CHOLESTEROL METABOLISM IN THE ENDOPLASMIC RETICULUM OF RAT LIVER

P.W.H. RAE

Ph.D. Thesis
University of Edinburgh
1980

This thesis was composed by myself, and the results described are the product of my own work.

Signed

ACKNOWLEDGEMENTS

The work described in this thesis was supported by a Medical Research Council Programme Grant under the supervision of Professor G.S. Boyd. The author held a University of Edinburgh Medical Faculty Scholarship.

The analyses performed at the Istituto di Farmacologia e Farmacognosia at the University of Milan were done in the laboratory of Professor G. Galli, with the assistance of Dr. Enrica Bosisio. This work was supported by the award of a University of Edinburgh Medical Faculty Travelling Scholarship to the author.

I am grateful to the following colleagues for many helpful discussions - Dr. Enrica Bosisio, Dr. Kathleen Botham, Dr. Iain Craig, Dr. Robert Hume, Miss Margaret Lawson, Dr. Denis Mathé, Dr. Shigeru Ozasa and Dr. Keith Suckling.

I am especially grateful to Professor G.S. Boyd for his advice, guidance and supervision throughout the course of the work described in this thesis.

Abbreviations

ATP	adenosine 5'-triphosphate
$NAD(P)^+$	nicotinamide adenine dinucleotide (phosphate)
cholesterol	5-cholesten-3β-ol
7α-hydroxycholesterol	cholest-5-ene-3β,7α-diol
7-ketocholesterol	cholest-5-ene-3β-ol-7-one
VLDL	very low density lipoprotein
LDL	low density lipoprotein
IDL	intermediate density lipoprotein
HDL	high density lipoprotein
CoA	coenzyme A
HMGCoA	3-hydroxy-3-methylglutaryl-coenzyme A

Enzymes

cholesterol 7α-hydroxylase E.C.1.14.13.17

acyl-coenzyme A:cholesterol acyltransferase E.C.2.3.1.26

lecithin:cholesterol acyltransferase E.C.2.3.1.43

glucose-6-phosphate dehydrogenase E.C.1.1.1.49

3-hydroxy-3-methylglutaryl-coenzyme A reductase E.C.1.1.1.34

cholesterol oxidase E.C.1.1.3.6

NADPH cytochrome c reductase E.C.1.6.2.4

fatty acyl coenzyme A desaturase E.C.1.14.99.5

cholesterol ester hydrolase E.C.3.1.1.13

acyl-coenzyme A synthetase E.C.6.2.1.3

Abstract

In work directed towards the purification of a liver microsomal cytochrome P-450 species capable of supporting cholesterol 7α-hydroxylase activity in a reconstituted system, the use of the detergent Renex 690 for the solubilisation of microsomal protein resulted in an unacceptable inhibition of cholesterol 7α-hydroxylation. This confirmed previous work, showing that Nonidet P42 is the optimum choice of solubilising agent for this purpose. DEAE-cellulose chromatography of microsomal protein solubilised with Nonidet P42 was confirmed to be a suitable first step in the purification of liver microsomal cytochrome P-450, since there is a good separation of this species from NADPH cytochrome c reductase activity. However, the recovery and purification of total cytochrome P-450 was low. Dithiothreitol, 4-phenyl-imidazole, diethyl-dithiocarbamate and glycerol, by themselves or in combination with each other, were shown to be useful agents in liver microsomal cytochrome P-450 purification. It was further demonstrated that increasing the recovery of cytochrome P-450 gave a concomitant improvement in its purification. Chromatography on quaternary aminoethyl-Sephadex or on carboxymethylcellulose did not result in any purification of cytochrome P-450. Hydroxyapatite chromatography of cytochrome P-450 - containing fractions from the DEAE-cellulose eluate gave a further small purification of cytochrome P-450. The use of chemical donors of "active oxygen" in the reconstitution of cholesterol 7α-hydroxylase activity with liver microsomal cytochrome P-450 was shown to be limited by the rapid destruction of the cytochrome by these agents.

An assay for cholesterol esterification by liver microsomal preparations was developed. This assay measured the fractional conversion of exogenous [4-^{14}C]-cholesterol to cholesterol ester in the

presence of coenzyme A and ATP, and therefore represents the combined activities of acyl-coenzyme A synthetase and acyl-coenzyme A:cholesterol acyltransferase. This activity was not a linear function of protein concentration, so the protein concentration used in subsequent experiments had to be maintained within a narrow range. The rate of esterification was linear up to twenty minutes and then declined. Esterification required the presence of coenzyme A, but the esterification rate was independent of coenzyme A concentration over a wide range. The coenzyme A analogues N-acetyl-cysteine and N-acetyl-cysteamine were not able to support cholesterol esterification. Perturbations of the rate of fatty acid desaturation by microsomal preparations had no effect on the rate of cholesterol esterification. Newly synthesised cholesterol ester sedimented with microsomes rather than forming a floating lipid layer. Butanol extraction or detergent solubilisation of microsomes resulted in large losses of cholesterol esterification activity.

The assay of acyl-coenzyme A:cholesterol acyltransferase throughout a twenty-four hour period indicated that this enzyme exhibited a small diurnal variation of activity with the maximum and minimum activities occurring three hours after the middle of the dark and light periods, respectively.

In the twenty-four hours after occlusion of the bile duct the plasma concentration and, by implication, the hepatic content of bile salts increased, as did the liver microsomal cholesterol content. There was no difference either in the size of the substrate pool for cholesterol 7α-hydroxylase or in the cholesterol 7α-hydroxylase activity between sham operated and bile duct ligated animals. The diurnal variation in cholesterol 7α-hydroxylase activity and in the size of its substrate pool persisted after bile duct occlusion. There was no difference in the size of the substrate pool for acyl-coenzyme A:

cholesterol acyltransferase between sham operated and bile duct ligated animals. After twenty-four hours the acyl-coenzyme A:cholesterol acyltransferase activity of bile duct ligated animals was greater than that of sham-operated controls. Plots of these enzyme activities against substrate pool size are consistent with the hypothesis that under these circumstances these enzymes are not saturated with substrate, and there is therefore a linear relationship between the amount of substrate accessible to the enzyme and the rate of conversion of substrate to product.

Feeding rats a diet containing 10% olive oil resulted in no detectable changes in either whole liver or liver microsomal free or esterified cholesterol contents. Feeding a diet containing 10% olive oil, 1% cholesterol gave a small increase in whole liver or liver microsomal free cholesterol and large increases in liver and liver microsomal cholesterol ester. The withdrawal of the cholesterol from the diet resulted in a rapid return of cholesterol ester contents to basal levels. Free cholesterol contents also returned to basal levels, but more slowly. The activity of acyl-coenzyme A:cholesterol acyltransferase increased on feeding either the olive oil or the olive oil plus cholesterol diet, but the rise in activity in the latter case started earlier and was more sustained. No increase in cholesterol 7α-hydroxylase was detected on feeding 10% olive oil, 1% cholesterol, despite an increase in the size of the substrate pool for this enzyme.

Acetone-extracted liver microsomes combined with liposomes made from microsomal lipid supplemented with increasing amounts of cholesterol exhibited increasing activities of acyl-coenzyme A:cholesterol acyltransferase. Saturation was not apparently achieved within the range of cholesterol content used.

Cholesterol-enriched serum was used to enrich liver microsomes with cholesterol. This resulted in an increase in acyl-coenzyme A:cholesterol acyltransferase activity, but no change in cholesterol-7α-hydroxylase activity.

The problems associated with the assay of enzymes that are membrane-bound and act on lipophilic substrates are discussed.

CONTENTS

Page No.

Acknowledgements i.

Abbreviations and Enzymes ii.

Abstract iii.

Section 1 Introduction 1

1.1 The metabolic and structural roles of cholesterol - an introduction 2

1.2 Cholesterol transport

(a) Lipoprotein composition and structure 4
(b) Lipoprotein metabolism 5

1.3 The cellular uptake of cholesterol 14

1.4 The functions of the hepatic endoplasmic reticulum in the metabolism of cholesterol 16

1.5 The function of cholesterol in biological membranes 22

1.6 The control of the activity of membrane-bound enzymes 24

Section 2 Materials and Methods 27

2.1 Animals and diets 28

2.2 Preparation of microsomes 29

2.3 Butanol extraction of rat liver microsomes 29

2.4 Solubilisation of microsomal proteins 29

2.5 Assay of cytochromes P-450 and b_5 30

2.6 Assay of NADPH-cytochrome c reductase 30

2.7 Assays for protein 31

2.8 Lipid extractions 31

2.9 Assay for phospholipid 31

2.10 Assays for cholesterol 32

2.11 Assays for cholesterol 7α-hydroxylase 33

2.12 Assays for acyl-coenzyme A:cholesterol acyltransferase 36

2.13 The calculation of a maximal estimate of substrate pool size for cholesterol 7α-hydroxylase or acyl-coenzyme A: cholesterol acyltransferase 38

2.14 Assay for flavins 39

Page No.

2.15 Liposome preparation 39

2.16 DEAE-cellulose 40

2.17 Statistics 40

2.18 Materials 40

Section 3 Approaches to the purification of a cytochrome P-450 species reconstitutively active in cholesterol 7α-hydroxylation 41

3.1 Introduction

(a) The requirement for a purified preparation of 7α-hydroxylase 42
(b) Purifications of microsomal cytochromes P-450 reconstitutively active in hydroxylations of substrates other than cholesterol 44
(c) Purifications of a cytochrome P-450 preparation capable of supporting cholesterol 7α-hydroxylation in a reconstituted system
(i) Procedures used in this laboratory 47
(ii) Procedures reported by other laboratories 48

3.2 The use of Renex 690 as a solubilising agent 49

3.3 DEAE-cellulose chromatography

(a) General description 50
(b) Control conditions 51
(c) The use of various protective agents
(i) Dithiothreitol 52
(ii) 4-Phenyl-imidazole 53
(iii) Diethyldithiocarbamate 54
(iv) 4-Phenyl-imidazole and diethyldithiocarbamate 55
(v) Glycerol 56
(vi) 1% Nonidet P42 57

3.4 Quaternary amino ethyl-Sephadex chromatography 58

3.5 Carboxymethylcellulose chromatography 58

3.6 Hydroxylapatite chromatography 59

3.7 Iodosobenzene as a donor of "active oxygen" for cholesterol 7α-hydroxylation 60

Section 4 The assay of acyl-coenzyme A:cholesterol acyltransferase 62

4.1 Introduction 63

4.2 The effect of variation of protein concentration 65

4.3 Time-course of acyl-coenzyme A:cholesterol acyl-transferase activity 67

4.4 The effect of variation of coenzyme A concentration 67

Section 5 Some in vitro studies of acyl-coenzyme A: cholesterol acyltransferase 68

5.1 Introduction 62

5.2 The use of N-acetyl-cysteine and N-acetyl-cysteamine as coenzyme A analogues 71

5.3 The effect of *in vitro* perturbations of fatty acyl-coenzyme A desaturase activity on cholesterol esterification 73

5.4 The fate of newly synthesised cholesterol ester 74

5.5 The effect of acyl-coenzyme A:cholesterol acyltransferase activity of freeze-drying and treatment with organic solvents 75

5.6 The effect of Nonidet P42 on acyl-coenzyme A: cholesterol acyltransferase activity 76

Section 6 Some in vivo studies of hepatic microsomal cholesterol metabolism 78

6.1 Introduction 79

6.2 The assay of acyl-coenzyme A:cholesterol acyltransferase activity through a twenty-four cycle 82

6.3 Assay of acyl-coenzyme A:cholesterol acyltransferase and cholesterol 7α-hydroxylase activities during the twenty-four hours following bile duct occlusion 84

6.4 The response of the activities of acyl-coenzyme A: cholesterol acyltransferase and cholesterol 7α-hydroxylase to feeding cholesterol-rich diets 87

(a) The hepatic effects of feeding and withdrawing a cholesterol-rich diet 88

(b) The effect of diet manipulation on hepatic cholesterol content and coenzyme A:cholesterol acyltransferase activity 91

(c) The effect of cholesterol feeding on cholesterol 7α-hydroxylase 95

Section 7 The response of cholesterol 7α-hydroxylase and acyl-coenzyme A:cholesterol acyltransferase to in vitro alterations of cholesterol concentration 97

7.1 Introduction 98

7.2 The effect of the recombination of acetone-extracted microsomes with microsomal lipid liposomes on acyl-coenzyme A:cholesterol acyltransferase activity 100

7.3 The effect of the recombination of acetone-extracted microsomes with microsomal lipid liposomes supplemented with varying amounts of cholesterol on acyl-coenzyme A: cholesterol activity 103

7.4 Acyl-coenzyme A:cholesterol acyltransferase and cholesterol 7α-hydroxylase activities in microsomes supplemented with cholesterol using cholesterol-enriched serum 105

7.5 The activity of ethylmorphine N-demethylase in microsomes supplemented with cholesterol using cholesterol-enriched serum 112

Section 8 Discussion 114

8.1 Introduction 115

8.2 The assay of the activity of enzymes that are membrane-bound and/or act on substrates that are lipophilic 117

8.3 The preparation of a cytochrome P-450 species reconstitutively active in cholesterol 7α-hydroxylation

(a) The existence of a cytochrome P-450 species specific for cholesterol 7α-hydroxylation 122
(b) The use of donors of "active oxygen" to reconstitute cholesterol 7α-hydroxylase activity 124
(c) The effect of increased recovery of cytochrome P-450 on its purification 125

8.4 The *in vivo* response of the liver to changes in its cholesterol supply

(a) Hepatic cholesterol metabolism during a twenty-four hour cycle, and during cholesterol feeding 126
(b) Hepatic cholesterol metabolism during the twenty-four hours following bile duct ligation 134

8.5 The response of hepatic microsomal cholesterol metabolism to *in vitro* alterations of the microsomal cholesterol content 136

Section 9 References 139

Section 1

Introduction

1.1 The metabolic and structural roles of cholesterol - an introduction

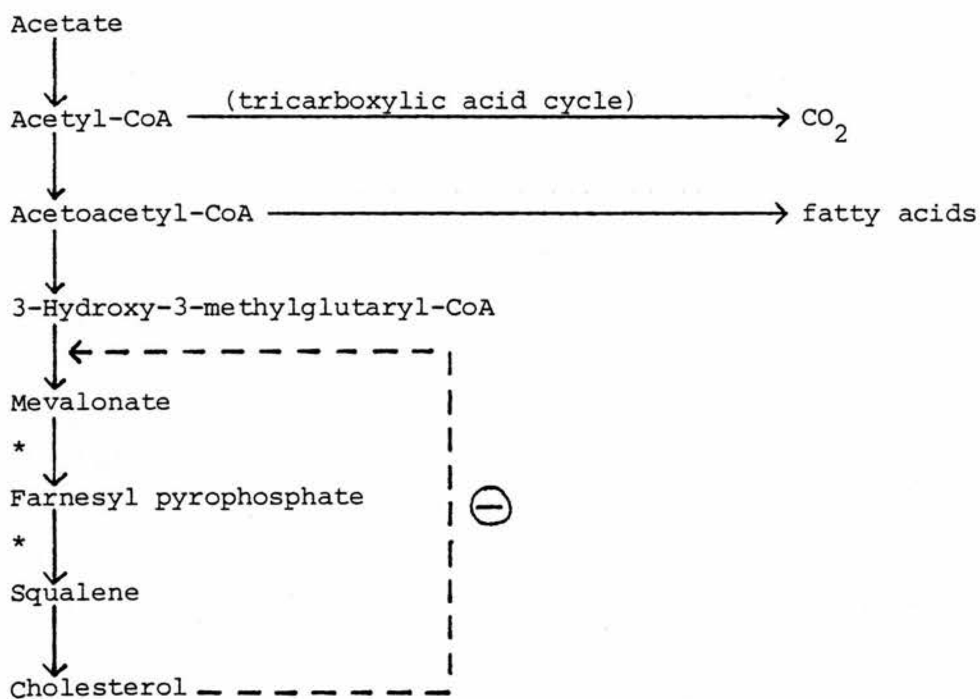
Cholesterol is found in varying amounts in all mammalian tissues. It is derived either from the diet, or by synthesis from simple two-carbon precursors (Figure 1.1). The absorption of cholesterol from the diet depends on various factors, a major influence simply being the amount of cholesterol in the diet (1). All tissues examined have the ability to synthesise cholesterol (2,3), but this activity is closely controlled by the supply of cholesterol (3,4).

In the absence of dietary cholesterol an adequate supply of cholesterol for the entire organism can be maintained by synthesis in the liver and the cells of the small intestine (3,5). The rate of cholesterol synthesis in the liver is determined by the dietary cholesterol intake (3,5,6), and the synthetic rate in all other tissues is normally maintained only at low levels (3,6). Such an arrangement has the advantage that the cholesterol metabolism of the whole organism is centrally controlled, but requires the export of cholesterol into the plasma, and a complex centrifugal transport system to carry the cholesterol from its sites of synthesis and absorption to the tissues where it is required (Section 1.2).

Cholesterol can be taken up into the cells of peripheral tissues both by non-specific exchange or diffusion processes (7-10) and by highly specific receptor-mediated transport mechanisms (Section 1.3). The advantage of the latter system is that it allows control of the quantity of cholesterol entering the cells of a tissue. Thus, once the cholesterol has entered the cells of its target tissue not only does it inhibit the rate of cholesterol synthesis in that tissue, but it also may inhibit the synthesis of the receptor required for its uptake. In addition, the conversion of cholesterol to cholesterol ester (presumed to act as an inert storage form of cholesterol) may be stimulated.

Figure 1.1

The synthesis of cholesterol



Each arrow in the sequence of metabolic conversions shown can represent one or more enzyme-catalysed reactions. The primary site of feedback regulation of cholesterol synthesis is indicated thus: --⊖--→
Secondary sites of regulation, exhibiting a decreased enzyme activity on prolonged cholesterol feeding are indicated thus: *.
(Adapted from reference 3).

This concerted control of cholesterol uptake, synthesis and storage has been best characterized for the fibroblast and has been embodied in the "low density lipoprotein receptor hypothesis" (Section 1.3).

In all tissues cholesterol is an essential structural component in both intracellular and extracellular membranes. The cholesterol content of membranes is critical in determining many of their properties such as structure, fluidity, transition temperature and enzyme activities (Sections 1.5 and 1.6).

In addition to this structural role which cholesterol fulfils in all tissues, in certain organs it serves specific metabolic purposes. In steroid hormone-producing tissues cholesterol is stored in the form of its ester. On the appropriate hormonal signal this ester is rapidly hydrolysed to free cholesterol and this is converted to steroid hormone (11-16). In the liver cholesterol can be converted to bile acids (Section 1.4). After conjugation with the bases glycine or taurine the resultant bile salts are secreted, with phospholipid and cholesterol, as bile. Finally, as has already been mentioned, the cholesterol may be esterified by combination with a fatty acid. Intracellularly, this fatty acid is derived from fatty acyl-coenzyme A, the enzyme being acyl-coenzyme A cholesterol acyltransferase (Sections 1.3 and 1.4); in plasma the fatty acid involved in esterification of cholesterol is derived from lecithin, the reaction being a transesterification catalyzed by the enzyme lecithin:cholesterol acyltransferase (Section 1.2(b)). The resultant cholesterol ester is the form in which cholesterol accumulates when it is present in excess. The site of this accumulation varies in different species, being the liver in rats, and is presumably the result of the relative activities of acyl-coenzyme A: cholesterol acyltransferase in each tissue and of lecithin:cholesterol acyltransferase.

The only major route for catabolism of cholesterol leads to the bile acids (Figure 1.2), and this occurs exclusively in the liver (17-21). In addition to the centrifugal cholesterol transport systems already mentioned there is therefore a need for a centripetal transport system to transfer excess cholesterol from peripheral tissues to the liver for catabolism and excretion (Section 1.2(b)).

From the foregoing outline of cholesterol transport, metabolism and function it can be concluded that the liver plays a central coordinating role, since it is capable of cholesterol synthesis, storage and catabolism, can secrete cholesterol into the blood and the bile, and can absorb cholesterol from the blood. This thesis examines some aspects of two of these processes - the first and rate-limiting step in the metabolism of cholesterol to bile acids, and the conversion of cholesterol to cholesterol ester. These reactions, however, must be considered in the context of overall cholesterol metabolism, transport and function described in the following paragraphs.

1.2 Cholesterol transport

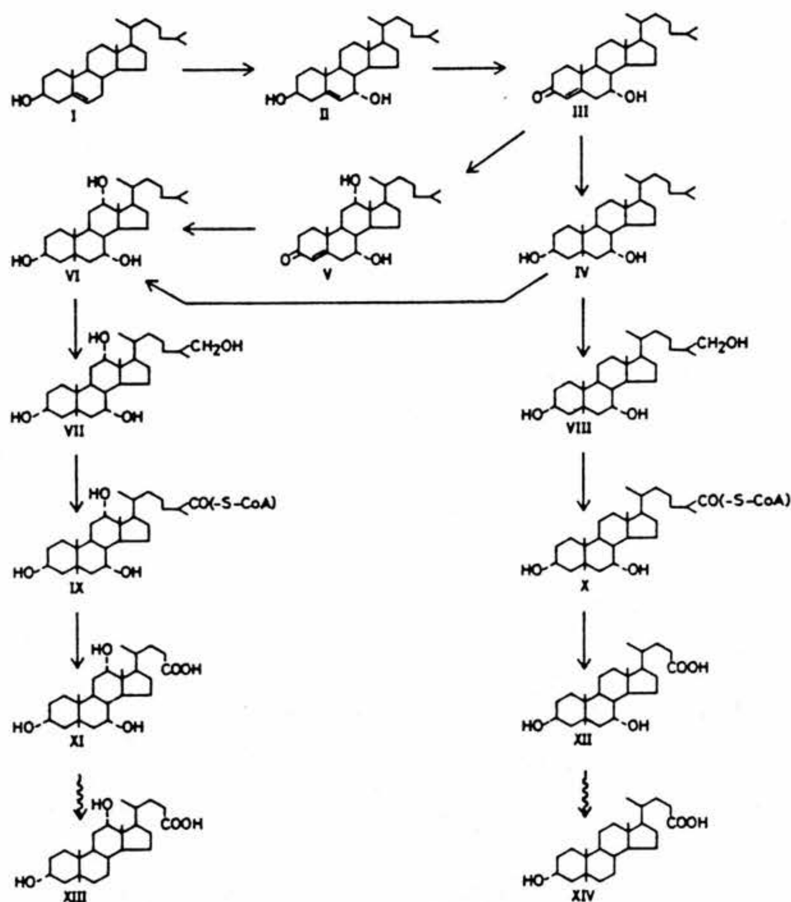
(a) Lipoprotein composition and structure

Like other lipid molecules cholesterol is transported in the blood by the serum lipoproteins, a heterogeneous class of particles composed of varying amounts of lipid (mainly triglyceride, phospholipid and free and esterified cholesterol) and protein. These proteins comprise three groups designated apoprotein A, apoprotein B and apoprotein C (apoA, apoB, apoC). Further subdivision of these apoprotein groups is denoted by a roman numeral suffix.

The lipoproteins are subdivided on the basis of their centrifugation characteristics into four main classes: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density

Figure 1.2

The metabolism of cholesterol to bile acids



Pathways for formation of bile acids. I, cholesterol; II, 7α-hydroxycholesterol (5-cholestene-3β, 7α-diol); III, 7α-hydroxy-4-cholesten-3-one; IV, 5β-cholestane-3α, 7α-diol; V, 7α, 12α-dihydroxy-4-cholesten-3-one; VI, 5β-cholestane-3α, 7α, 12α-triol; VII, 5β-cholestane-3α, 7α, 12α, 26-tetrol; VIII, 5β-cholestane-3α, 7α, 26-triol; IX, 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid; X, 3α, 7α-dihydroxy-5β-cholestanoic acid; XI, cholic acid; XII, chenodeoxycholic acid; XIII, deoxycholic acid; XIV, lithocholic acid. →, reactions catalyzed by liver enzymes; ⇝, reactions catalyzed by microbial enzymes.

(From reference 20).

lipoproteins (HDL). The size and composition of these species is summarised in Table 1.1 (22-25).

The structure of all the lipoproteins is such that non-polar molecules, or portions of molecules, are in the interior of the particle, shielded from the aqueous environment by a layer of polar molecules or parts of molecules. Thus, chylomicrons and VLDL have the structure of a large hydrophobic core of triglyceride (with some cholesterol ester, when present) surrounded by a monolayer of phospholipid and free cholesterol, the apoproteins being associated with this monolayer (22-25). LDL has a micellar structure, with an apolar core of cholesterol ester and triglyceride, surrounded by a monomolecular shell of phospholipid, free cholesterol and apoprotein (23). The structure of HDL will be discussed below, with the role of HDL in cholesterol transport.

(b) Lipoprotein metabolism

When a fat-containing meal is ingested, the lipid (mainly triglyceride) is hydrolyzed to free fatty acids and monoglycerides in the lumen of the small intestine and absorbed into the mucosal cells of the intestinal wall. These newly absorbed lipid components are used in the synthesis of triglyceride and phospholipid, which, with cholesterol and protein, are assembled into chylomicrons (23,26). VLDL synthesis uses fat derived from the diet and, in larger quantities, from endogenous synthesis, and occurs in the liver and the small intestine (27-30). These two lipoprotein species are usually considered to be assembled in a similar way, with lipid synthesis occurring in the smooth endoplasmic reticulum, protein synthesis in the rough endoplasmic reticulum, and the essentially completed lipoprotein then being transported to the Golgi apparatus and secreted by processes dependent on microtubules (31-42). Experiments performed to investigate the degree of interdependence of these various stages in synthesis and

Table 1.1

The characteristics of the major classes of plasma lipoproteins

Lipoprotein class	Density	Diameter (Å)	Composition (%)					Major apoproteins
			Triglyceride	Phospho-lipid	Free cholesterol	Cholesterol ester	Protein	
Chylomicrons	<0.95	>700	84	7	2	5	2	B, C-I, C-II, C-III
VLDL	0.95-1.006	250-700	50	18	7	12	8	B, C-I, C-II, C-III, E
LDL	1.006-1.063	180-260	11	22	8	37	21	B
HDL	1.063-1.210	40-100	5	25	5	15	50	A-I, A-II

secretion, using inhibitors of protein synthesis, perturbations of lipid synthesis rate and microtubule disrupting agents have tended to give equivocal results (43-46).

The intestinal triglyceride-rich lipoproteins are secreted into the lymph system and drain into the systemic blood circulation via the thoracic duct. The hepatic VLDL is secreted directly into the blood. When first secreted the intestinal particles do not have their full complement of apoC (29,44), but rapidly gain it from HDL, when in the blood (47,48).

Components of these lipoproteins are hydrolyzed by the action of lipoprotein lipase, an enzyme whose primary action is the hydrolysis of triglyceride, and which is situated on the luminal surface of capillary endothelium, being readily released into the circulation by heparin. Lipoprotein lipase is activated by apoC-II (48-50). Newly secreted chylomicrons and VLDL are therefore poor substrates for this enzyme until they have received apoC-II from HDL. The precise mechanism of action of lipoprotein lipase is not understood. In particular, it is not clear how it acts on triglyceride which is situated in the hydrophobic core of these lipoproteins, surrounded by a layer of polar lipid. However, it is known that the removal of triglyceride occurs in a number of steps, with repeated binding of lipoprotein to lipoprotein lipase, followed by enzyme activity with consequent loss of some triglyceride, and release into the circulation (51-53). This means that at any given time there will be circulating a spectrum of products of different sizes.

Over the range of chylomicron and VLDL concentration normally encountered _in vivo_, the lipoprotein lipase of the capillaries of heart muscle will be saturated with substrate, since it has a low K_m for the substrate, whereas the enzyme in the vessels of adipose tissue will not

be saturated, having a relatively high K_m (51). This has important consequences for the fate of triglyceride from triglyceride-rich lipoproteins. At all concentrations there will be a priority clearance, at a constant rate, of fatty substrate into tissues which require it for immediate utilization, while clearance into adipose tissue will be proportional to the concentration of circulating triglyceride.

This lipolysis results in the removal of lipid from the apolar core of the lipoprotein, with a resultant surplus of surface components. These must be removed if the particle is to remain stable. Various mechanisms have been proposed to achieve this removal. *In vitro* lipoprotein lipase exhibits some phospholipase activity towards the surface phospholipids of these lipoproteins (54,55), and this would have the desired effect of removing material from the external layer of the lipoproteins. However, this process has not been demonstrated *in vivo*. HDL plays an important role in the other mechanisms involved. Firstly, as triglyceride hydrolysis proceeds, chylomicrons and VLDL lose apoC peptides, and these return to HDL (47,48). Thus, in effect the plasma apoC pool cycles between HDL and triglyceride-rich lipoproteins, with the result that the fractional catabolic rate of VLDL apoC is considerably lower than that of apoB (24). In consequence these particles, as they lose their triglyceride content, eventually become sufficiently depleted in apoC-II that they cease to be effective substrates for lipoprotein lipase (48,56).

Secondly, it is well known that cholesterol can readily transfer between lipoprotein classes, and HDL, in conjunction with the activity of lecithin:cholesterol acyltransferase is particularly suited to remove cholesterol from particles or membranes where it is in excess (see below).

Thirdly, as the lipolysis of triglyceride-rich lipoproteins proceeds, there is a rapid appearance of phospholipid and protein (primarily apoA proteins) from these species in HDL-containing plasma fractions (57-60). Two mechanisms have been suggested to explain this observation. These species could be transferred to pre-existing HDL (57-60), the movement of phospholipid perhaps being facilitated by phospholipid exchange proteins (59). Alternatively, or in addition to this mechanism, there is the possibility that these compounds could themselves be a source of new HDL (61). Thus, VLDL lipolysis by the perfused rat heart yields particles very similar to discoidal HDL (58) in addition to the major product, triglyceride-depleted VLDL; selective delipidation of the chylomicron core results in the appearance of lamellar aggregates of the surface components similar to particles which appear in the HDL fraction shortly after the injection of chylomicrons into rats (62); finally, after _in vitro_ lipolysis of chylomicrons it is possible to observe protruding folds from the surface of the resulting particles presumably caused by a puckering of the surface layer as triglyceride is removed from the interior of the particle (63).

The depletion of the triglyceride content and the removal of surface components from chylomicrons and VLDL described above results in the formation of smaller lipid-containing particles known as remnants. Remnant particles derived from chylomicrons compare in size and composition with remnants derived from VLDL (sometimes referred to as intermediate density lipoprotein (IDL)), but are not metabolised in the same way in all species.

In all species investigated chylomicron remnants are rapidly cleared by the liver (64-66). Thus, following administration of labelled chylomicrons to rats, 80% of the cholesterol ester, but only 10% of the triglyceride is recovered in the liver (64). Also, in the intact rat

chylomicron remnants are not detected after the absorption of a fat-containing meal, but if the rat is hepatectomised before the intragastric administration of fat, or the intravenous injection of previously purified chylomicrons, remnants accumulate (64). The evidence suggests that the remnants are taken up intact (67), and by a high affinity, high specificity, saturable pathway (67-69). The recognition factor(s) for this uptake and for the discrimination between chylomicrons and chylomicron remnants are not known, but appear not to be particle size or composition (67,69). Finally, and in important contrast to the uptake of LDL by fibroblasts (see Section 1.3), the uptake of remnants into the liver is not in any way a function of the hepatic rate of cholesterogenesis (68,70).

In the rat remnants formed from VLDL lipolysis are also rapidly taken up by the liver in a manner similar to chylomicron remnants (71-73), only 10% of VLDL apoB remaining in the circulation, in LDL. By contrast, in the human VLDL remnants (or IDL) are not taken up by the liver, and there is a precursor-product relation between VLDL and LDL (74,75), with more than 90% of VLDL apoB being eventually recovered in LDL. The amount of apoB per particle remains relatively constant in the VLDL - IDL - LDL system, suggesting that it persists as a constant structural component in all the particles of this series. The remodelling of IDL to LDL primarily requires the removal of further triglyceride, and it can be speculated that this may be the function of hepatic lipase, an enzyme similar to lipoprotein lipase but situated on the endothelial surface of the hepatic blood vessels.

In the human the catabolism of VLDL can apparently account for all LDL synthesis (74), although alternative sources of LDL cannot be excluded. However, in the rat a further route must exist for LDL synthesis, and it is not clear where this route occurs (75). No LDL release has

been observed in the perfusate of the isolated liver, although the secretion of VLDL and HDL is readily detected (29,30,76). Nascent VLDL can be demonstrated in the rat liver Golgi apparatus (37,38), but no LDL-like particles can be seen. However, this does not exclude the possibility of direct hepatic LDL synthesis, since a relatively slow rate of synthesis could contribute significantly to the size of the plasma LDL pool because of the relatively slow turnover time of this lipoprotein.

LDL contains cholesterol, mainly as cholesterol ester, as a major component and is presumed to be the lipoprotein species responsible for the transport of cholesterol to extra-hepatic tissues. Thus, although uptake of cholesterol from LDL into the liver can be detected (70,77-79), in the pig hepatectomy does not result in an increase in the rate of removal of LDL from the plasma (80). Furthermore, the existence of a well characterised, highly specific pathway for LDL uptake and degradation in a variety of cells in culture (Section 1.3) strongly suggests that LDL is largely catabolized in the peripheral tissues. This therefore results in a net transfer of cholesterol from the organs of absorption (the intestine) and synthesis (the liver and the intestine) to tissues that require it either as a structural component or as a steroid hormone precursor.

There is much suggestive evidence that the role of HDL is to act in an opposite sense to LDL, to bring about the centripetal transport of cholesterol from the peripheral tissues to the site of cholesterol catabolism, the liver. Thus, there is strong negative correlation between plasma HDL cholesterol concentration and the size of the pools of tissue cholesterol both in normal and hyperlipidaemic man (81). There is an inverse relationship between the plasma HDL concentration and mortality from cardiovascular disease (81,82). Low levels of HDL predispose towards atherosclerosis (as do high levels of LDL (83,84)),

and high levels of HDL protect against it (81,82,85). In Tangier disease (HDL deficiency) there are cholesterol deposits in reticuloendothelial and other tissues (86). HDL cholesterol appears to be secreted in the bile in preference to LDL cholesterol (87). Finally, many studies _in vitro_ have shown that HDL has the ability to remove cholesterol from cells (88-91), from other lipoproteins and from cholesterol-rich lipid dispersions.

The ability of HDL to act in this way is intimately associated both with HDL structure and with the activity of lecithin:cholesterol acyltransferase. In addition to the possibility, mentioned above, that HDL arises from the excess surface components of chylomicrons and VLDL, HDL is synthesised by the liver (29,30,76) and by the intestine (28,29). Nascent HDL is a disc-shaped particle, apparently composed of a bilayer of phospholipid and free cholesterol, with apoA around the hydrophobic edge of the bilayer (61,76,92,93). This structure is analogous to that of the water-soluble lipid particles found in the bile, where the hydrophobic edges of the phospholipid-cholesterol bilayers are surrounded by bile salts. Thus, apoA (in particular, apoA-II) has a solubilising role, similar to that of bile salts. Unlike HDL isolated from plasma, these nascent HDL particles contain no cholesterol ester, and are relatively deficient in free cholesterol (61,76,93).

Cholesterol is known to readily exchange between different lipoprotein classes, cell membranes and phospholipid bilayers (7-10), presumably via the transient formation of a collision complex. The direction and extent of this transfer depends on the relative cholesterol concentrations of the particles involved. The relatively low concentration of free cholesterol in the surface layer of HDL (or the lipid bilayer of nascent HDL) is therefore particularly suited to promoting the movement of cholesterol onto HDL.

If cholesterol was only transported by the plasma lipoproteins as free cholesterol the carrying capacity of these particles would be severely limited by their surface area, since this is the thermodynamically favoured area for free cholesterol to occupy. (It is noteworthy that the bulk of the cholesterol in LDL, presumed to be the main transporter of cholesterol to the peripheral tissues, is present as cholesterol ester, which is situated within the core of the particle.) In the particular case of HDL and its ability to remove cholesterol from other particles or membranes, an equilibrium would rapidly be reached where the polar lipid layer was saturated with free cholesterol and was therefore unable to accept more cholesterol from potential donors.

The effectiveness of HDL in this function is considerably enhanced by the activity of lecithin:cholesterol acyltransferase (94,95). This is a plasma enzyme, synthesised in the liver (93,96), that transfers a fatty acid from the C-2' position of lecithin to cholesterol, giving lysolecithin and cholesterol ester. Lecithin:cholesterol acyltransferase is activated by apoA-I, one of the major apoproteins of HDL, and HDL is accordingly a particularly effective substrate (97).

The products of this reaction, lysolecithin and cholesterol ester, are no longer suited to the environment they occupied as substrates. Lysolecithin is removed as a complex with serum albumin. Cholesterol ester retreats from the polar surface of the particle into its hydrophobic core, and hence the disc-like nascent HDL is eventually converted to the spherical HDL observed to occur in plasma. This change of shape can be observed during the in vitro incubation of lecithin:cholesterol acyltransferase with nascent discoidal HDL (obtained from rat liver perfusions performed in the presence of an inhibitor of lecithin:cholesterol acyltransferase (93)).

As well as resulting in this change of shape as HDL matures, the esterification of cholesterol has the effect of maintaining a low concentration of free cholesterol at the surface of the particle. The ability of HDL to accept cholesterol from cholesterol-rich sources is thus prolonged. It should also be noted that as the other enzyme substrate, lecithin, becomes depleted it can be replenished by transferral of the surplus surface components generated during the lipolysis of the triglyceride-rich lipoproteins, possibly through the mediation of phospholipid exchange proteins, as described above.

HDL has been shown to be taken up and degraded by the liver (77,78,98-100). However, measurements of the rate of accumulation of cholesterol in liver cells, from HDL, indicate a plasma half-life for HDL cholesterol ester considerably shorter than that for HDL apoproteins (98,99). Various mechanisms could account for this. Firstly, HDL could circulate through the plasma, acquiring cholesterol from extrahepatic tissues and delivering this up at the liver without the uptake of the complete HDL particle. This process might occur several times before finally the entire particle was degraded. Secondly, the cholesterol ester-rich HDL particle might deliver its cholesterol to remnant lipoproteins derived from chylomicron or VLDL lipolysis. Two mechanisms might be envisaged for this. The process might be mediated by cholesterol ester exchange proteins, known to facilitate the transfer of cholesterol ester from HDL to VLDL or LDL, probably in exchange for triglyceride (101-103). Alternatively, HDL might give up some of its complement of apoA-I in the formation of new HDL from the excess of surface components of VLDL and chylomicrons. As this occurs the apoA-I-deficient HDL would become less stable in its aqueous environment, and would be likely to fuse with another lipoprotein (61). The fusion of this unstable HDL species with chylomicron remnants would provide a route for the transfer of HDL cholesterol ester to the liver.

In species such as man, where little or no cholesterol is secreted into lipoproteins as cholesterol ester, these mechanisms for ester transfer from HDL to other lipoproteins may be a major source of these lipoproteins' cholesterol ester content (104). In particular, the cholesterol ester of LDL may be derived directly by transfer to it, or indirectly by transfer to VLDL or its remnant. This is in addition to any low level of activity lecithin:cholesterol acyltransferase may display towards these particles. In the rat there is some evidence that, in addition to any plasma cholesterol ester generated by lecithin: cholesterol acyltransferase, there is some secretion of cholesterol ester in newly synthesised lipoproteins (105). This cholesterol ester is presumably synthesised by the intracellular enzyme acyl-coenzyme A: cholesterol acyltransferase.

1.3 The cellular uptake of cholesterol

Cholesterol can be taken up from the plasma lipoproteins into cells by at least two mechanisms. Firstly, cholesterol is relatively free to exchange between the lipoproteins and cell membranes. This exchange process is non-specific, and any net transfer of cholesterol depends solely on the existence of a difference in cholesterol concentration between the species concerned. Secondly, specific mechanisms exist for the high affinity, high specificity binding and internalisation of certain lipoproteins in certain tissues. Such mechanisms exist for the uptake of chylomicron remnants (and probably, in the rat, VLDL remnants), and possibly HDL, in the liver, and for LDL in extrahepatic tissues.

Of these specific systems, the clearance of chylomicron remnants has been studied to the extent of determining the kinetic characteristics and substrate preference of the mechanism (67-69).

Work on the uptake of LDL by tissue culture cells, however, has yielded information not just on the actual uptake mechanism, but also on the metabolic consequences to the cell. These results are usually presumed to be of general significance in the understanding of how individual cells regulate their cholesterol content to meet changing demands for cholesterol in the presence of a potentially fluctuating cholesterol supply. These observations have been embodied in the so-called "LDL receptor pathway" (83,106-109) which is briefly described below.

On the surface of cultured human fibroblasts there is a protein or glycoprotein receptor (110,111), which binds LDL with high specificity and high affinity (110,112,113), and which can be saturated (112,113). The binding requires apoB to be present in the lipoprotein (112,113) and probably involves an ionic interaction. The LDL-receptor complex then enters the cell by a process resembling endocytosis (112,114-116), and the endocytotic vesicles fuse with lysosomes with subsequent degradation of their components (113,117,118). This results in the release of amino acids into the culture medium (113) and the accumulation of free cholesterol within the cell, largely associated with cell membranes (108,117). This cholesterol can be used in any cellular requirement for the synthesis or regeneration of membranes.

In addition, the accumulation of free cholesterol has a regulatory effect on two endoplasmic reticulum enzymes, suppressing 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity and activating acyl-coenzyme A:cholesterol acyltransferase (119-123). The former enzyme is the rate-limiting enzyme in cholesterol synthesis, and the decrease in its activity accordingly results in the cessation of cellular cholesterol production. The latter enzyme catalyzes the esterification of cholesterol, and thus any free cholesterol not immediately required in membrane synthesis is converted to cholesterol ester which accumulates in the form of cytoplasmic lipid droplets (121-124).

A final, and most important, consequence of this binding and uptake of LDL is the suppression of LDL binding to receptors, apparently due to a decrease in the number of receptors on the cell surface (125-128). This is probably due to a suppression of the synthesis of receptor molecules (125,128).

Conversely, when this cholesterol-loaded cell experiences a demand for cholesterol, the cholesterol esters stored as cytoplasmic lipid droplets can be hydrolysed (83). These therefore act as a buffer in the cells' cholesterol metabolism. Once this supply is exhausted, the LDL receptor can again be synthesised to take advantage of any cholesterol supply from the extracellular medium, and a stimulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity can result in an increase in the intracellular production of cholesterol.

In the normal steady state in vivo it appears that most extra-hepatic tissues do not synthesise cholesterol (129-132), but rely instead for a supply delivered through the circulation from the diet or from the central organs of cholesterol synthesis (Section 1.2). The control of extra-hepatic cellular cholesterol content may well therefore be achieved by adjustments in the amount of the LDL receptor.

Although all the features of this pathway have been elaborated following work with the cultured human fibroblast, a similar mechanism operates in cultured human lymphoblasts (133) and aortic smooth muscle cells (134) and in circulating human lymphocytes (135) and in cultured mouse Y-1 adrenal cells (136).

1.4 The functions of the hepatic endoplasmic reticulum in the metabolism of cholesterol

The roles of the liver in cholesterol metabolism were described within the context of whole body cholesterol metabolism in Section 1.2,

and are diagrammatically shown in Figure 1.3. Section 1.3 outlined the pathway by which an alternative cell type controls its cholesterol uptake and metabolism, and at least some of the features of this system might be expected to be exhibited by the liver cell.

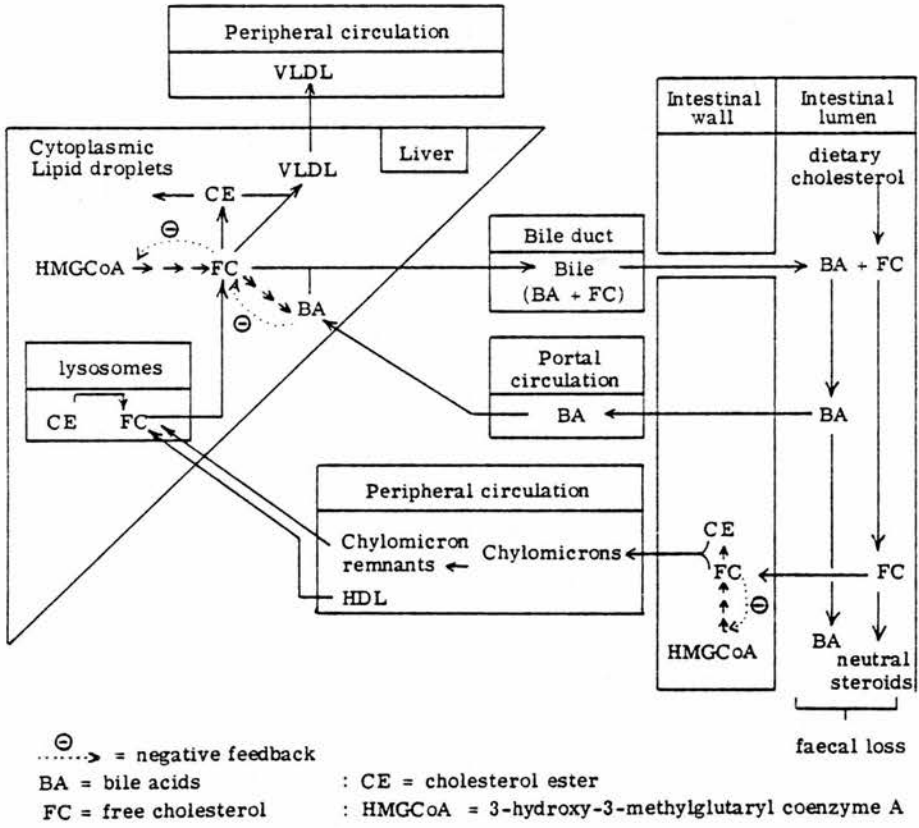
To summarise, those processes that might result in a net gain in the cholesterol content of liver cells are the synthesis of cholesterol and the uptake of lipoproteins from the bloodstream (in particular, the uptake of chylomicron remnants, but also probably the remnants of VLDL lipolysis and HDL and possibly even LDL). The uptake of lipoproteins will yield initially both free and esterified cholesterol, but if internalisation of lipoproteins is followed by lysosomal hydrolysis, as occurs in the uptake of LDL by fibroblasts, and as in some cases at least, also seems to occur in the liver (68,73,98,100,137,138), any cholesterol ester will be converted to free cholesterol before release into the cell.

Those processes resulting in no net change in cellular cholesterol content are cholesterol esterification and the hydrolysis of cholesterol ester. In the rat cholesterol esterification occurs both in the accumulation of cholesterol ester-rich cytoplasmic lipid droplets, and also contributes to the cholesterol ester content of lipoproteins. Whether cytoplasmic cholesterol ester can be used in the assembly of lipoproteins without prior hydrolysis and re-esterification is not known. In addition to lysosomal cholesterol ester hydrolysis, probably concerned exclusively with the uptake and disposal of lipoproteins from the plasma, this reaction can be catalysed by various other subcellular fractions of the liver, including microsomes, but the function of these other cholesterol ester hydrolases is at present not clear.

Processes resulting in a net loss of cholesterol from liver cells are the secretion of VLDL, HDL and also, in the rat, possibly LDL,

Figure 1.3

HEPATIC CHOLESTEROL METABOLISM



containing varying proportions of free and esterified cholesterol, the conversion of free cholesterol to bile acids, and the secretion of bile, containing bile acids and cholesterol. Figure 1.3 diagrammatically summarises all these hepatic functions in the metabolism and transport of cholesterol.

These functions can alternatively be divided into two major categories. First, metabolic transformations of cholesterol, such as synthesis, esterification and catabolism. Second,processes resulting in packaging or movement such as storage of lipid droplets, assembly and secretion of lipoproteins and bile, and uptake and degradation of lipoproteins.

Central to the metabolism of cholesterol by the liver are those processes occurring within the endoplasmic reticulum. These include the rate-determining reactions in the synthesis and catabolism of cholesterol, as well as many of the other enzymic steps in these pathways, cholesterol esterification, and the assembly of lipoproteins. The endoplasmic reticulum is a heterogeneous subcellular organelle largely recovered, on homogenisation and centrifugation, in the microsomal fraction (139-144).

This thesis concerns two of the processes described above as "metabolic transformations", namely the endoplasmic reticulum reactions catalysed by the cytochrome P-450-dependent enzyme cholesterol 7α-hydroxylase(145-147) (the rate-limiting reaction in bile acid synthesis) and by acyl-coenzyme A:cholesterol acyltransferase. However, although the work described concerns only these reactions and their control, implicit in it are the rates of synthesis of cholesterol, and the activity of the processes described as "packaging or movement", since, by analogy with the LDL receptor pathway, it can be expected that many of these systems will be regulated in a coordinated manner. The level of understanding of the regulation of these systems varies considerably.

Little is known about the production of cell surface receptors for the lipoproteins recognised by the liver. It can be speculated that the rate of production of these receptors will not be regulated because of the following observations. Firstly, the role of the liver as the major organ of cholesterol catabolism and the only organ of cholesterol excretion would perhaps preclude any regulation of the rate of hepatic cholesterol uptake from other tissues via the plasma. Secondly, the important part the liver plays in the adjustment of the whole body's cholesterol metabolism in sympathy with the cholesterol supply probably also suggests that any prevention of cholesterol uptake would be undesirable. Thirdly, the rate of clearance of chylomicron remnants by the liver does not appear to be a function of the hepatic rate of cholesterogenesis (68,70). Finally, when there is an excess supply of cholesterol to the liver, because of the ingestion of a cholesterol-rich diet, there is a considerable accumulation of cholesterol ester within the liver (148,149). In this respect, then, the hepatocyte appears to differ, or to be likely to differ, from the fibroblast.

Like the fibroblast, however, the hepatocyte does regulate its rate of cholesterol synthesis in accordance with the cholesterol supply (150-153). However, the function of the hepatocyte in the cholesterol transport system means that it senses the demands of all other tissues for cholesterol, in addition to taking account of its own requirements. Thus, when there is a surplus of cholesterol in the extrahepatic tissues, transfer of this excess cholesterol to HDL, and hence to the liver, will be high. Conversely, when there is a deficit in the cholesterol content of the extrahepatic tissues this transfer will be low. In addition, the uptake of LDL by extrahepatic tissues is dependant on their cholesterol content, being depressed when the content is adequate, and stimulated when cholesterol is required for membrane or steroid hormone synthesis

(Section 1.3). Since LDL uptake by the liver has been detected (70,77-79), it can be speculated that, under circumstances where the extrahepatic cholesterol supply is adequate and hence the extrahepatic removal of LDL from the plasma is low, excess LDL may be cleared by the liver. Therefore, by one or both of these mechanisms, the rate of return of cholesterol from the periphery to the liver reflects the cholesterol content of the peripheral tissues. Furthermore, the liver is the site for the clearance of chylomicron remnants, and also possibly VLDL remnants, from the plasma, and hence the hepatic uptake of cholesterol also reflects the intestinal absorption, and perhaps also the intestinal synthesis, of cholesterol. To summarise, these mechanisms mean that under circumstances of dietary intake of cholesterol and/or of adequate peripheral cholesterol content the supply of cholesterol to the liver will be high. When there is no dietary intake of cholesterol and/or there is a requirement for cholesterol in extrahepatic tissues the supply of cholesterol to the liver will be low. Cholesterol synthesis is particularly susceptible to feedback regulation, being strongly supressed in the presence of cholesterol (3,4,129,154-161). Thus, unlike the fibroblast which simply adjusts its cholesterogenesis to take account of its own requirements for, and supply of, cholesterol, the liver is in a position to regulate its cholesterogenesis in the light of the cholesterol supply and demand of the whole body.

The major site for the regulation of cholesterol synthesis is usually acknowledged to be at 3-hydroxy-3-methylglutaryl-coenzyme A reductase (3,4,154,155,162), although there is evidence for the coordinate regulation of other enzymes in the pathway. Mechanisms for the short term regulation of the enzyme involving feedback inhibition by cholesterol (149), phosphorylation/dephosphorylation (162-167), and the influence of the fluidity of the endoplasmic reticulum membrane

(168,169, and Section 1.6) have been proposed. The long term regulation of the activity of this enzyme is at the level of enzyme synthesis (4,155,170).

In the fibroblast, the accumulation of cholesterol ester in the presence of a rich supply of cholesterol is known to be accompanied by an increase in the activity of the cholesterol esterifying enzyme, acyl-coenzyme A:cholesterol acyltransferase (121,122). In the rat liver an increase in cholesterol supply is accompanied by an increase in the hepatic cholesterol ester content (148,149), but no reports of changes in acyl-coenzyme A:cholesterol acyltransferase activity had appeared at the time the work described here (Sections 6.4(a) and 6.4(b)) was undertaken.

Unlike fibroblasts, which have no specific mechanisms for cholesterol export other than passive loss from the membrane (presumably to HDL when *in vivo*), the liver cell loses cholesterol in lipoproteins, by conversion to bile acids, and, in association with phospholipids, in the bile.

Little is known about the control of the cholesterol content of either lipoproteins or bile (171). There is however a considerable body of information on the response of the rate of bile acid synthesis and cholesterol 7α-hydroxylase activity to a wide variety of experimental manipulations (Section 6.1).

Thus, the supply of a large amount of cholesterol to the liver of the rat tends to result in a stimulation of the rate of cholesterol conversion to bile acids (172,173), although the effect on cholesterol 7α-hydroxylase is disputed (147,161,174-176, and see Sections 6.1, 6.4(c) and 8.4(a)). Also, a high proportion of the bile salts secreted into the duodenum are re-absorbed in the terminal ileum and return to the liver through the hepatic portal circulation (177). The concentration of these returning bile acids is another determining factor in the rate

of bile acid synthesis and cholesterol 7α-hydroxylase activity. Thus, if this enterohepatic circulation is broken by the cannulation of the bile duct, or by feeding the bile acid-binding resin cholestyramine, the rates of bile acid synthesis and cholesterol 7α-hydroxylase activity both increase (147,174,178-182). The conversion of cholesterol to bile acids is thus largely determined by, among other factors, the presence of both the substrate and the end-product of this pathway.

