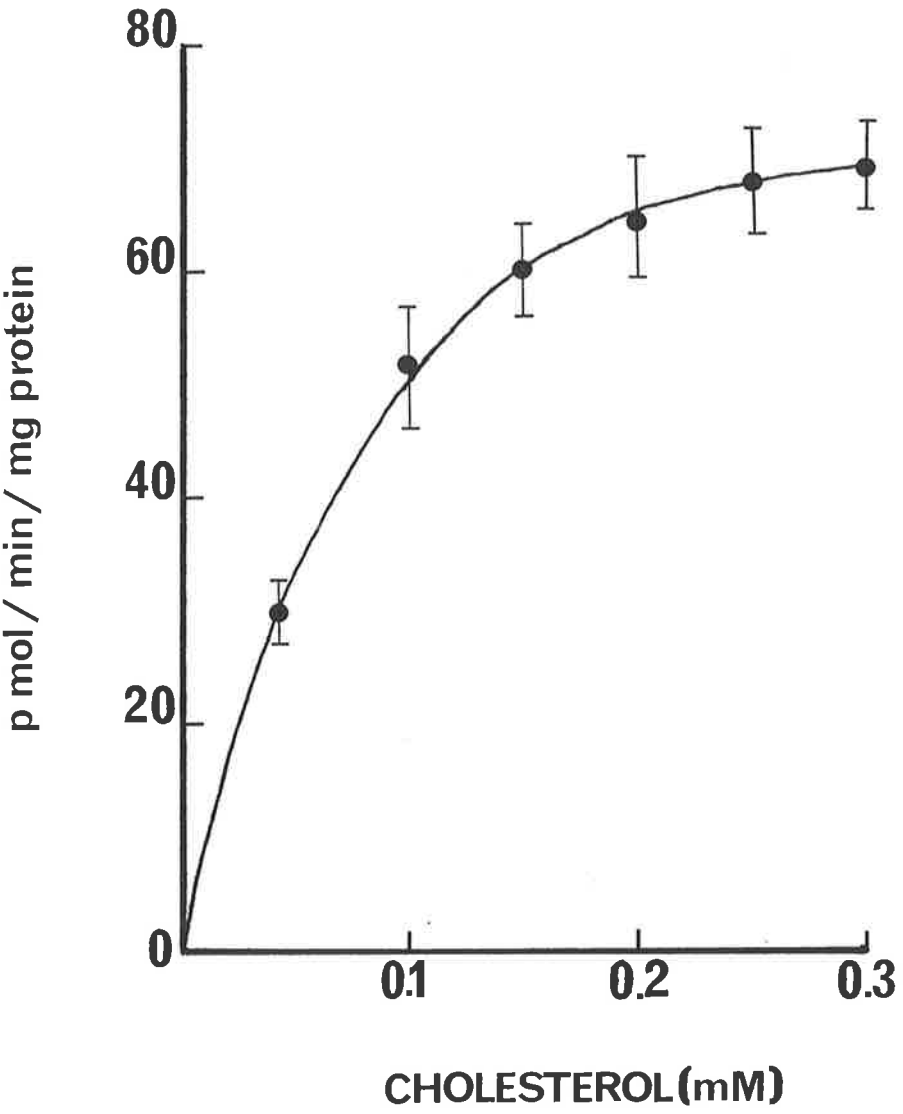


Figure 22

Effect of varying the substrate concentration on cholesterol 7α-hydroxylase activity. The freshly prepared ~~enzyme was~~ microsomes were assayed as described in section 7.2.1. except that the cholesterol concentration was varied as shown. Each point is the mean ± S.D. of duplicate measurements.

cholesterol or reference plus cholesterol diet (Table 15).

7.2.5. Arrhenius plot characteristics of cholesterol 7α-hydroxylase activity after dietary lipid supplementation

Arrhenius plots of cholesterol 7α-hydroxylase activity in liver microsomes of rats fed low cholesterol (REF, SO, CO) diets and these diets supplemented with 2% cholesterol are shown in Figures 23-25. Microsomal preparations from livers of rats fed the REF diet exhibited a sudden decrease in the enzyme activity at 27.6°C (Tc) resulting into an abrupt change in activation energy value from 7.1 K Cal/mole above the Tc to 15.7 K Cal/mole below the Tc (Table 16). The inclusion of 15% sunflower seed oil or 15% coconut oil in the REF diet increased the transition temperature observed in the Arrhenius plot of cholesterol 7α-hydroxylase activity. CO feeding had its main effect on the activation energy (Ea_1) value above the Tc, which was found to be higher than for the rats on REF diet. On the other hand, the effect of SO feeding is mainly to increase the activation energy (Ea_2) below the Tc (Table 16). The major effect of adding 2% cholesterol to the low cholesterol diets was to abolish the break point in Arrhenius plot leading to a constant activation energy (Ea) between 15 ↔ 37°C. This activation energy value was higher when rats were fed cholesterol along with high amounts of SO or CO in comparison to those fed 2% cholesterol without any added fat (Table 16).

7.2.6. Discussion

The results presented here have demonstrated that dietary fatty acids

Table 15. Cholesterol 7α - hydroxylase activity in liver microsomes of rats fed various lipid - supplemented diets.

2% Cholesterol	Reference diet	Sunflower seed oil diet	Coconut oil diet
	p mol cholesterol hydroxylated / mg protein / minute		
-	66.43 ± 3.15	52.07 ± 1.71[a**]	52.77 ± 3.42[a*]
+	114.86 ± 10.27[b***]	99.15 ± 4.89[b***]	111.64 ± 12.89[b***]

The experimental conditions are described in section 7.1 and the enzyme assay in section 7.2.1.

The values are the mean ± S.D. of triplicate measurements in liver microsomal preparations from rats (n = 4 - 5) in each dietary group.

a. Significantly different from the reference diet-fed animals : *, $P < 0.05$; **, $P < 0.01$.

b. Significantly different from the respective low cholesterol diet-fed animals : ***, $P < 0.001$

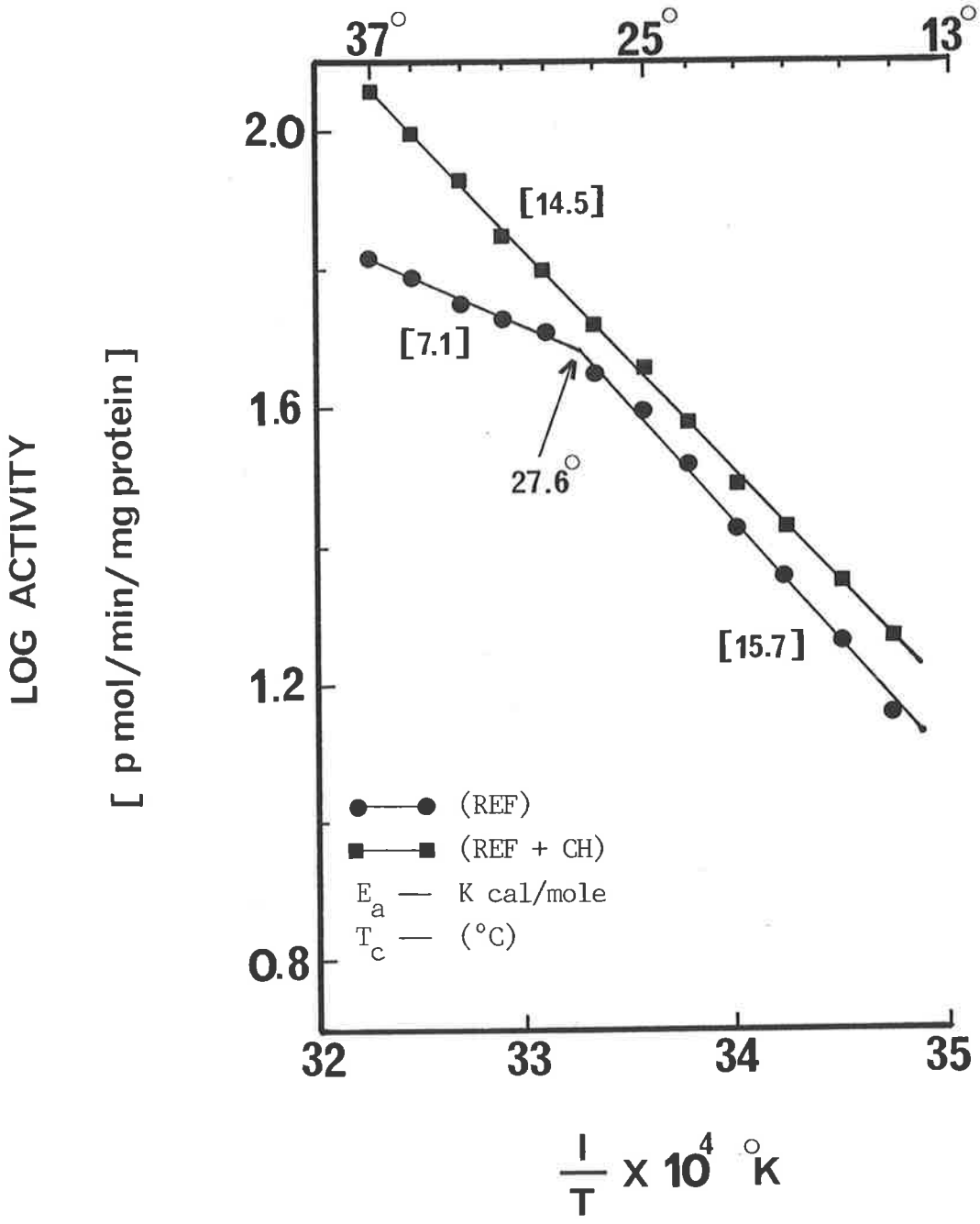


Figure 23

Arrhenius plots of liver microsomal cholesterol 7α- hydroxylase from rats fed a reference (REF) or 2% cholesterol (REF + CH) diet. The activation energy (E_a) values are shown in brackets and arrows indicate the transition temperatures (T_c).

