The Intracellular Control of Cholesterol Metabolism

by

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To my older brother, for putting up with me.

"When the going gets weird the weird turn pro."

Gonzo

(Hunter S. Thompson)

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Abbreviations

ACAT	acyl coenzyme A: cholesterol O-acyltransferase (E.C.2.3.1.26)
Azacholesterol	6-azacholes-4-en-3ß-ol-5-one
Chenodeoxycholic acid	3α, 7α dihydroxy -5ß cholanic acid
Cholesterol 7α-hyroxylase	cholesterol 7α-monoxygenase (E.C.1.14.13.17)
Cholic Acid	3α, 6ß, 7ß-trihydroxy-5ß-cholanic acid
d	density
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetracetic acid
FH	Familial hypercholesterolaemia
HDL	High density lipoprotein
HMG CoA	3-hydroxy-3-methyl glutaryl coenzyme A
HMG CoA Reductase	3-hydroxy-3-methyl glutaryl coenzyme A reductase (E.C.1.1.1.34)
IDL	Intermediate density lipoprotein
LCAT	Lecithin: cholesterol acyltransferase (E.C.2.3.1.43)
LDL	Low density lipoprotein (E.C.2.3.1.43)
LPL	Lipoprotein lipase
ß-Muricholic acid	3α, 6ß, 7ß-trihyroxy-5ß-cholanic acid.
NADH	Reduced nicotinamide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
PAGE	Polyacrylamide gel electrophoresis
RIA	Radioimmunoassay
r.p.m.	Revolutions per minute
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylene diamine
THF	Tetrahydrofuran
t.l.c.	Thin layer chromatography
VLDL	Very low density lipoproteins

WHHL	Watanabe heritable hyperlipidaemic
58-035	(3-[decyldimethylsiyl]-N-[2-(4-methylphenyl)-1-phenylethyl] propanamide

ABSTRACT

The liver has a major role in the metabolism of cholesterol, being the main site of lipoprotein assembly and degradation and the only tissue where the metabolism of cholesterol to bile acids occurs. This provides the major pathway for the removal of cholesterol from the body.

The results described in this thesis concern the use of specific enzyme inhibitors (58-035, Azacholesterol, Mevinolin) to determine the intracellular use of different sources of cholesterol in monolayers of rat hepatocytes. In particular, the fates of newly synthesized cholesterol from mevalonic acid and cholesterol derived from HDL_2 were investigated.

Incubation of hepatocyte monolayers with 58-035 resulted in the inhibition of esterification. In the presence of mevalonic acid as a cholesterol source, 58-035 stimulated bile acid synthesis. Azacholesterol inhibited bile acid synthesis, had no effect on cholesterol synthesis, and in the presence of mevalonic acid, stimulated secretion of cholesterol by the hepatocytes; it had no effect on cholesterol esterification. Mevinolin inhibited cholesterol synthesis and as a result inhibited esterification. HDL_2, in the presence of mevinolin, was used as a cholesterol source. It stimulated bile acid synthesis and cholesterol esterification. Addition of 58-035 to the system resulted in the inhibition of both esterification and bile acid synthesis. Overall, the results indicated that different intracllular pools of free cholesterol exist and that the inter-relationships of these pools give a complex pattern of flux of intracellular cholesterol between various pathways in the rat hepatocyte.

CONTENTS

CHAPTER 1

Introduction

1.1 General Introduction

Cholesterol, a necessary component of all mammalian tissues, has critical roles as a structural constituent of all membranes and lipoproteins and as an immediate precursor of steroid hormones, vitamins and bile acids. To meet these demands, a series of synthetic, transport and regulatory mechanisms have evolved to ensure a sufficient supply of cholesterol to the tissues. The total body pool of cholesterol is derived from two sources: dietary and synthetic. De novo synthesis of cholesterol can occur in all nucleated mammalian cell types; however most synthesis occurs in the liver and small intestine (Andersen and Dietschy, 1979; Dietschy and Wilson, 1970; Turley et al., 1981).

The combination of dietary uptake and de novo synthesis usually supplies more cholesterol than is utilized during normal metabolic turnover (Turley and Dietschy, 1982). It is thus important that regulatory mechanisms exist not only to ensure adequate provision, but also to prevent accumulation of cholesterol. For some time, it has been established that high levels of plasma cholesterol correlate positively with the incidence of ischaemic heart disease (Lipid Research Clinics Coronary Primary Prevention Trial Results, 1984), which indicates the seriousness of excess accumulation. The problem is that mammalian tissues are not capable of degrading the sterol nucleus. This knowledge has provoked extensive investigation into possible mechanisms of cholesterol removal from the body. Particularly, the synthesis and excretion of bile acids and the removal of plasma lipoproteins from the circulation have been closely studied.

As mentioned, the liver is a primary site of cholesterol synthesis; it is also a major site for the synthesis, uptake and degradation of plasma lipoproteins, and the only site of bile acid synthesis and excretion, which provides the major route for cholesterol removal from the body. Thus, the liver has a central role in the control of the total body pool of sterols.

1.2 Cholesterol Synthesis

One of the major sources of total body cholesterol is de novo synthesis. In the rat, cholesterol synthesis by the major organs and tissues has been studied, and it has been demonstrated that the liver accounts for most of the total body synthesis (Turley et al., 1981). The control and regulation of its synthesis has been shown to be very complex, and that the uptake of cholesterol via a lipoprotein carrier affects the rate of hepatic cholesterol synthesis (Andersen and Dietschy, 1977a). Chylomicron remnant or LDL uptake has been shown to suppress hepatic cholesterol synthesis, whilst HDL had no effect (Andersen and Dietschy, 1977b). The rate of cholesterol synthesis varies widely according to the stimuli.

The formation of cholesterol begins with acetyl CoA and proceeds through approximately 20 steps. The rate limiting step of its synthesis is the conversion of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) to mevalonic acid. The reaction is catalyzed by the enzyme HMG-CoA reductase, an enzyme that has been shown to undergo activation - inactivation by a dephosphorylation - phosphorylation regulatory mechanism (Nordstrom et al., 1977; Gibson and Ingebritsen, 1978; Beg et al., 1978). Mevalonic acid undergoes a series of condensation reactions to form squalene, which is cyclized to lanosterol. The latter is converted to cholesterol via a series of steps that modify the ring and side chain structure of the molecule.

The control and regulation of its synthesis has been shown to be very complex (Andersen and Dietschy 1977a). A rise in hepatic cholesterol exerts feedback inhibition of cholesterol synthesis, and the amount of HMG-CoA reductase is reduced by a high intracellular concentration of cholesterol. The precise mechanism by which cholesterol exerts this feedback control is not known, but some investigations have suggested that the regulating sterol is not cholesterol but an oxidation product such as 26-hydroxycholesterol (Kandutsch, 1978). Further to this, it has been shown that the uptake of cholesterol in the form of lipoproteins affects the rate of hepatic cholesterol synthesis. Chlyomicron remnants of LDL were shown to suppress hepatic cholesterol synthesis whilst HDL had no

effect (Andersen and Dietschy 1977b). Furthermore, the rate of cholesterol synthesis has been shown very widely depending upon the stimuli (Andersen and Dietschy, 1977a and 1977b; Koelz et al. 1982; Grundy, 1986)

A review by Turley and Dietschy (1982) outlines the rates of cholesterol synthesis in the liver and its contribution to whole body cholesterol in a number of species.

1.3 The Bile Acids

Bile acids are C-24 to C-28 carboxylic acids with a steroid nucleus derived from cholesterol, containing hydroxylic substituents and all or part of the side chain of 5ß - cholestane. Nearly all conjugated bile acids have 24 carbon atoms with the basic structure of 5ß - cholan-24-oic acid (figure 1.1). This type is the major one found in man and experimental animals. There are, however, C-27 bile alcohol sulphates to be found in lower vertebrates and taurine conjugates of C-27 and C-28 bile acids have been isolated from reptiles and amphibians (Haslewood, 1978).

The hydroxyl substituents of bile acids usually occur at C-3, C-7 and/or C-12 on the steroid nucleus. The two major bile acids found in most species are 3α, 7α 12α trihydroxy - 5ß cholan - 24 oic acid (cholic acid); 3α, 7α dihydroxy - 5ß cholan - 24 oic acid (chenodeoxycholic acid). There is a good deal of species variation that occurs. For instance, in man another major bile acid is deoxycholic acid, with small amounts of lithocholic acid also being synthesized; in rat, ß-muricholic acid is one of the primary bile acids synthesized.

Free, or unconjugated, bile acids are not usually present in the bile, but are enzymatically conjugated to glycine or taurine by peptide linkage at the C.24 position. As there is only one enzyme in the liver responsible for conjugation, the amount of glycine or taurine conjugated is dependent upon the substrate supply and the substrates differing affinities for the enzyme (Vessey, 1978).

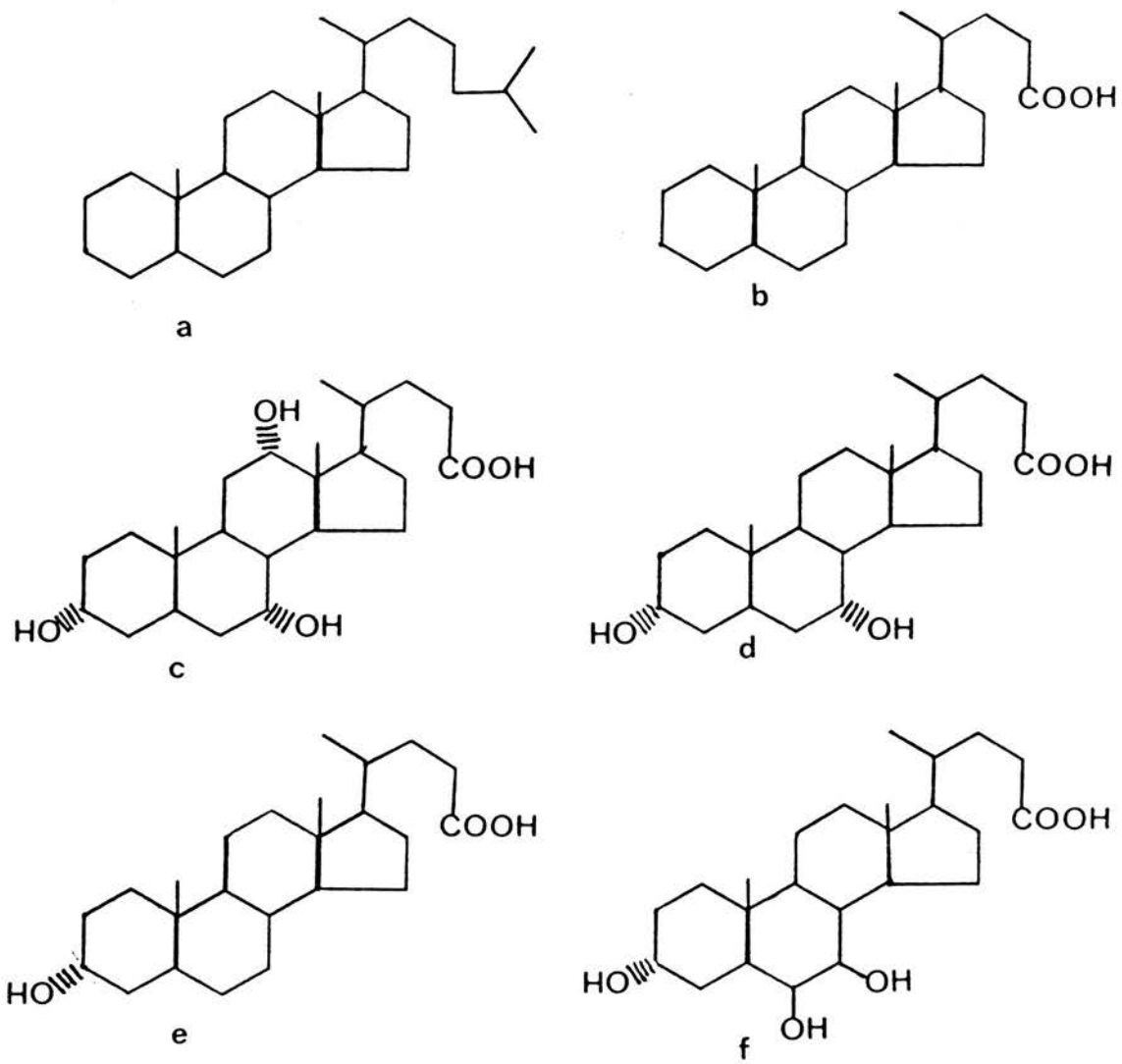


Figure 1.1 Chemical structures of (a) 5α-cholestane; (b) 5ß-cholan-24-oic acid; (c) cholic acid; (d) chenodeoxycholic acid; (e) lithocholic acid and (f) β-muricholic acid.

In vertebrates, formation of bile acids occurs in the liver via a series of reactions, initiated by the 7α - hydroxylation of cholesterol, (Danielsson and Sjovall, 1975; Salen and Shefer, 1983). They are then conjugated, secreted into the bile cannaliculi and thence to the biliary tract and the intestinal lumen. Once into the intestinal lumen, the bile acids facilitate the formation of the micellar phase that is necessary for the absorption of cholesterol, fat-soluble vitamins and the products of triglyceride hydrolysis formed during fat digestion.

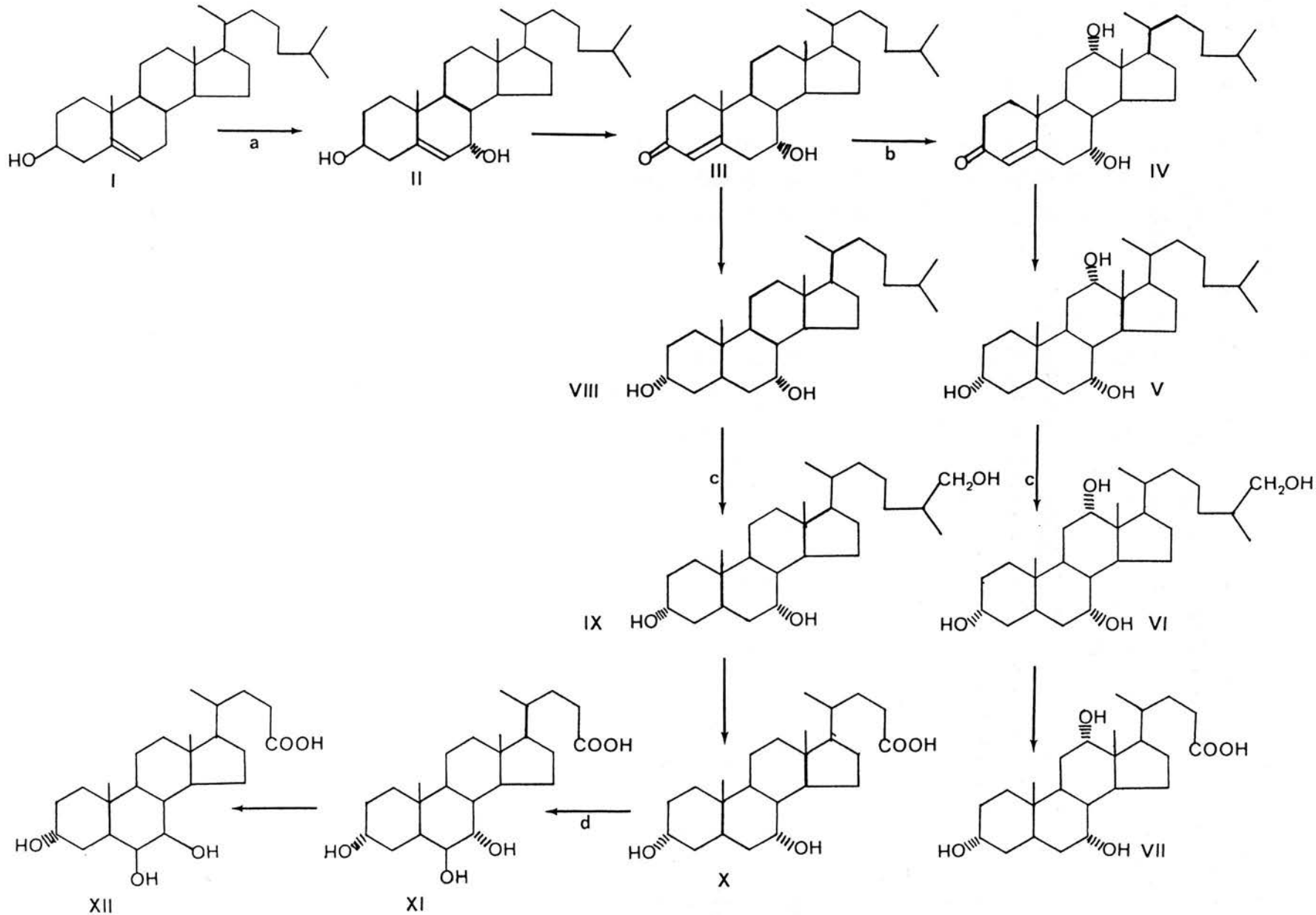
In mammalian species, the pathway of bile acid synthesis is essentially the same (Figure 1.2), but there are considerable variations in the end products (Elliot and Hyde, 1971). As mentioned, in most species cholic and chenodeoxycholic acids are the major bile acids produced; however, in rats, chenodeoxycholic acid can be further metablised to α - and ß-muricholic acids (Mahawold et al., 1957; Matschiner et al., 1971; Voight et al., 1968).

The first and rate controlling step in bile acid synthesis is the 7α hydroxylation of cholesterol to form 7α-hydroxycholesterol. The enzyme that catalyzes this step, cholesterol 7α-hydroxlyase, is found in the endoplasmic reticulum, and requires oxygen, NADPH and a thermostable co-factor (Scholan and Boyd, 1968; Boyd et al., 1969; Myant and Mitropoulos, 1977). The reaction is dependent upon a minor form of cytochrome P-450 (Danielsson and Wikvall, 1981). Further metabolism of 7α-hydroxycholesterol involves the oxidation of the 3 ß-hydroxyl group and isomerization of the double bond from C-5,6 to C-4,5, producing 7α-hydroxy-4-cholesten-3-one. This the final intermediate common to both cholic and chenodeoxycholic acid synthesis. This ketone can be 12α-hydroxylated to produce 7α, 12α-dihydroxy-4-cholesten-3-one, which then undergoes reduction of the double bond and C-3 ketone to form 5ß-cholestane-3α, 7α, 12α-triol. For chenodeoxycholic acid synthesis, the 7α-hydroxy-4-cholesten-3-one is reduced to produce 5ß-cholestone-3α, 7α diol. Both the diol and the triol undergo degradation of the cholestane side chain to produce chenodeoxycholic and cholic acid

Figure 1.2 The biosynthetic pathway of bile acids.

Structures: I) Cholesterol; II) 7α-hydroxycholesterol; III) 7α-hydroxy-4-cholesten-3-one; IV) 7α,12α-dihydroxy-4-cholesten-3-one; V) 5ß-cholestane-3α,7α,12α,26-tetrol; VII) cholic acid; VIII) 5ß-cholestane-3α,7α-diol; IX) 5ß-cholestane-3α,7α,26-triol; X) chenodeoxycholic acid; XI) α-muricholic acid XII) ß-muricholic acid

Enzymes: a) Cholesterol 7α-hydroxylase; b) 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase; c) 26-hydroxylase; d) chenodeoxycholic acid 6ß-hydroxylase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I
a
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O
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c
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IX
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X
HO
OH
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VII
HO
OH
COOH
d
COOH
XI
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OH
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XII
HO
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OH

respectively. In the rat, chenodeoxycholic acid is further hydroxylated to yield α- and ß-muricholic acid.

Two pathways have been proposed for the degradation of the cholestane side chain. They differ in the intracellular site of the initial hydroxylation. They are discussed below and outlined in Figure 1.3 for the synthesis of cholic acid.

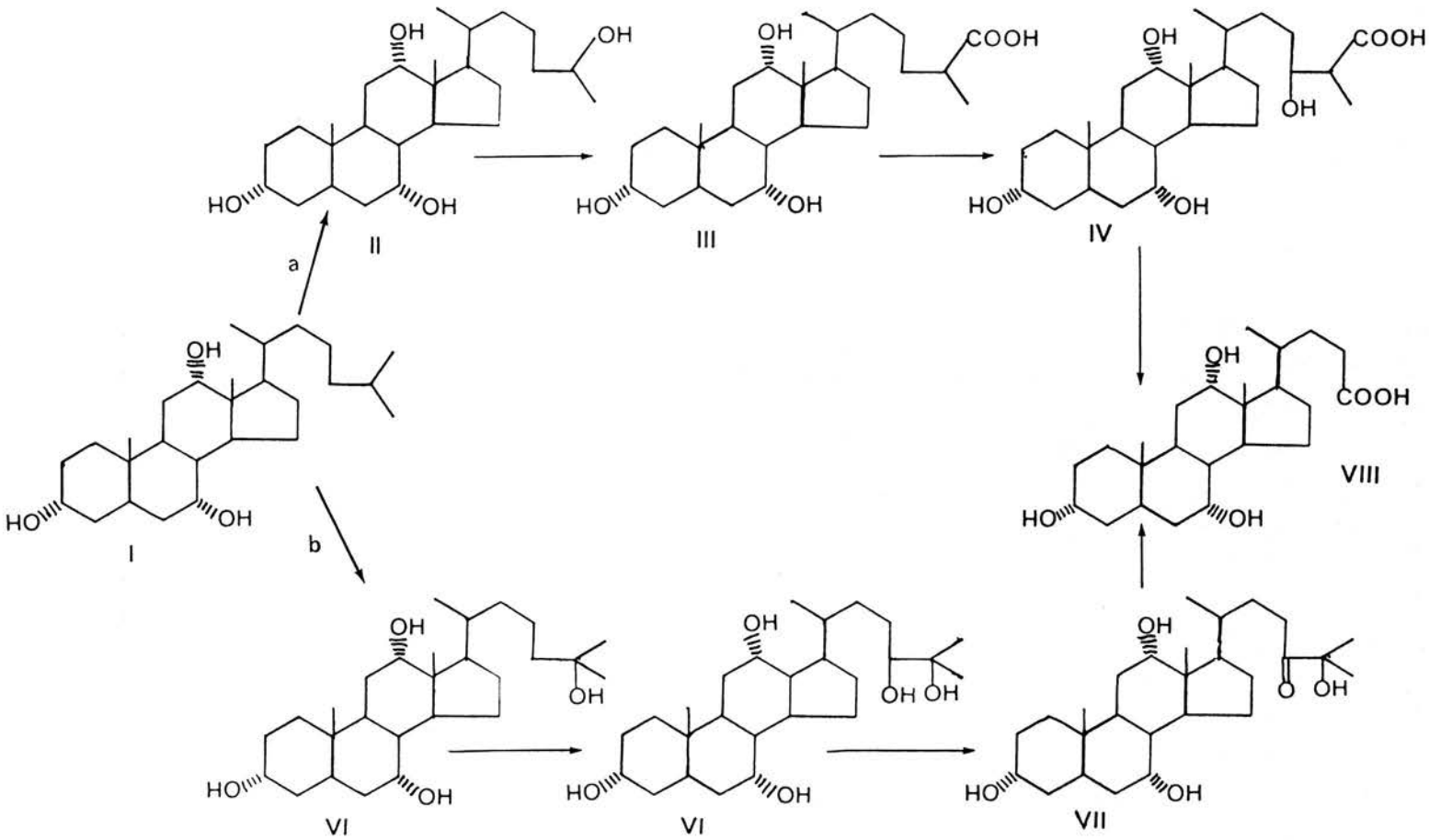
The mitochondrial fraction of both rat and human liver contains the enzyme 26-hydroxylase (Bjorkhem et al., 1975) which can convert 5ß-cholestane-3α, 7α, 12α triol to 5ß cholestane - 3α, 7α, 12α, 26-tetrol. The tetrol is then oxidized to 3α, 7α, 12α-trihydroxy-5ß-cholestane-26-oic acid (THCA) by the cytosol. This is followed by hydroxylation at C-24, yielding varanic acid, and then oxidized to shorten the side chain and yield cholic acid.

The alternative to this pathway, 25 hydroxylation, occurs in both rat and human liver (Shefer et al., 1975). The first step is the 25-hydroxylation of 5ß-cholestane - 3α, 7α, 12α-triol to give 5ß cholestane - 3α, 7α, 12α, 25 tetrol. The reaction is catalyzed by a microsomal enzyme system and is followed by 24-hydroxylation of the 5B-cholestane tetrol to yield 5ß-cholestane - 3α, 7α, 12α, 24, 25 - pentol. This is then oxidized to yield 3α, 7α, 12α, 25 - tetrahydroxy-5ß-cholestane-24-one (Shefer et al., 1982), which is then cleared by cytosolic enzymes to yield cholic acid and acetone.

After synthesis and secretion, bile acids are highly conserved. This occurs via the enterohepatic circulation and 95% of the bile salts that enter the duodenum in the normal state are recycled. To facilitate this, a specific transport system exists in the terminal portion of the small intestine (Dietschy, 1968). The 7α-hydroxylase enzyme system is sensitive to factors or events that modify the enterohepatic circulation. When bile acids are prevented from returning to the liver, the rate of bile acid synthesis increases. From this, the concept of negative feedback regulation of bile acid synthesis by bile acids was developed. It has been shown that the activity of cholesterol 7α-hydroxylase increases in parallel with bile acid synthesis and is affected by the levels of bile acids in the enterohepatic circulation (Myant and Mitropoulos, 1977).

Figure 1.3 The 26-hydroxylase (a) and the 25-hydroxylase (b) pathways for the degradation of the side chain of 5ß-cholestane-3α,7α 12α-triol (I) to form cholic acid (VII).

Structures: II) 5ß-cholestane-3α,7α,12α,26-tetrol; III) 3α,7α,12α-trihydroxy-5ß-cholestan-26-oic acid; IV) Vasanic acid; V) 5ß-cholestane-3α,7α,12α,26-tetrol; VI) 5ß cholestane-3α,7α,12α,24S,25-pentol; VII) 3α,7α,12α,25-tetrahydroxy-5ß-cholestan-24-one


OH
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II
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COOH
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Recently, studies with microsomal preparations have produced evidence that cholesterol 7α-hydroxylase exists in two forms, which are interconverted by cytosolic factors (Goodwin et al., 1982; Kwok et al., 1981a, Kwok et al., 1983). These factors may be a protein kinase and phosphatase, which have been proposed to regulate the enzyme by a phosphorylation (active form) - dephosphorylation (inactive form) mechanism (Sanghvi et al., 1981). Acyl - CoA cholesterol acyltransferase (ACAT), which utilizes cholesterol as a substrate, may be regulated in this manner, whilst 3-hydroxy-3-methyl-glutaryl CoA reductase (HMG-CoA reductase) is inhibited in the phosphorylated form. It has been postulated that short-term regulation of the concentration of unesterified cholesterol may be by coordinate control of these three enzymes by reversible phosphorylations (Scallen and Sanghvi, 1983).

Other investigators dispute this hypothesis, denying that cholesterol 7α-hydroxylase activity is modulated by phosphorylation - dephosphorylation (Danielsson et al., 1980; Danielsson et al., 1984). Berglund et al., (1986), have shown that under conditions favourable for phosphorylation - dephosphorylation, cholesterol 7α-hydroxylase activity was not affected. Under similar conditions, hepatic HMG-CoA reductase activity was modulated by phosphorylation - dephosphorylation as shown by other workers (Nordstrom et al., 1977; Gibson and Ingebritsen, 1978; Beg et al., 1978). Certainly there is no clear resolution to this question at this time. Considering that all the work so far deals with in vitro experimental systems which vary in design, no conclusions can yet be drawn as to what is occurring in vivo.

Another mechanism for the regulation of bile acid synthesis is through control of substrate availability. Newly synthesized cholesterol, preformed cholesterol existing in various functional pools within the cell and extracellular cholesterol in the form of plasma lipoproteins containing cholesterol arising from the diet(chylomicron remnants) or from other tissues in the body (LDL and HDL), are possible sources of cholesterol for bile acid synthesis. It has been shown that the preferred substrate for bile acid synthesis is newly synthesized cholesterol (Schwartz et al.,

1975; Bjorkhem and Lewenhaupt, 1979). It has also been shown that the preferred substrate for the synthesis of 7α-hydroxycholesterol is newly synthesized cholesterol (Myant and Mitropoulos, 1977; Bjorkhem and Danielsson, 1975; Ogura et al., 1976). These experiments do not state that newly synthesised cholesterol is the only substrate for bile acid synthesis, only that it is preferred; nor does it mean that most of the bile acids are synthesised from this source. In rats, it has been reported that only 25% of the bile acids are derived from newly synthesized cholesterol, the remaining 75% being derived from preformed cholesterol (Long et al., 1978). The contribution to bile acid synthesis of these different sources can change. A bile fistula was shown to increase the contribution of the newly synthesised cholesterol, whilst the mass of the alternate substrate pool remained unchanged (Long et al., 1978; Stange et al., 1985). The fistula interrupts the enterohepatic circulation and stimulates bile acid synthesis. These results reflect the livers' ability to alter the rate of cholesterol synthesis to meet the increase in bile acid synthesis. (Koelz et al., 1982). Kempen et al (1983), in experiments on the incorporation of tritiated water into cholesterol and bile acids by isolated hepatocytes from cholestyramine - fed rats, have estimated that during the first hour of incubation all the bile acid is formed from pre-existing cholesterol. After this time, the rate of bile acid synthesis dropped and newly synthesized cholesterol became more important as a substrate, probably because the sources of preformed cholesterol available have been exhausted.

Other investigations have looked at the possible preferential utilization of cholesterol from a particular lipoprotein source. Radioactive cholesterol in serum lipoproteins can be converted to bile acids when administered in vivo (Halloran et al., 1978, Miller et al., 1982, Price et al., 1985) and therefore contribute to total bile acid synthesis. High-density lipoprotein (HDL) is a better precursor for bile acids than low-density lipoproteins; both in man (Halloran et al., 1978, Price et al., 1985) and the rat (Miller et al., 1982). Recent work with cultured rat hepatocytes has found that bile acid synthesis and secretion was increased when the hepatocyte cholesterol pool was increased , either by addition of

mevalonic acid or lipoprotein fractions to the culture medium, or by feeding a cholesterol-rich diet (Davies et al., 1983b). Interestingly, bile acids did not have a direct inhibitory effect on bile acid synthesis in this experimental system (Davis et al., 1983a). This conflicts with the results of in vivo work that have suggested bile acids regulate their own synthesis by a negative feedback mechanism. It is possible, however, that an intestinally derived factor or metabolite may be required for this effect.

Further investigations into the existence of substrate pools for the synthesis of bile acids have suggested that individual bile acids may be derived from distinct substrate pools. A number of investigators have reported that cholic acid and chenodeoxycholic acid (Mitropoulos et al., 1974; Norman and Norum, 1976; Bjorkhem and Lewenhaupt, 1979; Long et al., 1978) or cholic acid and ß-muricholic acid (Kempen et al., 1983, Stange et al., 1985) are all derived from separate substrate pools.

1.4 The Plasma Lipoproteins

The plasma lipoproteins are water-soluble macromolecules (pseudomicellar particles) representing complexes of lipids (triglycerides, cholesterol and phospholipids) and one or more proteins referred to as apoproteins. The lipoproteins, of which there are five main classes, function to transport the afore mentioned lipids in the plasma. Certain of them act as vehicles for lipids entering the plasma from the intestine. Others function to transport lipids from one tissue to another, where the lipids can be used as structural components of the cell (e.g. cholesterol), or as precursors of tissue product (e.g. cholesterol for steroid hormones). Further to this, lipoproteins transport lipids to specific organs for storage (triglycerides to adipocytes) or for excretion from the body (cholesterol to the liver).

The five main classes of lipoproteins float within different density ranges under ultracentrifugation (Havel et al., 1955). These classes are chlyomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins

(LDL), and high density lipoproteins (HDL) (Table 1.1). They vary by their chemical composition as well as their physical properties (Table 1.1). The density ranges chosen for lipoprotein isolation are based on those of human plasma lipoproteins, a situation that does not apply to all species. In the rat, isolation of LDL and IDL is difficult (Chapman, 1980); the conventional density range of 1.006-1.063 g ml^{-1}, in which both LDL and IDL appear, is contaminated with apo E (Innerarity et al., 1980a).