However, despite the fact that the response of this pathway and cholesterol 7α-hydroxylase to these, and other, conditions are well known, the mechanisms by which these effects are mediated are still not clear.

1.5 The function of cholesterol in biological membranes

Natural membranes are complex associations of lipids and proteins, and in the study of the structure and function of membranes the use of simple models has frequently been of use. These models have included monolayers of lipid spread on water, black lipid membranes, and single or multiple walled liposomes, and these have been made of either single lipid species or of natural and artificial mixtures of lipids. Techniques used in the examination of membrane characteristics include x-ray crystallography, nuclear magnetic resonance, electron paramagnetic resonance, differential scanning calorimetry and the measurement of membrane permeability (183-189).

The result of this work is that membranes are now recognised to exist as bilayers of phospholipid arranged such that the fatty acid side chains of the lipid molecules are approximately aligned with each other and occupy the hydrophobic interior of the membrane, shielded from the aqueous environment by the polar head groups. Cholesterol is positioned with its hydroxyl group oriented towards the exterior polar layer while its nucleus and tail are sited within the hydrophobic interior. Protein molecules are embedded to varying degrees in the membrane, interacting with the different parts of the lipid environment by the appropriate hydrophobic or polar forces.

The single bonds of the fatty acid side chains of the phospholipids can exist in either the trans or the gauche conformation, and there can thus exist a degree of fluidity in the hydrophobic membrane interior. There is a gradient of probability of trans-gauche conformation changes within the membrane, and a corresponding fluidity gradient, the most fluid region being in the middle of the bilayer (190,191). In model membranes containing only one phospholipid species there is a characteristic transition temperature below which the fatty acid side chains exist in a rigid pseudo-crystalline state, with all trans conformation, and above which the lipids exist in a more fluid state (187). The change from pseudo-crystalline to fluid state is a co-operative phenomenon, with all the phospholipid molecules changing state simultaneously. The transition temperature is a function of phospholipid type, and fatty acid side chain length and saturation (192). In a simple mixture of lipids each lipid type condenses out at its own transition temperature, where these are sufficiently different from each other, and thus relatively extensive domains of rigid and fluid lipids can coexist (193-196).

The cholesterol molecule interacts with phospholipid in a non-stoichiometric fashion (188,197), with the hydroxyl group in the polar region of the membrane and the nucleus and the tail in the hydrophobic interior (188,198,199). Models of phospholipid-cholesterol complexes, incorporating highly specific interactions between the molecules, have been suggested (188,197-199), but not proved. However, various features of the cholesterol molecule have been demonstrated to result in an optimal interaction with phospholipids, including a planar nucleus (200) and tail length (201-203).

Addition of cholesterol to a lipid at a temperature below its phase transition temperature increases the probability of gauche

conformations and hence fluidity (184,187,204). Conversely, above the phase transition temperature cholesterol exerts a constraining effect, increasing the number of trans conformations, and decreasing the fluidity (184,187,204). Cholesterol thus induces the formation of a phase of fluidity intermediate between the pseudo-crystalline and liquid phases (187). In the presence of cholesterol it is not possible to detect a lipid phase transition temperature by differential scanning calorimetry (205), but laser Raman spectrometry suggests that a phase transition does occur, but is non-cooperative and is spread over a wide range of temperature (206,207).

Mixtures of phospholipids that differ sufficiently in their transition temperatures (for saturated phosphatidylcholine species this requires a difference in chain length of four or more carbons) show phase separation behaviour, as mentioned above. If cholesterol is added to such a mixture it is found to preferentially interact with the more fluid phospholipid (193). Thus, the non-random distribution of phospholipid can result in a non-random distribution of cholesterol. As well as depending on the fatty acid chain length, as in the above example, the distribution of cholesterol can depend on phospholipid type. Cholesterol tends to destabilize bilayers of phosphotidylethanolamine (208), and it is suggested that the phosphatidylethanolamine content of a membrane or of parts of a membrane may influence the cholesterol content of that membrane and its distribution within it.

1.6 The control of the activity of membrane-bound enzymes

The activity of membrane-bound enzymes can presumably be controlled by the same mechanisms that have been proposed for water-soluble enzymes. In addition, there is the possibility that some control may be exerted through the influence of membrane characteristics on enzyme activity

(209-212). Thus, in reconstitution studies many of these enzymes are inactivated on removal of lipid, activity being restored by the addition of lipid to the lipid-free preparation (213-216). Further, in intact preparations Arrhenius plots of enzyme activity as a function of the absolute temperature often show breaks at or near the lipid phase transition temperature (217-221). In the past these observations have been interpreted in terms of fluidity changes. It now appears that a better explanation may be in terms of lateral phase separations occurring at temperatures close to the transition temperature (211).

These observations have seldom been satisfactorily explained but possible roles for lipid in the mechanisms of action of membrane enzymes include the maintenance of enzyme configuration in an active form by binding the hydrophobic portions of the protein structure; ensuring the correct interaction of separate protein subunits of the enzyme, or the proximity of enzymes where more than one is required for overall activity; allowing the correct approach of substrate to enzyme, especially if the substrate partitions within the hydrophobic phase provided by the membrane interior.

Despite the fact that many membrane-bound enzymes are not disputed to either have an absolute requirement for lipid or to be influenced by the physical characteristics of the lipid matrix in which they are sited, there is little evidence for *in vivo* regulation of enzyme activity by changes in membrane properties in mammals. However, the possibility of such a control mechanism operating in the case of enzymes involved in cholesterol metabolism is particularly interesting, since cholesterol itself has such a profound influence on membranes' properties and hence, potentially, on enzyme activity. Such a mechanism has been proposed to operate in the case of the rate-determining enzyme of cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (168,169).

Factors influencing the activities of membrane-bound enzymes are further discussed in Section 8.2.

Section 2

Materials and Methods

2.1 Animals and diets

Female rats of the Wistar strain, in the weight range 150-250 g, were used throughout. These were bred at the Edinburgh University Small Animal Breeding Station. The rats were subjected to a controlled lighting schedule with a dark period, unless stated otherwise, between 1900 and 0700.

In dietary experiments, the control (or "soft") diet used was 70% wholemeal flour, 25% skimmed milk powder, 5% dried yeast. Experimental diets were made up by mixing the required components into this base. These additional components were the bile acid sequestering agent, cholestyramine, at a concentration of 4% w/w, or olive oil, at a concentration of 10% w/w, or cholesterol dissolved in olive oil, at final concentrations of 1% and 10% w/w respectively. When phenobarbital was given, to increase the hepatic content of cytochrome P-450, this was administered as a 0.1% solution in a 1% solution of glucose in the drinking water. Diets and drinking water were available *ad libitum*.

2.2 Preparation of microsomes

Rats were anaesthetised with ether, a cannula was inserted into the hepatic portal vein, and the liver was perfused with ice-cold 0.154 M potassium chloride. This had the effect of reducing contamination by haemoglobin and plasma enzymes, and also of rapidly cooling the liver.

The liver was removed, finely chopped with a pair of scissors, and homogenised with three passes of a Potter-Elvehjem homogeniser to give a 25% w/v homogenate in 0.154 M KCl. This was centrifuged at 8 000 x g to remove cell debris, nuclei and mitochondria. The resultant supernatant was subjected to further centrifugation at 105 000 x g, to sediment microsomes. This method is essentially that described by Ernster et al (222). When possible, fresh microsomes were prepared for each experiment.

If microsomes were to be stored for future use, they were suspended in a small quantity of distilled water, freeze-dried, and stored at $-20^{o}C$.

2.3 Butanol extraction of rat liver microsomes

Extraction of microsomes with butanol results in a large reduction of the lipid content, and especially the cholesterol content, of the preparation, with only minimal losses in the activity of cholesterol 7α-hydroxylase (223-226). All apparatus and solvents were pre-cooled to $-20^{o}C$, and strictly anhydrous conditions were maintained throughout. Freeze-dried microsomes were briefly homogenised in butanol and centrifuged at 12 000 x g for 2 minutes. The butanol was poured off, and the surface of the pellet was gently washed with acetone to remove excess butanol. The pellet was then resuspended in acetone, and the suspension was centrifuged at 2 000 x g for 30 s. The pellet was stirred into 200 ml acetone, and the acetone was then removed by filtration. The resultant powder was kept in the vacuum desiccator for 1 hr, and then stored at $-20^{o}C$.

2.4 Solubilisation of microsomal proteins

For solubilisation of microsomal protein, a freeze-dried powder of microsomes, or a butanol-extracted freeze-dried powder (a "butanol powder"), was suspended in an appropriate buffer at approximately 10 mg protein per ml. The buffer chosen depended on the starting conditions for the following column chromatography step. Nonidet P42 was added slowly to a final concentration of 4 µl per mg protein. After stirring on ice for 20 minutes, the solution was centrifuged at 100 000 x g for 1 hr, and the resultant supernatant was used for subsequent chromatography (225,226).

2.5 Assay for cytochromes P-450 and b_5

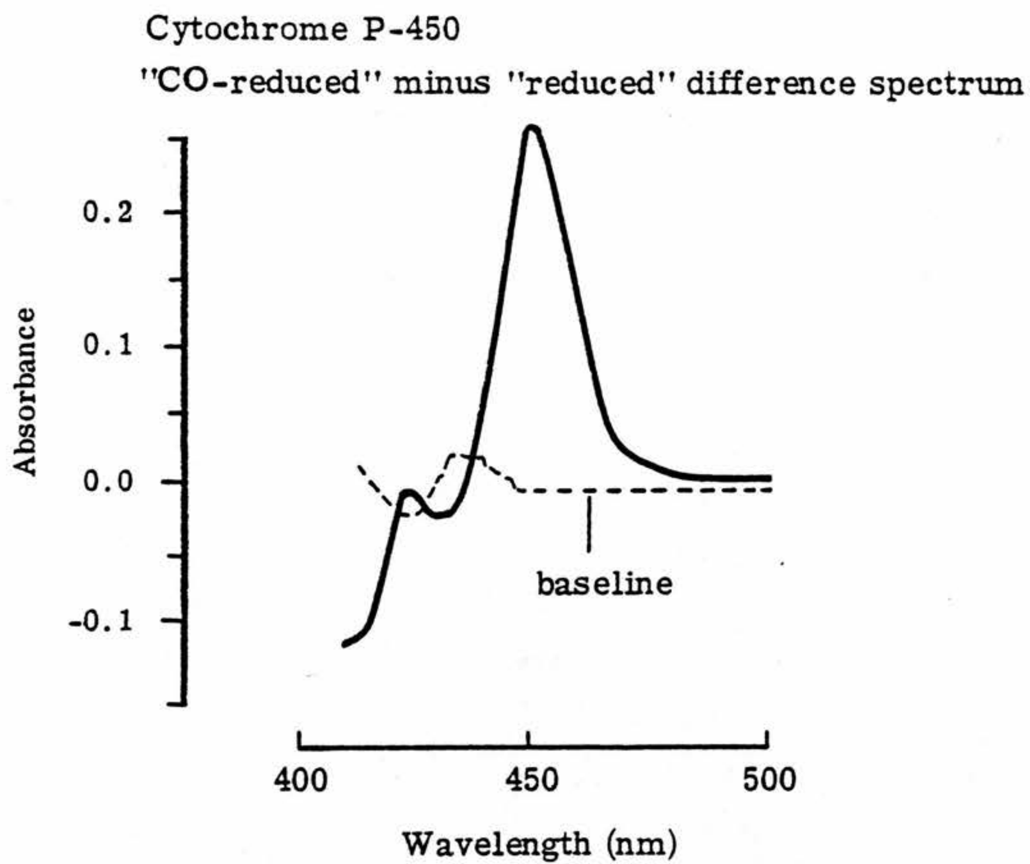
Difference spectra were recorded on a Unicam SP800 split beam spectrophotometer. Cytochrome P-450 concentration was determined by the method of Omura and Sato (227). A few grains of sodium dithionite were added to approximately 6 ml of sample solution. This was divided between two cuvettes and a base-line spectrum was recorded. Carbon monoxide was bubbled through the sample cuvette for 30 s, and a recording was then made of the "CO-reduced" minus "reduced" difference spectrum. The cytochrome P-450 concentration was calculated from the absorbance at 450 nm relative to 490 nm, using an extinction coefficient of 91 $mM^{-1}cm^{-1}$. Figure 2.1 shows a typical liver microsomal cytochrome P-450 spectrum.

Cytochrome b_5 was estimated from a "reduced minus oxidised" difference spectrum. Thus, after recording a base line spectrum, if cytochrome P-450 and NADH-cytochrome c reductase were present, a few grains of NADH were added to the sample, or in the absence of these components a few grains of sodium dithionite were added, and a difference spectrum was recorded. The cytochrome b_5 content was estimated from the difference in absorption at 424 nm and 409 nm, using an extinction coefficient of 185 $mM^{-1}cm^{-1}$ (228). A typical liver microsomal cytochrome b_5 spectrum is shown in Figure 2.2.

2.6 Assay for NADPH-cytochrome c reductase

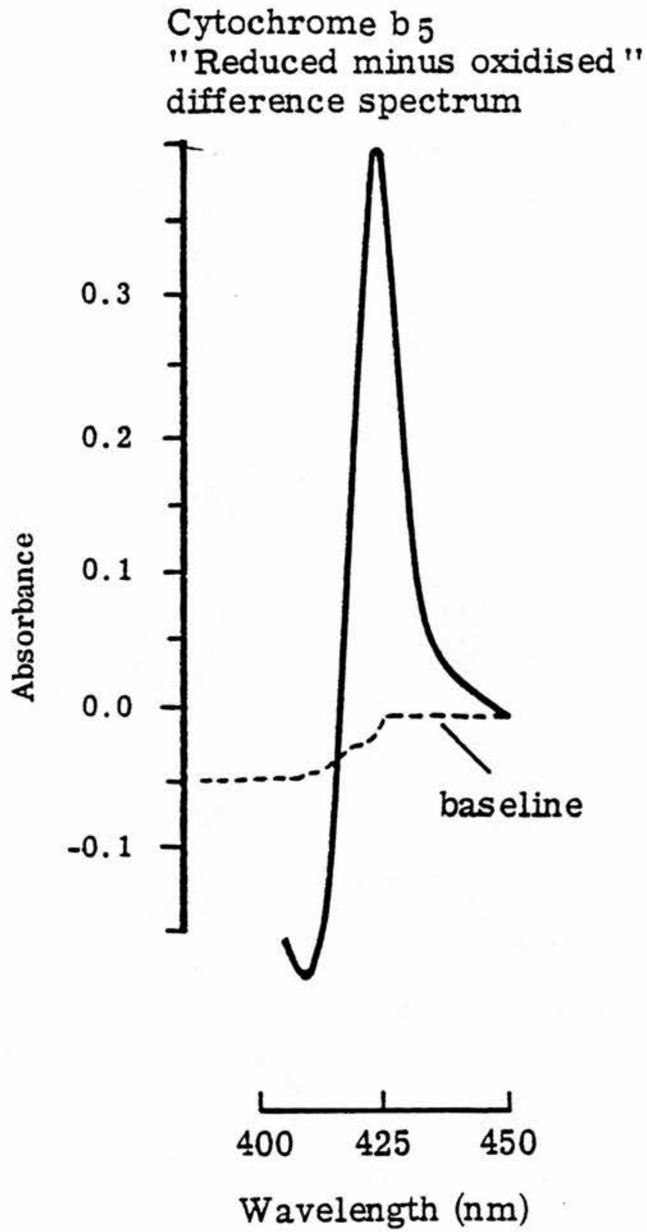
1 ml of a solution of 0.4 mg cytochrome c, 1 mg glucose-6-phosphate, 0.3 mg $NADP^+$ and 0.3 U glucose-6-phosphate dehydrogenase per ml 0.1 M potassium phosphate buffer, pH 7.7, was added to a 1 ml cuvette, in a water-jacketed cell-holder, maintained at 25°C in the Unicam SP800 spectrophotometer. Reaction was started by the addition of an appropriate amount of flavoprotein - containing sample. The NADPH-cytochrome c

Figure 2.1



(For method see text).

Figure 2.2



(For method see text).

reductase activity was determined by measuring the rate of change of absorbance at 550 nm against a suitable blank, and using an extinction coefficient for reduced minus oxidised cytochrome c at 550 nm of 19 $mM^{-1}cm^{-1}$ (229-231). The blank usually used was 1 ml of the assay solution without the addition of the sample. However, when relatively large volumes of turbid samples had to be used the blank used was 1 ml of assay solution made up without glucose-6-phosphate dehydrogenase. Equal volumes of the sample to be assayed were then added to the reference and sample cuvettes.

2.7 Assays for protein

Protein was usually assayed by the method of Lowry et al (232), or by the biuret method (233). In the presence of KCl or Nonidet P42, a slight turbidity developed when the Lowry assay was used. The use of suitable standard solutions containing appropriate concentrations of these components, or centrifugation to clear the solutions, was shown to allow accurate determination of protein concentration in these samples. When interference was very strong, for example in the presence of diethyldithiocarbamate, used in some column chromatography, an alternative assay based on protein-binding to the dye Coomassie Brilliant Blue G250 was used (234).

2.8 Lipid extractions

Where lipid contents of microsomal samples were to be measured, lipids were extracted from the samples prior to assay by the method of Folch et al (235), using a small aliquot of [4-^{14}C]-cholesterol to assess the recovery of lipid through the extraction procedure.

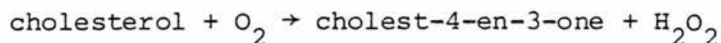
2.9 Assay of phospholipid

Phospholipid was assayed by the method of McClare (236). The assay reagent was made up by dissolving 2.5 g ammonium molybdate in water,

adding 20 ml perchloric acid, followed by 0.5 g ascorbic acid dissolved in water, and making up to 250 ml with water. (The order of mixing these compounds is important.) Perchloric acid (0.5 ml) was added to the dried lipid extract, and heated to 200°C for one hour, to convert all "lipid phosphate" (or organic phosphate) to free phosphate. After cooling, 9.5 ml assay reagent was added, the resultant mixture was incubated at 50°C for one hour, cooled to room temperature and the absorbance at 825 nm was measured. A standard curve was constructed using a standard solution of triphenylphosphine in ethanol. The assay was used in the range of 0.02 to 0.1 μmoles per assay tube.

2.10 Assays for cholesterol

Assays for cholesterol were usually enzymatic, using cholesterol oxidase from Nocardia sp., and were either colorimetric (237) or fluorimetric (238). Cholesterol oxidase releases hydrogen peroxide as one of the products of its reaction:



$$\text{cholesterol} + O_2 \rightarrow \text{cholest-4-en-3-one} + H_2O_2$$

This hydrogen peroxide can be estimated by its reaction, in the presence of peroxidase, with a suitable oxygen acceptor to form a species that can be detected either colorimetrically (in which case the reagent employed was 4-aminophenazone) or fluorimetrically (when p-hydroxyphenyl-acetic acid was used). Cholesterol ester contents were measured as the difference in free cholesterol contents before and after hydrolysis with 0.5 M KOH in 90% ethanol. Alternatively, during the mass fragmentographic assay for cholesterol 7α-hydroxylase, cholesterol contents were calculated from the total ionic current trace, which corresponds to a conventional gas chromatographic assay, using cholestane as an internal standard.

2.11 Assays for cholesterol 7α-hydroxylase

The activity of cholesterol 7α-hydroxylase was routinely assayed by determining the percentage conversion of [4-^{14}C]-cholesterol to [4-^{14}C]-7α-hydroxycholesterol on incubation with microsomes and the necessary cofactors. This assay has certain disadvantages, due to the dilution of the added tracer by endogenous cholesterol, and to the compartmentation of this endogenous cholesterol into pools of varying ability to act as substrate for cholesterol 7α-hydroxylase (239, and see Sections 2.12, 2.13, 4.1, 7.4 and 8.2). These factors prevent the calculation, from this assay, of enzyme activity in terms of actual production of 7α-hydroxycholesterol. However, this technique is convenient, requiring no complex or expensive equipment. Furthermore, under most circumstances, results obtained by this assay are in good agreement with results obtained by the determination of 7α-hydroxycholesterol production (240-242).

Assays which measure the actual production of 7α-hydroxycholesterol use gas-liquid chromatography (202,240,243) or combined gas-liquid chromatography and mass spectrometry (174,241,244) to directly measure the mass of 7α-hydroxycholesterol. More indirectly, assay incubations can be performed in the presence of [4-^{14}C]-cholesterol, enabling 7α-hydroxycholesterol (both labelled and unlabelled) to be readily detected on separation from other sterols by thin layer chromatography. The amount of this product can then be assessed by reaction with [^{3}H]-acetic anhydride of known specific radioactivity (245,246). Of these methods, gas-liquid chromatography has not proved to be reliable in this laboratory, apparently because of the breakdown of the sterol (whether derivatized or not) at the column temperatures used (Dr. I.F. Craig, personal communication); combined gas-liquid chromatography-mass spectrometry is perhaps the most satisfactory assay

of all, but requires very complex and expensive instrumentation, which was only available for a few experiments, and not for routine use; the use of [^{3}H]-acetic anhydride in the assay of 7α-hydroxycholesterol gives an assay which is extremely time-consuming to perform.

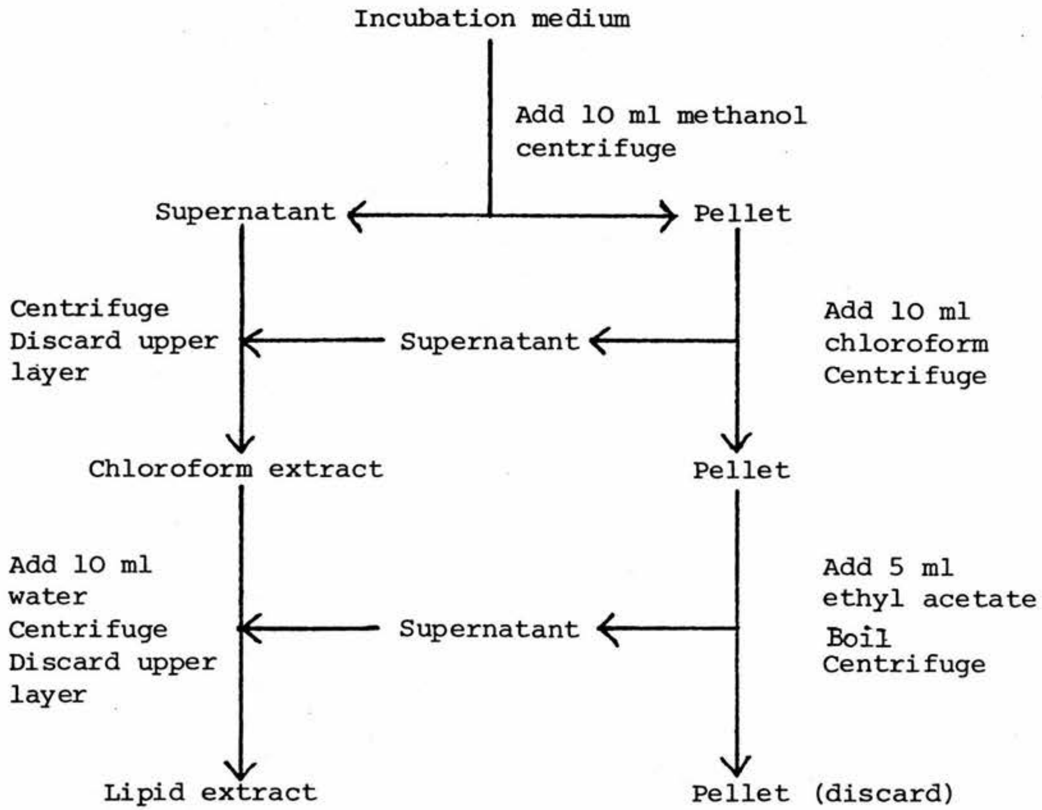
It was therefore decided that the use of the [4-^{14}C]-cholesterol-based assay, giving results in terms of per cent conversion to [4-^{14}C]-7α-hydroxycholesterol, was satisfactory for most experiments, provided that the limitations of this technique were appreciated. The conditions and method used were similar to those previously described by members of this laboratory (225,226,227).

The assay incubations contained 12.5 mg glucose-6-phosphate, 3.75 mg NADP$^+$, 8 mg β-mercaptoethylamine, 1.0 U glucose-6-phosphate dehydrogenase, approximately 10 mg microsomal protein (or the equivalent of 1 g liver) and 0.05 μCi radioactive cholesterol added in 50 μl acetone in a total of 7 ml 0.1 M potassium phosphate buffer, pH 7.4. Reaction was started by the addition either of the [4-^{14}C]-cholesterol or of the NADP$^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase mixture ("NADPH generator"). After 60 minutes in a shaking water bath at 37°, reaction was stopped by the addition of 5 ml methanol, and the solution was transferred to a glass-stoppered tube with the help of a further 5 ml methanol. Neutral lipid was extracted according to the scheme shown (Figure 2.3).

The reaction products were separated from each other and from the substrate by thin layer chromatography on silica gel, using a 7:13 mixture of benzene or toluene and ethylacetate as the solvent system. Peaks of radioactivity were located by scanning with a Panax thin layer radioactive scanner, and scraped directly into scintillation vails. 5 ml scintillant (4 g PPO, 30 mg POPOP per 1 toluene) was added, and radioactivity was determined in a Packard Tri-Carb scintillation spectrophotometer. Activity was expressed as the percentage of [4-^{14}C]-cholesterol converted to [4-^{14}C]-7α-hydroxycholesterol.

Figure 2.3

Lipid extraction scheme for cholesterol 7α-hydroxylase assay



In a few experiments, where the use of an assay giving activities as per cent conversions was particularly unsatisfactory, assays were performed using a mass fragmentographic procedure in collaboration with Professor Galli of the Institute of Pharmacology and Pharmacognosy at the University of Milan. For these assays, incubations were conducted as usual, with 10 mg microsomal protein per incubation flask when simultaneous radioactive assays were being performed on the same incubation, or with 5 mg microsomal protein if only the mass fragmentographic assay was to be performed. On stopping the reaction with methanol, cholestane (10 μg/mg protein) was added as an internal standard. Lipid extraction was as normal. If a simultaneous tracer assay was being performed, half the extract was then taken for thin layer chromatography. The lipid extract for mass fragmentography was stored, dry, in vials sealed under nitrogen.

A standard curve was prepared by pooling microsomal suspensions from all groups of animals in the experiment (for experiments lasting more than 12 hr, microsomes were stored frozen until the end of the experiment, and then thawed and pooled), and aliquots containing 5 mg protein were made up to a total of 7 ml with 0.1 M potassium phosphate buffer pH 7.4. Varying amounts of standard 7α-hydroxycholesterol and 50 μg cholestane were added to each flask. Extraction and storage was as above.

These vials were sent, or taken, to Milan for analysis. The conditions used were as have been described (244). The extracts were dissolved in ethyl acetate, and suitable aliquots were taken to dryness under nitrogen. After silylation with a 3:1 mixture of N,O-bis-trimethyl-silyl-trifluoroacetamide and pyridine, samples were injected into the Varian MAT 112 S gas chromatograph-mass spectrometer. The glass column, 2 m x 3 mm, was packed with 3% SE-30 on 100- to 120-mesh Gaschrom P

(Applied Science, State College, Pa.). The oven temperature was 280°C and the injector and detector were maintained at 290°C. The helium gas flow was 15 μl/min. The electron energy was set at 70 eV, the emission current at 1.5 mA, and the electron multiplier at 2.5 kV. The molecular separator and the ion source were at 280°C. The ions at m/e 546 (M^+ of di-trimethylsilylethers of cholestendiols) and at m/e 443 (M^+ -15 of trimethylsilylether of cholesterol, corresponding to the molecular fragment resulting from the loss of an angular methyl group) were focussed. The instrument allowed continuous monitoring of the total ionic current, and the ionic currents at m/e 546 and m/e 443. A typical example of the trace obtained is shown in Figure 2.4.

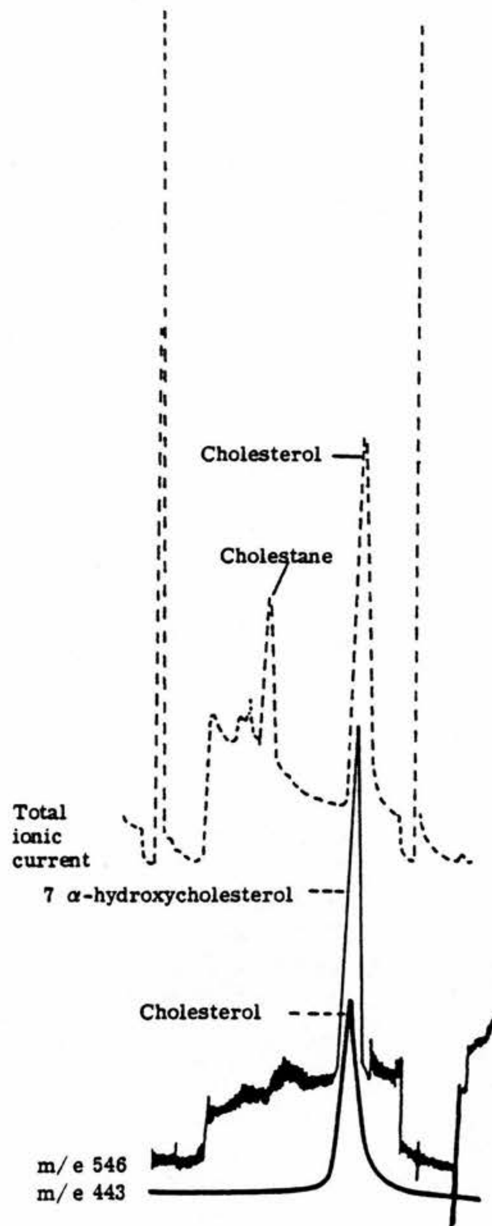
Comparison of the peak heights corresponding to cholesterol and to cholestane in the sample and in a standard 1:1 mixture of cholesterol and cholestane allows the calculation of cholesterol content. For the standard curve, a regression line is calculated for the ratio of the heights of the peaks at m/e 546 and m/e 443 against the ratio of the known amount of added 7α-hydroxycholesterol to the measured amount of cholesterol. Using the parameters of this regression line, for any sample with an unknown content of 7α-hydroxycholesterol, the ratio of the heights of the peaks at m/e 546 and m/e 443, and the measured cholesterol content,allows the determination of the 7α-hydroxycholesterol content.

2.12 Assays for acyl-coenzyme A:cholesterol acyltransferase

The conditions used for the assay of acyl-coenzyme A:cholesterol acyltransferase were essentially as described by Goodman et al (248) and by Goodman (249). For a further discussion of the choice of assay conditions see Section 4. The incubation mixture contained 2 mM ATP, 4 mM $MgCl_2$, 0.05 mM coenzyme A, 3.3 mM reduced glutathione and

Figure 2.4

Mass fragmentographic trace from cholesterol 7α-hydroxylase assay



(For sample preparation and running conditions see text. The gain in the signal at m/e 546 was 333.3 times that in the signal at m/e 443.)

approximately 100 μg of microsomal protein, in a total volume of 3 ml 0.1 M potassium phosphate buffer pH 7.1. Although a considerable range of protein concentration could be used for this assay, within any one experiment protein concentrations were kept as similar as possible because of the non-linearity of enzyme activity with protein concentration (further discussed in Sections 4.2 and 8.2). Reaction was timed from the addition of 0.05 μCi [4-^{14}C]-cholesterol, and was stopped after 20 minutes in a shaking water bath at 37°C, by the addition of 5 ml methanol. The mixture was brought briefly to the boil, and, after cooling, 10 ml chloroform was added. The upper, aqueous methanolic, phase was re-extracted with 20 ml of a 2:1 mixture of chloroform and methanol. The upper phase was discarded and the two chloroform phases were combined, taken to dryness, and subjected to thin layer chromatography on silica gel, using as developing solvent a 70:30:1 mixture of petroleum spirit, diethyl ether, acetic acid. The radioactive peaks were located and quantified as for the cholesterol 7α-hydroxylase assay. Activity was expressed as per cent conversion of [4-^{14}C]-cholesterol to [4-^{14}C]-cholesterol ester. This assay has the same drawbacks as the equivalent assay for cholesterol 7α-hydroxylase activity, in that this result cannot be converted to an actual rate of product formation, and is dependent on both enzyme activity and on the distribution of cholesterol between the various microsomal compartments.

In a few preliminary experiments, in order to measure ACAT activity in absolute terms, [1-^{14}C]-oleic acid was used as tracer instead of [4-^{14}C]- cholesterol. In this case, 1 mg bovine serum albumin and 0.05 μCi [1-^{14}C]-oleic acid was added per incubation and the reaction was started by the addition of the microsomal suspension. Activity was calculated from the radioactivity found as cholesteryl ester, and the specific radioactivity of the added oleic acid, assuming negligible dilution by endogenous fatty acids.

2.13 The calculation of a maximal estimate of substrate pool size for cholesterol 7α-hydroxylase or acyl-coenzyme A:cholesterol acyltransferase

There is evidence that not all microsomal cholesterol is available to act as substrate for either cholesterol 7α-hydroxylase (174,245,250,251) or acyl-coenzyme A:cholesterol acyltransferase (251-254). Where assays for these enzymes were performed both by measurement of the percent conversion of exogenous labelled cholesterol to product and by absolute measurement of the amount of product formed it is possible to obtain an estimate of the maximum possible size of the pool of microsomal cholesterol which acts as substrate (239,250).

If it is temporarily assumed that microsomal cholesterol exists as a single homogeneous pool, and that all exogenous labelled cholesterol partitions uniformly into this pool, it is possible to calculate an absolute enzyme activity by multiplying the percent conversion of labelled cholesterol to product by the total amount of microsomal cholesterol present. This will overestimate the actual enzyme activity by the same factor as that by which the total microsomal cholesterol is an over-estimate of the size of the substrate pool, as long as all the exogenous tracer cholesterol is available to the enzyme. If the actual enzyme activity is known by an independent absolute assay it is therefore possible to calculate the fraction of the total microsomal cholesterol that is in the enzyme's substrate pool. This estimate will always give the maximum possible substrate pool size, since it relies on the assumption that all exogenous tracer cholesterol enters the substrate pool, and the fraction of the tracer cholesterol which actually does enter this pool cannot be obtained.

This explanation of the justification for this calculation is an alternative expression of the directly analogous argument presented by Balasubramanian et al (250).

The dependence of this estimate of substrate pool size on experimental conditions and in particular on the vehicle used to add the cholesterol label to microsomal incubations (174,250), has led to the usefulness of the concept of the term "substrate pool" being questioned (174). However, despite the impossibility of determining the actual size of the substrate pool, the evidence suggests that such an entity exists, and that as long as experiments are performed under carefully controlled conditions, limited comparisons of estimated maximal substrate pool size within any one experiment may be useful (239).

2.14 Assay for flavins

Flavin adenine dinucleotide and flavin mononucleotide were assayed by the method of Faeder and Siegel (255).

2.15 Liposome preparation

Liposomes were prepared by the method of Bloj and Zilversmidt (256). A solution of the required amount of lipid was made up in chloroform. This was taken to dryness under nitrogen, and the last traces of organic solvent removed by storing in the vacuum desiccator for one hour. Buffer (chosen as appropriate for the remainder of the experiment) was added to give a final concentration of lecithin of approximately 3 mg/ml. The solution was then shaken with a vortex mixer for five minutes, to give multilamellar liposomes. When single-walled liposomes were required these were then subjected to sonication in an ice bath, either with the wide probe, power setting 8, of the Ultrasonics Ltd Rapidis 150 sonicator, output 20W, (2 minutes on, followed by 30 seconds off for 40 minutes), followed by centrifugation at 100 000 x g for 30 minutes to remove titanium particles, or with a sonication bath (Dawe Instruments Ltd, Sonicleaner Type 6443A) for 3-4 hours, until clear. These conditions have been well characterised as giving single-walled liposomes (202,257).

2.16 DEAE-cellulose

DEAE-cellulose for column chromatography was prepared, and linear gradients of KCl were generated according to Peterson and Soper (258).

2.17 Statistics

When estimating the significance of the difference of two results, Student's t test was used unless specifically stated to the contrary.

2.18 Materials

All common reagents were purchased from BDH or Sigma and were of Analar Grade.

Cytochrome c, glucose-6-phosphate, $NADP^+$, NADH, glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim GmbH, Mannheim.

Nonidet P42 was obtained from BDH Chemicals Ltd, Poole, England.

Renex 690 was obtained from Atlas Products, ICI United States, Inc.

PPO and POPOP were obtained from Koch-Light, England.

QAE - Sephadex was obtained from Pharmacia, Uppsala, Sweden.

DEAE-cellulose was obtained from Sigma.

CM-cellulose was obtained from Whatman, England.

Bio-Gel HTP (hydroxyapatite) was obtained from Bio-Rad, England.

Cholestyramine resin (Cuemid) and Kieselgel H (silica gel) were obtained from Merck, Sharp and Dohme.

[4-^{14}C]-Cholesterol and [1-^{14}C]-oleic acid were obtained from the Radiochemical Centre, Amersham. Before use, the [4-^{14}C]-cholesterol was purified by thin-layer chromatography on silica gel, using as the developing solvent a 70:30:2 mixture of di-isopropylether, petroleum spirit and acetic acid.

Section 3

Approaches to the Purification of a Cytochrome P-450 Species Reconstitutively Active in Cholesterol 7α-Hydroxylation

3.1 Introduction

(a) The requirement for a purified preparation of 7α-hydroxylase

Much work has been done on the *in vivo* regulation of bile acid synthesis and cholesterol 7α-hydroxylase activity. In order to extend further some of the ideas developed from the results of these experiments it will be necessary to examine the molecular mechanism of action of cholesterol 7α-hydroxylase and its control. This demands the use of a reasonably pure preparation of the enzyme. Among the reasons for this requirement are the following.

Under various circumstances HMGCoA-reductase and cholesterol 7α-hydroxylase, usually considered to be the rate-determining enzymes in cholesterol and bile acid synthesis, respectively, are regulated in coordination with each other (4,147,155,178,181,239,259-273). Since there is some evidence that the preferred substrate for bile acid production is newly synthesized cholesterol (174,250,274-276), it has proved difficult to separate the effects of these enzymes on the regulation of bile acid synthesis. The elucidation, at a molecular level, of a mechanism for the control of cholesterol 7α-hydroxylase in the absence of HMGCoA-reductase may help to resolve this problem.

A number of cytochrome P-450 species with different substrate specificities exist in the liver (277-283). At present there is no unequivocal evidence for the existence of a cytochrome P-450 species specific for cholesterol 7α-hydroxylase. Indeed, inducers of microsomal cytochrome P-450 have not been convincingly shown to induce cholesterol 7α-hydroxylase (266,284-287), cholestyramine feeding or the creation of a bile fistula results in an increase in cholesterol 7α-hydroxylase activity while the microsomal cytochrome P-450 content remains unchanged or decreases (146,266,287-289), and thyroxine treatment and thyroidectomy have opposite effects on liver microsomal cytochrome P-450 contents and

cholesterol 7α-hydroxylase activities (266-268). The reconstitution of enzyme activity with separate, pure components might allow an investigation of whether these results are best explained in terms of the existence of a cytochrome P-450 species specific for cholesterol 7α-hydroxylase, present in too small an amount for changes in its quantity to have a significant effect on total microsomal cytochrome P-450 content, or whether activity is dependent on some broad specificity cytochrome P-450, possibly in conjunction with one or more inducible protein or non-protein cofactors.

The reconstitution of drug hydroxylating activity from pure preparations of cytochrome P-450 and NADPH-cytochrome c reductase requires the presence of phospholipid (290), possibly to allow the correct interaction of these two components so that electron transfer can occur (290,291). There is some evidence that there is no such requirement for cholesterol 7α-hydroxylase activity (225). However, the enzyme preparations used were not pure enough to be free of contamination by phospholipid or detergent, especially since the solubilisation of microsomes by nonionic detergents does not allow good separation of phospholipid from cytochrome P-450, in contrast to the use of bile acid detergents (292,293). Further, nonionic detergents are not easily removed from protein preparations (294,295), and it is possible that detergent may substitute effectively for phospholipid in maintaining enzyme activity (213). The use of pure preparations of cholesterol 7α-hydroxylase might help to show whether this enzyme also requires phospholipid for electron transfer. It has been suggested that substrate binding to cytochrome P-450 is mediated by membrane lipids (195,296). The use of isolated enzyme components should demonstrate whether phospholipid is also required for the binding of cholesterol, an endogenous membrane component.

It is possible that cholesterol 7α-hydroxylase can respond to changes in the amount of its substrate in its environment, either because it is operating at substrate levels below saturation, or through some more subtle effect of cholesterol either directly on the enzyme, or through the mediation of changes in membrane fluidity. A particularly satisfactory way of investigating this would be to reconstitute the enzyme in liposomal membranes of varying cholesterol content.

Finally, by direct analogy with other microsomal cytochrome P-450-dependent enzymes, studies could be made of the substrate-binding, oxygen-binding and electron-accepting steps in the enzyme mechanism, of the interactions and stoichiometry of the cytochrome and its reductase, of the possible involvement of cytochrome b_5 in the enzyme mechanism, of the nature of the active site and many other unresolved problems.

Since there is evidence that the specificity for cholesterol 7α-hydroxylase activity resides in cytochrome P-450-containing fractions rather than NADPH-cytochrome c reductase-containing fractions in preliminary resolution experiments (225), the approach adopted in this laboratory has been to attempt to fractionate cytochrome P-450 for subsequent recombination with NADPH-cytochrome c reductase purified by published methods.

(b) Purifications of microsomal cytochromes P-450 reconstitutively active in hydroxylations of substrates other than cholesterol

Several groups of workers have now published schemes for the purification of cytochrome P-450 from the microsomes of livers of rats (297-301), rabbits (302-305), and mice (306) and the lungs of rats (307) and rabbits (308). Much of this work has been well summarised by Lu and Levin (292). Several important general points have emerged from this work.

Firstly, it is important to prevent extensive degradation of cytochrome P-450 to cytochrome P-420. Since the discovery that certain thiols and polyols promote the conversion of cytochrome P-420 to cytochrome P-450 (309), this has usually been effectively accomplished by the inclusion of glycerol, and frequently also dithiothreitol in buffers used in the purification of cytochrome P-450 (297,308,310).

Secondly, the choice of detergent used for the solubilisation of the microsomal membranes is crucial. The majority of detergents will inhibit hydroxylations to some extent, and some detergents are extremely inhibitory to some reactions. The degree of this inhibition needs to be established for any reaction of interest, with a wide variety of detergents. Ionic detergents, such as sodium cholate or deoxycholate, tend to have little inhibitory action on microsomal hydroxylations, and to allow a good separation of cytochrome P-450 from phospholipid, but not from NADPH-cytochrome c reductase. These detergents can be largely removed from protein preparations by extensive dialysis. Nonionic detergents are more likely to inhibit enzymic reactions and cannot easily be removed from the purified preparation because of their low critical micellar concentration, and large micelle size. However, in the presence of nonionic detergents the separation of cytochrome P-450 from NADPH-cytochrome c reductase is much improved.

An example of the use of sodium cholate for cytochrome P-450 preparation is offered by the early stages of the scheme reported by Lu and Levin (1972) (311). After solubilisation of microsomes with cholate, the resultant solution was fractionated with ammonium sulphate. Cytochrome P-450-containing fractions were redissolved, adsorbed to calcium phosphate gel and eluted with increasing concentrations of phosphate buffer. This gave a three-fold purification, with contamination by cytochrome b_5 and NADPH-cytochrome c reductase, but with low levels of

phospholipid. A similar preparation has been reported for rabbit liver cytochrome P-450, but with fractionation by polyethyleneglycol 6000 preceeding that by ammonium sulphate (312).

The exclusive use of nonionic detergents for cytochrome P-450 preparation has been reported by Miyake et al (Lubrol WX) (293), Fujita et al (Triton N-101) (313), and Sato et al (Emulgen 911) (314). The specific content of these preparations was not more than three to four times that of the starting material, although contamination by NADPH-cytochrome c reductase was low. However, where reported, phospholipid levels were high.

It has usually proved most satisfactory to use a combination of detergents for cytochrome P-450 purification. After solubilisation with a bile acid detergent and an initial fractionation with ammonium sulphate or polyethylene glycol, the resultant fractions are redissolved in a nonionic detergent-containing buffer before chromatography. Detergents used include Renex 690 (315,316), Emulgen 911 (299,311) and Nonidet P40 (305).

Most cytochrome P-450 purification schemes, after the initial fractionation and replacement of ionic with nonionic detergents, have involved some combination of chromatography on DEAE-cellulose with elution with increasing KCl concentrations, and adsorption to calcium phosphate gel, with elution by increasing concentrations of phosphate buffer (298,305,306,310,315,316). Some workers have found the use of octylamino-Sepharose to be satisfactory (303,307,308,317), and the use of lauric acid affinity chromatography has been reported (301).

It is of interest to note that the usually low overall recovery of cytochrome P-450 and the length of time involved in purification procedures consisting of many steps has recently resulted in increased attention to improving recoveries of cytochrome P-450 in the initial

steps of purification schemes. The use of affinity chromatography at a relatively early stage of a purification is often a particularly satisfactory solution to these problems.

(c) Purification of a cytochrome P-450 preparation capable of supporting cholesterol 7α-hydroxylation in a reconstituted system

(i) Procedures used in this laboratory

Dr. Hattersley, of this laboratory, tried many techniques, in various combinations, in attempts to purify preparations of cytochrome P-450 capable of supporting cholesterol 7α-hydroxylase activity in the presence of NADPH-cytochrome c reductase (225). Perhaps the most satisfactory preparation involved the solubilization of butanol-extracted microsomes with 4 μl Nonidet P42 per mg protein followed by centrifugation at 105 000 x g for 1 hour. This solubilised preparation was applied to a DEAE-cellulose column pre-equilibrated with 20 mM potassium phosphate buffer pH 7.7, 0.4% Nonidet P42. Cytochrome P-450 was eluted from this column unadsorbed in one major and two subsidiary peaks, largely separated from cytochrome b_5 and NADPH-cytochrome c reductase, which could be eluted by increasing the concentration of KCl in the elution buffer. Fractions containing cytochrome P-450 were pooled, diluted with glycerol to 20%, and added to a suspension of hydroxylapatite pre-equilibrated in 10 mM potassium phosphate pH 7.7. A batch-wise elution was carried out with increasing concentrations of potassium phosphate buffer pH 7.7, 20% glycerol, 1% Nonidet P42. Typically, the DEAE-cellulose chromatography gave less than a 50% recovery of cytochrome P-450 with a purification of 1.0- to 1.5-fold. About 80% of the cytochrome P-450 added to the hydroxylapatite was recovered, in a rather broad elution profile, with approximately half of this purified two-fold. This scheme therefore gives an overall purification of cytochrome P-450 of between two- and three-fold, with a recovery of about 15%.

Various aspects of this scheme are worth noting. Firstly, butanol-extracted microsomes were used. Butanol removes much of the phospholipid and, more especially, cholesterol from microsomal preparations. Since this low level of substrate might be helpful in various types of experiment, for example in reducing dilution of added radioactive cholesterol in cholesterol 7α-hydroxylase assays, and since these preparations retain activity well when stored at -20°C, butanol-extracted microsomes were rountinely used for all this work.

Secondly, the microsomal protein was solubilised with Nonidet P42. Most cytochrome P-450 purification schemes have involved an initial solubilisation with sodium cholate or deoxycholate, even if this is replaced by a nonionic detergent at a later stage of the preparation. These bile acids proved, perhaps unsurprisingly, to act as powerful inhibitors of cholesterol 7α-hydroxylase activity. The use of Nonidet is the only method of the many which have been tried that solubilises cholesterol 7α-hydroxylase without a substantial inhibition of its activity (225).

Thirdly, although the DEAE-cellulose chromatography gave no purification and relatively low recoveries of cytochrome P-450, the fairly good separation of cytochrome P-450 from cytochrome b_5 and NADPH-cytochrome c reductase makes this a useful first step in a purification scheme, and allows some investigations of the requirements for the reconstitution of enzyme activity to be done, even with such a relatively impure preparation (225,226,318).

(ii) Procedures reported by other laboratories

In 1974 the reconstitution of cholesterol 7α-hydroxylase activity from partially purified cytochrome P-450 and NADPH-cytochrome c reductase was reported by I. Bjorkhem, H. Danielsson and K. Wikvall (318). However, sodium cholate was used for the microsomal solubilisation,

although at a concentration three-fold less than that usually used, and this had a strong inhibitory effect on enzymic activity. Attempts at subsequent purification were generally unsuccessful, especially when involving the use of chromatographic procedures. It was possible to fractionate with polyethylene glycol or ammonium sulphate, but the extent of purification was low (319).

Since the work reported in subsequent parts of this Section was performed, Hansson and Wikvall have published schemes for the preparation of cytochrome P-450 active in 7α-hydroxylation, from rats and rabbits, to specific contents of 8.1 and 3.7 nmoles per mg protein, respectively (320). These procedures are summarised in Figure 3.1. It should be noted that Renex 690 was used for the solubilisation of rat liver microsomes, at a concentration of 1 μl per mg protein (see Section 3.2). Nonidet P42 was used for the solubilisation of rabbit liver microsomes at a concentration of 3 μl per mg protein.

3.2 The use of Renex 690 as a solubilising agent

Renex 690 has been much used in cytochrome P-450 purification work, usually after initial solubilisation by sodium cholate or deoxycholate (315,316), but also as the initial microsomal solubilising agent (320).

A 10% solution of Renex 690 was added to suspensions of butanol-extracted microsomes (100 mg protein per 8.7 ml, in centrifuge tubes) at detergent:protein ratios equivalent to 0.1 to 1.0 μl undiluted Renex 690 per mg protein. After 20 minutes on ice, with occasional shaking, these tubes were centrifuged at 105 000 x g for 1 hour. The supernatant was assayed for cytochromes P-450 and b_5. These results were expressed as a percentage of the concentrations in the original suspension (Figure 3.2). The decrease in the amount of cytochrome P-450 solubilised at higher detergent concentrations was at least partly due to a large conversion to cytochrome P-420.

Figure 3.1(a)

Purification of cytochrome P-450 from liver of cholestyramine-treated rats

MICROSOMES

↓ Renex, 1 μg/mg protein
protamine sulphate

DEAE-SEPHADEX
Tris-acetate, 0.1M + Renex, 0.1%

↓ Bio-Beads

HYDROXYLAPATITE
phosphate, 10 mM + Renex, 0.05%

phosphate, 10 mM	phosphate 0.1M	phosphate, 0.3M
↓ Bio-Beads	↓ Bio-Beads	↓ Bio-Beads
POLYETHYLENE GLYCOL 0-20%	POLYETHYLENE GLYCOL 0-20%	POLYETHYLENE GLYCOL 0-20%

(from the 0.1M phosphate branch) ↓

DEAE-SEPHADEX
Tris-acetate, 10 mM + Renex, 0.05%

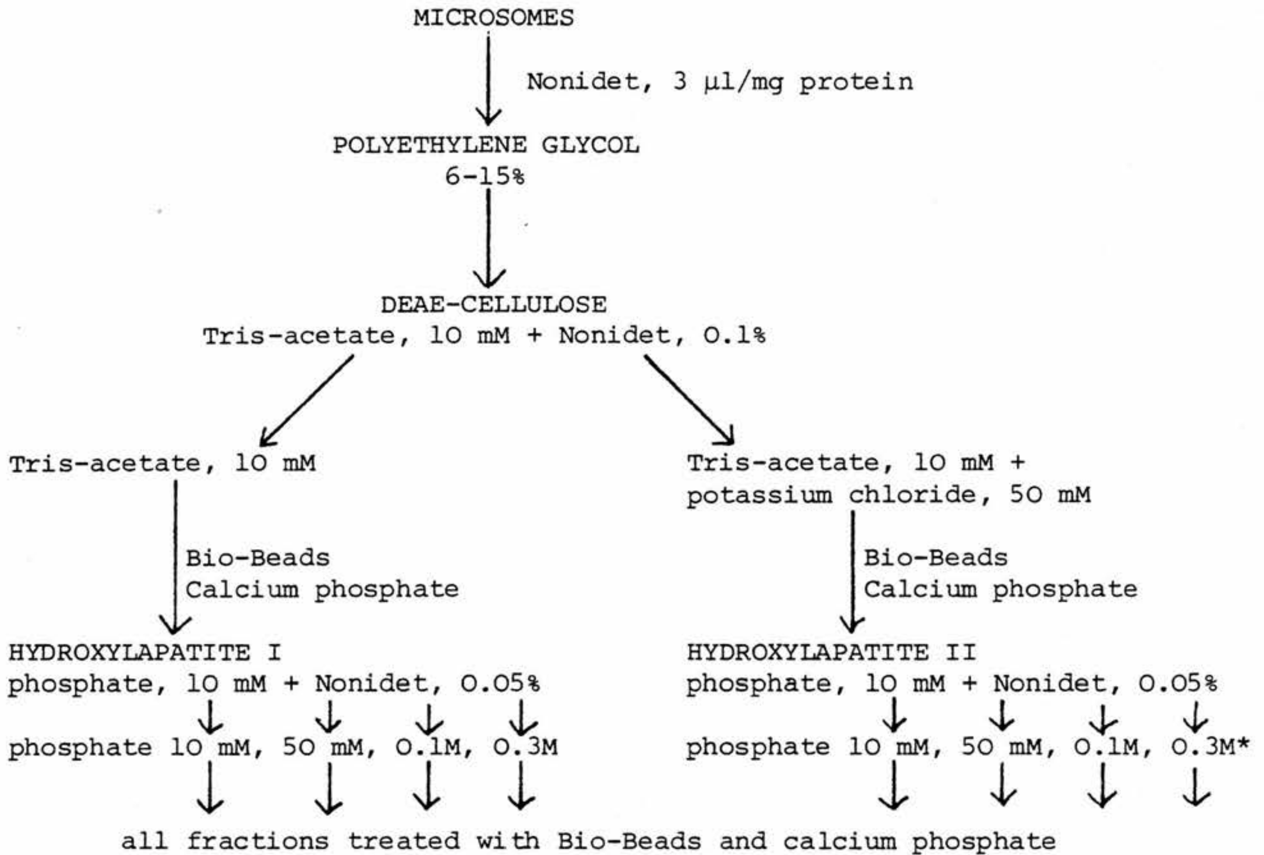
Tris-acetate, 10 mM	Tris-acetate, 10 mM + potassium chloride, 50 mM
↓	↓
Bio-Beads	Bio-Beads*

*The fraction indicated contained 3% of the total microsomal cytochrome P-450, with a specific content of 3.6 nmol/mg protein. In a reconstituted system cholesterol 7α-hydroxylase activity was 41 pmol/nmol cytochrome P-450/min. The microsomal cytochrome P-450 content was 0.5 nmol/mg protein, and the microsomal cholesterol 7α-hydroxylase activity was 63 pmol/nmol cytochrome P-450/min.