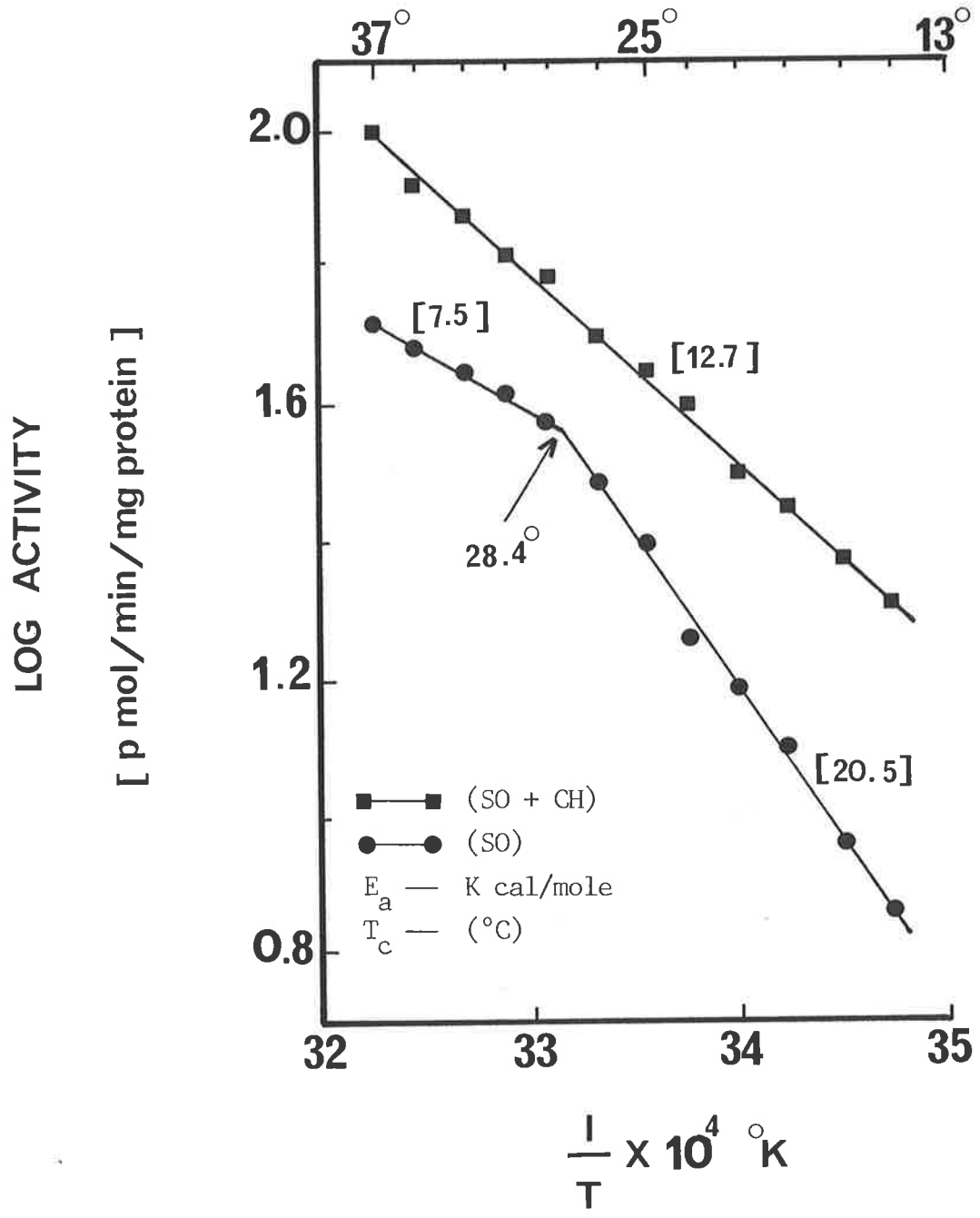


Figure 24

Arrhenius plots of liver microsomal cholesterol 7α- hydroxylase from rats fed a 15% sunflower seed oil (SO) or 15% sunflower seed oil plus 2% cholesterol (SO +CH) diet. The activation energy (E_a) values are shown in brackets and arrows indicate the transition temperatures (T_c).

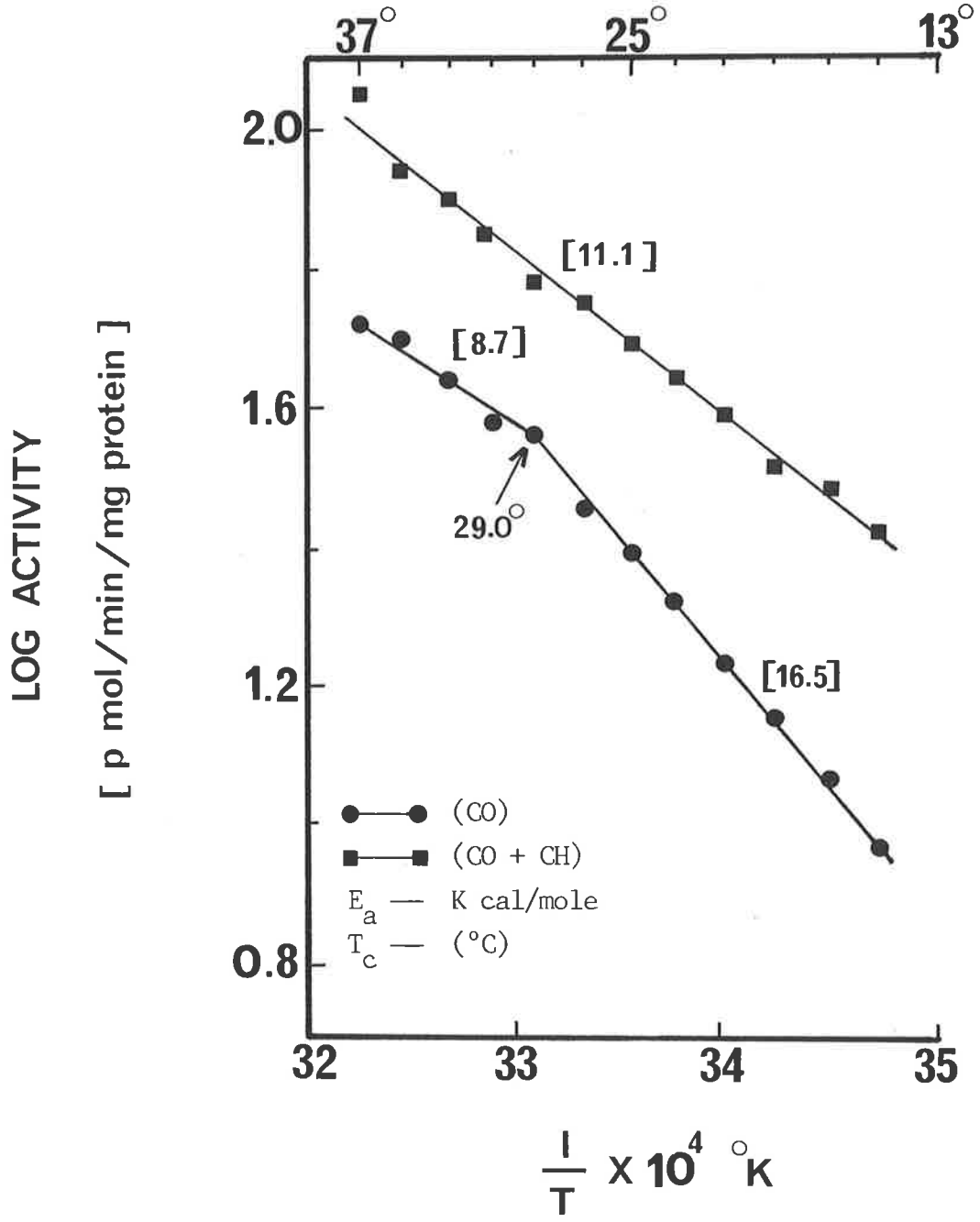


Figure 25

Arrhenius plots of liver microsomal cholesterol 7α- hydroxylase from rats fed a 15% coconut oil (CO) or 15% coconut oil plus 2% cholesterol (CO +CH) diet. The activation energy (E_a) values are shown in brackets and arrows indicate the transition temperatures (T_c).

Table 16. Arrhenius plot characteristics of cholesterol 7α - hydroxylase from liver microsomes of rats fed various lipid - supplemented diets.

Parameter	2% Cholesterol	REF	SO	CO
T_c	-	27.6	28.4	29.0
	+	-	-	-
Ea_1	-	7.1 ± 0.3	7.5 ± 0.2	8.7 ± 0.5[a*]
	+	14.5 ± 0.2	12.7 ± 0.2[b**]	11.1 ± 0.1[b***]
Ea_2	-	15.7 ± 0.1	20.5 ± 0.6[a**]	16.5 ± 0.2[a*]
	+	-	-	-

Arrhenius plot parameters were determined as described in section 7.1.2. T_c represent the transition temperature in degree centigrades. Ea_1 represents activation energy above the T_c and Ea_2 represents activation energy below the T_c and are expressed in K cals / mole.

a. Significantly different from reference diet - fed animals : *, $P < 0.05$; **, $P < 0.01$.

b. Significantly different from cholesterol plus reference diet - fed animals : **, $P < 0.01$; ***, $P < 0.001$.

and cholesterol, individually and together modify both cholesterol 7α-hydroxylase activity and its Arrhenius plot characteristics. For reasons in Section 7.2.2., the values of enzyme activity obtained are not absolute. It is likely that enzyme activity in cholesterol-fed rats was underestimated due to the dilution of exogenous cholesterol substrate by the endogenously increased microsomal cholesterol content following cholesterol feeding. Also, the critical assumption made during the calculations of cholesterol 7α-hydroxylase activity was that microsomal cholesterol esters do not participate in the 7α-hydroxylation reaction and these are present as much as 16 times more in (SO + CH) diet group than in the REF diet group, (Chapter 4). Both the saturated and unsaturated fat-enriched diets without any added cholesterol lowered the 7α-hydroxylation of cholesterol in liver microsomes. The unsaturated fat-enriched diet was rich in long chain unsaturated fatty acids (>57% of 18:2) and the saturated fat-enriched diet was rich in medium chain fatty acids (>55% of 8:0 + 10:0 + 12:0), whereas the REF diet contained no medium chain fatty acids and a relatively lesser amount of long chain unsaturated fatty acids (c.f. Chapter 4). It has been previously shown (Carrol, 1958; Bjorkhem et al, 1978) that the degree of absorption of medium chain saturated and long chain unsaturated fatty acids is much higher (more than 80%) than that of long chain saturated fatty acids (less than 55%). Thus it is likely that in the present study, the fatty acids of both the high fats diets were readily absorbed which can be associated with an increased number of circulations of each bile acid from the intestine. This increased enterophepatic circulation of bile acids may have inhibited the cholesterol 7α-hydroxylase activity in the liver by a feedback mechanism, as previously suggested by Bjorkhem et al (1978).

Because of increased bile circulation following administration of these high fat diets, a decrease in fecal sterol excretion would be anticipated in these animals. In this respect, the results are consistent with the fecal sterol excretion data of Bochenek and Rodgers (1978) who, using the same sources for saturated and unsaturated fats, demonstrated that the fecal excretion of both netural and acidic sterols was decreased when compared with a low fat, control diet.