This has been shown to be caused by an HDL subfraction rich in apo E. This is important because it is the chemical composition, and in particular the apoprotein content of a lipoprotein molecule that determines its function and subsequent catabolism.

1.4.1 The Lipoprotein Classes and their Metabolism

It was thought that plasma lipoproteins were synthesized by the liver and small intestine only. Evidence supports the idea that intestinal cells and hepatocytes are the source of virtually all the circulating chylomicrons and VLDL (Havel et al., 1980). Nascent HDL has been found primarily in hepatic perfusates in several mammals (Hamilton, 1984), and it appears that this is the major site of HDL production, although in the rat some HDL is synthesized in the intestine (Green and Glickman, 1981; Forester, et al., 1983). Recently, cells other than those of the small intestine and liver have been shown to synthesize some of the apolipoproteins. Apolipoprotein E is synthesized by the kidney and adrenal glands in the human (Blue et al., 1983), and the mRNA of apolipoprotein E has been found in the liver, adrenal glands, brain and spleen of other animals (Elshourbagy et al., 1985; Newman et al., 1984). Apo E is also secreted from mouse and human macrophages under certain conditions, such as cholesterol loading (Basu et al., 1982). The form in which this protein is secreted from these cells is not yet known nor has its function been determined.

(a) Chylomicrons

These are triglyceride rich particles formed in the small intestine

Lipoprotein Class	Electrophoretic Mobility	Diameter (nm)	Density	Molecular Weight	Chemical Composition (mg/100 mg) Lipoprotein Protein	TG[1]	CE	FC	PL
Chylomicrons	Origin	75-1200	<0.95	400,000K	5	83	5.0	2	5
VLDL	pre-ß	30-80	0.95-1.006	10-80000K	12.5	63	2.5	4	18
LDL	ß	18-25	1.006-1.019	2300K	23.5	20	27.5	8	21
HDL_1	α	9-12	1.063-1.085	360K	28.5	6	25.5	8	32
HDL_2	α	5-9	1.085-1.210	175K	40.5	3	26.0	3.5	27

Table 1.1

Physical and chemical properties of Rat Plasma lipoproteins. 1) abbreviations: TG - triglycerides; CE - cholesteryl ester; FC - free cholesterol; PL - phospholipids.

and secreted into the intestinal lymph during absorption of dietary fat. From here they are transported into the plasma compartment via the thoracic duct, and subsequently catabolised (Green and Glickman, 1982). The apoprotein constituents of plasma chylomicrons are apoproteins A-I (apo A-I), A-IV (apo A-IV), B (apo B), C (apo C) and E (apo E).

The C apoliproteins are represented by three low molecular weight apoproteins C-I, C-II, C-III. These proteins share the common property of redistributing among lipoprotein classes (Nestel & Fidge, 1982). These apoproteins preferentially redistribute to the surface of the triglyceride-rich chylomicrons during the initial synthesis of the lipoprotein molecule. As the triglyceride core of the chlyomicron is depleted during its catabolism, excess surface components are generated and the C apolipoproteins are transferred to HDL (Mahley et al., 1984). Individually, they have other functions. Large quantities of apo C-III relative to apo C inhibits the uptake of chlyomicron particles by the liver, thus allowing catabolism of dietary triglycerides (Redgrave, 1970). Apo C-II activates the enzyme lipoprotein lipase. LPL is bound to the capillary endothelium and is found in most extrahepatic tissues. It acts to hydrolyse the core triglyceride, releasing mono - and diacylglycerol and fatty acids. The mono - and diacylglycerol are either delivered to muscle and adipose tissue or further hydrolysed to fatty acids in the plasma. The fatty acids so released are picked up by albumin and transported to the liver. As the triglyceride core is depleted, surface compounds "bud-off", forming vesicles consisting of free cholesterol, phospholipid and apoproteins A-I, A-IV and C. These components are transferred to nascent HDL (Mahley et al., 1984), resulting in the formation of a cholesteryl ester rich particle termed a chylomicron remnant. The protein of the particle consists apo B and E, which results in the uptake of the particle by the hepatic apo E receptor (Havel, 1984; Cooper, 1985). The uptake of the chlyomicron remnants results in the protein and cholesteryl ester moieties entering into lysosomes where they are hydrolysed (van Dyke et al., 1985 and Jost-Vu, 1986).

(b) Very Low Density Lipoprotein (VLDL)

Very low density lipoproteins are synthesized by both the liver and small intestine. Intestinal VLDL functions in a similar way to chylomicrons, transporting dietary lipid into the plasma. Quantitatively, the chylomicrons are more important than the VLDL in terms of the absorption of dietary lipid. The nascent particles, on entering the plasma, transfer apo A-I and A-IV to HDL and acquire apo-E and C from HDL (Green et al., 1979; Green and Glickman, 1981). These exchanges produce the apoprotein profile of VLDL normally found in the plasma (Havel et al., 1980). In the rat, newly secreted VLDL contains a large amount of cholesteryl esters, synthesized by hepatic ACAT (Faergeman and Havel, 1975); however, it acquires little in the plasma due to the low cholesteryl ester/triglyceride transfer activity (Barter and Lally, 1978; Barter et al., 1982; Tall, 1986). In humans the situation is different; ACAT activity is low and cholesteryl ester/triglyceride transfer activity is high, thus VLDL acquires cholesteryl esters from HDL synthesized in the plasma by lecithin: cholesterol acyl transferase (LCAT) (Tall, 1986).

The initial step of VLDL metabolism is basically the same as for chylomicrons - binding to endothelial lipoprotein lipase, followed by hydrolysis of the bulk of the triglycerides. As with chlyomicrons, phospholipids and apo C apoproteins are transferred to HDL as hydrolysis proceeds; thus the products of LPL action, VLDL remnants, are depleted of triglycerides, phospholipids and C apoproteins, but retain apo B and E (Mjos et al., 1975; Havel, 1984). This hydrolysis results in delivery of mono - and diacylglycerol to the peripheral tissues. Surface components are then transferred from VLDL to HDL in the same manner as occurs with chylomicrons (Tall and Small, 1980, Gotto et al., 1986). The transfer of apoproteins A-I, A-IV and C, phospholipid and free cholesterol from VLDL to HDL produces VLDL remnants. In many species, most of the VLDL remnants are taken up by receptor-mediated endocytosis (Havel, 1984). The rat in particular removes the remnants from the circulation quite rapidly, allowing only 3% of the remnants to be converted to LDL (Faergeman and Havel, 1975). In humans, a large fraction, about one-half, is processed to LDL

Apoproteins	Distribution in Lipoproteins				Molecular	Origin	Function
	HDL	LDL	VLDL	Chylomicrons(mole %)	Weight		
A-I	97			3	28K	liver/intestine	Activation of LCAT
A-II	100			3	17K	liver/intestine	Activation of LPL?
A-IV	97				46K	liver/intestine	Activation of LCAT
B-48		98	2		264K	intestine	
B-100					550K	liver	Uptake of LDL
C-I	94		3	3	9.8K	liver/intestine	Activation of LCAT, Inhibits uptake E lipoprotein
C-II	60		20	20	9.8K	liver/intestine	Activation of LPL, Inhibits uptake of apo E lipoprotein
C-III	60		40		8.7K	liver	Inhibits uptake of apo E lipoprotein
D	100				20K	liver	Transfer of cholesteryl ester from HDL
E-II E-III E-IV	60		40		35K	liver	Uptake of lipoproteins via apo E receptor recognition

Table 1.2 The funtion, origin and distribution of Rat apoproteins

(Havel, 1984). Small VLDL yield small remnants, which are more likely to form LDL than larger remnants, whereas large VLDL is thought to produce large remnants that are more likely to be removed quickly by the liver, (Stalenhoef et al., 1984; Packard et al., 1984). The formation of LDL from VLDL occurs with further loss of triglycerides phospholipids and apo E, leaving only Apo B-100 in the LDL particles. In species with active cholesteryl ester/triglyceride transfer activity, processing of remnants to form LDL may be followed by redistribution of cholesteryl esters to larger paticles in exchange for triglycerides (Deckelbaum et al., 1982).

(c) Low Density Lipoproteins and Intermediate Density Lipoproteins

Both VLDL remnants and LDL, which contain apo B-100, are removed from the blood by interaction with LDL receptors (Goldstein et al., 1983), also referred to as apo B/E receptors. VLDL remnants are almost entirely removed by the liver, where efficient interaction between apo E and hepatic LDL receptors occurs (Havel, 1984). LDL, on the other hand, are removed less efficiently than VLDL through binding by the receptors to apo B-100, and are thus taken up by extrahepatic tissues that contain LDL receptors. From this, it can be seen that LDL functions to transport cholesterol to extrahepatic tissues, although the liver also contributes to the catabolism of LDL (Pittman et al., 1979; Attie et al., 1982; Stange and Dietschy, 1984).

In general,LDL uptake by extrahepatic tissues results in the inhibition of HMG -CoA reductase and stimulation of ACAT, thus reducing synthesis of cholesterol, and increasing the esterification of the cholesterol taken up by the tissue (Goldstein and Brown, 1974; Brown et al., 1975). LDL uptake occurs by both receptor (mentioned above) and non-receptor pathways, which will be discussed later.

IDL represents an intermediate in the conversion of VLDL to LDL by lipoprotein lipase. It contains relatively less triglyceride and cholesteryl ester compared to VLDL, although VLDL may contain particles that are functionally equivalent to IDL.

(d) High Density Lipoproteins

High density lipoprotein is synthesized by the liver and small intestine and enters the plasma as a discoidal nascent particle. These particles are composed of a bilayer of phospholipids (mainly lecithin); the proteins are associated mainly with the disk margin (Wlodauer et al., 1979; Hamilton, 1984). The metabolism of nascent HDL gives rise to the subfractions. In the rat, these are termed HDL_1 and HDL_2 (Oschry and Eisenberg, 1982); in man, the subfractions are HDL_3 and HDL_2 (Patsch et al., 1974). HDL_2 in rat and human are similar in their physical and chemical properties. Comparitively, the densities and sizes of the particles decreases from $HDL_3 \rightarrow HDL_2 \rightarrow HDL_1$. Chemically, the relative content of cholesteryl ester and apoE increases from $HDL_3 \rightarrow HDL_2 \rightarrow HDL_1$.

On entry into the plasma, cholesterol phospholipid, apoA-I, apoA-IV and apoC are transferred from the surface of chylomicrons and VLDL to nascent HDL, producing HDL_3 in humans, (HDL_2 in rats). The transfer of these components usually occurs during the hydrolysis of chylomicrons and VLDL by lipoprotein lipase (Havel, 1978; Patsch et al., 1978). The major apoprotein associated with HDL is apo A-I, the main activator of LCAT (Fielding and Fielding, 1980). The surface free cholesterol of HDL_2 is esterified via the transfer of a fatty acid from lecithin by LCAT. The lysolecithin formed is scavenged by albumin (Stein and Stein, 1966). Cholesterol esterification is followed by its movement into the core of the particle. Continuous esterification of the surface free cholsterol would lead to depletion of surface components, were it not for the transfer of cholesterol and phospholipid from VLDL and chylomicrons. Further esterification of the surface free cholesterol under these conditions leads to the formation of HDL, (Oschry and Eisenberg, 1982). In humans the process is similar for the conversion of HDL_3 to HDL_2.

It is thought that HDL functions to transport cholesterol from peripheral tissues to the liver and adrenal gland for degradation or use in other metabolic pathways (Tall and Small, 1980).

As mentioned, HDL catabolism results in depletion of surface free cholesterol and its replenishment by the transfer of surface components from VLDL and chylomicrons. It can also be replaced by the transfer of cholesterol from the plasma membranes of peripheral tissues to HDL (Rothblat and Phillips, 1982; Fielding and Fielding, 1981). In humans and other species, the presence of cholesteryl ester transfer proteins causes the redistribution of this accumulated cholesteryl ester from HDL to other lipoproteins of lower densities. These in turn may be picked up by the liver (Norum et al., 1983). Plasma of rats and dogs contains little or no transfer activity, so that cholesteryl ester produced by the action of LCAT, and cholesterol picked up from peripheral tissues largely remains in this lipoprotein class.

1.4.2 Mechanisms of Uptake of Plasma Lipoproteins

(a) Receptor-Mediated Uptake

The most well defined pathway for the uptake of lipoproteins in the LDL receptor pathway (Goldstein and Brown, 1977). The receptor, first discovered in cultured human skin fibroblasts, is capable of binding both apo B and apo E, although its affinity for apo E is more than 10 times its affinity for apo B (Pitas et al., 1980). Studies have shown that small lipoproteins containing several apoE molecules can bind to several sites (Innerarity et al., 1980a), whereas the apoB on an LDL particle probably interacts with a single binding site. It is probable that the higher affinity of VLDL remnants is due to the binding of the remnant to multiple sites on the receptor through its complement of apoE molecules (Windler et al., 1980). This higher binding affinity is probably responsible for the more efficient endocytosis of VLDL remnants than LDL in the liver.

The accessibility of apo E or apo B, and the size of the particle appear to influence the uptake of particles. The latter point may be a function of the greater number of apo E molecules on large VLDL remnants (Stalenhoef et al., 1984; Packard et al., 1984; Havel, 1984). These VLDL remnants and LDL compete for the apo B/E

receptor, to which remnant binding is the most efficient. Normal VLDL, contains apo B and apo E, neither of which effect binding. The apo E is inaccessible to the receptor (Bradley et al., 1984) and the apo B differs in its conformation and in its immunoreactivity from that of the apo B of LDL (Schonfeld et al., 1979). These factors ensure that it isn't taken up by the apo B/E receptor.

Chylomicron remnants contain apo B-48 not apo B-100, and are not taken up by the apo B/E receptor. Instead they have a distinct receptor (Havel, 1984) called the apo E receptor. The existence of a saturable high affinity pathway for chylomicron remnant uptake was shown by Sherrill and Dietschy (1978). The proof that this pathway had a specific receptor was shown in studies on patients with familial hypercholesterolaemia (FH) in which chylomicron remnant uptake was seen to be unaffected and in Watanabe heritable hyperlipidaemic (WHHL) rabbits (Brown et al.,1981; Kita et al., 1982). In both cases the number of apo B/E receptors is either halved - heterozygous FH - or abolished - homozygous FH. In the homozygous condition, LDL catabolism occurs via the receptor-independent pathway whilst chylomicron uptake remains unaffected, because the expression of the apo E receptor is unaffected in the above subjects (Goldstein et al., 1983). Binding of the chylomicron remnants is impeded by C apoproteins, which prevents premature uptake of the particles. Loss of these apoproteins during remnant formation ends triglyceride hydrolysis and facilitates binding to receptors (Havel, 1984).

The receptor-mediated pathways of LDL, VLDL and chylomicron catabolism are well understood. In vivo studies show that the liver is a major site for HDL catabolism (Stein et al., 1983). In studies conducted by Sigurdsson et al., (1979) using recirculating perfusion systems with the liver, it was shown that hepatic degredation of HDL was only 7% after four hours. Further studies in isolated hepatocytes demonstrated that the liver plays a major role in HDL catabolism (Drevon et al., 1977; Ghiselli et al., 1981). Both liver and steroidogenic cells contain an enzyme resembling hepatic lipase (Jackson, 1983). HDL binding to this enzyme could lead to endocytosis selective loss of lipid and then retroendocytosis of a

lipid depleted particle (Kinnunen et al., 1983). Interestingly, in the rat, HDL cholesteryl esters are taken up primarily by the liver, adrenal gland and gonads at a much greater rate than apo A-I (Stein et al., 1983; Glass et al.,1983; van't Hooft et al., 1981). This mechanism involves binding of the HDL to the cell surface, but receptor recycling and endocytosis do not appear to be involved (Pittman et al., 1987b).

In cultures of hepatocytes of steroidogenic cells, HDL binds to cell surfaces by a mechanism different from that of lipoprotein apo B-100. It is not dependent on calcium ion and is resistant to proteolysis (Norum et al., 1983; Havel, 1986; Gwynne and Strauss, 1982). This binding might promote influx or efflux of cholesterol or uptake of cholesteryl esters. In some, but not all cells, LDL and VLDL compete with HDL for the same site (Bachorik et al., 1982), and as such are also involved in this selective uptake, but to a much lesser degree (Pittman et al., 1987a). Further to this, it is likely the relative apo E content of the HDL particles might influence their interaction with the B/E or E receptor, and thus influence any receptor-mediated endocytosis. Certainly, the predominance of one pathway or route of catabolism has yet to be defined. HDL comprise a varied and adaptable class of lipoproteins that are involved in plasma cholesterol transport and are catabolised, at least in part, by a pathway involving receptor-mediated endocytosis, as well as other means.

Two other receptors, the ß-VLDL receptor and the scavenger receptor, are involved in the endocytosis of lipoproteins containing apo B, into cells of reticuloendothelial origin, e.g. macrophages, artery wall foam cells, endothelial cells (Brown and Goldstein, 1983; Baker et al., 1984). ß-VLDL receptors are specific for chylomicrons, large VLDL of hypertriyglyceridaemic patients and the cholesteryl ester rich, ß-migrating VLDL of cholesterol fed animals (Gianturco et al., 1982; VanLenten et al., 1983; Goldstein et al., 1980). These lipid rich particles are endocytosed, hydrolyzed in lysosomes, and the lipid re-sterified and stored in the cytoplasm, giving the cells a foam cell appearance (Brown and Goldstein, 1983).

The scavenger receptor is involved in the uptake of chemically modified proteins; modified to increase their negative charge, as occurs by acetylation (Brown and Goldstein, 1983). This receptor, also termed the acetyl-LDL receptor, is not affected by cellular cholesterol levels, as is the LDL receptor (Mazzone and Chait, 1982). The function of this receptor and the ß-VLDL receptor is probably to clear abnormal lipoproteins and they may also be involved in the deposition of cholesterol in developing atherosclerotic plaques.

(b) Non-Receptor-Mediated or Receptor-Independent Uptake

Some LDL in liver and other tissues are endocytosed by pathways not involving receptor-mediated endocytosis. Fibroblasts from homozygous FH subjects and hepatocytes from WHHL rabbits are capable of substantial LDL catabolism although they lack the apo B/E receptor (Goldstein and Brown, 1974; Attie et al., 1982). This can be shown in vivo by the high catabolic rates for LDL seen in receptor-deficient animals (Bilheimer et al., 1982). Binding studies have demonstrated the existence of a low-affinity site through which the pathway is mediated (Kita et al., 1981). Quantitatively, this route have a high capacity for lipoprotein removal, and Shepherd et al., (1980) has suggested that two-thirds of LDL catabolism occurs by this route. The exact mechanism of this pathway is not yet fully understood. Certainly, bulk fluid endocytosis (pinocytoses), common to all tissues, contributes little to LDL uptake by FH fibroblasts (Attie et al., 1982), and is no longer considered. The LDL might absorb to low-affinity binding sites and be taken up during the course of membrane turnover. This has yet to be demonstrated conclusively (Attie et al., 1982; Ho et al., 1977).

1.4.3 Lipoprotein Lipase and Transfer Factors

Lipoprotein lipase (LPL) is the enzyme responsible for the hydrolysis of the triglycerides and phospholipids of chylomicrons and VLDL, and is activated by apo C-II (Havel et al., 1970; La Rosa et al., 1970). LPL is bound to the capillary endothelium and found

in most extrahepatic tissues. Hepatic lipase, first isolated in the perfusate of livers treated with heparin, also hydrolyses triglycerides and phospholipids (Nilsson-Ehle et al., 1980). It is immunologically distinct from LPL (Jansen and Hulsmann, 1975) and is not activated by apo C-II. Lipoprotein lipase is involved in the metabolism of chlyomicrons and VLDL. Its activity is influenced by several hormones of which insulin is the most important for short-term regulation (Jackson, 1983). The function of hepatic lipase is less clear. It is known that high hepatic lipase levels decrease plasma HDL-cholesterol whilst low hepatic lipase activity corresponds to an increase in the rate at which tissues acquire cholesterol. A role for hepatic lipase in the degradation of IDL as well as HDL has been speculated (Jackson, 1983; Jansen and Hulsmann, 1985).

Lecithin:cholesterol acyl transferase (LCAT) is another enzyme involved in plasma lipid metabolism. Its substrates are phosphatidylcholine and cholesterol, and it produces cholesteryl esters and lysolecithin. Both hydrolysis and transesterification are activated by apo A-I, the major protein of HDL (Aron, 1978). Apo C-I and apo A-IV also activate LCAT. The enzyme acts on fatty acids on the sn-2 position of phosphatidylcholine. It preferentially cleaves unsaturated fatty acids and its specificity for the sn-2 position is partly influenced by the high relative abundance of sn-1-saturated-sn-2-unsaturated phosphatidylcholines (Gotto et al., 1986).

Acyl: coenzyme A: cholesterol o-acyltransferase (ACAT) is an intracellular enzyme located on the endoplasmic reticulum (Goodman et al., 1964). Initially, it was found in rat liver (Murkherjee et al., 1958), but has since been found in most tissues. It is responsible for the intracellular formation of cholesteryl esters from free cholesterol and fatty acids. The activity of the enzyme may be regulated by an ATP-dependent phosphorylation mechanism (Suckling et al., 1983). As mentioned earlier, HMG-CoA reductase is regulated by such a mechanism and it is postulated that so to is cholesterol 7α-hydroxylase. Thus, three enzymes of intracellular cholesterol metabolism may be regulated together.

Lastly, there is a group of proteins, the lipid-transfer proteins, that mobilize lipids from one compartment to another. Lipid transfer among plasma lipoproteins and cell membranes is mediated by a series of specific protein factors in the plasma. Brewster et al., (1978) first demonstrated this for the transfer of phosphatidylcholine. A similar factor is responsible for the transfer of cholesteryl esters (Zilversmitt et al., 1975). Exchange proteins tend to equilibrate the fatty acid composition of lipids among the lipoproteins. Net transfer of and not just simple exchange also occurs, and is an important means of transfer of lipids from lipoprotein to lipoprotein (Tall, 1986). Phospholipid exchange proteins are active in all mammalian systems looked at; however, cholesteryl ester/triglyceride exchange protein activity varies from species to species. This particular protein has been intensively investigated, such that its cDNA sequence was recently eluciated (Drayna, 1987). In the rat however, it is absent and the type of cholestryl ester transfer it is involved in other species is not of importance in rat lipoprotein metabolism.

1.5 Summary

In the preceding sections, I have attempted to describe hepatic cholesterol metabolism, of which the overall picture is complex. There are a number of sources of cholesterol available to become intracellular free cholesterol, namely endogenous synthesis, and uptake of plasma lipoproteins, by either receptor or non-receptor mediated pathways. In the rat, the HDL subfraction is of particular improtance due to its potential role in delivery of cholesterol from peripheral tissues and because of the absence of LDL. Each of these present a different sound and potentially a different, discrete pool of free cholesterol.

Once in these potential pools the free cholesterol can then be used in a number of pathways: secretion into bile or plasma with lipoproteins, esterification and storage or secretion with lipoproteins, and finally further metabolism into bile acids and then subsequent secretion. Again, each of these products might derive from different precursor pools of free cholesterol. It is

thus of interest to define the fates of different cholesterol sources, and the inter-relationship of any potential precursor pools.

Important in the control of these processes and inter-relationships are three enzymes, all of which are located microsomally and possibly subject to reciprocal metabolic control: HMG-CoA reductase, ACAT and cholesterol 7α-hydroxylase. They are key enzymes in the synthesis, esterification and conversion to bile acids of cholesterol and its flux. Within the scope of this thesis the influence of these enzymes on cholesterol metabolism in conjunction with the effect of different cholesterol sources (newly-synthesised and HDL-derived) will be investigated. Using specific inhibitors of the above enzymes, singly and in combination, the effect on bile acid production and cholesterol and cholesteryl ester secretion was investigated. This strategy has allowed for the isolation of particular pathways to see if any relationships of cholesterol flux occur between said pathways or precursor pools. For instance, by inhibiting ACAT activity, we can see if either cholesterol secretion or bile acid synthesis is changed, and if so, to what degree. Further to this, using a source of newly-synthesised cholesterol such as mevalonate, its effect in the above situation can be determined. From this approach we hope to show these different intracellular pools of cholesterol which though metabolically inter-related all have distinct functions.

CHAPTER 2

Materials and Methods

2.1 Materials

Galactose, gentamycin sulphate, insulin, ornithine hydrochloride, activated charcoal, reduced nicotinamide adenine dinucleotide, cholesterol, oleic acid, Coomasie brilliant blue G were obtained from Sigma Chemical Company, Poole, Dorset, U.K.

Sodium pyruvate, cholesterol oxidase, cholesterol esterase, horseradish peroxidase, bovine serum albumin fraction V pwoder, bovine serum albimin fraction V powder (fatty acid free) were from Boehringer Corporation (London) Ltd., Lowes, East Sussex, U.K.

Trypan blue, polyethylene glycol, mercaptoethanol, polyacrylamide and sodium dodecyl sulphate were from BDH Chemicals Ltd., Poole, Dorset, U.K.

Non-essential amino-acids, foetal calf serum, newborn bovine serum albumin were from Flow Laboratories Ltd, Irvine, Scotland, U.K.

Dubbecco's modified Eagle's medium and streptomycin/penicillin and nystatin were from GIBCO (Europe) Ltd., Paisley, Scotland, U.K.

Percoll and 4-30% acylamide gradient gels were from Pharmacia (Great Britain) Ltd., Milton Keynes, Middlesex. U.K.

Cholestyramine was provided by Bristol-Myers Company (U.K.) Ltd., Cramlington, Northumberland. U.K.

58-035 was provided by Dr John Heider of Sandoz Research Institute, East Hanover, New Jersey, U.S.A.

Mevinolin was provided by Mr A. Alberts, Merck-Sharpe and Dohme, Rahway, New Jersey. U.S.A.

Bond-eluts were obtained from Jones Chromatography, Hengoed, Mid-Glamorgan, U.K.

All radiochemicals and radionuclides were obtained from Amersham International p.l.c., Amersham, Bucks. U.K.

All other chemicals and reagents were of analytical grade.

2.2 Animals

Female Wistar rats (200-250g) were used in all experiments. Animals were fed either a soft diet, comprising 70% wholemeal flour, 25% skimmed milk powder and 5% yeast or the soft diet supplemented with 4% cholestyramine (referred to a "diet Z").

2.3 Preparation of Glassware Used in the Isolation of Hepatocytes

To prevent adhesion of hepatocytes to glassware, any glassware that was used with hepatocytes was treated as follows. Glassware was washed with chromic acid (80-85ml saturated sodium chromate in 2.2 l concentrated sulphuric acid), rinsed in double distilled water, and allowed to dry. This was followed by treatment with Dri-Film SC87 (10% v/v in petroleum ether). The glassware was then drained, washed with hot water and dried in an oven at 60°C for 2 hours.

2.4 Preparation and Maintenance of I solated Hepatocytes

2.4.1 Solutions Required For Isolation of Hepatocytes

Glucose and Calcium Free Locke's Solution:

Sodium chloride (9 g l^{-1}) and potassium chloride (0.24 g l^{-1}) in double distilled water.

Glucose and Calcium Free Hank's Solution:

Sodium chloride (8 g l^{-1}), potassium chloride (0.4 g l^{-1}), magnesium sulphate.7 H_2O (0.2 g l^{-1}), potassium dihydrogen

phosphate (0.06 g l^{-1}) and phenol red (0.02% w/v) in 0.01M sodium phosphate buffer, pH 7.4. Phenol red was used in order to monitor the pH throughout the perfusion.

2.4.2 Preparation of Isolated Hepatocytes

The method used for isolation of hepatocytes was upon that developed by Quistorff et al. (1973), Botham and Boyd (1979) and Ford (1984).

Rats were anaethetised with diethyl ether. A longitudinal incision in the abdomen was made and the intestines pushed to one side. The hepatic portal vein was cannulated and the liver flushed through with calcium - and glucose-free Locke's solution. Once the liver had blanched completely the perfusion was stopped, the liver removed and placed in the perfusion hood (figure 2.1). The liver was then washed for a further 2 minutes prior to perfusion with glucose - and calcium-free Hank's solution supplemented with 0.5 mM calcium chloride and containing 1-1.5 mg ml^{-1} collagenase for 12-16 minutes. The amount of collagenase used depended upon the commercial preparation obtained. Finally, the liver was perfused with glucose - and calcium-free Locke's solution containing 0.2mM ethylenediaminetetra-acetic acid (EDTA) for 5 minutes. All perfusates were oxygenated during perfusion and were supplemented with 2% (w/v) bovine serum albumin and 38 mM sodium bicarbonate. The pH of the perfusates was adjusted to pH 7.4 with sodium bicarbonate if necessary. The procedure was carried out at 30-35°C.

After perfusion, the cells were dispersed in glucose - and calcium-free Hank's solution by gentle teasing with forceps in a glass petri dish, and immediately poured through a 200µm nylon mesh filter into a glass beaker. The isolated hepatocytes were sedimented by centrifugation and resuspended in 10 ml of culture medium, consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.4 mM ornithine hydrochloride, non-essential amino acids, gentamycin sulphate (100 mg l^{-1}), penicillin/ streptomycin (10,000 IU l^{-1}) nystatin (20 U ml^{-1}) and galactose (1 g l^{-1}) containing 2% (w/v) charcoal treated bovine serum albumin. The resuspended hepatocytes were then applied to two 35 ml

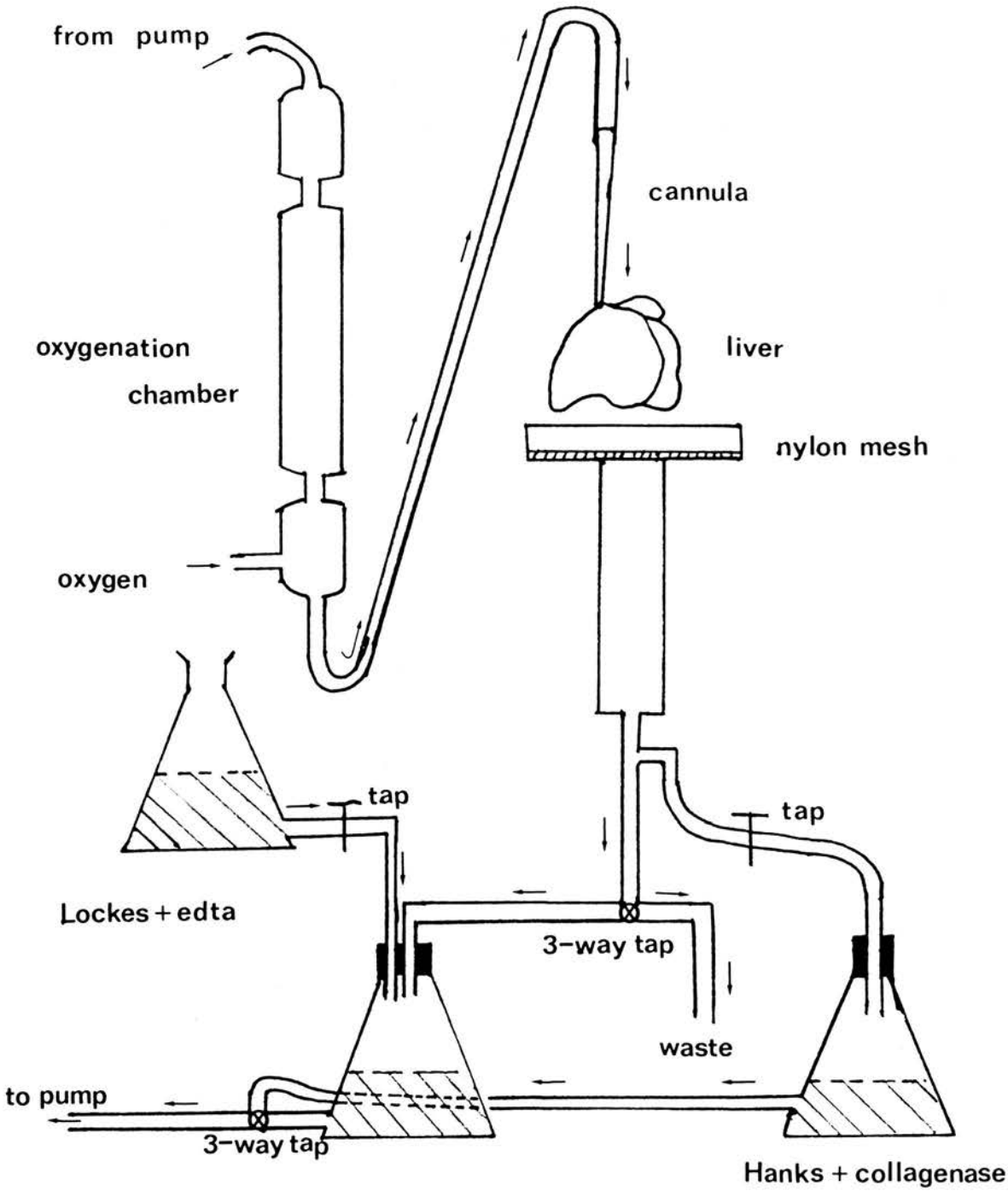


Figure 2.1 Liver perfusion apparatus

'Percoll' density gradients (0-70% (v/v) 'Percoll' in supplemented DMEM, containing 2% bovine serum albumin). Continuous gradients were formed using a gradient mixer. Viable cells were separated from non-viable cells by centrifugation at 50 xg for 15 minutes. The viable cells formed a pellet or lower band of cells, were removed and washed with 2 x 15 ml supplemented DMEM with 2% (w/v) charcoal treated bovine serum albumin. Hepatocytes were finally resuspended in 10 ml of supplemented DMEM, containing 20% (v/v) foetal calf serum and insulin (100 U l^{-1}). The complement of foetal calf serum was inactivated by heating tO 56°C for 1 hour. The viability of this final suspension, determined by trypan blue exclusion, was routinely greater than 95%.