(Reference 320).

Figure 3.1(b)

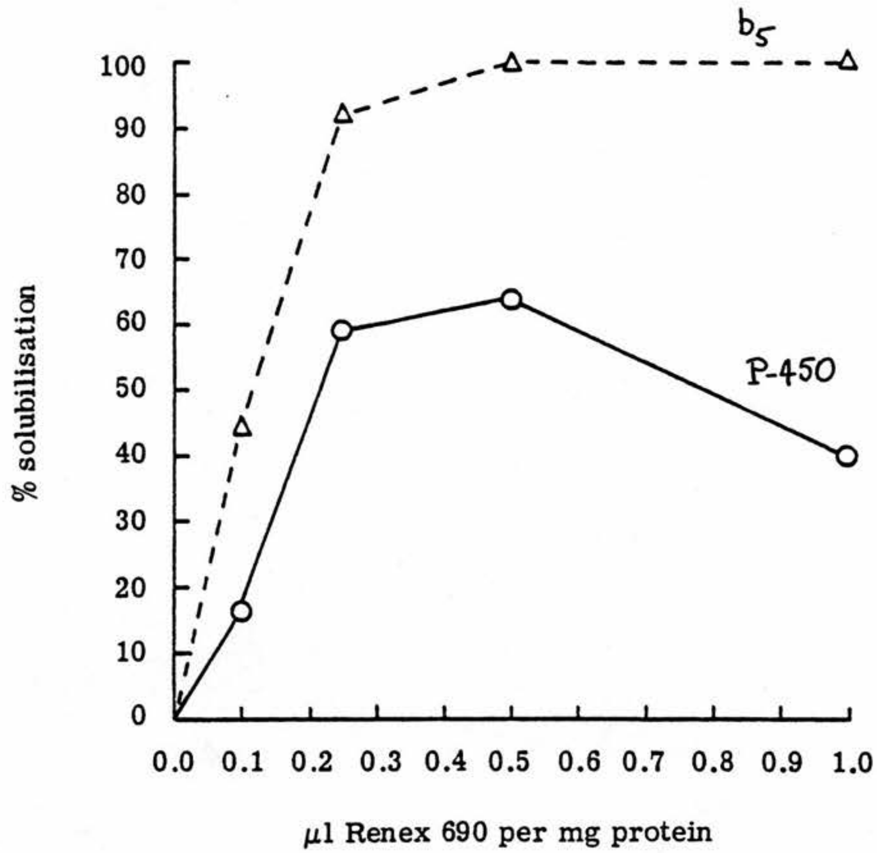
Purification of cytochrome P-450 from liver of cholestyramine-treated rabbits



*The fraction indicated contained 2% of the total microsomal cytochrome P-450, with a specific content of 3.7 nmol/mg protein. In a reconstituted system cholesterol 7α-hydroxylase activity was 4 pmol/nmol cytochrome P-450/min. The microsomal cytochrome P-450 content was 0.4 nmol/mg protein, and the microsomal cholesterol 7α-hydroxylase activity was 12 pmol/nmol cytochrome P-450/min.

(Reference 320).

Figure 3.2



(For method see text).

The effect of Renex 690 on cholesterol 7α-hydroxylase activity was investigated by adding various concentrations of the detergent to enzyme assay incubations. The results are shown in Figure 3.3. The presence of 0.5 μl Renex 690 per mg protein depressed cholesterol 7α-hydroxylase activity to less than 50% of control values.

It was concluded that Renex 690 was not a useful detergent for cholesterol 7α-hydroxylase or cytochrome P-450 solubilisation.

3.3 DEAE-cellulose chromatography

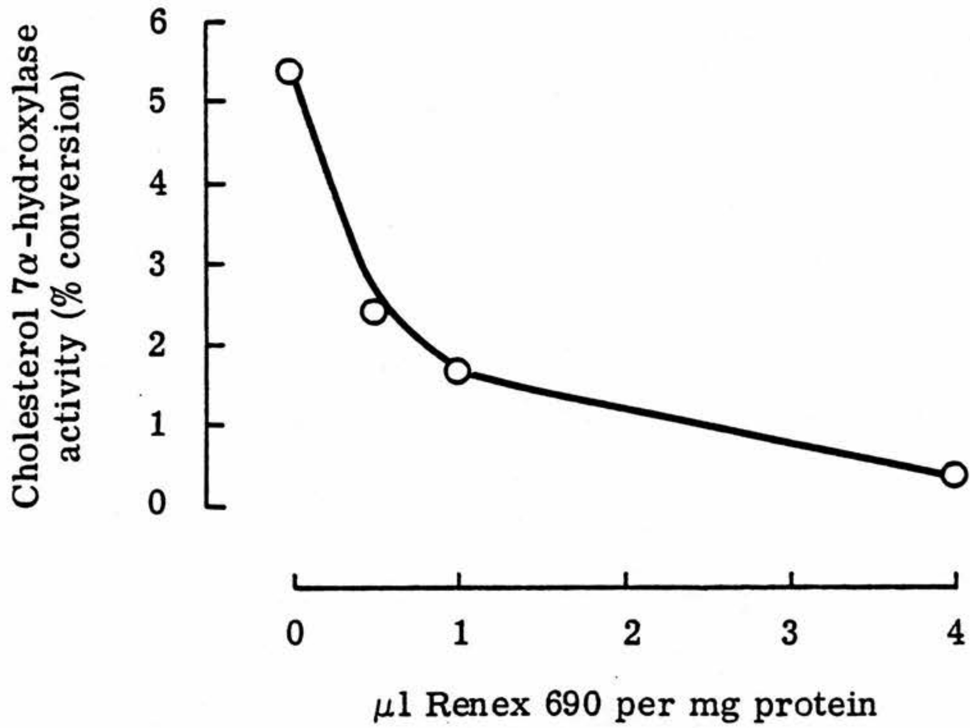
(a) General description

Microsomes prepared from rats fed on 4% cholestyramine for one week were freeze-dried and extracted with butanol at $-20^{\circ}C$ as described in the methods section. This butanol powder was suspended at a concentration of 50 mg/ml in buffer and solubilised by the addition of 4 μl Nonidet P42 per mg protein. After centrifugation at 105 000 x g for 1 hour, a suitably-sized sample was applied to the DEAE-cellulose column, which had been pre-equilibrated by washing with at least 5 column volumes of a solution of 0.4% Nonidet P42 in the buffer used for suspension.

The column was then washed with three column volumes of this equilibration buffer. This eluted cytochrome P-450 in one large unbound peak, and a smaller, slightly retarded, subsidiary peak, which usually appeared as a shoulder on the major peak.

The remaining monooxygenase components were usually eluted with a linear gradient of KCl in the range of 0-0.4 M in the equilibration buffer. This eluted first cytochrome b_5 in a fairly broad band, followed by, and overlapping with, NADPH-cytochrome c reductase. These components were alternatively occasionally eluted by stepwise increases of the concentration of KCl in the equilibration buffer. The KCl concentrations

Figure 3.3



(For method see text).

were chosen such that the first increase would hopefully elute any cytochrome P-450 still adsorbed to the column, uncontaminated by cytochrome b_5 (in fact, no cytochrome P-450 was ever detected in such a fraction), the second would elute cytochrome b_5 largely uncontaminated by NADPH-cytochrome c reductase, and the third would elute the reductase largely free of cytochrome b_5.

The appearance of cytochromes from the column was often conveniently monitored by the absorbance of the eluate at 419 nm, the wavelength of their Soret absorption band. The concentrations of monooxygenase components and of protein were determined either in each fraction or in suitably pooled groups of fractions, and recoveries and purifications were calculated.

(b) Control conditions

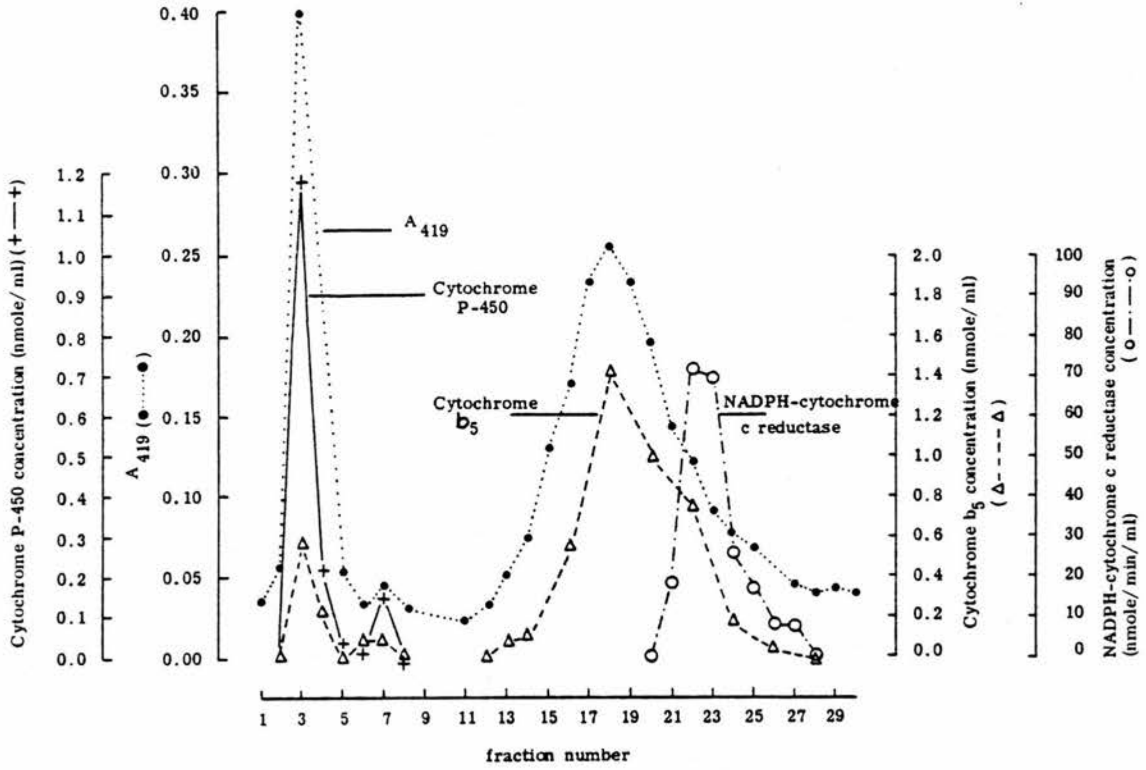
In control experiments the buffer used for suspension of the butanol powder was 20 mM potassium phosphate pH 7.7, and the DEAE-cellulose column was pre-equilibrated with 20 mM potassium phosphate pH 7.7, 0.4% Nonidet P42. A typical elution profile of a column eluted with a linear gradient of KCl concentration is shown in Figure 3.4.

For five experiments, the mean recovery of cytochrome P-450 was 43%, and the mean purification (the ratio of nmoles cytochrome P-450 per mg protein in the pooled cytochrome P-450-containing fractions to that in the applied sample) was 1.1-fold, and in some of the experiments actually fell to values less than 1.0. Contamination of this fraction by NADPH-cytochrome c reductase was frequently undetectable, and at most represented less than 5% of the total activity applied to the column. The amount of cytochrome b_5 in this cytochrome P-450-containing fraction varied, and was typically 10%, but under some conditions could be up to 35%, of the cytochrome b_5 eluted from the column.

EX BIBL UN V EDINBURGEN

Figure 3.4

DEAE - cellulose chromatography of solubilised rat liver microsomes



(For microsomal protein solubilisation see Section 2.4. The column volume was 15 ml; 39 mg microsomal protein in a volume of 3 ml was applied to the column; elution was with 30 ml 20 mM potassium phosphate, pH 7.7, 0.4% Nonidet, followed by a 0-0.5 M potassium chloride gradient in this buffer; 4.5 ml fractions were collected.)

The main conclusions from this work were therefore that DEAE-cellulose chromatography gives no increase in the specific content of cytochrome P-450, and that less than 50% of the cytochrome P-450 applied to the column was detectable in the eluate. It was felt that these facts were linked, and were likely to be a consequence of loss of haem from the cytochrome to give a spectrally undetectable species, and therefore that any attempts to raise the recovery of cytochrome P-450 could be hoped to bring a concomitant increase in its purification. It was considered worthwhile to look for conditions resulting in this increase in recovery and purification, rather than to try completely alternative techniques, since in principle DEAE-cellulose chromatography is a good first step in a purification scheme for cytochrome P-450, because of the satisfactory separation from NADPH-cytochrome c reductase. For a first step in such a scheme, however, the low recovery obtained was particularly unacceptable.

(c) The use of various protective agents

The results of these experiments are summarised in Table 3.1.

(i) Dithiothreitol

The effect of polyols and certain thiols in promoting the reconversion of cytochrome P-420 to cytochrome P-450 is well known (309). For this reason, many workers in the field of microsomal cytochrome P-450 purification routinely add glycerol and/or a thiol, commonly dithiothreitol, to the buffers used (297,308,310).

Dithiothreitol was therefore added to the buffers used for solubilisation and chromatography, at a concentration of 0.1 mM. Recovery and purification increased slightly, to 49% (probably still within the range of controls) and 1.8-fold respectively.

Table 3.1

Summary of results of DEAE-cellulose chromatography

Buffer	No. of experiments	Recovery (%)	Purification (- fold)	See paragraph
20 mM potassium phosphate pH 7.7, 0.4% Nonidet P42	5	43	1.1	3.3(b)
20 mM potassium phosphate pH 7.7, 0.4% Nonidet P42, 0.1 mM dithiothreitol	1	49	1.8	3.3(c)(i)
20 mM potassium phosphate pH 7.7, 0.4% Nonidet P42, 1.0 mM 4-phenyl-imidazole	3	70	1.9	3.3(c)(ii)
20 mM potassium phosphate pH 7.7, 0.4% Nonidet P42, 10.0 mM diethyldithiocarbamate	1	54	2.4	3.3(c)(iii)
20 mM potassium phosphate pH 7.7, 0.4% Nonidet P42, 1.0 mM 4-phenyl-imidazole, 10.0 mM diethyldithiocarbamate	1	54	1.2	3.3(c)(iv)
10 mM potassium phosphate pH 7.7, 0.4% Nonidet P42, 1.0 mM 4-phenyl-imidazole, 10.0 mM diethyldithiocarbamate	1	26	2.1	3.3(c)(iv)
20 mM potassium phosphate pH 7.7, 0.4% Nonidet P42, 10.0 mM diethyldithiocarbamate, 10% glycerol	3	46	0.9	3.3(c)(v)
20 mM potassium phosphate pH 7.7, 0.4% Nonidet P42, 10.0 mM diethyldithiocarbamate, 1.0 mM 4-phenyl-imidazole, 10% glycerol	2	39	1.2	3.3(c)(v)
20 mM potassium phosphate pH 7.7, 1.0% Nonidet P42, 10.0 mM diethyldithiocarbamate, 1.0 mM 4-phenyl-imidazole, 10% glycerol	1	-	-	3.3(c)(vi)

(ii) <u>4-Phenyl-imidazole</u>

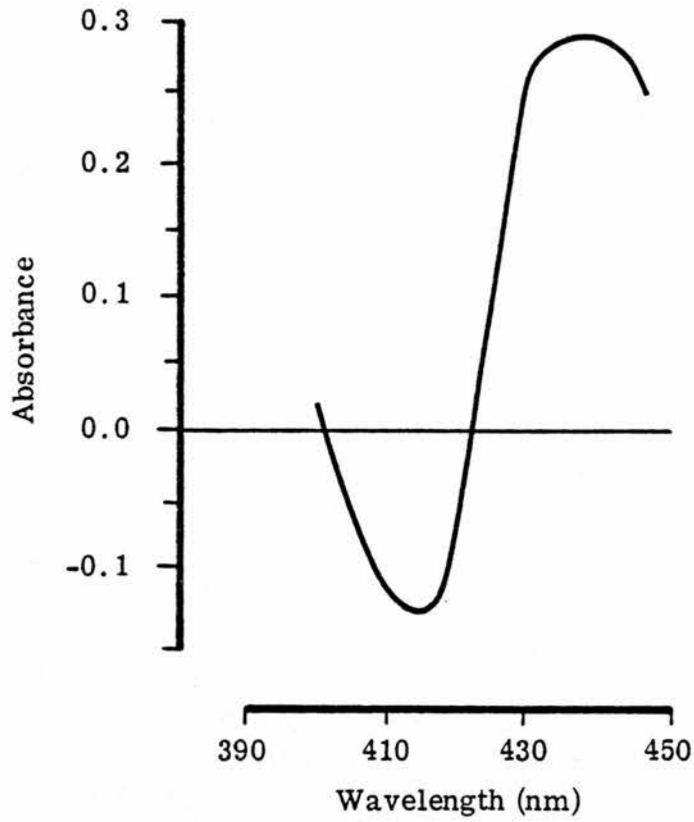
The cytochrome P-450 isolated from Psueodmonas putida grown in the presence of camphor as the sole carbon source is extremely stable in the presence of relatively low concentrations of its substrate, camphor, presumably because of some conformational effect of having the substrate binding site occupied. In the purification of the cytochrome P-450 species of bovine adrenal cortical mitochondria that is responsible for the cleavage of the side-chain of cholesterol, the use of a cytochrome P-450 ligand, 4-phenyl-imidazole, has proved to be of considerable benefit, presumably because of a similar stabilising action (Dr. R. Hume, personal communication).

Figure 3.5 illustrates the difference spectrum observed on the addition of 4-phenyl-imidazole to a suspension of butanol-extracted microsomes. The addition of increasing amounts of 4-phenyl-imidazole allows the calculation of an apparent spectral binding constant, K_s. The variation of the value of this constant with cytochrome P-450 concentration is plotted in Figure 3.6.

In the range of cytochrome P-450 concentrations encountered in column chromatography eluates (up to 1.5 nmoles/ml) the apparent K_s varied up to about 20 μM. In order that the cytochrome should be saturated with ligand at these concentrations (and also at the higher concentrations used during solubilisation, when it should be noted, however, there was little loss of cytochrome), 4-phenyl-imidazole was added to all solutions used for solubilisation and chromatography to a final concentration of 1.0 mM. This resulted in an increase in the recovery of cytochrome P-450 to 70%, and an increase in the purification to 1.9-fold.

Figure 3.5

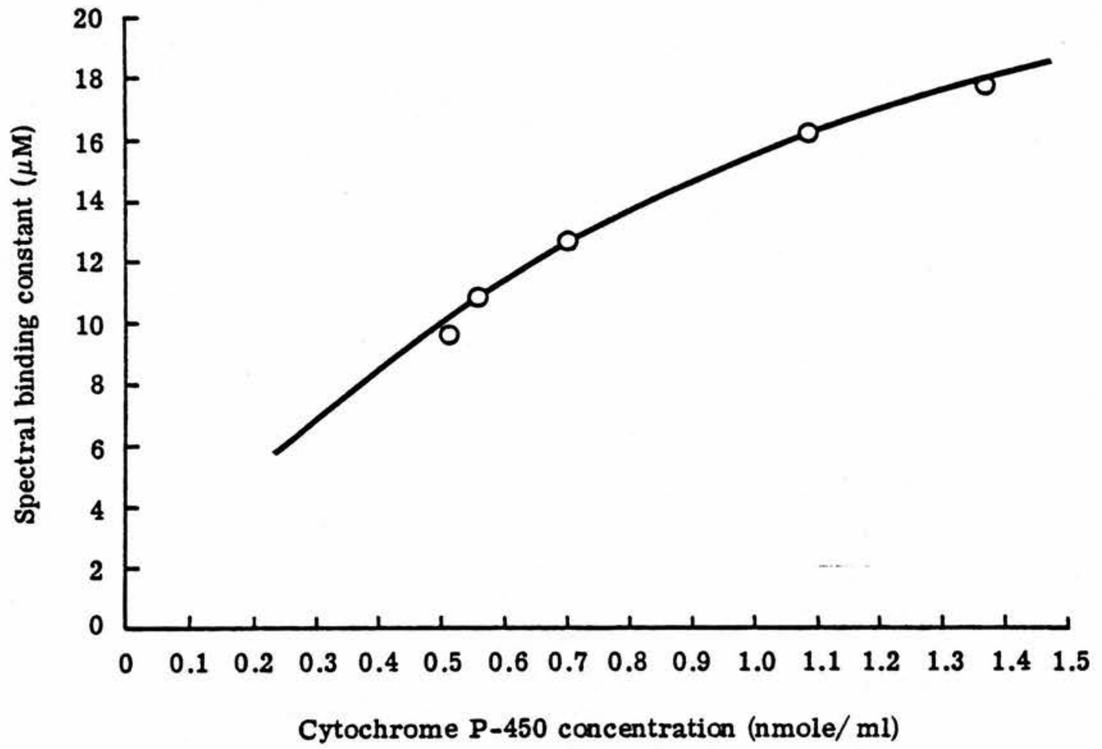
CYTOCHROME P-450 DIFFERENCE SPECTRUM
OBTAINED WITH 4-PHENYL-IMIDAZOLE



Cytochrome P-450 concentration = 1.08 nmol/ ml
4-phenyl-imidazole concentration = 60 nmol/ ml

Figure 3.6

The variation of the spectral binding constant of 4-phenyl-imidazole with cytochrome P-450 concentration



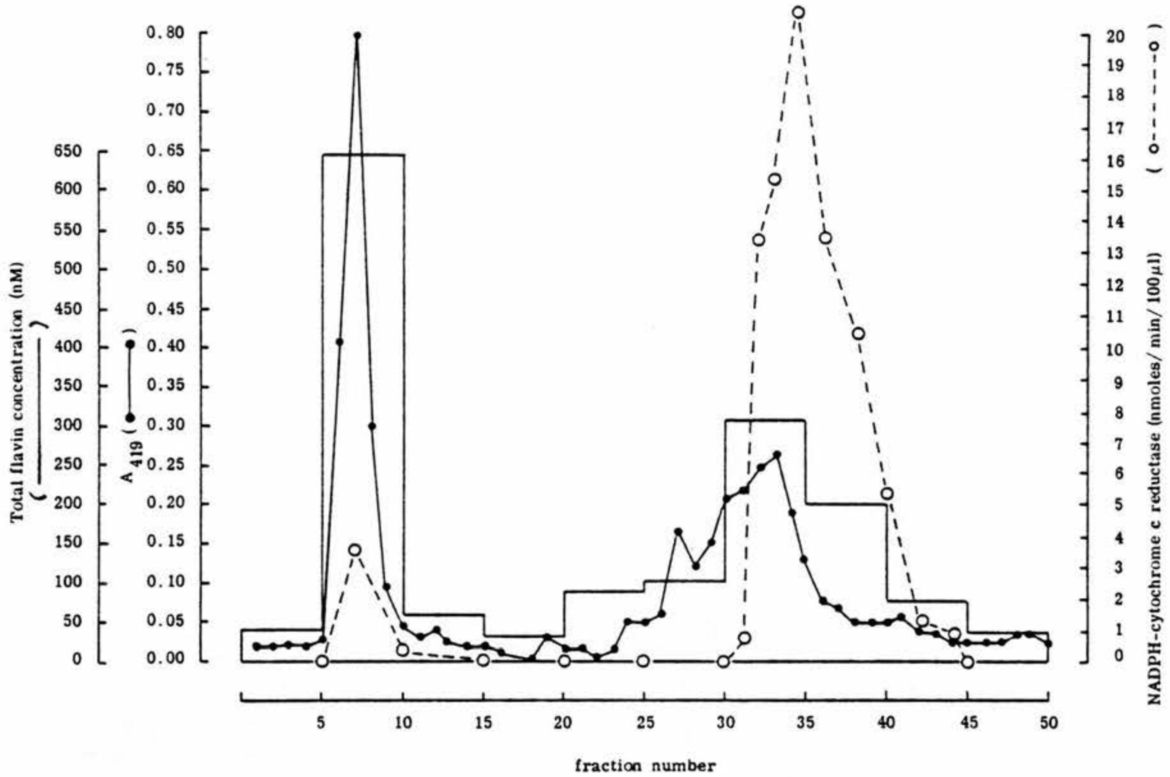
(iii) Diethyldithiocarbamate

In the preparation of the prostaglandin-synthesising cyclooxygenase diethyldithiocarbamate has been used to increase enzyme recoveries (321). This compound is a copper chelator, but its mode of action in increasing recoveries of this enzyme is obscure, since the purified cyclooxygenase contains no copper. It is possible to speculate that the removal of trace amounts of copper from the enzyme solutions might prevent autoxidative processes which might be promoted in the presence of some transition metal ions. Alternatively, the particular combination of hydrophobic properties and mercaptan groups may be involved.

The use of 10.0 mM diethyldithiocarbamate in the buffers used for solubilisation and chromatography of microsomal protein gave a recovery of cytochrome P-450 of 54%, and a purification of more than 2-fold.

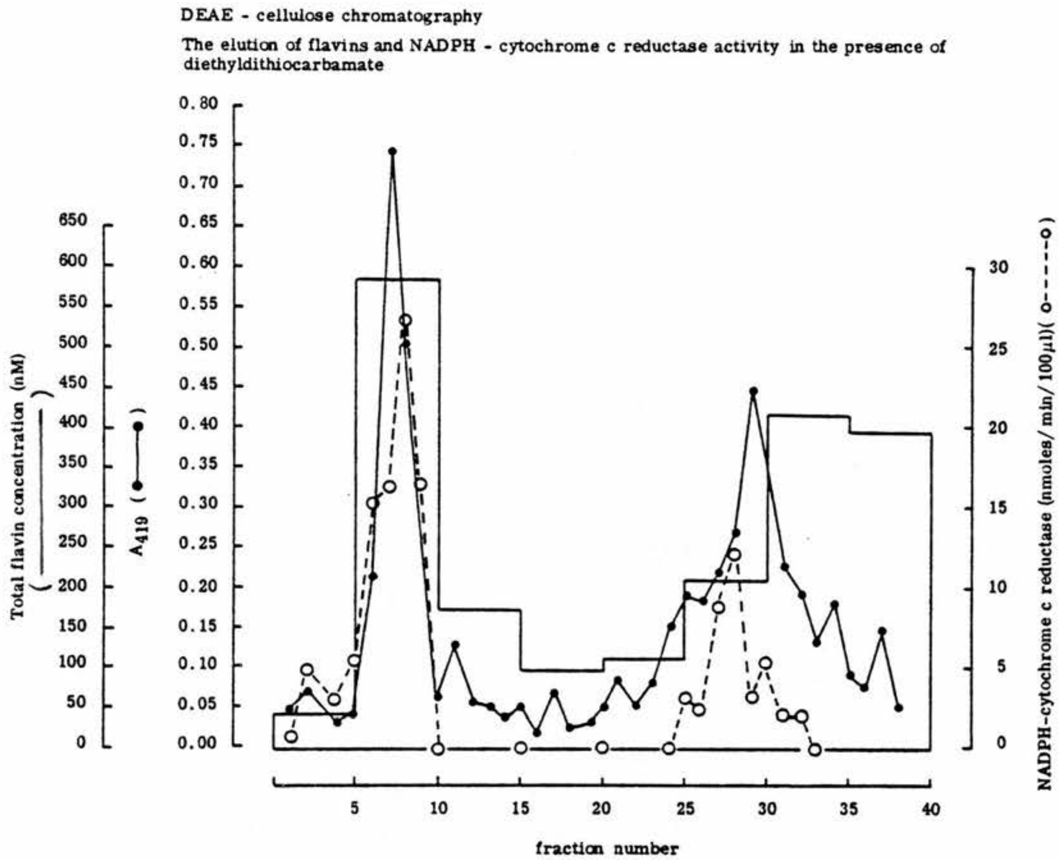
Diethyldithiocarbamate was able to reduce cytochrome c, and in assays for NADPH-cytochrome c reductase this background rate of reduction was therefore subtracted from all reaction rates to obtain values for enzymic reduction rates. The elution profile of apparent NADPH-cytochrome c reductase activity was profoundly altered by the presence of diethyldithiocarbamate, when a large peak of activity eluted with cytochrome P-450. In an attempt to resolve whether this activity was genuinely due to the presence of NADPH-cytochrome c reductase, or was an artefact, the flavin content (flavin adenine dinucleotide plus flavin mononucleotide) of pooled groups of fractions of eluate from columns run in the presence and absence of diethyldithiocarbamate was measured (Figure 3.7). This did not succeed in resolving the problem, since a large peak of flavin elutes in the void volume of the column, with cytochrome P-450, whether or not diethyldithiocarbamate is present. There is, however, little apparent change in flavin elution profile, which is suggestive that the change in the elution profile of apparent

Figure 3.7(a)



(For microsomal protein solubilisation see Section 2.4. The column volume was 30 ml; 84 mg microsomal protein in a volume of 4 ml was applied to the column; elution was with 60 ml 20 mM potassium phosphate, pH 7.7, 10% glycerol, 0.4% Nonidet, followed by a 0-0.5 M potassium chloride gradient in this buffer; 4.2 ml fractions were collected.)

Figure 3.7(b)



(Conditions were as specified for Figure 3.7(a), except all buffers contained 10 mM diethyldithiocarbamate, and 89 mg microsomal protein was applied to the column.)

activity of NADPH-cytochrome c reductase is not the result of a change in the elution pattern of the enzyme itself.

Further evidence, again inconclusive, for this statement is offered by the results summarised in Table 3.2. In this experiment 1 ml 0.1 M potassium phosphate buffer pH 7.4 containing 0.4 mg cytochrome c, 1 mg glucose-6-phosphate and 0.3 mg $NADP^+$ was pipetted into a cuvette, followed by additions of 100 μl 10 mM diethyldithiocarbamate, 1.0 U glucose-6-phosphate dehydrogenase, and 100 μl of a 2.55 nmole/ml solution of cytochrome P-450, as indicated in the table. (The cytochrome P-450 solution was a partially purified sample of bovine adrenal mitochondrial cytochrome P-450, kindly supplied by Dr. R. Hume, and contained no detectable NADPH-cytochrome c reductase activity.) The rate of reduction of cytochrome c was measured. It is apparent that cytochrome c is reduced by diethyldithiocarbamate, and the rate of this reduction is enhanced by the presence of cytochrome P-450. The presence of glucose-6-phosphate dehydrogenase, and hence of NADPH, makes little difference to the observed reduction rates.

These results demonstrate that a peak of NADPH-cytochrome c reductase activity would be detected as coeluting with cytochrome P-450 in the presence of diethyldithiocarbamate, even if no enzyme was in fact present. It was not, however, possible to demonstrate unequivocally that the observed peak was not due to the presence of NADPH-cytochrome c reductase.

(iv) 4-Phenyl-imidazole and diethyldithiocarbamate

When a combination of 1.0 mM 4-phenyl-imidazole and 10.0 mM diethyldithiocarbamate was used in the solubilisation and chromatography buffers the recovery and purification of cytochrome P-450 were 54% and 1.2-fold, respectively.

Table 3.2

Reduction of cytochrome c by diethyldithiocarbamate

Additions*	Cytochrome c reduction rate (nmoles/min)	
	No G6PDH†	1.0 U G6PDH
1.0 μmol DDC†	3.99	4.22
0.255 nmol P-450†	0.44	1.55
1.0 μmol DDC + 0.255 nmol P-450	7.33	7.77

*All cuvettes contained 0.4 mg cytochrome c, 1 mg glucose-6-phosphate, 0.3 mg $NADP^+$ in 1 ml 0.1M potassium phosphate buffer, pH 7.4, with the specified additions.

†Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; DDC, diethyldithiocarbamate; P-450, bovine adrenal mitochondrial cytochrome P-450, free of detectable NADPH-cytochrome c reductase activity.

Some cytochrome b_5 always elutes from a DEAE-cellulose chromatography column with the main peak of cytochrome P-450, although the main peak of cytochrome b_5 does not elute until the ionic strength of the eluting buffer is increased. There is no evidence for a heterogeneous population of cytochrome b_5 in liver microsomes. In order to try to adsorb all the cytochrome b_5 to the DEAE-cellulose column while cytochrome P-450 eluted unadsorbed, and also, perhaps, to achieve a better separation of the different cytochrome P-450 peaks, solubilisation and chromatography were performed in buffers of reduced ionic strength. (It should be noted that this approach makes the implicit assumption that adsorption to the ion-exchange column is dependent on protein structure. Since some lipid remains firmly bound to protein during chromatography, protein adsorption could be due to charged groups on these lipid molecules, and hence variations in adsorption could be caused by differing numbers of lipid molecules bound to the protein.)

Butanol-extracted microsomes were suspended in 10 mM potassium phosphate pH 7.7, 10 mM 4-phenyl-imidazole, 10.0 mM diethyldithiocarbamate and chromatography was performed in a 0.4% solution of Nonidet P42 in this buffer. The recovery of cytochrome P-450 was relatively low (26%), but, since the amount of protein eluting in this first, unadsorbed, peak was also low, the specific content of cytochrome P-450 was high (2.3-nmoles/mg protein) and the purification was high (2.3-fold). However, 8% of the cytochrome b_5 applied to the column eluted with the cytochrome P-450, and under these circumstances of low cytochrome P-450 recovery this totalled a quantity greater than 65% of the amount of cytochrome P-450 present in this fraction.

(v) Glycerol

Since the observation that the presence of polyols and certain thiols promotes the reconversion of cytochrome P-420 to cytochrome P-450

(309), many workers in the field of hepatic microsomal cytochrome P-450 purification have routinely added various concentrations of glycerol to the buffers used in their purification schemes (297,308,309).

Solubilisation was performed in 20 mM potassium phosphate pH 7.7, 10.0 mM diethyldithiocarbamate, 10% glycerol, and DEAE-cellulose chromatography was run in a 0.4% Nonidet solution in this buffer. Recovery and purification were 46% and 0.86-fold, respectively. These relatively low results could possibly be ascribed to the increased running time of the column, because of the higher viscosity of the buffers. It was noticeable, however, that cytochrome P-450 spectra were more satisfactory, exhibiting only a small shoulder at 420 nm.

Because of this advantage of running the chromatography in a glycerol-containing buffer a further attempt was made to find satisfactory conditions for chromatography in the presence of glycerol. 20 mM Potassium phosphate buffer pH 7.7, 10 mM diethyldithiocarbamate, 10% glycerol 1.0 mM 4-phenyl-imidazole was used for solubilisation, and a 0.4% solution of Nonidet P42 in this buffer for chromatography. This gave a 39% recovery and a 1.1-fold purification.

(vi) 1% Nonidet P42

There is a possibility that the elution profile of solubilised microsomal protein from a DEAE-cellulose column depends not only on charged groups on the protein, but also on charged groups on the lipids bound to the protein. If this is so, it might be possible to alter the elution of microsomal proteins by performing solubilisation and chromatography at raised detergent concentrations if this resulted in a displacement of a greater number of charged lipids from the protein, and their replacement by uncharged detergent molecules.

Butanol-extracted microsomes were therefore suspended in 20 mM potassium phosphate buffer pH 7.7, 10.0 mM diethyldithiocarbamate,

1.0 mM 4-phenyl-imidazole, 10% glycerol and solubilised with 5 µl Nonidet P42 per mg protein. Chromatography was carried out in a 1% Nonidet P42 solution in the buffer used for suspension. This resulted in the loss of 90% of the cytochrome P-450, principally to some spectrally undetectable species, but also to cytochrome P-420.

3.4 Quaternary amino ethyl-Sephadex chromatography

Under all conditions used for DEAE-cellulose chromatography a substantial proportion of the cytochrome b_5 applied to the column elutes in cytochrome P-450-containing fractions. Because of the controversy surrounding the possible role of cytochrome b_5 in microsomal cytochrome P-450-dependent hydroxylations this is a particularly undesirable contaminant. It was therefore decided to try the use of a stronger anion exchanger, quaternary amino ethyl Sephadex (QAE-Sephadex), as an alternative first step in cytochrome P-450 purification.

Butanol-extracted microsomes were suspended in 20 mM potassium phosphate pH 7.7, 1.0 mM 4-phenyl-imidazole, 10% glycerol and solubilised with 4 µl Nonidet P42 per mg protein. Chromatography was run in 0.4% Nonidet P42 in the buffer used for suspension, with elution by a linear gradient of KCl.

The elution profile of this QAE-Sephadex column was the same as that of a DEAE-cellulose column, with cytochrome P-450 eluting unabsorbed, followed by cytochrome b_5 and NADPH-cytochrome c reductase at raised ionic strengths. There was a 38% recovery, the specific content of the recovered cytochrome P-450 fell to 0.7-fold that of the applied sample, and 40% of the recovered cytochrome b_5 eluted with cytochrome P-450.

3.5 Carboxymethylcellulose chromatography

Since cytochrome P-450 elutes unbound from a DEAE-cellulose column it was decided to try the use of a cation exchange column as an alternative

first step. Various cation exchanges were used under various conditions by N.G. Hattersley, but always after DEAE-cellulose chromatography, when no purification of cytochrome P-450 was observed(225).

In 20 mM potassium phosphate pH 7.7, 10% glycerol, 0.4% Nonidet P42, cytochromes P-450 and b_5 and NADPH-cytochrome c reductase eluted together, unbound (Figure 3.8). All the cytochrome P-450 was recovered, with a 1.3-fold increase in specific content. Similar results were obtained when the buffer concentration was decreased to 5 mM.

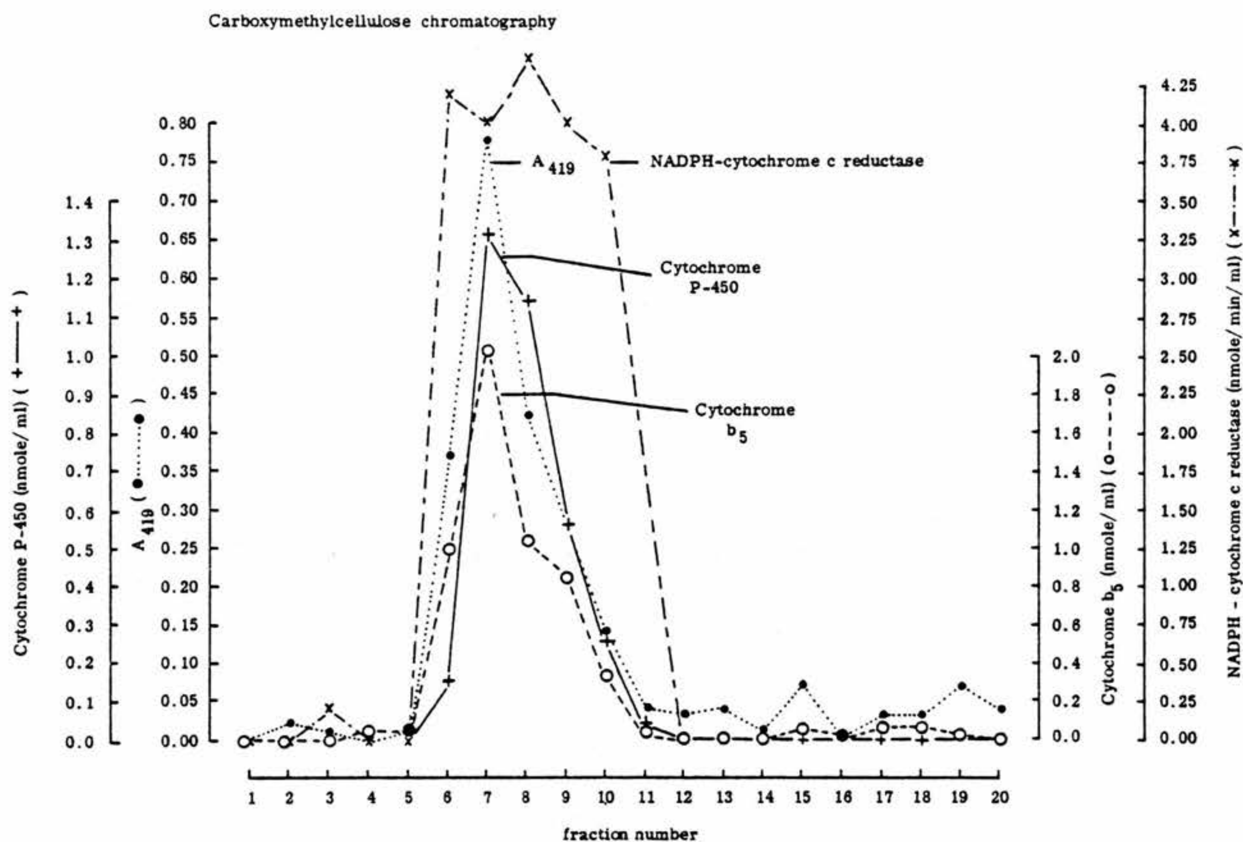
When 5 mM potassium acetate, pH 6.2, 10% glycerol, 0.4% Nonidet P42 was used cytochrome P-450 bound to the column. When the ionic strength of the eluting buffer was raised by means of a linear KCl gradient monooxygenase components again eluted together. The recovery of cytochrome P-450 was low (25%) and its specific content was decreased to 0.4 times that of the applied material.

3.6 Hydroxylapatite chromatography

In the light of the DEAE-cellulose experiments described above, where increased detergent concentrations resulted in large losses of cytochrome P-450 and the presence of glycerol tended to depress recoveries even while minimising degradation to cytochrome P-420, the requirement for performing hydroxylapatite chromatography in phosphate buffers containing 20% glycerol and 1% Nonidet P42 was investigated.

The cytochrome P-450-containing fractions from a DEAE-cellulose column were pooled and stirred into a suspension of washed hydroxylapatite in the first elution buffer, at a ratio of 1 g hydroxylapatite per 5 mg protein. After 10 minutes stirring at 4°C the hydroxylapatite was sedimented by centrifugation, and resuspended in a volume of the next elution buffer equal to two times the volume of packed hydroxylapatite.

Figure 3.8



(For microsomal protein solubilisation see Section 2.4. The column volume was 50 ml; 71.4 mg microsomal protein in a volume of 6 ml was applied to the column; elution was with 100 ml 20 mM potassium phosphate, pH 7.7, 10% glycerol, 0.4% Nonidet, followed by a 0-0.5 M potassium phosphate gradient in this buffer; 5.4 ml fractions were collected.)

This procedure was repeated with the buffer concentrations specified in Table 3.3.

The results of three experiments are given in Table 3.3, with some results reported by N.G. Hattersley for comparison. The omission of glycerol and reduction of Nonidet P42 concentration to 0.4% reduces the total cytochrome P-450 recovery to 33%. In the absence of glycerol and high concentrations of Nonidet P42 cytochrome P-450 bound much more strongly to the hydroxylapatite, only being eluted at high concentrations of potassium phosphate.

On increasing the concentration of Nonidet P42 to 1.0% the cytochrome P-450 eluted at slightly lower phosphate buffer concentrations. Recovery of cytochrome P-450 summed over all recovered fractions was 100%, and 55% of this recovered cytochrome P-450 was purified by more than 5-fold. When glycerol was added to a total concentration of 10% recovery of cytochrome fell to 65%. There was however a greater than 7-fold purification of 16% of the applied cytochrome P-450.

3.7 Iodosobenzene as a donor of "active oxygen" for cholesterol 7α-hydroxylation

These studies on the resolution of cholesterol 7α-hydroxylase components have concentrated on cytochrome P-450, since there is evidence that this is where the specificity of this enzyme resides (225). There is a need, however, to demonstrate that the cytochrome P-450 preparations obtained are capable of supporting cholesterol 7α-hydroxylase activity. One way of doing this involves the use of a pure preparation of NADPH-cytochrome c reductase which would act by reducing the cytochrome P-450-substrate-oxygen complex, and thereby "activating" the oxygen to a species capable of reacting with the substrate cholesterol.

Table 3.3

Hydroxylapatite chromatography. Recovery and purification of cytochrome P-450

Elution with[1]:	20% glycerol, 1% Nonidet P42[2]		0.4% Nonidet P42		1.0% Nonidet P42		10% glycerol, 1.0% Nonidet P42, 10.0 mM DDC	
	Total P-450 (nmoles)	nmoles P-450 per mg protein	Total P-450 (nmoles)	nmoles P-450 per mg protein	Total P-450 (nmoles)	nmoles P-450 per mg protein	Total P-450 (nmoles)	nmoles P-450 per mg protein
Applied sample	470 (100)[3]	1.25 (1.0)	15.6 (100)	0.45 (1.0)	6.55 (100)	0.27 (1.0)	3.75 (100)	0.21 (1.0)
X = 20[1]	n.d.[4]	-	n.d.	-	n.d.	-	0.76 (20)	0.90 (4.3)
50	56 (12)	1.05 (0.8)	n.d.	-	n.d.	-	1.08 (29)	0.45 (2.1)
80	99 (21)	1.65 (1.3)	n.d.	-	n.d.	-	0.61 (16)	1.50 (7.1)
150	205 (44)	2.62 (2.1)	n.d.	-	2.97 (45)	1.08 (4.0)	n.d.	-
220	69 (15)	1.39 (1.1)	1.6 (10)	0.40 (0.9)	3.63 (55)	1.56 (5.7)	n.d.	-
400	20 (4)	0.59 (0.5)	3.5 (23)	1.03 (2.3)	n.d.	-	n.d.	-
Total P-450 recovered	449 (95)		5.1 (33)		6.60 (100)		2.45 (65)	

[1]Elution was with X mM potassium phosphate buffer pH 7.7 with the specified additions.

[2]Results of N.G. Hattersley

[3]Figures in brackets refer to percent recoveries or purification ratios, as appropriate.

[4]n.d. = not detectable.

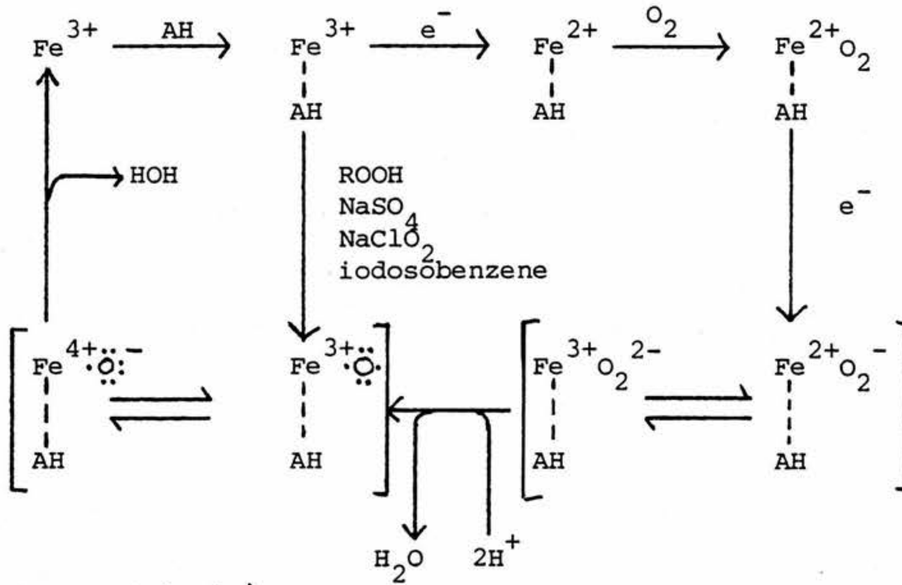
An alternative approach would be to use one of those compounds that have been used to investigate the nature of the "active oxygen" species involved in cytochrome P-450-catalysed reactions and which are able to support reaction in the absence of molecular oxygen or NADPH (322-329). The type of reaction mechanism by which these compounds are thought to work is shown in Figure 3.9.

Iodosobenzene was synthesised and shown to be more than 98% pure, with contamination by iodoxybenzene less than 0.5%. Persistent attempts to demonstrate cholesterol 7α-hydroxylase activity supported by iodosobenzene were unsuccessful, both under aerobic and anaerobic conditions, and at times from 10 s to 1 hr. A low rate of $NaIO_4$-supported reaction was detectable (1.5% conversion of tracer cholesterol to 7α-hydroxy-cholesterol in 1 hr), but was concomitant with a high level of cholesterol autoxidation, principally to 7-ketocholesterol.

It has been suggested that iodosobenzene-supported reaction proceeds with destruction of cytochrome P-450 (328). This observation was confirmed by the present work, where a suspension of butanol-extracted microsomes was placed in the sample and reference cuvettes of the Unicam SP800 difference spectrometer. The addition of iodosobenzene to the sample cuvette caused the appearance of a broad trough in the difference spectrum, centred at approximately 420 nm. This trough had completely formed within five minutes of adding iodosobenzene, and the reaction was 60% complete after one minute, and 80% complete after two minutes. This result was not affected by the use of anaerobic conditions, or by the use of a solubilised cytochrome P-450 preparation free of detectable NADPH-cytochrome c reductase activity.

Figure 3.9

A reaction mechanism for cytochrome P-450 showing a possible site of action of donors of "active oxygen"



(AH = substrate)

(From reference 325).

Section 4

The Assay for Acyl-Coenzyme A: Cholesterol Acyltransferase

4.1 Introduction

All assays for acyl-coenzyme A:cholesterol acyltransferases follow, with no or only minor modifications, that published by Goodman et al (248), described in more detail by Goodman (249). In this assay, enzyme activity is measured by the conversion of exogenous radioactive cholesterol to cholesterol ester in an *in vitro* incubation of microsomes. These incubations can be performed in two ways. Fatty-acyl-coenzyme A can be used as cosubstrate for the esterification, the activity thus measured being a function of only one enzyme-catalysed reaction, that catalysed by acyl-coenzyme A:cholesterol acyltransferase. Alternatively, a combination of ATP, coenzyme A and potassium oleate can be used, when a combination of two reactions is measured, the activation of fatty acid to fatty-acyl coenzyme A, and the transfer of the fatty acid moiety of this species to cholesterol. Since the point of interest of these investigations was the rate of the net conversion of cholesterol to cholesterol ester in rat liver endoplasmic reticulum, and this rate will depend on both these reactions, this latter approach was adopted, with the following modification. The measured activity in the presence of ATP, coenzyme A and potassium oleate has been shown by Goodman et al (248) to be slightly greater than in the absence of potassium oleate (13.3% conversion of free [4-^{14}C]-cholesterol to cholesterol ester rather than 12.1% conversion). Since this stimulation as measured by these authors is relatively small, and proved difficult to reproduce (results not shown), potassium oleate was not added to incubations.

This approach to the assay of acyl-coenzyme A:cholesterol acyltransferase suffers from the same problems as the similar assay for cholesterol 7α-hydroxylase, discussed in detail in Sections 2.11, 2.13, 7.4 and 8.2 and elsewhere (174,245,250-253), namely (i) the insolubility of cholesterol and hence the difficulty of delivering the exogenous radioactive

cholesterol to the enzyme without the use of concentrations of either detergent or organic solvent that raise the possibility that the enzyme or its membranous environment is disturbed; (ii) the cholesterol of microsomes exists in more than one compartment, and these compartments are not necessarily equally accessible to the enzyme; (iii) added, radioactive cholesterol does not necessarily partition into each of these compartments to an equal extent.

It is in an attempt to avoid these problems, and to arrive at absolute values for acyl-coenzyme A:cholesterol acyltransferase rather than merely fractional conversions of exogenous labelled cholesterol, that most of the published modifications of the original assay of Goodman et al (248) have been used.

These modifications have involved the use of labelled fatty acyl-coenzyme A (usually [1-^{14}C]-oleoyl-coenzyme A (330,331), or [1-^{14}C]-palmitoyl-coenzyme A (332,333,334)) or of [1-^{14}C]-oleic acid in the presence of ATP and coenzyme A (122,252,335). In each case the assumptions are made that the added labelled substrate will exist in the incubation as a single homogeneous pool, and that the amount of endogenous unlabelled fatty acid or fatty acyl-coenzyme A is negligible. This means that the exogenous labelled substrate will not be diluted to a significant extent on addition to the incubation, and hence that the specific radioactivity of cholesterol esters formed during incubation will be the same as that of the substrate. Thus, an absolute rate of cholesterol ester production can be calculated.

This approach was not adopted for the preliminary experiments on acyl-coenzyme A:cholesterol acyltransferase for various reasons. Among these reasons was the fact that activities were to be compared with those of cholesterol 7α-hydroxylase under various conditions. Since absolute activities for cholesterol 7α-hydroxylase are not

readily obtainable, and it was desirable that the enzyme activities should be expressed in a similar form, it was necessary to determine activities for esterification in terms of a fractional conversion of exogenous labelled free cholesterol to cholesterol ester. Also, it was hoped that eventually estimates would be made of the size of the cholesterol pool acting as substrate for esterification, and this requires values for acyl-coenzyme A:cholesterol acyltransferase activity expressed both in absolute terms and as a fractional conversion of added tracer cholesterol to cholesterol ester.