Addition of 2% cholesterol in the REF or saturated fat-enriched diet accelerated the rate of cholesterol hydroxylation in the liver microsomes to the same extent, but this accerlation was relatively slower when cholesterol was fed with unsaturated fat. All three high cholesterol diets raised the free cholesterol content of liver microsomes to the same extent, however the esterified cholesterol content was very much different in the three high cholesterol diet groups and is in the order (SO + CH) > (CO + CH) > (REF + CH) diet (Chapter 4). Spector et al (1980) have shown that a PUFA-enriched diet elevates the acylcoenzyme A : cholesterol acyltransferase (ACAT) activity in the liver microsomes, in comparison to a saturated fat-enriched diet. Thus, the conditions for cholesterol esterification, which is an alternative route of cholesterol metabolism in the liver, are more favourable when cholesterol is fed with unsaturated rather than saturated fat. Consequently, liver cholesterol in the (SO + CH) group has a greater tendency to be esterified and a lower tendency to be hydroxylated with a lower cholesterol 7α-hydroxylase activity as compared to (CO + CH) or (REF + CH) groups. These results on cholesterol 7α-hydroxylase activity are

again consistent with the observations of Bochenek and Rodgers (1978), who showed that cholesterol feeding with saturated fat increased the fecal excretion of netural sterols by a factor of 30, while the value for high cholesterol-unsaturated fat group was only 15 times the value observed for the respective low cholesterol diet.

No consistent relationship between dietary fat, membrane lipid composition and Arrhenius plot parameters of cholesterol 7α-hydroxylase activity was found. Regardless of the type and amount of fat fed, the point to note is that, like HMG-CoA reductase, the Arrhenius plots of cholesterol 7α-hydroxylase from livers of rats fed 2% cholesterol were linear within the temperature range of 15 - 37°C. The DSC scans of microsomal membrane lipids from livers of rats exhibited two clear separate transitions inspite of cholesterol enrichment of these membranes following cholesterol feeding (Chapter 6). This indicates that like HMG-CoA reductase, cholesterol 7α-hydroxylase also has a specific microenvironment ("boundary lipid") which has a higher cholesterol content than that of the bulk lipids of the microsomal membranes. Mitropoulos et al (1978) have even suggested that the pool of cholesterol in the immediate environment of HMG-CoA reductase that directly affects the enzyme activity, and the cholesterol pool that acts a substrate for cholesterol 7α-hydroxylase, are the same.

In brief then, these results demonstrate that the enzyme cholesterol 7α-hydroxylase, appears to co-operate with HMG-CoA reductase for the maintenance of cholesterol homeostasis in the microsomal membranes. Under the conditions of increased cholesterol in the liver microsomes

the activity of HMG-CoA reductase is inhibited and concomitantly, the rate of hydroxylation of cholesterol is enhanced so as to affect the removal of the excess.

7.3. Acylcoenzyme A: cholesterol acyltransferase (ACAT) (EC 2.3.1.26) and Acylcoenzyme A hydrolase (ACH) (EC 3.1.2.2)

ACAT catalyzes the transfer of a long chain fatty acid to the 3β-hydroxyl position of cholesterol molecule from appropriate fatty acyl CoA molecule. Like most of the acyltransferases that catalyze lipid ester synthesis, ACAT is also bound tightly to the intracellular membranes, recovered primarily in the rough endoplasmic reticulum (Balasubramaniam et al, 1978) and is located topologically on the cytoplasmic surface (Hashimoto and Fogelman, 1980; Lichtenstein and Brecher, 1980). In the liver, changes in the activity of this enzyme may be partly responsible for the maintenance of the cholesterol ester content in conjunction with other processes such as the hydrolysis of esters and their transfer into and out of the liver. The close relationship between free and esterified cholesterol has been discussed in Section 2.8.2.4. The maintenance of a balance between non-esterified cholesterol concentration and that of cholesterol esters appears to be important in cellular cholesterol homeostasis.

In view of the possible involvement of ACAT in the accumulation of cholesterol esters in the arterial wall during the development of atherosclerosis (Hashimoto and Dayton, 1977; Brecher and Chobanian, 1974) it is important to determine the factors that regulate the activity of this enzyme. In addition, ACAT might be important in

regulating the activity of HMG-CoA reductase, as both of these enzymes are present on the same membranes and the rate of cholesterol ester formation has been shown to be regulated under conditions associated with changes in the rate of cholesterol synthesis (Brown et al, 1975; Faust et al, 1977; Balasubramaniam et al, 1977).

Acylcoenzyme A hydrolase (ACH) catalyzes the hydrolysis of long chain acylcoenzyme A thioester to CoASH and fatty acid. ACH activity has been detected in cytoplasm (Bedord et al, 1978), mitochondrial and microsomal fractions (Berge and Farstad, 1979) and peroxisomes (Berge et al, 1982). However, the major functional roles of these enzyme activities are still not clear. Interest in ACH in the present exercise is mainly because the end product of hydrolase action, i.e. free fatty acid, has been shown to be inhibitory for ACAT activity (Goodman et al, 1964). Thus the microsomal ACH activity must be considered when ACAT activity is measured.

7.3.1. ACAT and ACH assays

Rats were fed the experimental diets explained in Section 3.2.1. for 28 days. Microsomes from livers of these animals were isolated according to the procedure described in Section 3.2.3. and microsomal pellet resuspended in 0.1 M K_2HPO_4 buffer (pH 7.2). ACAT and ACH were assayed simultaneously in the same reaction mixture essentially by the method of Spector et al (1980).

The reaction mixture contained in a final volume of 0.5 ml; 100-200 µg of microsomal protein, 0.1 M K_2HPO_4 (pH7.2) and 1 mM dithiothreitol.

The reaction was started by the addition of 10 nmoles of [1 - ^{14}C] palmitoyl coenzyme A (≃ 0.05 μCi). The incubations were carried out for 5 minutes at 37°C with shaking and the reaction was terminated with 2 ml of choloroform : methanol (2:1, v/v) containing 0.005% (w/v) butylated hydroxytoluene as an antioxidant.

Lipids from the samples were extracted into the chloroform phase by the use of a mechanical shaker. Extraction of the lipids was essentially complete, as no significant radioactivity was detectable in the residual aqueous layer. Cholesterol esters and free fatty acids produced as a result of ACAT and ACH actions, respectively, were separated by thin layer chromatography on silica gel G plates as previously described in Section 3.2. The zones along the chomatographic plate corresponding to cholesterol esters and free fatty acids were scrapped off directly into the scintillation vials containing 10 ml of toluene fluor (5 g PPO, 0.1 g POPOP in 1 litre of toluene). Using the known specific activity of [1 - ^{14}C] palmitoyl CoA substrate, the ACAT activity was expressed as p moles of cholesterol palmitate formed per minute per mg microsomal protein and ACH activity as p moles of palmitic acid formed per minute per mg microsomal protein.

7.3.2. Assay conditions for ACAT

The time course for the formation of cholesteryl palmitate shown in Figure 26 illustrates that linear rates were obtained during the first 6 minutes of incubation with [1-C^{14}] palmitoyl CoA as the substrate. Thus a 5 minute incubation time was employed in the subsequent assays.

Enzyme assays were carried out with 10 nmol of palmitoyl CoA (20 μM) as the substrate, however similar results were obtained when palmitoyl CoA concentration was varied between 10 and 40 μM (Figure 27). Under these conditions, the assay is linear over the range of 30-180 μg microsomal protein (Figure 28). These observations are in agreement with those of Spector et al (1980).

7.3.3. Effect of dietary fatty acids and/or cholesterol on ACAT and ACH activity

ACAT activity in liver microsomes of rats fed the experimental diets is shown in Table 17. The rate of cholesterol ester formation was significantly elevated in the liver microsomes of rats fed 15% sunflower seed oil while 15% coconut oil diet had no appreciable effect in comparison to a low fat, reference diet. Administration of 2% cholesterol into the REF diet increased the ACAT, but the difference did not reach to a significant level ($P > 0.05$) (Table 17). On the other hand, feeding 2% cholesterol along with high fats significantly raised the ACAT activity. Within the high cholesterol-high fat groups, the animals on cholesterol plus sunflower seed oil (SO + CH) diet exhibited higher ACAT activity than those on cholesterol plus coconut oil (CO +CH) diet, however the difference did not reach a significant level (Table 17). Acyl-CoA hydrolase (ACH) activity in the liver microsomes was not affected to any significant level by any of the experimental diets (Table 18).

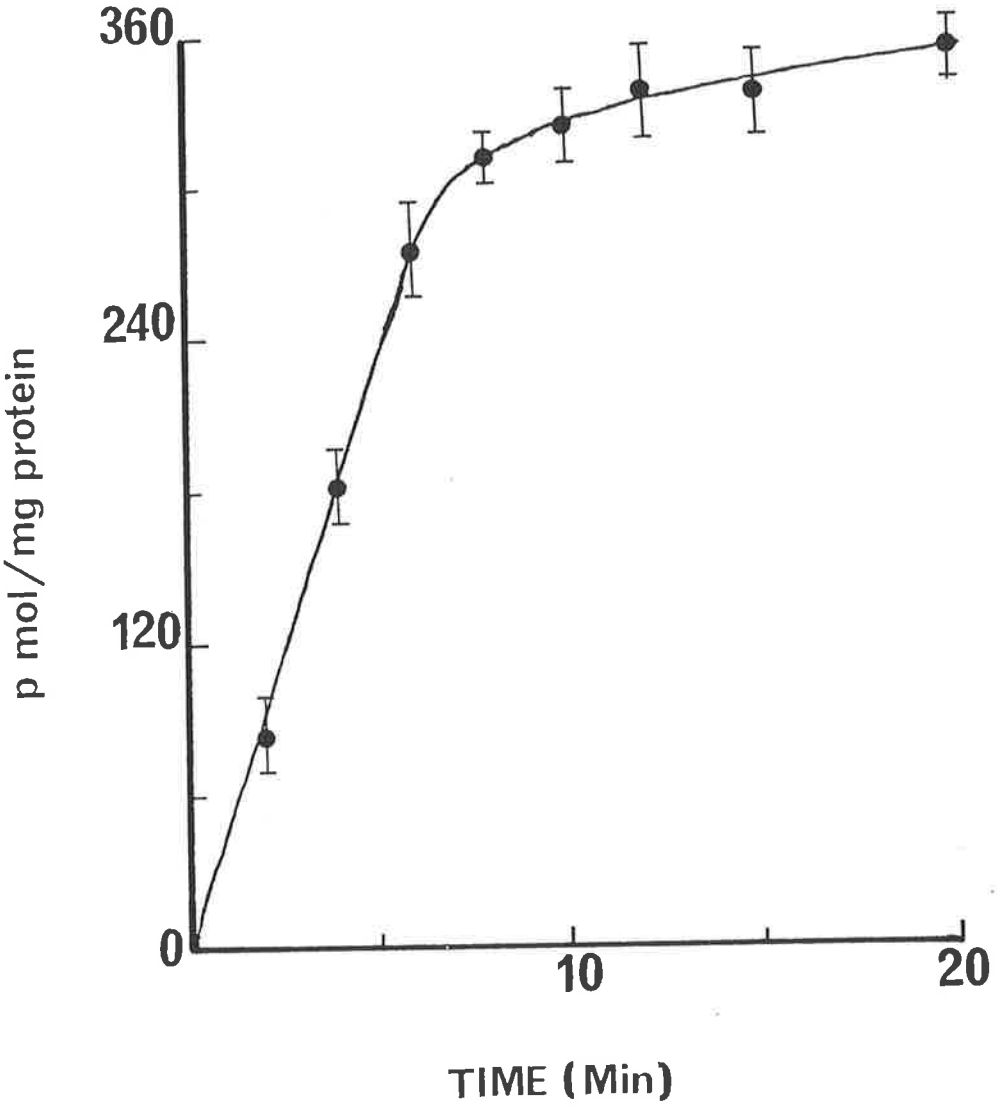


Figure 26

Time course of ACAT activity. The freshly prepared microsomal preparation was assayed as described in section 7.3.1. except that the incubation time was varied as shown. Each point on the curve represents the mean ± S.D. of duplicate measurements.