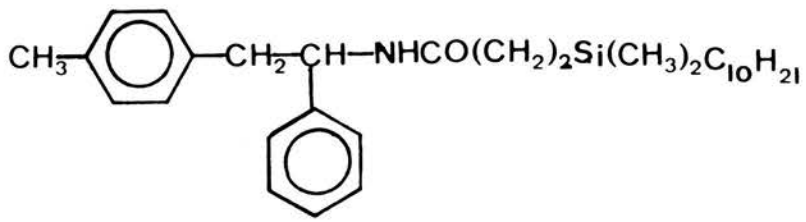
2.4.3 Maintenance of Isolated Hepatocytes

The hepatocyte suspension was diluted with supplemented DMEM containing 20% (v/v) foetal calf serum and insulin (100 U^{-1}) to give a final cell density of approximately 1 x 10^6 cells ml^{-1}. The cells were incubated for 3-3.5 hours in 50 mm plastic petri dishes (2.5-3.0 x 10^6 cells 3 ml^{-1}), during which time cell adhesion occurred. The medium was then removed and replaced with 2 ml of supplemented DMEM or 2 ml of supplemented DMEM with 2% (w/v) charcoal treated bovine serum albumin, depending on the experimental conditions required.

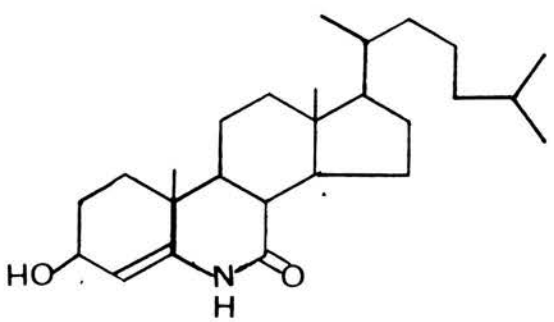
2.5 Procedures for the Use of Inhibitory Compounds

2.5.1 Experimental Use of 58-035

58-035, an extremely hydrophobic compound, was dissolved in dimethyl sulphoxide (DMSO) and stock solutions of 1 mg ml^{-1} and 10 mg ml^{-1} were prepared. Aliquots of between 10-50 µl of these stocks were used. The hepatocyte monolayer cultures were pre-incubated with an aliquot of 58-035 and 2 ml of supplemented DMEM with 2% charcoal treated bovine serum albumin, for 2 hours to facilitate uptake of the compound. The medium was then changed to that required by the given experiment, usually 2 ml of supplemented DMEM.



a



b

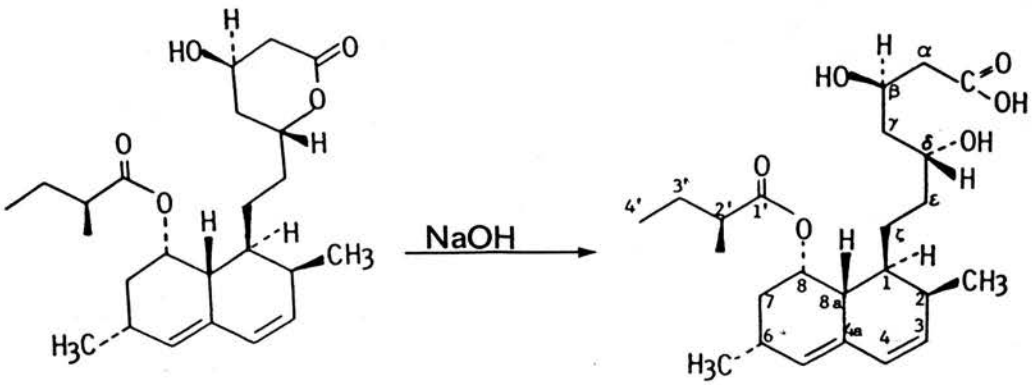
Figure 2.2 Chemical structures of a) 58-035 and b) azacholesterol

2.5.2 Experimental Use of 6-Azacholes-4-en-3ß-ol-7-one (Azacholesterol)

As with 58-035, azacholesterol was dissolved in DMSO, and stock solutions of 5 mM, 10 mM and 20 mM were prepared. Aliquots of between 10-50 µl from these stocks were used. The hepatocyte monolayer cultures were presented with an aliquot of azacholesterol and 2 ml of supplemented DMEM with 2% (w/v) charcoal treated bovine serum albumin. Pre-incubation of the azacholesterol with the hepatocytes prior to the start of the experiment was not necessary.

2.5.3 Experimental Use of Mevinolin

Mevinolin is transformed from its lactone form by heating at 50°C for 2 hours in 0.1N sodium hydroxide as shown in reaction (1) (Brown et al, 1978).



Mevinolin Mevinolic Acid

8.091 mg of mevinolin was dissolved in 25 ml of 0.1N sodium hydroxide, and heated. The resultant solution was neutralised with hydrochloric acid, diluted to 100 ml with 75 ml of 59 mM sodium bicarbonate, and adjusted to pH 7.4. This produced a stock of 200 µM mevinolin in 44 mM sodium bicarbonate. Aliquots of between 10-50 µl of this stock were used. Hepatocytes were presented with an aliquot of this compound and 2 ml of supplemented DMEM, and pre-incubated for 1 hours. After this time, the medium was changed to that required by the experiment, and a fresh aliquot of mevinolin added.

2.6 The Viability of Hepatocytes Maintained as Monolayers

Hepatocyte viability prior to plating was determined by trypan blue exclusion. After treatment with DMSO, 58-035, azacholesterol and mevinolin, viability was assessed by trypan blue exclusion and leakage of lactate dehydrogenase.

2.6.1 Exclusion of Trypan Blue

Prior to plating, viability was determined by mixing 100 µl of the hepatocyte suspension, 300 µl culture medium, and 400 µl 0.4% (w/v) trypan blue solution. An aliquot of the resultant mixuture was added to a Hawkesly Haemocytometer and the viable and non-viable cells counted. Monolayer viability was determined by addition of 0.2 ml 0.4% (w/v) trypan blue in 5 ml of culture medium. Monolayers were incubated for 5 minutes, after which the trypan blue was removed and percentage viability assessed. In both procedures, viability was expressed as the percent of cells excluding the dye.

2.6.2 Leakage of Lactate Dehydrogenase

Lactate dehydrogenase was determined spectrophotometrically with pyruvate and reduced nicotine adenine dinucleotide according to Vassault (1982). The activity of lactate dehydrogenase was determined from the rate of oxidation of NADH, by measuring the decrease in absorbance at 340 nm.

To determine the leakage of lactate dehydrogenase, the enzyme was assayed in both the culture medium and the disrupted cells. Cells were disrupted by sonication of approximately 1-1.5 mg ml^{-1} of cellular protein in phosphate buffered saline, pH 7.4. An aliquot was assayed for lactate dehydrogenase activity. The culture medium was assayed directly. Percentage viability was expressed at [1-(total units lactate dehyrodgenase in the medium - total units in the cells)] x 100. The effect on lactate dehydrogenase leakage of DMSO, 58-035, azacholesterol and mevinolin was assessed after 0 to 5 hours. Addition of the above compounds was a described previously. After the allotted time period, cells were scraped off the plates into 1 ml of phosphate buffered saline, pH 7.4, and used as stated above.

2.7 Determination of Conjugated Bile Acids

Determination of conjugated cholic, chenodeoxycholic and ß-muricholic acids was by radioimmunoassay (Becket et al., 1978; Beckett et al., 1979 and Botham et al., 1983). Bile acid content of the culture medium was measured directly, using the antisera described in the above 3 papers.

Cellular bile acid content was determined by first extracting the cells with 1M sodium hydroxide for 24 hours. This procedure has been demonstrated to extract 100% of bile acid from tissue samples (Strange et al., 1979)

2.7.1 Preparation of Immunogens

Immunogens in the following bile salt : bovine serum albumin molar ratios, cholate (17:1), chenodeoxycholate (18:1) and ß-muricholate (10:1), when prepared by the mixed anhydride method of Erlanger et al. (1957) as modified by Murphy et al. (1974).

2.7.2 Preparation of Anti-serum

1 mg of each bile acid - bovine serum albumin immunogen in 1 ml of 1:1 (v/v) emulsion of Freund's complete adjuvant in saline was injected sub-cutaneously into individual New Zealand white rabbits, a booster injection was given six weeks later using 1 mg of each conjugate in Freunds incomplete adjuvant emulsion. Blood was obtained from the ear vein and serum separated from blood by centrifugation. The anti-serum from these animals was used without further treatment.

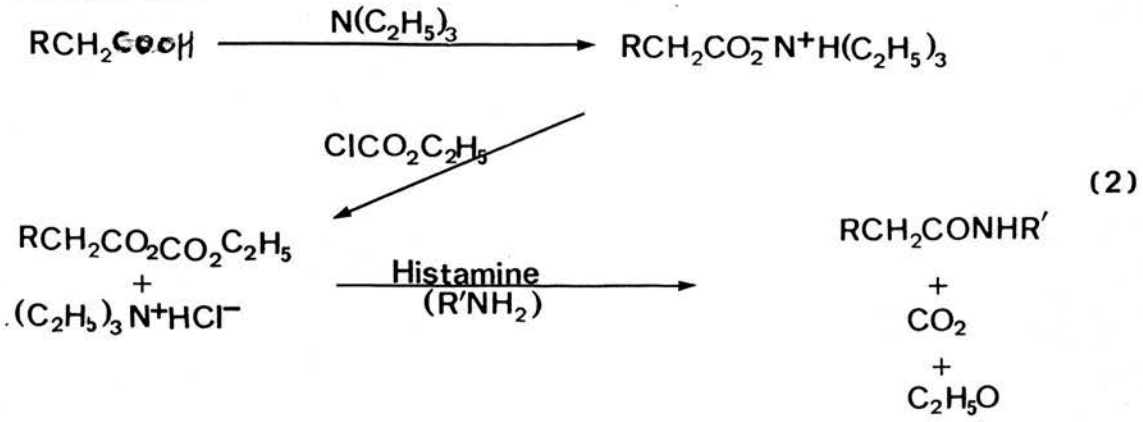
2.7.3 Determination of the Optimum Dilution of The Anti-serum

The dilution of anti-serum used in the assay of each bile acid was determined by incubation of [^{125}I]-labelled bile acid histamine conjugates wiht serial dilutions of the homologous antibody in the presence and absence of the highest standard used in each assay. Binding of the [^{125}I]-labelled ligand to the anti-serum is described in section 2.7.6. The

anti-serum dilutions that gave the greatest range of binding were subsequently used in each assay. This produced the most sensitive assay conditions (Beckett et al., 1978).

2.7.4 Preparation of Bile Acid-Histamine Conjugates

Bile acid-histamine were prepared by the mixed anhydride method outlined by Feisen and Feisen (1967). The process is shown in reation (2).



(2)

The following proportions of reactants were used; bile acid, x moles; triethylamine, 1.2xmoles; ethylchloroformate, 1.2 x moles; histamine, 1.5xmoles. A given bile acid was dissolved in a small volume (less than 5 mls) of sodium-dried tetrahydrofuran. (THF) Triethylamine was added, and the mixture cooled at 4°C. Ethylchloroformate was added dropwise. The reaction mixture was stirred for 30 minutes at 4°C, and then histamine (in tetrahydrofuran) was added. The mixture was stirred at 4°C for 1 hour, and then left stirring at room temperature overnight.

The conjugates produced tended to precipitate out of THF, so all the products and reaction mixture were dissolved in methanol. The methanol and residual tetrahydrofuran was then removed by rotary evaporation.

This left a gummy residue of bile acid-histamine conjugate, unreacted starting materials and triethylamine hydrochloride. In the case of the chenodeoxycholate conjugate, the gum was re-dissolved in methanol : water (1:1 (v/v)) and the methanol

evaporated off by rotary evaporation. The conjugate precipitated out of solution, and the water removed by vacuum filtration. Figure 2.3 shows the NMR spectrum of this conjugate.

For the conjugate of cholic and ß-muricholic acid purification was performed differently because of their greater solubility in water. The gummy residues were dissolved in methanol and applied to silical gel columns. The conjugates were eluted off the column using chloroform : methanol (10:3, v/v). The eluants were rotary evaporated, leaving behind crystalline residues which were the conjugates in question. These conjugates were also characterised by NMR.

2.7.5 Iodination of Bile Acid-Histamine Conjugates

Iodination of conjugates was performed by a method based on that described by Hunter et al. (1975). 50 nmol of each bile acid histamine conjugate was mixed wiht 1 mCi of sodium [^{125}I]-iodine and 20 µl of 20 mM chloramine T in 0.5 M phosphate buffer, pH 7.9. The [^{125}I]-labelled bile acid histamine conjugate was then extracted by addition of 0.2 ml of water followed by 0.5 ml of ethyl acetate. The ethyl acetate layer was collected and the water removed by drying with anhydrous sodium sulphate. The ethyl acetate extract was transferred to a silica gel thin layer chromatography plate and developed in ethyl acetate/toluene/triethylamine (50:25:25:10, v/v). This separated unreacted [^{125}I] iodide from the radiolabelled conjugate. After scanning, the area of the plate corresponding to the radiolabelled conjugate was extracted with methanol.

2.7.6 Assay Procedure

Bile salts were determined by using a specific anti-serum with a homologous bile acid histamine ligand. The assay was performed by mixing 10 µl of the sample with 200 µl of radio-immunoassay buffer (100 mM sodium phosphate buffer, 15 mM sodium azide, 15 mM sodium chloride, pH 7.4) containing newborn bovine serum (10% v/v), [^{125}I]-labelled bile acid histamine conjugate and appropriate anti-serum. Anti-serum was diluted to give optimum assay conditions

Figure 2.3

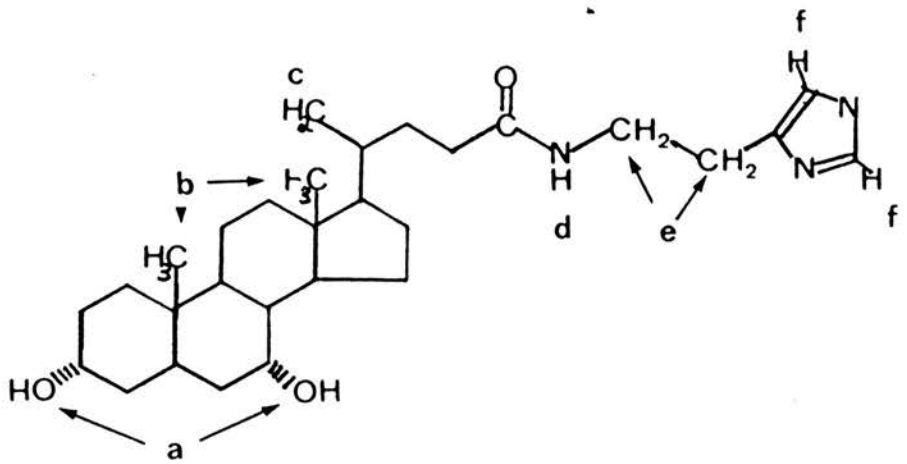
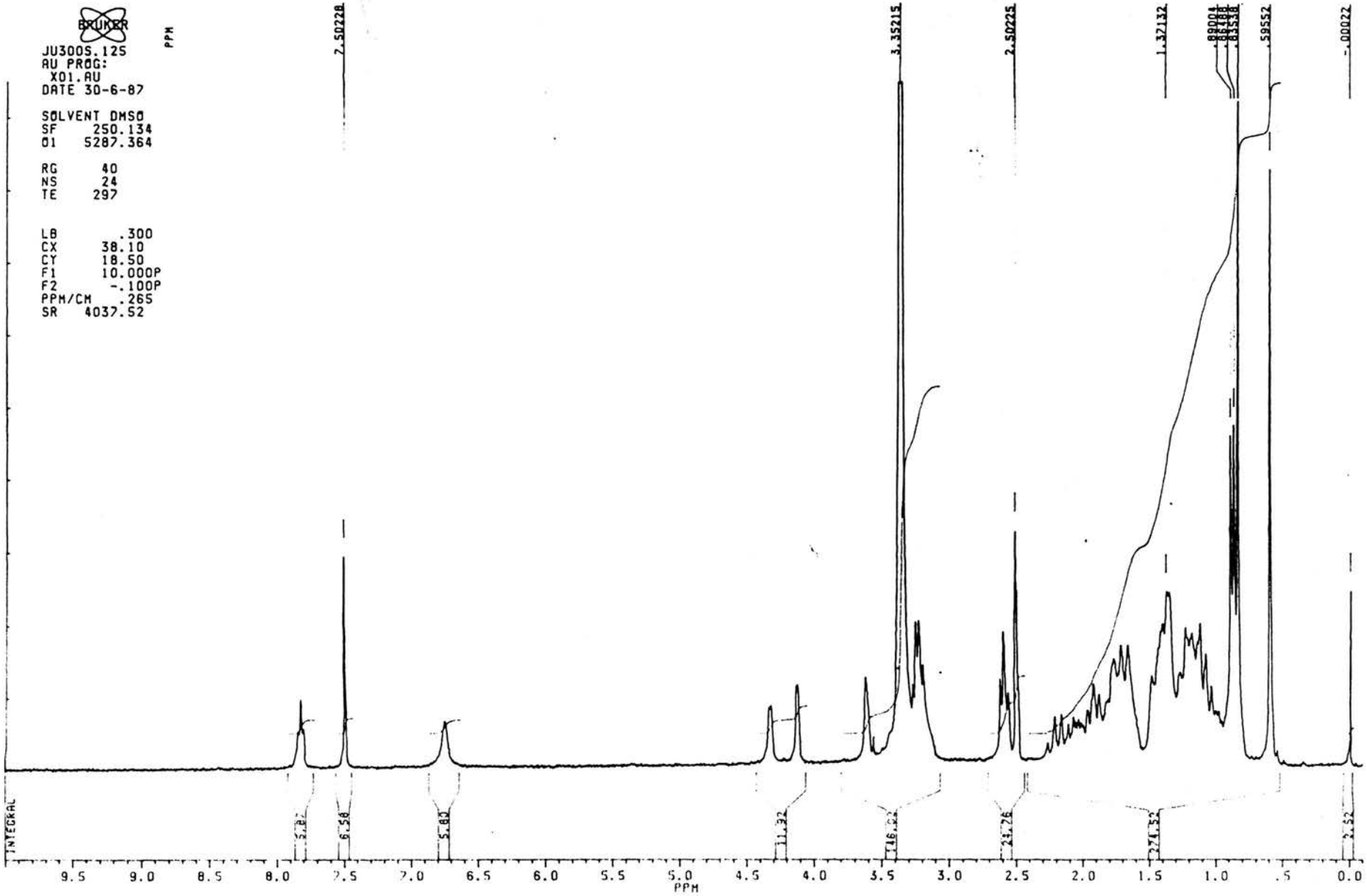


Figure 2.3 Structure and NMR Sprectum of Chenodeoxycholate-Histamine Conjugates. The groups indicated above corresponding to the following peaks on the spectrum:

a) 3α and 7α-hydroxyl groups at 4.1 and 4.35 ppm.
b) CH_3 groups at positions 18 and 19 at 0.6 and 0.8 ppm.
c) CH_3 grop at position 21, doublet peak at 0.9 ppm.
d) Amide group at 7.8 ppm.
e) $-CH_2-CH_2-$, triplet peaks at 2.6 and 3.2 ppm.
f) Imidazole hydrogens at 6.75 and 7.5 ppm.

No contamination of the above product by unreacted starting materials or reaction by products is evident in this spectrum.

F22651 123/2


BRUKER
JU300S.12S
AU PROG:
X01.AU
DATE 30-6-87
SOLVENT DMSO
SF 250.134
01 5287.364
RG 40
NS 24
TE 297
LB .300
CX 38.10
CY 18.50
F1 10.000P
F2 -.100P
PPM/CM .26S
SR 4037.52
PPM
2.50228
3.35215
2.50225
1.32132
.59552
-.00022
INTEGRAL
9.5
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5.0
4.5
4.0
3.5
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2.5
2.0
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1.0
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0.0
PPM

(section 2.7.3). Samples were incubated at room temperature for at least 3 hours. The bound ligand was separated from the free by precipitation with 3 ml 2.5% (w/v) ammonium sulphate, followed by centrifugation at 3,000 r.p.m. (2560 xg) for 15 minutes at 4°C. The precipitate was counted using a gamma counter. The bile salt content was determined from standard curves.

2.8 Separation and Measurement of Cholesterol, Cholesteryl Ester and Bile Salts After Incubation of Hepatocyte Monolayer Cultures with Radioactive Precursors

Following extraction of samples, cholesterol, cholestryl ester and bile salts were separated by thin layer chromatography (t.l.c.) or by elution on silica gel columns.

2.8.1 Extraction of Samples

Lipids and bile acids were exracted from samples after the addition of sodium hydroxide (final concentration, 1M) using chloroform/ methanol (2:1 v/v) according to the procedure of Folch et al. (1957). Cholesterol and cholesteryl ester were partitioned in the non-aqueous phase. THis was blown down to dryness and the residue re-dissolved in a known volume of chloroform. The bile salts were partitioned in the aqueous phase. Sodium hydroxide addition before extraction improved the recovery of bile salts. The protein precipitate present in the aqueous phase was removed by a centrifugation and washed with 2 x 2 ml methanol. The washes were pooled with the original supernatant, the solvent evaporated and the residue taken up in known volume of methanol.

2.8.2 Thin Layer Chromatography of Non-aqueous and Aqueous Extracts

The non-aqueous extracts, containing cholesterol and cholesteryl ester were developed in the following solvent systems:

1. For separation of cholesteryl esters only: petroleum ether/diethyl ether/acetic acid (glacial) (95:5:0.5, v/v). Rf value for cholesteryl esters is 0.875.

2. For separation of cholesteryl esters and cholesterol: diisopropyl ether/petroleum ether/acetic acid (glacial) (70:30:2, v/v). Rf values for cholesteryl esters and cholesterol are 1.00 and 0.60, respectively.

Samples of the bile salt were developed in ethyl acetate/methanol/acetic acid/water (35:12:2:2, v/v). Recovery of radioactivity from the extraction and t.l.c. procedures was followed using known quantities of appropriate radioactive tracers. Identification of cholesteryl ester, cholesterol and bile salts was determined either by scanning the plates on a t.l.c. plate scanner or by running marker plates with non-radioactive standards. The standards were visualised by spraying with 1% (v/v) phosphomolybdic acid in methanol, followed by incubating in an oven for 10 minutes at 70°C. Bands corresponding to the standards were scraped off the sample plates into scintillation vials and counted.

2.8.3 Separation of Cholesterol and Cholesteryl Ester on Silica Gel Columns

A new procedure, faster and more convenient than t.l.c. plates, was developed using silica gel columns (Anachem "bond-elut" columns, size 3cc, bonded phase SI). The columns were prepared from the assay by addition of 1 ml hexane/diethyl ether (3:1, v/v). The residues of dried non-aqueous extracts, containing cholesterol and cholesteryl esters, were taken up in 0.5 ml of hexane/diethyl ether (3:1, v/v), and applied to the columns. A further 0.5 ml of hexane/diethyl ether (3:1, v/v) was used to wash out each sample tube and then applied to the columns. Once the samples were loaded onto the columns, a further 1 ml of hexane/diethyl ether (3:1, v/v) was added to each, and the eluants collected in scintillation vials. Next, 3 mls of hexane/diethyl ether (1:1, v/v) was added to each column, and the resulting eluants collected in scintillation vials. The cholesteryl ester was extracted from the column into the first eluant collection and the cholesterol into the second eluant collection.

This system brings about the complete separation of cholesterol from cholesterol ester, but is limited to experimental situations were there are no significant levels of radioactive label incorporation either into triglycerides or long chain free fatty acids.

2.8.4 Assay of Radioactivity in T.L.C. or Silica Gel Chromatography Samples

Radioactivity present in bands scraped from t.l.c. plates or in the eluants of silica gel columns wereassayed by addition of 10 ml of Pico-fluor 15 scintillant, and counted in a Packard scintillation spectrophotometer.

2.8.5 Determination of Recovery Efficiency

In certain experiments, where the synthesis of products from radioactive precurssors occurred, prior to extraction and separation, the exact amount of activity attributable to the products was unknown. To ensure that the recovery efficiency of these products could be determined, known quantities of appropriate radioactive recovery markers were added to samples prior to extraction, e.g. for a [^{14}C] cholesterol product, [^{3}H] cholesterol was the recovery marker.

The percent recovery was determined and used to estimate the activity of the products prior to extraction.

2.9 Determination of Cholesterol

Free and total cholesterol were determined by the fluorimetric method of Gamble *et al*. (1978).

After extraction of the samples with chloroform/methanol (2:1, v/v), the non-aqueous infranatant were removed, the solvent evaporated and the residue taken up in chloroform. Free (unesterified) cholesterol is oxidised to 4-cholestene-3-one with concomitant formation of hydrogen peroxide is a measure of the amount of cholesterol present and is estimated with p-hydroxyphenylacetic acid. Oxidation of

p-hydroxyphenylacetic acid yields a fluorescent quinone. Fluorescent quinone. Fluorescence was detected using a Perkin-Elmer 3000 fluorescence spectrophotometer.

Determination of total cholesterol was carried by first hydrolysing the cholesteryl esters using cholesterol esterase, and then estimating the cholesterol present as outlined above.

2.10 Determination of Protein

Protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

2.11 Preparation of a Microsomal Fraction from Hepatocyte Monolayer Cultures for the Determination of Acyl CoA : Cholesterol Acyltransferase Activity

Having prepared monolayer cultures of hepatocytes, as described in section 2.4, certain experiments required the preparation of a microsomal fraction from the cultured cells.

After adhesion of the cells to the plates, the medium was changed to 2 ml supplemented DMEM and the cells incubated overnight. The medium was then changed to 2 ml supplemented DMEM containing 20% foetal calf serum (v/v), 58-035 was added in solution in DMSO (20 µl) to give a final concentration of up to 100 µg/ml of inhibitor. After a further 2 hours, the medium was removed and the cells washed and scraped off the plates using 2 x 0.5 ml 0.1 M potassium phosphate buffer. The resulting suspensions were individually homogenised, 10 µl removed for protein determination , and the remainder transferred to Eppendorf tubes and centrifuged at 8,800 x g in a microfuge for 5 minutes. 100 µl of bovine serum albumin (fatty acid free) and glutathione solution in potassium phosphate buffer (each 10 mg/ml) was added to 850 µl of the supernatant. After 30 minutes preincubation at 37°C [^{14}C] oleoyl CoA (150 µl, final concentration 42 µM) was added and the mixture incubated for 1 minute. The reaction was stopped by the addition of 2 ml methanol. Extraction and analysis of products has been described in sections

2.8.1 and 2.8.2. Acyl CoA : cholesterol acyltransferase activity was taken as a measure of the amount of [^{14}C]oleoyl CoA incorporated into cholesterol ester mg^{-1} cellular protein minute^{-1}.

2.12 Non-enzymatic Hydrolysis of Cholesterol Ester to Determine Incorporation of a Radioactive Label Into The Fatty Acids of Cholesterol

In certain experiments synthesis of cholesterol and cholesteryl esters were measured by observing the incorporation of radioactive precursssors (acetate, mevalonate) into the molecules in question. In particular, it was necessary to know the amount, if any, of radioactive label appearing in the fatty acyl group of cholesteryl esters synthesised from [^{3}H] [2- ^{14}C] mevalonic acid.

250 µl of media, collected from hepatocyte monolayer cultures incubated with [^{3}H] mevalonic acid, was added to 1 ml of alcoholic potassium hydroxide, and heated to 50°C for 1 hour. This was then followed by extraction with chloroform/methanol (2:1, v/v) (section 2.8.1) and separation on silica gel columns (section 2.8.3). Prior to hydrolysis a known amount of [^{14}C] cholesterol was added to each sample to act as a recovery marker. After hydrolysis, a known quantity of [^{14}C] cholesteryl oleate was added to each sample to act as a recovery marker for the esters.

Parallel to this, a further 250 µl of the media samples, with recovery markers added, were extracted (section 2.10.1) and cholesterol and cholesteryl esters separated on silica gel columns (section 2.8.3).

The following equation was determined:-

$$HFC = FC + xCE$$

The amount of radioactive label incorporated into the hydrolysed free cholesterol (HFC) should equal the free cholesterol (FC) plus the cholesterol ester if none of the label is present in the fatty

acyl group of the ester. If some label has been incorporated into the fatty acyl group, then the hydrolysed free cholesterol will be less than expected i.e. by the factor x.

The hydrolysed free cholesterol (HFC) was determined from the hydrolysed samples, and the free cholesterol and cholesteryl esters were determined from non-hydrolysed samples.

2.13 Isolation of Rat Plasma High Density Lipoprotein Subfraction (HDL_2)

Rat HDL_2 was isolated by density gradient ultracentrifugation (Havel et al, 1955; Jansen et al, 1983). The density range used for isolation of HDL_2 was 1.085 - 1.21 g ml^{-1}. (Oschry and Eisenberg, 1982).

Blood was obtained from ether anaethetised, female Wistar rats via the inferior vena cava and cardiac puncture, and collected in 10 ml polypropylene tubes containing 40 mg EDTA to prevent clotting. Blood cells were separated from plasma by centrifugation at 2,000 r.p.m. (1140 xg) in a Beckman J-6-B centrifuge using a JS-3.0 rotor, at 4°C.

The rat plasma obtained was adjusted to a density of 1.090 g ml^{-1} by mixing with a high density salt solution (1.346 g ml^{-1}) containing sodium chloride (158 g l^{-1}), pottasium bromide (354 g l^{-1}) and EDTA (1 g l^{-1}), pH 7.2. This mixture was added to a Beckman "Quick-Seal" centrifuge tube and over-layered with a salt solution of a density of 1.080 g ml^{-1}. This latter solution was prepared by mixing appropriate volumes of the high density salt solution with a low density salt solution (1.006 g ml^{-1}) containing sodium chloride (9 g l^{-1}), EDTA (1 g l^{-1}) and sodium azide (0.2 g l^{-1}), pH 7.2. The tubes were sealed and then centrifuged at 49,000 r.p.m. (200,000 xg) in a Beckman L8-70M ultracentrifuge using a 50.2 Ti rotor for 22 hours at 12°C. After centrifugation the tubes were sliced open, and the higher density fraction removed, adjusted to a density of 1.23 g ml^{-1} as described previously, added to Beckman "Quick-Seal" tubes, and over-layered with a salt solution of 1.21 g ml^{-1}.

The tubes were sealed and centrifuged at 49,000 r.p.m. (200,000 xg), as described earlier. On completion, the remaining lipoproteins had floated to the top of the centrifuge tube, and were extracted by slicing open the centrifuge tube at the appropriate level. The fraction collected was dialysed overnight against 2 changes of 4 litre water and then reverse phase dialysed using [carbo-wax, solid polyethylene glycol].