4.2 The effect of variation of protein concentration

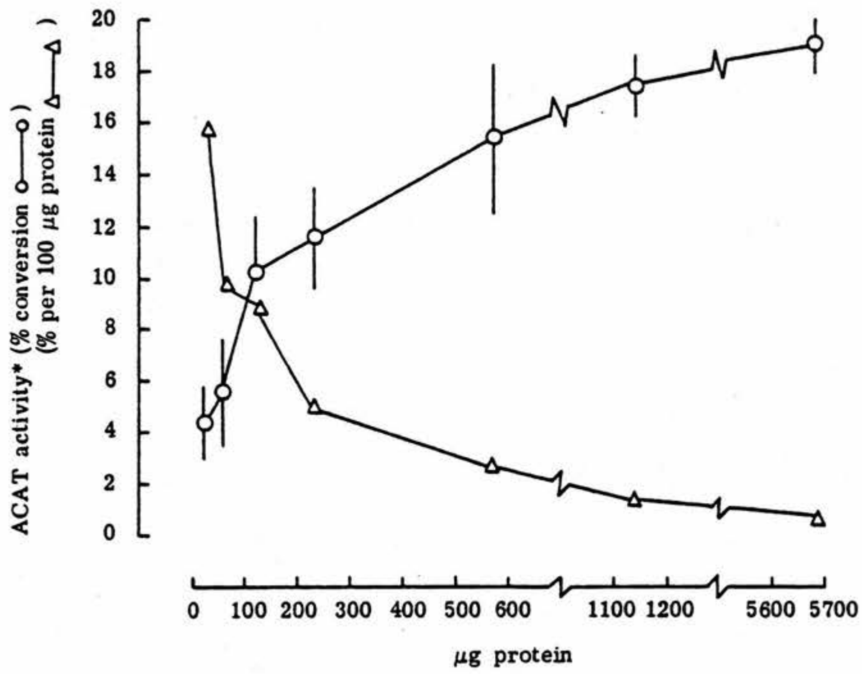
Varying amounts of microsomes from the pooled livers of three rats fed cholesterol-rich diet for eight days were incubated for 1 hour at 37°, in the presence of 0.5 mM coenzyme A, 2 mM ATP, 4 mM $MgCl_2$, 3.3 mM reduced glutathione and 0.05 μCi [4-^{14}C]-cholesterol in a total of 3 ml 0.1 M potassium phosphate buffer, pH 7.1. Figure 4.1 shows the observed percentage conversion of [4-^{14}C]-cholesterol to [4-^{14}C]-cholesterol ester, both in absolute terms and per 100 μg protein added.

The non-linearity of activity and the non-constancy of specific activity when the enzyme concentration is varied illustrate well the inherent problems in the assay of enzymes where changes in enzyme concentration bring concomitant changes in endogenous substrate concentration and hence variations in the dilution of exogenous tracer substrate.

A further complication is introduced by the possibility that there exist several microsomal cholesterol pools and that these are not equally accessible to the action of acyl-coenzyme A:cholesterol acyl-transferase. Over the very wide range of microsomal protein concentration used for this experiment it is quite possible that the exogenous tracer

Figure 4.1

The effect of protein concentration on acyl-coenzyme A : cholesterol acyltransferase activity



* ACAT = acyl-coenzyme A :cholesterol acyltransferase

(For method see text).

(Mean of 5 assays ± S.D.)

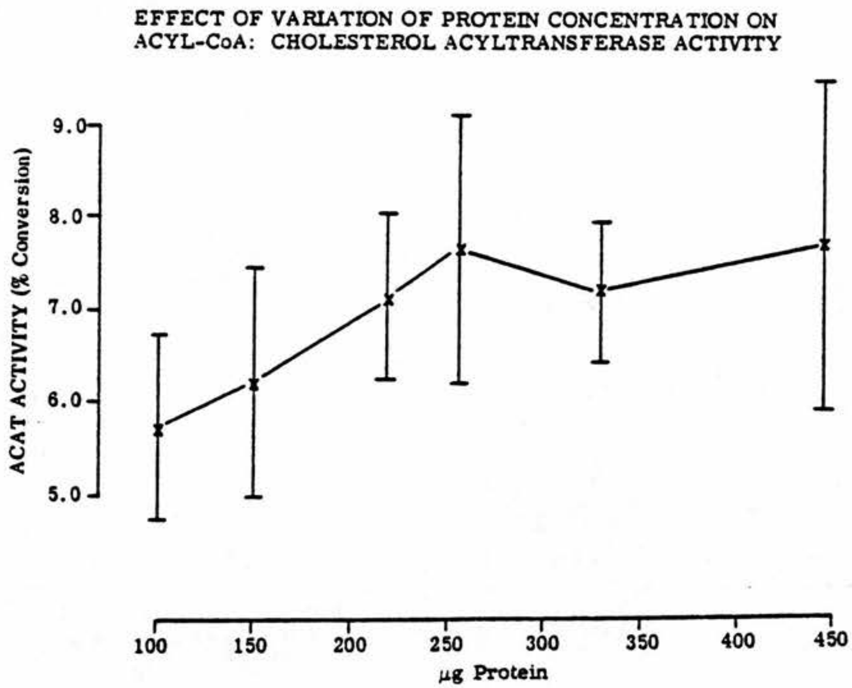
substrate will equilibrate into these different cholesterol pools to varying extents, providing a further reason for the observed non-linearity.

Another experiment was therefore performed to examine the effect of the variation of protein concentration over a much smaller range. Incubations were for 20 minutes at 37°, and contained 2 mM ATP, 4 mM $MgCl_2$, 3.3 mM reduced glutathione and 0.05 mM coenzyme A in a total volume of 3 ml 0.1 M potassium phosphate pH 7.1 (Figure 4.2). Little variation of percent conversion of [4-^{14}C]-cholesterol to [4-^{14}C]-cholesterol ester was detected. This is as would be expected if (a) the specific activity of the enzyme (i.e. the activity expressed as moles of product produced per mg protein) was constant, (b) the fraction of the total microsomal cholesterol in the cholesterol pool preferentially used as the enzyme substrate was constant and (c) a constant fraction of tracer cholesterol enters the enzyme substrate pool.

If it is assumed that microsomal cholesterol exists as a single pool, and exogenously added radioactive cholesterol equilibrates completely into this pool, it is possible to calculate a specific radioactivity of the substrate cholesterol and hence of the product cholesterol ester. Thus, by multiplying the percentage conversion of [4-^{14}C]-cholesterol to [4-^{14}C]-cholesterol ester by the amount of endogenous cholesterol present in the incubation it is possible to obtain an estimate of the absolute enzyme activity. This estimate will always consistently overestimate the actual activity by factors related to the fraction of total microsomal cholesterol in the substrate pool and the equilibration of radioactive cholesterol into this pool. (This problem is discussed in detail in Section 2.13.)

Figure 4.3 shows the results of Figure 4.2 reworked in this manner, when a satisfactory straight line is obtained.

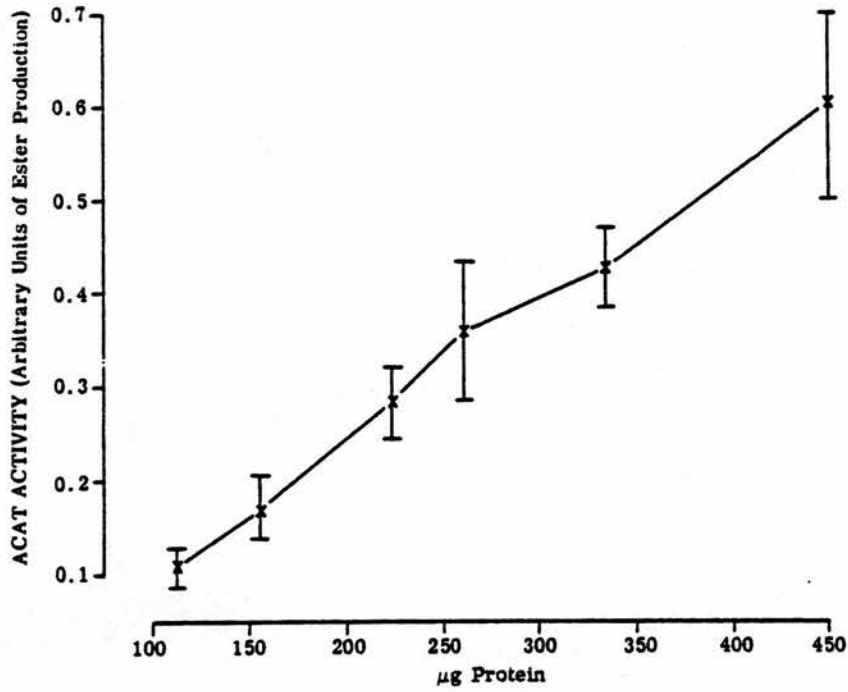
Figure 4.2



(For method see text).

(Mean of 5 assays ± S.D.)

Figure 4.3



(For method see text).

(Mean of 5 assays ± S.D.)

The main conclusion to be drawn from these experiments where protein concentration was varied was that when acyl-coenzyme A:cholesterol acyltransferase activities of a number of preparations were to be compared it is desirable to keep the protein concentrations of all samples as closely similar as possible, because of the non-linearity of activity with protein concentrations.

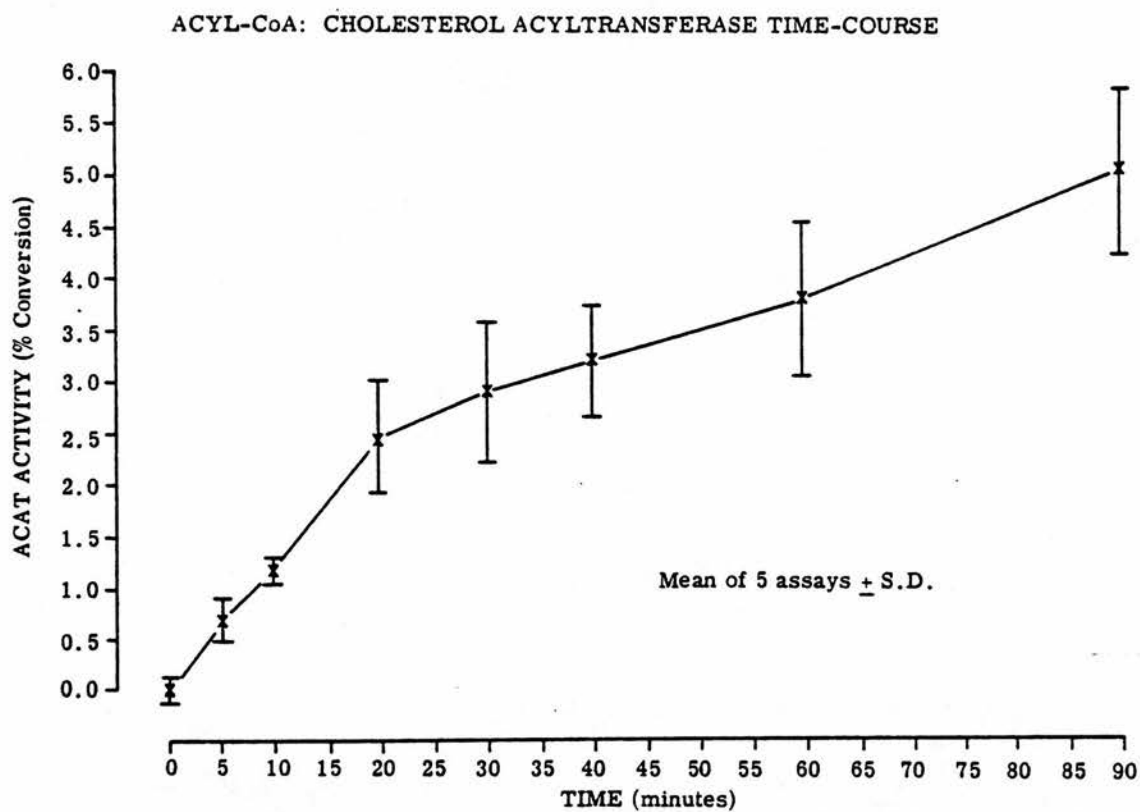
4.3 Time-course of acyl-coenzyme A:cholesterol acyltransferase activity

Microsomes were prepared from the livers of rats fed cholesterol for 10 days. Incubations of 130 μg microsomal protein were performed at 37° for varying lengths of time in the presence of 0.5 mM coenzyme A, 2 mM ATP, 4 mM $MgCl_2$, 3.3 mM reduced glutathione. The result is shown in Figure 4.4. The rate of cholesterol ester production was linear and relatively high up to 20 minutes, after which the rate declined. This result is closely similar to that of Balesubramanian et al (252), who interpret the break in activity at 20 minutes as indicating that the cholesterol in the enzyme substrate pool has become sufficiently depleted that enzyme activity becomes limited by the migration of cholesterol into this pool from other pools within the microsomal membrane.

4.4 The effect of variation of coenzyme A concentration

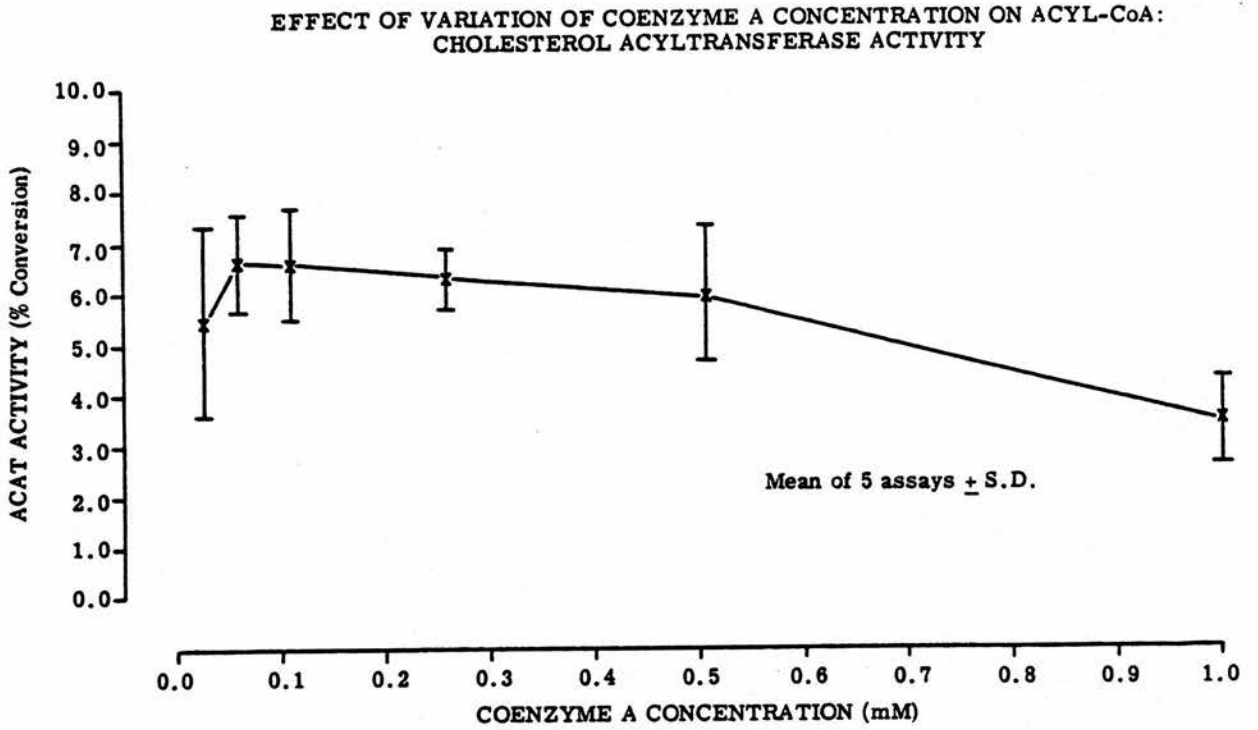
Microsomes were prepared from rats fed a cholesterol diet for 8 days. Microsomal protein (100 μg) was incubated at 37° for 20 minutes with 2 mM ATP, 4 mM $MgCl_2$, 3.3 mM reduced glutathione and various concentrations of coenzyme A. Figure 4.5 demonstrates that there is no significant variation in the activity of acyl-coenzyme A:cholesterol acyltransferase over a wide range of coenzyme A concentration. As a result of this experiment the concentration of coenzyme A routinely used in assays was decreased from 0.5 mM to 0.05 mM.

Figure 4.4



(For method see text).

Figure 4.5



(For method see text)

Section 5

Some In Vitro Studies of Acyl-Coenzyme A: Cholesterol Acyltransferase

5.1 Introduction

Little work has been done to extend the original observations of Mukherjee et al (336) and of Goodman et al (248) on hepatic acyl-coenzyme A:cholesterol acyltransferase. These were principally the demonstration of the requirement for ATP and coenzyme A, or alternatively for fatty acyl-coenzyme A, if cholesterol esterification is to proceed at significant rates and the investigation of the specificity of the enzyme towards a series of free fatty acids of varying length and saturation.

In work on the fatty acid synthetase enzyme complex the use of coenzyme A analogues has proved very useful (337,338). These could be substituted for coenzyme A for various types of study, though usually, because of the low affinities of the enzyme for these compounds, the concentrations used had to be many times higher than the concentration of coenzyme A for similar reaction rates to be observed (338). One of the most successfully used analogues has been N-acetyl-cysteamine, whose structure, together with that of coenzyme A, is given in Figure 5.1.

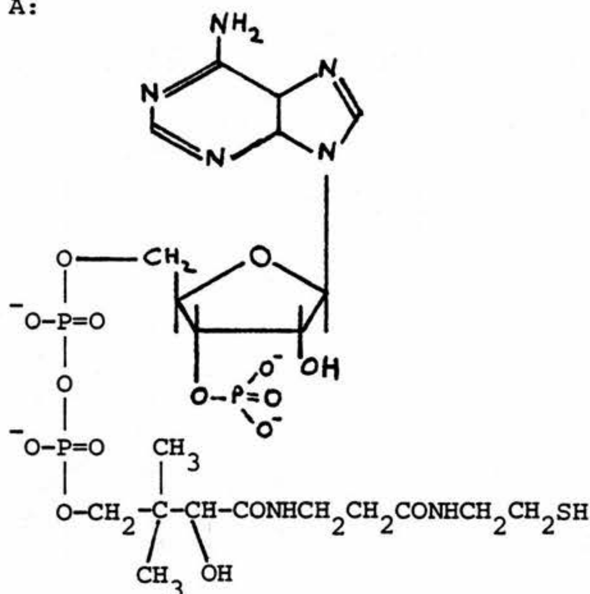
The ability of N-acetyl-cyteamine and N-acetyl-cysteine to support cholesterol esterification by microsomes in the presence of ATP was therefore investigated.

Liver acyl-coenzyme A:cholesterol acyltransferase has a substrate preference for fatty acids that are mono-unsaturated, oleic acid being the optimum (248). In liver microsomes there exists a cytochrome b_5-dependent fatty acyl-coenzyme A desaturase system. Since very little is known of the source of the fatty acid for cholesterol esterification, the effect of a series of conditions designed to perturb the activity of the desaturase on the rate of cholesterol esterification was investigated. This was in the hope that, in the absence of exogenous fatty acid, variations in the rate of the desaturase might alter the

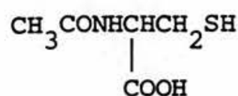
Figure 5.1

The structures of coenzyme A and some possible coenzyme A analogues

Coenzyme A:

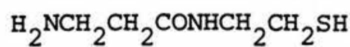


N-acetyl-cysteine:



N-acetyl-cysteamine:

N-(β-alanyl)-cysteamine:



size of the endogenous microsomal fatty acid pool and hence the rate of cholesterol esterification (Figure 5.2).

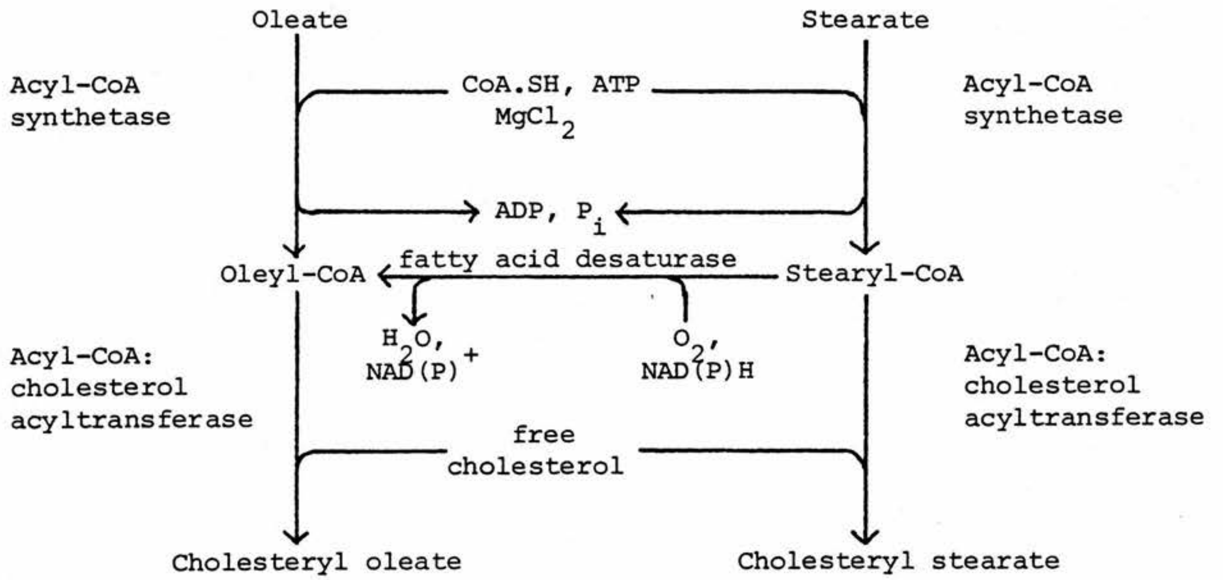
Acyl-coenzyme A:cholesterol acyltransferase is found in the rough endoplasmic reticulum enzyme (339,340), and sediments with the microsomal fraction when centrifuged at 105 000 x g for 1 hour. However, in cholesterol-fed rats, the cholesterol ester which accumulates as lipid droplets in the liver does not sediment with the microsomes on centrifugation, but forms a floating layer at the surface of the supernatant.

Cholesterol ester is soluble in unilamellar egg yolk lecithin liposomes to 5 moles per cent (341), probably in a "horseshoe" structure with the ester linkage near the lipid-water interface and the cholesterol and fatty acid moieties buried within the hydrophobic membrane core (341). A small amount of cholesterol ester sediments with microsomes, but the proportion of this ester dissolved within the membrane in this manner, as opposed to being associated with proteins within the lumen of the microsomal vesicles (for example, nascent lipoproteins) is unknown. Indeed, it is possible that all of the sedimented cholesterol ester is associated with protein rather than with membrane lipid.

Two possibilities for the immediate fate of newly synthesised cholesterol ester are therefore that it is dissolved within the microsomal membrane, or that it is extruded into the intravesicular space to become associated with a protein. A third, perhaps less likely, alternative is that the cholesterol ester is extruded into the extravesicular space. That is, in an intact cell the cholesterol ester would be secreted directly into the cytoplasm to form lipid droplets.

Figure 5.2

The interrelation of acyl-coenzyme A:cholesterol acyltransferase and fatty acyl-coenzyme A desaturase activities



As a preliminary investigation into these possibilities the sedimentation behaviour of [4-^{14}C]-cholesterol ester newly synthesised from [4-^{14}C]-cholesterol was observed.

5.2 The use of N-acetyl-cysteine and N-acetyl-cysteamine as coenzyme A analogues

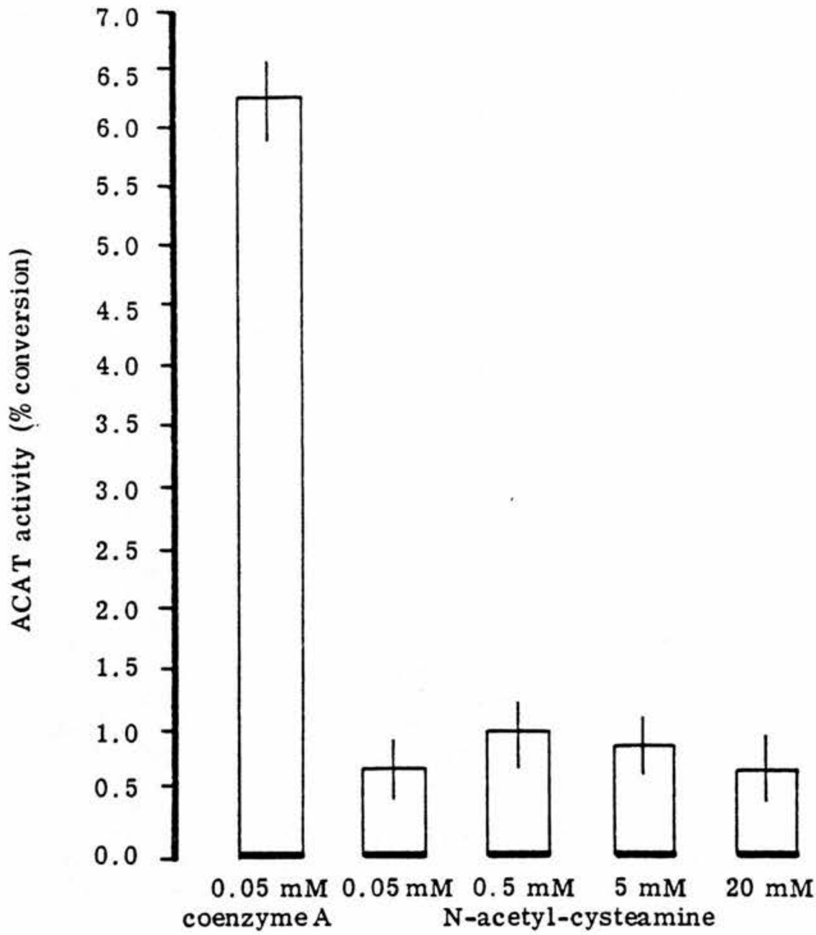
The use of N-acetyl-cysteine at concentrations ranging from 50 μM to 50 mM in incubations of 186 μg microsomal protein from cholesterol-fed rats in the presence of ATP, $MgCl_2$ and reduced glutathione did not produce detectable esterification of exogenous [4-^{14}C]-cholesterol.

N-acetyl-cysteamine more closely resembles the structure of the active thiol end of coenzyme A than N-acetyl-cysteine, and has been shown to be a more satisfactory coenzyme A analogue in the work on the fatty acid synthase system (337,338).

Figure 5.3 shows the effect of incubating 240 μg microsomal protein from cholesterol-fed rats either with 0.05 mM coenzyme A, or with various concentrations of N-acetyl-cysteamine, in the presence of ATP, $MgCl_2$ and reduced glutathione. The radioactivity recovered in the area corresponding to cholesterol ester from the thin layer chromatogram in the presence of 0.05 mM or 20 mM N-acetyl-cysteamine was not above the level of background radioactivity, which will account for approximately 0.75% of the total recovered radioactivity. The activity observed in the presence of 0.5 mM and 5 mM N-acetyl-cysteamine, although resulting in clearly observable cholesterol ester peaks in the radioactivity scans of the thin layer chromatography plates was probably not significantly above background levels.

This experiment suggests that, while it might have been expected that high concentrations of N-acetyl-cysteamine would be required to observe reaction rates comparable to those obtained in the presence of

Figure 5.3



(Mean of 5 assays ± S.E.M.)

(For method see text).

coenzyme A, further increases in concentration above 20 mM are unlikely to increase activity above the low values detected at 0.5 mM. If N-acetyl-cysteamine was to be used as an analogue for coenzyme A in the study of acyl-coenzyme A:cholesterol acyltransferase the main requirement would thus be the use of more sophisticated means for assessing whether observed reaction rates were above background levels. However, in this range the detection of small changes in enzyme activity would be extremely difficult.

More fruitful approaches would probably be the use of analogues likely to have a higher affinity for the active site of the enzyme. These analogues could be designed to resemble a greater part of the coenzyme A molecule than N-acetyl-cysteamine does - an example would be the use of N-(β-alanyl)-cysteamine.

An alternative would be to investigate the ability of fatty acyl thioesters of N-acetyl-cysteamine to support cholesterol esterification in the presence of microsomes.

5.3 The effect of in vitro perturbations of fatty acyl-coenzyme A desaturase activity on cholesterol esterification

Various approaches to altering microsomal fatty-acyl-coenzyme A desaturase were used. These included bubbling either oxygen or oxygen-free argon through the microsomal suspension both before and during the enzyme incubation, intended to, respectively, stimulate the enzyme activity (in the presence of NADPH) and inhibit it. These conditions were not wholly satisfactory, since the measured acyl-coenzyme A: cholesterol acyltransferase activity of all samples that had been bubbled with gas was lower than that of controls. This was probably attributable to problems associated with frothing of the samples.

The more satisfactory approach was therefore to use a high concentration of potassium cyanide (1 mM) to completely inhibit any desaturatase activity (342), and to use an NADPH-generating system to support activity. (The natural reducing agent for fatty-acyl-coenzyme A desaturase is NADH, but there is a considerable degree of cross-specificity for NADPH (342).) It should be noted that the presence of NADPH will allow cholesterol 7α-hydroxylase activity. The presence of oxygenated sterols will not affect the actual measurement of esterification activity, since, in the solvent system used for chromatography, these polar cholesterol derivatives will migrate with cholesterol. It is possible that an active cholesterol 7α-hydroxylase may influence esterifying activity, perhaps by altering the amount of cholesterol available for esterification. However, some preliminary experiments suggest that this is not so (Dr. K.E. Suckling, personal communication), and in the absence of cysteamine cholesterol 7α-hydroxylase activity will anyway be limited.

Table 5.1 shows the results obtained on the addition of either 1.0 mM potassium cyanide or NADPH generator to a standard assay for acyl-coenzyme A:cholesterol acyltransferase activity. It can be seen that potassium cyanide had no inhibitory effect and NADPH no stimulatory effect on esterification activity. Under these conditions, then, agents chosen to perturb the activity of fatty-acyl-coenzyme A desaturase *in vitro* have no effect on acyl-coenzyme A:cholesterol acyltransferase activity. This suggests either that the product of the desaturase does not directly enter the substrate pool for esterification or that esterification activity is not limited by the supply of its preferred substrate.

Table 5.1

The effect of in vitro perturbations of fatty-acyl-coenzyme A desaturase activity on acyl-coenzyme A:cholesterol acyltransferase activity

Addition to* incubation	ACAT activity (% conversion)≠ Experiment 1	Experiment 2
None	11.3 (1)†	11.3 (5)
KCN	11.2 (3)	12.6 (3)
NADPH	10.0 (3)	9.4 (2)

*The incubation contained 2 mM ATP, 4 mM $MgCl_2$, 0.5 mM coenzyme A, 3.3 mM reduced glutathione and 350 μg protein (experiment 1) or 460 μg protein (experiment 2) in a total of 3 ml 0.1M potassium phosphate buffer pH 7.4. KCN was added to a final concentration of 1.0 mM. The NADPH-generating system was made up as for cholesterol 7α-hydroxylase assays, and was added to give, per incubation, 12.5 mg glucose-6-phosphate, 3.75 mg $NADPH^+$, 1.0 U glucose-6-phosphate, dehydrogenase. Incubation was for 1 hour at 37°.

≠ACAT = acyl-coenzyme A:cholesterol acyltransferase.

†The figure in brackets indicates the number of replicates included in the reported mean.

5.4 The fate of newly synthesised cholesterol ester

Acyl-coenzyme A:cholesterol acyltransferase incubations were performed using 460 μg protein and the usual concentrations of cofactors. Incubations were started at various times such that a time-course of activity could be obtained, with all incubations being stopped at the same time. The microsomal suspension was added to the solution of cofactors in the incubation flask and the flask put into the incubation bath five minutes before incubation was due to start, in order to allow temperature equilibration. Incubation was started by the addition of [4-^{14}C]-cholesterol. Reaction in all flasks was stopped simultaneously by transferring the flasks to ice. The contents of the flasks were transferred to centrifuge tubes, with washing with two 1 ml aliquots of phosphate buffer. These tubes were centrifuged at 105 000 x g for 1 hour.

There was no visible floating lipid layer at the end of centrifugation. The supernatants were transferred by aspiration (in an attempt to prevent any unseen floating lipid adhering to the sides of the centrifuge tubes) directly into extraction tubes containing a 2:1 mixture of chloroform and methanol. The tubes and the surfaces of the pellets were gently washed with two 500 μl aliquots of buffer, which were also transferred to the extraction tubes.

The pellets were then resuspended in 2 ml phosphate buffer with the use of a vortex mixer, and the suspension transferred to extraction tubes as above. This was repeated twice with 1 ml aliquots of phosphate buffer.

Extractions and chromatography were as usual in assays for acyl-coenzyme A:cholesterol acyltransferase activity. The results are shown in Table 5.2. These indicate that cholesterol ester newly synthesised from exogenous radioactive free cholesterol is entirely associated with the microsomal pellet, despite the fact that a considerable proportion

Table 5.2

The fate of newly synthesised cholesterol ester*

Incubation time (min)	Free cholesterol		Cholesterol ester		ACAT activity†
	Super-natant	Pellet	Super-natant	Pellet	
0	35.5	61.6	0.3	1.1	1.4
15	16.5	74.5	0.4	7.4	7.8
30	17.7	65.2	0.7	12.6	13.3
45	19.5	59.7	0.7	18.7	19.4
60	11.5	64.5	0.7	20.9	21.6

*For method,see text. The results are the percent of the total recovered radioactivity found as free cholesterol or cholesterol ester in the supernatant and pellet after centrifugation, and are the means of two incubations.

†ACAT = acyl-coenzyme A:cholesterol acyltransferase. Activity is the percent of total recovered radioactivity as cholesterol ester.

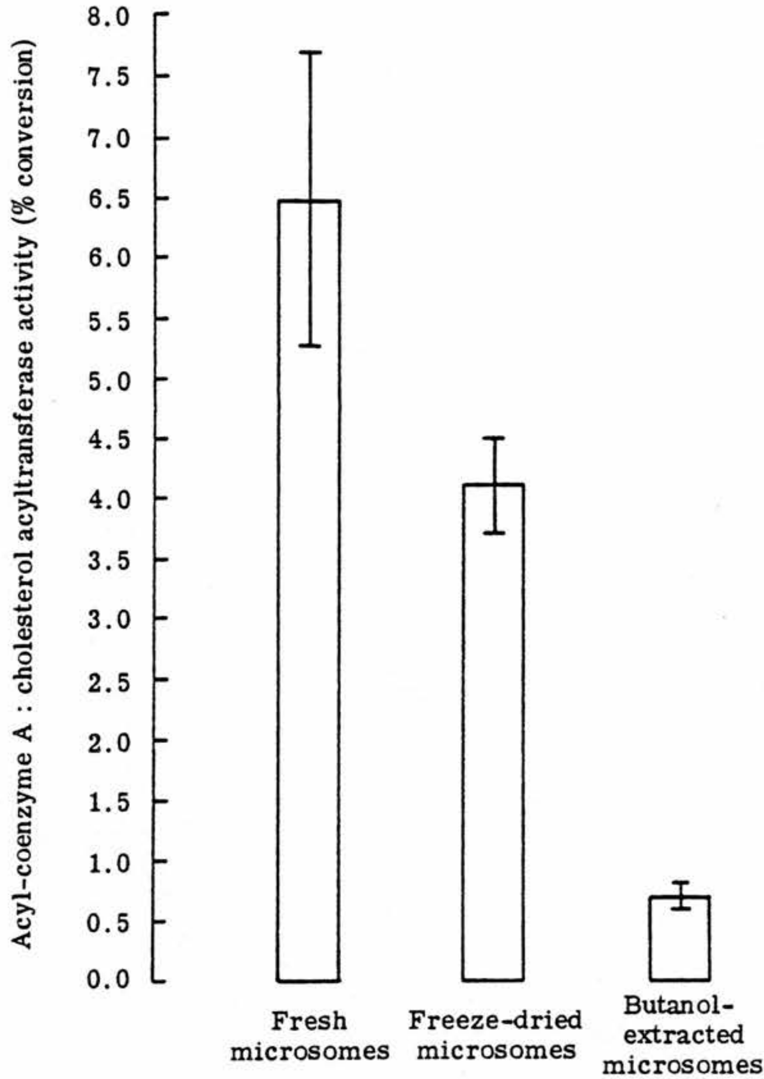
of the added free cholesterol is found in the supernatant. This suggests that newly synthesised cholesterol ester either is capable of dissolving within the microsomal membrane, or becomes associated with protein trapped within the microsomal vesicles.

5.5 Effect on the acyl-CoA:cholesterol acyltransferase activity of microsomes of freeze-drying and treatment with organic solvents

A convenient source of enzyme for experiments on cholesterol 7α-hydroxylase is a freeze-dried powder of microsomes, which can be stored at -20°. Butanol extraction of this freeze-dried powder both lengthens the time for which the powder can be stored, and helps the interpretation of the results of certain experiments, where it is an advantage to have minimum dilution of added radioactive cholesterol by endogenous cholesterol.

The effect of freeze-drying and butanol extraction on microsomal acyl-coenzyme A:cholesterol acyltransferase activity was therefore investigated (Figure 5.4). Freeze-drying caused a loss of acyl-coenzyme A: cholesterol acyltransferase activity to 60% of that of fresh microsomes. Butanol extraction reduced activity to barely detectable levels (11% of the activity measured in fresh microsomes, 17% of the activity of a freeze-dried powder). If the argument described in Section 4.2 is used to allow the calculation of actual enzyme activities, the lack of activity of the butanol-extracted preparation is considerably emphasised, since the cholesterol content of this sample is so low. Thus, the activity of the butanol-extracted preparation is 0.50 ng cholesterol converted to cholesterol ester per 100 µg protein per 20 minutes, compared to a control activity for the freeze-dried microsomes of 74 ng cholesterol converted per 100 µg protein per 20 minutes.

Figure 5.4



(Mean of 5 assays ± S.E.M.)

(Acyl-coenzyme A:cholesterol acyltransferase assays were performed as described in Section 2.12. Assays contained the following amounts of protein: fresh microsomes 79.5 µg; freeze-dried microsomes 186 µg; butanol-extracted microsomes 153 µg.)

Extraction of freeze-dried microsomes with acetone at -20° removes considerably less lipid than extraction with butanol. The examination of acyl-coenzyme A:cholesterol acyltransferase activities of freeze-dried and acetone-extracted microsomes showed losses of enzyme activity on acetone extraction, to 20-40% of control values. These results are further discussed in Section 7.

5.6 The effect of Nonidet P42 on acyl-coenzyme A:cholesterol acyltransferase activity

For many experiments it would be an advantage to use a reasonably pure preparation of acyl-coenzyme A:cholesterol acyl transferase, and for this purpose it is necessary to solubilise the enzyme, while retaining enzyme activity.

Although no results have been published, it appears that the acyl-coenzyme A:cholesterol acyltransferase of aortic microsomes has proved difficult to solubilise with retention of enzyme activity. ("Attempts to study the enzyme in isolation...have been unsuccessful." (343).) Similarly, no work has been published on the preparation of the liver enzyme. Since Nonidet P42 has been shown to be successful in the solubilisation of another liver microsomal enzyme, cholesterol 7α-hydroxylase, without loss of activity (226), its effect on acyl-coenzyme A:cholesterol acyltransferase was investigated.

Table 5.3 shows the effect of adding varying amounts of Nonidet to a suspension of microsomes on their acyl-coenzyme A:cholesterol acyltransferase activity. At low detergent concentrations there is a slight increase in enzyme activity but at higher detergent concentration the measured activity rapidly falls off. When these microsomal samples with varying detergent concentrations were centrifuged at 105 000 x g for one hour no enzymic activity was detectable in the supernatants obtained.

Table 5.3

The effect of the solubilisation of microsomal protein with Nonidet P42 on acyl coenzyme A:cholesterol acyltransferase activity

Nonidet P42 concentration (μl/mg protein)	Acyl-coenzyme A:cholesterol acyltransferase activity (% conversion)	
	Uncentrifuged sample	105,000 x g supernatant
-	2.8	-
0.25	2.8	0.4
0.51	3.8	0.5
1.02	0.9	0.6
2.04	0.4	0.5
4.08	0.4	0.2

(After this experiment had been performed, Kaduce et al (344) reported on the use of 2% Triton X-100 in the solubilisation of acyl-coenzyme A:cholesterol acyltransferase from Ehrlich ascites cell microsomes. This resulted in the loss of enzyme activity to levels of 1-2% of the activity of untreated controls. Removal of detergent by the use of SM-2 BioBeads restored activity to 40% of control values, and this activity could be further enhanced by up to 2.5-fold, by incorporating this solubilised protein into liposomes.)

Section 6

Some In Vivo Studies of Hepatic Microsomal Cholesterol Metabolism

6.1 Introduction

The liver plays a central controlling role in cholesterol metabolism, as discussed in Section 1. In particular, the rates of synthesis and degradation of cholesterol, and the activities of the enzymes catalysing rate-determining reactions in these processes (3-hydroxy-3-methylglutaryl-coenzyme A reductase, and cholesterol 7α-hydroxylase) display a diurnal variation (4,129,154,155,262,263,272,345,346), and a response to cholesterol absorption (4,129,147,154,155,159-161,174,176,347) and to the prevention of cholesterol and bile acid secretion into bile by occlusion of the bile duct (160,348). In addition, in the rat the liver is able to accumulate large quantities of cholesterol ester on cholesterol feeding (148,149). Experiments were undertaken to investigate some of these aspects of the control of liver microsomal cholesterol metabolism.

Although cholesterol synthesis (and 3-hydroxy-3-methyl-glutaryl coenzyme A reductase) and bile acid production (and cholesterol 7α-hydroxylase) are known to vary during a twenty-four hour cycle, no diurnal measurements of the other microsomal enzyme of cholesterol metabolism (acyl-coenzyme A:cholesterol acyltransferase) have been reported. In this context, it has been shown that, if the cholesterol synthesis rate in hepatocyte suspensions is forced to increase by the addition of mevalonic acid, cholesterol esterification is stimulated (349), though this is difficult to reconcile with the lack of effect on ACAT activity *in vivo* when mevalonic acid is administered intravenously (350). (It should be noted that while it is recognised that hepatic ACAT activity and cholesterol ester accumulation increase after intragastric administration of mevalonic acid or mevalonolactone (331, 350,351), this is likely to be due to an increased delivery of cholesterol to the liver, after synthesis in the intestinal tract, rather than to an increased local, intrahepatic, cholesterol synthesis (350).)

This report of a connection between cholesterol synthesis rate and cholesterol esterification confirmed the need to investigate the possibility that acyl-coenzyme A:cholesterol acyltransferase might exhibit a diurnal variation on its activity. While this experiment was being planned, a report appeared claiming that this enzyme shows no diurnal variation of activity (252), but this conclusion was based only on measurements at noon and midnight.

The liver secretes bile which contains cholesterol, phospholipid and bile salts, by a poorly understood mechanism. If the bile duct is occluded by ligation, during the following few days the hepatic contents of free cholesterol and bile salts rise (352,353), there is an increase in serum free cholesterol (353), an increase in the rate of hepatic synthesis of cholesterol (160,353), a decrease in overall bile salt synthesis (354), and a change in the pattern of bile salts synthesised (348,352,355). There is a small decrease in cholesterol 7α-hydroxylase activity in the first twenty-four hours after bile duct ligation, with a large increase in the activity of this enzyme after two days (348). It is not clear how this increase in activity accords with the observed decrease in bile acid synthesis. No attempt was made in this report to differentiate the relatively small changes in enzyme activity within the first twenty-four hours from the expected diurnal rhythm in activity. In addition the assays were performed by measuring the conversion of exogenous labelled cholesterol to 7α-hydroxycholesterol, but the results were reported in terms of actual production of 7α-hydroxycholesterol. In view of the increase in hepatic cholesterol content, the probability of a change in the size of the substrate pool for cholesterol 7α-hydroxylase makes the validity of this practice dubious. An experiment was therefore undertaken to measure cholesterol 7α-hydroxylase activity by an absolute method and also, by simultaneously measuring the activity

by the conversion of labelled substrate to product, to obtain an estimate of the size of the cholesterol pool accessible to the enzyme. This was done for the twenty-four hour period after bile duct ligation, and the observed changes in activity were differentiated from the normal diurnal variation by the use of sham-operated control animals.

Hepatic acyl-coenzyme A:cholesterol acyltransferase has been shown to be affected by the supply of cholesterol, as follows. Hepatic cholesterol esterification increases on intragastric mevalonic acid administration (331,350,351), and on cholesterol feeding (252,331,351), and esterification in isolated hepatocytes is stimulated by addition of mevalonic acid (349). In addition, the stimulatory influence of an increase in cholesterol supply on acyl-coenzyme A:cholesterol acyltransferase activity in fibroblasts (106,121,123), and in rat hepatoma Fu5AH cells (333,356-358), has been well characterised. The response of hepatic acyl-coenzyme A:cholesterol acyltransferase activity to the increase in liver cholesterol content achieved by bile duct ligation was therefore also investigated.

Because of the central controlling role played by the liver in cholesterol metabolism it is reasonable to expect that hepatic cholesterol metabolising enzymes will show adaptational responses to the large increases in cholesterol absorption caused by feeding cholesterol-rich diets. It is widely acknowledged that cholesterol feeding in the rat leads to an increase in bile acid synthesis (172,173). However, the position with regard to cholesterol 7α-hydroxylase activity is considerably confused, with reports of no increase in enzyme activity (174,175), and reports of a stimulation of enzyme activity (147,161,176). There has also been a suggestion that increased cholesterol 7α-hydroxylase activity in some circumstances may be due not to an increased amount or increased intrinsic activity of the enzyme itself, but to an increase in the size

of the enzyme's substrate pool (176). This observation is complicated by the fact that these authors observed a variation in substrate pool size at 1.0 a.m. and 1.0 p.m. that was in synchrony with the activity of cholesterol 7α-hydroxylase, with high values of each at 1.0 a.m. in cholesterol-fed rats (176); when they had previously measured enzyme activity and pool size throughout one day in control diet-fed animals the variations in each were out of phase, with the maximum enzyme activity coinciding with the minimum size of substrate pool and vice versa (263). The relationship between cholesterol supply and cholesterol 7α-hydroxylase activity in cholesterol feeding is thus by no means clear.

Cholesterol-fed rats have long been known to accumulate large quantities of cholesterol esters in their livers (148,149). As briefly mentioned above, there have been reports of a stimulatory effect of cholesterol feeding on acyl-coenzyme A:cholesterol acyltransferase activity (252,331,351). However, in one of these reports, animals were killed after 12 hours of feeding diets of varying cholesterol contents (252), and the others contain no mention of the fat content of the diet (331,351). No reports attempt to correlate the rapid decrease in liver cholesterol ester contents on withdrawal of cholesterol-rich diets with changes in the activities of enzymes of cholesterol esterification or cholesterol ester hydrolysis.

Three experiments were therefore performed to investigate the effects of cholesterol-rich diets on the liver microsomal enzymes of cholesterol metabolism and on the sizes of the pools of cholesterol accessible to these enzymes.

6.2 The assay of acyl-coenzyme A:cholesterol acyl-transferase activity through a twenty-four hour cycle

The rats for this experiment were divided into two groups one of which was kept in the animal house where they experienced a lighting

schedule with twelve hours light between 0800 and 2000. The other group was kept in a light-proof cabinet with the lighting schedule altered to give twelve hours light between 1700 and 0500. This cabinet was housed within the same animal house room as the other group of rats to minimise the effects of any other environmental influences. The rats were fed the cholesterol-rich diet.

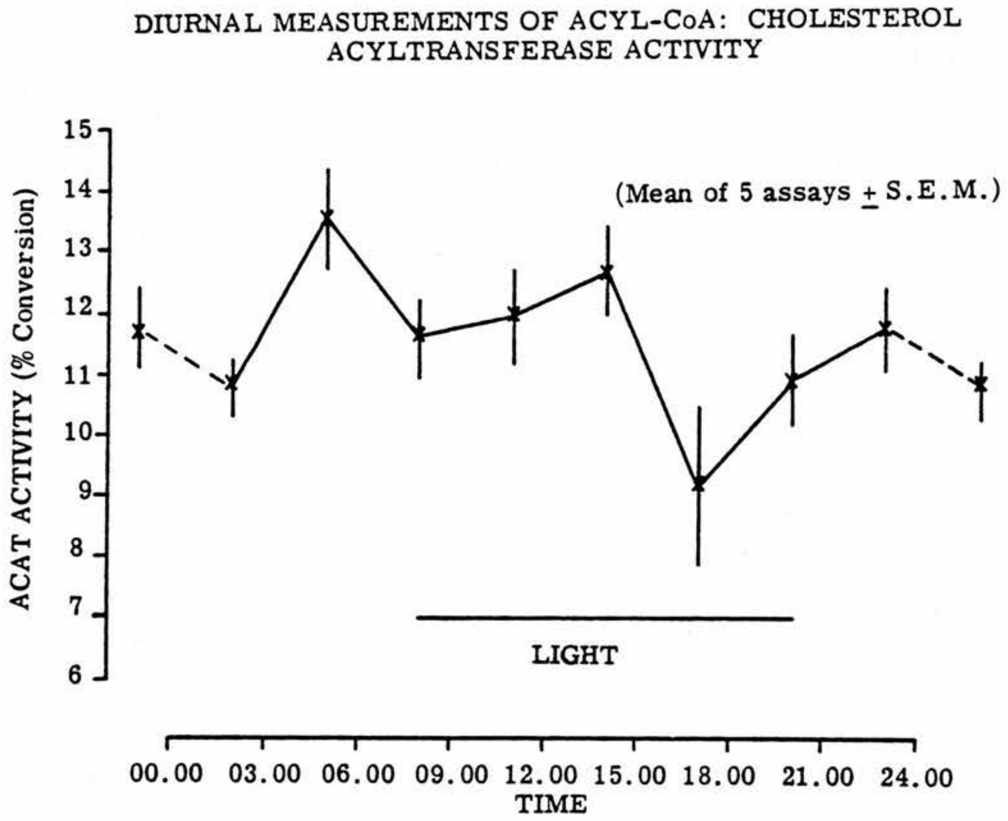
Pairs of rats were killed from each group throughout a twelve hour period, microsomes were prepared immediately and resuspended in 20 ml water. After assay for protein, suitable amounts of microsomal suspension were taken for assay of acyl-coenzyme A:cholesterol acyltransferase activity.

In view of the non-linearity of this assay the amount of protein was kept as constant as practicable, and was always between 175 μg and 250 μg, a difference which might be expected to account for an increase in activity of approximately 1% (see Figure 4.2).

The results are shown in Figure 6.1. In this figure the results from both groups of rats have been plotted on a time-scale where the lighting was on between 0800 and 2000. A peak of activity was obtained at 0500 and a trough at 1700. These times are separated by twelve hours - the duration of the light and dark periods - and can be hypothesised to occur at the times of maximal feeding and maximal fasting, respectively (D. Mathé, personal communication).

The significance of the difference of the results at 0500 and 1700 is $P<0.01$ (by Student's t test), or $P<0.025$ (by the Welch-Aspin test, which does not assume two identical normal distributions). However, the validity of the result of this experiment is cast in doubt when protein concentrations are taken into account. The protein concentration had been allowed to vary within the limits of 175 μg and 250 μg per assay because of the relatively small difference in measured ACAT activity

Figure 6.1



(For method see text).

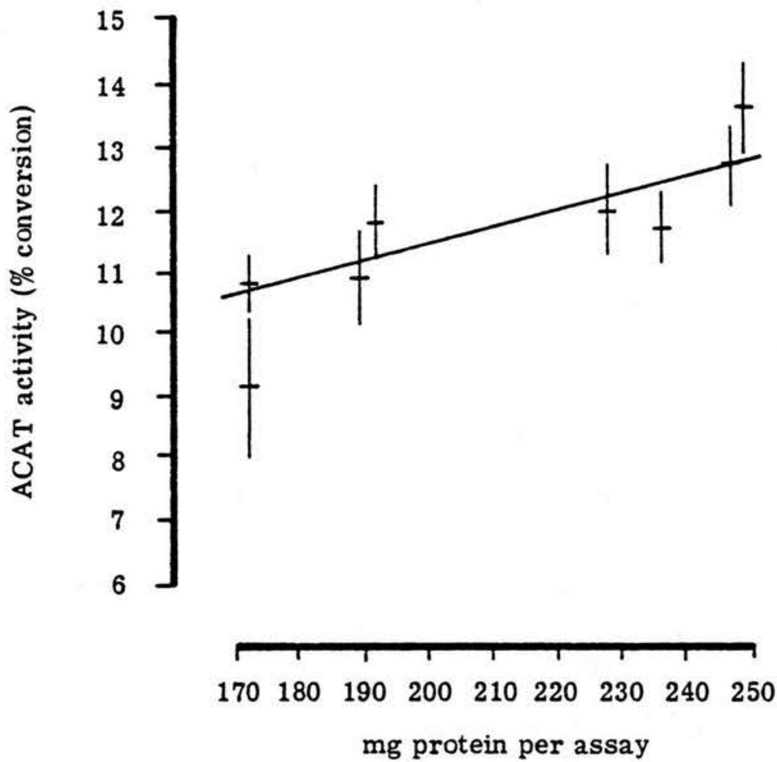
this would be expected to cause, as long as activity is measured in terms of percent conversion of tracer substrate to product (see Figures 4.1 and 4.2). The result of plotting the acyl-coenzyme A:cholesterol acyltransferase activities obtained in this experiment against the amount of protein per incubation is shown in Figure 6.2. Here it can be seen that much of the variation in measured acyl-coenzyme A:cholesterol acyltransferase activity in this experiment can be accounted for by variations in the amount of protein used. It is however apparent that the values obtained at 0500 and 1700 do in fact lie furthest from the line obtained from this plot, so the possibility remains that there is indeed a diurnal variation in acyl-coenzyme A:cholesterol acyltransferase activity, with the high and low values being obtained at these times. This experiment has been repeated using the more satisfactory assay based on [1-^{14}C]-palmitoyl-coenzyme A, and the presence of a diurnal variation in acyl-coenzyme A:cholesterol acyltransferase activity has been confirmed (Dr. K.E. Suckling, personal communication).