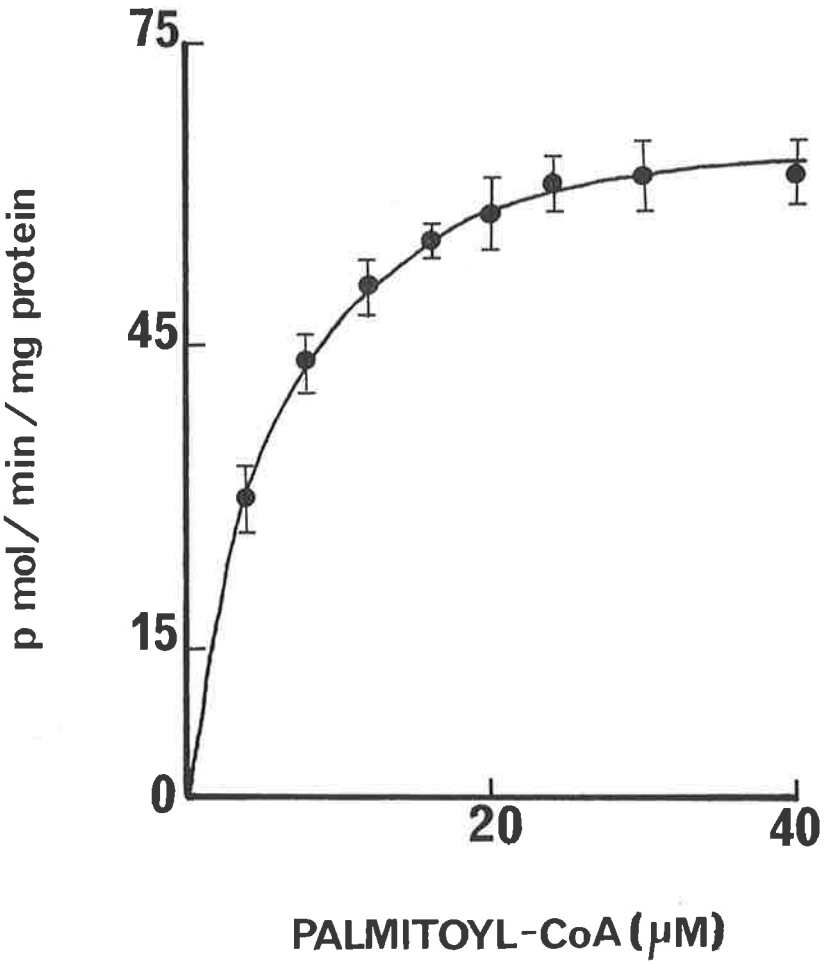


Figure 27

Effect of varying palmitoyl-CoA concentration on ACAT activity. The freshly prepared microsomal ~~enzyme~~ preparation was assayed as described in section 7.3.1. except that the concentration of palmitoyl-CoA was varied as shown. Each point on the curve is the mean ± S.D. of duplicate measurements.

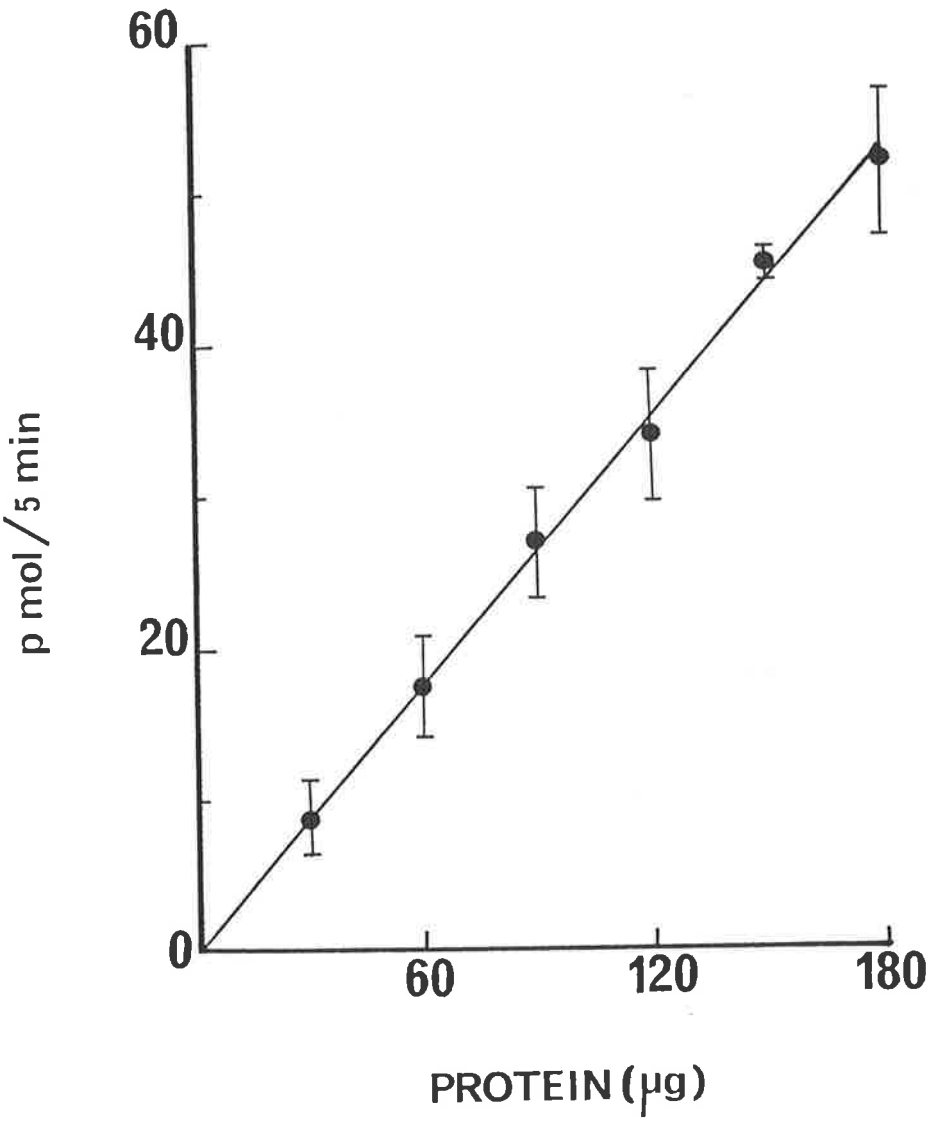


Figure 28

Effect of varying enzyme concentration on ACAT activity. The freshly prepared microsomal ~~enzyme~~ preparation was assayed as described in section 7.3.1. except that the microsomal protein concentration was varied as shown. Each point represents the mean ± S.D of duplicate measurements.

Table 17. Acylcoenzyme A : Cholesterol acyltransferase activity in liver microsomes of rats fed various lipid - supplemented diets.

2% Cholesterol	Reference diet	Sunflower seed oil diet	Coconut oil diet
	p mol cholesterol palmitate formed / mg protein / minute		
-	46.77 ± 5.43	75.86 ± 2.73[a***]	53.70 ± 3.90
+	54.95 ± 4.54	99.67 ± 3.76[b***]	91.20 ± 2.88[b***]

Experimental conditions are described in section 7.1 and the enzyme assay in section 7.3.1.

The values are the mean ± S.D. of triplicate measurements in liver microsomal preparations from rats (n = 4 - 5) in each dietary group.

a. Significantly different from reference or coconut oil diet-fed animals : ***, $P < 0.001$

b. Significantly different from the respective low cholesterol diet-fed animals :***, $P < 0.001$.

Table 18. Acylcoenzyme A hydrolase activity in liver microsomes of rats fed various lipid - supplemented diets.

2% Cholesterol	Reference diet	Sunflower seed oil diet	Coconut oil diet
	p mol palmitic acid formed / mg protein / minute		
-	624 ± 86	709 ± 60	684 ± 20
+	551 ± 33	650 ± 62	736 ± 73

The experimental conditions are described in section 7.1 and the enzyme assay in section 7.3.1.

The values are the mean ± S.D. of triplicate measurements in liver microsomal preparations from rats (n = 4 - 5) in each dietary group.

None of the values in experimental groups is significantly different from reference diet - fed animals : $P > 0.05$.

7.3.4. Arrhenius behaviour of ACAT after dietary lipid supplementation

Arrhenius plots of liver microsomal ACAT from rats fed various high lipid diets are shown in figures 29-31. Animals fed a REF diet exhibited Arrhenius plots which showed that there was a sudden decline in the enzyme activity at 28.5°C (Tc) resulting in an abrupt increase of activation energy from 13.9 K Cal / mole (Ea_1) above this temperature to 31.7 K Cal/mole (Ea_2) below this temperature (Table 19). The addition of either sunflower seed oil or coconut oil to the reference diet had no appreciable effect on the transition temperature (Tc). The activation energy values (Ea_1) above the Tc were also essentially unaffected however activation energy (Ea_2) below the Tc was significantly lower in the high fat-diet groups (Table 19). The magnitude of decrease was significantly higher when rats were supplemented with 15% sunflower seed oil rather than 15% coconut oil (Table 19).

Addition of 2% cholesterol to the reference diet had no significant effect on either the Tc or the activation energy values observed in the Arrhenius plot of ACAT activity. Both the sunflower seed oil and coconut oil feeding with 2% cholesterol decreased the transition temperature (Tc) observed in the Arrhenius plot of enzyme activity from rats fed the respective low cholesterol diet (Table 19). Again Ea_1 is not affected to any considerable extent but Ea_2 values were elevated in high cholesterol-high fat groups in comparison to their respective low cholesterol groups (Table 19).