2.14 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis of HDL_2 Apoproteins

SDS-discontinuous Slab-gel electrophoresis was performed according to the method of Laemmli (1970) with modifications as described by Douglas and Butow (1976). Gradient gel electrophoresis with a continuous buffer system was performed according to the method outlined by Hames and Rickwood (1981).

2.14.1 Solutions Required for SDS-discontinuous Gel Electrophoresis

A) Separating gel buffer : 15 M Tris HCl, 8mM EDTA, 0.4% SDS (w/v), pH 8.8.

B) Stock acrylamide : 30% (w/v) acrylamide, 0.8% (w/v) N,N'-methylene bisacrylamide dissolved in water, filtered through glass wool and stored in the dark at 4°C.

C) Stacking gel buffer : 0.5M Tris HCl, 8mM EDTA, 0.4% (w/v) SDS, pH 6.8.

D) Polyacrylamide : 1.5% (w/v) polyacrylamide dissolved slowly in water containing 1mM sodium azide and 1mM sodium fluoride.

E) Concentrated sample buffer 0.2M Tris HCl buffer, pH 6.8, 8mM EDTA 8% (w/v) SDS, 40% (v/v) glycol, 4% (v/v) 2-mercaptoethanol, 0.0025% (w/v) bromophenol blue. Samples were solubilised in the sample buffer and the diluted 4 times.

F) Electrode buffer : 50mM Tris-glycine buffer, pH 8.6, 2mM EDTA, 0.1% (w/v) SDS.

2.14.2 Solutions Required for SDS-Continuous Gel-Electrophoresis

A) Electrophoresis buffer : 40mM Tris HCl, 20mM sodium acetate, 2mM EDTA, 0.2% (w/v) SDS, pH 7.4

B) Concentrated sample buffer : refer to section 2.14.1 E.

2.14.3 Preparation of a Separating Gel

A gel cassette was assembled which comprised two 18 x 18 cm glass plates separated by two spacers smeared with vacuum grease placed at both sides of the plates. The cassette was then placed in a 'foil boat' and the bottom sealed by pouring 12 ml of a solution comprising 8.75ml solution B, 2.38 ml 2M Tris HCl, pH 8.8, 0.25 ml 10% (w/v) SDS and 1.2 ml water, to which was added 250 µl 10% (w/v) ammonium persulphate and 10 µl TEMED. TEMED was added to initiate polymerization. After 30 minutes the gel had set and the foil boat and excess gel was removed from the bottom of the cassette.

A 15% polyacrylamide gel was then prepared by pouring 30 ml of a separating gel mixture consisting of 7.5 ml solution A, 15.0 ml solution B, 5.0 ml D, 2.2 ml water and 0.3 ml 10% (w/v) ammonium persulphate to which 15 µl of TEMED had been added, into the cassette. Secondary butanol, saturated with water was carefully layered onto the separating gel, which was then left to set. After 1 hour, the secondary butanol was removed and a stacking gel prepared. The stacking gel mixture consisted of 1.2 ml solution B, 2.0 ml solution C, 1.3 ml solution D, 3.4 ml water, 15 µl TEMED. A toothed comb was inserted into the top of the cassette, and the stacking gel allowed to set. Whem polymerisation was complete the toothed comb was carefully removed and samples placed into sample holders.

The gradient gels used were 4%-30% polyacrylamide, purchased from Pharmacia.

CHAPTER 3

The Viability of Rat Hepatocyte Monolayers

3.1 Introduction

The work initially performed by Berry and Friend (1969) on the isolation of hepatocytes and that by Bissell *et al.*, (1973) on culturing hepatocytes in non-proliferating monolayers were the first to demonstrate the feasibility of this approach in the study of functional aspects of the liver. Investigations over short time periods of a few hours use parenchymal cells in suspension, whilst longer term studies use monolayer cultures. Both of these techniques have been useful in studying certain aspects of cholesterol and bile acid metabolism.

The obvious advantages of these procedures are that a number of experiments can be performed on cells from the same animal, whilst use of a defined culture medium allows control of the extracellular enviroment. Particularly, investigations involving the catabolism of different lipoprotein classes are not hindered by the problem of in vivo exchange of the lipid and protein moieties between said classes. As with all models of biological systems there are disadvantages in using these techniques .

Firstly, there is the physical consideration that the cells are not in their normal orientations. In vivo, hepatocytes are in contact with three compartments:-

i) the perisinusoidal or space of Disse.
ii) the intercellular space.
iii) the bile canaliculi.

Studies on isolated plasma membrane fractions (Wisher and Evans, 1975) have demonstrated functional differentiation of the hepatocyte plasma membrane. Bile acids are taken up on the sinusoidal surface and secreted on the canalicular side. This distinct membrane polarity is lost once the hepatocytes have been isolated, which

might cause certain problems. For example, the hepatocytes will synthesize and secrete bile acids and lipoproteins into the culture medium from where they can be taken up (Iga and Klaassen, 1982) which in turn might effect the control mechanisms under study. Furthermore, it is not possible to determine into which compartment, bile or plasma, various secretions are made.

Some circulating hormones normally present in the plasma are known to influence the metabolism of cholesterol. These normal metabolic interactions are not catered for using defined culture medium.

Also, whilst this procedure allows for the investigation of the catabolism of defined lipoprotein classes, it is likely that transfer and exchange of components between said classes provide a mechanism by which lipids are removed from plasma. Thus, it is essential to consider these problems when trying to use isolated hepatocytes to answer specific metabolic questions.

Along with the theoretical considerations there are also practical problems in using rat hepatocytes. Work done previously (Ford, 1984; Ford et al., 1985a) outlined and defined many of these details. Isolation of the hepatocytes really starts with the digestion of the intercellular matrix by collagenase, first described by Berry and Friend, (1969), and later modified by a range of investigators (Quirstorff et al., 1973; Seglen, 1973). Most modifications are concerned with the preparation of crude collagenase used in the isolation procedure. The varying digestive powers of the collagenase obtained by these methods will have a marked effect on the initial viability of the hepatocytes. From my experience, a good commercial preparation or batch of collagenase should be obtained. Certainly the efficiency of digestion should be such that no more than 1.5 mg collagenase per ml of perfusate should be necessary, nor should the perfusion time exceed 16 minutes.

Maintenance of the hepatocytes once isolated presents the next series of problems. The culture media used in many experiments required supplementation with foetal calf serum (heat-inactivated) and insulin to ensure adhesion of the hepatocytes to the culture

surface. After over- or under- digestion of the liver, resulting in total non-viability, lack of adhesion of the hepatocytes, was the cause of experimental failure, usually due to the inactivity of the insulin used.

Ford (1984) in his work on characterisation of rat hepatocyte monolayers investigated a number of methods for assessing hepatocyte viability. The methods chosen by Ford; trypan blue exclusion, LDH leakage, protein synthesis and measurement of cellular content of cytochrome P-450, are only a few of the criteria available (Crisp and Pogson, 1972; Baur et al., 1975). It is clear from his work that the plasma membranes of hepatocytes remain intact and the hepatocytes maintain protein synthesis over 24hours. Thus for the system of rat hepatocyte monolayer cultures used in the following work, trypan blue exclusion was used as the criteria for assessing viability in all experiments. Preliminary viability experiments with the compounds used with the heptocytes were conducted using both trypan blue exclusion and LDH leakage.

3.2 Exclusion of Trypan Blue by Rat Hepatocyte Monolayers

Viability was determined at 0 and 5 hours under the variety of conditions required for later experiments (refer to Chaper 2.5.1 - 2.6.1). Thus the effects of mevinolin, 58-035, 58-035 + mevinolin, DMSO, and azacholesterol on the ability of hepatocyte monolayers to exclude trypan blue was assessed.

From Table 3.1, the results show that over the time period of 5 hours, viability of cells adhered to the plates remained greater than 90% and none of the culture conditions differed significantly from the control values. During the period of incubation an apparent loss of cells occurred on some plates usually of the order of 6.0×10^4 cells, less than 3% of cells originally plated out. This loss was neither significant nor apparently related to the particular experimental conditions.

Experiment	Time (Hours)	% Viability Trypan Blue Exclusion	Total LDH Activity (U per 2.5×10^6 cells) Cells & Media	% LDH Activity Relative to Control	
				Cells	Media
a) Control	0	96.5	2.71	100	0
	5	95	2.79	68.8	31.2
b) DMSO	0	96	2.76	100	0
	5	93	2.80	70.9	29.1
c) 58-035	0	96	2.69	100	0
	5	95.5	2.79	71.3	28.7
d) Mevinolin	0	99	2.84	100	0
	5	96.5	2.91	73.8	26.2
e) Mevinolin + 58-035	0	96	2.61	100	0
	5	95.5	2.86	72.7	27.3
f) Azacholesterol	0	97.5	2.82	69.1	30.1

Table 3.1

Assessment of the viability of hepatocyte monolayers by trypan blue exlusion (expressed as a percentage) and LDH leakage after 0 and 5 hours as affected by treatment with : a) Control, 2 mls DMEM with 2% bovine serum albumin (fatty acid free); b) Control + 20 µl DMSO; c) Control + 200 µg 58-035 in 20 µl DMSO; d) Control + mevinolin (5 µM); e) Control + 58035 (200 µg) + mevinolin (5 µM); f) Control + Azacholesterol (500 µM).

3.3 Leakage of Lactate Dehydrogenase by Rat Hepatocyte Monolayers

The results as determined by the measurement of LDH in the cellular and media compartments (Table 3.1) indicate that 30% of cellular LDH leaks into the media over 5 hours for both the control and the experimental conditions. This leakage of LDH was to be expected when compared to previous investigations (Ford, 1984). Interestingly no difference occurs between control and experimental samples, indicating that the compounds used have not affected the cells viability.

3.4 Discussion

The various experimental conditions to which the hepatocyte monolayers were subjected did not affect their viabilities. There was no difference between the results obtained for the control and experimental samples as measured by either trypan blue exclusion or LDH leakage. Although, leakage of LDH did occur for both experimentals and controls, this was within expected values and not due to any experimental treatment.

Thus, for later experiments trypan blue exclusion alone was used for assessment of cells used for culture, and was considered a sufficient guide to their viability.

CHAPTER 4

The Effect of Compound 58-035 on Cholesterol Metabolism and Bile Acid Synthesis

4.1 Introduction

In Chapter 1, cholesterol metabolism was discussed fully and the fates of hepatic cholesterol described. As follows, free cholesterol can be secreted either into the plasma associated with lipoproteins, or into the bile canaliculi. It can be further metabolised into bile acids and secreted into bile; and can be esterified and stored or secreted into the plasma with lipoproteins. The existence of these different metabolic roles for cholesterol raises the possibility that different intracellular pools of free cholesterol are responsible for the various discrete products (Stone et al., 1985; Stone et al., 1987; Botham, 1986; Ford et al., 1985a).

Esterification of cholesterol occurs by the action of the microsomal enzyme Acyl-CoA: cholesterol acyl transferase. (Suckling and Stange, 1985). It is thought that any modifications of the activity of this enzyme may have important consequences for the fate of free cholesterol. Certainly, if the activity of ACAT is inhibited, the possibility exists that the free cholesterol normally utilized would be used elsewhere. It is thus important to determine the fate of cholesterol under these conditions, as it would elucidate the interconnection of different pathways of cholesterol metabolism.

The Sandoz compound 58-035 has been shown to be a potent inhibitor of this enzyme, and is thus of use in studying intracellular cholesterol metabolism. It has been shown to inhibit ACAT activity in hepatoma cells, gut cells, ovarian cells, macrophages, bovine adrenal cortical cells and fibroblasts (Ross et al., 1984, Ross & Rowes, 1984; Veldhuis et al., 1985; Jamal et al., 1985; Havekes, 1987; Middleton, 1987).

The effect that the inhibition of ACAT has on intracellular cholesterol metabolism varies depending upon the cell type. In macrophages, 58-035 enhanced the down-regulation of LDL receptors and HMG-CoA reductase by LDL, and decreased cellular accumulation of cholesterol derived from LDL (Tabas et al., 1986); whereas in fibroblasts, 58-035 inhibited ACAT without affecting receptor numbers (Middleton, 1987). In macrophages ACAT competes for a regulatory pool of cholesterol that mediates LDL receptor down-regulation, which isn't the case in fibroblasts. Middleton (1987) suggests that fibroblasts have two interconnected but distinct pools of cholesterol, one involved in LDL-receptor down-regulation; the other providing substrate for ACAT.

Inhibition of ACAT in two types of steroidogenic tissues, ovarian and adrenal cortical cells, resulted in increased progesterone and cortisol secretion respectively, indicating an INVERSE correlation between the pool or pools of cholesterol for esterification and steroidogenesis (Jamal et al., 1985; Veldhuis et al., 1985). The situation is not as uniform as it might seem from the above, for cellular responses to trophic hormones and other factors differ in these cells. In the ovarian cells, 58-035 enhances the effect of trophic hormones but in combination with LDL does not increase steroidogenesis above that seen with 58-035 alone. In the adrenal cortical cells, 58-035 did not augment the effect of a trophic hormone.

These examples demonstrate that although the basic action of 58-035, inhibition of ACAT, occurs in all tissues investigated, the overall effect on intracellular cholesterol metabolism varies. This variation will depend largely upon the basic functions of different cell types. These functional differences may in turn be seen in differences in the intracellular architecture and organisation, resulting in the existence of different cholesterol pools with distinct functions depending upon the cell type.

The following experiments were performed to characterise the effects of 58-035 in rat hepatocytes in monolayer culture.

Initial in vitro work on rat liver microsomes showed that 58-035 inhibited ACAT activity to 12% of controls. Stimulation of ACAT activity

by a number of means, by 25-hydroxycholesterol, by an ATP dependent process and by addition of cholesterol in a number of ways, was carried out. In each case 58-035 inhibited the activated system to levels similar to those of control assays containing 58-035 and microsomes alone. 58-035 is therefore able to inhibit ACAT activity whatever the state of the enzyme in vitro. It did not inhibit rat liver microsomal 7α-hydroxylase at the concentrations required to inhibit esterification, or at concentrations 40-times that (Sampson et al., 1987a). With this information in mind, it was decided to determine the effect of 58-035 on the fate of newly synthesized cholesterol within the hepatocytes from mevalonate. This approach has been used to study cholesterol esterification and VLDL secretion and assembly in monolayer cultures of rat hepatocytes, Drevon et al., 1980a; Drevon et al., 1980b; Davis et al., 1982); and to study the dependence of bile acid synthesis on substrate supply in the same system (Davis et al., 1983a; Davis et al., 1983b). In work already mentioned, mevalonate provides a substrate for cholesterol synthesis that by-passes the enzyme HMG-CoA reductase, ensuring synthesis of free cholesterol without the effects of normal metabolic regulation. Although not as close to a physiological model as work with lipoproteins, this approach allows the definition of intracellular cholesterol metabolism under mechanisms of substrate supply which are both understood and defined (Drevon et al., 1980a). This will thus provide the background for investigations involving lipoproteins described in Chapter 6.

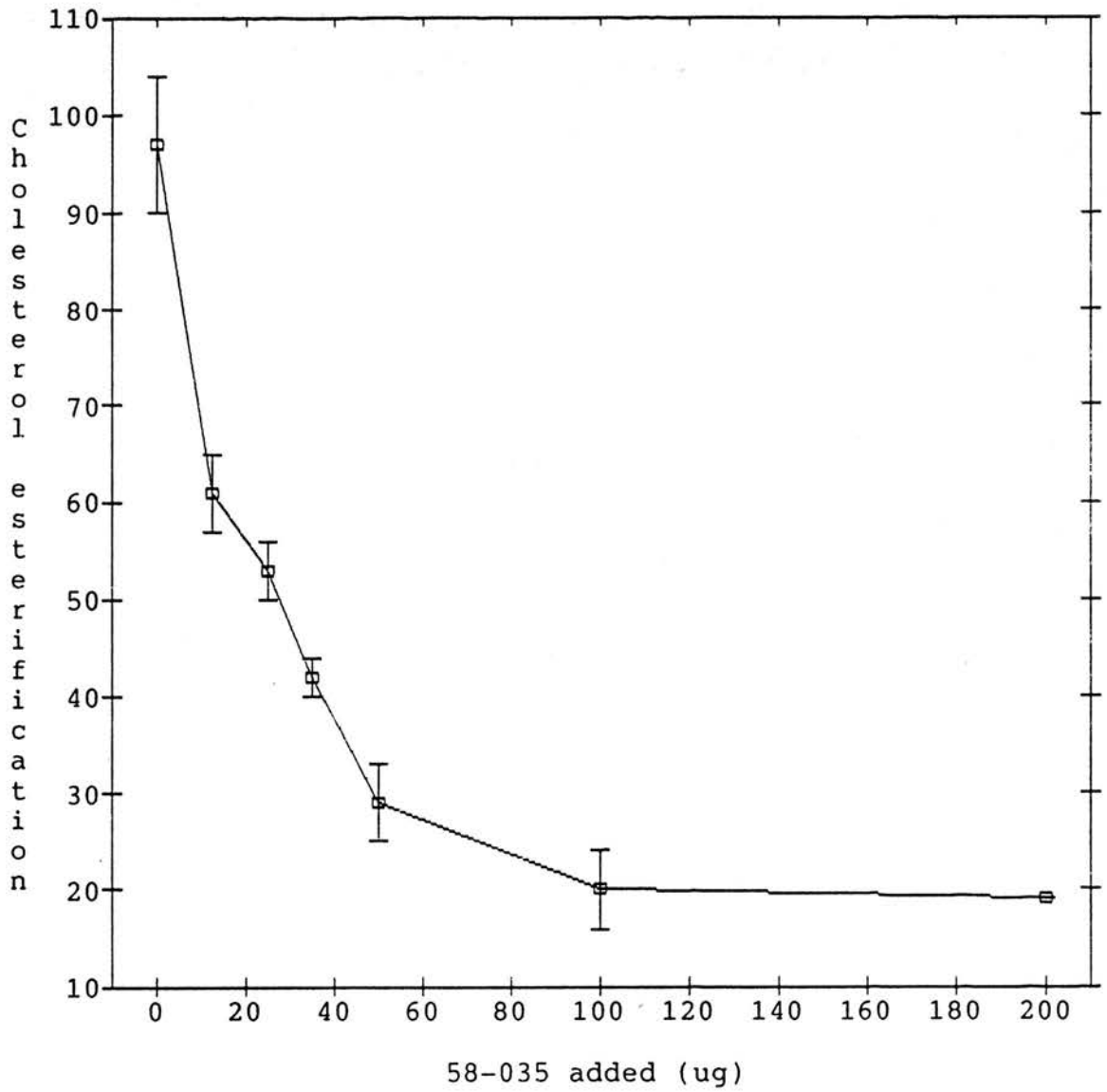
4.2 Effect of 58-035 on Cholesterol Esterification in Rat Hepatocyte Monolayers

These initial experiments were to determine whether 58-035 could enter the intact cells and inhibit cholesterol esterification. Rat hepatocyte monolayers were prepared as described in Chapter 2. 58-035 was presented to the cells as a solution in DMSO (0-200 µg/dish in 20 µl) in the presence of bovine serum albumin (2-5%

Figure 4.1

Inhibition of cholesterol esterification in monolayers of rat hepaotcytes by 58-035. 58-035 was added in amounts ranging from 0 to 200µg in 20µl DMSO, as stated in Chapter 2.5.1. After the 2 hour pre-incubation microsomal supernatants were prepared from the cultures as described in Chapter 2.11, and ACAT activity measured. The results are in units of pmole min^{-1} mg^{-1} cellular protein, ± S.E. of 4 independent experiments.

Figure 4.1


Cholesterol esterification
110
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
100
120
140
160
180
200
58-035 added (ug)

(w/v)). Following a 2 hour incubation with the inhibitor, the formation of cholesteryl [^{14}C] oleate from [^{14}C] oleoyl-CoA in the 8800 x g supernatant prepared from the cells (Chapter 2.11). Fig 4.1 shows that inhibition of esterification occurs under the condition used, reaching a maximum of approximately 80% at doses of 100 µg/dish or greater. Experiments carried out in which bovine serum albumin was absent from the culture medium failed to produce inhibition of esterification any greater than 30% at doses of up to 300 µg/dish, indicating that for efficient delivery of 58-035 to its target enzyme, protein in the culture medium is essential.

In these experiments and those following, controls with and without DMSO were used, showing no effect of DMSO on cholesteryl ester formation and secretion or bile acid synthesis and secretion. Also after the 2 hour incubation with 200 µg per dish of 58-035 in the presence of 2% BSA, the medium was changed, fresh medium without inhibitor added and the cells incubated for up to 23 hours, followed by preparation of the 8800 x g supernatant and assay of ACAT activity. Under these conditions, inhibition was still observable, remaining at approximately 80%. This indicates that 58-035 remains associated with the cells even after the donor medium is removed, given a sufficient (2 hours) incubation time.

To confirm that inhibition of esterification occurs in intact cells, the incorporation of potassium [^{3}H] oleate into cholesteryl oleate was measured. Following incubation for 2 hours with 58-035 (100 or 200 µg/dish) in the presence of 2% (w/v) bovine serum albumin, the medium was changed, potassium [^{3}H] oleate (1 mM) and 25-hydroxycholesterol (20 µg/dish in 10 µl of ethanol) in the presence of 5% (w/v) bovine serum albumin was added, and the cells incubated for 1.5 hours.

Table 4.1 Shows that 58-035 effectively inhibits cholesterol esterification in intact cells. It also shows that addition of 25-hydroxycholesterol stimulates cholesteryl ester formation (Drevon et al., 1980a). This stimulation was inhibited by 58-035, but in each case 25-hydroxycholesterol increased the extent ester formation by about 2.8 fold.

Experiment	Cholesterol Esterification p mole per hour per dish	% of control
a) Control	205 ± 8	100
b) DMSO	198 ± 6	96.6
c) 25-OH	555 ± 43	271
d) 25-OH + DMSO	541 ± 25	264
e) 100µg 58-035	35 ± 4	17.1
f) 200µg 58-035	34 ± 1	16.6
g) 100µg 58-035 + 25-OH	103 ± 5	50.2
h) 200µg 58-035 + 25-OH	100 ± 11	48.8

Table 4.1

The effects of 58-035 and 25-hydroxycholesterol on cholesterol esterification in rat hepatocyte monolayers. Cholesterol esterification in intact cells was measured by following the incorporation of potassium [^{3}H] oleate into cholesteryl ester under the following conditions: a) control, b) 20µl DMSO added to the medium, c) 25-hydroxycholesterol (10µg ml^{-1}), d) 25-hydroxy cholesterol + DMSO, e) 100µg 58-035 in 20µl DMSO, f) 200µg 58-035 in 20µl DMSO, g) 100µg 58-035 + 25-hydroxycholesterol and h) 200µg 58-035 + hydroxy cholesterol. The table shows the mean ± S.D. (n=4).

4.3 Effect of 58-035 on the Secretion of Bile Acids by Rat Hepatocyte Monolayers

Previous work has shown that monolayers of rat hepatocytes secrete cholate, chenodeoxycholate and ß-muricholate over periods up to 18 hours in culture and that these represent more than 90% of bile acids synthesized by rat hepatocytes. The greatest rate of synthesis occurs over the first 5 hours of incubation. The amount of bile acids secreted by a preparation of hepatocytes can be varied by altering the diet of the rats concerned (Ford et al., 1985a). The diets used then comprised a soft diet (fibre free), pellet diet, and soft diet supplemented with 4% cholestyramine, (a bile-acid-binding resin). Synthesis and secretion of bile acids was highest in hepatocytes derived from rats fed the diet containing cholestyramine and lowest in cells from the rats fed the soft diet. In the following experiments, rats fed on both the soft diet and the cholestyramine-supplemented diet Z were used.

Evidence suggests that the rate of bile-acid synthesis, regulated by the activity of cholesterol 7α-hydroxylase (Chapter 1.3), is dependent upon the supply of substrate cholesterol (Ford et al., 1985b, Davis et al., 1983 a and b). To investigate the role played by ACAT in the rat hepatocytes, monolayers were incubated with and without 58-035 in the presence of increasing concentrations of mevalonic acid (0-10 mM), used to provide a substantial source of intracellular cholesterol. Secretion of the 3 bile acids into the cultured medium was measured after 5 hours by radioimmunoassay. Nine animals, 4 fed the soft and 5 fed diet Z, with basal rates of bile acid secretion ranging from 0.2 nmol/mg cell protein to 4 nmol in 5 hours, were studied. The results obtained were treated in 2 ways due to the wide variation in the basal rates. Firstly, the amounts of the bile acids secreted in 5 hour were expressed as the increase in bile acid secretion over that obtained with the control incubations in which no 58-035 or mevalonic acid had been added. The mean of these values was calculated and statistical significance between the incubations with 58-035 present and absent was evaluated using the Students t-test. These results are shown in Tables 4.2 and 4.3. Alternatively, the data from paired incubations with and

Diet and animal number	Experiment	[Mevalonic Acid](mM) 0	1	5*	10**
All animals (9)	Control	1 ± 0	1.175 ± 0.057	1.066 ± 0.074	1.182 ± 0.079
	Inhibitor	1.066 ± 0.06	1.246 ± 0.099	1.342 ± 0.064	1.474 ± 0.148
Soft diet (4)	Control	1 ± 0	1.131 ± 0.099	1.047 ± 0.133	1.174 ± 0.105
	Inhibitor	1.051 ± 0.03	1.165 ± 0.091	1.261 ± 0.085	1.334 ± 0.120
Diet Z (5)	Control	1 ± 0	1.210 ± 0.072	1.081 ± 0.108	1.189 ± 0.125
	Inhibitor	1.077 ± 0.112	1.310 ± 0.158	1.407 ± 0.089	1.586 ± 0.251

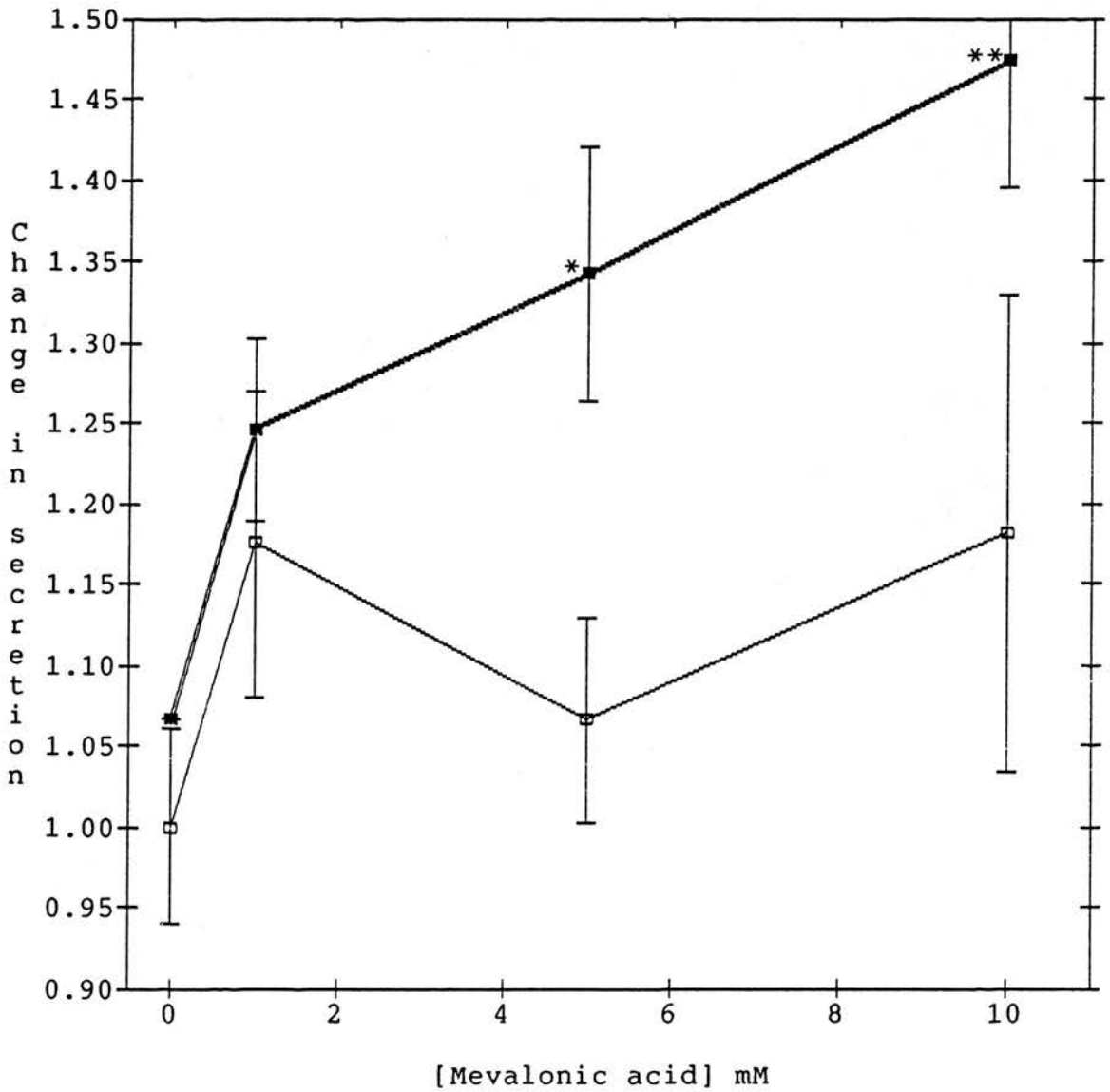
Table 4.2

The effect of 58-035 on bile acid secretion in the presence of mevalonic acid in hepatocyte monolayers. 200µg of 58-035 per dish of 2.5 x 10^6 cells was added and incubated for 5 hours, and the bile acids measured by radioimmunoassay. Raw data showed a significant difference between control and inhibitor experiments at *5mM mevalonic acid ($p=0.195\%$, one-tailed) and **10mM mevalonic acid ($p=0.39\%$, one-tailed), when analysed by the Wilcoxon signed rank test. Students t-tests of the paired normalised data data above also showed significant differences at 5 and 10mM mevalonic acid ($p<0.05$). The table shows the mean ± S.E. of the normalised values.

Figure 4.2

The effect of 58-035 on bile acid secretion in the presence of mevalonic acid in hepatocyte monolayers. For details refer to the legend on Table 4.2 (□) control experiments; (■) experiments in the presence of 200µg per dish of 58-035.

Figure 4.2


1.50
1.45
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
Change in secretion
*
**
0
2
4
6
8
10
[Mevalonic acid] mM

without 58035 were compared directly by the Wilcoxon signed rank test. These results are included in the legends of Tables 4.2 and 4.3

As data had been collected from animals on 2 different diets, and on 3 individual bile acids, a number of comparisons were possible. Firstly, the effects of 58-035 and mevalonate on total bile acid secretion in all animals combined was looked at, and then the effects of the different diets were looked at to see if any difference due to the activity of cholesterol 7α-hydroxylase could be discerned. Secondly, the secretion of the 3 bile acids were looked at to see if one or other was more effected by the experimental conditions. Cholate was looked at individually, whereas chenodeoxycholate and ß-muricholate were combined because they are both synthesized on the same branch of the bile acid pathway (Chapter 1.3 and Figure 1.2).

Whichever method of analysing the results was chosen, when looking at the combined data from all animals, 58-035 was found to cause a highly significant increase in the secretion of bile acids from the cells at concentrations of mevalonic acid over 5 mM (1.26-times control at 5 mM and 1.25 - times at 10 mM). No significant increase was seen in absence of mevalonic acid. (Table 4.2 and Figure 4.2). When examining total bile acid secretion with respect to the diet of the animals, the Wilcoxon signed rank test was not applicable, as the sample size was not large enough, so only the Student's t-test comparison was undertaken. The results (Table 4.2) show that the increase in bile acid secretion due to 58-035 is most marked in cells from animals on diet Z, where the basal rate of bile acid secretions was high.