It should be noted that, while contradicting the conclusions of Balasubramanian et al (252) that there is no diurnal variation in the activity of acyl-coenzyme A:cholesterol acyltransferase, these results fully explain the observation of these authors, since they based their deduction on assays performed only at midday and midnight. At these times there is certainly no difference in observed activity.

6.3 Assay of acyl-coenzyme A:cholesterol acyltransferase and cholesterol 7α-hydroxylase activities during the twenty-four hours following bile duct occlusion

Rats were prefed on a 10% olive oil 1% cholesterol diet for 10 days. At 0900 four rats were killed. The remaining animals were divided into two groups. The animals in one of these groups were anaesthetised and their bile ducts were ligated proximally and distally. The animals in

Figure 6.2



(Mean of 5 assays ± S.E.M.)

(The results of the acyl-coenzyme A:cholesterol acyltransferase assays obtained throughout a 24 hour cycle are here plotted against the amount of protein used per assay.)

the other group were sham operated. At 2, 4, 8, 16 and 24 hours after operation pairs of experimental rats and pairs of control rats were killed.

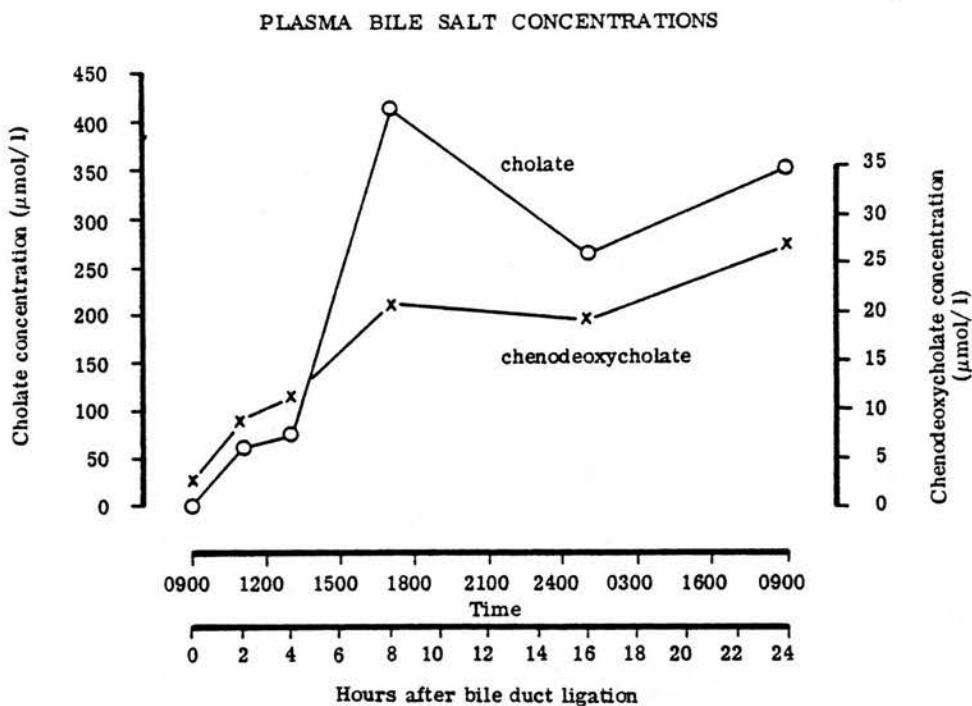
In all cases samples of systemic blood were obtained and plasma was assayed for conjugated cholic acid and conjugated chenodeoxycholic acid by Dr. G.J. Beckett of the Department of Clinical Chemistry, University of Edinburgh. Liver microsomes were prepared, and assayed for acyl-coenzyme A:cholesterol acyltransferase and cholesterol 7α-hydroxylase.

In order that estimates of the sizes of the substrate pools for these enzymes could be obtained parallel sets of assays were performed. Thus, acyl-coenzyme A:cholesterol acyltransferase was assayed using both [4-^{14}C]-cholesterol and [1-^{14}C]-oleic acid as the tracer substrate, and cholesterol 7α-hydroxylase was assayed both in terms of percent conversion of tracer substrate to product and by absolute measurement of 7α-hydroxycholesterol production by combined gas chromatography - mass spectrometry. This latter technique also provided an assay of the cholesterol content of the microsomes.

Figure 6.3 shows the plasma concentrations of cholic acid and chenodeoxycholic acid following bile duct ligation. Two hours after ligation conjugated cholic acid and conjugated chenodeoxycholic acid levels had increased by twenty-fold and three-fold respectively. Eight hours after ligation the concentrations of conjugated cholic acid and conjugated chenodeoxycholic acid were respectively one hundred and thirty-fold and eight-fold greater than control values. These results allow confidence that the ligations of the bile ducts were successful in achieving its occlusion.

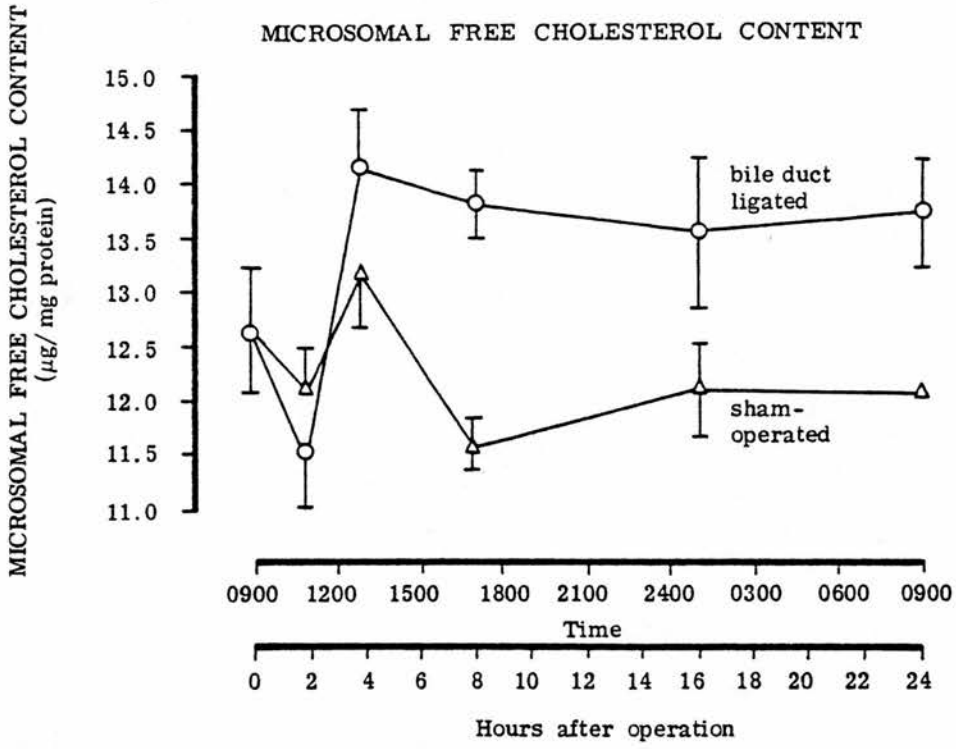
Figure 6.4 shows hepatic microsomal free cholesterol contents, expressed relative to protein. Eight hours after operation the level of

Figure 6.3



(For method see text).

Figure 6.4



(Mean of 5 assays ± S.E.M.)

cholesterol in the experimental animals was 5.7 nmol (2.20 μg) per mg protein greater than in the controls, and this difference was significant ($P<0.025$). At later times the difference between experimental and control groups was approximately maintained, although the significance levels declined somewhat.

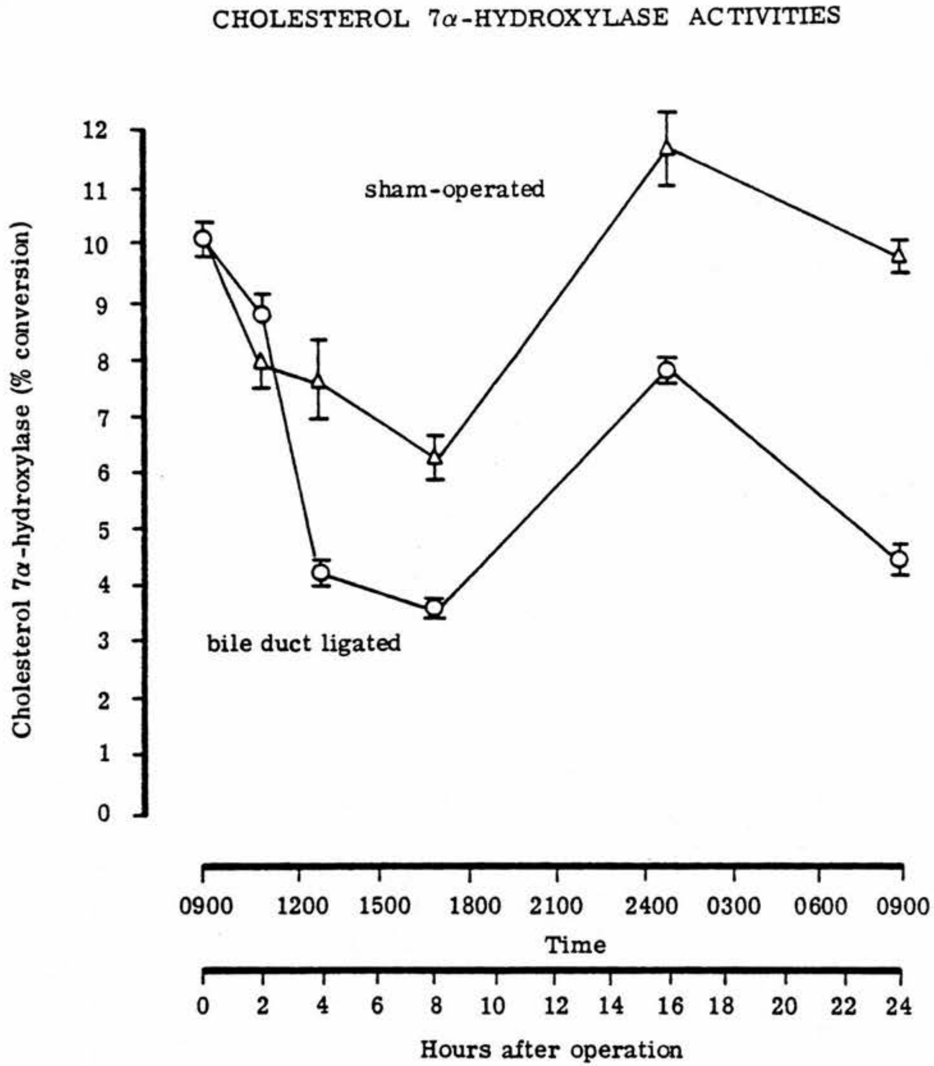
Figure 6.5 shows the results of assays for cholesterol 7α-hydroxylase activity, both in terms of percent conversion of exogenous tracer cholesterol to tracer product, and in terms of actual production of 7α-hydroxycholesterol. In terms of the percent conversion assay (Figure 6.5(a)), the enzyme activity of the ligated rats was significantly less than that of the sham-operated rats after 4 hr ($P<0.05$), and remained so for the duration of the experiment. The normal diurnal rhythm in cholesterol 7α-hydroxylase activity was maintained, superimposed on the change observed.

In contrast, absolute measurement of enzyme activity shows that at no time during the experiment was there a significant difference between the activities of microsomal preparations from ligated and sham operated rats (Figure 6.5(b)). Again, the diurnal variation in activity can be seen to persist.

Estimates of the maximum possible size of the substrate pool can be derived from these results. Figure 6.6 shows substrate pool size, both in absolute terms and as a fraction of total microsomal free cholesterol, plotted against time. There is a suggestion that the absolute size of the pool in the experimental animals may be elevated above that in control animals, but this difference does not approach significance, the greatest difference being after 8 hours ($0.05<P<0.1$).

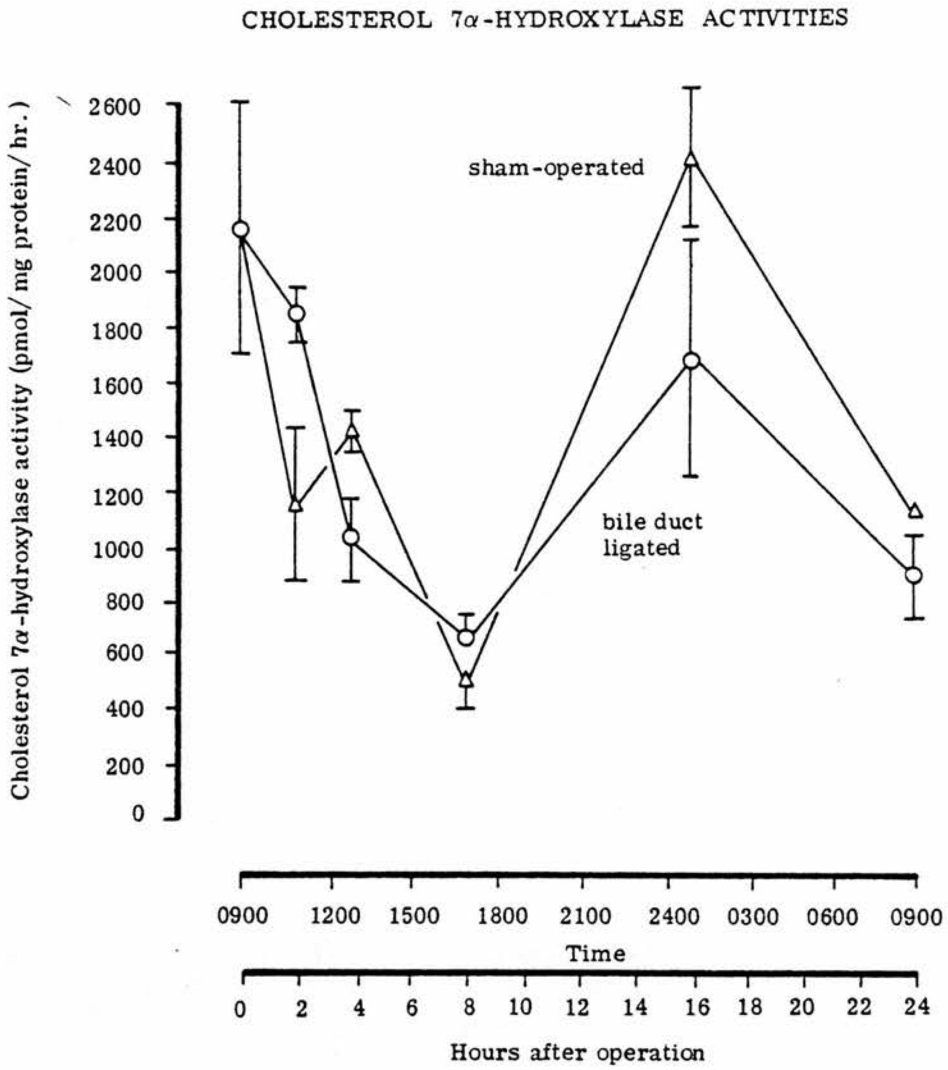
The results of both absolute and percent conversion assays for acyl-coenzyme A:cholesterol acyltransferase are shown in Figure 6.7. The percent conversion of exogenous labelled free cholesterol to

Figure 6.5(a)



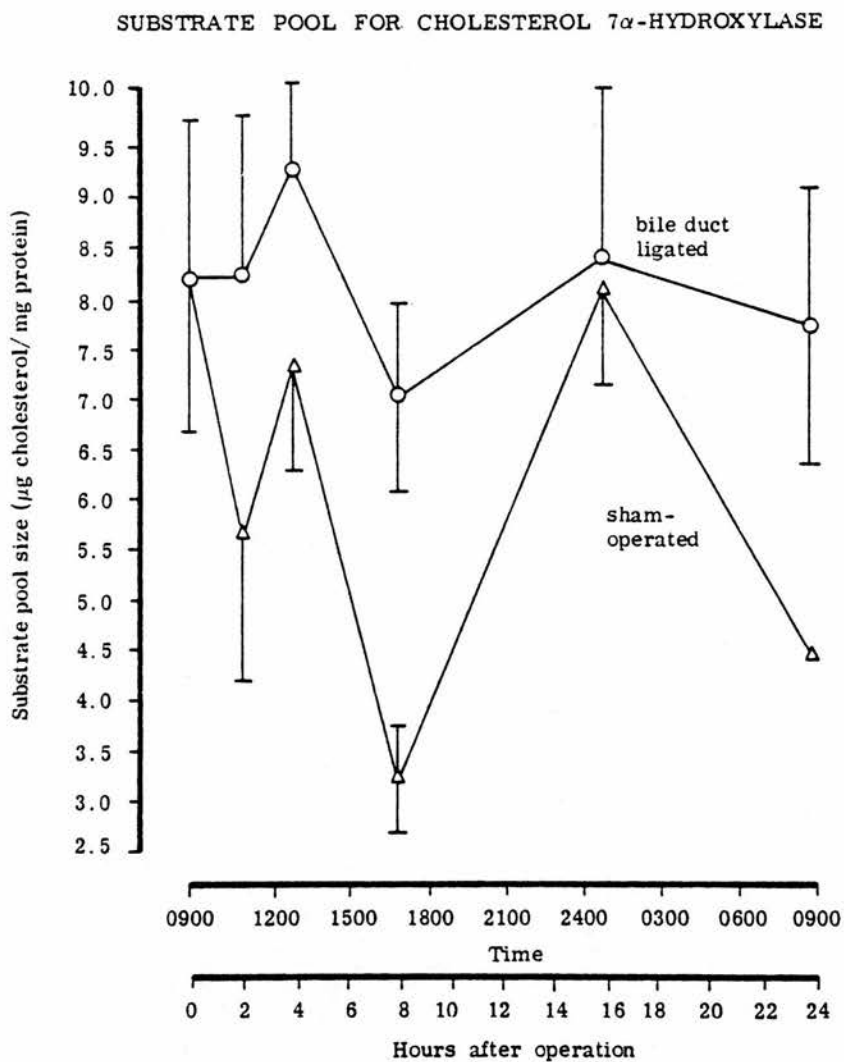
(Mean of 5 assays ± S.E.M.)

Figure 6.5(b)



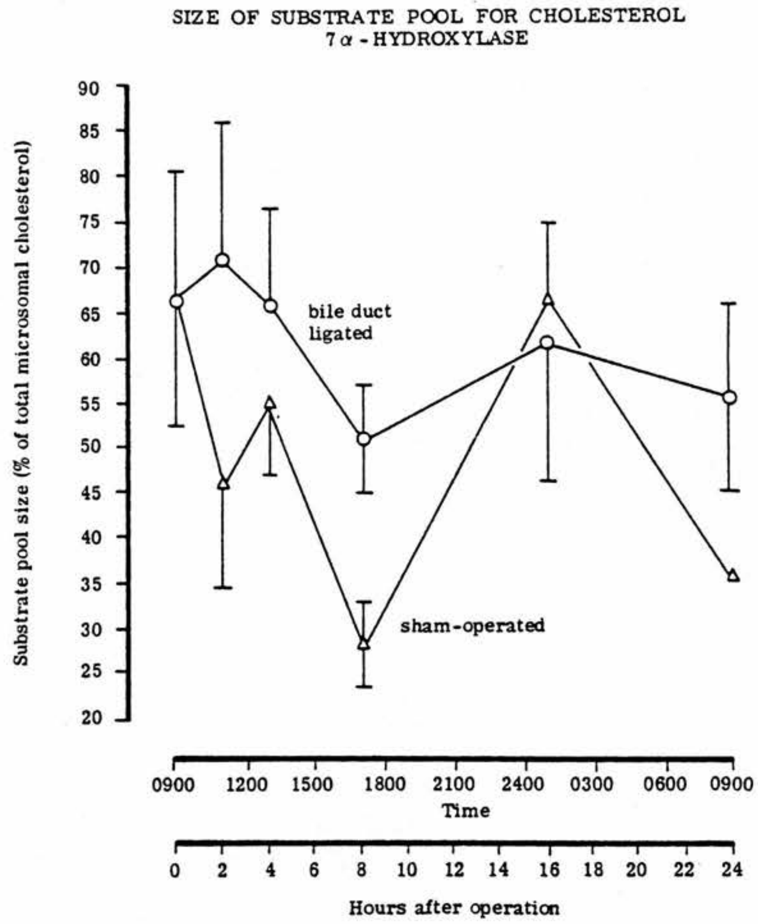
(Mean of 5 assays ± S.E.M.)

Figure 6.6(a)



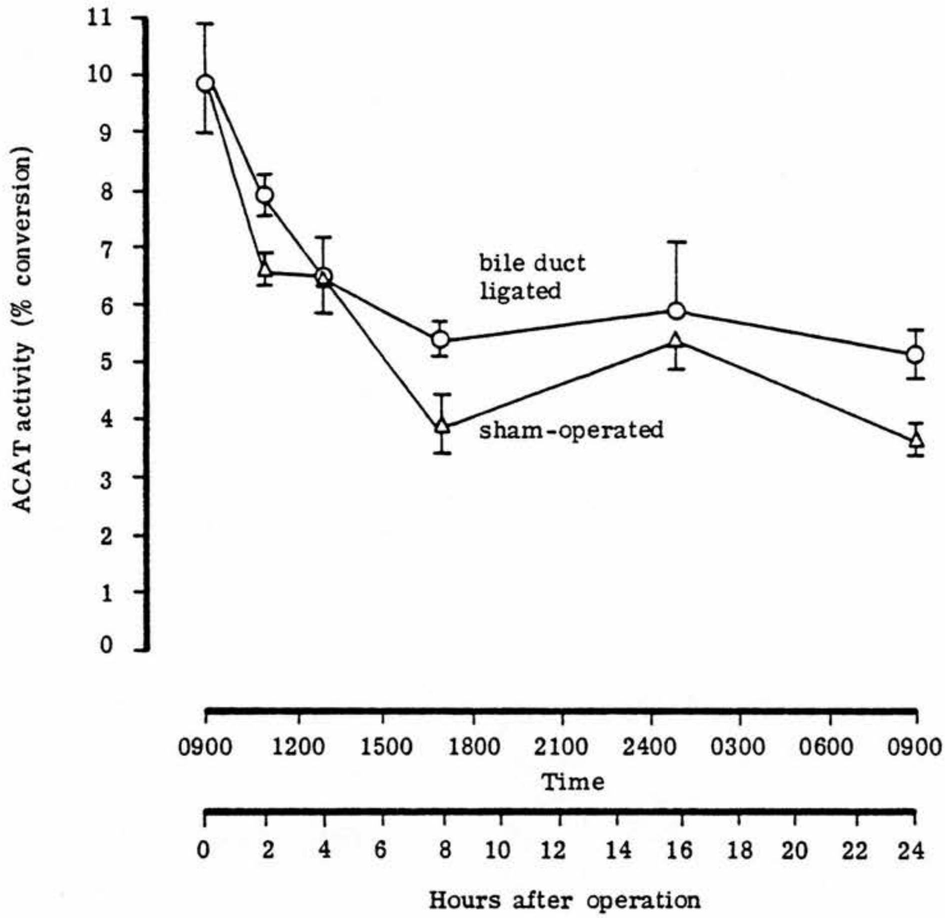
(Mean of 5 assays ± S.E.M.)

Figure 6.6(b)



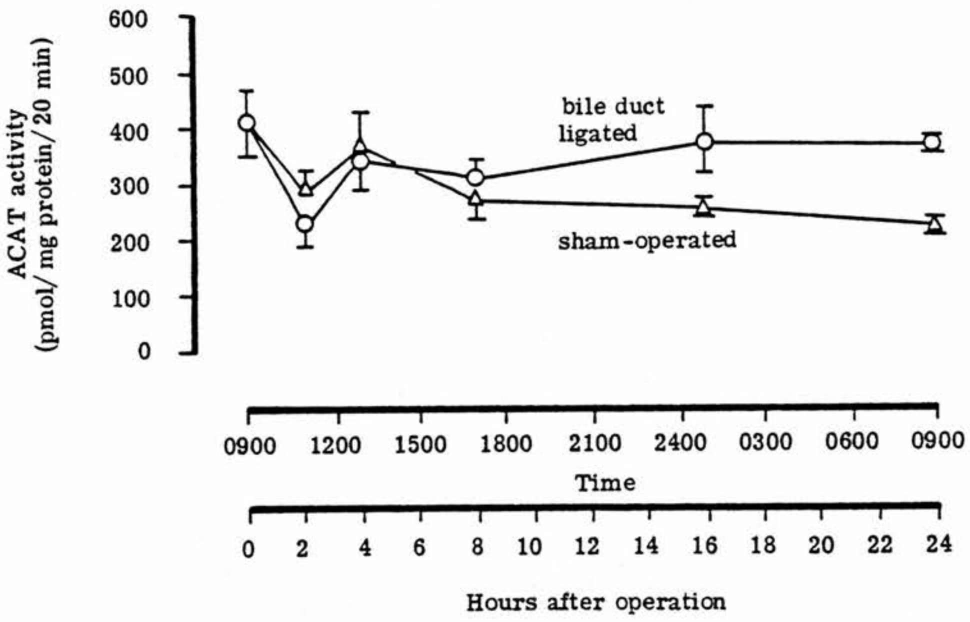
(Mean of 5 assays ± S.E.M.)

Figure 6.7(a)



(Mean of 5 assays ± S.E.M.)

Figure 6.7(b)



(Mean of 5 assays ± S.E.M.)

cholesterol ester declined steadily during the first 8 hours of the experiment and thereafter remained approximately constant (Figure 6.7(a)). There was no difference in activity between animals with occluded bile ducts and sham operated controls.

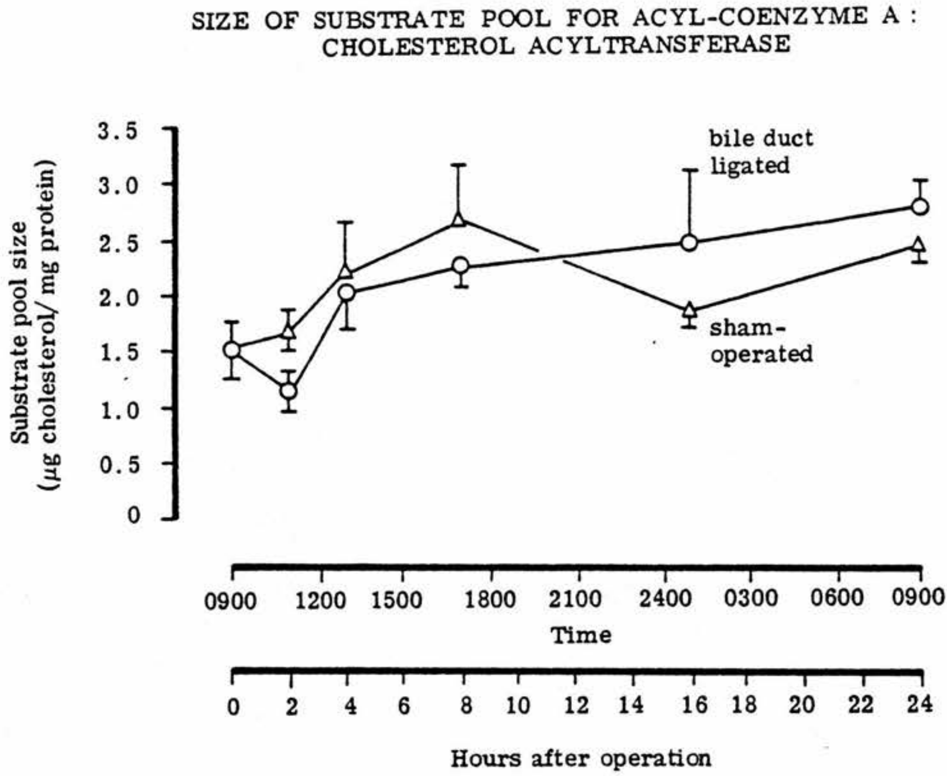
When acyl-coenzyme A:cholesterol acyltransferase was assayed using [1-^{14}C]-oleic acid there was little change in activity detectable (Figure 6.7(b)). After 24 hours the enzyme activity of microsomes from ligated animals was greater than the controls ($P<0.01$), although neither of these activities was significantly different from that measured at the beginning of the experiment.

Figure 6.8 shows the values derived for the maximum size of the pool of substrate for acyl-coenzyme A:cholesterol acyltransferase, both in absolute terms, and as a fraction of the total microsomal free cholesterol. In neither case was there a significant difference between the substrate pool size of ligated and sham operated rats. There was a suggestion that substrate pool size increased somewhat with time, both in actual size and relative to microsomal free cholesterol levels. However, in no instance did this increase quite approach significance. (For the difference between values at the beginning of the experiment and after 24 hr, $0.05<P<0.1$.)

6.4 The response of the activities of acyl-coenzyme A:cholesterol acyltransferase and cholesterol 7α-hydroxylase to feeding cholesterol-rich diets

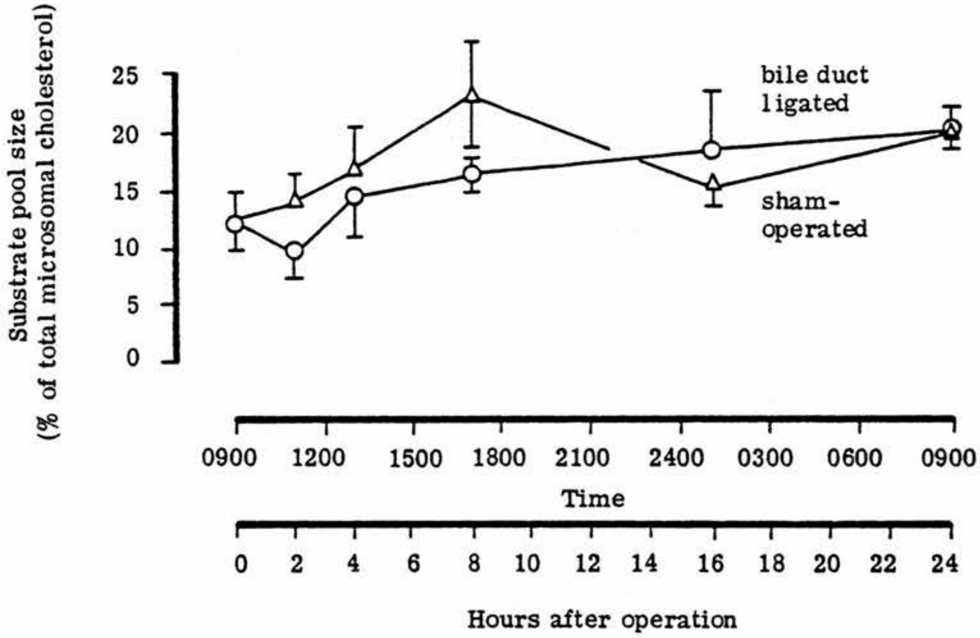
Three experiments were performed. In the first one the response of both enzymes to feeding and then withdrawing a cholesterol-rich diet was investigated. Aspects of the response of each enzyme were investigated in more detail in the subsequent two experiments. (For details of diets, see Materials and Methods.)

Figure 6.8(a)



(Mean of 5 assays ± S.E.M.)

Figure 6.8(b)



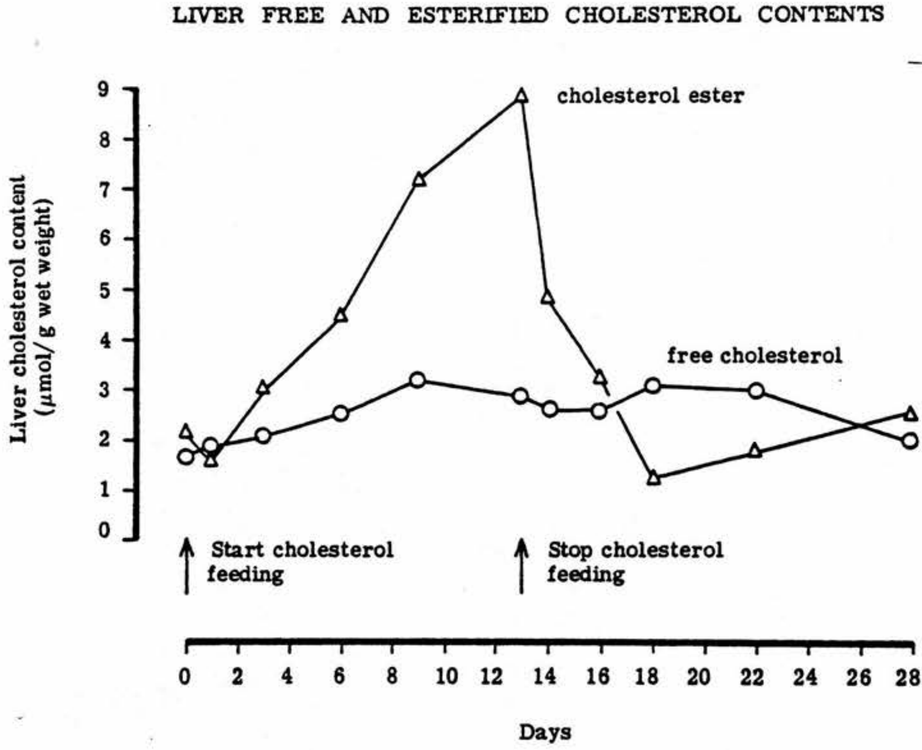
(Mean of 5 assays ± S.E.M.)

(a) The hepatic effects of feeding and withdrawing a cholesterol-rich diet

Rats were fed for one week on a 10% olive oil diet. On Day 0 of the experiment the diet was changed to 1% cholesterol, 10% olive oil, and the animals were maintained on this diet until Day 13, when they reverted to 10% olive oil. On the days shown in the diagrams pairs of rats were killed. Samples of liver were homogenised in a small volume of water and freeze-dried. Lipids were extracted from the freeze-dried powder, using an aliquot of [4-^{14}C]-cholesterol to assess recovery, and free and total cholesterol were assayed. The rest of the liver was used for the preparation of microsomes. These were assayed for cholesterol 7α-hydroxylase and acyl-coenzyme A:cholesterol acyltransferase, and samples were extracted as above for the assay of free and total cholesterol.

The effect of cholesterol feeding on whole liver free and esterified cholesterol contents is shown in Figure 6.9. Free cholesterol levels increased moderately from about 2.1 μmol per g liver and appeared to reach a new plateau value of about 3.1 μmol per g liver after 9 days of cholesterol feeding. On withdrawing the cholesterol-containing diet there was no fall in free cholesterol for at least 9 days, after which free cholesterol returned to approximately the starting level within the following 6 days. On cholesterol feeding the cholesterol ester content increased rapidly from its starting value of about 1.55 μmol per g liver, and had reached 8.8 μmol per g liver and was still apparently increasing when cholesterol feeding ceased. The decline in cholesterol ester on removing the cholesterol-rich diet was similarly rapid, with a drop from 8.8 to 4.9 μmol per g liver within one day, and a return to the basal starting level within five days. There was a subsequent small increase in hepatic cholesterol ester that was maintained over the following ten days.

Figure 6.9



Similar results were obtained for the microsomal contents of free and esterified cholesterol (Figure 6.10). There was a relatively small (15%) increase in free cholesterol that was probably non-significant. After an initial drop on withdrawing the cholesterol-containing diet there was a small but steady increase in microsomal free cholesterol for the following eight days. There was no alteration in microsomal cholesterol ester content from basal levels of 5.2 nmol per mg protein for three days following the change to the cholesterol-rich diet, but there was then a large (six-fold) increase up to 38.8 nmol per mg protein. On returning to the cholesterol-free diet cholesterol ester rapidly fell to low levels.

On the basis of a typical microsomal phospholipid content of 0.52 μmoles per mg protein it is possible to estimate that the microsomal cholesterol ester to phospholipid ratio increased from 1:101 to 1:13.4. Studies with lipid model systems containing phospholipid, cholesterol and cholesterol ester, but no protein, have shown that cholesterol ester can pack into phospholipid layers at molar ratios of cholesterol ester to phospholipid of from 1:50 to 1:20 (phospholipid bilayers of varying degrees of hydration (359)) or 1:20 (unilamellar liposomes (341)). It is thus possible that cholesterol feeding has resulted in microsomal cholesterol ester levels in excess of those that might be accommodated within the microsomal membrane. The location of this cholesterol ester and its spatial and temporal relation to the cholesterol ester secreted in lipoproteins and accumulated as cytoplasmic lipid droplets is not yet clear.

Figure 6.11 shows the activities of cholesterol 7α-hydroxylase and acyl-coenzyme A:cholesterol acyltransferase throughout this experiment. There was no statistically significant change in cholesterol 7α-hydroxylase activity as measured here by percent conversion of exogenous

Figure 6.10

MICROSOMAL FREE AND ESTERIFIED CHOLESTEROL CONTENTS

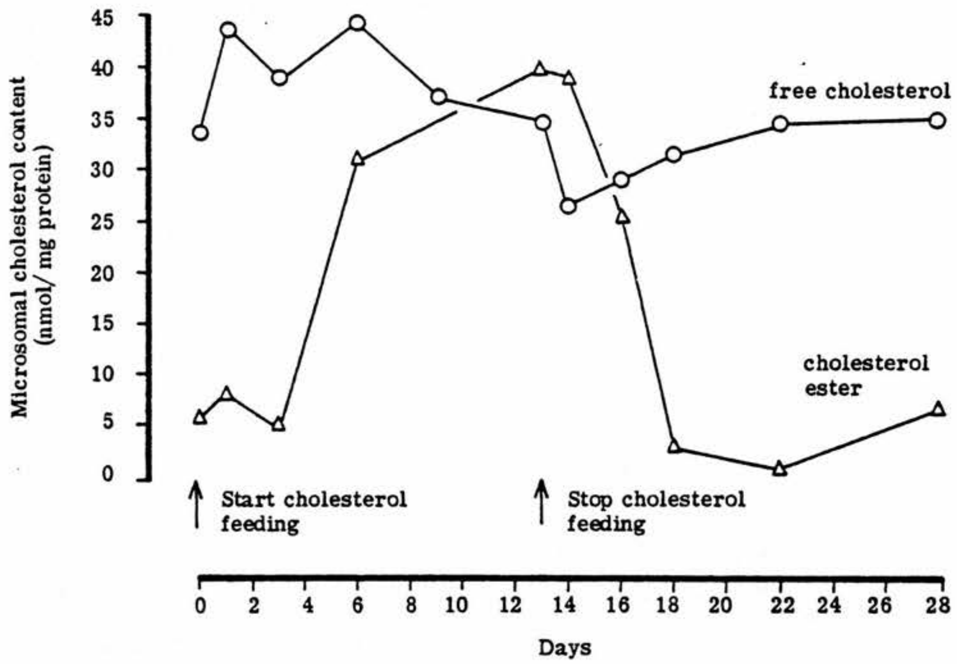
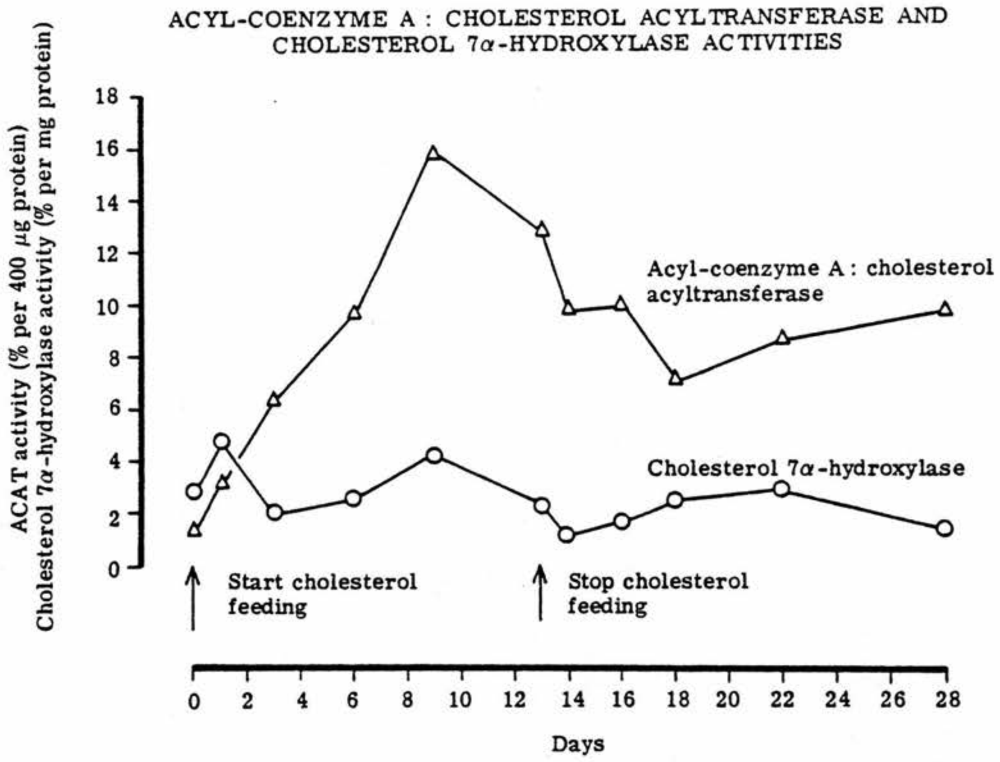


Figure 6.11



labelled cholesterol to 7α-hydroxycholesterol (see also Section 6.4(c)). In contrast the activity of acyl-coenzyme A:cholesterol acyltransferase increased from a starting level of less than 2% conversion of labelled cholesterol to cholesterol ester per 400 mg protein per hour to a maximum value of more than 15% conversion after nine days of cholesterol feeding. This activity fell slightly to 12.6% by the last day of cholesterol feeding (Day 13). The fall in activity on withdrawing the cholesterol-rich diet was relatively small and slow compared with the observed increase, and resulted in an activity of 10% after one day and 7% after five days. However, after this time there was a small rise in activity for the rest of the experiment with a final activity of 10% being measured.

Thus, although acyl-coenzyme A:cholesterol acyltransferase activity was observed to increase on feeding the 1% cholesterol 10% olive oil diet, the activity did not return equally rapidly to basal levels on returning to the 10% olive oil diet. Various explanations were considered for this. It is possible that during the feeding of the cholesterol-containing diet there had been some cholesterol deposition in peripheral tissues. On withdrawing the diet the rapid loss of cholesterol from the liver might have been followed by a slower return of this peripheral cholesterol to the liver and its subsequent excretion as neutral sterols or bile acids. However, the evidence is that cholesterol feeding in the rat results in little or no increase in carcass cholesterol and only a slight increase in serum cholesterol and that the only organ showing a large increase in cholesterol content is the liver (148).

Alternatively, failure of acyl-coenzyme A:cholesterol acyltransferase to return to basal levels on removing the cholesterol-rich diet may be related to the use of the 10% olive oil control diet. This possibility was further investigated (See Section 6.4(b)).

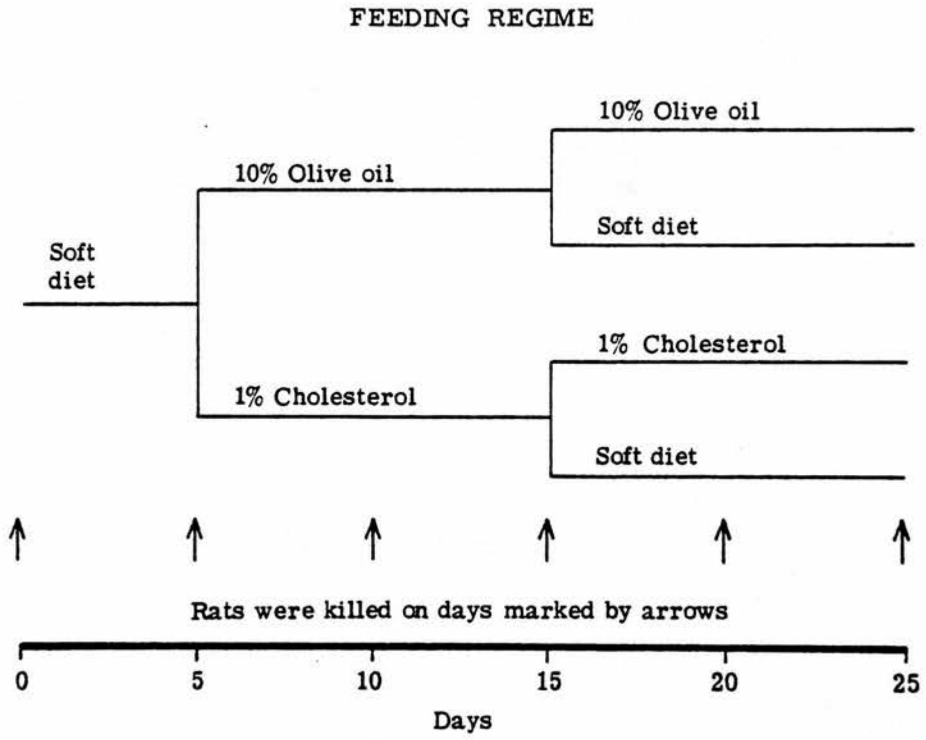
The possibility had been considered that on cholesterol feeding and withdrawal the observed changes in the level of cholesterol ester might be a result not just of changes in the supply of substrate for esterification and the activity of acyl-coenzyme A:cholesterol acyltransferase, but also of concomitant changes in the activity of cholesterol ester hydrolase. In particular, the rapid decline in hepatic cholesterol ester content might have been associated with an increase in the activity of this enzyme. On every occasion that animals were killed during the course of this experiment samples of microsomes and cytoplasm were assayed for cholesterol ester hydrolase activity by Dr. A.M.S. Gorban of this laboratory. These assays showed no significant change in activity.

(b) The effect of diet manipulation on hepatic cholesterol content and acyl-coenzyme A:cholesterol acyltransferase activity

Rats were fed for one week on soft diet before the first group were killed. After this first day of the experiment the remaining rats were maintained on soft diet for a further five days. The animals were then divided into two groups, one of which received soft diet supplemented with 10% olive oil, the other being given soft diet supplemented with 10% olive oil and 1% cholesterol. After ten days these groups were each further subdivided into two groups one of which continued to eat either the olive oil diet or the cholesterol plus olive oil diet, as appropriate, the other reverting to soft diet. This feeding regime is schematically represented in Figure 6.12.

At each point indicated in the figures three rats were killed, their livers were pooled and homogenised, and microsomes were prepared. Lipid was extracted from duplicate samples of liver and microsomes, and each lipid extract was assayed for total and free cholesterol in triplicate. Microsomes were assayed for protein and acyl-coenzyme A:

Figure 6.12



cholesterol acyltransferase activity. Each reported enzyme activity is the mean of five assays.

The hepatic and microsomal free and esterified cholesterol contents are shown in Figures 6.13 and 6.14. Because of the large number of points error bars are not included, to avoid confusion, but, typically, standard errors of the mean amounted to some 15% of the mean value. Cholesterol feeding resulted in liver and microsomal free cholesterol contents that were consistently greater than those of the olive oil-fed rats, but the significance of this difference never exceeded $P<0.1$.

Cholesterol feeding resulted in a slow but steady increase in whole liver free cholesterol. This increase was paralleled by a similar rise from the fifth day onwards of olive oil feeding. Similar trends were observed for microsomal free cholesterol.

The whole liver free cholesterol content of olive oil fed rats showed little change when these rats reverted to eating the soft diet. When the cholesterol-fed rats reverted to the soft diet the mean values for whole liver free cholesterol declined to values similar to those for rats fed no cholesterol, but again the drop was not statistically significant. There was no change in the hepatic microsomal free cholesterol level within the first five days of olive oil- or cholesterol-fed animals reverting to the soft diet, but in the second five days there was an apparent increase. This increase was confirmed by repeated cholesterol assays on the same lipid extracts, but never attained statistical significance.

The results of assays for esterified cholesterol were much more clear cut. Feeding cholesterol resulted in an accumulation of cholesterol ester. This seems to reach a plateau value of 38.8-46.5 nmol/mg protein in microsomes, but accumulation continued for the duration of cholesterol feeding for whole liver. When cholesterol-fed animals reverted to

Figure 6.13(a)

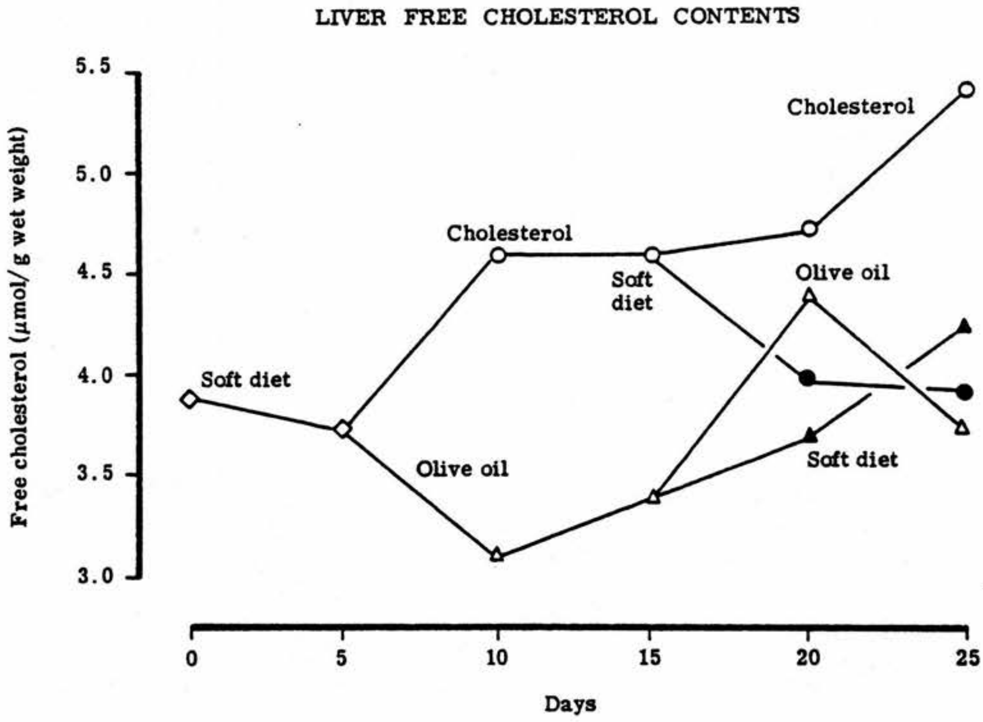


Figure 6.13(b)

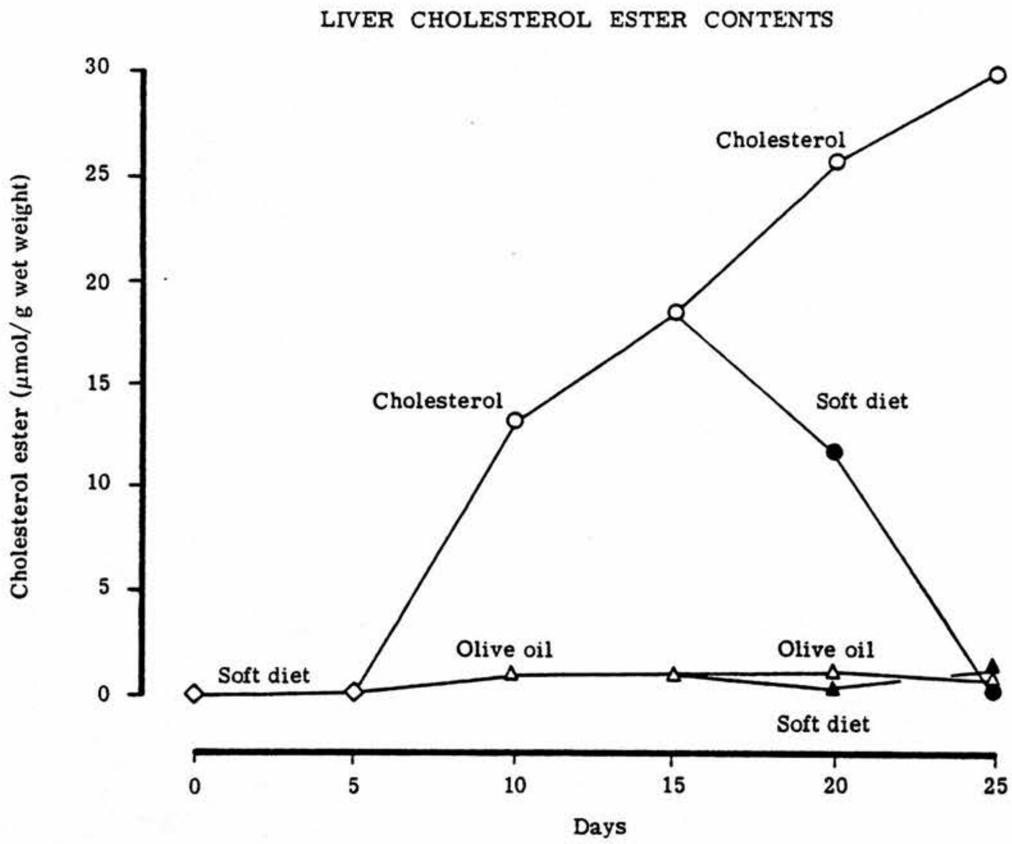


Figure 6.14(a)

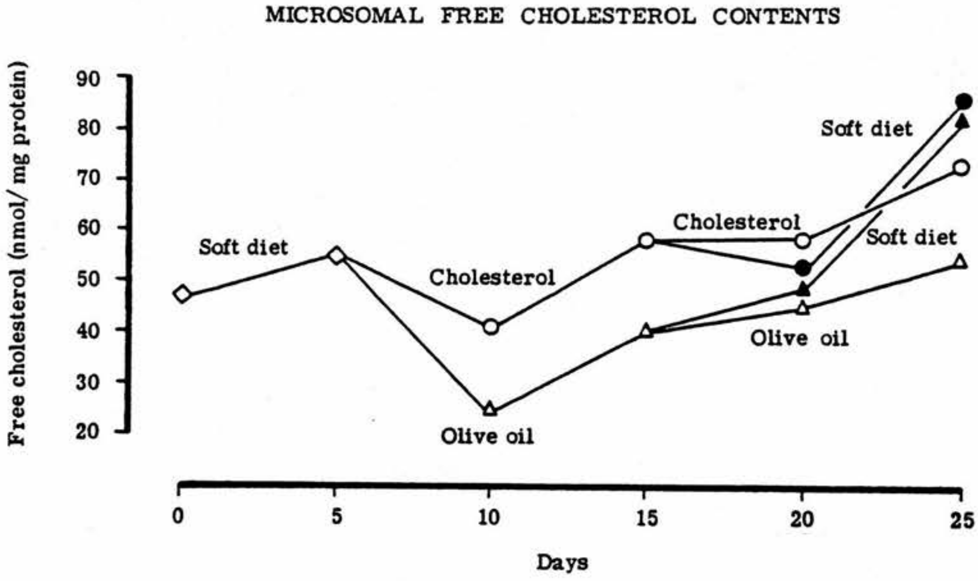
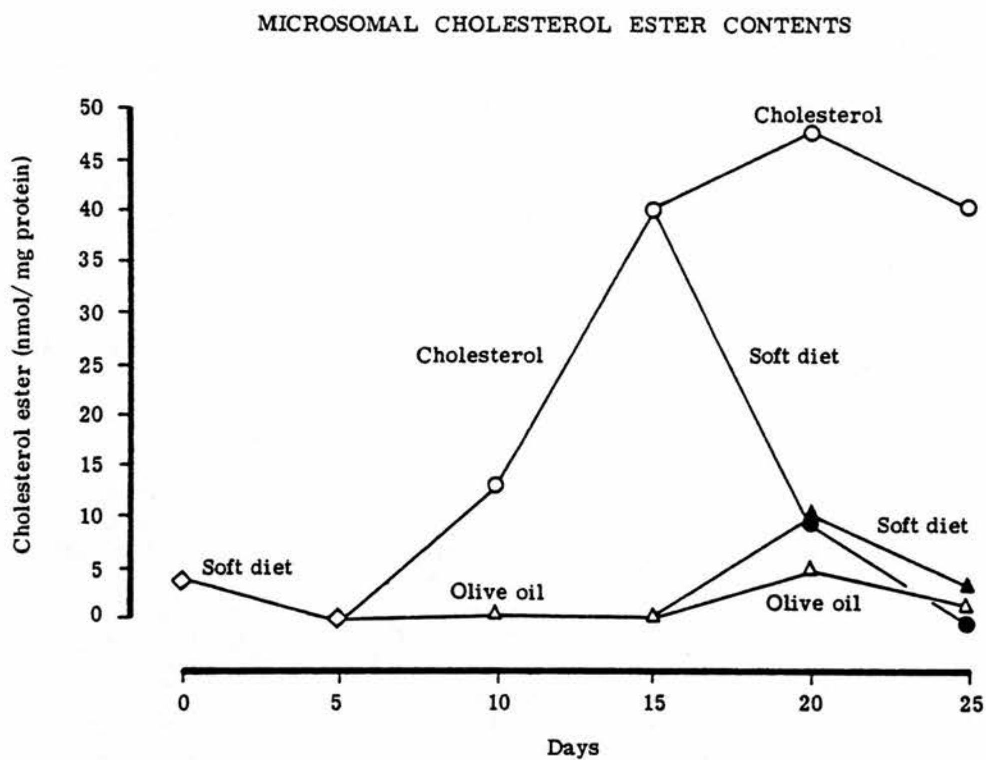


Figure 6.14(b)

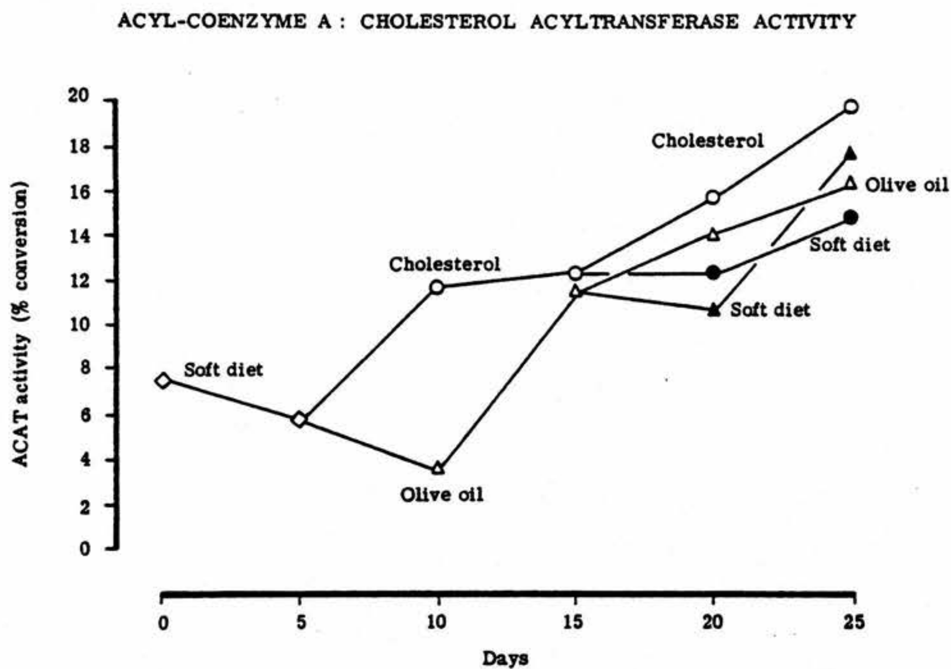


eating the soft diet, cholesterol ester levels rapidly returned to control values. Little or no cholesterol ester accumulated on olive oil feeding.