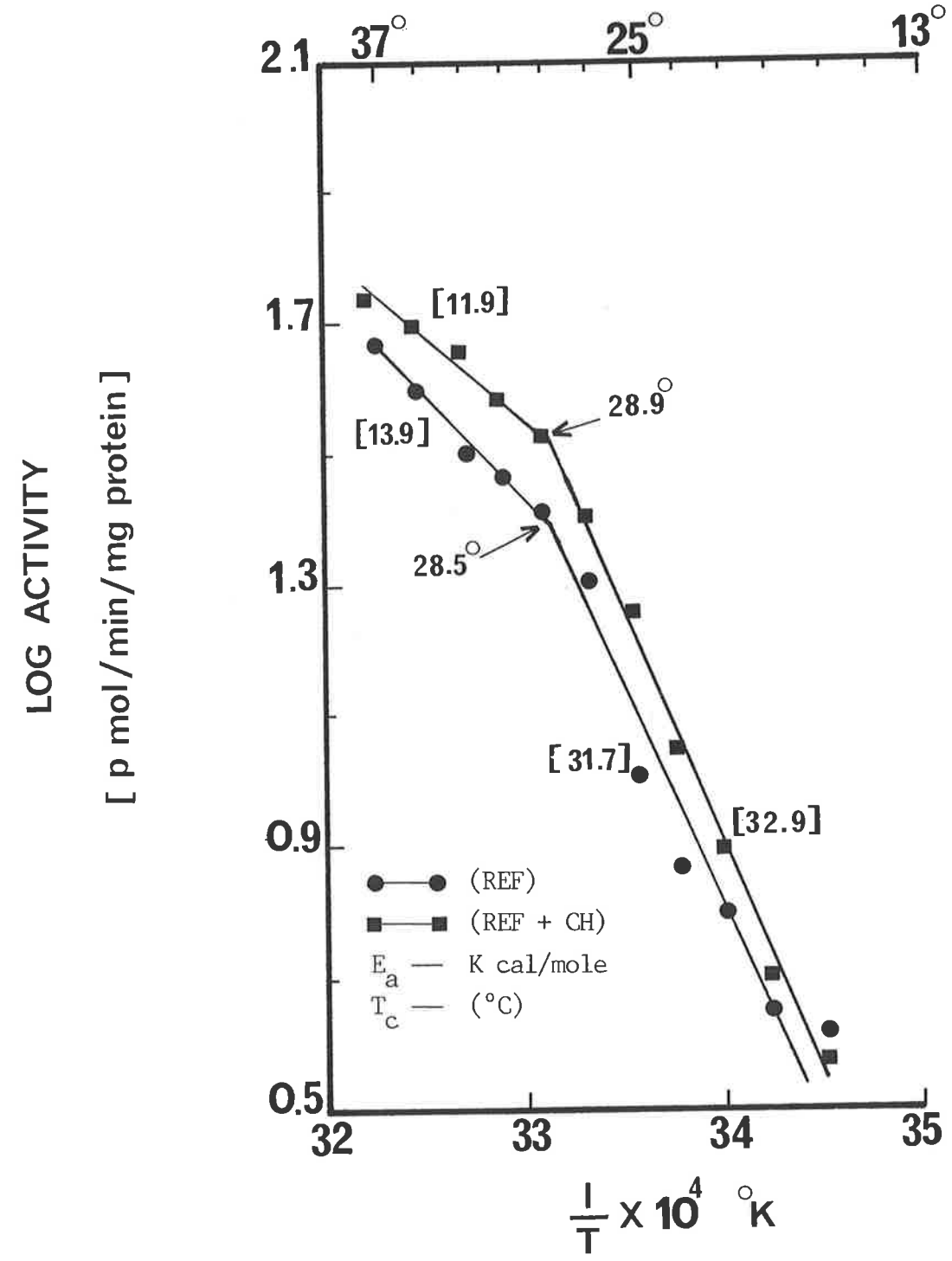


Figure 29

Arrhenius plots of liver microsomal ACAT from rats fed a reference (REF) or 2% cholesterol (REF + CH) diet. The activation energy (E_a) values are shown in the brackets and arrows indicate the transition temperatures (T_c).

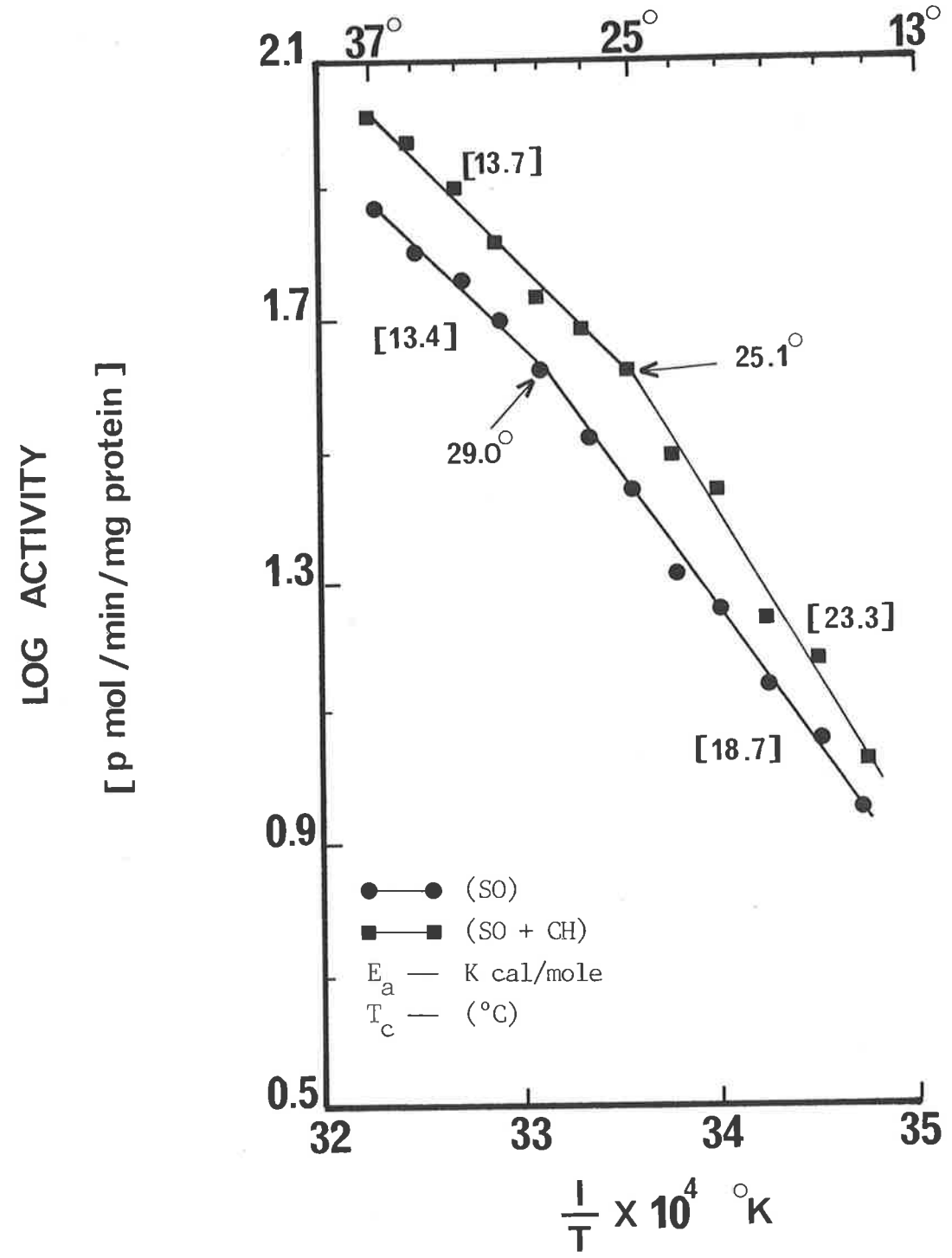


Figure 30

Arrhenius plots of liver microsomal ACAT from rats fed a 15% sunflower seed oil (SO) or 15% sunflower seed oil plus 2% cholesterol (SO + CH) diet. The activation energy (E_a) values are shown in brackets and arrows indicate the transition temperatures (T_c).

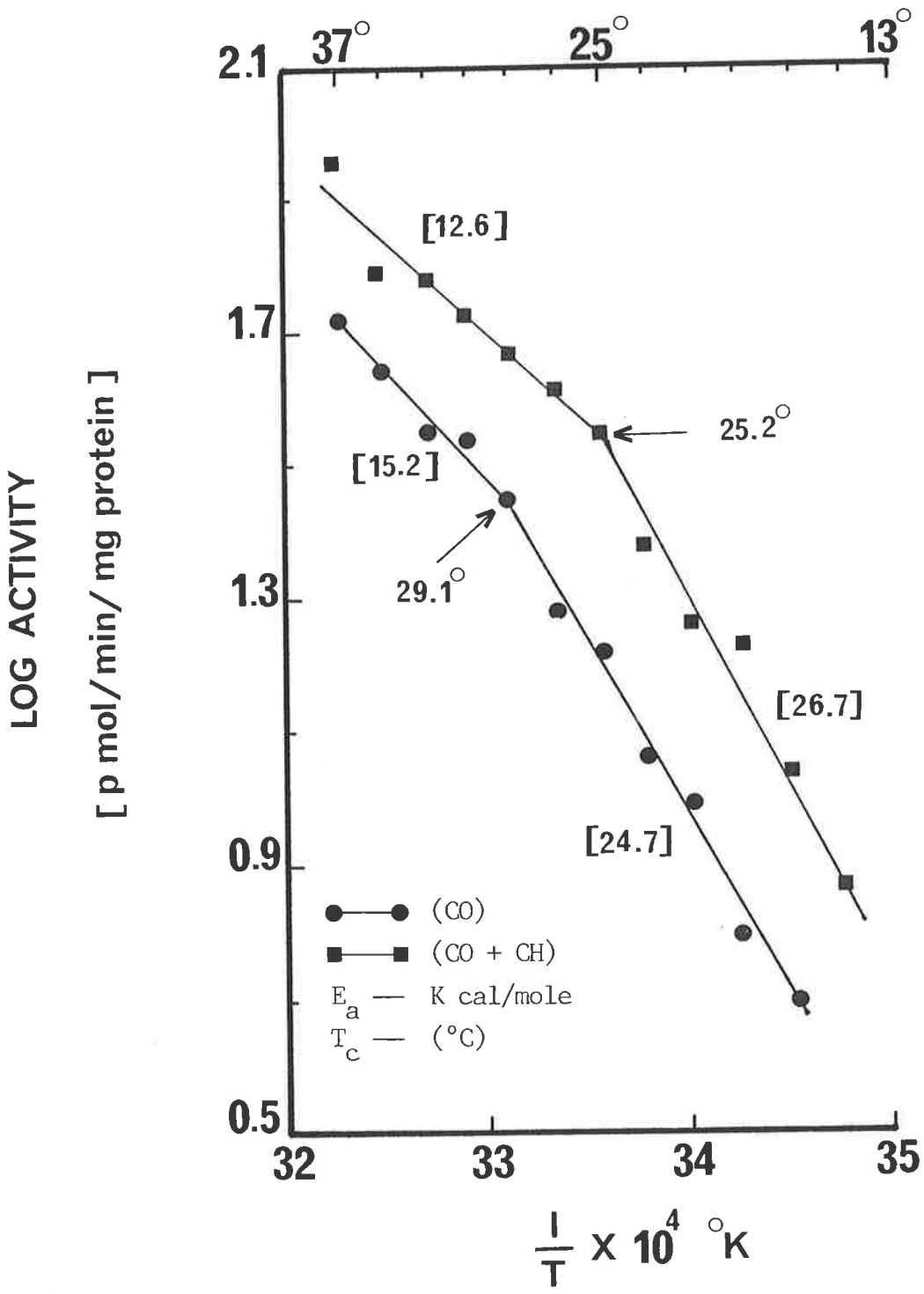


Figure 31

Arrhenius plots of liver microsomal ACAT from rats fed a 15% coconut oil (CO) or 15% coconut oil plus 2% cholesterol (CO + CH) diet. The activation energy (E_a) values are shown in brackets and arrows indicate the transition temperatures (T_c).