Looking at the data for cholate and [chenodeoxycholate + ß-muricholate] as outlined in Tables 4.3 and 4.4, one can see the same trends as shown by total bile acid secretion (Table 4.2) are apparent in both. This indicates that 58-035 is having the same effect, increasing bile acid production in the presence of 5mM and 10mM mevalonic acid, but not in its absence, on both branches of the bile acid production. There is little difference between cholate or

Table 4.3

The effect of 58-035 on the secretion of the bile acids (refer to the legend of Table 4.2). a) cholic acid and b) [chenodeoxycholate + ß-muricholate]. Significance as tested by the Wilcoxon signed rank test showed differences between control and inhibitor experiments raw data at *5 and**10mM mevalonic acid for a) cholate at p = 0.195% and p= 4.89% respectively; b) [chenodeoxycholate + ß-muricholate] at p = 0.195% and p = 0.390%.

Diet and Animal #	Experiment	[Mevalonic acid](mM) 0	1	5*	10**
a)					
All animals (9)	Control	1 ± 0	1.079 ± 0.069	0.990 ± 0.980	1.163 ± 0.091
	Inhibitor	0.999 ± 0.052	1.125 ± 0.105	1.214 ± 0.065	1.374 ± 0.143
Soft diet (4)	Control	1 ± 0	1.038 ± 0.151	0.971 ± 0.160	1.091 ± 0.114
	Inhibitor	0.986 ± 0.085	1.066 ± 0.143	1.176 ± 0.119	1.300 ± 0.118
Diet Z (5)	Control	1 ± 0	1.111 ± 0.054	1.006 ± 0.086	1.220 ± 0.142
	Inhibitor	1.008 ± 0.072	1.173 ± 0.162	1.265 ± 0.091	1.434 ± 0.192
b)					
All animals (9)	Control	1.0 ± 0	1.213 ± 0.051	1.065 ± 0.064	1.192 ± 0.086
	Inhibitor	1.083 ± 0.081	1.291 ± 0.076	1.383 ± 0.048	1.491 ± 0.107
Soft diet (4)	Control	1.0 ± 0	1.203 ± 0.097	1.104 ± 0.142	1.257 ± 0.119
	Inhibitor	1.101 ± 0.063	1.244 ± 0.088	1.338 ± 0.079	1.394 ± 0.159
Diet Z (5)	Control	1.0 ± 0	1.221 ± 0.062	1.033 ± 0.046	1.140 ± 0.129
	Inhibitor	1.069 ± 0.145	1.329 ± 0.124	1.420 ± 0.063	1.568 ± 0.152

[chenodeoxycholate + ß-muricholate] in terms of the relative increases in secretion, and increased secretion of one bile acid alone cannot account for the increase in total bile acid secretion.

In a later experiment, there were no differential effects observed between cholate or [chenodeoxycholate + ß-muricholate] production by any of the conditions used, so only effects on total bile acid production will be discussed. Certainly, under the conditions looked at, the effects are evenly distributed over all bile acid secretion.

4.4 Effect of 58-035 on the Secretion of Cholesteryl Esters by Rat Hepatocyte Monolayers

In conjunction with the work conducted on the secretion of bile acids just outlined, the secretion of cholesteryl ester into the medium was also examined. Firstly, the nature of the lipoprotein particles in which the cholesteryl ester was secreted was determined. It was believed that ester would be secreted with VLDL-like particles of d<1.03 (Davis et al., 1979; Davis, et al., 1980; Davis et al., 1982; Drevon et al; 1980a). This fraction was prepared by ultracentrifugation of media from incubations of rat hepatocyte monolayers for over 5 hours.

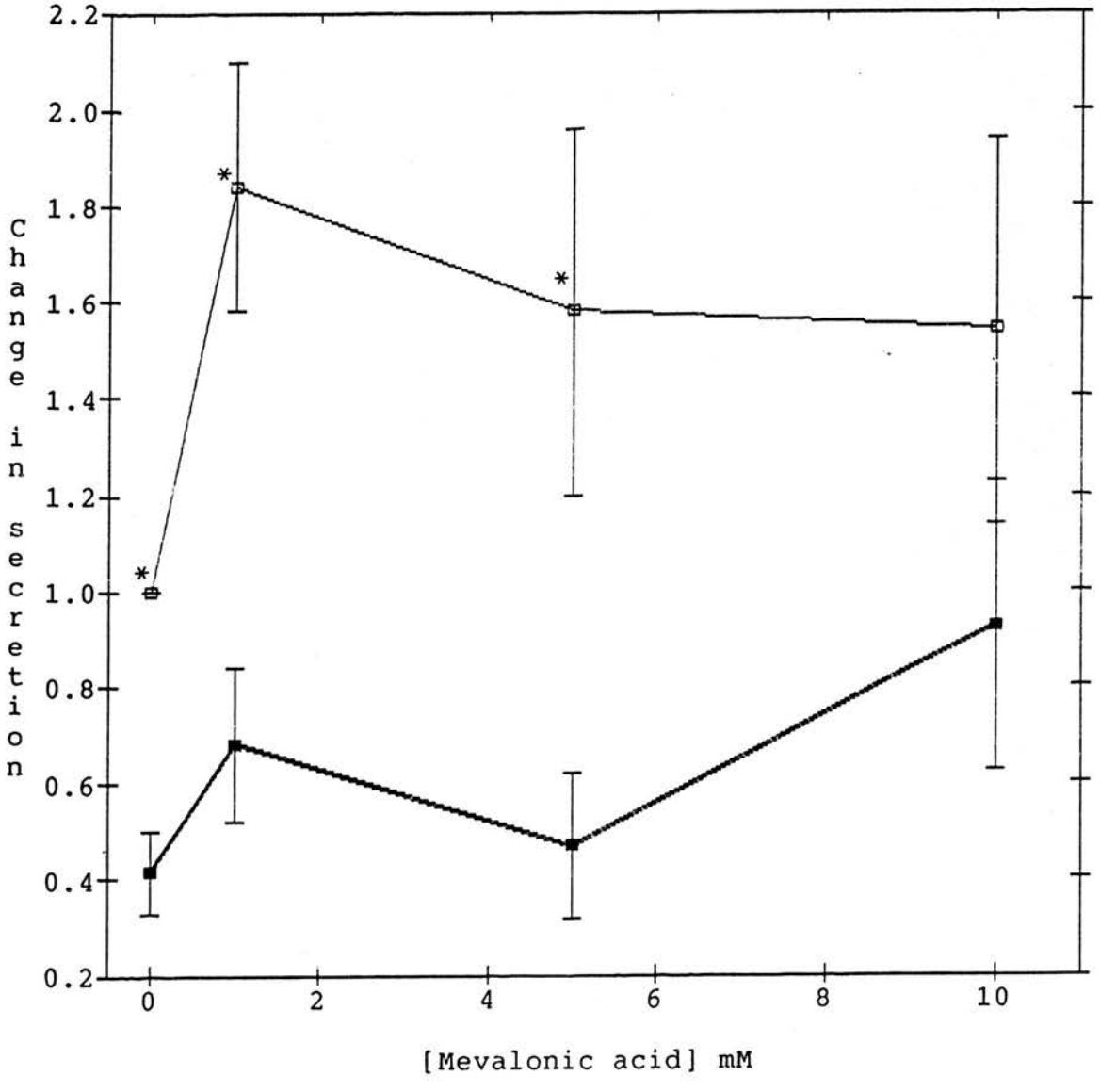
The fraction of density less than 1.03 was analysed for lipid composition and apolipoprotein content by SDS-polyacrylamide gel electrophoresis. The lipid composition was triacylglycerol: phospholipid: free cholesterol: cholesteryl ester; 100: 20: 10: 14: and apoproteins, apoB, apoC and apoE were detected on the gels. The amount of cholesteryl ester in this fraction was 90% of the total cholesteryl ester in the medium.

The presence of mevalonic acid resulted in an increase in cholesterol ester secretion, as expected (Drevon et al., 1980 a and b). The secreted ester was now to be found in lipoprotein density fractions other than d<1.03. 27% was present in the d< 1.03 fraction, 22% was present in the fraction of range 1.03 - 1.08 and the final 27% was located in the fractions of range 1.08 - 1.23.

Figure 4.3

Effect of 58-035 on cholesteryl ester secretion in the presence of mevalonic acid in hepatocyte monolayer cultures. The points show the cholesteryl ester secreted relative to the control and are the mean ± S.E. of eight independent experiments. Comparison of paired data for each animal by the Wilcoxon signed rank test showed a significant decrease in cholesteryl ester over controls at 0mM ($p=0.78\%$), 1mM ($p=3.9\%$) and 5mM ($p=3.1\%$). *Means are significantly different ($p<5\%$) from control (calculated by Student's t-test on the normalised data. (□) control; (■) + 200µg of 58-035.

Figure 4.3


2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Change in secretion
0
2
4
6
8
10
[Mevalonic acid] mM
*
*
*

Thus, all the cholesteryl ester secreted was accounted for in one of the lipoprotein fractions. Considering that the densities quoted, d<1.03, 1.03 - 1.08, and 1.08 - 1.23, correspond to the lipoproteins fractions VLDL, LDL, and HDL, it appears that the increase in cholesteryl ester secretion is being distributed amongst a wider range of lipoproteins and that, in the case of HDL, an increase in the secretion of other lipoproteins is also occurring. It may well be that the increased levels of secreted cholesteryl ester, once exchange between the lipoprotein moieties has equilibrated, are responsible for this difference of proportional distribution. This is unlikely, however, as the results in the absence of mevalonate would not show such of distribution of cholesteryl ester. More than likely, during the incubation period, some of the particles of d<1.03 are metabolised by interaction with the hepatocytes and by some exchange of lipids/cholesteryl ester with other secreted lipoproteins to form the group of particles making up the density range 1.03 - 1.08. Along with this there will be probably an increase in the secretion of nascent HDL particles which will interact with the secreted VLDL and exchange protein/lipid factors, contributing to the range 1.03 - 1.08 and making up the range 1.08 - 1.23.

As observed with the bile acid secretion data, the rates of secretion of cholesteryl ester by the cells varied widely, from 0.36 to 10.4 nmol/mg cell protein in 5 hours. The amounts of cholesteryl ester secreted by the cells in the presence and absence of 58-035, and of increasing concentrations of mevalonic acid (0-10 mM) were compared as outlined for the bile acids in Chapter 4.3. Cholesteryl ester secretion was increased 1.5 times by the addition of mevalonic acid. The presence of 58-035 inhibited ester secretion at concentrations of mevalonic acid from 0 to 5 mM (42% of control at zero; 37% at 5 mM). The effect of 10 mM mevalonic acid, in the presence and absence of 58-035 was not significant. Since inhibition of ACAT is approximately 80% complete in these experiments, it is probable that any effect of 58-035 at 10mM mevalonic acid was probably negated by the large excess of free cholesterol present in the cells.

No effect of 58-035 was seen on the secretion of free cholesterol, the free cholesterol secreted in the presence of 58-035 in all experiments was 1.14 ± 0.35 (SD., n = 21) of the corresponding incubations without 58-035. The free cholesterol secreted by the hepatocytes could be for secretion either into bile of plasma compartments. Unfortunately, due to this ambiguity inherent in both the monolayer and suspension techniques, no conclusions with regards to the fate of the secreted free cholesterol can be drawn from the data obtained.

4.5 Discussion

The preceding experiments have shown that 58-035 is an effective inhibitor of ACAT in both microsomal preparations from rat hepatocytes and the intact cell, inhibiting ACAT activity by approximately 80%. The use of this compound has allowed a comparison of the availability of newly synthesized free cholesterol in the hepatocyte for esterification and secretion of lipoproteins with its use for the synthesis of bile acids. Under the experimental conditions used, bile acid secretion was increased in the presence of 58-035, but only when an excess of free cholesterol was available. Conversely, ester secretion was decreased as expected, but at a high concentration of mevalonate this effect was not significant; free cholesterol secretion was unaffected by 58-035.

Thus, in the presence of 58-035 ester secretion drops and accumulation of intracellular free cholesterol occurs due to mevalonate, before the increase in bile acid secretion becomes significant. It can be seen that the substrate pools of cholesterol for bile-acid synthesis and for esterification can interact. Although the changes in bile-acid synthesis and esterification are in opposite directions, there is not a molar correlation of the two trends. This may be because neither substrate pool are saturated (Botham, 1986; Suckling, and Stange, 1985), which would appear to be the case under the conditions in these experiments, as the addition of mevalonic acid alone inceased secretion of both products. The trends observed are confirmed by other studies in rat hepatocytes

that show that bile acid synthesis is positively correlated with the amount of free cholesterol in the cell, but negatively correlated to with the amount of cholesteryl ester present (Botham and Boyd, 1987).

Thus, the data obtained demonstrates the possibilities for the movement of newly-synthesized free cholesterol within the hepatocyte from one pathway to another. Later experiments will investigate whether similar effects occur with cholesterol derived from lipoproteins.

CHAPTER 5

The Effects of 6-Azacholes-4-en-3ß-ol-7-one on Cholesterol Metabolism and Bile Acid Synthesis

5.1 Introduction

In Chapter 1.3, bile acid synthesis and its regulations was discussed. As mentioned, 7α-hydroxylation is the first and rate controlling step in the synthesis of bile acids using the enzyme: cholesterol 7α-hydroxylase. This enzyme is located in the endoplasmic reticulum of hepatocytes, requires oxygen, NADPH and a thermostable co-factor and is dependent upon a minor species of cytochrome P-450. The activity of this enzyme is influenced by a number of factors. Feed back inhibition occurs in vivo, but not in in vitro culture systems, (Davis et al, 1983a), suggesting that a metabolite of bile acids produced in the intestinal lumen is responsible for this control. There is also the possibility of a phosphorylation-dephosphorylation (active-inactive) mechanism in operation, but this is disputed (Kwok et al., 1983; Berglund et al., 1986).

In the following work, the same approach as used in Chapter 4 was applied to cholesterol 7α-hydroxylase. By blocking the activity of a key enzyme such as cholesterol 7α-hydroxylase, it might be possible to determine the fate of the accumulated excess free cholesterol, and as such, elucidate any interconnections between the substrate pool or pools of free cholesterol used in bile acid synthesis or alternative pathways.

In vivo work using a similar approach, examined the role of ACAT in hepatic cholesterol esterification and its relationship with the secretion of cholesterol into bile. (Nervi et al., 1984) and found that a reciprocal relationship exists between ACAT activity and biliary free cholesterol output. The work by Stone et al. (1987) determined that the majority of cholesterol delivered to the liver in cholesterol-rich lipoproteins is either esterified and stored or resecreted as free and esterified cholesterol and does not alter biliary secretion of cholesterol.

The work outlined in the previous chapter demonstrates that inhibition of ACAT activity stimulates bile acid synthesis from newly-synthesized cholesterol from excess mevalonic acid, indicating an inter-relationship between the two pathways open to newly-synthesized cholesterol. Recently, work by Mackinnon et al. (1987), shows that HDL taken up by the receptor-mediated pathway provides cholesterol for bile acid synthesis and cholesterol esterification, in line with the work of Ford et al. (1985b), and that presented in Chapter 6.

Thus one can see that recent work has produced a number of contrasting results in the determination of the use free cholesterol. Certainly they show that free cholesterol derived from different sources appears to have different fates. The experiments involving HDL show that the free cholesterol provided is used in cholesterol esterification, but they differ in their results on the secretion of free cholesterol. The in vivo work will be hampered by the interactions that occur between different lipoprotein classes and by the uptake of HDL-cholesterol by more than one pathway, whereas the use of cultures of hepatocytes is free from these problems but does not allow for the importance of normal plasma interactions nor differentiation between secretion into bile of plasma compartments. Thus different approaches to the question of cholesterol metabolism may give contrasting results due to the limitations of the techniques used. Furthermore each approach may be defining certain conditions that favour the determination of specific intracellular pools of free cholesterol which have particular uses, indicating the existence of both complex compartmentalization of intracellular free cholesterol and a number of different intracellular pools of free cholesterol. It is therefore necessary to investigate the effects of bile acid synthesis on intracellular free cholesterol to provide a complete study of hepatic cholesterol metabolism.

Until recently, there were no specific inhibitors of cholesterol 7α-hydroxylase so work involving the inhibition of bile acid synthesis was not possible. The compound ketoconazole has now been used for this purpose (Princen et al., 1986)., but it has certain

disadvantages. It is known to inhibit several cytochrome P-450 dependent enzymes and as such inhibits cholesterol synthesis (Gupta et al., 1986; Kraemer and Spilman, 1986; Baldwin, 1983). Thus studies where the fate of newly-synthesized cholesterol is being investigated, it is not of use. Brown et al., (1987) have produced a compound, 6-azacholest-4-en-3ß-ol-7-one (azacholesterol), shown in figure 2.2b, which is a specific inhibitor of cholesterol 7α-hydroxylase (Sampson et al., 1987b). Studies on rat liver microsomal fractions with azacholesterol show that it is effective in inhibiting cholesterol 7α-hydroxylase by a non-competitive mechanism, and that it does not inhibit either HMG-CoA reductase or ACAT (Sampson et al., 1987b). This compound has allowed me to determine the effect that the inhibition of bile acid synthesis has upon esterification of cholesterol derived from newly-synthesized cholesterol.

5.2 Effect of Azacholesterol on HMG-CoA Reductase and ACAT in Rat Hepatocyte Monolayers

Primary cultures of hepatocytes prepared from rats fed diet Z (Chapter 2.2) were used in these and subsequent experiments, to ensure that bile acid synthesis was sufficiently active. 200 nm of azacholesterol in 20µl DMSO were added to the cells (100 µM) in the presence of 2% or 5% bovine serum albumin. HMG-CoA reductase activity was assessed by addition of [^{14}C] acetate (3.6 µM), and measurement of its incorporation into cholesterol and cholesterol ester after 1 and 2 hours. Table 5.1 shows that azacholesterol has no effect on the synthesis of cholesterol from [^{14}C] acetate nor does it affect [^{14}C] acetate incorporation into cholesterol ester.

The above experiment was repeated using [^{3}H] potassium oleate (40 µM) instead of [^{14}C] acetate, in order to assess the effect of azacholesterol on cholesterol esterification independent of cholesterol and triglyceride synthesis. The results here show that azacholesterol has no effect on cholesterol esterification.

As mentioned, ketonazole inhibits cholesterol biosynthesis, acting as a ligand for several forms of cytochrome P-450, through its

imidazole ring system (Baldwin,1983). Evidence that azacholesterol might also inhibit other cytochromes P-450 involved in the conversion of lanosterol to cholesterol was looked for by examining the sterols synthesized in cells incubated with azacholesterol and [^{3}H] mevalonic acid. In the a presence of mevalonic acid any effects on HMG-CoA-reductase are avoided and effects later in the pathway will be more apparent. Incubation of monolayers of rat hepatocytes with [^{3}H] mevalonic acid and subsequent analysis by t.l.c. showed that cholesterol accounted for 92 ± 6% of the total radioactivity in mono-hydroxy sterols secreted into the culture medium and in the presence of 100 µM azacholesterol 83 ± 9% of the total label was located in the cholesterol fraction. These values are not significantly different from each other under the experimental conditions outlined, indicating that azacholesterol does not inhibit the later oxidative steps in cholesterol synthesis.

5.3 Effect of Azacholesterol on Bile Acid Synthesis in Rat Hepatocyte Monolayers

Azacholesterol (concentration range 0 to 500 µM) in the presence of 2% bovine serum albumin was incubated with rat hepatocyte monolayers for 5 hours. The bile acids produced in the cells and medium were analysed by radioimmunoassay. Figure 5.1 shows a dose-dependent inhibition of bile acid synthesis and secretion, with a maximum inhibition of approximately 90% at concentrations of 100 µM of azacholesterol or greater. Thus, azacholesterol is an effective inhibitor of bile acid synthesis in cultures of hepatocytes, without the concomitant inhibition of cholesterol synthesis, as seen with ketoconazole, and may be used as a probe for cholesterol flux to this enzyme.

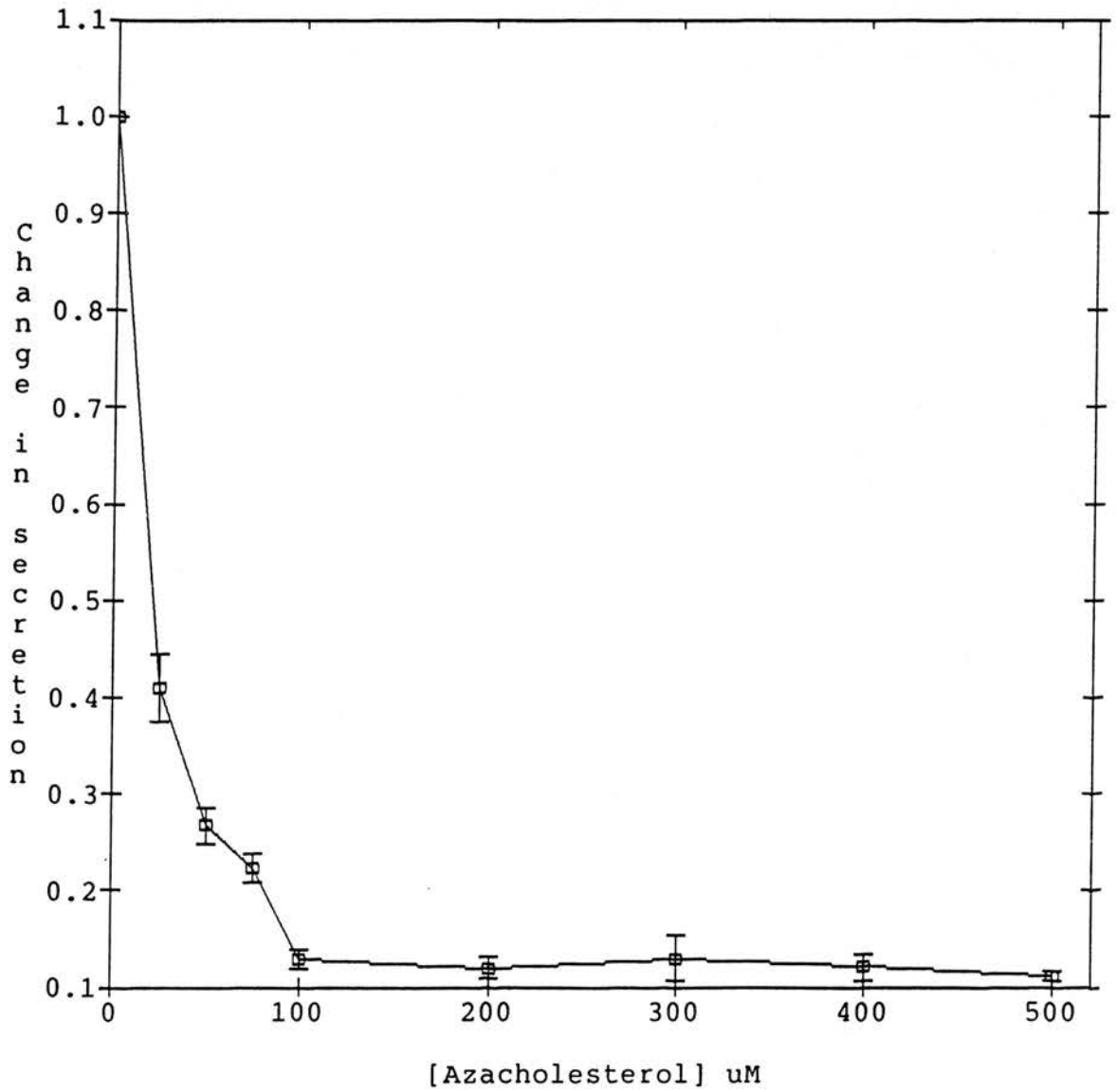
5.4 Effect of Azacholesterol on the Fate of Newly-Synthesized Cholesterol in Rat Hepatocyte Monolayers

Following approximately the same procedure used in Chapter 4.2 to investigate the role played by cholesterol 7α-hydroxylase in determining cholesterol fluxes within the hepatocyte, monolayers were incubated with and without azacholesterol in the presence of increasing concentrations of [^{3}H] mevalonic acid (0 to 10 mM) used

Figure 5.1

Inhibition of bile acid synthesis in rat hepatocyte monolayers by azacholesterol. Azacholesterol was added in the concentrations shown in 10 to 50µl of DMSO to dishes containing 2.5×10^6 cells in 2ml DMEM containing 2% bovine serum albumin (fatty acid free). After incubation for 5 hours, bile acids in the cells and medium were determined by radioimmunoassay. The precision of all measurements was ± 7%.

Figure 5.1


1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Change in secretion
0
100
200
300
400
500
[Azacholesterol] uM

to provide a substantial source of intracellular cholesterol. The masses of cholesterol and cholesterol ester secreted into the medium were calculated from the known specific activity of the mevalonic acid. Although 6 molecules of mevalonic acid are used to produce cholesterol, only 5 of the [^{3}H] labels are incorporated, due to the loss of one of them during folding of squalene. Thus the number of moles of cholesterol produced is 1/5 not 1/6 of the number of moles of mevalonate used as determined from the tracer.

To check this, samples with a level cholesterol determined by the above formula were assayed for cholesterol using a fluormetric method (Chapter 2.10). The results of this showed that the incorporation of labelled mevalonate occurred at a rate of approximately 4.9 moles mevalonate into 1 mole of cholesterol, only slightly less than the theoretical value of 5:1. Also, the cholesterol ester fraction was subjected to hydrolysis in 5N alcoholic potassium hydroxide and subsequently re-extracted. This showed that less than 10% of the radioactivity in the cholesterol ester fraction was located in the fatty acyl component.

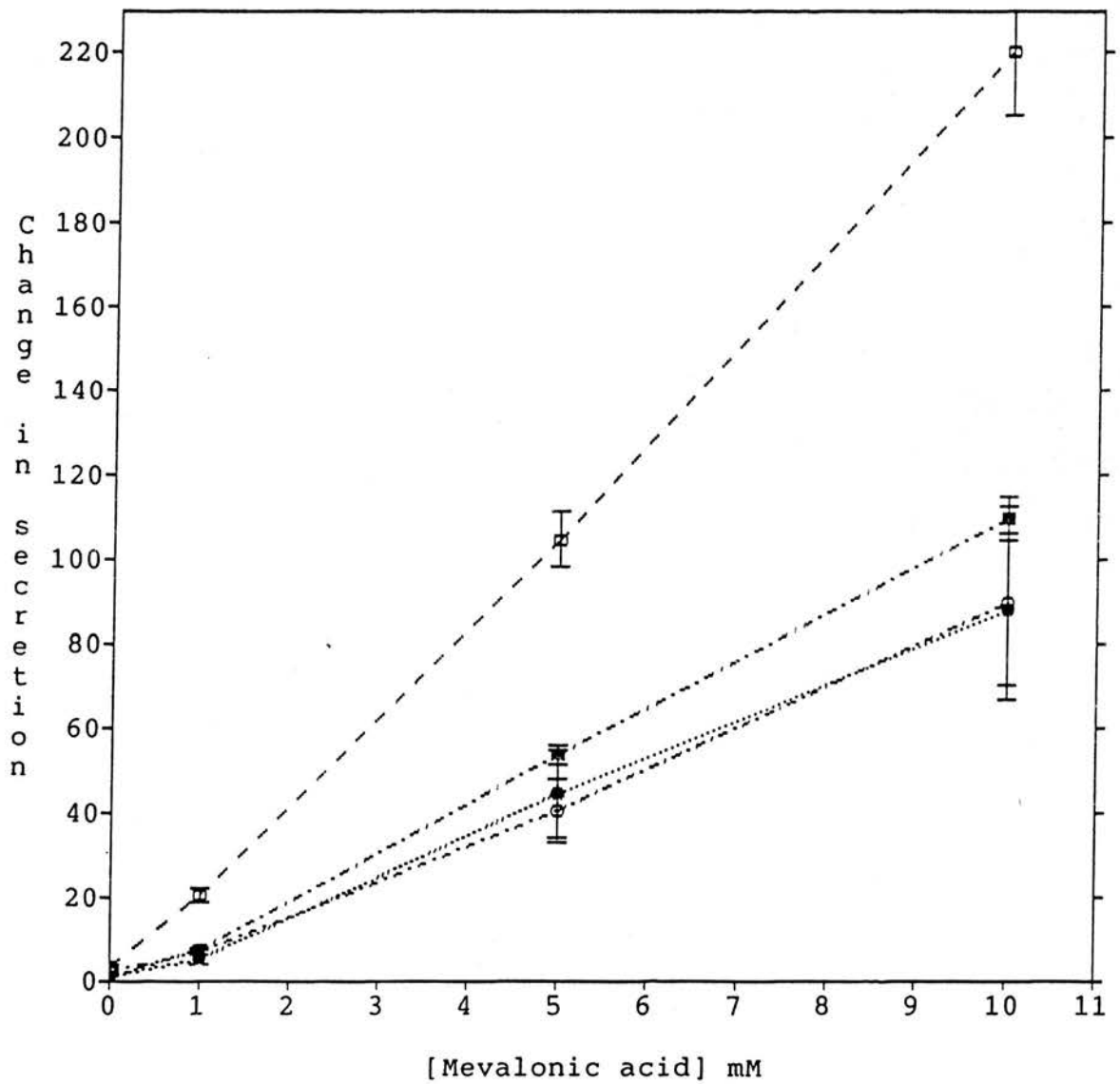
The results shown in Figure 5.2 are from studies using cells derived from 4 separate animals. Azacholesterol did not effect cholesterol ester secretion at any concentration of mevalonic acid. Instead, it caused a significant increase in the secretion of free cholesterol into the medium at all levels of mevalonate used. The mean increase of free cholesterol secretion in the presence of the inhibitor was 2.6 ± 0.7 times the control in the absence of the inhibitor.

In conjunction with the above experiments, the bile acids produced in the cells and the medium were measured (Table 5.2). These results show that across all concentrations of mevalonic acid, in the presence of azacholesterol, total bile acid synthesis is inhibited by between 84-88%. Thus, increasing accumulation of free cholesterol must be occurring, and this excess is seen in the increase in free cholesterol secretion only.

Figure 5.2

Effect of azacholesterol on the secretion of free and esterified cholesterol newly synthesised by rat hepatocyte monolayers in the presence of [^{3}H] mevalonic acid. Azacholesterol was added to give a concentration of 200μM, producing 86% inhibition of bile acid synthesis (Table 5.3). Labelled mevalonic acid was added at tracer concentrations and at 1, 5 and 10mM. After 5 hours the medium was extracted and the radioactivity in free cholesterol and cholesteryl ester determined. Secretion of free cholesterol was significantly increased ($p<0.01$) by the presence of azacholesterol by a factor of 3.48 ± 0.9 at tracer levels of mevalonate, 2.8 ± 0.4 at 1mM, 2.0 ± 0.2 at 5mM and 2.0 ± 0.3 at 10mM. Secretion measurements are expressed in units of n mole in 5 hours per 2.5 x 10^6 cells.
(○) cholesteryl ester secretion in control experiments, (●) cholesteryl ester secreted in the presence of azacholesterol, (■) free cholesterol in control experiments, (□) free cholesterol secreted the presence of azacholesterol.

Figure 5.2


Change in secretion
220
200
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
11
[Mevalonic acid] mM

Experiment	[Mevalonic acid] mM 0	1	5	10
Control (a)	4.05±1.08	5.13±1.44	5.19±1.46	5.39±1.39
azacholesterol (a)	0.63±0.18	0.59±0.19	0.70±0.25	0.86±0.29
Control (b)	1.00±0	1.258±0.064	1.284±0.072	1.334±0.062
azacholesterol (b)	0.156±0.024	0.151±0.041	0.177±0.044	0.213±0.038

Table 5.2

Effect of Azacholesterol on bile acid synthesis in the presence of [^{3}H] mevalonic acid in rat hepatocye monolayers. Experimental conditions are as outlined for Figure 5.2. The data above is presented in 2 ways: a) Raw data in n moles in 5 hours per 2.5×10^{6} cells; b) as normalised data, relative to the control samples with neither mevalonate nor azacholesterol. Data is expressed as mean ± S.D. (n=4).