The results of acyl-coenzyme A:cholesterol acyltransferase assays are shown in Figure 6.15. Again, to avoid confusion, error bars are not shown. (The average of the standard deviations for all assays was $\pm$ 1.8% conversion of exogenous labelled free cholesterol to cholesterol ester.) This experiment again demonstrated that feeding the 1% cholesterol 10% olive oil diet resulted in an increase in the measured acyl-coenzyme A:cholesterol acyltransferase activity. However, feeding 10% olive oil also resulted in increased values for esterification activity, there being no statistically significant difference between the activities of microsomes from oil-fed and cholesterol-plus-oil-fed animals at ten and fifteen days of feeding. However, a lag in the increase of acyl-coenzyme A:cholesterol acyltransferase activity on olive oil feeding resulted in a significant rise in activity of cholesterol-fed animals over olive oil-fed animals at five days of feeding ($P<0.0025$). The difference between activities at twenty days feeding was also significant ($P<0.01$).

No decrease in esterification activity was detected when animals reverted to the soft diet. However, there was no further significant increase of activity in animals that had been previously fed on cholesterol, with a resultant significant difference between their activity after ten days and that of animals that continued to eat the cholesterol diet ($P<0.005$). On the other hand, when olive oil-fed animals reverted to eating soft diet, there was a significant difference between their esterification activity and that of animals that continued on the 10% olive oil diet at five days ($P<0.01$), but not at ten days.

Figure 6.15



In summary it thus appears that feeding cholesterol plus olive oil resulted in an increase in cholesterol esterification activity, but that this increase was only greater than that produced by feeding olive oil alone at certain stages. One of these stages was defined by the lag before any increase was detectable on feeding olive oil. On reverting to low fat, low cholesterol diets there was no immediate reduction in the ability of liver microsomes to esterify cholesterol.

The reasons for this stimulation of measured esterification activity on olive oil feeding, and for the maintenance of high levels of activity on the removal of the dietary stimuli initially causing them, are unclear.

The failure of the liver to accumulate cholesterol ester under these circumstances presumably is at least partly explained by the lack of substrate cholesterol in the quantities available in cholesterol-fed animals.

It has been shown that feeding high fat diets to rats results in a stimulation of hepatic cholesterol synthesis (360,361). It is possible that this will result in a perturbation of the relative sizes of microsomal cholesterol pools and/or of the partitioning of exogenous labelled cholesterol between these pools. This will influence the activity of acyl-coenzyme A:cholesterol acyltransferase as measured by esterification of exogenous labelled cholesterol. A comparison of results obtained as in this experiment with results obtained using a more sophisticated assay, which measured actual cholesterol ester production, might therefore help to explain some of these problems by enabling an estimate of maximal substrate pool size to be made.

Alternatively, there is some conflicting evidence for the role of the cytoplasm in hepatic cholesterol esterification, with some reports of a stimulation of microsomal cholesterol esterification on the inclusion

of cytoplasmic protein in the assay incubations (362,363), and some reports claiming no such stimulatory effect (248), ~~and some reports claiming no such stimulatory effect (248)~~. Similarly, work with rabbit aorta preparations has failed to demonstrate the presence of any cytoplasmic inhibitory or stimulatory factors from normal or atherosclerotic animals (332). It is thus possible that, if some cytoplasmic factor is important for enzyme activity, by assaying microsomes alone, in common with most other workers in this field, a true assay of the ability of the liver to catalyse cholesterol esterification is not being obtained.

Finally, the modification of microsomal membrane fatty acid composition *in vitro* with rat liver microsomes (364), or *in vivo* with Ehrlich ascites microsomes (365) has been shown to influence acyl-coenzyme A:cholesterol acyltransferase activity. It is not known what influence the olive oil diet had on the microsomes prepared from the livers of rats fed on it, but it is possible that part of the change in esterification activity may have been caused by some change in microsomal phospholipid fatty acid composition.

(c) The effect of cholesterol feeding on cholesterol 7α-hydroxylase

There are ambiguities inherent in measuring cholesterol 7α-hydroxylase activity in terms of conversion of exogenous labelled cholesterol under circumstances when it is likely that the total microsomal cholesterol content or the size of the cholesterol pool accessible to the enzyme is changing. Because of the reported confusion over the effect of cholesterol feeding on the activity of this enzyme an experiment was undertaken to measure cholesterol 7α-hydroxylase, both by fractional conversion of tracer cholesterol and by absolute measurement of 7α-hydroxy-cholesterol production, in the days following a change to a cholesterol-rich diet.

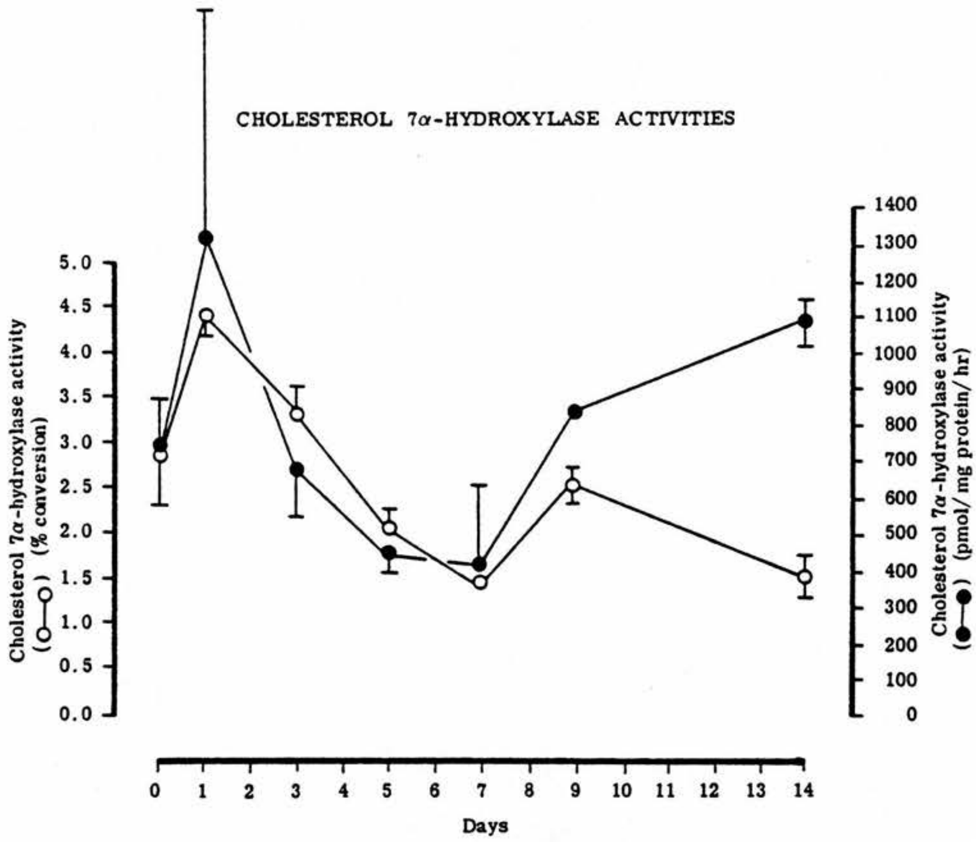
Rats were fed for ten days on the 10% olive oil diet. At the beginning of the experiment the diet was changed to 10% olive oil, 1% cholesterol. On each day indicated in the Figures three rats were killed, their livers were pooled, and microsomes were prepared. Microsomal suspensions were assayed for protein. Triplicate incubations were performed for the measurement of cholesterol 7α-hydroxylase both in percentage and absolute terms. The gas chromatography traces obtained during the absolute measurement of 7α-hydroxycholesterol production allowed calculation of the microsomal cholesterol contents.

Mean values for cholesterol 7α-hydroxylase activities, measured by both assays, indicated the possibility of a rise in activity after one day with a subsequent steady decline over the following week (Figure 6.16), a result similar to that shown in Figure 6.11. However, as in that experiment, none of the assay results showed a significant difference from that obtained before cholesterol feeding began.

A drop in microsomal cholesterol content after one day of cholesterol feeding just attained significance ($P<0.05$) (Figure 6.17). This result is unexpected, and possibly needs to be explained in terms of some physiological response not controlled in the experiment. (An example might be that the rats detected the substitution of the cholesterol-containing diet for the control diet and had not yet adapted to this change and were thus partially starved.) After fourteen days of cholesterol feeding, microsomal cholesterol levels were significantly greater than at the beginning of the experiment ($P<0.05$).

The maximal size of the substrate pool for cholesterol 7α-hydroxylase was calculated, and its variation with cholesterol feeding is shown in Figure 6.18. The substrate pool size, both in terms of actual size and as a fraction of total microsomal free cholesterol, after an initial delay, showed a steady increase to reach a size significantly greater than that on day 0 ($P<0.05$).

Figure 6.16



(Mean of 3 assays ± S.E.M.)

Figure 6.17

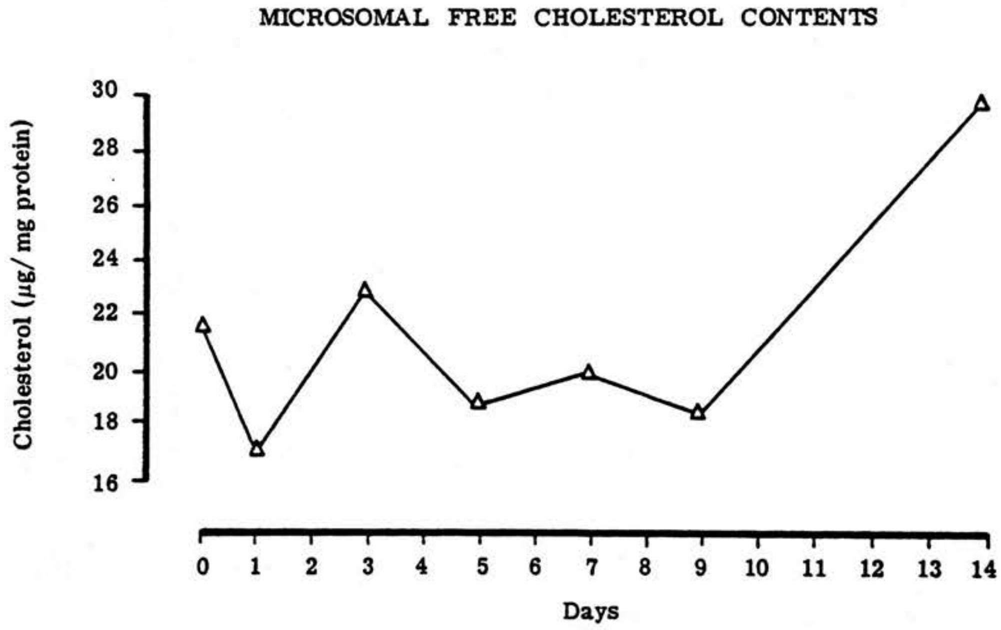
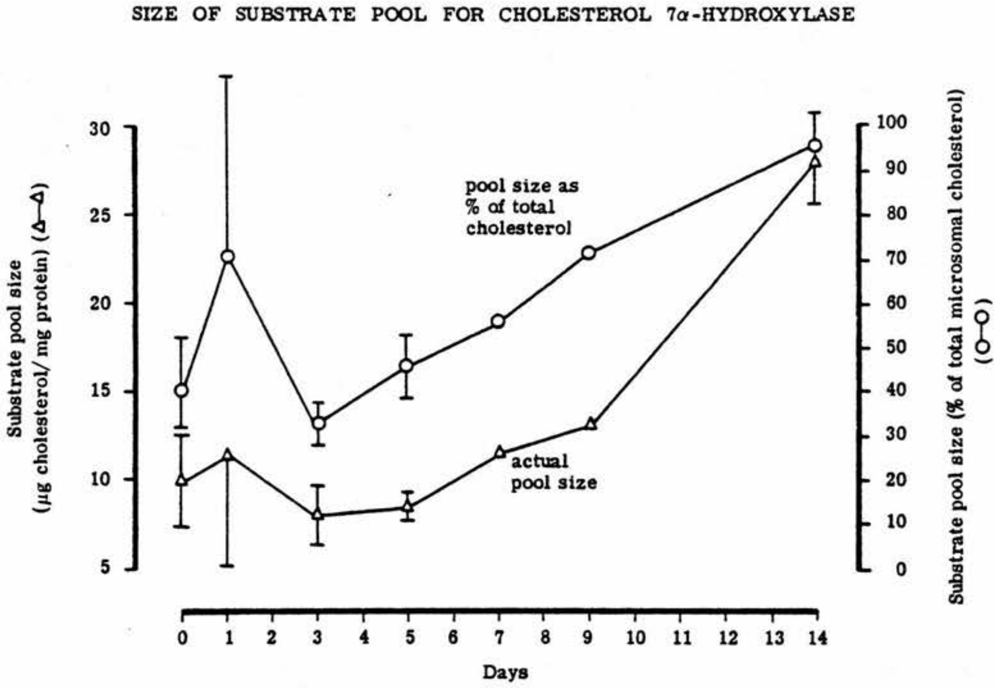


Figure 6.18



(Mean of 3 assays ± S.E.M.)

Section 7

The Response of Cholesterol 7α-Hydroxylase and Acyl-Coenzyme A:Cholesterol Acyltransferase to In Vitro Alterations of Cholesterol Concentration

7.1 Introduction

Increased delivery of cholesterol to rat liver is associated with a decrease in microsomal 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase activity (129,159,160), with an increase in microsomal acyl-coenzyme A: cholesterol acyltransferase activity (252,331,349-351), and with an increase in bile acid synthesis (172,173), although the effect on the microsomal rate-limiting enzyme in this latter process, cholesterol 7α-hydroxylase, is not clear (147,161,174-176, and see Sections 6 and 8). The possibility presumably exists that these effects could all be at least in part mediated at the level of protein synthesis, by the induction of, or repression of the production of, the enzyme or some protein or non-protein cofactor.

However, in the case of 3-hydroxy-3-methylglutaryl-coenzyme A reductase there is evidence that the phase of inhibition due to the repression of enzyme synthesis is preceded by a phase of inhibition that is independent of protein synthesis or its inhibition (170). Since this phase is not accompanied by a change in the K_m of the enzyme (366), it has been suggested that this effect is mediated not by a classical allosteric inhibition but via a decrease in the fluidity of the endoplasmic reticulum membrane caused by an increase in its free cholesterol content (168,169,350). Such an effect of membrane lipid composition on the activity and the control of a number of other membrane-bound enzymes has been well documented (209-211). (Without eliminating the possibility that such a mechanism may indeed operate, it should however be noted that there has been increasing evidence for the acute control of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity by a mechanism involving phosphorylation and dephosphorylation (162-167). In addition, the half-life of the enzyme is sufficiently short (2-4 hours) (366) that quite rapid and fine control could be exerted at the level of protein synthesis (166).)

In the case of another liver microsomal enzyme, the cytochrome P-450-dependent drug hydroxylation system, the critical effect of membrane fluidity on substrate binding (217,296,367), on the interaction of the cytochrome P-450 species with NADPH-cytochrome c reductase, (213,218,368, 369), and on overall enzyme activity (214-216,218-221) is recognised, as is the influence of membrane components on the cytochrome spin state (370). It is possible that the cytochrome P-450-dependent cholesterol 7α-hydroxylase reaction may display a similar sensitivity to membrane fluidity and that this may mediate any effects of increased cholesterol concentration on the enzyme. A similar mechanism may operate for acyl-coenzyme A:cholesterol acyltransferase.

In addition to this type of mechanism it is possible that neither of these enzymes is saturated with substrate at the cholesterol to phospholipid ratios encountered in physiological membranes. It has been shown that when cholesterol is added to liver microsomes the estimated substrate pool size (250), and the production of 7α-hydroxycholesterol (174,250) both increase, but in these studies cholesterol was added by means of organic solvents or detergents. These vehicles are unlikely to mimic physiological means of cholesterol delivery to the endoplasmic reticulum. It is, indeed, plausible that the vehicle used for cholesterol addition, being either lipophilic or surface active, may have a perturbing effect on the disposition of cholesterol between different microsomal compartments.

However, these studies indicate that the size of the substrate pool appears to be too small to saturate the enzyme, and saturation was not achieved even at the highest amounts of exogenous cholesterol used (174,250). Similar results have been demonstrated for the acyl-coenzyme A: cholesterol acyltransferase of normal and atherosclerotic aortas (254) of Ehrlich ascites cells (344) and of Fu5AH rat hepatoma cells (333).

It is important once more to emphasise that the lipids in both model and biological membranes (193,208), and in particular in the membrane of the endoplasmic reticulum (194-196), do not exist in a homogeneous state. It has repreatedly been shown that not all the microsomal cholesterol acts as substrate for cholesterol 7α-hydroxylase (174,242,250,275,371) and there is evidence that this is also true for acyl-coenzyme A:cholesterol acyltransferase (252,254). In addition, it appears that the composition and structure of the membrane phospholipids in the vicinity of cytochrome P-450 may differ from the remainder of the endoplasmic reticulum (217,372).

This has various consequences. In particular, a change in overall microsomal membrane properties need not necessarily be reflected by a similar change in the vicinity of a particular microsomal enzyme, and, conversely, the absence of a detectable change in the microsomal fraction does not rule out the possibility of local changes around a particular enzyme. Also, since cholesterol 7α-hydroxylase and acyl-coenzyme A: cholesterol acyltransferase are located in different microsomal subfractions, even if both enzymes were found to respond in a similar way to local increases in membrane cholesterol levels, they need not respond in a similar way to an increase in cholesterol supply to the endoplasmic reticulum from any one particular source.

7.2 The effect of the recombination of acetone-extracted microsomes with microsomal lipid liposomes on acyl-coenzyme A:cholesterol acyltransferase activity

This experiment was simultaneously performed with rats fed on soft diet and with rats fed on cholesterol-rich diet. Three rats from each group were killed and liver microsomes were prepared and freeze-dried. Aliquots of these freeze-dried powders (200 mg) were homogenised in 20 ml acetone at -20°C, and filtered rapidly on apparatus chilled to -20°C, with the use of a further 100 ml acetone. After one hour in the vacuum desiccator the acetone-extracted powders were stored at -20°C. The yield

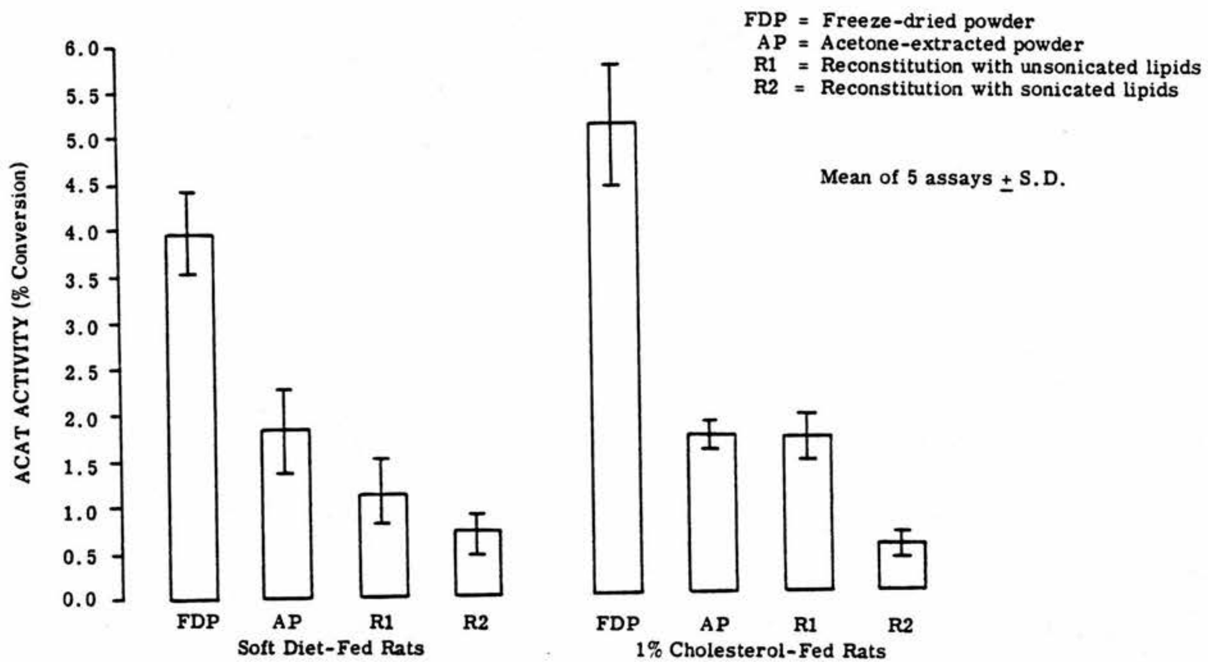
of each acetone powder was approximately 150 mg. The acetone filtrate from each powder was taken to dryness on the rotary evaporator, dissolved in chloroform, and divided into six equal aliquots. One of these aliquots was used to prepare multilamellar liposomes and one to prepare unilamellar liposomes, using 5 ml 0.1 M potassium phosphate buffer, pH 7.1 (see Section 2.14).

At the end of the liposomal preparation, 20 mg acetone powder was added to each liposome suspension. After thorough vortexing the resultant mixture was incubated in a shaking water-bath at 37°C for 45 minutes. Controls were prepared by similarly suspending and incubating 20 mg of freeze-dried powder and acetone powder in 5 ml buffer. All samples were assayed for protein and acyl-coenzyme A:cholesterol acyltransferase activity (Figure 7.1).

The freeze-dried microsomes retained cholesterol esterificaction activity, and those prepared from cholesterol-fed animals had a greater activity than those from soft diet-fed animals ($P<0.0125$). Acetone extraction resulted in a fall in activity to 46% (soft diet-fed rats) and 34% (cholesterol-fed rats) of the activities of the freeze-dried microsomes. These activities were, however, still readily measurable, and were greater than those obtained on butanol extraction (see Section 4.5, and Figure 4.6). There was no longer any difference in activity between preparations from cholesterol-fed and control rats.

Recombining the acetone-extracted microsomal protein with the microsomal lipid failed to restore the measured activity to the levels observed for freeze-dried microsomes. When multilamellar liposomes were used, there was a decrease observed in the activity of preparations from soft diet-fed rats ($P<0.05$), and no change in that of preparations from cholesterol-fed rats. The use of unilamellar liposomes gave activities that were in both cases significantly lower than the activities recorded with multilamellar liposomes ($P<0.05$ and $P<0.0005$ for soft diet- and cholesterol-fed rats, respectively).

Figure 7.1



The observation that the unilamellar liposome preparations gave lower activities than the multilamellar preparations suggests that a major problem for the interpretation of this experiment is posed by the creation of additional compartments into which the exogenous labelled cholesterol used for the assay can partition. Thus, although the multilamellar and unilamellar liposomes were made from identical amounts of lipid, the size of the lipid compartments immediately accessible to the cholesterol label will be related to the amount of lipid in the outermost layer of the liposomes. This will amount to essentially all the lipid for the unilamellar liposomes, but only a fraction of it for the multilamellar liposomes. Thus, in the systems reconstituted using unilamellar liposomes the compartment into which the cholesterol label can rapidly partition is larger than in the systems using multilamellar liposomes. This will result in lower measured activities whether or not all of this compartment is accessible to the enzyme, since there is the possibility both of greater dilution of the tracer substrate, and of a proportion of this substrate being sequestered away from the enzyme.

This suggestion will adequately explain the measured activity of the preparations using unilamellar liposomes being lower than those using multilamellar liposomes, and also means that the lack of observed enhancement of activities to levels greater than those of the acetone powders may be an artefact of the assay method.

However, despite these difficulties, the following results remain. The difference in activities of the preparations from the two groups of rats was abolished on lipid extraction. The addition of microsomal lipids, as multilamellar liposomes, back to delipidated microsomal protein resulted in no decrease in activity for preparations from cholesterol-fed animals although there was a decrease in activity for preparations from soft diet-fed animals. These observations tend to suggest that at least some changes in the activity of acyl-coenzyme A:cholesterol acyltransferase might be due to changes in the lipid fraction of microsomes (possibly their cholesterol content) rather than to changes in the amount of protein.

7.3 The effect of the recombination of acetone-extracted microsomes with microsomal lipid liposomes supplemented with varying amounts of cholesterol on acyl-coenzyme A:cholesterol acyltransferase activity

Freeze-dried liver microsomes were prepared from three rats fed the cholesterol-rich diet for six weeks. An amount of this freeze-dried powder equivalent to 20 g liver was extracted with acetone as described in Section 7.2. Aliquots of the acetone filtrate equivalent to 2 g liver, supplemented with 0, 250, 500 or 1000 μg cholesterol, were taken to dryness and used in the preparation of unilamellar and multilamellar liposomes in 1.0 ml 0.1 M Tris-chloride buffer, 0.15 M KCl, pH 7.4 at 4°C.

Freeze-dried microsomes and acetone-extracted microsomes were suspended at a concentration such that 1.0 ml contained microsomal protein derived from 2.0 g liver. When the preparation of the liposomes was complete, 1.0 ml of the acetone powder suspension was added to each sample of liposomes. Controls were prepared by the addition of 1.0 ml of the freeze-dried and acetone powder suspensions to 1.0 ml buffer. Thus, all preparations now contained protein equivalent to 2 g liver, and all liposome-containing preparations contained lipid equivalent to 2 g liver with or without additional cholesterol, in a total of 2 ml. All preparations were shaken for 2½ hours at 4°C, transferred to centrifuge tubes with the help of an additional 5 ml buffer, and centrifuged at 105 000 x g for 1 hr.

The resultant pellets were resuspended and assayed for protein and acyl-coenzyme A:cholesterol acyltransferase activity. This assay was performed using [1-^{14}C]-oleic acid as tracer. Samples were taken for organic extraction and measurement of cholesterol and phospholipid contents (Figure 7.2 and Table 7.1).

It should be noted that after the very long exposure to the cholesterol-rich diet both the activity of acyl-coenzyme A:cholesterol

Figure 7.2

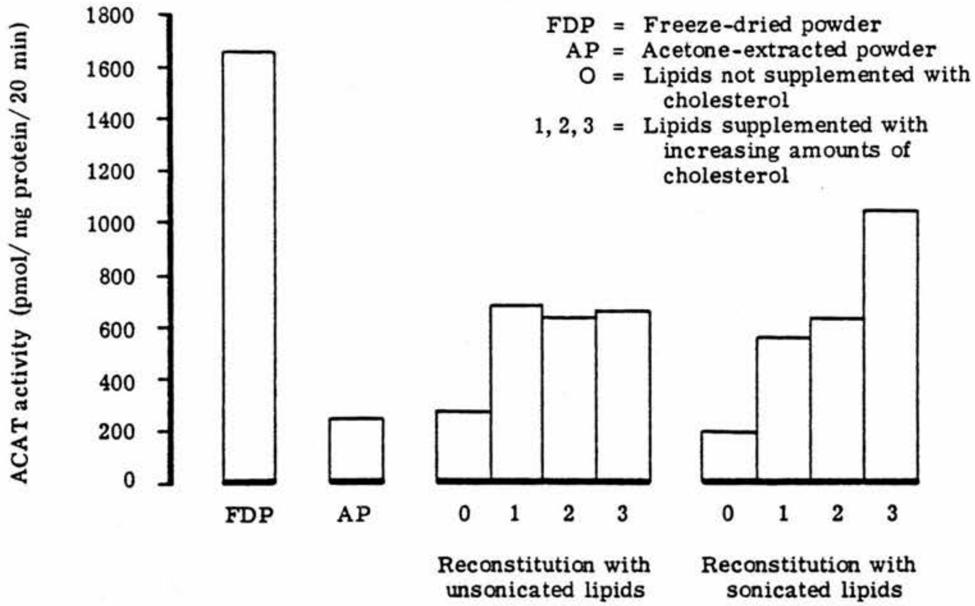


Table 7.1

Lipid compositions

Sample:	Freeze-dried powder	Acetone powder	Preparations reconstituted from unsonicated liposomes				Preparations reconstituted from sonicated liposomes			
Additions*	-	-	0	1	2	3	0	1	2	3
Protein (mg/ml)	2.46	2.76	2.76	2.34	2.32	1.98	3.06	2.46	2.40	1.52
Cholesterol (μmol/ml)	0.50	0.16	0.33	0.45	0.45	0.47	0.25	0.52	0.72	0.87
Phospholipid (μmol/ml)	0.86	1.03	1.04	1.08	0.99	0.93	1.07	1.03	1.20	0.63
Cholesterol: phospholipid ratio	0.58	0.15	0.32	0.42	0.46	0.50	0.23	0.50	0.60	1.39

*0 = lipids used in liposome preparation not supplemented with cholesterol.
1,2,3 = lipids used in liposome preparation supplemented with increasing amounts of cholesterol.

acyltransferase and the free cholesterol to phospholipid ratio were high, being elevated some two-fold and four-fold, respectively, over typical values obtained after one week of cholesterol feeding.

Extraction with acetone once more caused a large decrease in activity, to 15% of that of the freeze-dried microsomes. Recombination of this acetone-extracted powder with liposomes with no additional cholesterol caused a small increase ($P<0.025$) and a small decrease ($P<0.0025$) in activity for multilamellar and unilamellar liposomes respectively.

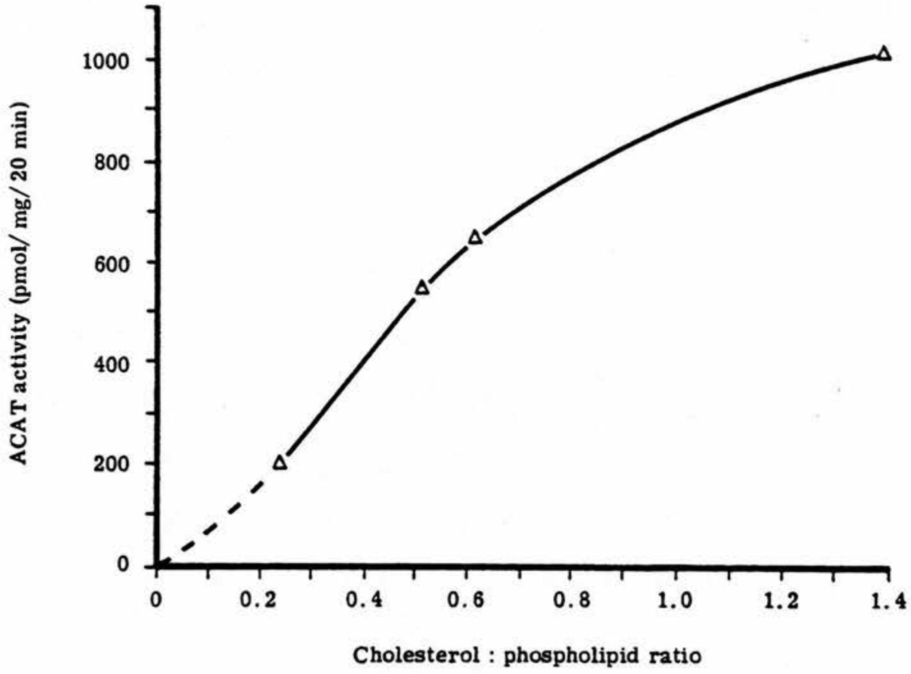
The activities of preparations reconstituted using multilamellar liposomes supplemented with varying amounts of cholesterol were greater than when no additional cholesterol was present, but were closely similar to each other. This is explained by the cholesterol to phospholipid ratios of the final, washed preparation, which demonstrate that these liposomes failed to take up the increasing amounts of cholesterol that were used in their preparation.

In contrast, the cholesterol contents of the unilamellar liposomes were found to reflect the increasing amounts used in their preparation. As the amount of cholesterol in these liposomes increased, their esterification activity increased. This is best shown in the plot of activity against cholesterol to phospholipid ratio in Figure 7.3. This shows that there is a relationship between the amount of cholesterol in membranes accessible to acyl-coenzyme A:cholesterol acyltransferase and the rate of esterification. In particular, an apparent saturation is not reached at the cholesterol concentrations normally found in the endoplasmic reticulum, or even at the very high cholesterol concentration found in this particular group of rats which had been fed on a cholesterol-rich diet for an extensive period.

However, supplementing the cholesterol content of these preparations failed to restore esterification activity to levels observed in

Figure 7.3

ACETONE-EXTRACTED LIVER MICROSOMAL ACYL-COENZYME A: CHOLESTEROL ACYLTRANSFERASE ACTIVITY ON RECONSTITUTION WITH VARYING AMOUNTS OF CHOLESTEROL



preparations which had not been subjected to the relatively harsh procedure of organic solvent extraction. Thus, despite the advantages of this system (e.g. the ability to readily examine the effects of cholesterol concentrations lower as well as greater than usual, and the ease with which the method could have been extended to explore the effect of different phospholipid fatty acids) it was felt that alternative methods based on the use of cholesterol-enriched serum should be investigated.

7.4 Acyl-coenzyme A:cholesterol acyltransferase and cholesterol 7α-hydroxylase activities in microsomes supplemented with cholesterol using cholesterol-enriched serum

Rats were fed on soft diet, 1% cholesterol diet or 4% cholestyramine diet for one week. Three rats from each group were killed and liver microsomes were prepared. The microsomal pellet was incubated at $4^{o}C$ for three hours suspended in normal horse serum, in horse serum enriched *in vitro* with cholesterol (373), or in 0.1 M tris-chloride buffer pH 7.4, at a concentration of 5 mg microsomal protein per ml. At the end of this incubation the microsomes were sedimented by centrifugation at 105 000 x g for one hour, and washed free of any adherent cholesterol-containing serum particles by resuspending and resedimenting a further twice.

The microsomes were finally suspended in 0.1 M tris-chloride buffer pH 7.4. Aliquots were taken for assays for protein, acyl-coenzyme A: cholesterol acyltransferase and cholesterol 7α-hydroxylase and for organic solvent extraction and subsequent assay for cholesterol and phospholipid. (In these experiments the incubation of microsomes with the cholesterol-enriching and the control media and the assays for protein, cholesterol, and phospholipid were performed by Dr. K.E. Suckling and Mr. H.A.F. Blair.)

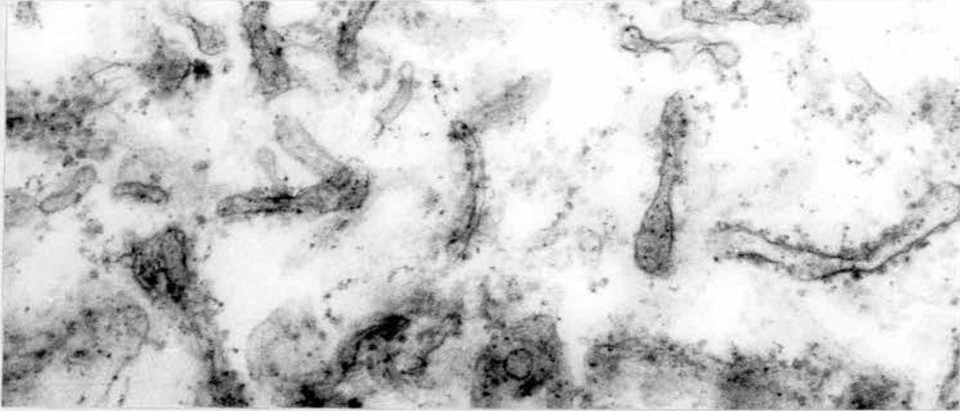
Various experiments were undertaken to ensure that the additional cholesterol detectable in the preparations incubated with the cholesterol-enriched serum was indeed actually within the microsomal membrane rather than being due to cholesterol-rich particles adsorbed to the membrane. These particles could be hypothesised to be cholesterol-rich serum lipoproteins or even cholesterol crystals formed as an artefact during the preparation of the cholesterol enriched serum. Electron micrographs of thin sections of glutaraldehyde-fixed cholesterol-enriched and control microsomes (provided by Dr. A. Whyllie, Department of Pathology, University of Edinburgh) showed no difference in appearance (Figure 7.4). The physical state of a membrane can be investigated by means of a suitable lipid-partitioning paramagnetic molecule, such as the fatty acid derivative 5-doxyl stearate. Electron paramagnetic resonance spectroscopy then allows the measurement of an order parameter for this molecule, which in turn reflects the ordering of the membrane. When cholesterol is incorporated into membranes an increase in the order parameter for such a molecule can be detected. The order parameter of 5-doxyl stearate added to microsomes incubated with cholesterol-enriched serum showed an increase over values obtained for microsomes incubated with control serum. This is consistent with the suggestion that at least some of the additional cholesterol detectable in these preparations is located within the membrane (Dr. K.E. Suckling, unpublished results).

The results of a typical experiment are shown in Figure 7.5 and Table 7.2. Incubation of microsomes with control serum resulted in small increases in both cholesterol and phospholipid contents expressed relative to protein, the increase in cholesterol being slightly greater than that in phospholipid as demonstrated by the increase in cholesterol to phospholipid ratio. Incubation with cholesterol-enriched serum

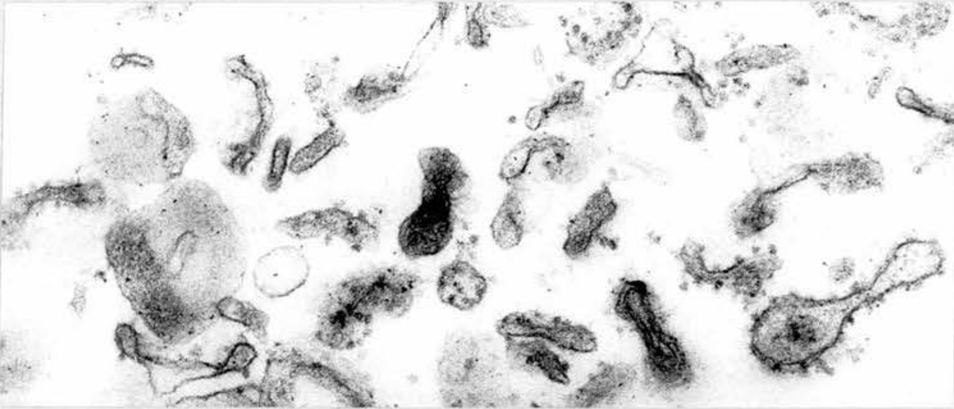
Figure 7.4

Electron micrographs of microsomal preparations

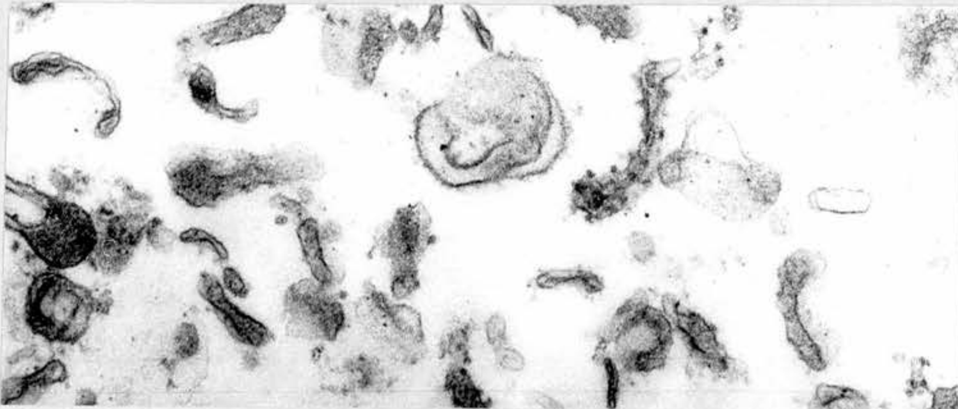
(a)



(b)



(c)



Magnification = 75,000 x

(a) Fresh microsomes

(b) Microsomes incubated with control serum

(c) Microsomes incubated with cholesterol-enriched serum.

(The preparations were fixed in glutaraldehyde and stained with uranyl acetate and lead nitrate.)

Figure 7.5

THE ACTIVITIES OF CHOLESTEROL 7α-HYDROXYLASE AND ACYL-COENZYME A : CHOLESTEROL ACYLTRANSFERASE OF LIVER MICROSOMES INCUBATED WITH CONTROL OR CHOLESTEROL-ENRICHED SERUM

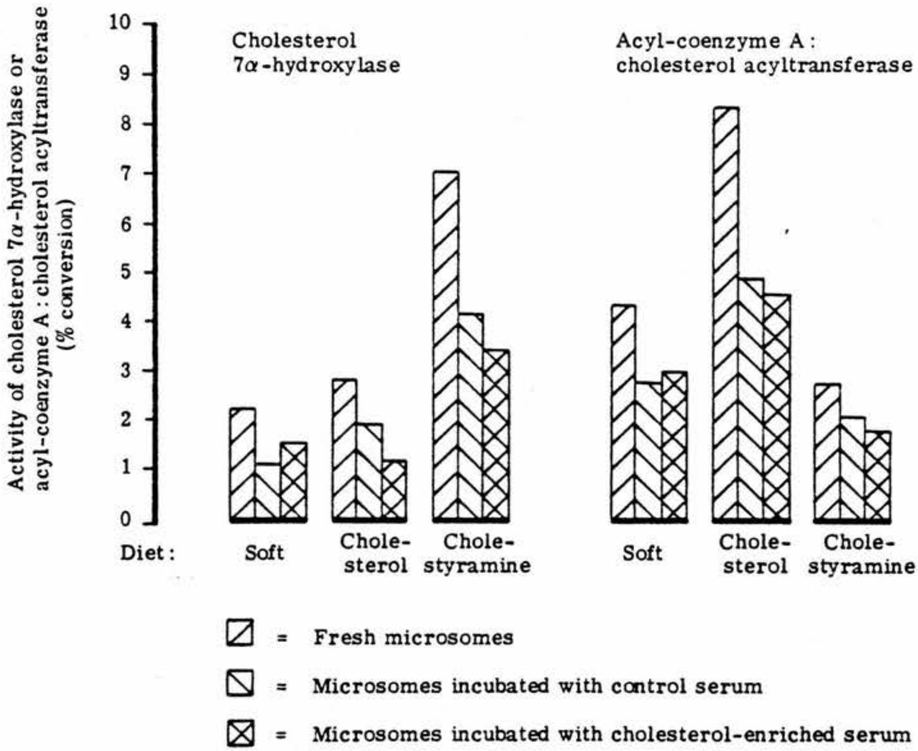


Table 7.2

The lipid compositions of liver microsome preparations after incubation with control or cholesterol-enriched serum

Diet	Sample	Protein (mg/ml)	Phospholipid (μmol/ml)	Cholesterol (μmol/ml)	Cholesterol: phospholipid ratio	Cholesterol: protein ratio	Phospholipid: protein ratio
Soft	Fresh microsomes	6.40	2.84	0.413	0.415	0.0645	0.444
	Microsomes incubated with control serum	4.68	2.77	0.393	0.142	0.0840	0.592
	Microsomes incubated with cholesterol-enriched serum	4.68	2.87	1.806	0.629	0.386	0.613
1% cholesterol 10% olive oil	Fresh microsomes	6.56	3.42	0.493	0.144	0.0752	0.521
	Microsomes incubated with control serum	5.64	3.26	0.576	0.177	0.102	0.578
	Microsomes incubated with cholesterol-enriched serum	5.48	3.13	1.884	0.602	0.344	0.571
4% cholestyramine	Fresh microsomes	5.48	2.55	0.354	0.139	0.0646	0.465
	Microsomes incubated with control serum	4.32	2.55	0.470	0.184	0.108	0.590
	Microsomes incubated with cholesterol-enriched serum	4.16	2.48	1.832	0.739	0.440	0.574

resulted in little or no further increase in the phospholipid content of the microsomal preparations, but a large (five-fold or more) increase in the cholesterol content.

When enzyme activities were measured by the fractional conversion of labelled cholesterol to product, there was little difference between the activities measured after incubation with the control serum and the cholesterol-enriched serum, but these activities were somewhat less than those of preparations incubated in the absence of serum. The interpretation of this finding is considerably complicated by the varying cholesterol contents of these preparations. Also, since there is some evidence that cholesterol 7α-hydroxylase and acyl-coenzyme A:cholesterol acyltransferase exist in different microsomal subfractions (253), the same explanation need not apply to both enzymes.

Explanations of this finding are illustrated diagrammatically in Figure 7.6 and are as follows. The first possibility is that the additional serum-derived cholesterol entered a pool within the microsomal preparation not accessible to the enzyme, and which therefore does not act as a substrate for the enzyme. Two alternative suggestions could then be put forward to explain the observed lack of change in conversion of labelled cholesterol to labelled product. Firstly, the added tracer entered the enzyme's substrate pool to give the same specific radioactivity in each case (that is, the additional serum-derived cholesterol caused no dilution of the added cholesterol label within the substrate pool) (Figure 7.6(b)(i)). In this case, the unchanged per cent conversion between preparations incubated with control serum and cholesterol-enriched serum would reflect an unchanged enzyme activity. Alternatively, the added tracer entered the substrate pool to a greater or lesser extent than in the unsupplemented case (Figure 7.6(b)(ii) and (iii)). (It could be suggested that more labelled cholesterol might be available

Figure 7.6

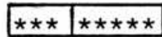
Diagrammatic representation of microsomal cholesterol enrichment experiments

Left hand compartment = pool of cholesterol available to act as enzyme substrate

Right hand compartment = pool of cholesterol not available to act as enzyme substrate

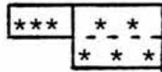
* = [4-^{14}C]-cholesterol

(a) Normal microsomes

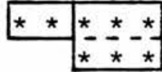


(b) Additional cholesterol enters non-substrate pool

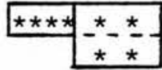
(i) No change in substrate specific radioactivity



(ii) Decrease in substrate specific radioactivity

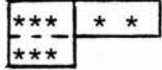


(iii) Increase in substrate specific radioactivity

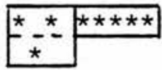


(c) Additional cholesterol enters substrate pool

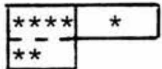
(i) No change in substrate specific radioactivity



(ii) Decrease in substrate specific radioactivity



(iii) Increase in substrate specific radioactivity



to enter this pool because of the "saturation" of membranes elsewhere with the additional cholesterol, or that less might be available because of the creation of a new alternative pool into which some of the tracer might be diverted.) If the resultant specific radioactivity of the substrate was greater than in the preparations not supplemented with cholesterol, a decreased enzyme activity would be necessary to account for the similar per cent conversion in each case. Conversely, if the specific radioactivity of the substrate was less than in the unsupplemented case, an increased enzyme activity would be needed to explain the observed results.

The second possibility is that the additional cholesterol entered the enzyme's substrate pool to a greater or lesser extent and hence caused an expansion in its size. If, then, the amount of tracer cholesterol entering this pool increased similarly, such that the specific radioactivity of the substrate remained approximately constant (Figure 7.6(c)(i)), the unchanged per cent conversion of labelled substrate to product would reflect no change in actual enzyme activity. If, however, an approximately similar amount of tracer cholesterol entered the substrate pool in the control and the cholesterol-enriched preparations, or at least if the tracer in the substrate pool is diluted to a greater extent than in the unsupplemented preparation, the unchanged per cent conversion suggests an increased enzyme activity (Figure 7.6(c)(ii)).

In summary, so long as the addition of the unlabelled serum-derived cholesterol caused a decrease in the specific radioactivity of the cholesterol in the substrate pool of the enzyme, it was necessary to postulate an increase in the actual activity of the enzyme in order to explain the absence of a decrease in the observed per cent conversion of labelled cholesterol to labelled product. In view of the five-fold increase in the cholesterol content of preparations incubated with

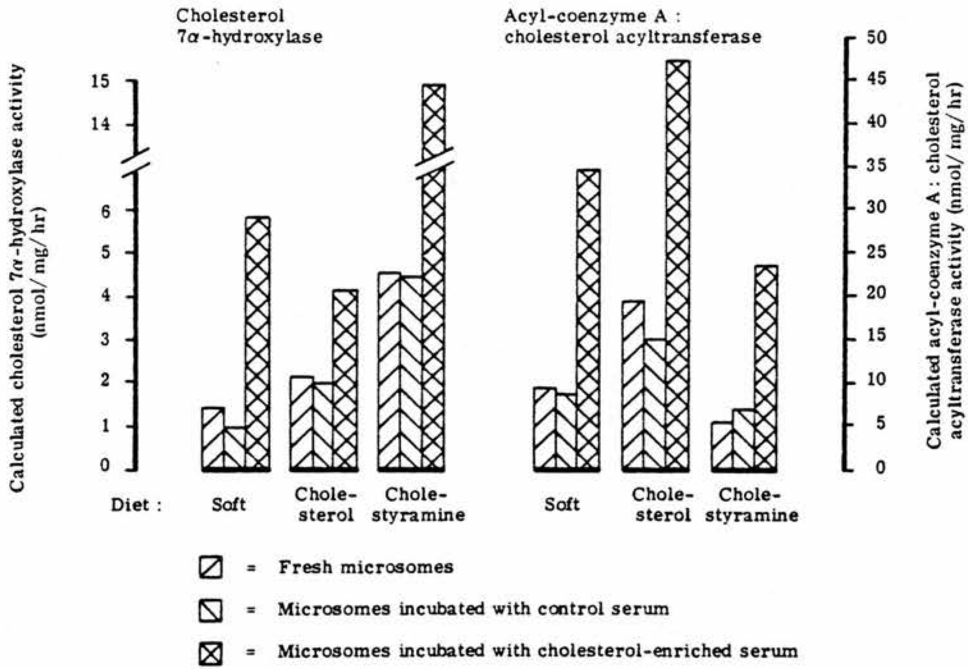
cholesterol-enriched serum and the relatively lengthy incubation and washing procedure involved, it was felt most likely that an equilibrium would be reached when the size of all cholesterol pools within the preparation would be increased to a greater or lesser extent. This would then imply that there would have to be an increase in enzyme activity in order to obtain the observed result.