Table 19. Arrhenius plot characteristics of Acylcoenzyme A : cholesterol acyltransferase from liver microsomes of rats fed cholesterol- supplemented diets.

Parameter	2% Cholesterol	REF	SO	CO
T_c	-	28.5	29.0	29.1
	+	28.9	25.1	25.2
Ea_1	-	13.9 ± 0.3	13.4 ± 0.4	15.2 ± 0.6
	+	11.9 ± 0.4[a*]	13.7 ± 0.1	12.6 ± 0.1[a*]
Ea_2	-	31.7 ± 0.4	18.7 ± 0.2[b***]	24.7 ± 0.3[b**]
	+	32.9 ± 1.1	23.3 ± 0.2[c**]	26.7 ± 0.9[c*]

Arrhenius plot parameters were determined as described in section 7.1.2. T_c represents the transition temperature in degree centigrades. Ea_1 represents activation energy above the T_c and Ea_2 represents activation energy below the T_c and are expressed in Kcals / mole.

a. Significantly different from respective low cholesterol diet - fed animals : *, $P < 0.05$.

b. Significantly different from reference diet - fed animals : **, $P < 0.01$; ***, $P < 0.001$.

c. Significantly different from cholesterol plus reference diet - fed animals : *, $P < 0.05$; **, $P < 0.01$.

7.3.5. Discussion

It is quite clear from the present exercise that liver microsomal ACAT activity responds to the amount of cholesterol present in the diet. In addition, the results demonstrate that the type of fatty acids fed with or without exogenous cholesterol has a remarkable effect on ACAT activity and its Arrhenius plot characteristics. There was a 1.5 fold increase in the enzyme activity following the feeding of a diet rich in PUFA in comparison to a diet rich in SFA. A similar enhancement of ACAT activity in response to a PUFA - enriched diet has been reported in microsomes from rabbit intestine (Field and Salome, 1982) and rat liver (Spector et al, 1980). In the present study, the increase in ACAT activity as a result of PUFA feeding was accompanied by an elevation in the cholesterol ester content of liver microsomes (c.f. Chapter 4) and of whole liver (c.f. Chapter 8). However, Spector et al (1980) failed to detect any such increase in free or esterified cholesterol content in liver or liver microsomes. It has been already discussed (Chapter 4) that the conditions for the cholesterol transport from gut to the liver are more favourable when a diet rich in PUFA is fed. Therefore it is possible that the observed increase in ACAT activity in liver microsomes after PUFA feeding was due to an increased availability of cholesterol as a substrate. A much larger (more than 3 times) accumulation of cholesterol esters occurred in the liver microsomes, when 2% cholesterol was fed along with unsaturated instead of saturated fat (c.f. Chapter 4). The greater enhancement of ACAT activity following cholesterol feeding with unsaturated fat is also consistent with the idea that unsaturated fat increases the availability of cholesterol for ACAT action.

It is possible that different fatty acids and/or cholesterol feeding resulted in different amounts of free fatty acids in the liver microsomes which have been shown to be inhibitory for ACAT activity (Goodman et al, 1964). However this seems unlikely in view of the Acyl CoA hydrolase results. Microsomal preparations from all the dietary groups hydrolyzed palmitoyl CoA to the same extent, indicating that the concentration of free fatty acids in the liver microsomes was similar in all the experimental groups. The possibility that acyl CoA hydrolase has a different affinity for fatty acyl CoA, other than palmitoyl CoA, however cannot be ignored.

The Arrhenius plots of ACAT activity show that the transition temperature (Tc) was unaffected by the type of dietary fat-fed. Also the activation energy (Ea_1) above the Tc is unaltered in both the cases. On the other hand, DSC scans of microsomal membrane lipids from rats fed saturated and unsaturated fats exhibited different thermotropic properties. The phase transition in the saturated fat group occured at a higher temperature than in the unsaturated fat group (c.f. Chapter 5). If the ACAT has any relation with the changes in microsomal lipid fluidity, then the results presented are suggestive evidence for the existence of a specific micro-environment (boundary lipids) around ACAT which has different physical properties than that of the bulk lipids of microsomal membranes. Such a concept of micro-environment for ACAT in liver microsomes has been previously postulated (Spector et al, 1980). Unlike HMG-CoA reductase and cholesterol 7α-hydroxylase, the Arrhenius plots of ACAT in the liver microsomes of rats fed high cholesterol diets were non-linear inspite

of the cholesterol enrichment of microsomal membranes. The break point temperature was, however lowered followed by dietary cholesterol treatment. These results are consistent with the DSC results which indicated that dietary cholesterol causes the lowering of transition temperature in the lipids of liver microsomal membranes (c.f. Chapter 5). Also the results are in line with the observation of Balasubramaniam et al (1978) who demonstrated that the microsomal pool of cholesterol that acts as substrate for ACAT is very small. If the pool of cholesterol around ACAT would have been large, then a linear Arrhenius plot of the enzyme would be anticipated as has been observed for HMG-CoA reductase and cholesterol 7α-hydroxylase. The occurence of the transition at a lower temperature following cholesterol feeding indicates that cholesterol concentration in the immediate environment of ACAT is increased, which results in an increased ACAT activity. However this increase in cholesterol concentration is not enough to abolish the break temperature of the Arrhenius plot.

Thus under the conditions of the present study, it appears that the alterations in the ACAT activity were a consequence of a change in the rate of flow of cholesterol to liver from the plasma. Hence the increased availability of cholesterol as a substrate for ACAT is important for the regulation of this enzyme. The results also demonstrate that ACAT co-operates with HMG-CoA reductase and cholesterol 7α-hydroxylase for the maintenance of cholesterol homeostasis in the microsomal membranes. Under the conditions of increased microsomal cholesterol following either unsaturated fat feeding or dietary cholesterol treatment, the activity of HMG-CoA

reductase is inhibited. On the other hand, increased microsomal cholesterol content enhances ACAT to form more of cholesterol esters for transport to the plasma as very low density lipoproteins.

CHAPTER 8. EFFECT OF DIETARY CHOLESTEROL ON LIPID COMPOSITION OF LIVER AND PLASMA OF RATS FED VARIOUS FATTY ACID DIETS

Attempts to elucidate the mechanism(s) responsible for the hypocholesterolemic effect of polyunsaturated fat feeding in man (Spritz et al, 1965; Grundy and Ahrens, 1970) and animals (Bieberdorf and Wilson, 1965; Corey et al, 1976) have, in part, lead to the hypothesis that polyunsaturated fats cause a shift of cholesterol from plasma to tissue pools. In order to test this hypothesis, numerous studies have examined the effects of polyunsaturated fatty acids (PUFA) on plasma and tissue cholesterol level (Avigan and Steinberg, 1958; Gran and Nicolaysen, 1966; Kellogg, 1974). On the other hand, only a few studies have examined the reverse effect i.e. of exogenous cholesterol on PUFA levels and profiles of liver and/or plasma (Morin et al, 1962; Bochenek and Rodgers, 1978).

It is evident from Chapter 4 that dietary cholesterol produces significant changes in cholesterol content and fatty acid composition of rat liver microsomes and that the type of fatty acid supplement has a pronounced effect on this process. The enzymes responsible for bringing about these alterations in lipid composition are located on the microsomal membranes. Therefore, it is likely that the changes observed in lipid composition of liver microsomes are reflected in the whole liver and/or plasma. The main object of this present exercise was to examine simultaneously the behaviour of liver and plasma lipids of rats under the dietary conditions explained in Section 3.2.2. It was also hoped that the results might throw some light on the mechanism(s) involved in the modification of lipid composition

(cholesterol content and fatty acid profiles) of liver microsomal membranes by dietary cholesterol.

8.1. Enrichment of liver and plasma with cholesterol after dietary cholesterol treatment

After extraction, liver and plasma total lipids were fractionated into phospholipids, free cholesterol, triacylglycerols and cholesterol esters as previously explained in Section 3.2.5. The effect of dietary cholesterol on phospholipid, free and esterified cholesterol content as well as on the fatty acid profiles of major lipid classes was determined.

Table 20 contains the data on cholesterol content of both plasma and liver lipids. Unexpectedly, the excess cholesterol in the diet, had no significant effect on the plasma cholesterol level, when supplemented to the reference (REF) diet. When 2% cholesterol was fed in combination with sunflower seed or coconut oil, the cholesterol level in the plasma was significantly elevated but not to the extent expected from a diet containing 2% cholesterol (Table 20). On the other hand, cholesterol content of the liver was increased from 1.5 mg/g to 2.7 mg/g of liver when 2% cholesterol was included in the REF diet. The increase in cholesterol content of liver was much higher when cholesterol was fed either with coconut oil (4.5 mg/g) or sunflower seed oil (12.0 mg/g) (Table 20). Both the free and esterified cholesterol contents were elevated but the increase was predominantly in the ester fraction. When cholesterol was fed in combination with sunflower seed oil, cholesterol esters were

accumulated in the liver, 16 times more than that of the REF diet group whereas feeding of cholesterol with coconut oil could accumulate only 5 times more than that of the REF diet group (Table 20).

8.2. Phospholipid content of plasma and liver of rats fed cholesterol-supplemented diets

Table 20 also shows the data on total phospholipid content of liver and plasma of rats fed various lipid-supplemented diets. None of the experimental groups exhibited any significant variation in the phospholipid content of liver or plasma. Therefore, the cholesterol enrichment of liver following cholesterol feeding resulted in an increase of cholesterol/phospholipid (C/P) molar ratios in these animals. The C/P ratio was found to be highest when cholesterol was fed in combination with sunflower seed oil (1.378) followed by cholesterol plus coconut oil (0.506) and cholesterol plus reference diet group (0.313) (Table 20).