5.5 Effect of Azacholesterol and 7α-Hydroxycholesterol on Bile Acid Synthesis in Rat Hepatocyte Monolayers

7α-Hydroxycholesterol is the product formed by the action of cholesterol 7α-hydroxylase on cholesterol. Thus, addition of this compound to the culture medium in the presence of azacholesterol should overcome the inhibition of bile acid synthesis. That is assuming that azacholesterol only affects cholesterol 7α-hydroxylase and no other enzymes of bile acid synthesis. 7α-hydroxy cholesterol in ethanol was added to the culture medium (concentration range: 0 to 2mM) in the presence of 100 μM azacholesterol and 2% bovine serum albumin, and the cells cultured for 5 hours. The bile acids produced in the cells and medium were analysed by radioimmunoassay. Table 5.3 shows that addition of 7α-hydroxycholesterol increases bile acid synthesis in the presence of azacholesterol indicating that the subsequent stages in the synthesis of bile acids are not affected.

5.6 Discussion

In Chapter 4, I found that bile acid synthesis was increased in the presence of excess newly-synthesized free cholesterol by inhibiting ACAT activity. Under these conditions free cholesterol secretion was not effected. There appeared to be a reciprocal relationship between bile acid synthesis and cholesterol esterification. In the experiments just presented, bile acid synthesis in the presence of a range of mevalonic acid concentrations was inhibited by azacholesterol; however, cholesterol esterification did not increase in the presence of the inhibitor, indicating that the pool of newly-synthesized free cholesterol used for bile acid synthesis was not related to the pool of free cholesterol use for esterification. In the presence of azacholesterol free cholesterol secretion did increase; however, given the experimental conditions used, it is not possible to determine if this increased secretion of free cholesterol would be into the biliary or plasma (lipoprotein) compartments. From this work it appears that the bile acid synthesis and free cholesterol secretion are reciprocally related.

Experiment	Bile Acid Synthesis (p mole in 5 hours per 2.5×10^6 cells)	% Inhibition
a) control	4.21 ± 0.23	0
b) Azacholesterol	0.70 ± 0.10	88.4
c) Azacholesterol + 7x-hydroxycholesterol	1.22 ± 0.12	71.0
d) Azacholesterol + 7x-hydroxycholesterol	2.03 ± 0.26	51.8

Table 5.3

Effect of 7α-hydroxycholesterol on azacholesterol-induced inhibition of bile acid synthesis. Experiments were incubated for 5 hours and the bile acids measured in cells and medium by radioimmunoassay. Azacholesterol was added at a concentration of 200μM and 7α-hydroxy cholesterol was added at 2 concentrations: c) 100μM and d) 200μM. Results are expressed as the mean ± S.D. (n=3).

It is possible to argue that under the conditions used, the pathway for cholesterol esterification was saturated with excess free cholesterol derived from mevalonate. The data presented in Table 5.2 and Figure 5.2 shows that esterification did not become saturated until between 5 and 10 mM mevalonic acid. this is an agreement with the data presented in Figure 4.3, where the controls show that saturation of esterification occurs between 5 and 10 mM mevalonic acid. Yet the effect of azacholesterol was the same at all levels of mevalonic acid, thus supporting the above argument and providing evidence of stricter compartmentation of the movement of intracellular free cholesterol in the rat hepatocyte.

These results contrast with those obtained in Chapter 4, indicating a high degree of complexity in the organisation and use newly-synthesized free cholesterol. Both studies indicate different interrelationships between the pathways of cholesterol use, and different patterns of cholesterol flux. The following experiments use an exogenous source of cholesterol, HDL_2, to attempt to further define the interactions of the various pathways.

CHAPTER 6

The Effect of HDL_2 on Cholesterol Esterification and Bile Acid Synthesis

6.1 Introduction

The synthesis of bile acids is thought to be regulated by the availability of cholesterol (Davis et al., 1983b). Synthesis can be stimulated by a number of sources of cholesterol derived from both lipoproteins and the endogenous synthesis of free cholesterol (Chapter 1.3). In the preceeding chapters I have examined the effect of newly-synthesized free cholesterol derived from mevalonic acid on both cholesterol esterification and bile acid synthesis. In the proceeding chapter, I will discuss the role of cholesterol derived from the lipoprotein subfraction HDL_2 in bile acid synthesis and cholesterol esterification.

In the rat, HDL_2 contains apoproteins A-I, A-II, C-I, C-II, C-III D and E, cholesterol, cholesteryl ester and phospholipid, with a very minor amount of triglyceride. It has been postulated that HDL is involved in the transfer of cholesterol from peripheral tissues to the liver and adrenal gland (Tall and Small, 1980). Thus, the subfraction may be involved in regulating the size of the extrahepatic pool of total body cholesterol and involved in the removal of cholesterol via either its secretion into bile or its metabolism into bile acids. Thus, it is of interest to determine the relationship between HDL-cholesterol and bile acid synthesis.

Work by Ford et al., (1985b), showed that HDL_2 can stimulate bile acid synthesis in mono-layers of rat hepatocytes. Recently this was confirmed in part by the work of Mackinnon et al., (1987). They showed that different types of HDL containing apo E can stimulate bile acid synthesis and that reductive methylation of the HDL, causing inhibition of apo E binding to both apo B/E and apo E receptors (Hui et al., 1986), blocked receptor-mediated uptake and stimulation of bile acid synthesis.

Also, apo-E rich HDL was shown to inhibit cholesterol synthesis and cause a net accummulation of cholesteryl esters in hepatocytes. Thus, HDL-cholesterol appears to be available for both cholesterol esterification and bile acid synthesis.

In the following study, the effect of HDL_2 on bile acid synthesis and cholesterol esterification will be discussed using inhibitors of ACAT and HMG-CoA reductase. 58-035 and its effects have already been described in Chapter 4. Mevinolin, the other compound used, is a competitive inhibitor of HMG-CoA reductase, and this of cholesterol synthesis (Kita et al., 1980; Alberts et al., 1980). Davis et al., (1983a) demonstrated that in cultures of rat hepatocytes, mevinolin inhibits bile acid synthesis, linking the pool of newly-synthesized cholesterol with that used by cholesterol 7α-hydroxylase. Other recent work has shown that the effect of mevinolin on cholesterol synthesis has effects on the levels of the mRNA of both lipoprotein receptors and certain apoproteins (Ma et al., 1986; Monge et al., 1985).

By using mevinolin to inhibit HMG-CoA reductase, therefore, I was able to decrease the pool of newly-synthesized free cholesterol, making the hepatocytes dependent on an exogenous source (HDL_2) for free cholesterol. By then inhibiting the activity of ACAT using 58-035, it was hoped that the pattern of intracellular flux so far determined could be further elucidated by observing the uptake of HDL_2.

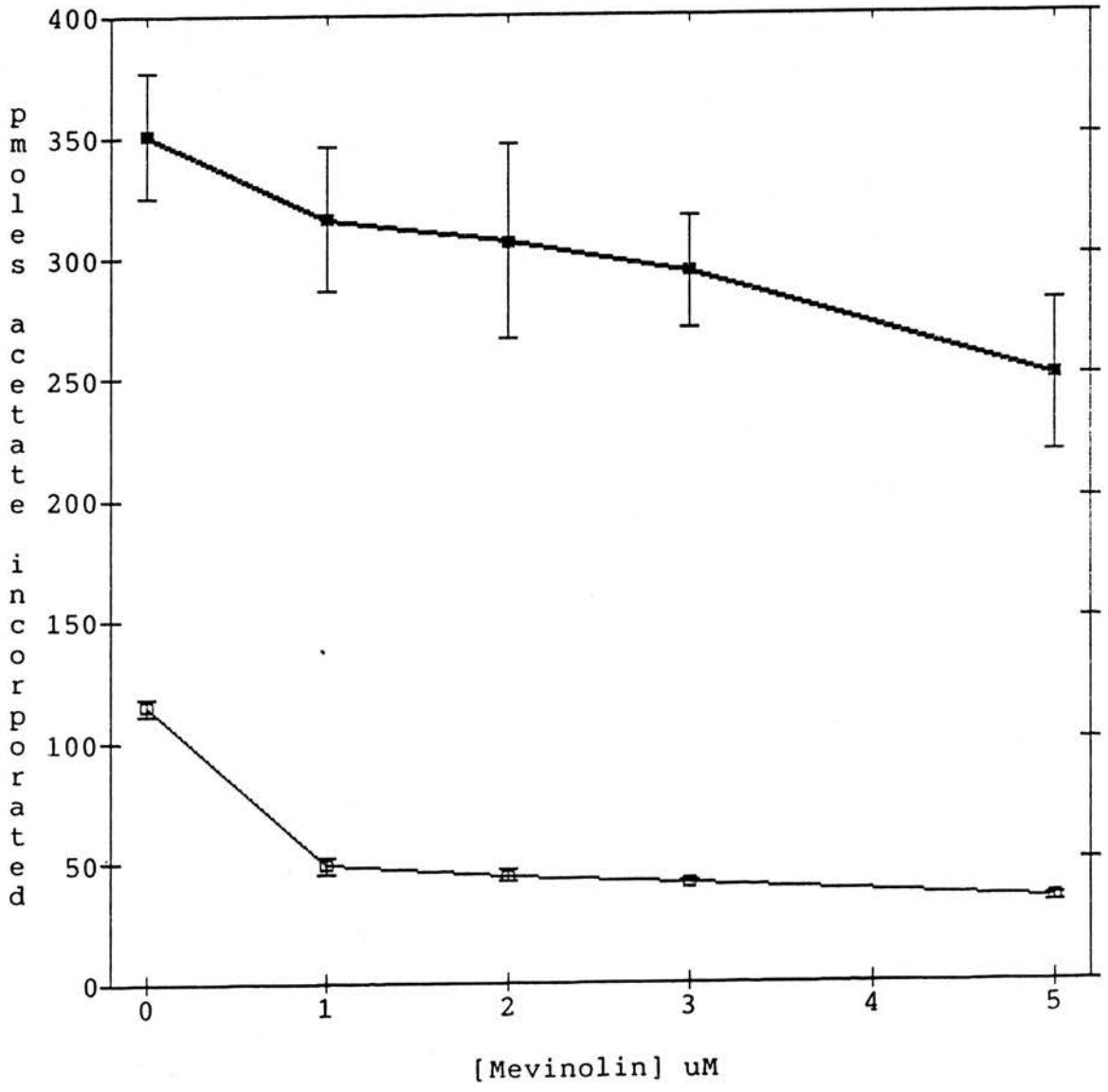
6.2 Effect of Mevinolin on Cholesterol Synthesis and Esterification in Rat Hepatocyte Monolayers

Primary cultures of hepatocytes prepared from rats fed diet Z (Chapter 2.2) were used in this and subsequent experiments. Aliquots of mevinolin (10 to 50 µl) were added to the culture medium in the presence of 2% bovine serum albumin giving a concentration range of 0 to 5 µM, as described in Chapter 2.5.3. The cultures were then incubated for 5 hours. Cholesterol synthesis and esterification were assessed by measuring the incorporation of [^{14}C] acetate (3.6 µM). Figure 6.1 shows the dose-dependent

Figure 6.1

Effect of mevinolin on cholesterol synthesis and esterification in rat hepatocyte monolayers. Mevinolin was added to the cultures in the range of 0 to 5 μM, and synthesis and esterification determined by following the incorporation of [^{14}C]acetate into cholesterol and cholesteryl ester over 5 hours. Results are in p moles in 5 hours per 2.5 x 10^6 cells (n=2)
(□) cholesterol; (■) cholesteryl ester.

Figure 6.1


400
350
300
250
200
150
100
50
0
pmoles acetate incorporated
0
1
2
3
4
5
[Mevinolin] uM

effect of mevinolin on both synthesis and esterification. It inhibits cholesterol synthesis by about 70% at concentrations of 2 μM or greater, confirming the report of Pullinger and Gibbons (1982). Other experiments by our group involving monolayer cultures of bovine adrenocortical cells also achieved maximal inhibition at concentrations of greater than 2 μM; however, the level of inhibition was approximately 80% (Ochoa and Suckling, 1987). This difference may be due to metabolic differences in the different tissues.

In terms of cholesterol esterification, the maximum level of inhibition is about 30%; this can be compared to 40% obtained with the bovine adrenal cortical cells. Both results indicate that mevinolin affects esterification, but not as markedly as synthesis. The reason for this is simply that the cholesteryl ester contains radioactive label in both fatty acyl and cholesterol components. As mevinolin does not act on fatty acid synthesis, then incorporation of label into fatty acids may actually increase due to increased availability of substrate. The fatty acids produced will esterify with any available cholesterol giving the effect seen. Work by Ochoa (1986) confirmed this.

6.3 Isolation and Characterization of Lipoprotein Subfraction HDL_2 from Rat Plasma

As described in Chapter 2.13, HDL_2, a lipoprotein subfraction of density range 1.085 - 1.21 was prepared by sequential density gradient ultracentrifugation (Jansen *et al.*, 1983), dialysed against distilled water to remove the salts, and then reverse-phase dialysed against carbowax. Samples of this preparation were freeze dried and then reconstituted in a smaller volume producing a concentrated sample for analysis by SDS-page. The results of this are shown in Figure 6.2. The major proteins identified were apo A-I, apo A-IV and apo E, and trace amounts of the C and D apoproteins are also visible. Apo B is absent, indicating no contamination with LDL, VLDL or chylomicrons. Table 6.1 shows the protein and cholesterol composition of the subfraction, which is similar to that obtained by Oschry and Eisenberg (1982) but differs from the results of Jansen

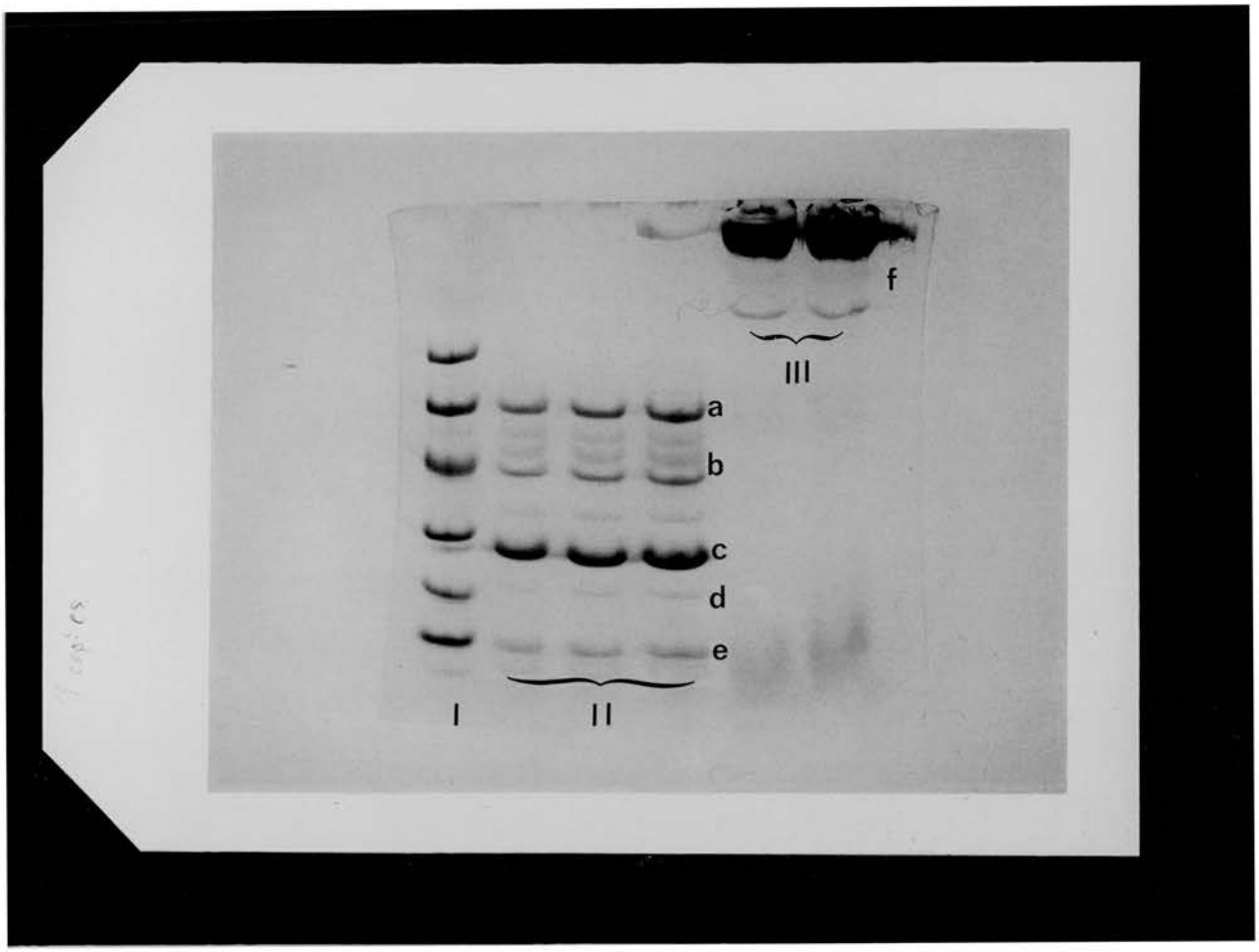


Figure 6.2 SDS-PAGE of HDL_2 from rat plasma.

Column I: Molecular weight markers.

Column II: HDL_2 apoproteins a) apo A-IV; b) apo E; c) apo A-I; d) apo D or apo A-II; e) apo C-I/C-II/C-III

Column III: LDL sample f) apo B present as aggregates (upper band) and as monomers (lower band).

HDL_2 samples show no contamination with other lipoproteins (VLDL or LDL) due to the absence of apo B.

Source	Protein	FC	TC	FC/TC	TC/Protein
ug/100µl of	251	20	180	0.111	0.72
mg/100mg Oschry and Eisenberg (1982)	40.5	3.6	29.5	0.12	0.73
µg/ml	336	34	180	0.19	0.54
% (w/w) Jansen et al (1984)	46.1	4.6	25.6	0.19	0.53

Table 6.1

The protein and cholesterol content of rat HDL_2. The results are compared with those of Oschry and Eisenberg (1982) and Jansen et al., (1984). FC - free cholesterol; TC - total cholesterol.

et al., 1983. The differences are due to Jansen and co-workers defining HDL_2 as a subfraction of density range 1.112-1.128, a narrower range than used by myself or Oschry and Eisenberg (1982), which will have a higher amount of lipids relative to its protein content.

Thus, 200 µl of this preparation was added per dish in subsequent experiments to provide an approximate physiological concentration of 500 µg of HDL_2 protein per dish. (Oschry and Eisenberg, 1982; Chapman, 1980).

6.4 Effect of HDL_2 on Cholesterol Esterification in Rat Hepatocyte Monolayers

Monolayer cultures of hepatocytes were prepared from 4 rats fed diet Z (Chapter 2.2). The cells were pre-treated with 58-035 (200 µg per dish) or control solvent (DMSO), and mevinolin was added (5 µM) to inhibit cholesterol synthesis. Incubations were carried out for a total of 5 hours. During the final 2 hours, potassium [^{3}H] oleate was added (1 mM) to determine the activity of ACAT in the cultures.

Table 6.2 shows that HDL_2 stimulated cholesterol esterification by 1.32-times the control. Addition of 58-035 inhibited esterification both in the presence and absence of HDL_2; although HDL_2 in the presence of 58-035 appears to stimulate esterification 1.33-times when compared to 58-035 alone. These results indicate that cholesterol derived from HDL_2 is available for esterification. The experimental system had a homologous source for the hepatocytes and lipoproteins, using a specific subfraction, and produced results not previously recorded. Although a large body of work has been done with lipoproteins, usually the cells and lipoproteins are derived from different species, and may produce results characteristic to their experimental systems only. The results obtained in this study do not indicate whether uptake of HDL_2 is occurring via a receptor or non-receptor mediated mechanism. Work by Jensen et al (1985 and 1987) determined that LDL and chylomicron uptake by rat hepatocyte monolayers was cell-density-dependent. To

Experiment	Cholesterol Esterification pmole/hour/2.5 x 10^6 cells	% of Control
a) control	118 ± 6.5	100
b) 58035	26 ± 3	22
c) HDL_2	156 ± 9	132
d) 58-035 + HDL_2	35 ± 5.5	30

Table 6.2

Esterification of potassium [^{3}H] oleate in the presence of 58-035 and HDL_2. Control experiments had 2ml DMEM with 2% bovine serum albumin (fatty acid free) and 5μM mevinolin; b) as the controls with 200μg of 58035 per dish; c) as the controls with 500μg of HDL_2 per dish; d) as the controls with both HDL_2 and 58-035 added. Cultures where incubated for 3 hours and then potassium [^{3}H] oleate (40μm) was added for the final 2 hours. Results are expressed as the means ± S.D. (n=4).

ensure that the results I obtained were not affected by this, only cultures of approximately 2.5 x 10^6 cells and 1.5 mg of cell protein per dish were used.

6.5 Effect of HDL_2 on Bile Acid Synthesis in Rat Hepatocyte Monolayers

Monolayer cultures of rat hepatocytes were prepared from four rats fed on diet Z (Chapter 2.2). The cells were pre-treated with 58-035 (200 µg per dish) or control solvent (DMSO), and mevinolin was added (5 µM) to inhibit cholesterol synthesis. Incubations were carried out for a total of 5 hours, and bile acids estimated in the cells and medium by radioimmunoassay.

The basal rate of synthesis of bile acids was 2.47 ± 0.46 per 2.5 x 10^6 cells. The changes caused by addition of HDL_2 and 58-035 are shown in Table 6.3 and Figure 6.3. The data shows that HDL_2 stimulates bile acid synthesis, as seen in previous work (Ford et al., 1985b) and recently shown by Mackinnon et al. (1987). The increase seen was about 13%. Addition of 58-035 inhibits bile acid synthesis by about 10% compared to the controls without either HDL_2 or 58-035. In combination 58-035 and HDL_2 also inhibit bile acid synthesis by greater than 10%. Comparison of the absolute values of these differences by the two-tailed paired t-test showed that the changes were significant with $p < 0.05$.

From these studies, it appears that cholesterol derived from HDL_2 can be used by cholesterol 7α-hydroxylase, but that inhibiting ACAT inhibits the flux of such cholesterol through to bile acid synthesis. This indicates that prior esterification and subsequent ester hydrolysis must occur before cholesterol derived from HDL_2 can be used by cholesterol 7α-hydroxylase.

6.6 Discussion

The combination of 58-035 and mevinolin has allowed me to study the fate of cholesterol derived from HDL_2 and in particular its availability for bile acid synthesis. In rat hepatocyte monolayers,

Experiment	Bile Acid Syntheses nmole per 5 hours per 2.5×10^6 cells	Normalised Data
a) control	2.47 ± 0.40	1 ± 0
b) 58-035	2.21 ± 0.40	0.893 ± 0.039
c) HDL_2	2.71 ± 0.44	1.133 ± 0.039
d) 58-035 + HDL_2	2.10 ± 0.43	0.854 ± 0.044

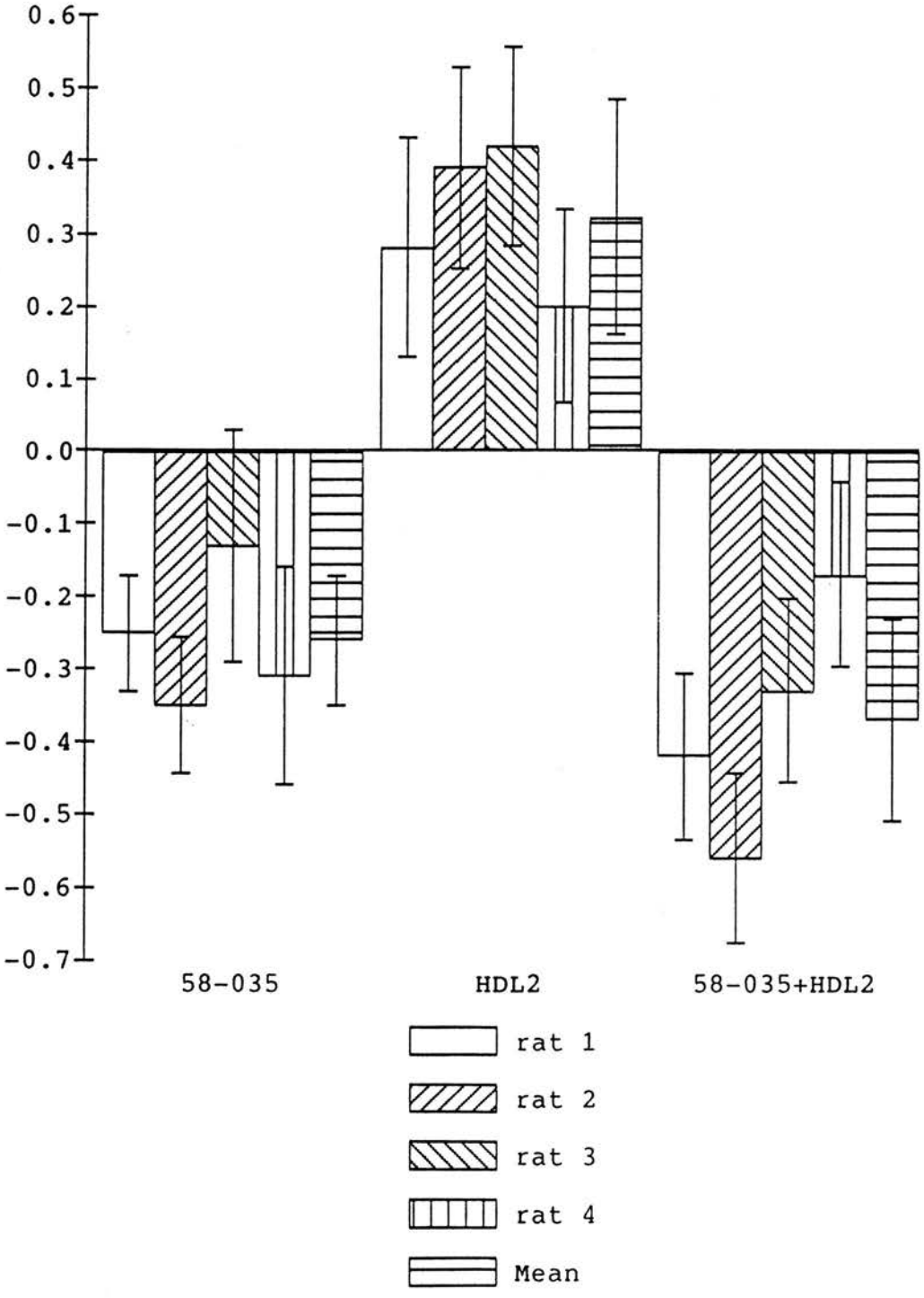
Table 6.3

Effect of mevinolin on bile acid synthesis in rat hepatocyte monolayers. a) control samples had 2ml DMEM with 2% bovine serum albumin (fatty acid free) and mevinolin (5μm); b) as the controls with 200μg of 58-035 per dish; c) as the controls with 500μg of HDL_2 per dish; d) as the controls with both HDL_2 and 58-035. Cultures were incubated for 5 hours, and bile acids were measured in cells and medium by radioimmunoassay. Results are expressed as nmole in 5 hours per 2.5×10^6 cells ± S.D. (n=4) or as normalised data ± S.D. (n=4). Comparisons of (b) to (a), (c) to (a), (d) to (a), and (d) to (c) by the paired t-test showed significant differences in cell comparisons ($p<0.05$). Refer to Figure 6.3.

Figure 6.3

Effect of 58-035 and HDL_2 on bile acid synthesis in rat hepatocyte monolayers. for details refer to the legend of Table 6.3. Results are expressed as the change in bile acid synthesis from control values i.e. change in nmoles in 5 hours per 2.5×10^6 cells ± S.D. (n=4). All comparisons between controls and treated samples by the paired t-test were significant ($P<0.05$).

Figure 6.3


0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
58-035
HDL2
58-035+HDL2
rat 1
rat 2
rat 3
rat 4
Mean

this cholesterol is available for both esterification and 7α-hydroxylation; however, it appears that any HDL_2-derived

cholesterol used for bile acid synthesis must first be esterified and then hydrolysed before it is available for that purpose. Certainly, the results indicate that the immediate fate of exogenous cholesterol is esterification, under the experimental conditions used.

These experiments are more closely related to the in vivo situation than those in Chapters 4 and 5, although use of a single lipoprotein subfraction does limit the normal exchange processes that might occur between lipoprotein classes. A preliminary experiment using a preparation containing all the defined rat plasma lipoprotein classes produced similar results for bile acid synthesis as obtained by using HDL_2 alone.

In my earlier experiments, intracellular transport and regulatory mechanisms were probably saturated by the excess free cholesterol from the added mevalonate, and uptake mechanisms and the regulatory effect were by-passed. This system concentrates on an external source of cholesterol and enhances the suppressant effect that lipoprotein uptake has on HMG-CoA reductase and cholesterol synthesis, by use of the inhibitor, mevinolin. Thus it may provide a better estimate of the in vivo situation, when dietary cholesterol ester is an important consideration.

CHAPTER 7

Discussion

The major aim of this body of work was to investigate and determine the intracellular control of cholesterol metabolism in the hepatocyte. The strategy employed involved the use of rat hepatocytes maintained in monolayers, exposed to specific inhibitors of different key enzymes of cholesterol metabolism and to different sources of cholesterol. The rationale for this approach was that by using isolated hepatocytes the extracellular environment could be controlled, so that the effects of the inhibitors and substrates would be more easily elucidated.

Inhibiting specific pathways of cholesterol utilization would potentially increase the flux of cholesterol into other pathways of cholesterol utilization; furthermore, not only are the pathways themselves important but so too is the source of the cholesterol used. Thus by manipulating both the pathways and sources of cholesterol, inter-relationships that might exist between the pathways of cholesterol metabolism and their cholesterol sources could be elucidated.

Certainly, previous studies have attempted to define the sources of free cholesterol used by specific pathways of cholesterol metabolism. Schwartz *et al*. (1975) and Bjorkhem and Lewenhaupt (1979) showed that the preferred substrate for bile acid synthesis is newly synthesised cholesterol; a view supported by the work of Bjorkhem and Danielsson (1975) and Myant and Mitropoulos (1977). Such findings have led to the idea that discrete pools of free cholesterol exist within the hepatocyte which contribute different amounts of substrate to different pathways of cholesterol metabolism (Stange, 1987). It is of importance to realise that the term preferred substrate when applied to bile acid synthesis means that newly synthesised cholesterol is converted to bile acids in preference to equilibrating with other cellular pathways, but it does not mean that most of the bile acids are derived from this source.

In rats, it has been reported that only 25% of bile acids synthesised are derived from newly-synthesised cholesterol (Long et al., 1978); the remainder are synthesised from cholesterol derived from lipoproteins. It is obvious from this that depending upon the experimental conditions defined the contribution of these two sources of substrate to the synthesis of bile acids can change. It has been shown that cholesterol derived both from endogenous synthesis and from certain lipoprotein fractions can be used to produce cholesteryl esters and bile acids and can be secreted into plasma with lipoproteins and secreted into bile (Turley and Dietschy, 1982; Stone et al, 1987; Mackinnon et al 1987; Ford et al, 1985b). As such, the term 'preferred substrate' is potentially misleading.

The microsomal enzyme systems used in cholesterol metabolism are unlikely to be able to determine any difference between free cholesterol derived from either endogenous or exogenous sources. What is most likely is that such preferences are dependent upon the intracellular architecture of the hepatocyte. The hepatocyte, in vivo, has 3 distinct surfaces at which different processes occur. The cannalicular surface is involved in the secretion of bile acids and free cholesterol into bile, and the sinusoidal surface is involved in the uptake of certain lipoproteins and returning bile acids and the secretion of other lipoproteins. I think it is probable that the cell is organized in such a way that its internal structure reflects the functions of its various aspects. Thus, bile acid metabolism may be organized within the endoplasmic reticulum near to or involved with the cannalicular surface and cholesterol ester secretion in lipoproteins may be organized near the sinusoidal surface. This leads to the idea that it would be convenient for those secretory processes that occur at the sinusoidal surface to derive their cholesterol from exogenous sources and those that occur at the cannalicular surface to derive their free cholesterol from endogenous sources. This would require the existence of different intracellular compartments of free cholesterol, but which can and do equilibrate with each other depending upon the metabolic state of the hepatocyte at a given time. Certainly, other workers have evidence for compartmentalisation both in the hepatocyte and

intestinal cells (Stange, 1987) but no consideration has been given to their intracellular situation or organization.