It is therefore of interest to calculate enzyme activities from the product of the observed per cent conversion of labelled cholesterol to cholesterol ester or 7α-hydroxycholesterol and the microsomal free cholesterol content (Figure 7.7). It should be noted that the results thus obtained require the assumption that all the added cholesterol tracer was accessible to the enzyme, and will then be overestimates of the true activity by the same factor as that by which the total microsomal free cholesterol is an overestimate of the substrate pool size. These results then, while overestimating the actual activities in all cases, give an indication of the relative size of increase that will occur on cholesterol enrichment, so long as the proportion of the total cholesterol that is in the substrate pool remains constant. If the fraction of the total cholesterol that is in the substrate pool increases or decreases when the overall cholesterol content increases, then this calculated relative increase in enzyme activity will be respectively an overestimate or an underestimate of the actual relative increase in enzyme activity.

Because of the large number of uncertainties involved, too much importance should not be placed on these calculations. However, some preliminary results, described below, have indicated that the actual activity of acyl-coenzyme A:cholesterol acyltransferase does indeed increase when the microsomal cholesterol content is increased, although the position for cholesterol 7α-hydroxylase is not yet clear.

Figure 7.7

THE CALCULATED ACTIVITIES OF CHOLESTEROL 7α-HYDROXYLASE AND ACYL-COENZYME A : CHOLESTEROL ACYLTRANSFERASE OF LIVER MICROSOMES INCUBATED WITH CONTROL OR CHOLESTEROL-ENRICHED SERUM



The result of an experiment where acyl-coenzyme A:cholesterol acyltransferase was assayed in microsomes incubated with control and with cholesterol-enriched serum, using the [1-^{14}C]-oleic acid-based assay, is shown in Table 7.3. This shows that despite a 34% decrease in the enzyme activity measured by the [4-^{14}C]-cholesterol-based assay on cholesterol enrichment, there was a two-fold increase in the activity measured by cholesterol ester production from [1-^{14}C]-oleic acid. On the basis of these results it is possible to calculate that the maximum estimate of the enzyme's substrate pool size increased three-fold, while the proportion of the total microsomal cholesterol that it constituted fell from 19% to 7%.

After this experiment was performed, a similar experiment was reported (374), showing that incubation of rabbit liver microsomes with cholesterol-rich lipoproteins resulted in an increase in acyl-coenzyme A: cholesterol acyltransferase activity, and that this activity correlated well with the microsomal free cholesterol content.

During the assay of cholesterol 7α-hydroxylase activity in the experiment summarised in Figure 7.5 and Table 7.2, at the end of the incubation with the cofactors required for cholesterol 7α-hydroxylase activity, an aliquot of cholestane was added to assess recovery, lipids were extracted, and the lipid extract was divided in two. One of these lipid samples was subjected to thin-layer chromatography for the conventional [4-^{14}C]-cholesterol-based assay of cholesterol 7α-hydroxylase activity. The other sample was stored under nitrogen for eventual assay of 7α-hydroxycholesterol production by coupled gas chromatography-mass spectrometry.

However, problems were encountered in the assay of 7α-hydroxy-cholesterol because of the large amount of cholesterol present. The peaks due to cholesterol (on the total ionic current channel of the

Table 7.3

The assay of acyl-coenzyme A:cholesterol acyltransferase activity in cholesterol-enriched microsomes

Sample	:	Control	Cholesterol-enriched
Cholesterol content			
μmoles/mg protein	:	0.32	1.76
Acyl-coenzyme A:cholesterol acyltransferase activities -			
% conversion/20 min*	:	6.18	4.07
pmoles/mg protein/20 min†	:	781	1541
Substrate pool size -			
nmoles/mg protein	:	12.6	37.9
% of total microsomal cholesterol	:	19	7

*Mean of three assays measuring the % conversion of exogenous [4-^{14}C]-cholesterol to cholesterol ester.

†Mean of three assays measuring production of cholesterol ester from [1-^{14}C]-oleic acid.

instrument (equivalent to the gas chromatography trace) and at m/e 443 (the major fragmentation product derived from cholesterol)) were asymmetrical due to overloading of the gas chromatography column. This made the measurement of the cholesterol concentration (required for the eventual calculation of the 7α-hydroxycholesterol concentration) unreliable, and also affected the peak at m/e 546 (the principal fragmentation product from 7α-hydroxycholesterol) which has a similar retention time (see Figure 2.4).

In order to reduce the amount of cholesterol present all samples were taken to dryness, and, after a known amount of tritiated cholesterol was added to assess recoveries, applied to columns of 0.5 g silicic acid (Biosil A, 100-200 mesh), and cholesterol was partially eluted using 8 ml of an 80:20 mixture of petroleum spirit (60°-80°) and diethyl ether. The remaining sterols were eluted with 10 ml ethyl acetate. Samples were taken up in ethyl acetate, radioactive recoveries were measured, and a C_{29} sterol was added as a new internal standard. The assay then proceeded as normal.

After the number of experimental manipulations involved in these measurements, without the possibility of preliminary assessments of the techniques involved, the errors in the final results were high, with large disagreements between duplicates (Table 7.4). Insofar as it is possible to tell from these results, there was no increase in enzyme activity when the microsomal cholesterol content was increased, although in each case the enzyme's apparent substrate pool size in the cholesterol-enriched microsomes was enlarged, compared to the native microsomes. The results obtained for the soft diet-fed rats' microsomes incubated with control serum, and for the cholestyramine-fed rats' microsomes whether native or incubated with control serum, are anomalous in that the calculated maximal substrate pool size is greater than the

Table 7.4

The cholesterol 7α-hydroxylase activity and the size of the cholesterol 7α-hydroxylase substrate pool of liver microsomes after incubation with control or cholesterol--enriched serum

		Cholesterol 7α-hydroxylase		Substrate pool size	
Diet	Sample	% conversion of [4-^{14}C]-cholesterol	pmol/mg protein/hr	μg cholesterol per mg protein	% of total microsomal cholesterol
Soft	Fresh microsomes	2.20	840, 1340	19.2	77
	Microsomes incubated with control serum	1.09	2520	89.4	275
	Microsomes incubated with cholesterol-enriched serum	1.51	1210, 1800	38.5	26
1% cholesterol 10% olive oil	Fresh microsomes	2.84	1510, 500	13.7	47
	Microsomes incubated with control serum	1.90	1150, 870	20.6	52
	Microsomes incubated with cholesterol-enriched serum	1.20	880, 1240	34.3	26
4% cholestyr-amine	Fresh microsomes	7.03	7810, 11040	51.8	207
	Microsomes incubated with control serum	4.11	7960, 4070	56.6	135
	Microsomes incubated with cholesterol-enriched serum	3.39	8750, 6230	85.4	50

total free cholesterol present. This could be due to the breakdown of the assumption required in the calculation of maximal substrate pool sizes, that all added tracer cholesterol is accessible to the enzyme. However, in this experiment the possibility remains that this problem is due to experimental error.

The repetition of these experiments by Dr. K.E. Suckling has confirmed these results, showing that the incubation of microsomes with cholesterol-enriched serum results in an increase in acyl-coenzyme A: cholesterol acyltransferase activity and no change in cholesterol 7α-hydroxylase activity. Further, if microsomes are incubated with serum enriched with [1α, 2α(n)-^{3}H]-cholesterol in proportions that allow cholesterol exchange but no net increase in its microsomal concentration, and then enzyme assays are performed, radioactivity can be recovered as cholesterol ester, but relatively little is found in 7α-hydroxycholesterol. This is despite the similar activities of these two enzymes. This result tends to support the idea that these two enzymes are present in different microsomal subfractions. In addition, it appears that the sub-fraction containing acyl-coenzyme A:cholesterol acyltransferase is better able to accept cholesterol from cholesterol-rich lipoproteins than that subfraction which contains cholesterol 7α-hydroxylase.

7.5 The activity of ethylmorphine N-demethylase in microsomes supplemented with cholesterol using cholesterol-enriched serum

Microsomes were prepared from two groups of rats fed on soft diet for one week, one of these groups having received 0.1% phenobarbitone, 1% glucose in their drinking water. These microsomes were incubated with control serum and cholesterol-enriched serum and washed as described above (Section 7.4). Assays were performed for cytochrome P-450, NADPH-cytochrome c reductase, protein, cholesterol and phospholipid

(Table 7.5), and for ethylmorphine N-demethylase (time-course shown in Figure 7.8, and results calculated on the basis of the first five minutes of activity in Table 7.5). The relatively large sample size required for the ethylmorphine N-demethylase assay and the relatively small amount of microsomes that could be treated at one time with the serum preparations meant that only one assay could be performed per preparation. However, replicate assays performed on another sample of microsomes indicated that the standard error of the mean of five assays was typically $\pm$ 10% of the mean value.

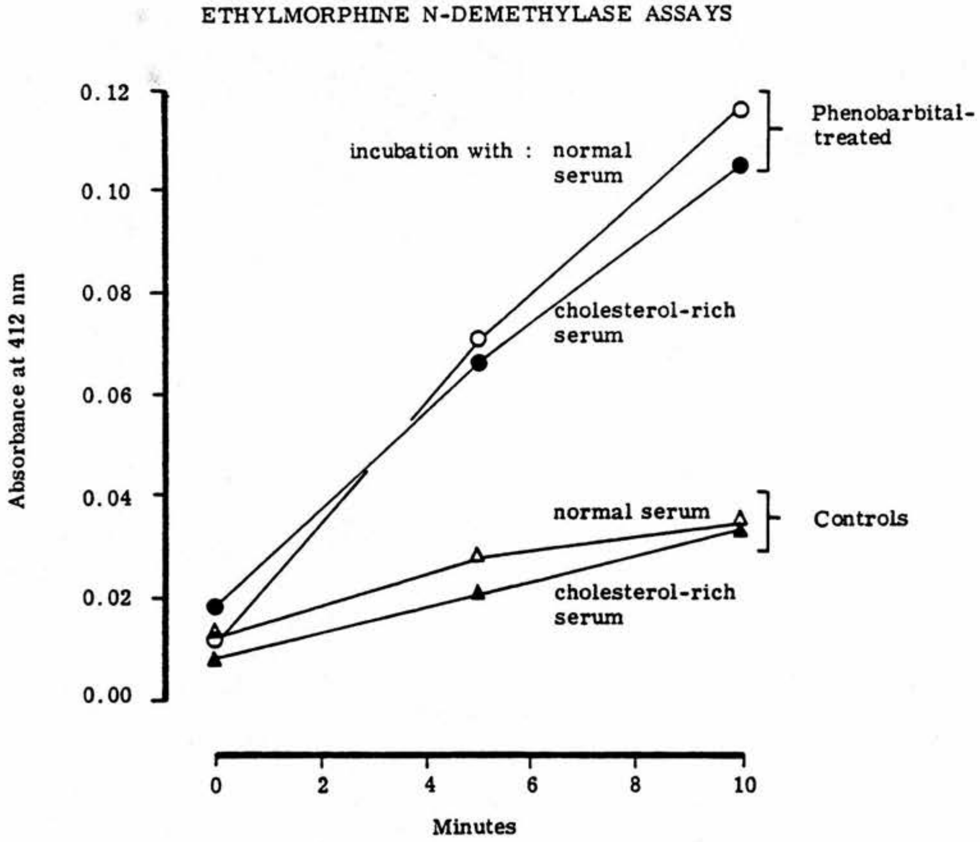
These results showed a small increase in NADPH-cytochrome c reductase activity on cholesterol enrichment. The observed small decrease in ethylmorphine N-demethylase activity was not significant at the proposed standard errors.

Table 7.5

The ethylmorphine N-demethylase activity of microsomes incubated with control or cholesterol-enriched serum

Animal treatment	Microsome treatment	Cytochrome P-450 concentration (nmol/mg protein)	NADPH-cytochrome c reductase activity (μmol cytochrome c reduced/min/mg protein)	Cholesterol: phospholipid ratio	Ethylmorphine N-demethylase activity (nmol formaldehyde released/min/mg protein)
Controls	Incubation with control serum	0.66	76	0.132	1.56
	Incubation with cholesterol-enriched serum	0.57	99	0.735	1.44
0.1% phenobarbitone in drinking water	Incubation with control serum	1.00	69	0.128	4.56
	Incubation with cholesterol-enriched serum	1.06	89	0.614	4.26

Figure 7.8



Section 8

Discussion

8.1 Introduction

The work described in this thesis was performed with the intention of elucidating some of the mechanisms involved in the short and long term regulation of cholesterol-metabolising processes of the rat liver endoplasmic reticulum, and in particular the responses of these processes to alterations in the supply of cholesterol.

Although many details in the mechanism have still to be worked out, the activity of endoplasmic reticulum 3-hydroxy-3-methylglutaryl-coenzyme A reductase and the overall rate of hepatic cholesterol synthesis are acknowledged to be closely controlled by the availability of cholesterol. Attention was accordingly focussed on the activities of cholesterol 7α-hydroxylase and acyl-coenzyme A:cholesterol acyltransferase, and, by implication, on the processes of cholesterol excretion and cholesterol storage.

Perhaps the ideal way to perform a study on any membrane-bound enzyme, whose activity is likely to be influenced by the properties of the membrane in which it is situated, is to at least partially purify the detergent-solubilised enzyme. This preparation can then be recombined with lipids of known composition in order to unambiguously investigate the effects of varying lipid composition on enzyme activity. Such an approach would be especially useful for enzymes, such as the two under consideration here, where the enzyme substrate is an integral membrane component, and, until there is evidence to the contrary, can be presumed to be likely to approach the enzyme's active site from within the membrane.

Cholesterol 7α-hydroxylase activity depends on at least two membrane-bound proteins, cytochrome P-450 and NADPH-cytochrome P-450 reductase, the interaction of which is required for enzymic activity of the analogous drug hydroxylases. Overall cholesterol esterification by the endoplasmic

reticulum similarly requires the activity of two enzymes, acyl-coenzyme A synthetase and acyl-coenzyme A:cholesterol acyltransferase. At present there is no evidence to suggest a close relationship between these two enzymes of cholesterol esterification. The interaction between the components of cholesterol 7α-hydroxylase, and any possible interaction between the enzymes involved in cholesterol esterification, are likely to be influenced by membrane properties. Again, such effects are conveniently studied by the recombination of resolved, lipid-free, preparations with lipid of known composition.

Such *in vitro* studies are most usefully performed if the experimental conditions used are chosen in the light of the results of *in vivo* experiments. Accordingly, the experiments performed were intended to further purify a cytochrome P-450 species able to support cholesterol 7α-hydroxylase activity in a reconstituted system (Section 3), to establish and characterise an assay for acyl-coenzyme A:cholesterol acyltransferase (Section 4), to investigate the possibility of preparing purified acyl-coenzyme A:cholesterol acyltransferase (Section 5.6), to determine the activities of cholesterol 7α-hydroxylase and acyl-coenzyme A:cholesterol acyltransferase in *in vivo* conditions where the delivery of cholesterol to the liver and/or the lipid composition of the endoplasmic reticulum were likely to be altered (Section 6), and, finally, to investigate the effects on the activities of these enzymes of *in vitro* perturbations of the cholesterol content of their membrane environment (Section 7).

In all of this work an appreciation of the practical and theoretical problems associated with the assay of these enzymes is essential.

8.2 The assay of the activity of enzymes that are membrane-bound and/or have lipophilic substrates

The interpretation of many of the experiments described has been considerably complicated by the nature of the enzymes being assayed (375-379). Firstly, these enzymes are membrane-bound. This is presumably mediated by the insertion of a hydrophobic portion of the protein structure into the membrane bilayer (380-382), and thus not only can the membrane exert an influence on the enzyme (213-221,296, 367-370), but also the enzyme can affect, at least locally, the membrane characteristics (217,372,384). Secondly, these enzymes act on a lipophilic, membrane-soluble substrate that is, furthermore, an essential membrane component whose concentration has a profound influence on membrane characteristics (184).

The types of problems that arise in the interpretation of the results of assays of these enzymes are broadly divisible into two main categories. The first set of problems is associated in particular with the use of assays based on the measurement of the conversion of radioactively labelled substrate to product. These difficulties arise from the large amount of endogenous substrate, and from the existence of this substrate not in one homogeneous pool, but in a series of pools of differing ability to act as enzyme substrate (174,242,250,252,254,275,371). Thus the radioactive substrate is diluted by non-radioactive substrate, and the specific radioactivity of the substrate pool directly accessible to the enzyme cannot be known. This means that this most straightforward of assays cannot give activities in terms of an amount of substrate converted to product, and, further, that the results obtained are a function not only of enzyme activity but also of the amount of endogenous substrate, and its distribution among the various compartments in which it exists. These difficulties can all be circumvented by the use of

assays which measure the actual amount of product formed. However, these assays are technically more difficult and, so long as the drawbacks of the simple radioactive tracer-based assay are appreciated, satisfactory conclusions can often be obtained. (These points have been mentioned or discussed in more detail in Sections 2.11, 2.12, 2.13, 4.1 and 7.4 and are implicit in many of the experiments described in Sections 6 and 7.)

There is a further set of problems in the assay of these enzymes that is not so amenable to solution by the use of more sophisticated assay techniques. Thus, there is a difficulty in how best to express concentrations, especially of substrate. These problems are of two kinds. Firstly, membrane-bound enzymes acting on lipophilic substrates are operating within an essentially two-dimensional system, and the expression of concentration in terms of bulk volume units is accordingly inappropriate. However, the most suitable and practicable expression of concentration is by no means clear. One possibility would be in terms of the membrane surface area, but this measurement would present considerable difficulties. Also, in these terms the concentration of all components would be apparently temperature-dependent, undergoing an abrupt change where there is a well-defined membrane transition temperature, or a more gradual change in complex mixtures of membrane lipids. Thus, even if this measurement were readily available, its use would be fraught with difficulties. Because of this perhaps a more satisfactory alternative would be to use the mole fraction of substrate in the membrane phase. Again, this is not a quantity that lends itself to simple experimental determination, especially in membranes such as the endoplasmic reticulum where there is a relatively large amount of protein. However, if phospholipid is taken to constitute the matrix of the membrane and is present in large amounts, then perhaps a relatively acceptable alternative to the mole fraction of substrate

would be the substrate to phospholipid ratio. Alternatively, where the protein-to-phospholipid ratio remains constant, the substrate concentration could be expressed per unit weight of protein.

Secondly, even if satisfactory units for substrate concentration are found, there are doubts about the adequacy of statistical averages of numbers of molecules in expressing substrate concentrations for enzymes acting at interfaces or within membranes, because of the probability that these enzymes are operating within a microenvironment differing in its characteristics from the bulk solution or from the rest of the membrane. In particular, there is evidence that membrane-bound enzymes sequester around themselves an annulus of lipids differing in their characteristics and composition from the membrane in general (217,372,384); membrane lipids can undergo lateral phase separation, resulting in areas with concentrations of particular lipids different from their overall average concentration (193-196,208); microsomes contain more than one pool of cholesterol, and the cholesterol pools are not equally accessible to cholesterol 7α-hydroxylase and acyl-coenzyme A: cholesterol acyltransferase (174,242,250,252,254,275,371); finally, if the diffusion of substrate within the membrane is relatively slow compared to enzyme activity, substrate concentrations in the vicinity of the enzyme could be affected (see below).

These discussions of the problems involved in the expression and use of concentrations in the context of these enzyme systems have so far been concerned with lipophilic components that will be expected to partition into the membrane bilayer. In addition to these, the concentration of charged species and in particular the pH, at the phospholipid-water interface will not be the same as the concentration in the bulk solution and the water concentration within the membrane bilayer will be far removed from that of an aqueous solution.

Both protons and water participate in the reactions of hydroxylation and esterification as either substrates or products, and the local concentration of these components in the vicinity of the enzymes (as distinct from the overall concentration) is therefore of interest.

In addition to these difficulties associated with the determination of local concentrations in complex membranes, there is the possibility that the movement of substrate from distant sites within the membrane to the vicinity of the enzyme may be slow relative to the enzyme activity rate. This will result in a concentration gradient of substrate within the membrane with lower concentrations of substrate in the vicinity of the enzyme than in the rest of the membrane. Conversely, if the rate of product diffusion is slow relative to the reaction rate, there will be a gradient of product concentration with a high concentration around the enzyme.

In the case of an enzyme which is saturated with substrate at low substrate concentrations the reaction rate will then depend on the diffusion rate of the substrate within the membrane. For enzymes not saturated at endogenous substrate concentrations the relationship between reaction rate and diffusion rate will be more complex, since the reaction rate and the diffusion rate will both be among the determinants of the substrate concentration close to the enzyme, and this in turn will affect the reaction rate.

The product diffusion rate becomes important when the enzyme is inhibited by the product (as is cholesterol 7α-hydroxylase (146)). In this case, if the relative rates of product production and diffusion are such that a concentration gradient is formed, the enzyme activity will be in part determined by the inhibition constant of the product and its local concentration which in turn depends on the rates of product formation and diffusion. (*In vivo*, the relative activities of

cholesterol 7α-hydroxylase and the following enzymes in the pathway of bile acid synthesis are such that 7α-hydroxycholesterol is unlikely to accumulate to any appreciable extent. However, the in vitro assay for cholesterol 7α-hydroxylase of necessity does not include the NAD^+ required for the activity of the 3β-ol dehydrogenase whose substrate 7α-hydroxycholesterol is. The amount of 7α-hydroxycholesterol formed during an assay for cholesterol 7α-hydroxylase (1-10 nmol per mg protein) is just sufficient to achieve a detectable inhibition of enzyme activity (146).)

The foregoing discussion seeks to establish that, for enzymes such as the ones under consideration, the measured enzymic activity whether actual (measured by product formation) or apparent (measured by fractional conversion of radioactive substrate to product) is determined not only by the intrinsic enzymic activity but also by the disposition of substrate between different parts of the membrane and its rate of movement between these sites. Factors affecting these will therefore influence the measured enzymic activity. Examples of such factors include temperature, the presence of organic solvents or detergents (one or other of these is usually used in the addition of radioactive substrate to such enzymes for their assay), and the lipid composition of the membrane. This last category is of particular importance, since cholesterol is not only the substrate of the two enzymes of interest, but is also a membrane component whose concentration has a marked influence on membrane characteristics including transition temperatures and fluidity (184).

(It should be noted that these effects of the membrane on membrane-bound enzyme activity are quite distinct from any direct effect of membrane lipid on enzymic activity - for example, the frequent requirement of purified membrane-bound enzymes for lipid if activity

is to be detected (209-211,213), or the influence of the membrane on the interaction of different protein molecules required for overall enzyme activity (215,219,385-387).)

8.3 The preparation of a cytochrome P-450 species reconstitutively active in cholesterol 7α-hydroxylation

(a) The existence of a cytochrome P-450 species specific for cholesterol 7α-hydroxylation

The existence of a cytochrome P-450 species specific for cholesterol 7α-hydroxylation has yet to be conclusively proved. In particular there is no clear correlation between hepatic microsomal cytochrome P-450 content and cholesterol 7α-hydroxylase activity (266-268,286-289). One possible explanation for this observation might be that there exists some protein or non-protein cofactor for the cholesterol 7α-hydroxylase system whose induction or repression, rather than that of cytochrome P-450, controls enzyme activity. An alternative hypothesis, which does not require the postulation of an entity for which there is no experimental evidence, suggests that hepatic microsomal cytochrome P-450 exists in multiple forms (277-283) and that the form specific for cholesterol 7α-hydroxylase only represents a relatively small fraction of the total microsomal cytochrome P-450 content. This would then mean that changes in this species sufficient to account for observed changes in cholesterol 7α-hydroxylase activity would be difficult to detect against the large background cytochrome P-450 content. Conversely, the large inductions of drug hydroxylation-specific cytochrome P-450 that can be observed on pre-treating animals with appropriate drugs would not be expected to have any effect on cholesterol 7α-hydroxylase.

A simple calculation can demonstrate that such a suggestion is reasonable. In a purified, reconstituted system the activity of

benzphetamine demethylase is 50 nmol per minute per nmol pure, specific cytochrome P-450 (299). In experiments reported in this thesis cholesterol 7α-hydroxylase activities in the range of 1-10 nmol per hour per mg protein were observed, the lower values being obtained on feeding the control or the cholesterol-containing diets, and the higher values on feeding the 4% cholestyramine diet. On the basis of a typical microsomal cytochrome P-450 content of 0.5 nmol per mg protein, this activity is approximately in the range 0.05-0.5 nmol per minute per nmol total microsomal cytochrome P-450. Therefore, if the two enzymes were to operate at the same rate per nmol of specific cytochrome P-450, cholesterol 7α-hydroxylase-specific cytochrome P-450 need only account for 0.1-1% of the total microsomal cytochrome P-450. It is quite possible that the intrinsic activity of the cholesterol 7α-hydroxylase system is lower than that of drug hydroxylations. However, even if the cholesterol 7α-hydroxylase operated at a rate 10-fold slower than drug hydroxylases, it need still account for no more than 1-10% of the total microsomal cytochrome P-450 in the uninduced and induced examples, respectively.

The resolution of microsomal cytochrome P-450 into several fractions which vary in their ability to support cholesterol 7α-hydroxylase activity in a reconstituted system (320, and S. Ozasa, personal communication) tends to confirm the existence of a cholesterol 7α-hydroxylase-specific cytochrome P-450. However, these observations do not unambiguously prove the existence of such a cytochrome P-450 species, since it is possible that a cytochrome P-450 of more general specificity could be co-purifying with some further protein subunit capable of conferring specificity for cholesterol 7α-hydroxylation.

The suggestion that there is a cytochrome P-450 specific for cholesterol 7α-hydroxylation and that this is present as a relatively

small fraction of the total microsomal cytochrome P-450 has two important consequences. Firstly, at each stage of a purification procedure it is necessary to use any cytochrome P-450 fraction(s) obtained to reconstitute cholesterol 7α-hydroxylase activity, to ensure that this activity has been retained, and to test whether any fraction shows an enhanced ability to support this activity. Secondly, if there is only a small amount of the specific cytochrome P-450 in the microsomal fraction even in animals where enzyme activity has been induced by feeding cholestyramine, it is desirable that as little of the cytochrome should be lost as possible, especially in the initial stages of the purification.

(b) The use of donors of "active oxygen" to reconstitute cholesterol 7α-hydroxylase activity

It was felt that a particularly convenient approach to the reconstitution of hydroxylating activity with a series of cytochrome P-450 fractions would involve the use of one of the chemical donors of "active oxygen" which have been demonstrated to support a variety of cytochrome P-450-catalysed drug and steroid hydroxylations in the absence of NADPH or molecular oxygen (322-329). The experiments performed (Section 3.7) showed that it was not possible to detect any cholesterol 7α-hydroxylation catalysed by a microsomal suspension in the presence of iodosobenzene under a range of experimental conditions, and only a very low rate of $NaIO_4$-supported activity was observed.

At least part of the reason for the former result was the rapid destruction of cytochrome P-450 by iodosobenzene which occurs concomitantly with activity (328, and Section 3.6). Such an effect may be tolerable in the study of enzyme activities where there are sensitive assays for the enzymic reaction product, so that the initial rate of reaction can be measured during the first few minutes of incubation, but poses a considerable problem for the assay of cholesterol 7α-hydroxylase activity.

An approximate calculation demonstrates the problems involved. A typical cholesterol 7α-hydroxylase assay incubation contains 4 nmoles microsomal cytochrome P-450. If it is assumed that 5% of this is specific for cholesterol 7α-hydroxylation, and that each cytochrome molecule turns over once, and in doing so produces one molecule of 7α-hydroxycholesterol and is itself destroyed, then 200 pmoles 7α-hydroxycholesterol will be produced during the course of the incubation. This amount of oxygenated sterol is within the detection limits of the gas chromatography-mass spectrometry assay, but this was not available for routine use. If the problems introduced by the possibilities of cholesterol compartmentation are ignored this production of 7α-hydroxycholesterol represents a 0.00005% conversion of endogenous cholesterol if native microsomes were used, or a 0.001% conversion if a butanol-extracted preparation was used. Such conversions are not detectable by the [4-^{14}C]-cholesterol-based assay.

The use of KIO_4 was judged to be unsatisfactory because of the large amounts of 7-ketocholesterol produced even in the presence of β-mercaptoethylamine. 7-Ketocholesterol is a potent inhibitor of cholesterol 7α-hydroxylase.

(c) <u>The effect of increased recovery of cytochrome P-450 on its purification</u>

Because of the small amounts of putative cholesterol 7α-hydroxylase-specific cytochrome P-450 that are likely to be present in microsomes and the low recoveries of total microsomal cytochrome P-450 that were obtained on DEAE-cellulose chromatography, it was considered that a search for methods to improve the recovery of cytochrome P-450 at this initial step would be useful. Furthermore, it was considered that if the recoveries of cytochrome P-450 could be improved a more satisfactory purification might result.

The results obtained with a series of protective agents in various combinations are tabulated in Table 3.1. Increasing the detergent concentration or decreasing the buffer concentration caused large losses of cytochrome P-450. The purifications of total cytochrome P-450 obtained on using the various combinations of protective agents in 20 μM potassium phosphate pH 7.7, 0.4% Nonidet are plotted against recoveries in Figure 8.1. This demonstrates that this approach did indeed tend to achieve improved purifications of cytochrome P-450 with increased recoveries. On the basis of these results it would appear that the use of 10 mM diethydithiocarbamate, with or without the additional use of 10 mM 4-phenyl-imidazole, is a useful precaution in any further work aimed at preparing cytochrome P-450.

8.4 The in vivo response of the liver to changes in its cholesterol supply

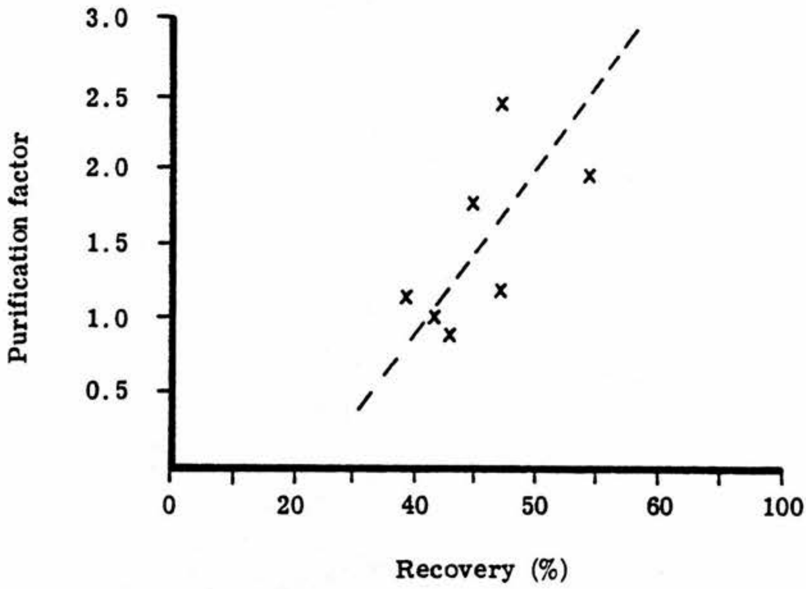
(a) Hepatic cholesterol metabolism during a twenty-four hour cycle, and during cholesterol feeding

The most straightforward way of rationalising the diurnal changes in hepatic cholesterol metabolism is to relate them to the periods of maximum intake of food. Thus, rats feed most actively during the dark phase of a twenty-four hour cycle, and the cholesterol metabolism of the animal has to be controlled, largely in the liver, to take account of this.

During the period of maximum food ingestion, in order to ensure absorption of fat, the rat must increase its synthesis of bile acids in order that bile salts can be secreted into the intestinal tract. This requirement for enhanced bile acid synthesis is a direct consequence of the absence of any storage capacity for bile in the rat. As would therefore be expected, bile acid synthesis, and the activity of cholesterol 7α-hydroxylase, increase at times approximately coinciding with the

Figure 8.1

THE PURIFICATION AND RECOVERY OF LIVER MICROSOMAL CYTOCHROME P-450 ON DEAE-CELLULOSE CHROMATOGRAPHY UNDER VARIOUS CONDITIONS



times of maximum food intake (176,263,272,346). (It is of interest to note that cholesterol 7α-hydroxylase is the only cytochrome P-450-dependent enzyme activity known to exhibit such a variation.)

The relation between this fluctuation in cholesterol 7α-hydroxylase activity and the variations detected in the size of the microsomal cholesterol pool which preferentially acts as substrate for the enzyme is not simple. When rats are fed a cholesterol-free diet, the fluctuations in these quantities are directly out of phase with each other, with a peak in enzyme activity during the dark period coinciding with a trough in substrate pool size (263). This result raises the possibility that the activity of cholesterol 7α-hydroxylase may itself be one of the determining factors for the size of the cholesterol substrate pool for bile acid synthesis, by controlling the rate of efflux from the pool. On the other hand, when a cholesterol-rich diet is fed, the fluctuations in cholesterol 7α-hydroxylase activity and in the size of its substrate pool are in phase with each other, with maxima in both being detected in the middle of the dark phase (176). This observation can perhaps be rationalised in terms of the large absorption of cholesterol occurring at this time resulting in an increase in hepatic microsomal cholesterol, and, in particular, in an increase in the amount of cholesterol in the pool that can act as substrate for cholesterol 7α-hydroxylase. This increased delivery of cholesterol into this pool could be envisaged to overwhelm the previously observed decrease in pool size which was perhaps due to an increased efflux of cholesterol from the pool.

When the diet contains no cholesterol, the liver can make use of the increased supply of dietary substrates that occurs during the dark phase for the *de novo* synthesis of cholesterol, and, accordingly, peaks of cholesterol synthesis and 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity can be detected (4,129,154-158,262,263,272,345,346).

This newly synthesised cholesterol can be used to supply the body's needs for this compound via lipoprotein (principally VLDL) synthesis and export, or, since at this time there is a potential drain on the body's cholesterol supply because of active bile acid synthesis, can act as the preferred substrate for cholesterol 7α-hydroxylase (174,250,274,275).

Under these conditions the supply of cholesterol from intrahepatic synthesis can be matched to cholesterol loss, and there is unlikely to be any accumulation, temporary or long term, of free cholesterol in the liver. If it is postulated that one of the functions of hepatic acyl-coenzyme A:cholesterol acyltransferase activity is to deal with excess free cholesterol by converting it to cholesterol ester for storage, then there is no need for an enhancement of this enzyme's activity at any time. However, in the rat, as well as this function, hepatic acyl-coenzyme A:cholesterol acyltransferase probably synthesises some of the cholesterol ester present in very low density lipoprotein (VLDL) (388, 389). If there is a diurnal variation in the rate of VLDL synthesis, it is possible that the activity of acyl-coenzyme A:cholesterol acyltransferase may vary in synchrony with this.

If, on the other hand, the rat was to eat a cholesterol-rich diet, there would be an uncontrollable entry of cholesterol into this system, and, furthermore, this entry would occur in a cyclic fashion, with maxima at, or shortly after, times of maximum food intake. Known responses to this are an inhibition of 3-hydroxy-3-methylgutaryl coenzyme A reductase and a reduction in the rate of cholesterol synthesis (4,129, 154-162,262,347), and an enhanced rate of bile acid synthesis (172,173). The effect on cholesterol 7α-hydroxylase is disputed, with reports of an increased activity and of no change in activity (147,174,175, and see Sections 6.4(a), and 6.4(c)). However, if this enzyme was not saturated with substrate an enhanced conversion of substrate to product

will result from the increase in substrate supply, in the absence of any stimulation of activity (176, and see Sections 7 and 8.4(a)).

It could be envisaged that if the diet only contained a small amount of cholesterol these mechanisms could be sufficient to prevent cholesterol accumulation. However, if the cholesterol delivery is in excess of the amount that can be converted to bile acids, it is apparent that there will be a net increase in whole body cholesterol, and it is well known that in the rat this increase occurs almost exclusively in the liver, and in the form of cholesterol ester (148,149). (The tissue localisation of this accumulated cholesterol does not concern the present work, but is probably a result of a complex interaction between the amounts of the different classes of lipoproteins, the activity of plasma lecithin:cholesterol acyltransferase, and the activity of acyl-coenzyme A: cholesterol acyltransferase in the liver and the peripheral tissues.) On the basis of the foregoing discussion, it might be predicted that this increase in cholesterol ester might occur in a cyclical fashion, with maxima at, or shortly after, times of maximum cholesterol ingestion.

It is widely accepted that intracellular cholesterol ester accumulation is catalysed by acyl-coenzyme A:cholesterol acyltransferase activity, and, in the fibroblast, at least, is associated with an increase in this enzyme's activity (106,121,123). However, there is no absolute requirement for any enhancement of enzymic activity in order to obtain the observed accumulation of cholesterol ester on increasing the substrate supply to the enzyme if the enzyme is not saturated with substrate, at the normal endoplasmic reticulum membrane cholesterol concentration. Before the present work was undertaken it was not known whether hepatic acyl-coenzyme A:cholesterol acyltransferase was saturated with substrate at normal endoplasmic reticulum cholesterol levels (that is, whether any observed changes in esterification rate

could be fully accounted for by changes in substrate supply to the enzyme), or whether changes in esterification rate were due to changes in enzyme activity. As discussed above, of particular interest were the possibilities that there might be a cyclical variation in activity during a twenty-four hour period, associated with feeding, and that there might be a stimulation of activity over a longer period on eating a cholesterol-rich diet.

The experiments performed demonstrated that there does indeed appear to be a diurnal variation in acyl-coenzyme A:cholesterol acyltransferase activity, and this observation has been confirmed in subsequent experiments using the assay based on [1-^{14}C]-palmitoyl-coenzyme A. This result contrasts with published results which claim to show that there is no diurnal variation in the activity of acyl-coenzyme A:cholesterol acyltransferase (252). However, these authors based their conclusions on assays for enzymic activity performed at midday and midnight. These times were chosen to approximately coincide with the maxima and minima in the diurnal rhythms in the rates of cholesterol and bile acid synthesis. However, the experiment presented here (Section 6.2 and Figure 6.1) showed the small peak and trough of acyl-coenzyme A:cholesterol acyltransferase activity occurred somewhat later than these times, and that, at the pair of times chosen by these authors, no difference in enzymic activity would be expected. Indeed, from the description of hepatic cholesterol metabolism presented above, it would appear quite reasonable that any peak in esterification activity should follow, rather than coincide with, peaks of cholesterol and bile acid synthesis.

It was also demonstrated that the increase in hepatic cholesterol ester content on cholesterol feeding is accompanied by an increase in acyl-coenzyme A:cholesterol acyltransferase activity. However, it was shown that care was needed in the interpretation of this result,

since feeding the olive oil-containing control diet also caused the activity of this enzyme to increase. However, as is shown in Figure 6.15, the activity of acyl-coenzyme A:cholesterol acyltransferase in the liver microsomes of rats fed the 1% cholesterol, 10% olive oil diet was greater than in those fed the 10% olive oil diet, but only significantly greater at certain times. Within the relatively limited time-scale that was studied, these times seemed to be defined by an early lag phase before any increase in activity was detectable in the olive oil-fed animals, and a later period when the enzymic activity of the cholesterol-fed animals appeared to be increasing at a faster rate than that of the olive oil-fed animals.

The results of these cholesterol-feeding experiments raise several questions. These include the reason for the observed increase in cholesterol esterification activity on olive oil-feeding, the failure to accumulate cholesterol ester despite this increased activity, and the lack of any observed decrease in activity on withdrawing the cholesterol or olive oil diets.

The reason for an enhancement of microsomal cholesterol esterification activity on feeding olive oil is not clear, but there are three main alternative explanations. Firstly, for some as yet unexplainable reason, there may be an enhanced induction of acyl-coenzyme A:cholesterol acyltransferase on olive oil feeding, and the raised activity would then be a consequence of an increase in the amount of this enzyme. Secondly, feeding olive oil may alter the fatty acid composition of the phospholipids of the microsomal membranes, and this may influence acyl-coenzyme A: cholesterol acyltransferase activity through physico-chemical effects on the environment of the enzyme. Thirdly, olive oil is particularly rich in oleic acid and this is the favoured fatty acid for cholesterol esterification by acyl-coenzyme A:cholesterol acyltransferase (248).

The increased esterification of cholesterol by incubations of microsomes from olive oil-fed rats could therefore possibly be explained by an increased supply of this cosubstrate. With reference to these possibilities there is the observation, published since these experiments were performed, that feeding rats diets of different fatty acid composition results in a change in microsomal phospholipid fatty acid composition, and also in altered acyl-coenzyme A:cholesterol acyl-transferase activity (389-391). Although it was not proved, the implication of this work was that the altered enzyme activity was in some way caused by the changed physicochemical properties of the membranes.

The failure of the livers of olive oil-fed rats to accumulate cholesterol ester despite the enhancement of measured esterification activity is presumably explained by the lack of an increase in cholesterol supply to the livers of these rats compared to the large increase in supply to the livers of cholesterol-fed rats. This observation once more serves to emphasise that the actual activity of enzymes such as these is a function not only of measurable enzyme activity, but also of the rate of supply of substrate to the vicinity of the enzyme.

In no experiment where a dietary stimulus resulted in an increase in acyl-coenzyme A:cholesterol acyltransferase activity did removal of that diet result in activity returning to basal levels. The effect of olive oil feeding on the activity of this enzyme provides a satisfactory explanation of these results. Thus, in the experiment described in Section 6.4(a) and Figures 6.9-6.11, the animals were fed with the 10% olive oil diet for seven days before transferring to the 1% cholesterol, 10% olive oil diet. In the light of the following experiment (Section 6.4(b), Figures 6.12-6.15) this period of olive oil feeding would be expected to result in little or no change in esterification activity, and the activity on day 0 of the experiment was accordingly low.

However, after feeding the 1% cholesterol, 10% olive oil diet for thirteen days, the animals would have been eating a diet rich in olive oil for twenty days, and any effect due to this component of the diet might be expected to have stabilised. Thus, on removing the cholesterol from the diet, there was a small decrease in activity, followed by a relatively constant level of activity for the remainder of the duration of the experiment.

If the effect of feeding olive oil on acyl-coenzyme A:cholesterol acyltransferase activity is indeed mediated by changes in the microsomal lipid composition, then these changes would be expected to be relatively slow, as would their reversal on removing olive oil from the diet. This probably explains the lack of any observable effect on acyl-coenzyme A:cholesterol acyltransferase activity in the ten days following the removal of either the 10% olive oil diet or the 1% cholesterol, 10% olive oil diet (Section 6.4(b), Figures 6.12-6.15).

On no occasion was it possible to demonstrate an increase in cholesterol 7α-hydroxylase activity on cholesterol feeding (Sections 6.4(a) and 6.4(c), Figures 6.11 and 6.16), despite the known enhancement of bile acid synthesis under these conditions (172,173), and the role of cholesterol 7α-hydroxylase as the rate-limiting enzyme in this process (392-397). This again reinforces the suggestion that substrate supply as well as enzyme activity is important in the control of the activity of this enzyme as well as that of acyl-coenzyme A:cholesterol acyltransferase. Thus, an increased rate of product formation appears to be obtainable by increasing the supply of substrate in the absence of increased amounts of enzyme activity. However, in the converse situation, where, on cholestyramine feeding, an increase in enzymic product formation is required in the absence of an increase in substrate supply, then an increase in enzyme activity would be predicted to be necessary, and is,

indeed, observed (146,288,289). It is of interest to contrast this observation with the comparable situation where acyl-coenzyme A: cholesterol acyltransferase activity is enhanced in the absence of increased supply of substrate on olive oil feeding, when as yet no increase in cholesterol ester formation (or, at least, accumulation) has been detected.

(b) Hepatic cholesterol metabolism during the twenty-four hours following bile duct ligation

Having examined *in vivo* conditions where the rate of cholesterol supply to the liver was varied, a condition was sought where cholesterol loss from the liver was prevented, at least in part. The two main routes of loss of cholesterol from the liver are via lipoprotein synthesis and secretion, and via the secretion of free cholesterol and bile salts in the bile.

The former can be prevented by the use of drugs including protein synthesis inhibitors and 4-aminopyrazolo-[3,4-d]-pyrimidine (31,130-132, 180,393-395). These compounds have considerable toxic effects both on the whole animal and on the liver. It was therefore decided to prevent cholesterol loss into the bile, by occlusion of the bile duct, since the effects of this are likely to be less immediately deleterious to the health of the animal, thus simplifying the interpretation of the results obtained. Furthermore, bile duct occlusion will raise hepatic cholesterol levels, which might be expected to affect the activities of cholesterol 7α-hydroxylase and acyl-coenzyme A:cholesterol acyltransferase, and will also increase the hepatic contents of bile salts, which are known, at least *in vitro*, to inhibit both these enzymes (178,272,396,397). Whether the bile salt concentration ever reaches levels inhibitory to these enzymes *in vivo* is not clear, because of the existence of cytosolic bile salt-binding proteins in the hepatocytes (398). Because of the

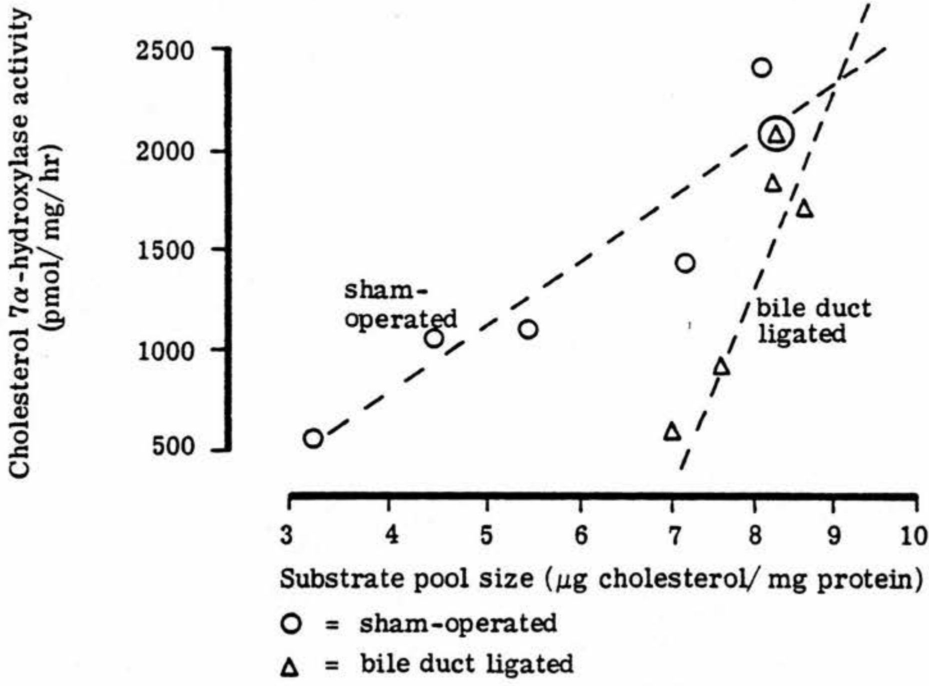
existence of these potentially antagonistic effects, it was decided that it was of interest to examine the consequences of occluding the bile duct by ligation.

The experiment described in Section 6.3 and Figures 6.3-6.8 demonstrated that microsomal free cholesterol levels did indeed increase following bile duct ligation. The concentration of bile salts in the systemic circulation also increased, and it is a reasonable assumption that this was reflected by an increased hepatic concentration of bile salts, although whether these were free or were protein-bound is not clear.

The calculated size of the substrate pool for cholesterol 7α-hydroxylase exhibited a diurnal variation that was in phase with the variation in enzyme activity, as would be expected for cholesterol-fed animals (176). The effect of ligating the bile duct appeared to be to diminish the size of the variation in pool size with the result that the pool size was usually somewhat greater than in sham operated animals (Figure 6.6). Cholesterol 7α-hydroxylase activity was observed to decrease on bile duct ligation despite this increase in substrate supply, presumably because of the inhibitory effect of raised hepatic bile salt levels (Figure 6.5).

If it is assumed that from the first time-point after bile duct ligation onwards the inhibitory effect on cholesterol 7α-hydroxylase of the increased bile salt concentration was constant, it is possible usefully to plot cholesterol 7α-hydroxylase activity against substrate pool size for both the sham operated and the bile duct ligated animals (Figure 8.2(a)). This plot demonstrates a straight line relationship between enzyme activity and substrate pool size for each group of animals. (The usefulness of this plot tends to be confirmed by a plot of enzyme activity against total microsomal free cholesterol, which

Figure 8.2(a)



exhibits no obvious correlation at all (Figure 8.2(b)). The line obtained for the group of animals with ligated bile ducts is displaced to the right and has a steeper gradient. These observations are consistent with the following model. Cholesterol 7α-hydroxylase is not saturated with its substrate, and increasing the amount of cholesterol in the vicinity of this enzyme therefore results in an increased production of 7α-hydroxycholesterol. Cholesterol 7α-hydroxylase is inhibited by bile salts, and this inhibition is of competitive type, since it can be overcome by increasing the concentration of substrate.

The calculated size of the substrate pool for acyl-coenzyme A: cholesterol acyltransferase increased both on sham operation and on bile duct ligation, the increase being somewhat larger in the latter case (Figure 6.8). There was relatively little change in acyl-coenzyme A: cholesterol acyltransferase activity during the course of this experiment, but twenty-four hours after ligation the enzyme activity was greater in the bile duct ligated than in the sham operated animals (Figure 6.7), this being due to a drop in activity in the sham operated controls rather than to an increased activity in the bile duct ligated animals. Again, enzyme activity can be plotted against substrate pool size (Figure 8.3(a)). A straight line is obtained for the plot of results from bile duct ligated animals. No obvious relationship exists for the sham operated animals, or for plots of enzyme activity against total microsomal free cholesterol (Figure 8.3(b)).