8.3. Fatty acid profiles of plasma and liver lipids after dietary cholesterol treatment

The fatty acid composition of total lipids from plasma of rats fed the various experimental diets is given in Table 21. Administration of 15% sunflower seed oil to the reference diet resulted in an increase of 18:2 fatty acid in the plasma at the cost of 16:0 and 18:1. On the other hand, coconut oil feeding reduced the level of 18:2 and,20:4 in the plasma, with accompanying increase of medium chain (12:0 and 14:0) fatty acids (Table 21). Addition of 2% cholesterol to the reference

Table 20. Effects of dietary cholesterol on plasma and liver cholesterol contents and liver phospholipids.

	R	CH	SO	(SO + CH)	CO	(CO + CH)
Plasma CH (mg/dl)	56.5 ± 6.0	60.2 ± 4.3	58.3 ± 3.7	94.1 ± 11.1[c]	62.6 ± 8.1	83.5 ± 10.9[a]
Liver CH (mg/g)	1.5 ± 0.1	2.7 ± 0.1[c]	2.0 ± 0.1	12.0 ± 0.8[c]	1.2 ± 0.2	4.5 ± 0.4[c]
Free (mg/g)	1.00	1.76[b]	1.21	3.54[c]	0.80	1.88[c]
Esterified (mg/g)	0.51	0.90[a]	0.76	8.49[c]	0.40	2.60[c]
Liver PL (mg/g)	15.8 ± 1.3	16.5 ± 0.8	16.5 ± 1.2	17.0 ± 1.3	16.8 ± 1.0	17.2 ± 0.9
C/P (mole/mole)	0.184	0.313	0.235	1.378	0.138	0.506

Values with a superscript are significantly different from their corresponding low cholesterol diet - fed animals : a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$.

diet resulted in the accumulation of 16:1 and/or 18:1, and 18:2 with concomitant depletion of 18:0 and 20:4. Similar results were obtained when cholesterol was fed in combination with either coconut or sunflower seed oil (Table 21).

Plasma lipids were fractionated into phospholipids, triacylglycerols and cholesterol esters and fatty acid composition of individual fractions was determined. The data on the major fatty acids of these plasma lipid fractions has been summarized in Figure 32. It is evident that triacylglycerols were devoid of 20:4, whereas this is the principal fatty acid present in the cholesterol esters. Inclusion of 2% cholesterol in the diets increased the proportion of monounsaturated (16:1 plus 18:1) and diunsaturated (18:2) fatty acids with a simultaneous decrease of saturated (12:0, 14:0, 16:0 and 18:0) and polyunsaturated (20:4) fatty acids in phospholipid and triacylglycerol fractions of plasma (Figure 32). In the cholesterol ester fraction, however, a decrease in 20:4 after cholesterol feeding was accompanied by an increase in saturated fatty acids, in addition to the increase in monounsaturated and diunsaturated fatty acids (Figure 32).

In the livers of rats fed 2% cholesterol, the percentage of 18:1 and 18:2 was increased with accompanying decrease of 18:0 and 20:4, irrespective of the fatty acid supplement (Table 22). These changes in fatty acid composition were associated more or less with all the major lipid fractions of the liver, however cholesterol esters again exhibited the largest differences (Figure 33).

Table 21. Effect of dietary cholesterol on fatty acid composition of plasma total lipids.(%)

Fatty acid[1,2]	R	CH	SO	(SO + CH)	(CO)	(CO + CH)
12:0	-	-	-	-	11.9 ± 0.3	10.9 ± 0.3
14:0	-	-	-	-	8.1 ± 0.1	8.7 ± 0.3
16:0	25.3 ± 1.1	23.2 ± 0.5	16.0 ± 0.7	12.7 ± 0.1[a]	20.7 ± 0.3	19.0 ± 0.6
16:1 (ω7)	T	3.8 ± 0.2[c]	-	-	T	T
18:0	10.1 ± 0.4	3.9 ± 0.1[c]	10.6 ± 0.4	8.7 ± 0.1[a]	10.6 ± 0.2	8.3 ± 0.1[b]
18:1 (ω9)	18.0 ± 0.5	23.8 ± 0.5[a]	15.2 ± 0.3	17.7 ± 0.2[a]	15.6 ± 0.5	19.6 ± 0.2[b]
18:2 (ω6)	33.5 ± 0.2	36.9 ± 0.3[a]	45.5 ± 1.7	53.1 ± 0.4[b]	24.4 ± 0.4	26.9 ± 0.6[a]
18:3 (ω3)	2.0 ± 0.1	1.8 ± 0.2	-	-	-	1.1 ± 0.2
20:4 (ω6)	10.3 ± 0.4	6.3 ± 0.4[b]	12.6 ± 0.6	7.5 ± 0.2[b]	8.6 ± 0.1	5.4 ± 0.2[c]

1. Only major individual fatty acids have been listed.

2. Values with a superscript are significantly different from their corresponding low cholesterol diet-fed animals : a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$.

FIGURE 32: Fatty acid profiles of plasma lipid fractions from rats fed: R - reference; CH - cholesterol; S - sunflower seed oil; SCH - sunflower seed oil plus cholesterol; C - coconut oil; CCH - coconut oil plus cholesterol, diets. The values with a superscript are significantly different from their respective low cholesterol diet groups: a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.

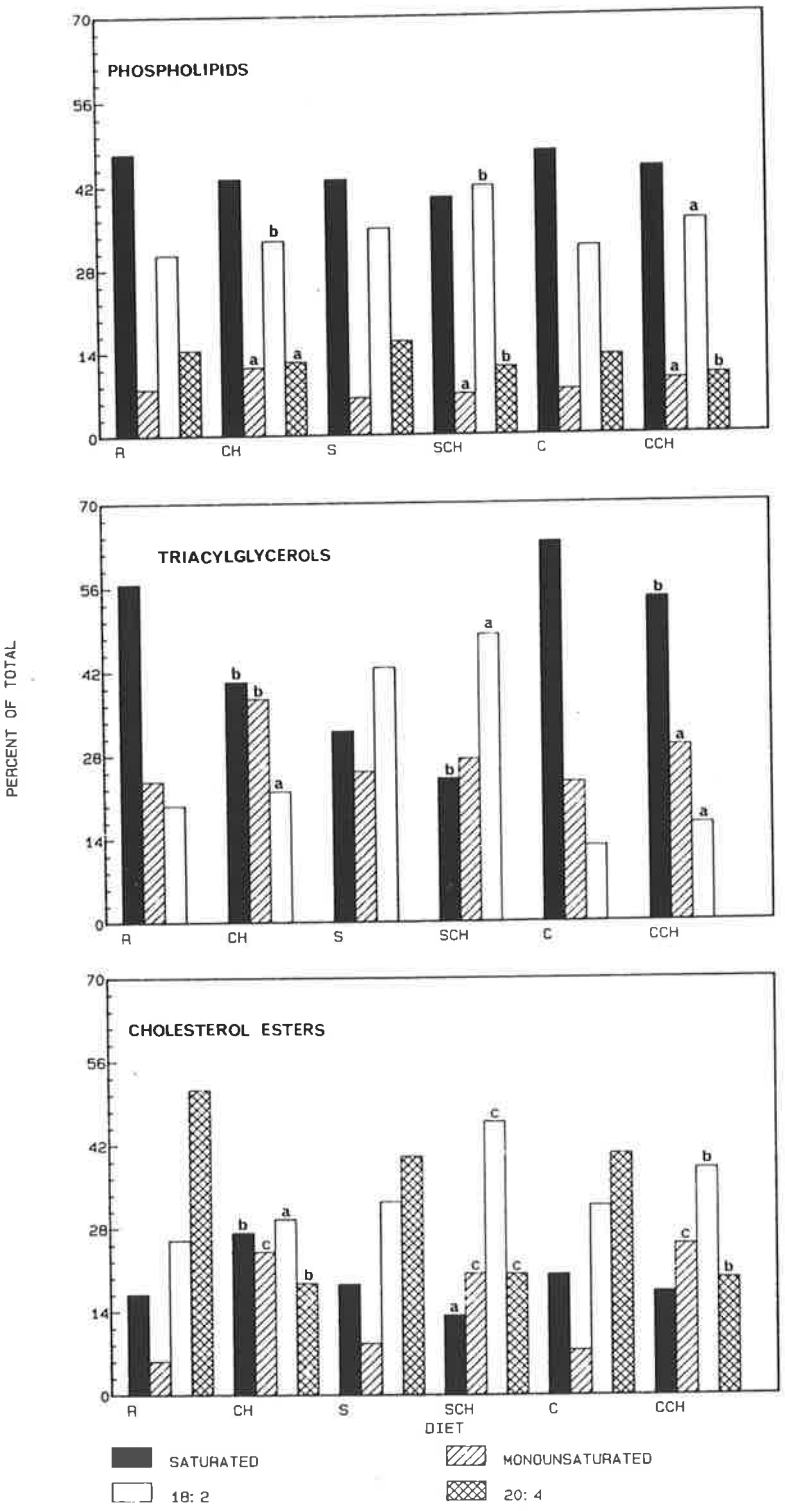

PHOSPHOLIPIDS
TRIACYLGLYCEROLS
CHOLESTEROL ESTERS
PERCENT OF TOTAL
70
56
42
28
14
0
R
CH
S
SCH
C
CCH
DIET
SATURATED
18: 2
MONOUNSATURATED
20: 4