The results of the experiments presented in this thesis tend to support the idea of compartmentalisation, when looked at as a whole. In Chapter 4, the effect of ACAT inhibition was examined using the compound 58-035 with mevalonic acid as a cholesterol source. The results of the various experiments show that 58-035 is an effective inhibitor of ACAT and does not inhibit cholesterol 7α-hydroxylase. When used in conjunction with mevalonic acid, a stimulation of bile acid secretion occurred, but no increase in the secretion of free cholesterol was observed, whilst cholesterol ester secretion was inhibited. Although there was no direct quantifiable relationship between bile acid and cholesteryl ester secretion, the trends were in opposite directions. Thus, inhibition of ACAT decreased cholesterol esterification, resulting in an accumulation of free cholesterol that was used in bile acid synthesis. This indicates that newly synthesized cholesterol can be used for both esterification and bile acid synthesis but is not secreted. Explained in terms of intracellular pools, as seen in figure 7.1 newly synthesized cholesterol forms a specific pool of free cholesterol from which ACAT cholesterol and 7α-hydroxylase can derive their substrate but which is separate from the pool of free cholesterol used for secretion into bile or into plasma with lipoproteins. The limitations of rat hepatocyte monolayers do not allow for differentiation between secretion of free cholesterol into bile or plasma because the normal anatomical relationship of the cells has been disrupted. Thus, inhibition of ACAT accumulates free cholesterol which is used by cholesterol 7α-hydroxylase. Possibly further accumulation of free cholesterol would result in transfer from pool FC_1, to FC_2 and then increases in free cholesterol secretion would be seen, but this was not seen at the level of cholesterol synthesis caused by the addition of mevalonic acid. Figure 7.1 and subsequent figures are schematic representations of intracellular cholesterol flux. They are independent of each other and apply to particular experimental data or combinations of data.
In Chapter 5 the results turned out a little differently. Should the scheme shown in figure 7.1 be correct, then use of an inhibitor

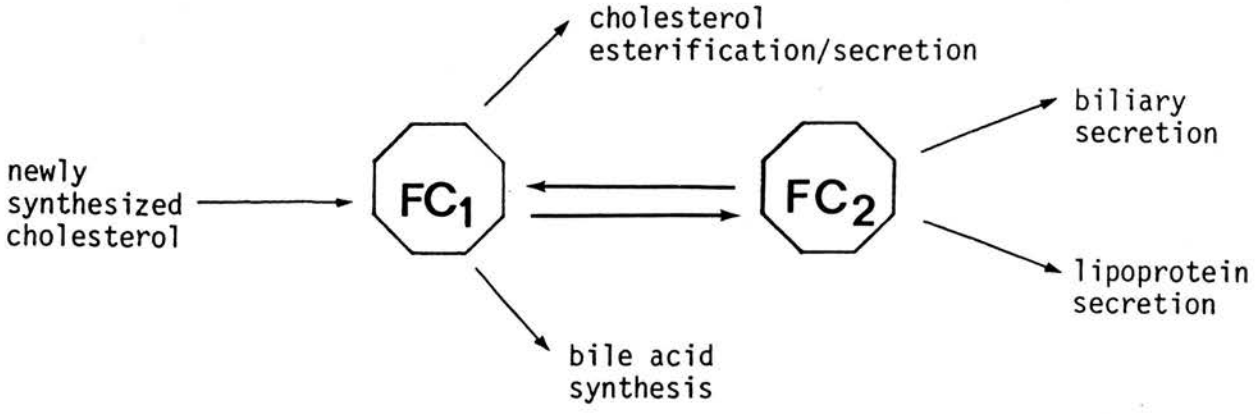


Figure 7.1

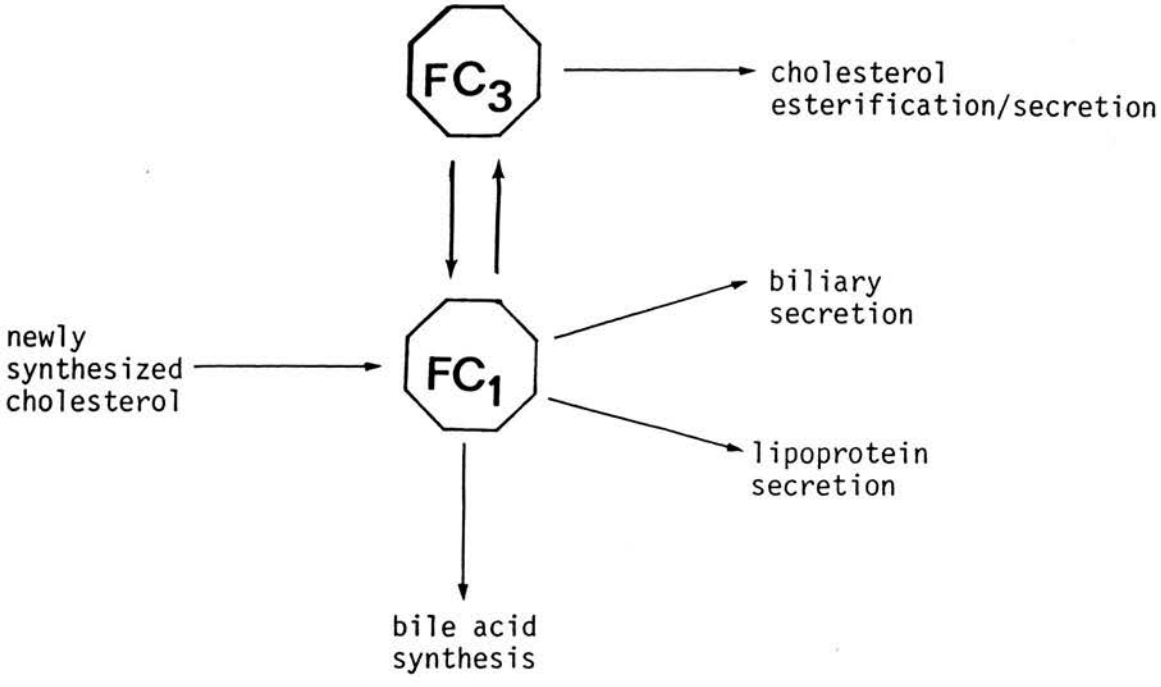


Figure 7.2

of bile acid synthesis would have produced an increase in esterification and no increase in free cholesterol secretion. This was not the case. Having shown that the compound azacholesterol does not affect the enzymes of cholesterol synthesis and esterification , it was used in a parallel fashion to a 58-035. Bile acid synthesis was inhibited at all levels of mevalonic acid used, indicating that the free cholesterol normally used should be accumulating; cholesterol esterification was not effected by this but free cholesterol secretion was increased. Although I suspect that this increase in secretion was into the bile, I have no proof of this. Again, the relationship between the decrease in bile acid synthesis and the increase in free cholesterol secretion was not directly quantifiable. Figure 7.2 attempts to outline these findings. Inhibition of bile acid synthesis produces an accumulation of free cholesterol in pool FC_1, which produces an increase in free cholesterol secretion; potentially, further accumulation may result in transfer to pool FC_3 and cause an increase in cholesterol esterification. This model allows for the results seen and for some equilibration between pools FC_1 and FC_3, and thus allows for some cholesteryl ester to be derived from newly synthesized cholesterol. When taken together, the results so far discussed produce an apparently contradictory picture. Figure 7.3 is an attempt to resolve these differences. Inhibition of ACAT and cholesterol esterifications causes an accumulation of free cholesterol in pool FC_3 and thus in pool FC_1 also. The degree of cholesterol 7α-hydroxylase activity is such that any free cholesterol accumulated is used in bile acid synthesis rather than being transferred to pool FC_2. Inhibition of cholesterol 7α-hydroxylase and bile acid synthesis causes accumulation of cholesterol in pool FC_1. If the kinetics of transfer of cholesterol from pool FC_1 favours its delivery to pool FC_2 over pool FC_3, then an increase in free cholesterol secretion will be seen. Thus, this model accounts for the combined results of chapters 4 and 5, but does not determine, as the results do not, the fate of the secreted free cholesterol.

Having seen the effects of an endogenous source of cholesterol, an exogenous source was used in the experiments in Chapter 6. Results

concerning both bile acid synthesis and cholesterol esterification were determined, but determination of free cholesterol secretion was not possible. Mevinolin, an inhibitor of cholesterol synthesis, was used to ensure that the only cholesterol available was that derived from HDL_2. 58-035 was used in its capacity as an inhibitor of ACAT. HDL_2 lipoprotein sub-fraction was used as a source of cholesterol. The results show that cholesterol derived from HDL_2 increases cholesterol esterification confirming recent reports (Mackinnon et al., 1987; Stone et al, 1987). It also stimulates bile acid synthesis. Thus, it appears that there is a connection between these two pathways. From the models outlined in figures 7.1 - 7.3, transfer of free cholesterol from the pool used for esterification to that used for synthesis of bile acids is to be expected. The situation is not quite as simple as that, for addition of 58-035 to the system caused an inhibition of cholesterol esterification, as expected, and inhibited bile acid synthesis. This indicates that the cholesterol derived from HDL_2 must first be esterified and then hydrolysed before it can be used in bile acid synthesis. This idea is supported when one compares the bile acid secretion results obtained in the presence of 58-035 with the control values. As cholesterol synthesis is inhibited, then bile acid synthesis will also be inhibited due to lack of substrate (Davis et al., 1983a). Potentially the only sources of free cholesterol will be the small amount being synthesized and any stored in the pool derived from lipoproteins prior to isolation of the hepatocytes from the rat. As flux of cholesterol does occur from lipoprotein-derived cholesterol, then it is reasonable to assume that the controls in these experiments are deriving some cholesterol from the newly-synthesised pool which is depleted due to inhibition of cholesterol synthesis, and some is from the lipoprotein-derived pool. Addition of 58-035 will cut off this latter source, reducing bile acid synthesis even more, as was seen. Thus, bile acid synthesis is stimulated by cholesterol derived from HDL_2, but the flux of this cholesterol follows an intracellular route that is inhibited by 58-035. This is outlined in figure 7.4.

These results taken in conjunction with those of chapters 4 and 5 would indicate that newly-synthesised cholesterol enters pool FC_1

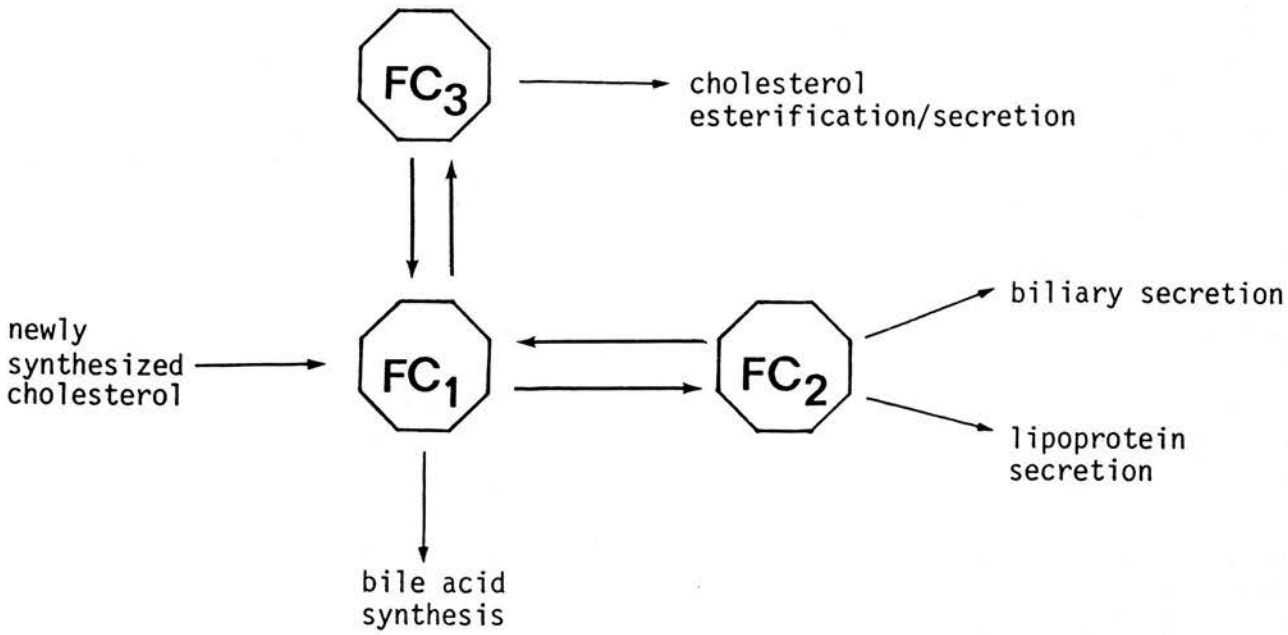


Figure 7.3

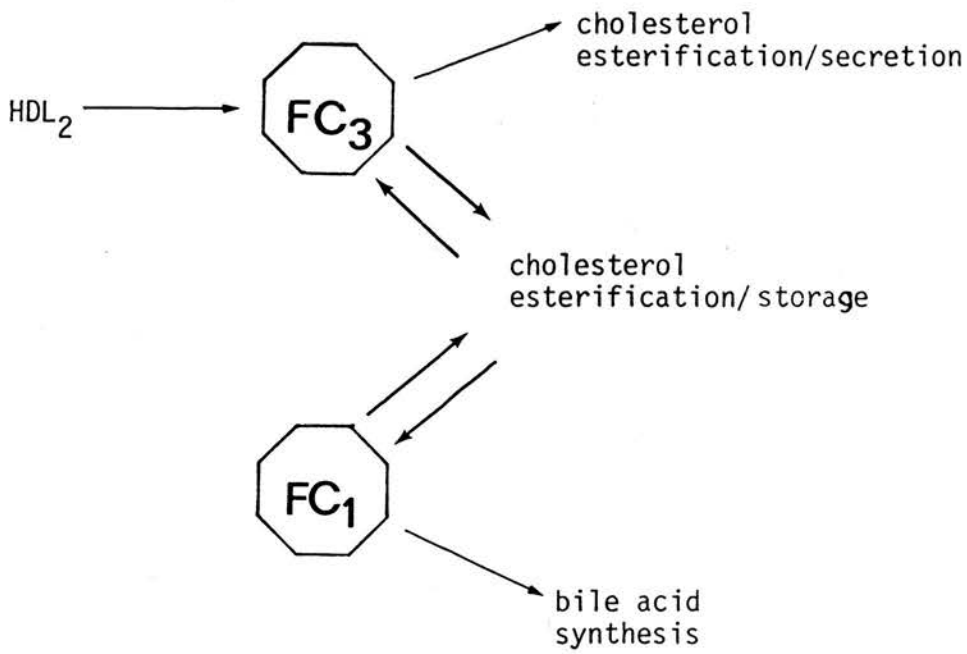


Figure 7.4

and cholesterol derived from HDL_2 (the contribution of receptor and non-receptor mediated uptake to these results is not known) enters pool FC_3 and that transfer of cholesterol between these pools occurs via a cholesteryl ester pool. Figure 7.5 summarises the various ideas presented here, and takes into account the different uses of the cholesterol sources. Unfortunately, location of the pools responsible for biliary and lipoprotein secretion of free cholesterol is not possible, as secretion into the culture medium cannot differentiated along those lines. Thus, the figures show both pathways deriving from the same pool, which may not be the case.

Comparison of this figure with figure 7.6, taken from Stange (1987) shows that there are a number of differences between the 2 schemes. Firstly, newly-synthesised cholesterol proceeds to both bile acid synthesis and to a metabolically active pool of free cholesterol from where it is esterified and secreted, rather than through the postulated cholesteryl ester storage pool determined from my results. Secondly, an HDL-cholesterol pool is directly linked to bile acid synthesis as well as ester secretion, which does not fit with the results determined in Chapter 6. Newly-synthesized cholesterol is shown to be secreted into both bile and lipoproteins, indirectly indicated by my results.

The compartmentalisation described by Stange is drawn from the work of a number of investigators using different animal systems, whereas the work presented here was done exclusively in the rat, where differences in the end products of bile acid synthesis are seen, and where the LDL lipoprotein fraction is metabolically insignificant. Thus in comparison to man and other species, the rat has a potentially different pattern of intracellular cholesterol metabolism than the composite scheme shown in figure 7.6. Furthermore, the literature shows that depending on the experimental conditions used certain inter-relationships are shown in preference to others. Certainly the picture of the intracellular organisation of hepatic cholesterol metabolism is complex. It may well be that the number of distinct substrate pools of free cholesterol may be larger than has been postulated so far. Possibly, there are a

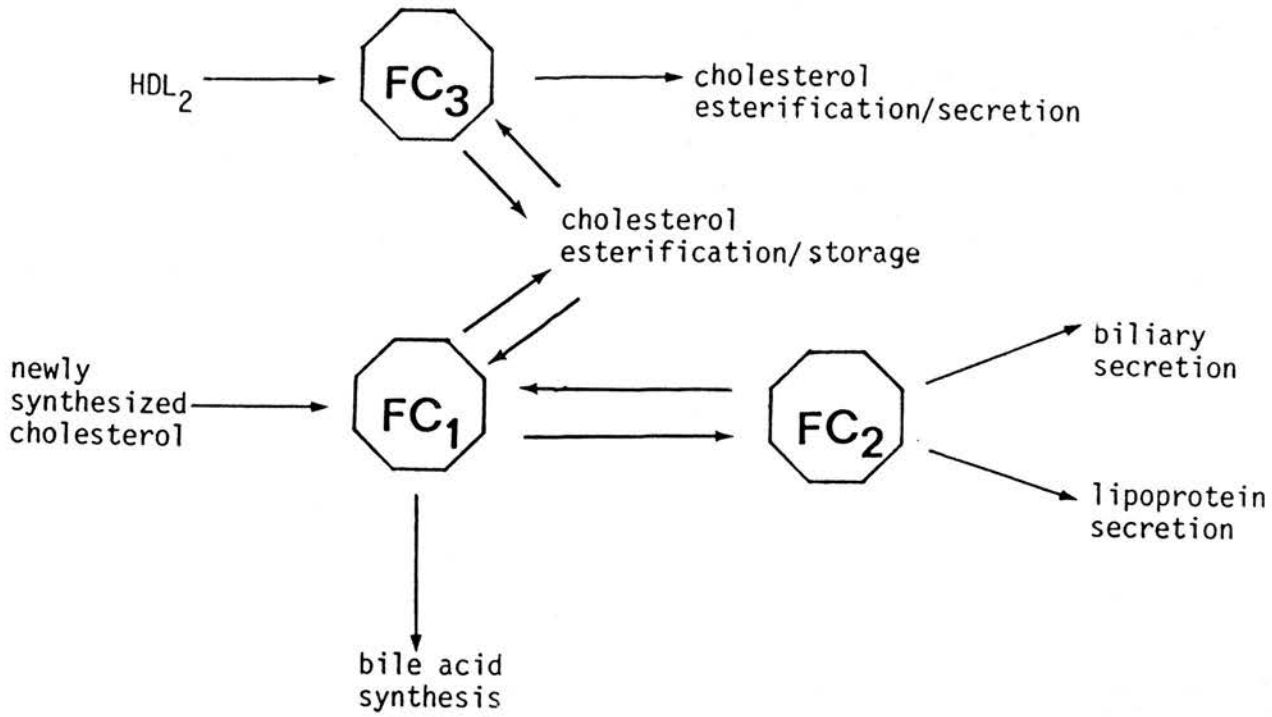


Figure 7.5

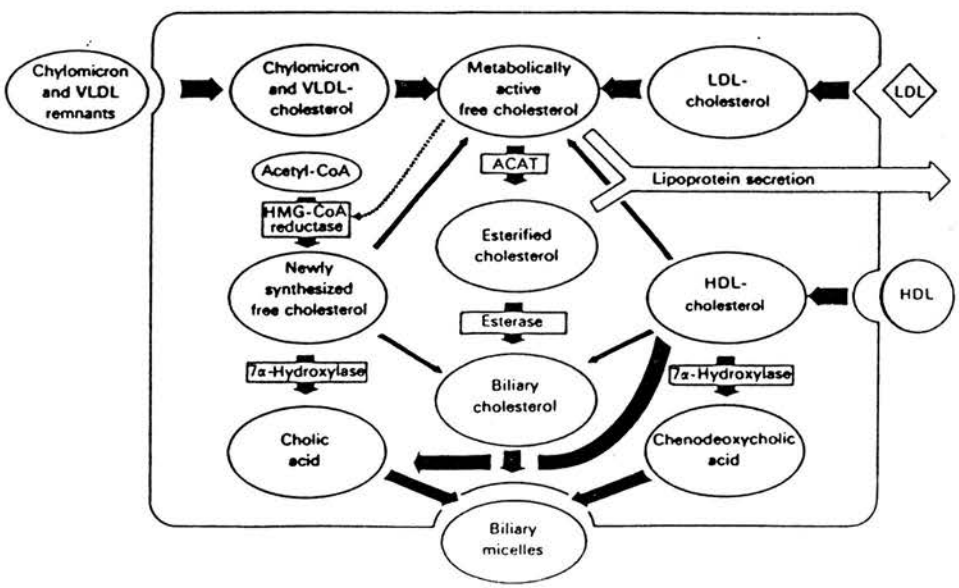


Figure 7.6

number of products, (cholesteryl ester for storage or lipoprotein secretion; free cholesterol for biliary or lipoprotein secretion and use in the cell membrane; bile acid for secretion) each with a specific precursor pool. These pools will in turn, have inputs from a number of sources (endogenous synthesis; uptake from different lipoproteins by receptor and non-receptor mediated routes). Each pool in turn may be able to equilibrate with a number of the others so that transfer of free cholesterol between the pools can occur. However, due to physical and spatial/vectoral restraints, the kinetics of transfer may favour certain inter-relationships. Thus, blocking the formation of one product does not result in an even redistribution of the accumulated free cholesterol between the other pools, but rather to those pools which are most readily available.

In terms of the experiments defined in this thesis, a number of refinements could be attempted to clarify the situation in the rat hepatocyte. Repeating the experiments of chapter 6 using HDL_2 labelled in either its free cholesterol or cholesteryl ester moiety, would provide further information concerning the partion of the lipoprotein cholesterol between esterification, secretion and bile acid synthesis, and further define the effects of 58-035. It would also be of interest to examine the effects of chylomicron and VLDL remnants and determine if cholesterol delivered by them follows the same intracellular routes as that from HDL_2, providing information on the metabolic roles of apo B/E (chylomicron, VLDL, HDL) receptors and apo E (HDL) receptors in cholesterol metabolism. Also, techniques could be employed to investigate non-receptor mediated uptake of HDL-cholesterol and determine its intracellular fate.

As stated earlier, the problem with monolayer cultures is that they do not have the normal anatomical arrangement seen in vivo, where the hepatocytes are in contact with 3 extracellular compartments. Culturing of the hepatocytes on a membrane system would allow for the maintenance of 2 separate compartments from which medium can be removed or added. Thus, secretion into both compartments could be examined to ascertain if the cells develop a polarity closer to that seen in vivo, and be of use in defining the nature of the secretion of the free cholesterol.

The discussion has so far limited itself to work on hepatic cholesterol metabolism and not considered any other of the tissues in which cholesterol metabolism is of significance. In two cell types, macrophages and bovine adrenal cortical cells, a cholesteryl ester cycle has been postulated (Goldstein and Brown, 1977; Brown et al., 1980; Jamal et al., 1985). Cultured macrophages have a continuous energy-consuming (futile) cycle of esterification of cholesterol (Brown et al., 1980), which is performed by a non-lysosomal cholesteryl ester hydrolase and ACAT. It appears to be interrupted by HDL which promotes removal of free cholesterol from the cell. This leads to the idea that one of the roles of HDL is the removal of cholesterol from peripheral tissues, in particular macrophages, as mentioned in Chapter 1. Cultured cells of the bovine adrenal cortex have a cycle similar to that of macrophages. Both newly synthesised and lipoprotein cholesterol are available for synthesis of cholesteryl ester, which can be hydrolysed and the cholesterol used either in steroidogenesis or in cholesteryl ester synthesis. One further cell type, the Fu5AH hepatoma cell, is said to have such a cholesteryl ester cycle (Glick et al., 1987). Such a situation conflicts with the compartmentalisation seen in normal non-transformed hepatocytes, presenting a much simpler picture of intracellular hepatic cholesterol metabolism. This conflict is easily resolved when one considers that bile acid synthesis does not occur Fu5AH hepatoma cells, and thus one important pathway of cholesterol utilization is missing. As such, the cells are primarily concerned with the synthesis and secretion of free and esterified cholesterol with lipoproteins, and will have a different intracellular organization of cholesterol metabolism.

Intestinal epithelial cells on the other hand show evidence for compartmentalisation of intracellular free cholesterol, recently reviewed by Stange (1987). In this case, 2 pools, one of absorbed free cholesterol, the other of newly-synthesised cholesterol, contributes to a third pool, which provides free cholesterol for both a cell membrane pool and a chylomicron pool. Also, the absorbed cholesterol pool provides the substrate for cholesteryl esterification and secretion. In this model, stricter roles for the various sources of free cholesterol exist than occur in any model

seen for the hepatocyte; furthermore, the pattern of intracellular cholesterol flux is apparently less complex than that of the hepatocyte. Thus, the organisation of intracellular cholesterol metabolism differs depending on the cell type and the complexity of its function.

The importance of the cell types mentioned, in particular hepatocytes, in the metabolism of cholesterol, is seen when one considers certain disease states related to the excess accumulation of cholesterol, such as atherosclerosis. Intestinal cells are the absorption site of dietary cholesterol; macrophages are involved directly in the formation of atherosclerotic lesions via their transformation into foam cells; and adrenocortical and other steroidogenic tissues metabolise cholesterol to specific products. Each of these is involved in cholesterol homeostasis. The liver, also involved in cholesterol homeostasis, is the only site of the metabolism of cholesterol to bile acids and their subsequent excretion. Thus, it represents the only significant route for the removal of cholesterol and the decrease of the total body pool of cholesterol, potentially preventing accumulation of excess steroid. Understanding intracellular hepatic cholesterol metabolism may show a way to more directly controlling cholesterol homeostasis, providing a route for medical intervention in certain disease states.

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"And on the pedestal, these words appear:
My name is Ozymandias, King of Kings,
Look on my Works, ye Mighty and despair!
Nothing beside remains..."

Shelley

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The role of acyl-CoA : cholesterol acyltransferase in the metabolism of free cholesterol to cholesteryl esters or bile acids in primary cultures of rat hepatocytes

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Key words: Acyl-CoA : cholesterol acyltransferase; Cholesterol metabolism; Bile acid; (Rat hepatocyte)

Sandoz compound 58-035 has been shown to inhibit acyl-CoA : cholesterol acyltransferase activity in a variety of cell types. We have shown that it does not inhibit rat liver microsomal cholesterol 7α-hydroxylase, the rate-limiting enzyme of bile-acid synthesis, but it does inhibit acyl-CoA : cholesterol acyltransferase in both the microsomal fraction and in rat hepatocyte monolayers. To test the role of acyl-CoA : cholesterol acyltransferase in these cells, monolayers were incubated over 5 h in the presence and absence of 58-035 and in the presence of increasing amounts of mevalonic acid to provide a source of cholesterol. The addition of mevalonic acid increased the secretion of bile acids by the cells, and this was further increased by the addition of 58-035. The secretion of cholesteryl esters was conversely inhibited by the addition of 58-035. The results help define the role of acyl-CoA : cholesterol acyltransferase in determining the fate of intracellular cholesterol.

Introduction

The liver presents one of the most complex patterns of intracellular cholesterol metabolism of all tissues. It can take up cholesterol from the blood in lipoproteins derived from endogenous sources (LDL, IDL) and from exogenous sources (chylomicron remnants) [1]. The liver is also an important tissue for synthesis of cholesterol [2]. Cholesterol is secreted from the liver in the free and esterified form in nascent lipoproteins, particularly VLDL. It is also secreted into bile in combination with phospholipids and with bile acids, which are themselves synthesised from hepatic cholesterol [3]. The existence of these different metabolic roles for hepatic cholesterol raises the possibility that certain discrete intracellular pools of cholesterol are present that are specific for one or other of these functions. There is some evidence that such pools exist and interact. For example the pool of cholesterol that provides the free sterol secreted into bile appears to be related to the substrate pool for esterification of cholesterol [4,5]. In other cases, for example the cholesterol that serves as substrate for bile-acid

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Abbreviations: IDL, intermediate-density, lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; 58-035, 3-(decyldimethylsilyl)-*n*-[2-(4-methylphenyl)-1-phenylethyl]propanamide.

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synthesis, the source of cholesterol is less clear. Newly synthesised cholesterol has often been regarded as the 'preferred' source [6]. More recently, lipoproteins have been shown to provide substrate cholesterol for bile-acid synthesis in the rat [7]. In man either the free [8] or esterified [9] form may be important. In rats and in many other animals, especially in the fed state, the cholesteryl ester that is secreted into the blood from the liver is formed under the catalysis of the microsomal enzyme acyl-CoA : cholesterol acyltransferase [10]. This is a key enzyme in the intracellular exchanges of free cholesterol in the liver, since it determines the fate of a certain proportion of the cellular cholesterol.

Recently, a useful tool, the Sandoz compound 58-035, has become available for studying the role of this enzyme in cellular cholesterol metabolism. It has been shown to inhibit acyl-CoA : cholesterol acyltransferase activity in hepatoma cells, gut cells, ovarian cells, macrophages and in bovine adrenal cortical cells [11–15]. We have used this compound in studies of the control of cholesterol movement in the adrenal cortex. By inhibition of either cholesterol esterification (58-035) or mitochondrial oxidation of cholesterol, we have been able to describe the changes in the pathway followed by cholesterol derived from lipoproteins in these cells when they are stimulated with a tropic hormone [14,16]. In this paper we describe the first stages of the parallel studies of the more complex pathway open to intracellular cholesterol in the liver [10]. In these new studies esterification of cholesterol is again inhibited by 58-035, and oxidation of cholesterol (in studies to be reported elsewhere) by a new specific inhibitor of cholesterol 7α-hydroxylase [17]. Because several lipoprotein particles can be taken up by the liver, by mechanisms not limited to receptor-mediated endocytosis [18], and because the effects of 58-035 have not yet been characterised in rat hepatocytes in monolayer culture, we chose first to study the effect of 58-035 on the fate of cholesterol newly synthesised within the liver from mevalonate. This is an approach that has often been used in studies of cholesterol esterification in liver [19,20] and it has also been useful in describing the dependence of bile-acid synthesis on substrate supply in rat hepatocytes [21,22]. We believe that this is a reasonable first approach, although it is not as close to the physiological prototype as a study with defined lipoprotein fractions. However, the effects of mevalonic acid on cholesterol metabolism are broadly understood [19–22], whereas the situation with regard to lipoproteins is not. The present paper, therefore, describes the effects of 58-035 on hepatic cholesterol metabolism in the presence of mevalonic acid as a simple first step to set the scene for a broader study to follow.

Materials and Methods

Preparation of hepatocytes

Rat hepatocytes were prepared by the collagenase perfusion technique described previously [7] from rats fed either a control diet or the same diet containing 4% (w/w) cholestyramine.

Effect of 58-035 on cholesteryl ester formation in monolayers of rat hepatocytes

The effect of the acyl-CoA : cholesterol acyltransferase inhibitor 58-035 on cholesteryl ester formation in these cells was determined in two ways. Firstly, after adhesion of the cells to the dishes the medium was changed to 2 ml supplemented Dulbecco's modified Eagle's medium [7] and the cells incubated overnight. The medium was then changed to 2 ml supplemented Dulbecco's modified Eagle's medium [7] containing 20% (v/v) foetal calf serum. 58-035 was added in solution in dimethyl sulphoxide (20 μl) to give a final concentration of up to 100 μg/ml inhibitor. After a further 2 h incubation the medium was removed and the cells were washed in 0.1 M potassium phosphate buffer (pH 7.4). The cells were then scraped off the dishes with a rubber policeman using 2×0.5 ml aliquots of potassium phosphate buffer. The resulting suspensions were individually homogenised, 10 μl were removed for analysis of protein, and the remainder transferred to Eppendorf tubes and centrifuged at $8\,800 \times g$ in a microfuge for 5 min. 100 μl of bovine serum albumin (fatty-acid free) and glutathione solution in potassium phosphate buffer (each 10 mg/ml) was added to 850 μl of the supernatant. After 30 min preincubation at 37° C [^{14}C]oleoyl-CoA (150 μl, final concentration 42 μM) was added and the mixture incubated for 1 min. The reaction was

stopped by the addition of 2 ml methanol. Extraction and analysis of products was carried out as described previously [23].