8.5 The response of hepatic microsomal cholesterol metabolism to in vitro alterations of the microsomal cholesterol content

The experiments performed *in vivo* having tended to support the idea that changes in the activities of cholesterol 7α-hydroxylase and acyl-coenzyme A:cholesterol acyltransferase could under some

Figure 8.2(b)

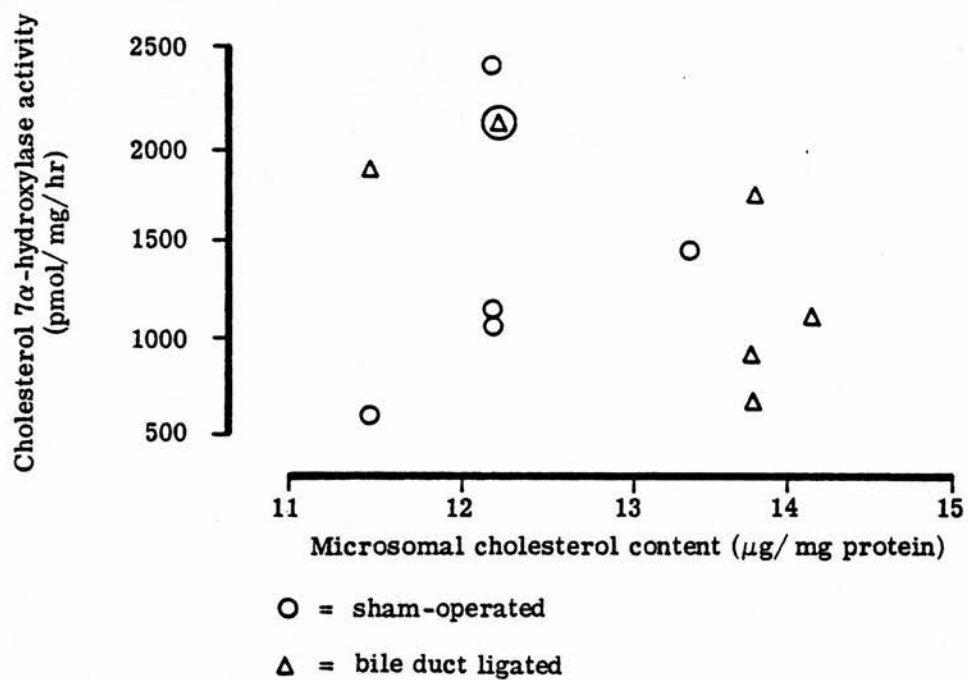


Figure 8.3(a)

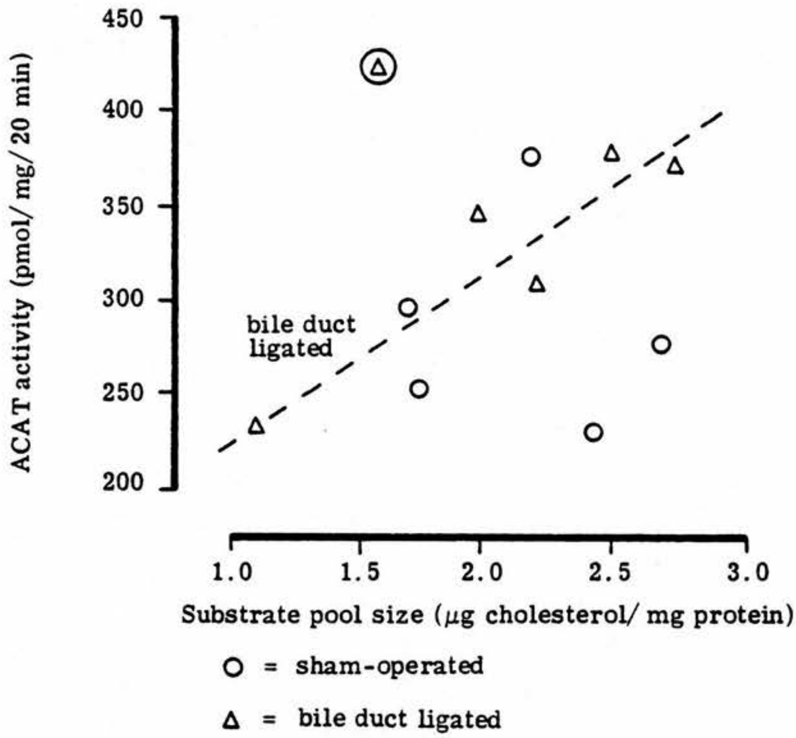
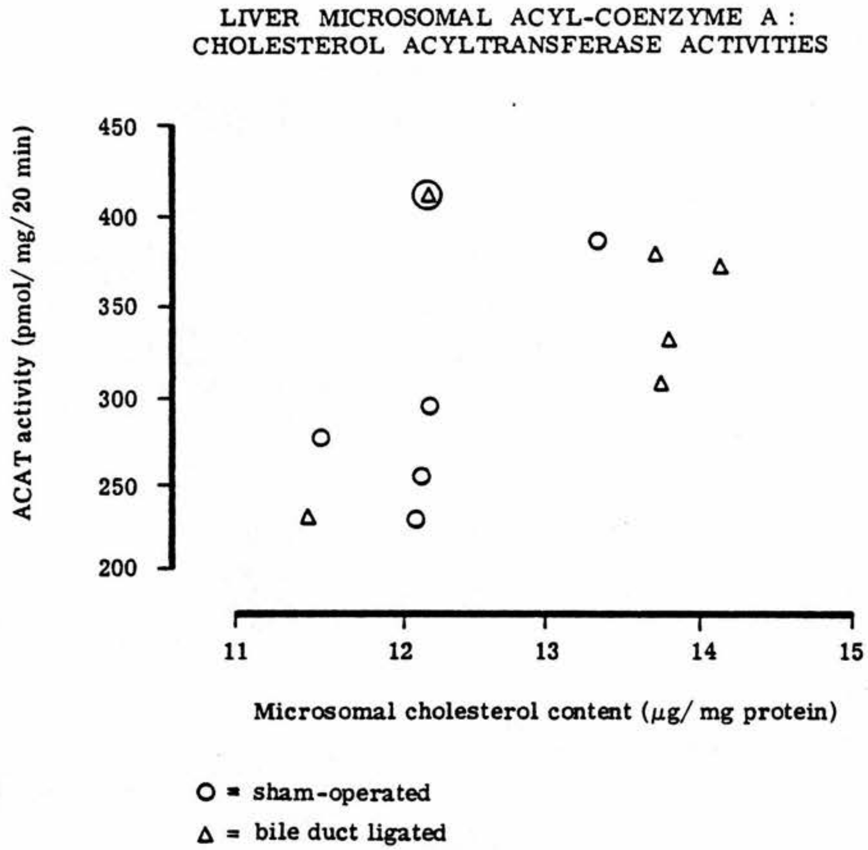


Figure 8.3(b)



circumstances be caused by changes in delivery of cholesterol to these enzymes, a direct *in vitro* demonstration of this was sought.

Two types of approach to this problem are possible. The microsomal preparation can be first delipidated, using either organic solvent or detergent, and then reconstituted using lipid of known composition, or alternatively, the lipid composition can be altered by exchange processes with liposomes or serum lipoproteins. The former type of technique allows lipids of completely defined composition to be used, but often results in large losses of enzyme activity; the latter approach, being less harsh, is less likely to cause loss of enzyme activity, but does not so easily enable lipids of predetermined composition to be used.

It was shown in Section 5.6 that detergent solubilisation destroys microsomal cholesterol esterification activity (although it might be anticipated that this activity could be at least in part restored on reconstitution with liposomes (344)), and, furthermore, traces of non-ionic detergents can be extremely difficult to remove from protein preparations, and may influence enzymic activity or membrane properties. Accordingly, the first approaches towards investigating the effects of altered lipid composition on the activities of these enzymes utilised extraction of freeze-dried microsomes with acetone.

The results of the experiment described in Section 7.2 and Figure 7.1 contain the suggestion that at least part of the difference in cholesterol esterification activity between soft diet-fed and cholesterol-fed animals was caused by some factor that was extracted with acetone. However, the interpetation of results obtained using [4-^{14}C]-cholesterol-based assays was considerably complicated by the presence of additional lipid pools created by the presence of the liposomes. This problem could be completely circumvented by the use of an absolute assay for acyl-coenzyme A:cholesterol acyltransferase.

This was used in the following experiment where the cholesterol content of the liposomes was varied (Section 7.3, Figures 7.2 and 7.3, and Table 7.1). This unequivocally demonstrated that an increase in the cholesterol supply to the enzyme resulted in an increase in the rate of cholesterol esterification. However, enzyme activity was not restored to the levels that existed before the acetone extraction.

The use of the gentler technique of incubation with cholesterol-enriched serum was therefore tried, in collaboration with Dr. K.E. Suckling. Again, the use of [4-^{14}C]-cholesterol-based assays yielded results that proved difficult to interpret, but suggested that an increase in cholesterol supply resulted in increased activities of these two enzymes. This result tended to be confirmed by preliminary experiments using absolute assays for enzyme activity.

Section 9

References

1. Borgström, B. (1968) J. Lipid Res. 9, 473-481.

2. Popják, G. and Beeckmans, M.-L. (1950) Biochem. J. 47, 233-238.

3. Dietschy, J.M. and Wilson, J.D. (1970) New Engl. J. Med. 282, 1128-1138, 1179-1183, 1241-1249.

4. Dempsey, M.E. (1974) Ann. Rev. Biochem. 43, 967-990.

5. Cayen, M.N. (1971) J. Lipid Res. 12, 482-490.

6. Jeske, D.J. and Dietschy, J.M. (1979) Fed. Proc. 38, 632.

7. Basford, J.M., Glover, J. and Green, C. (1964) Biochim. Biophys. Acta 84, 764-766.

8. Bruckdorfer, K.R. and Green, C. (1967) Biochem. J. 104, 270-277.

9. Bailey, J.M. and Butler, J. (1973) Arch. Biochem. Biophys. 159, 580-581.

10. Kovanen, P.T. and Nikkilä, E.A. (1976) Biochim. Biophys. Acta 441, 357-369.

11. Davis, W.W. and Garren, L.D. (1966) Biochem. Biophys. Res. Comm. 24, 805-810.

12. Beckett, G.J. and Boyd, G.S. (1977) Eur. J. Biochem. 72, 223-233.

13. Beckett, G.J. and Boyd, G.S. (1975) Eur. J. Biochem. 53, 335-342.

14. Beckett, G.J. (1975) Ph.D. Thesis, University of Edinburgh.

15. Trzeciak, W.H. and Boyd, G.S. (1974) Eur. J. Biochem. 46, 201-207.

16. Gorban, A.M.S. (1980) Ph.D. Thesis, University of Edinburgh.

17. Björkhem, I. and Danielsson, H. (1974) Mol. Cell. Biochem. 4, 79-95.

18. Boyd, G.S. and Percy-Robb, I.W. (1971) Am. J. Med. 51, 580-587.

19. Danielsson, H. (1973) in the Bile Acids (Nair, P.P. and Kritchevsky, D., eds.), Vol. 2, pp. 1-32, Plenum, New York.

20. Danielsson, H. and Sjövall, J. (1975) Ann. Rev. Biochem. 44, 233-253.

21. Elliott, W.H. and Hyde, P.M. (1971) Am. J. Med. 51, 568-579.

22. Eisenberg, S. and Levy, R.I. (1975) Adv. Lipid Res. 13, 1-89.

23. Jackson, R.L., Morriset, J.D. and Gotto, A.M. (1976) Physiol. Rev. 56, 259-316.

24. Lewis, B. (1977) Biochem. Soc. Trans. 5, 589-601.

25. Schaefer, E.J., Eisenberg, S. and Levy, R.I. (1978) J. Lipid Res. 19, 667-687.

26. Zilversmit, D.B. (1967) Fed. Proc. 26, 1599-1605.

27. Roheim, P.S., Gidez, L.I. and Eder, H.A. (1966) J. Clin. Invest. 45, 297-300.

28. Windmueller, H.G., Lindgren, F.T., Lossow, W.J. and Levy, R.I. (1970) Biochim. Biophys. Acta 202, 507-516.

29. Windmueller, H.G., Herbert, P.N. and Levy, R.I. (1973) J. Lipid Res. 14, 215-223.

30. Marsh, J. (1976) J. Lipid Res. 17, 85-90.

31. Bar-On, H., Kook, A.I., Stein, O. and Stein, Y. (1973) Biochim. Biophys. Acta 306, 106-114.

32. Cardell, R.R., Badenhausen, S. and Porter, K.R. (1967) J. Cell Biol. 34, 123-155.

33. Chapman, M.J., Mills, G.L. and Tayleur, C.E. (1972) Biochem. J. 128, 779-787.

34. Chapman, M.J., Mills, G.L. and Tayleur, C.E. (1973) Biochem. J. 131, 177-185.

35. Hamilton, R.L., Regen, D.M., Gray, M.E. and LeQuire, V.S. (1967) Lab. Invest. 16, 305-319.

36. LeMarchand, Y., Singh, A., Assimacopoulos-Jeannet, F., Orci, L., Rouiller, C. and Jeanrenaud, B. (1973) J. Biol. Chem. 248, 6862-6870.

37. Mahley, R.W., Bennett, B.D., Morré, D.J., Gray, M.E., Thistlethwaite, W. and LeQuire, V.S. (1971) Lab. Invest. 25, 435-444.

38. Mahley, R.W., Bersot, T.P., LeQuire, V.S., Levy, R.I., Windmueller, H.G. and Brown, W.V. (1970) Science 168, 380-382.

39. Oledzka-Slotwinska, H. and Desmet, V.J. (1971) Histochemie 28, 276-287.

40. Stein, O. and Stein, Y. (1967) J. Cell Biol. 33, 319-339.

41. Stein, O. and Stein, Y. (1973) Biochim. Biophys. Acta 306, 142-147.

42. Glaumann, H., Bergstrand, A. and Ericsson, J.C.E. (1975) J. Cell Biol. 64, 356-377.

43. Glickman, R.M., Kilgore, A., Khorana, J. (1978) J. Lipid Res. 19, 260-268.

44. Glickman, R.M. and Kirsch, K. (1973) J. Clin. Invest. 52, 2910-2920.

45. Redgrave, T.G. and Zilversmit, D.B. (1969) Am. J. Physiol. 217, 336-340.

46. Sabestin, S.M. and Isselbacher, K.J. (1965) Science 147, 1149-1151.

47. Havel, R.J., Kane, J.P. and Kashyap, M.L. (1973) J. Clin. Invest. 52, 32-38.

48. Havel, R.J., Fielding, C.J., Olivecrona, T., Shore, V.G., Fielding, P.E. and Egelrud, T. (1973) Biochem. 12, 1828-1833.

49. Mjos, O.D., Faergman, O., Hamilton, R.L. and Havel, R.J. (1975) J. Clin. Invest. 56, 603-615.

50. LaRosa, J.C., Levy, R.I., Herbert, P., Lux, S.E. and Fredrickson, D.S. (1970) Biochem. Biophys. Res. Comm. 41, 57-62.

51. Fielding, C.J. (1978) in Disturbances in Lipid and Lipoprotein Metabolism (Dietschy, J.M., Gotto, A.M. and Ontko, J.A., eds.), pp. 83-98, Am. Physiol. Soc., Bethesda.

52. Eisenberg, S., Bilheimer, D.W., Levy, R.I. and Lindgren, R.T. (1973) Biochim. Biophys. Acta 326, 361-377.

53. Higgins, J.M. and Fielding, C.J. (1975) Biochem. 14, 2288-2293.

54. Fielding, P.E., Shore, V.G. and Fielding, C.J. (1977) Biochem. 16, 1896-1900.

55. Scow, R.O. and Egelrud, T. (1976) Biochim. Biophys. Acta 431, 538-549.

56. Fielding, C.J. (1973) Biochim. Biophys. Acta 316, 66-75.

57. Tall, A.R., Green, P.H.R., Glickman, R.M. and Riley, J.W. (1979) J. Clin. Invest. 64, 977-989.

58. Chajek, T. and Eisenberg, S. (1978) J. Clin. Invest. 62, 1654-1665.

59. Eisenberg, S. (1978) J. Lipid Res. 19, 229-236.

60. Schaefer, E.J., Jenkins, L.L. and Brewster, B.B. (1978) Biochem. Biophys. Res. Comm. 80, 405-412.

61. Tall, A.R. and Small, D.M. (1978) New Engl. J. Med. 299, 1232-1236.

62. Tall, A.R., Green, P.H.R., Abreu, E. and Glickman, R. (1978) Circulation 58, Suppl. 2, II-15.

63. Blanchette-Mackie, E.J. and Scow, R.O. (1976) J. Lipid Res. 17, 57-67.

64. Redgrave, T.G. (1970) J. Clin. Invest. 49, 465-471.

65. Bergman, E.N., Havel, R.J., Wolfe, B.M. and Bohmer, T. (1971) J. Clin. Invest. 50, 1831-1839.

66. Quarfordt, S.H. and Goodman, D.S. (1967) J. Lipid Res. 8, 264-273.

67. Sherrill, B.C. (1978) in Disturbances in Lipid and Lipoprotein Metabolism (Dietschy, J.M., Gotto, A.M. and Ontko, J.A., eds.), pp. 99-109, Am. Physiol. Soc., Bethesda.

68. Cooper, A.D. and Yu, P.Y.S. (1978) J. Lipid Res. 19, 635-643.

69. Sherrill, B.C. and Dietschy, J.M. (1978) J. Biol. Chem. 253, 1859-1867.

70. Anderson, J.M., Nervi, F.O. and Dietschy, J.M. (1977) Biochim. Biophys. Acta 486, 298-307.

71. Eisenberg, S. and Rachmilewitz, D. (1973) Biochim. Biophys. Acta 326, 378-390.

72. Eisenberg, S. and Rachmilewitz, D. (1973) Biochim. Biophys. Acta 326, 391-405.

73. Stein, O., Rachmilewitz, D., Sanger, L., Eisenberg, S. and Stein, Y. (1974) Biochim. Biophys. Acta 360, 205-216.

74. Siguardsson, G., Nicoll, A. and Lewis, B. (1975) J. Clin. Invest. 56, 1481-1490.

75. Fidge, N.H. and Poulis, P. (1978) J. Lipid Res. 19, 342-349.

76. Hamilton, R.L., Williams, M.C., Fielding, C.J. and Havel, R.J. (1976) J. Clin. Invest. 58, 667-680.

77. van Berkel, T.J.C. and van Tol, A. (1978) Biochim. Biophys. Acta 530, 299-304.

78. van Berkel, T.J.C., van Tol, A. and Koster, J.F. (1978) Biochim. Biophys. Acta 529, 138-146.

79. Sigurdsson, G., Noel, S.-P. and Havel, R.J. (1978) J. Lipid Res. 19, 628-634.

80. Sniderman, A.D., Carew, T.E., Chandler, J.G. and Steinberg, D. (1974) Science 183, 526-528.

81. Miller, G.J. and Miller, N.E. (1975) Lancet 1, 16-19.

82. Miller, G.J., Fjorde, O.H., Thelle, D.S. and Mjos, O.D. (1977) Lancet 1, 965-968.

83. Goldstein, J.L. and Brown, M.S. (1977) Ann. Rev. Biochem. 46, 897-930.

84. Brattsand, R. (1976) Atherosclerosis 23, 97-110.

85. Rhoads, G.G., Gulbrandsen, C.L. and Kagan, A. (1976) New Engl. J. Med. 294, 293-298.

86. Fredrickson, D.S., Gotto, A.M. and Levy, R.I. (1972) in The Metabolic Basis of Inherited Disease (Stanbury, J.B., Wyngaarden, J.B. and Fredrickson, D.S., eds.), pp. 493-530, McGraw-Hill, New York.

87. Schwarz, C.C., Halloran, L.G., Vlahcevic, Z.R., Gregory, D.H. and Swell, L. (1978) Science 200, 62-64.

88. Bates, S.R. and Rothblat, G.H. (1974) Biochim. Biophys. Acta 360, 38-55.

89. Miller, N.E. (1978) Biochim. Biophys. Acta 529, 131-137.

90. Stein, O. and Stein, Y. (1973) Biochim. Biophys. Acta 326, 232-244.

91. Stein, O., Vanderhoek, J. and Stein, Y. (1976) Biochim. Biophys. Acta 431, 347-358.

92. Green, P.H.R., Tall, A.R. and Glickman, R.M. (1978) J. Clin. Invest. 61, 528-534.

93. Hamilton, R.L. (1978) in Disturbances in Lipid and Lipoprotein Metabolism (Dietschy, J.M., Gotto, A.M. and Ontko, J.A., eds.), pp. 155-171, Am. Physiol. Soc., Bethesda.

94. Glomset, J.A. and Norum, K.R. (1973) Adv. Lipid Res. 11, 1-65.

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

96. Simon, J.B. and Boyer, J.L. (1971) Biochim. Biophys. Acta 218, 549-551.

97. Soutar, A.K., Garner, C.W., Baker, H.N., Sparrow, J.T., Jackson, R.L., Gotto, A.M. and Smith, L.C. (1975) Biochem. 14, 3057-3064.

98. Drevon, C.A., Berg, T. and Norum, K.R. (1977) Biochim. Biophys. Acta 487, 122-136.

99. Roheim, P.S., Rachmilewitz, D., Stein, O. and Stein, Y. (1971) Biochim. Biophys. Acta 248, 315-329.

100. Rachmilewitz, D., Stein, O., Roheim, P.S. and Stein, Y. (1972) Biochim. Biophys. Acta 270, 414-425.

101. Barter, P.J. and Lally, J.I. (1978) Biochim. Biophys. Acta 531, 233-236.

102. Chajek, T. and Fielding, C.J. (1978) Proc. Natl. Acad. Sci. U.S. 75, 3445-3449.

103. Nestel, P.J., Reardon, M. and Billington, T. (1979) Biochim. Biophys. Acta 573, 403-407.

104. Barter, P.J. (1974) J. Lipid Res. 15, 234-242.

105. Roheim, P.S., Haft, D.E., Gidez, L.I., White, A. and Eder, H.A. (1963) J. Clin. Invest. 42, 1277-1285.

106. Brown, M.S. and Goldstein, J.L. (1976) Science 191, 150-154.

107. Brown, M.S. and Goldstein, J.L. (1978) in Disturbances in Lipid and Lipoprotein Metabolism (Dietschy, J.M., Gotto, A.M. and Ontko, J.A., eds.), pp. 173-180, Am. Physiol. Soc., Bethesda.

108. Goldstein, J.L. and Brown, M.S. (1976) Curr. Top. Cell. Regul. 11, 147-182.

109. Goldstein, J.L. and Brown, M.S. (1975) Am. J. Med. 58, 147-150.

110. Anderson, R.G.W., Goldstein, J.L. and Brown, M.S. (1976) Proc. Natl. Acad. Sci. U.S. 73, 2434-2438.

111. Di Ferrante, N., Donelly, P.V., Di Ferrante, D.T., Toma, S. and Gotto, A.M. (1978) Biochem. Biophys. Res. Comm. 84, 366-373.

112. Brown, M.S. and Goldstein, J.L. (1974) Proc. Natl. Acad. Sci. U.S. 71, 788-792.

113. Goldstein, J.L. and Brown, M.S. (1974) J. Biol. Chem. 249, 5153-5162.

114. Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1979) Nature 279, 679-685.

115. Heuser, J. and Evans, L. (1980) J. Cell Biol. 84, 560-583.

116. Miller, N.E. and Yin, J.A. (1978) Biochim. Biophys. Acta 530, 145-150.

117. Brown, M.S., Dana, S.E. and Goldstein, J.L. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2925-2929.

118. Goldstein, J.L., Dana, S.E., Faust, J.R., Beaudet, A.L. and Brown, M.S. (1975) J. Biol. Chem. 250, 8487-8495.

119. Brown, M.S., Dana, S.E. and Goldstein, J.L. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2162-2166.

120. Brown, M.S., Dana, S.E. and Goldstein, J.L. (1974) J. Biol. Chem. 249, 789-796.

121. Goldstein, J.L., Dana, S.E. and Brown, M.S. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4288-4292.

122. Brown, M.S., Dana, S.E. and Goldstein, J.L. (1975) J. Biol. Chem. 250, 4025-4027.

123. Goldstein, J.L., Dana, S.E., Brunschede, G.Y. and Brown, M.S. (1975) Proc. Natl. Acad. Sci. U.S. 72, 1092-1096.

124. Brown, M.S., Faust, J.R. and Goldstein, J.L. (1975) J. Clin. Invest. 55, 783-793.

125. Brown, M.S. and Goldstein, J.L. (1975) Cell 6, 307-316.

126. Goldstein, J.L., Sobhani, M.K., Faust, J.R. and Brown, M.S. (1976) Cell 9, 195-203.

127. Basu, S.K., Goldstein, J.L. and Brown, M.S. (1978) J. Biol. Chem. 253, 3852-3856.

128. Brown, M.S. and Goldstein, J.L. (1976) Cell 9, 663-674.

129. Andersen, J.M. and Dietschy, J.M. (1977) J. Biol. Chem. 252, 3646-3651.

130. Andersen, J.M. and Dietschy, J.M. (1976) Science 193, 903-905.

131. Andersen, J.M. and Dietschy, J.M. (1977) J. Biol. Chem. 252, 3652-3659.

132. Balasubramanian, S., Goldstein, J.L., Faust, J.R. and Brown, M.S. (1976) Proc. Natl. Acad. Sci. U.S. 73, 2564-2568.

133. Ho, Y.K., Brown, M.S., Kayden, H.J. and Goldstein, J.L. (1976) J. Exp. Med. 144, 444-455.

134. Brown, M.S., Anderson, R.G.W. and Goldstein, J.L. (1977) J. Supramol. Struct. 6, 85-94.

135. Ho, Y.K., Brown, M.S., Bilheimer, D.W. and Goldstein, J.L. (1976) J. Clin. Invest. 58, 1465-1474.

136. Faust, J.R., Goldstein, J.L. and Brown, M.S. (1977) J. Biol. Chem. 252, 4861-4871.

137. Nilsson, A., Norden, H. and Wilhelmsson, L. (1973) Biochim. Biophys. Acta 296, 593-603.

138. Stokke, K.T. (1972) Biochim. Biophys. Acta 280, 329-335.

139. Beaufay, H., Amar-Costesec, A., Thinès-Sempoux, D., Wibo, M., Robbi, M. and Berthet, J. (1974) J. Cell Biol. 61, 213-231.

140. Dallman, P.R., Dallner, G., Bergstrand, A. and Ernster, L. (1969) J. Cell Biol. 41, 357-377.

141. Dallner, G. and Ernster, L. (1968) J. Histochem. Cytochem. 16, 611-632.

142. Dallner, G. and Ericsson, J.L.E. (1976) in Progress in Liver Diseases (Popper, H. and Schaffner, F., eds.), Vol. 5, pp. 35-50, Grune and Stratton, New York.

143. Palade, G.E. and Siekevitz, P. (1956) J. Biophys. Biochem. Cytol. 2, 171-199.

144. Siekevitz, P. (1965) Fed. Proc. 24, 1153-1155.

145. Wada, F., Hirata, K., Nakao, K. and Sakamoto, Y. (1969) J. Biochem. 66, 699-703.

146. Boyd, G.S., Grimwade, A.M. and Lawson, M.E. (1973) Eur. J. Biochem. 37, 334-340.

147. Boyd, G.S., Scholan, N.A. and Mitton, J.R. (1969) Adv. Exp. Med. Biol. 4, 443-456.

148. Beher, W.T., Baker, G.D. and Penney, D.G. (1963) J. Nutr. 79, 523-530.

149. Gould, R.G. (1977) in Cholesterol Metabolism and Lipolytic Enzymes (J. Polonovski, ed.), pp. 13-38, Masson Publishing, Inc., New York.

150. Nervi, F.O. and Dietschy, J.M. (1975) J. Biol. Chem. 250, 8704-8711.

151. Nervi, F.O., Weis, H.J. and Dietschy, J.M. (1975) J. Biol. Chem. 250, 4145-4151.

152. Anderson, J.M., Turley, S.D. and Dietschy, J.M. (1979) Proc. Natl. Acad. Sci. U.S. 76, 165-169.

153. Nervi, F.O. and Dietschy, J.M. (1978) J. Clin. Invest. 61, 895-909.

154. Gaylor, J.L. (1972) Adv. Lipid Res. 10, 89-141.

155. Rodwell, V.W., Nordstrom, J.L. and Mitschelen, J.J. (1976) Adv. Lipid Res. 14, 1-74.

156. Spence, J.T. and Gaylor, J.L. (1977) J. Biol. Chem. 252, 5852-5858.

157. Siperstein, M.D. and Fagan, V.M. (1966) J. Biol. Chem. 241, 602-609.

158. Edwards, P.A., Popják, G., Fogelman, A.M. and Edmond, J. (1977) J. Biol. Chem. 252, 1057-1063.

159. Edwards, P.A., Fogelman, A.M. and Popják, G. (1976) Biochem. Biophys. Res. Comm. 68, 64-69.

160. Harry, D.S., Dini, M. and McIntyre, N. (1973) Biochim. Biophys. Acta 296, 209-220.

161. Raicht, R.F., Cohen, B.I., Shefer, S. and Mosbach, E.H. (1975) Biochim. Biophys. Acta 388, 374-384.

162. Brown, M.S. and Goldstein, J.L. (1980) J. Lipid Res. 21, 505-517.

163. Nordstrom, J.L., Mitschelen, J.J. and Rodwell, V.W. (1976) Fed. Proc. 35, 1471.

164. Chow, J.C., Higgins, M. and Rudney, H. (1975) Biochem. Biophys. Res. Comm. 63, 1077-1084.

165. Beg, Z.H., Allman, D.W. and Gibson, D.M. (1973) Biochem. Biophys. Res. Comm. 54, 1362-1369.

166. Brown, M.S., Goldstein, J.L. and Dietschy, J.M. (1979) J. Biol. Chem. 254, 5144-5149.

167. Hunter, C.F. and Rodwell, V.W. (1980) J. Lipid Res. 21, 399-405.

168. Sabine, J.R. and James, M.J. (1976) Life Sci. 18, 1185-1192.

169. Mitropoulos, K.A. and Venkatesan, S. (1977) Biochim. Biophys. Acta 489, 126-142.

170. Higgins, M. and Rudney, H. (1973) Nature 246, 60-61.

171. Turley, S.D. and Dietschy, J.M. (1979) J. Lipid Res. 20, 923-934.

172. Wilson, J.D. (1964) J. Lipid Res. 5, 409-417.

173. Wilson, J.D. (1962) Am. J. Physiol. 203, 1029-1032.

174. Björkhem, I. and Danielsson, H. (1975) Eur. J. Biochem. 53, 63-70.

175. Mosbach, E.H. (1972) in Bile Acids in Human Diseases (Back, P. and Gerok, W., eds.), pp. 89-96, F.K. Schattauer Verlag, Stuttgart.

176. Mitropoulos, K.A., Balasubramanian, S. and Myant, N.B. (1973) Biochim. Biophys. Acta 326, 428-438.

177. Hanson, R.F. and Pries, J.M. (1977) Gastroenterology 73, 611-618.

178. Shefer, S., Hauser, S. and Mosbach, E.H. (1968) J. Lipid Res. 9, 328-333.

179. Shefer, S., Hauser, S., Bekersky, I. and Mosbach, E.H. (1969) J. Lipid Res. 10, 646-655.

180. Shefer, S., Hauser, S., Bekersky, I. and Mosbach, E.H. (1970) J. Lipid Res. 11, 404-411.

181. Danielsson, H., Einarsson, K. and Johansson, G. (1967) Eur. J. Biochem. 2, 44-49.

182. Johansson, G. (1970) Eur. J. Biochem. 17, 292-295.

183. Bangham, A.D. (1972) Ann. Rev. Biochem. 41, 753-776.

184. Demel, R.A. and de Kruyff, B. (1976) Biochim. Biophys. Acta 457, 109-132.

185. Hendler, R.W. (1971) Physiol. Rev. 51, 66-97.

186. Oldfield, E. (1973) Science 180, 982-983.

187. Oldfield, E. and Chapman, D. (1972) FEBS Lett. 23, 285-297.

188. Rothman, J.E. and Engelman, D.M. (1972) Nature 237, 42-44.

189. Siekevitz, P. (1972) Ann. Rev. Physiol. 34, 117-140.

190. Hubbell, W.L. and McConnel, H.M. (1971) J. Am. Chem. Soc. 93, 314-326.

191. Opella, S.J., Yesinowski, J.P. and Waugh, J.S. (1976) Proc. Natl. Acad. Sci. U.S. 73, 3812-3815.

192. Lancee-Hermkens, A.M.W. and de Kruyff, B. (1977) Biochim. Biophys. Acta 470, 141-151.

193. de Kruyff, B., van Dijck, P.W.M., Demel, R.A., Schuijff, A., Brants, F. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 356, 1-7.

194. Depierre, J. and Dallner, G. (1975) Biochim. Biophys. Acta 415, 411-472.

195. Depierre, J. and Ernster, L. (1977) Ann. Rev. Biochem. 46, 201-262.

196. Duppel, W. and Dahl, G. (1976) Biochim. Biophys. Acta 426, 408-417.

197. Huang, C. (1977) Lipids 12, 348-356.

198. Darke, A., Finn, E.G., Flovk, A.K. and Phillips, M.C. (1972) J. Mol. Biol. 63, 265-279.

199. Cullis, P.R., de Kruyff, B. and Richards, R.E. (1975) Biochim. Biophys. Acta 426, 433-446.

200. Demel, R.A., Bruckdorfer, K.R. and van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 311-320.

201. Suckling, K.E. and Boyd, G.S. (1976) Biochim. Biophys. Acta 436, 295-300.

202. Craig, I.F. (1978) Ph.D. Thesis, University of Edinburgh.

203. Craig, I.F., Boyd, G.S. and Suckling, K.E. (1978) Biochim. Biophys. Acta 508, 418-421.

204. Schreier-Muccillo, S., Marsh, D., Dugas, H., Schneider, H. and Smith, I.C.P. (1973) Chem. Phys. Lipids 10, 11-27.

205. Ladbrooke, B.D., Williams, R.M. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333-340.

206. Lippert, J.L. and Peticolas, W.L. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1572-1576.

207. Spiker, R.C. and Levin, I.W. (1976) Biochim. Biophys. Acta 433, 457-468.

208. Cullis, P.R. and de Kruyff, B. (1978) Biochim. Biophys. Acta 507, 207-218.

209. Fourcans, B. and Jain, M.K. (1974) Adv. Lipid Res. 12, 147-226.

210. Farias, R.N., Bloj, B., Morero, R.D., Sineriz, F. and Trucco, R.E. (1975) Biochim. Biophys. Acta 415, 231-251.

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

212. Madden, T.D., Chapman, D. and Quinn, P.J. (1979) Nature 279, 538-540.

213. Ingelman-Sundberg, M. (1977) Biochim. Biophys. Acta 488, 225-234.

214. Duppel, W., Poensgen, J. and Ullrich, V. (1976) Hoppe Seylers Z. Physiol. Chem. 357, 1025.

215. Ingelman-Sundberg, M. and Glaumann, H. (1977) FEBS Lett. 78, 72-78.

216. Vore, M., Hamilton, J.G. and Lu, A.Y.H. (1974) Biochem. Biophys. Res. Comm. 56, 1038-1044.

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

218. Duppel, W. and Ullrich, V. (1976) Biochim. Biophys. Acta 426, 399-407.

219. Becker, J.F., Meehan, T. and Bartholomew, J.C. (1978) Biochim. Biophys. Acta 512, 136-146.

220. Yang, C.S., Strickhart, F.S. and Kicha, L.P. (1977) Biochim. Biophys. Acta 465, 362-370.

221. Stier, A. (1976) Biochem. Pharmacol. 25, 109-113.

222. Ernster, L., Siekevitz, P. and Palade, G.E. (1962) J. Cell Biol. 15, 541-562.

223. Morton, R.K. (1950) Nature 166, 1092.

224. Morton, R.K. (1955) in Methods in Enzymology (Kolowick, S.P. and Kaplan, N.O., eds.), Vol. 1, pp. 25-51, Academic Press, New York.

225. Hattersley, N.G. (1975) Ph.D. Thesis, University of Edinburgh.

226. Hattersley, N.G. and Boyd, G.S. (1975) FEBS Lett. 53, 80-83.

227. Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370-2378.

228. Estabrook, R.W. and Werringloer, J. (1978) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 52, pp. 212-220, Academic Press, New York.

229. Strobel, H.W. and Dignam, J.D. (1978) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 52, pp. 89-96, Academic Press, New York.

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

231. Williams, C.H. and Kamin, H. (1962) J. Biol. Chem. 237, 587-595.

232. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

233. Layne, E. (1957) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 3, pp. 450-451, Academic Press, New York.

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

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

236. McClare, C.W.F. (1971) Anal. Biochem. 39, 527-530.

237. Trinder, P. (1969) Ann. Clin. Biochem. 6, 24-27.

238. Gamble, W., Vaughan, M., Kruth, H.S. and Avigan, J. (1978) J. Lipid Res. 19, 1068-1070.

239. Myant, N.B. and Mitropoulos, K.A. (1977) J. Lipid Res. 18, 135-153.

240. Brown, M.J.G. (1972) Ph.D. Thesis, University of Edinburgh.

241. Björkhem, I. and Danielsson, H. (1974) Anal. Biochem. 59, 508-516.

242. van Cantfort, J. and Gielen, J. (1975) Eur. J. Biochem. 55, 33-40.

243. Malinov, N.R., McLaughlin, P., Papworth, L. and Kittinger, G.W. (1975) Steroids 25, 663-672.

244. Sanghvi, A., Kienle, M.G. and Galli, G. (1978) Anal. Biochem. 85, 430-436.

245. Mitropoulos, K.A. and Balasubramanian, S. (1972) Biochem. J. 128, 1-9.

246. Shefer, S., Nicolau, G. and Mosbach, E.H. (1975) J. Lipid Res. 16, 92-96.

247. Mitton, J.R., Scholan, N.A. and Boyd, G.S. (1971) Eur. J. Biochem. 20, 569-579.

248. Goodman, D.S., Deykin, D. and Shiratori, T. (1964) J. Biol. Chem. 239, 1335-1345.

249. Goodman, D.S. (1969) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 15, pp. 522-537, Academic Press, New York.

250. Balasubramanian, S., Mitropoulos, K.A. and Myant, N.B. (1973) Eur. J. Biochem. 34, 77-83.

251. Balasubramanian, S., Mitropoulos, K.A. and Myant, N.B. (1975) Biochim. Biophys. Acta 398, 172-177.

252. Balasubramanian, S., Mitropoulos, K.A. and Venkatesan, S. (1978) Eur. J. Biochem. 90, 377-383.

253. Balasubramanian, S., Venkatesan, S., Mitropoulos, K.A. and Peters, T.J. (1978) Biochem. J. 174, 863-872.

254. Hashimoto, S. and Dayton, S. (1977) Atherosclerosis 28, 447-452.

255. Faeder, E.J. and Siegel, L.M. (1973) Anal. Biochem. 53, 332-336.

256. Bloj, B. and Zilversmit, D.B. (1977) Biochem. 16, 3943-3948.

257. Huang, C. (1969) Biochem. 8, 344-351.

258. Peterson, E.A. and Soper, H.A. (1962) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 5, pp. 3-27, Academic Press, New York.

259. Myant, N.B. and Eder, H.A. (1961) J. Lipid Res. 2, 363-368.

260. Boyd, G.S. and Lawson, M.E. (1976) FEBS Lett. 64, 435-439.

261. Balasubramanian, S., Press, C.M., Mitropoulos, K.A., Magide, A.A. and Myant, N.B. (1976) Biochim. Biophys. Acta 441, 308-315.

262. Danielsson, H. (1972) Steroids 20, 63-72.

263. Mitropoulos, K.A., Balasubramanian, S., Gibbons, G.F. and Reeves, B.E.A. (1972) FEBS Lett. 27, 203-206.

264. Back, P., Hamprecht, B. and Lynen, F. (1969) Arch. Biochem. Biophys. 133, 11-21.

265. Kandutsch, A.A. and Saucier, S.E. (1969) J. Biol. Chem. 244, 2299-2305.

266. Balasubramanian, S. and Mitropoulos, K.A. (1975) Biochem. Soc. Trans. 3, 363-368.

267. Balasubramanian, S., Mitropoulos, K.A. and Myant, N.B. (1975) in Advances in Bile Acid Research (Matern, S., Hackenschmidt, J., Back, P. and Gerok, W., eds.), pp. 61-67, F.K. Schattauer Verlag, Stuttgart.

268. Mayer, D. (1975) in Advances in Bile Acid Research (Matern, S., Hackenschmidt, J., Back, P. and Gerok, W., eds.), pp. 53-59, F.K. Schattauer Verlag, Stuttgart.

269. Guder, W., Notte, I. and Wieland, O. (1968) Eur. J. Biochem. 4, 273-278.

270. Edwards, P.A. (1973) J. Biol. Chem. 248, 2912-2917.

271. Takeuchi, N., Ito, M. and Yamamura, Y. (1974) Atherosclerosis 20, 481-494.

272. Mayer, D. (1972) in Bile Acids in Human Diseases (Back, P. and Gerok, W., eds.), pp. 103-109, F.K. Schattauer Verlag, Stuttgart.

273. Bucher, N.L.R., Overoth, P. and Lynen, F. (1960) Biochim. Biophys. Acta 40, 491-501.

274. Mitropoulos, K.A., Myant, N.B., Gibbons, G.F., Balasubramanian, S. and Reeves, B.E.A. (1974) J. Biol. Chem. 249, 6052-6056.

275. Björkhem, I. and Lewenhaupt, A. (1979) J. Biol. Chem. 254, 5252-5256.

276. Mitropoulos, K.A., Venkatesan, S.,Balasubramanian, S. and Peters, T.J. (1978) Eur. J. Biochem. 82, 419-429.

277. Ryan, D., Lu, A.Y.H., West, S. and Levin, W. (1974) J. Biol. Chem. 250, 2157-2163.

278. Lu, A.Y.H., Kuntzman, R., West, S., Jacobson, M. and Conney, A.H. (1972) J. Biol. Chem. 247, 1727-1734.

279. Lotliker, P.D. and Dwyer, E.N. (1976) Biochem. J. 160, 821-824.

280. Dean, W.L. and Coon, M.J. (1977) J. Biol. Chem. 252, 3255-3261.

281. Alvares, A.P. and Siekevitz, P. (1973) Biochem. Biophys. Res. Comm. 54, 923-929.

282. Alvares, A.P., Schilling, G., Levin, W. and Kuntzman, R. (1967) Biochem. Biophys. Res. Comm. 29, 521-526.

283. Lu, A.Y.H., Levin, W., West, S.B., Jacobson, M., Ryan, D., Kuntzman, R. and Conney, A.H. (1973) J. Biol. Chem. 248, 456-460.

284. Scholan, N.A. (1969) Ph.D. Thesis, University of Edinburgh.

285. Einarsson, K. and Johansson, G. (1968) Eur. J. Biochem. 6, 293-298.

286. Shefer, S., Hauser, S. and Mosbach, E.H. (1972) J. Lipid Res. 13, 69-70.

287. Mellon, W.S., Witiak, D.T. and Feller, D.R. (1978) Biochem. Pharmacol. 27, 1055-1062.

288. Brown, M.J.G. and Boyd, G.S. (1974) Eur. J. Biochem. 44, 37-47.

289. Boyd, G.S., Brown, M.J.G., Hattersley, N.G., Lawson, M.E. and Suckling, K.E. (1975) in Advances in Bile Acid Research (Matern, S., Hackenschmidt, J., Back, P. and Gerok, W., eds.), pp. 45-51, F.K. Schattauer Verlag, Stuttgart.

290. Strobel, H.W., Lu, A.Y.H., Heidema, J. and Coon, M.J. (1970) J. Biol. Chem. 245, 4851-4854.

291. Coon, M.J., Strobel, H.W., Autor, A.P., Heidema, J. and Duppel, W. (1972) in Biological Hydroxylation Mechanisms (Boyd, G.S. and Smellie, R.M.S., eds.), pp. 45-54, Academic Press, London.

292. Lu, A.Y.H. and Levin, W. (1974) Biochim. Biophys. Acta 344, 205-240.

293. Miyake, Y., Gaylor, J.L. and Mason, H.S. (1968) J. Biol. Chem. 243, 5788-5797.

294. Razin, S. (1972) Biochim. Biophys. Acta 265, 241-296.

295. Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29-79.

296. Ebel, R.E., O'Keefe, D.H. and Peterson, J.A. (1978) J. Biol. Chem. 253, 3888-3897.

297. Comai, K. and Gaylor, J.L. (1973) J. Biol. Chem. 248, 4947-4955.

298. Dialameh, G.H. (1978) Biochem. Biophys. Res. Comm. 81, 19-23.

299. Levin, W., Ryan, D., West, S. and Lu, A.Y.H. (1974) J. Biol. Chem. 249, 1747-1754.

300. Autor, A.P., Kaschnitz, R.M., Heidema, J.K. and Coon, M.J. (1973) Mol. Pharmacol. 9, 93-104.

301. Gibson, G. and Schenkman, J.B. (1978) J. Biol. Chem. 253, 5957-5963.

302. Ichikawa, Y. and Yamano, T. (1970) Biochim. Biophys. Acta 200, 220-240.

303. Imai, Y. and Sato, R. (1974) Biochem. Biophys. Res. Comm. 60, 8-14.

304. Ingelman-Sundberg, M. and Gustafsson, J.-A. (1977) FEBS Lett. 74, 103-106.

305. Johnson, E.F. and Muller-Eberhard, U. (1977) J. Biol. Chem. 252, 2839-2845.

306. Huang, M.-T., West, S.B. and Lu, A.Y.H. (1976) J. Biol. Chem. 251, 4659-4665.

307. Jernström, B., Capdevila, J., Jakobsson, S. and Orrenius, S. (1975) Biochem. Biophys. Res. Comm. 64, 815-822.

308. Guengerich, F.P. (1977) Mol. Pharmacol. 13, 911-923.

309. Ichikawa, Y. and Yamano, T. (1967) Biochim. Biophys. Acta 131, 490-497.

310. Lu, A.Y.H. and Coon, M.J. (1968) J. Biol. Chem. 243, 1331-1332.

311. Lu, A.Y.H. and Levin, W. (1972) Biochem. Biophys. Res. Comm. 46, 1334-1339.

312. van der Hoeven, T.A., Heidema, J.K., Michniewicz, B.M. and Coon, M.J. (1973) Fed. Proc. 32, 631.

313. Fujita, T., Shoeman, D.W. and Mannering, G.J. (1973) J. Biol. Chem. 248, 2192-2201.

314. Sato, R., Satake, H. and Imai, Y. (1973) Drug Metab. Dispos. 1, 6-13.

315. van der Hoeven, T.A. and Coon, M.J. (1974) J. Biol. Chem. 249, 6302-6310.

316. Haugen, D.A. and Coon, M.J. (1976) J. Biol. Chem. 251, 7929-7939.

317. Imai, Y. and Sato, R. (1974) J. Biochem. 75, 689-697.

318. Björkhem, I., Danielsson, H. and Wikvall, K. (1974) Biochem. Biophys. Res. Comm. 61, 934-941.

319. Cottman, J., Danielsson, H., Hansson, R. and Wikvall, K. (1977) in Bile Acid Metabolism in Health and Disease (Paumgartner, G. and Stiehl, A., eds.), pp. 1-10, MTP Press, Lancaster.

320. Hansson, R. and Wikvall, K. (1979) Eur. J. Biochem. 419-426.

321. Hemler, M., Lands, W.E.M. and Smith, W.L. (1976) J. Biol. Chem. 251, 5575-5579.

322. Coon, M.J., Nordbloom, G.D., White, R.E. and Haugen, D.A. (1975) Biochem. Soc. Trans. 3, 813-817.

323. Coon, M.J., White, R.E., Nordbloom, G.D., Ballou, D.P. and Guengerich, F.P. (1977) Croatica Chemica Acta 49, 163-177.

324. Gustafsson, J.-A., Hrycay, E.G. and Ernster, L. (1976) Arch. Biochem. Biophys. 174, 440-453.

325. Hrycay, E.G., Gustafsson, J.-A., Ingelman-Sundberg, M. and Ernster, L. (1975) FEBS Lett. 56, 161-165.

326. Hrycay, E.G., Gustafsson, J.-A., Ingelman-Sundberg, M. and Ernster, L. (1976) Eur. J. Biochem. 61, 43-52.

327. Lichtenberger, F., Nastainczyk, W. and Ullrich, V. (1976) Biochem. Biophys. Res. Comm. 70, 939-946.

328. Lichtenberger, F. and Ullrich, V. (1976) Hoppe Seylers Z. Physiol. Chem. 357, 1037.

329. Rahimtula, A.D. and O'Brien, P.J. (1974) Biochem. Biophys. Res. Comm. 60, 440-447.

330. Norum, K.R., Lilljeqvist, A.-C. and Drevon, C.A. (1977) Scand. J. Gastroenterol. 12, 281-288.

331. Erickson, S.K., Shrewsbury, M.A. and Gould, R.G. (1978) Circulation 58, Supplement 4, II-169.

332. Hashimoto, S., Dayton, S., Alfin-Slater, R.B., Bui, P.T., Baker, N. and Wilson, L. (1974) Circulation Res. 34, 176-183.

333. Rothblat, G.H., Naftulin, M. and Arbogast, L.Y. (1977) Proc. Soc. Exp. Biol. Med. 155, 501-506.

334. Spector, A.A., Mathur, S.N. and Kaduce, T.L. (1979) Prog. Lipid Res. 18, 31-53.

335. Day, A.J. and Proudlock, J.W. (1974) Atherosclerosis 19, 253-258.

336. Mukherjee, S., Kunitake, G. and Alfin-Slater, R.B. (1958) J. Biol. Chem. 230, 91-96.

337. Lynen, F. (1953) Fed. Proc. 12, 683-691.

338. Lynen, F. (1961) Fed. Proc. 20, 941-951.

339. Stokke, K.T. and Norum, K.R. (1970) Biochim. Biophys. Acta 210, 202-204.

340. Chesterton, C.J. (1968) J. Biol. Chem. 243, 1147-1151.

341. Grover, A.K., Forrest, B.J. Buchinski, R.K. and Cushley, R.J. (1979) Biochim. Biophys. Acta 550, 212-221.

342. Oshino, N., Imai, Y. and Sato, R. (1966) Biochim. Biophys. Acta 128, 13-28.

343. Kritchevsky, D. and Kothari, H.V. (1978) Adv. Lipid Res. 16, 221-266.

344. Kaduce, T.L., Schmidt, R.W. and Spector, A.A. (1978) Biochem. Biophys. Acta 81, 462-468.

345. Hamprecht, B., Nüssler, C. and Lynen, F. (1969) FEBS Lett. 4, 117-121.

346. Balasubramanian, S., Mitropoulos, K.A. and Myant, N.B. (1972) in Bile Acids in Human Diseases (Back, P. and Gerok, W., eds.), pp. 97-102, F.K. Schattauer Verlag, Stuttgart.

347. Romsos, D.R. and Leveille, G.A. (1974) Adv. Lipid Res. 12, 97-146.

348. Danielsson, H. (1973) Steroids 22, 567-579.

349. Nilsson, A. (1975) Eur. J. Biochem. 51, 337-342.

350. Mitropoulos, K.A., Balasubramanian, S., Venkatesan, S. and Reeves, B.E.A. (1978) Biochim. Biophys. Acta 530, 99-111.

351. Erickson, S.K. (1978) Fed. Proc. 37, 1428.

352. Greim, H., Trülzsch, D., Roboz, J., Dreissler, K., Czygan, P., Hutterer, F., Schaffner, F. and Popper, H. (1972) Gastroenterology 63, 837-845.

353. Lutton, C., Mathé, D. and Chevallier, F. (1973) Biochim. Biophys. Acta 306, 483-496.

354. Boyd, G.S., Eastwood, M.A. and MacLean, N. (1966) J. Lipid Res. 7, 83-94.

355. Gustafsson, J. (1978) J. Lipid Res. 19, 237-243.

356. Rosen, J.M. and Rothblat, G.H. (1977) Lipids 12, 222-227.

357. Rothblat, G.H. (1974) Lipids 9, 526-535.

358. Rothblat, G.H., Arbogast, L., Kritchevsky, D. and Naftulin, M. (1976) Lipids 11, 97-108.

359. Janiak, M.J., Loomis, C.R., Shipley, G.G. and Small, D.M. (1974) J. Mol. Biol. 86, 325-339.

360. Craig, M.C., Dugan, R.E., Muesing, R.A., Slakey, L.L. and Porter, J.W. (1972) Arch. Biochem. Biophys. 151, 128-136.

361. Goldfarb, S. and Pitot, H.C. (1972) J. Lipid Res. 13, 797-801.

362. Swell, L. and Treadwell, C.R. (1962) Proc. Soc. Exp. Biol. Med. 110, 55-57.

363. Gavey, K.L. (1979) Fed. Proc. 38, 785.

364. Hashimoto, S. and Dayton, S. (1978) Biochem. Biophys. Res. Comm. 82, 1111-1120.

365. Brenneman, D.E., Kaduce, T. and Spector, A.A. (1977) Lipid Res. 18, 582-591.

366. Rodwell, V.W., McNamara, D.J. and Shapiro, D.J. (1973) Adv. Enzymology 38, 373-412.

367. Parry, G., Palmer, D.N. and Williams, D.J. (1976) FEBS Lett. 67, 123-129.

368. Taniguchi, H., Imai, Y., Iyanagi, T. and Sato, R. (1979) Biochim. Biophys. Acta 550, 341-356.

369. Yang, C.S. (1975) FEBS Lett. 54, 61-64.

370. Gibson, G.G., Cinti, D.L., Sligar, S.G. and Schenkman, J.B. (1980) J. Biol. Chem. 255, 1867-1873.

371. Ogura, M., Ayaki, Y. and Goto, M. (1976) J. Biochem. 80, 537-545.

372. Stier, A., Finch, S.A.E. and Bösterling, B. (1978) FEBS Lett. 91, 109-112.

373. Shinitzky, M. (1978) FEBS Lett. 85, 317-320.

374. Hashimoto, S. and Dayton, S. (1979) Biochim. Biophys. Acta 573, 354-360.

375. McLaren, A.D. and Packer, L. (1970) Adv. Enzymol. 33, 245-308.

376. MacRitchie, F. (1978) Adv. Protein Chem. 32, 283-326.

377. Gatt, S. and Barenholz, Y. (1973) Ann. Rev. Biochem. 42, 61-90.

378. Gatt, S. and Bartfai, T. (1977) Biochim. Biophys. Acta 488, 1-12.

379. Gatt, S. and Bartfai, T. (1977) Biochim. Biophys. Acta 488, 13-24.

380. Nicolson, G.L. and Singer, S.J. (1971) Science 175, 720-731.

381. Guidotti, G. (1972) Ann. Rev. Biochem. 41, 731-752.

382. Coleman, R. (1973) Biochim. Biophys. Acta 300, 1-30.

383. Haugen, D.A., Armes, L.G., Yasunobu, K.T. and Coon, M.J. (1977) Biochem. Biophys. Res. Comm. 77, 967-973.

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

385. Rogers, M.J. and Strittmatter, P. (1975) J. Biol. Chem. 250, 5713-5718.

386. Rogers, M.J. and Strittmatter, P. (1974) J. Biol. Chem. 249, 895-900.

387. Okuda, T., Mihara, K. and Sato, R. (1972) J. Biochem. 72, 987-992.

388. Barter, P.J. (1974) J. Lipid Res. 15, 11-19.

389. Mitropoulos, K.A., Venkatesan, S. and Balasubramanian, S. (1980) Biochim. Biophys. Acta 619, 247-257.

390. Gidez, L.I., Roheim, P.S. and Eder, H.A. (1965) J. Lipid Res. 6, 377-382.

391. Spector, A.A., Kaduce, T.L. and Dane, R.W. (1980) J. Lipid Res. 21, 169-179.

392. Lindstedt, S. (1957) Acta Chem. Scand. 11, 417-420.

393. Davis, R.A., Engelhorn, S.C., Pangburn, S.H., Weinstein, D.B. and Steinberg, D. (1979) J. Biol. Chem. 254, 2010-2016.

394. Stein, O., Bar-On, H. and Stein, Y. (1972) in Progress in Liver Diseases (Popper, H. and Schaffner, F., eds.), Vol. 4, pp. 45-62, Grune and Stratton, London.

395. Balasubramanian, S., Goldstein, J.L., Faust, J.R., Brunschede, G.Y. and Brown, M.S. (1977) J. Biol. Chem. 252, 1771-1779.

396. Neelon, V.J. and Lack, L. (1977) Biochim. Biophys. Acta 487, 137-144.

397. Mosbach, E.H. (1972) Arch. Internal Med. 130, 478-487.

398. Strange, R.C., Nimmo, I.A. and Percy-Robb, I.W. (1976) Biochem. J. 156, 427-433.