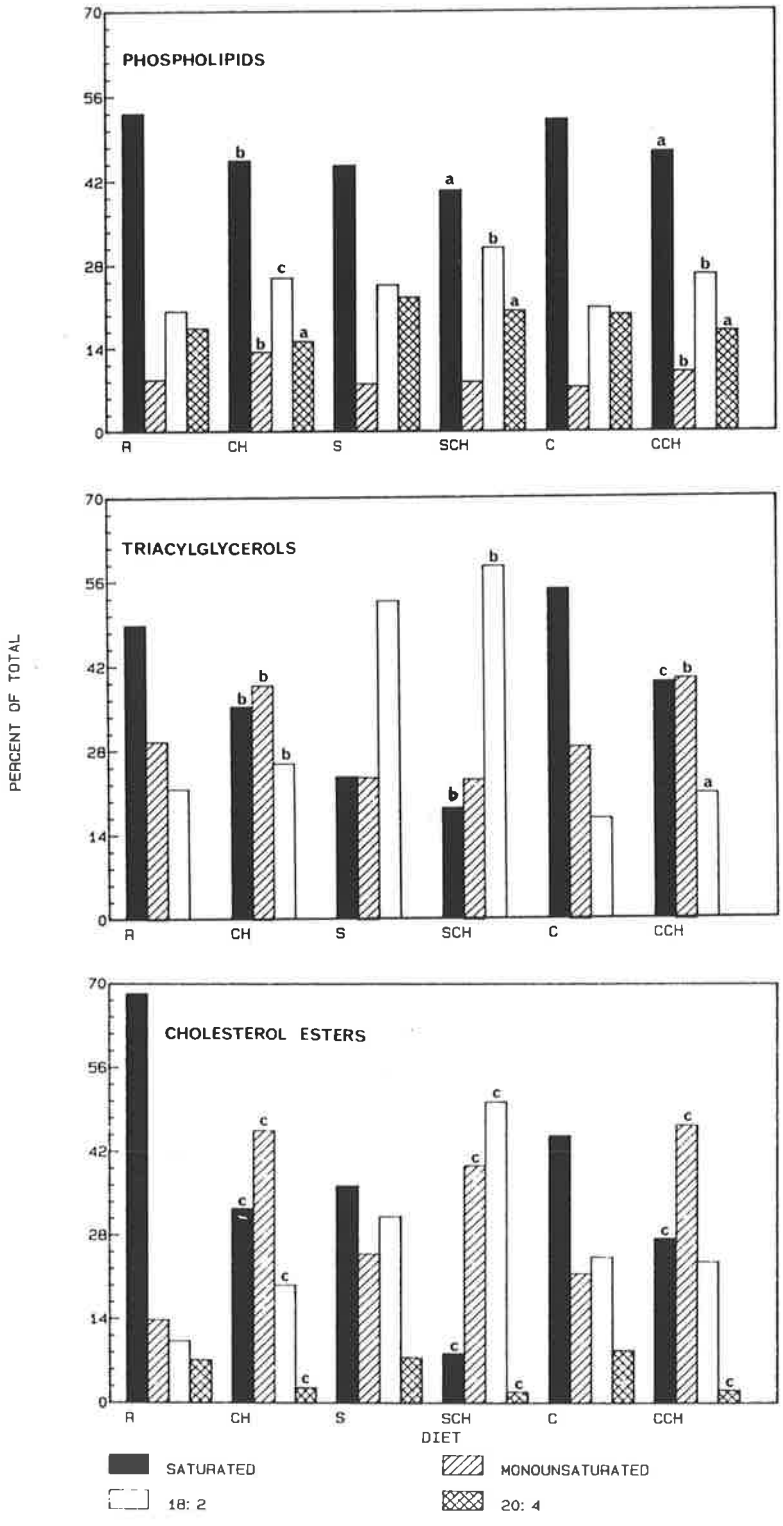
Table 22. Effect of dietary cholesterol on fatty acid composition of liver total lipids.

Fatty acid[1,2]	R	CH	SO	(SO + CH)	(CO)	(CO + CH)
14:0	-	-	-	-	3.8 ± 0.3	2.9 ± 0.0[a]
16:0	31.5 ± 1.4	31.7 ± 2.3	17.9 ± 1.2	12.9 ± 0.1[a]	26.5 ± 0.6	23.4 ± 0.2[a]
16:1	-	-	-	-	1.1 ± 0.0	2.8 ± 0.1[c]
18:0	19.8 ± 0.3	13.7 ± 0.4[b]	15.4 ± 0.8	7.8 ± 0.2[b]	18.0 ± 0.4	11.6 ± 0.3[a]
18:1	11.0 ± 0.8	18.9 ± 0.5[b]	14.4 ± 0.7	24.9 ± 0.3[c]	16.9 ± 0.5	27.9 ± 0.3[c]
18:2	20.8 ± 0.1	25.4 ± 0.6[b]	36.1 ± 0.6	46.2 ± 0.4[b]	18.8 ± 0.1	22.8 ± 0.1[c]
20:4	16.8 ± 0.3	10.4 ± 2.1[a]	16.4 ± 0.6	8.2 ± 0.1[b]	14.7 ± 0.2	8.4 ± 0.1[c]

1. Only major individual fatty acids have been listed.

2. Values with a superscript are significantly different from their corresponding low cholesterol diet-fed animals : a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$.

FIGURE 33: Fatty acid profiles of liver lipid fractions from rats fed: R - reference; CH - cholesterol; S - sunflower seed oil; SCH - sunflower seed oil plus cholesterol; C - coconut oil; CCH - coconut oil plus cholesterol diets. The values with a superscript are significantly different from their respective low cholesterol diet groups: a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.


PHOSPHOLIPIDS
TRIACYLGLYCEROLS
CHOLESTEROL ESTERS
PERCENT OF TOTAL
70
56
42
28
14
0
R
CH
S
SCH
C
CCH
DIET
SATURATED
MONOUNSATURATED
18: 2
20: 4

8.4. Discussion

The results presented in this Chapter and Chapter 4 leave no doubt that dietary cholesterol treatment alters the cholesterol metabolism of rat liver and that the quality and quantity of fatty acid supplement has a pronounced effect on this process. Cholesterol-supplemented diets increased the cholesterol content of liver, predominantly as cholesterol esters and significantly more so when cholesterol was fed in combination with unsaturated fat. Even without added cholesterol, unsaturated fat elevated the liver cholesterol level. On the other hand, plasma cholesterol concentration was affected only to a smaller extent inspite of a large (2%) dietary load. Therefore, in these rats, liver cholesterol, not plasma cholesterol, appears to be directly correlated with the amount of cholesterol intake, the extent depending upon the type of fatty acid supplement. These results suggest that whatever cholesterol is absorbed into the blood from the gut, is immediately removed from circulation by the liver and possibly also by other tissues. As is previously discussed (Chapter 4), consumption of unsaturated fat might enhance the absorption of cholesterol from the intestine into the blood and also accelerate the transport of cholesterol from blood to the liver, which can well result in an accumulation of cholesterol in this organ. Accumulation of cholesterol esters to the greatest extent following cholesterol plus unsaturated fat feeding is also consistent with the greatest enhancement of liver microsomal ACAT activity (c.f. Section 7.3.).

The decrease in saturated fatty acids with an accompanying increase in

unsaturated fatty acids is consistent with the enhancement of Δ^9-desaturase activity of rat liver. On the other hand, the decrease in 20:4 with a concomitant increase in 18:2 is consistent with the inhibition of Δ^5- and Δ^6-desaturase activities after cholesterol feeding (c.f. Chapter 6).

Regardless of the mechanism(s) by which dietary cholesterol brings about the changes in lipid composition of rat liver and plasma, the results presented indicate that plasma cholesterol is not a suitable measure of the changes in cholesterol concentrations in the animals as a whole nor in individual tissues. This is in agreement with earlier suggestions of Gerson et al (1961). Further, major variations can exist in the amount of cholesterol contained in the major pools of the body without having any necessary relationship to the level of cholesterol in the plasma, as previously pointed out by Nestel et al (1969). In considering various possibilities for the hypocholesterolemic effects of unsaturated fats, these results would support the theory that this effect is mediated via a shift of cholesterol from plasma to tissue pools.

CHAPTER 9. CONCLUSION

The main aim of this work was to examine the validity of the concept of membrane homeostasis (Sabine, 1983). The dietary cholesterol and/or fatty acid - induced alterations in the activity of the enzymes, responsible for producing changes in cholesterol content and fatty acid composition of rat liver microsomal membranes, when considered in unison, clearly underline this validity. The results already presented in Chapters 4, 5, 6, 7 and 8 and summarized in Table 23 lead to the following major conclusions.

Under a high dietary load of cholesterol (2%), the microsomal membranes appear to be responding in two ways; an inhibition of HMG-CoA reductase activity, and an acceleration of cholesterol 7α-hydroxylase and ACAT activities, both tending to nullify the effect of a high cholesterol intake. This reduced endogenous synthesis and increased removal of excess cholesterol however is not enough to prevent the enrichment of these membranes with cholesterol. Then, to compensate for this accumulated cholesterol, the fatty acyl desaturase activities and thus the fatty acid composition of microsomal membrane is modified in such a manner that the overall unsaturation of the membrane lipids (unsaturated index) is decreased. Therefore, the cholesterol enrichment of the microsomal membranes appears to be the major primary event occuring following cholesterol feeding. This is followed by a modification of the enzymes of lipid metabolism, which in turn has a tendency to cancel the effect of increased cholesterol on membrane fluidity.

Table 23. Summary table.

Parameter Studied	Diet					
	REF	(REF + CH)	SO	(SO + CH)	CO	(CO + CH)
Cholesterol Content	100	141	129	349	94	200
Phospholipid Content	100	96	83	97	101	103
HMG-CoA Reductase	100	52	77	38	95	63
Cholesterol 7α - hydroxylase	100	173	78	149	79	168
Acyl-CoA : Cholesterol Acyltransferase	100	117	162	213	115	195
U.I. (Phospholipids)	100	92	98	92	96	92
U.I. (Triacylglycerols)	100	134	181	196	96	110
U.I. (cholesterol esters)	100	135	168	218	97	114
Transition Temperature (DSC)	100	42	103	13	177	136

REF, Reference diet; (REF + CH), Reference + 2% Cholesterol diet; SO, 15% Sunflower seed oil diet; (SO + CH), 15% Sunflower seed oil + 2% Cholesterol diet; CO, 15% Coconut oil diet; (CO + CH), 15% Coconut oil + 2% Cholesterol diet.

Further, it appears that the neutral lipids of the micrsomal membranes act as a buffer for the phospholipids. When 15% coconut oil or 15% sunflower seed oil is fed in the diet, the unsaturation index of membrane phospholipids remains essentially unaltered, whereas the expected changes in the fatty acid composition are expressed within the neutral lipids. Administration of 2% cholesterol in the diet puts a demand on the membrane to decrease its phospholipid unsaturation (unsaturation index) for the maintenance of lipid fluidity. Therefore, the unsaturation index of membrane phospholipids is actually found to be decreased following cholesterol feeding, and the unsaturation index of triacylglycerols and cholesterol esters is increased.

If the temperature discontinuity in the Arrhenius plots of microsomal enzymes actually represents a phase change in membrane lipids, then the results presented in Chapter 7 suggest that the enzymes, HMG-CoA reductase, cholesterol 7α-hydroxylase and ACAT have a specific microenvironment within the microsomal membranes, which appears to have a different lipid composition than that of the bulk lipids of the membranes. However, the fact that microsomal lipids include plasma membrane and golgi apparatus lipids in addition to the endoplasmic-reticulum lipids and thus that the pure endoplasmic-reticulum lipids might exhibit an entirely distinct thermal behaviour, cannot be ignored.

These studies also support the theory that the hypocholesterolemic effect of PUFA may be due to a shift of cholesterol from plasma to the tissue pools, and not due to any overall decrease of total body

cholesterol. If the later explanation would have been true, then the administration of PUFA in the diet would be expected to increase cholesterol synthesis and cholesterol hydroxylation in the rat liver. But in the present study, both HMG-CoA reductase and cholesterol 7α-hydroxylase activities of liver microsomes were found to be decreased following PUFA feeding (Chapter 7). On the other hand, accumulation of cholesterol in the liver is suggestive evidence that there is a more efficient transport of cholesterol from plasma to the liver when PUFA are fed in the diet.

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