In the second group of experiments the formation of cholesteryl ester was measured directly in the cells. After 2 h preincubation in the presence of 58-035 (100 or 200 μg dish added in dimethyl sulphoxide) or dimethyl sulphoxide alone the medium was removed and 2 ml supplemented Dulbecco's modified Eagle's medium containing 5% bovine serum albumin and potassium [^{3}H]oleate (40 μM, 50 dpm/pmol) was added. 25-Hydroxycholesterol was added to some incubations as a solution in ethanol (10 mg/ml) to give a final concentration of 10 μg/ml medium [14]. Potassium [^{3}H]oleate was prepared from oleic acid, following Ref. 20. After 1.5 h incubation, the medium was removed and the cells scraped off into potassium phosphate buffer. Both the cells and the media were extracted with chloroform/methanol (2 : 1 (v/v)) and the radioactivity in the cholesteryl ester fraction was determined as described previously [23].

Effect of 58-035 on secretion of bile acids and cholesteryl ester by monolayers of rat hepatocytes

After the cells had adhered to the culture dishes the medium was replaced with 2 ml supplemented Dulbecco's modified Eagle's medium containing 2% w/v bovine serum albumin. 200 μg 58-035 in 20 μl dimethyl sulphoxide was added to some of the dishes; either dimethyl sulphoxide (20 μl) or no additions were amde to control dishes. The dishes were further incubated for 2 h. After this the medium was replaced with 2 ml supplemented Dulbecco's modified Eagle's medium containing 0–10 mM mevalonolactone and without bovine serum albumin. After a 5 h incubation the media were removed and stored for determination of bile acid and cholesteryl esters. The cells were scraped off the plates into 1 ml potassium phosphate buffer, and a homogenate of this mixture was used for protein determination.

Conjugated cholic, chenodeoxycholic and β-muricholic acids were determined in the medium by radioimmunoassay [24–26]. Cholesterol and cholesteryl ester in the medium were determined fluorimetrically [27]. In other experiments the distribution of cholesterol, cholesteryl ester, triacylglycerol and phospholipid was determined in the pooled media from several culture dishes. The media were separated into fractions of different buoyant density [28], and the lipids in the various fractions analysed by fluorimetry for cholesterol and cholesteryl ester and on a centrifugal autoanalyser for triacylglycerol and phospholipid. SDS-polyacrylamide gel electrophoresis [29] was used to characterise the apoproteins present in the lipoprotein fraction $d < 1.03$ g/ml.

Cholesterol 7α-hydroxylase activity was determined, using [^{14}C]cholesterol added to a microsomal preparation of rat liver [30]. Protein was determined by the dye-binding method of Bradford [31]. Acyl-CoA : cholesterol acyltransferase activity was determined in microsomal fractions of rat liver as described previously [23], using oleoyl-CoA.

Collagenase, galactose, gentamycin sulphate, insulin and mevalonolactone and bovine serum albumin fraction V powder were obtained from Sigma (Poole, U.K.). Other preparations of collagenase and bovine serum albumin were obtained from Boehringer-Mannheim (London, U.K.). The bovine serum albumin was pretreated with charcoal to remove bile acids. 25-Hydroxycholesterol was purchased from Steraloids (Croydon, U.K.). Dulbecco's modified Eagle's medium and penicillin/streptomycin were obtained from Gibco Europe (Paisley, U.K.). Non-essential amino acids and foetal calf serum were obtained from Flow Laboratories (Irvine, U.K.). Foetal calf serum was heat-inactivated by incubation at 56 °C for 30 min. Cholestyramine (Cuemid) was supplied by Bristol-Myers (Uxbridge, U.K.). Percoll was a product of Pharmacia (Milton Keynes, U.K.). Radiochemicals were obtained from Amersham International (Amersham, U.K.). 58-035 (3-(decyldimethylsilyl)-*n*-[2-(4-methylphenyl)-1-phenylethyl]propanamide) was a gift of Dr John Heider, Sandoz Inc., East Hanover, NJ, U.S.A.

Results

Effect of 58-035 on acyl-CoA : cholesterol acyltransferase activity in vitro

Since the effect and specificity of 58-035 had not previously been reported in rat liver, we carried out preliminary experiments to test the effect

of 58-035 on acyl-CoA : cholesterol acyltransferase activity in rat liver microsomes in vitro. 58-035 added in solution in acetone or dimethyl sulphoxide inhibited acyl-CoA : cholesterol acyltransferase activity to 12% of controls containing the solvents at a concentration of 0.6 μg 58-035 to 500 mg microsomal protein. Acyl-CoA : cholesterol acyltransferase activity can be increased in vitro by a variety of methods, by 25-hydroxycholesterol, by an ATP-dependent process and by addition of free cholesterol in a number of ways [10]. In each case 58-035 inhibited the 'activated' system to levels similar to those of control assays containing 58-035 and microsomes alone. Thus, 58-035 is able to inhibit acyl-CoA : cholesterol acyltransferase activity whatever the state of the enzyme in vitro, and this is consistent with the inhibitor's acting directly on the enzyme. 58-035 did not inhibit rat liver microsomal cholesterol 7α-hydroxylase at concentrations equivalent to those capable of inhibiting cholesterol esterification or at concentrations 40-times that. 58-035 is also known not to inhibit cholesterol synthesis in bovine adrenal cortical cells [14], although this point is not important in the present work, since most of the studies were carried out in the presence of mevalonic acid, to provide a substantial supply of intracellular cholesterol.

Effect of 58-035 on cholesterol esterification in rat hepatocyte monolayers

We then carried out parallel experiments in monolayers of rat hepatocytes to determine whether 58-035 could enter the cells and efficiently inhibit cholesterol esterification. The inhibitor was most effectively presented to the cells as a solution in dimethyl sulphoxide (up to 200 μg/dish in 20 μl) in the presence of bovine serum albumin (2–5% (w/v)). Under these conditions the inhibition of the formation of cholesteryl [^{14}C]oleate from [^{14}C]oleoyl-CoA in the 8 800 $\times$ g supernatant prepared from the cells followed the dose-response curve shown in Fig. 1. These observations were confirmed by measuring the incorpotation of potassium [^{3}H]oleate into cholesteryl oleate in the intact cells over 1.5 h (Table I). Table I also shows that addition of 25-hydroxycholesterol to the cells stimulated formation of cholesteryl ester [19]. The stimulation by 25-hydroxycholesterol was inhibited by 58-035, but in each case 25-hydroxycholesterol increased the extent of cholesteryl ester formation by the same amount, about 2.8-fold. In these experiments and those that follow, the solvent, dimethyl sulphoxide, had no effect on cholesteryl ester formation or secretion or bile-acid synthesis and secretion.

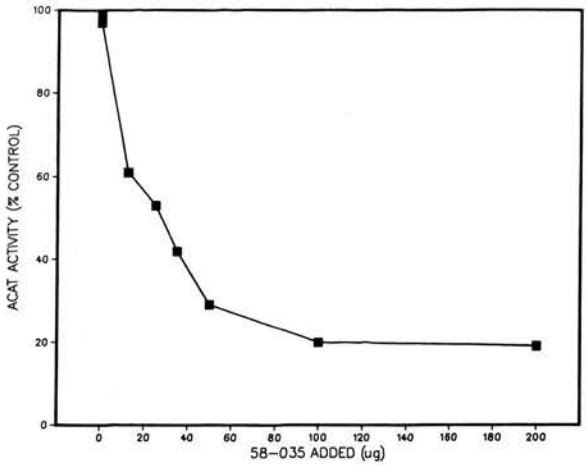

Fig. 1. Dose-response curve for the inhibition of acyl-CoA : cholesterol acyltransferase activity in the 8 800 $\times$ g supernatant prepared from monolayers of rat hepatocytes treated with the inhibitor 58-035. See Materials and Methods for experimental details. ACAT, acyl-CoA : cholesterol acyltransferase.

TABLE I

INCORPORATION OF POTASSIUM [^{3}H]OLEATE INTO CHOLESTERYL ESTER IN MONOLAYERS OF RAT HEPATOCYTES IN THE PRESENCE OF 58-035 AND 25-HYDROXYCHOLESTEROL

Monolayers of rat hepatocytes were incubated in the presence of potassium [^{3}H]oleate with 58-035 and 25-hydroxycholesterol under the conditions described in Materials and Methods. The table shows the mean $\pm$ S.D. (n = 4).

		Cholesteryl oleate formed (pmol/h per dish)
1	Control	205 $\pm$ 8
2	Control + dimethyl sulphoxide	198 $\pm$ 6
3	1 + 25-Hydroxycholesterol	555 $\pm$ 43
4	2 + 25-Hydroxycholesterol	541 $\pm$ 25
5	1 + 100 μg 58-035	35 $\pm$ 4
6	2 + 200 μg 58-035	34 $\pm$ 1
7	5 + 25-Hydroxycholesterol	103 $\pm$ 5
8	6 + 25-Hydroxycholesterol	100 $\pm$ 11

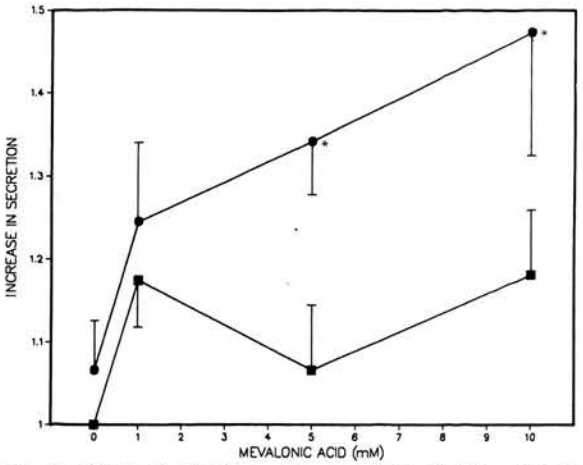

Fig. 2. Effect of 58-035 on the secretion of bile acids by monolayers of rat hepatocytes in the presence of increasing concentrations of mevalonic acid. The points show the bile acids secreted relative to control and are the mean ± S.E. from nine independent experiments. Comparison of the paired data for each animal by the Wilcoxon signed rank test showed a significant increase in bile-acid secretion in the presence of 58-035 over controls at 5 mM mevalonic acid ($P = 1.95\%$) and at 10 mM ($P = 3.9\%$). ■, control; ●, 58-035 added. * Means are significantly different ($P < 5\%$) from control (calculated by Student's t-test on the normalised data).

Changing the medium from that containing the inhibitor to fresh medium without inhibitor and further incubation of the cells for up to 16 h followed by preparation of the $8\,800 \times g$ supernatant fraction and assay of acyl-CoA : cholesterol acyltransferase activity showed that the inhibition was still observable (data not shown). It appears that the highly hydrophobic 58-035 remains associated with the cells even when the donor medium is removed. These studies and those described above together with published work [11–15] show that 58-035 is a suitable compound for studying the effects of inhibition of cholesterol esterification in monolayers of rat hepatocytes.

Effect of 58-035 on secretion of bile acids by rat hepatocyte monolayers

We have shown previously that monolayers of rat hepatocytes secrete cholate, chenodeoxycholate and β-muricholate over periods of up to 18 h in culture. The greatest rate of synthesis is observed over the first 5 h of incubation. The three bile acids measured by radioimmunoassay represent over 90% of those synthesised by the hepatocytes [32]. The amount of bile acids secreted by a preparation of hepatocytes can be increased by feeding the rats from which the cells are to be prepared a diet containing the bile-acid-binding resin cholestyramine [32].

There is evidence from studies in isolated cells [7,21] and also in microsomal preparations [6] that the rate of bile-acid synthesis, controlled by the activity of cholesterol 7α-hydroxylase, is dependent on the supply of substrate cholesterol. To investigate the role of acyl-CoA : cholesterol acyltransferase in the rat hepatocytes, we therefore incubated monolayers with and without 58-035 in the presence of increasing concentrations of mevalonic acid to provide a substantial intracellular source of cholesterol. The secretion of the three bile acid into the culture medium was measured after 5 h by radioimmunoassay. Nine animals were independently studied with basal rates of bile-acid synthesis ranging from 0.2 nmol/mg cell protein to 4 nmol in 5 h, depending on the diet used. Because of this wide variation the results were treated in two ways. Firstly the amounts of the bile acids secreted in 5 h were expressed as the increase in bile-acid secretion over that obtained with the control incubations in which no 58-035 or mevalonate had been added. The mean of these values was calculated and statistical significance between the incubations with 58-035 present and absent was evaluated using Student's t-test. The results of this analysis are shown in Fig. 2. Alternatively, the data from paired incubations with and without 58-035 were compared directly by the Wilcoxon signed rank test. The results of this comparison are given in the legend to Fig. 2.

Whichever method of analysing the results for significance was chosen, 58-035 was found to cause a highly significant increase in the secretion of bile acids from the cells at concentrations of mevalonic acid over 5 mM (1.26-times control at 5 mM and 1.25-times at 10 mM). No significant increase was observed in the absence of mevalonic acid. A small increase in the secretion of bile acids was observed before [21]. The increase in bile-acid secretion due to 58-035 was significant, taking all the nine animals studied as a group, but it was most marked in the situations where the basal rate of bile-acid secretion was high.

Effect of 58-035 on the secretion of cholesteryl esters by monolayers of rat hepatocytes

To parallel the observations on the secretion of bile acids just outlined, we examined the secretion of cholesteryl ester into the medium in the same preparations of cells as those studied for bile-acid secretion. Firstly we characterised the nature of the lipoprotein particles in which the cholesteryl ester was secreted from the cells. Based on previous work, it was thought to be likely that the cholesteryl ester would mainly be secreted into particles of density less than 1.03 [28]. This fraction was prepared by ultracentrifugation of media from incubations of rat hepatocyte monolayers for over 5 h. The fraction of density less than 1.03 was analysed for lipid composition and for apolipoproteins by SDS-polyacrylamide gel electrophoresis. The lipid composition was found to be triacylglycerol : phospholipid : free cholesterol : cholesteryl ester 100 : 20 : 10 : 14 and the apolipoproteins, apo-B, apo-C and apo-E were detected on the gels. The amount of cholesteryl ester in this fraction was about 90% of the total in the medium.

In the presence of mevalonic acid the secretion of cholesteryl ester would be expected to increase [19,20]. This was observed and the secretion of ester now was found in other lipoprotein density fractions as well as $d < 1.03$. 27% of the total cholesterol ester in the medium was found in the $d < 1.03$ fraction. A further 22% was present in the range 1.03–1.08 and the remaining 51% in the range 1.08–1.23. Thus, all the cholesteryl ester secreted from the cells was present in a lipoprotein fraction. As with the secretion rates of the bile acids, the rates of secretion of cholesteryl ester by

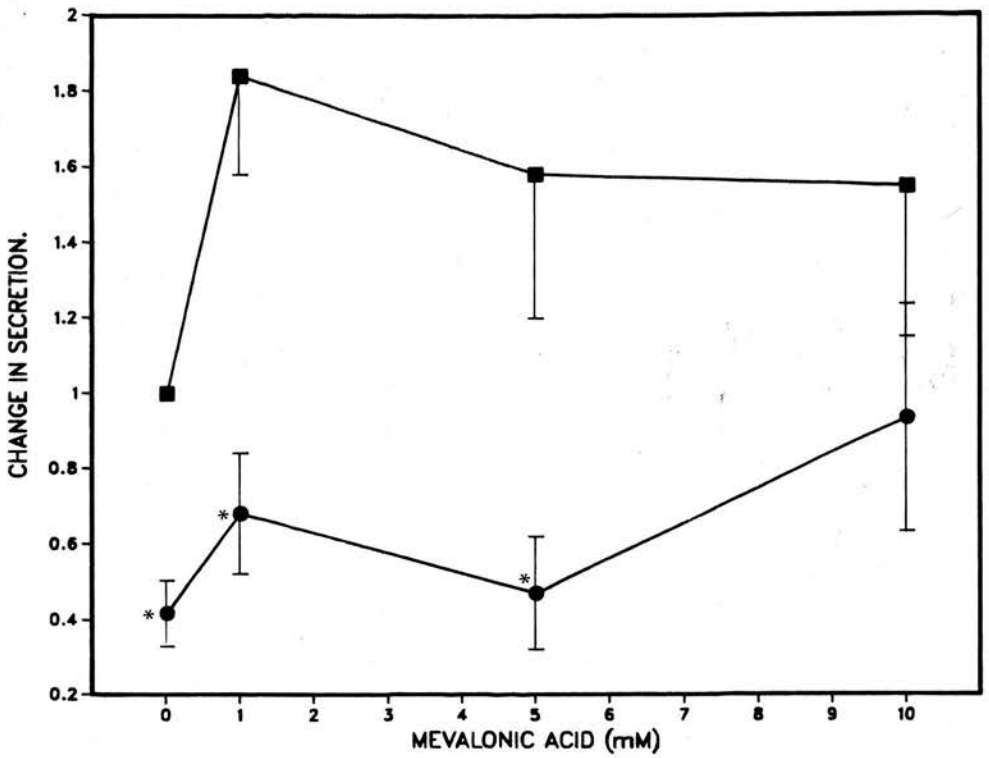

Fig. 3. Effect of 58-035 on the secretion of cholesteryl ester by monolayers of rat hepatocytes in the presence of increasing concentrations of mevalonic acid. The points show the cholesteryl ester secreted relative to control and are the mean ± S.E. of eight independent experiments. Comparison of the paired data for each animal by the Wilcoxon signed rank test showed a significant decrease in the secretion of cholesteryl ester over controls at 0 mM mevalonic acid ($P = 0.78\%$), 1 mM ($P = 3.9\%$) and 5 mM ($P = 3.1\%$), ■, control; ●, 58-035 added. * Means are significantly different ($P < 5\%$) from control (calculated by Student's t-test on the normalised data).

the cells varied over a wide range, from 10.4 to 0.36 nmol/mg cell protein in 5 h. The amounts of cholesteryl ester secreted by the cells in the presence and absence of 58-035, and of varying concentrations of mevalonic acid were compared in the same way as the bile-acid data using normalised data, and by the Wilcoxon signed rank test (Fig. 3). Secretion of cholesteryl ester was increased by mevalonic acid 1.5-fold, as would be expected. The presence of 58-035 inhibited the secretion of cholesteryl ester at concentrations of mevalonic acid from 0 to 5 mM (42% of control at zero mevalonate and 37 °C at 5 mM). The effect of 10 mM was not significant. Since inhibition of acyl-CoA : cholesterol acyltransferase is about 80% complete in these experiments (Fig. 1), any effect at 10 mM mevalonic acid was probably overcome by the large excess of free cholesterol present in the cells.

No effect of 58-035 was observed on the secretion of free cholesterol. The free cholesterol secreted in the presence of 58-035 in all the experiments was 1.14 ± 0.35 (S.D., $n = 21$) of the corresponding incubations without 58-035. The free cholesterol secreted from monolayer cultures of hepatocytes could derive from either secretion into bile or plasma compartments. Because of this ambiguity inherent in the monolayer technique, no firm conclusions can be drawn from this observation.

In combination, the experiments in Fig. 2 and 3 show that inhibition of acyl-CoA : cholesterol acyltransferase causes an increase in bile-acid secretion and a corresponding decrease in secretion of cholesteryl esters in those cells which had a high availability of free cholesterol.

Discussion

The use of the acyl-CoA : cholesterol acyltransferase inhibitor 58-035 in the experiments described here has allowed a comparison of the availability of free cholesterol in the hepatocyte for esterification and secretion in lipoproteins with its use as a precursor for bile acids. It is clear that under the present conditions the substrate pools of cholesterol for bile-acid synthesis and for esterification can interact: they may be regarded as part of the same large intracellular metabolically active pool. The increase in bile-acid synthesis in the presence of 58-035 does not show a molar correlation with the decrease in the secretion of ester, although clearly the broad trends are in opposite directions. Other studies in rat hepatocytes show that the synthesis of bile acids is positively correlated with the amounts of free cholesterol in the cells, but negatively correlated with the amounts of cholesteryl ester present [33]. This is in agreement with the pattern shown by the current studies where the increased supply of free cholesterol caused by the inhibition of acyl-CoA : cholesterol acyltransferase leads to an increased production of bile acids. Other recent studies [34,35] relate to the question of substrate supply for cholesterol esterification and bile-acid synthesis in rat liver. These are long-term in vivo studies (several days) and cannot be compared with the present work where processes occurring in a few hours and under special conditions were examined. Many studies do lead to the conclusion that in liver the substrate pools for bile-acid synthesis and cholesterol esterification are probably not saturated [6,10]. This would also appear to be the case in our cells under the conditions of these experiments, since the addition of mevalonic acid alone increased the secretion of both products.

The pattern of the intracellular cholesterol flux described here is similar to that we observed in parallel experiments in the bovine adrenal cortical cells [14]. The excess cholesterol produced in the cells is used according to the capacity of the enzyme systems that are present. However, it is clearly difficult to extrapolate from the present data to the situation in vivo. The present data demonstrate the possibilities for the movement of free cholesterol within the hepatocyte. It is of particular importance to discover whether similar effects occur with cholesterol derived from lipoproteins. The result may well differ for lipoproteins of different sources and density ranges, and with the mechanism of uptake of cholesterol and cholesteryl ester.

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The effect of inhibition of cholesterol esterification on the fate of cholesterol derived from HDL in rat hepatocyte monolayers

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Rat HDL_2 is known to stimulate bile acid synthesis in rat hepatocyte monolayers. The intracellular fate of the cholesterol derived from the HDL_2 was studied using the inhibitor of cholesterol esterification, Sandoz compound 58-035. Rat HDL_2 added to rat hepatocyte monolayers caused a stimulation of cholesterol esterification of 32%. This stimulation could be inhibited by 58-035. A small significant increase in bile acid synthesis was also observed in cells in the presence of HDL_2, confirming our earlier observations. 58-035 prevented this increase. These observations imply that cholesterol entering the cell from HDL_2 is first esterified and can only enter the substrate pool for bile acid synthesis after subsequent intracellular hydrolysis.

Bile acid; HDL; Acyl-CoA:cholesterol acyltransferase; Lipoprotein; Cholesterol metabolism; (Rat hepatocyte)

1. INTRODUCTION

Lipoproteins are taken up by cells by a variety of mechanisms. Of these the best characterised is receptor-mediated endocytosis by the LDL pathway [1]. In some tissues non-receptor mediated uptake also contributes, and mechanisms in which the apoprotein of HDL is taken up at a slower rate than its lipid core have also been described [2]. In all these cases some of the metabolic consequences of uptake of cholesterol are known but it is not clear whether cholesterol entering a cell by one of these routes is constrained to a particular cellular pathway or whether it can immediately equilibrate with the cellular metabolically active pool [3,4]. In some tissues, such as the bovine adrenal cortex, it has been possible to demonstrate the route followed by cholesterol derived from LDL within the cell under different metabolic conditions [5,6]. In other tissues, such as the liver, there are indications that there may be compartmentation of cholesterol metabolism [3,4] but there has been no direct evidence of this.

Free cholesterol derived from lipoproteins in the liver may have a number of fates. This free cholesterol may be secreted into the plasma as a component of lipoproteins or, with phospholipid and bile acids, into bile. Alternatively it may be used as a substrate for bile acid synthesis or be esterified by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) [4]. We demonstrated a few years ago that a rat lipoprotein fraction, HDL_2 can stimulate the production of bile acids in monolayers of rat hepatocytes [7]. Here we show that this stimulation can be completely blocked by the inhibition of cholesterol esterification in the cells by the Sandoz compound 58-035.

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2. MATERIALS AND METHODS

Rat hepatocyte monolayers were prepared by collagenase perfusion of livers obtained from rats that had been maintained on

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a diet containing 4% cholestyramine to induce bile acid synthesis. The culture conditions and the measurement of bile acids by radioimmunoassay were carried out as described [8]. The ACAT inhibitor, Sandoz compound 58-035, provided by Dr J. Heider (Sandoz Research Institute, E. Hanover, NJ), was added to cell monolayers dissolved in dimethyl sulphoxide (200 μg per 2 ml dish in 20 μl dimethyl sulphoxide [9]), and cholesterol esterification was measured by determining the incorporation of [^{3}H]oleic acid into cholesteryl esters [9].

Hepatocyte monolayers were prepared independently from 4 rats. The cells were pretreated with 58-035 or control solvent (dimethyl sulphoxide) as described [9]. Mevinolin (obtained from Mr A. Alberts, Merck Sharp & Dohme, Rahway, NJ) was added (5 μM) to remove the effect of endogenous cholesterol synthesis, making the cells more dependent on added lipoprotein. Incubations were carried out for 5 h and bile acids estimated in both cells and medium as described in [7]. In some of the incubations [^{3}H]oleic acid was added to estimate cholesterol esterification during the final 2 h of incubation [9]. HDL_2 was added to incubations at a concentration of 500 μg per dish, about that corresponding to the plasma concentration in rats [7,10]. The lipoprotein fraction was prepared by standard centrifugation techniques from rat plasma [11], the fraction of density 1.085–1.210 being used for the experiments. This fraction contained 251 μg protein, 160 μg cholesteryl ester and 20 μg free cholesterol in 100 μl of the preparation that was added to cells. The composition of this fraction is close to that reported in [12]. Analysis of this fraction by SDS-gel electrophoresis showed that apo-A-I was the main apolipoprotein present. There were also significant amounts of apo-E and apo-A-IV and trace amounts of apo-C-II and apo-D. No apo-B was present.

3. RESULTS AND DISCUSSION

The addition of HDL_2 to the cells in culture caused an increase in the synthesis of cholesteryl ester of 32% (table 1). Esterification could be inhibited by the addition of 58-035, as we had observed previously [9]. This increase, which is significant ($p < 0.01$), provides a scale over which the changes due to HDL_2 can be compared. The data in table 1 suggest that the cholesterol present in the HDL_2 was able to enter the cell and increase the supply of substrate to the pool for esterification, which is probably not saturated under normal conditions [4]. In other experiments we have shown that HDL_2 labelled with [^{14}C]cholesterol in either the unesterified or esterified form can enter rat hepatocytes in monolayer culture and provide substrate for bile acid synthesis (Ford, R.P., unpublished). In related work in vivo and in the perfused liver Stone et al. [13] have shown that cholesteryl ester-rich rat HDL can stimulate hepatic ACAT activity by 45%, which is a comparable increase to that found in our experiments

Table 1

Esterification of [^{3}H]oleate by rat hepatocyte monolayers in the presence of 58-035 and rat HDL_2

Additions	pmol cholesteryl ester formed
1. Control (5 μM mevinolin)	237 ± 13
2. 1 + 58-035	52 ± 6
3. 1 + HDL_2	312 ± 18
4. 2 + 58-035	69 ± 10

Cells (4 independent preparations) were incubated in the presence of additions for 5 h. During the last 2 h of this period potassium [^{3}H]oleate was added. At the end of the incubations the lipids were extracted and the radioactivity in the cholesteryl esters determined after separation on silica Bond-Elut columns. Data are the means ± SD of 4 independent preparations assayed in duplicate culture dishes

in hepatocytes. However, unlike the experiments in cell culture [7,10], no increase in bile salt synthesis was observed.

We have shown previously that 58-035 does not inhibit cholesterol 7α-hydroxylase [7] or HMG-CoA reductase [5]. Other enzymes of cholesterol metabolism in certain cell types (e.g. cholesteryl ester hydrolase in bovine adrenal cortical cells) are also unaffected by 58-035 [5]. In the present experiments cholesterol synthesis in the cultured hepatocytes was inhibited by mevinolin, so any indirect effects of 58-035 on HMG-CoA reductase activity over the 5 h incubation period would not be of significance. The basal rate of synthesis and secretion of bile acids by the cells in 5 h was 2.47 ± 0.46 nmol per 2×10^6 cells. The changes caused by the addition of HDL_2 and 58-035 in each of the 4 animals studied (and the corresponding mean values) are shown in fig.1. Comparison of the absolute values from which these differences were calculated by the two-tailed paired t-test showed that the changes were significant with $p < 0.05$.

The data in fig.1 show that, in addition to causing an increase in cholesterol esterification, the added HDL_2 caused a small but significant stimulation of bile acid synthesis. This observation was reported previously from our group [7] and has been confirmed under slightly different experimental conditions [10]. Thus cholesterol derived from the added lipoprotein was available as substrate for both ACAT and cholesterol 7α-hydroxylase, the initial step in bile acid synthesis [4].

Free cholesterol is required as the substrate for

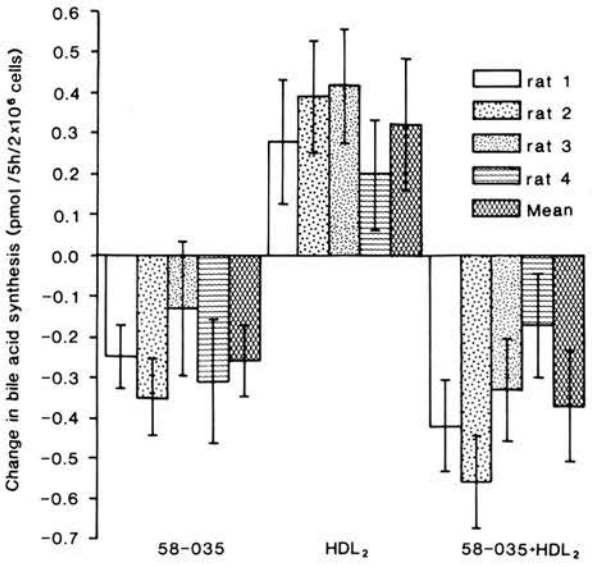

Fig.1. Change in bile acid synthesis by rat hepatocyte monolayers in the presence of mevinolin, 58-035 and rat HDL_2. Rat hepatocytes were incubated with the additions shown under the conditions described in section 2. The data shown are the changes in bile acid synthesis compared with the control value for preparations of cells from each of 4 rats. Error bars show the standard deviation of triplicate determinations of the secreted bile acids by radioimmunoassay [8].

cholesterol 7α-hydroxylase [14]. Therefore cholesterol entering the cell as cholesteryl ester must be hydrolysed at some stage before it enters the substrate pool for synthesis of bile acids. Free cholesterol entering the cell could in principle penetrate either directly to the substrate pool for bile acid synthesis or be esterified by ACAT. If the route followed in the cell by either free cholesterol or cholesteryl ester from the lipoprotein leads compulsorily through esterification by ACAT, this should be detectable by inhibition of this activity by 58-035. Fig.1 shows that the increase in bile acid synthesis due to the addition of HDL_2 is completely blocked by the inhibition of ACAT (table 1). Under the present conditions, therefore, the immediate fate of the cholesterol taken up by the cells from HDL_2 must be esterification.

This observation is in contrast to our recent experiments in which the fate of excess cholesterol newly synthesised from endogenous mevalonate was studied [9]. Here cholesterol esterification was stimulated about 2-fold by the addition of mevalonic acid and bile acid synthesis was also slightly stimulated. However, in this case 58-035 caused a significant increase in the secretion of bile acids, implying that excess cholesterol could be diverted to bile acid synthesis from the ACAT substrate pool.

It is clearly important in experiments with hepatocyte monolayers to attempt to correlate them with the in vivo situation. In the present experiments HDL_2, a specific homologous lipoprotein fraction which carries a substantial amount of cholesterol in the rat, can deliver cholesterol to the hepatocyte. This cholesterol follows a defined intracellular pathway, through ACAT to form the ester, followed by hydrolysis before it can become a substrate for bile acid synthesis. The limiting step in this system is probably the uptake of the lipoprotein by the cultured cells. This experimental design represents a close approximation in a hepatocyte monolayer culture to the situation in vivo. In our earlier experiments [9] the intracellular transport and regulatory mechanisms were probably saturated by the excess cholesterol synthesised from the added mevalonate. Uptake mechanisms were by-passed.

It is possible in cell culture experiments to devise conditions that will channel cholesterol from different sources in any desired way. Such control can be carried out with bovine adrenal cortical cells, which can use cholesterol from any of several sources as a substrate for synthesis of cortisol [4,6]. Studies such as those reported here show what pathways are possible for movement of cholesterol in the cultured cell. Other techniques would be required to demonstrate the operation of these pathways in an intact animal.

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