THE CATABOLISM OF CHOLESTEROL TO BILE SALTS BY RAT HEPATOCYTES MAINTAINED IN MONOLAYERS

by

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

TO MUM AND DAD FOR THEIR LOVE,

SUPPORT AND ENCOURAGEMENT

PROFESSOR GEORGE S. BOYD (1924 - 1983)

At the commencement of my Ph.D. I had the pleasure of working under the supervision of Professor George S. Boyd. His untimely death was a shock to both past and present members of the M.R.C. Steroid Metabolism Group. Although I worked with Professor Boyd for only 15 months I will always remember him for his kindness, sense of humour and infectious enthusiasm which will always remain a constant spur in my future career. I will always be extremely grateful for the many hours of helpful discussion and for the useful advice he was always so willing to supply.

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Publications

"The effect of a rat plasma high density lipoprotein subfraction on the synthesis of bile salts by rat hepatocyte monolayers".

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Biochem. Soc. Trans. (1984) in the press.

Abbreviations

BSA - bovine serum albumin.

chenodeoxycholic acid - 3α, 7α-dihydroxy - 5β-cholanic acid.

cholesterol - 5-cholesten - 3β-ol.

cholic acid - 3α,7α, 12α-trihydroxy - 5β-cholanic acid.

c.p.m. - counts per minute.

DMEM - Dulbecco's modified Eagle's medium.

EDTA - ethylenediaminotetraacetic acid.

h - hour (s).

HDL - high density lipoprotein.

HDL_2 - subfraction of HDL isolated by rate zonal ultracentrifugation.

7α-hydroxycholesterol - cholest - 5-ene - 3β, 7α-diol.

IU - international units.

LDL - low density lipoprotein.
LPL-lipoprotein lipase
min - minute (s).

α-muricholic acid - 3α, 6β, 7α-trihydroxy - 5β-cholanic acid.

β-muricholic acid - 3α 6β 7β-trihydroxy - 5β-cholanic acid.

NADH - reduced nicotinamide adenine dinucleotide.

NAD^+ - oxidised nicotinamide adenine dinucleotide.

PAGE - polyacrylamide gel electrophoresis.

r.p.m. - revolutions per minute.

sec - second (s).

SDS - sodium dodecyl sulphate.

t.l.c. - thin layer chromatography.

TEMED - N, N, N', N' - tetramethylethylene diamine.

U - units.

VLDL - very low density lipoprotein.

Enzymes

Acyl coenzyme A: cholesterol o-acyltransferase (ACAT) (E.C.2.3.1.26).

Chenodeoxycholic acid 6β-hydroxylase (E.C. not yet assigned).

Cholesterol esterase, cholesterol ester hydrolase or sterol ester acylhydrolase (CEH) (E.C.3.1.1.13).

Cholesterol 7α-hydroxylase or cholesterol 7α-monoxygenase (E.C.1.14.13.17).

Cholesterol oxidase or cholesterol : oxygen reductase (E.C.1.1.3.6.).

7α-hydroxycholest - 4-en - 3-one 12α-hydroxylase (E.C. not yet assigned).

Hydroxymethylglutaryl coenzyme A (HMG CoA) reductase or mevalonate : NADP oxidoreductase (coenzyme A acylating) (E.C.1.1.1.34).

Lactate dehydrogenase or L-lactate : NAD oxidoreductase (E.C. 1.1.1.27).

Lecithin : cholesterol acyltransferase (LCAT) (E.C.2.3.1.43).

ABSTRACT

The liver plays a central role in the metabolism of cholesterol being the major site at which lipoproteins are both assembled and degraded and the only organ where cholesterol can be degraded to bile salts. The synthesis of bile salts by the liver provides the major pathway for the removal of cholesterol from the body.

The results in this thesis describe the characterisation of a rat hepatocyte monolayer system, suitable for studying the synthesis of bile salts. The utilization of the cholesterol derived from a high density lipoprotein subfraction (HDL_2) for the synthesis of bile salts was also investigated.

Following the isolation of a viable cell preparation, hepatocytes were maintained in monolayers for up to 24h. During this period hepatocytes were shown to maintain their viability and to synthesise and secrete bile salts, as determined by radio-immunoassay of conjugated cholic, chenodeoxycholic and β-muricholic acids. The rate of synthesis of these bile salts by hepatocytes was increased by feeding rats cholestyramine for at least 5 days prior to the preparation of hepatocyte monolayers.

Incubation of hepatocyte monolayers with rat HDL_2 had no effect on the synthesis of the three bile acid conjugates measured when the cells were obtained from rats fed the pellet diet. However, when the experiment was repeated using hepatocytes obtained from rats fed cholestyramine, HDL_2 was found to increase the synthesis of the bile salts measured. This is the first report that a defined lipoprotein fraction can increase the synthesis of bile salts.

In an attempt to ascertain the reason for the increase in the synthesis of bile salts, hepatocytes isolated from cholestyramine-

fed rats were incubated in the presence of HDL_2 radiolabelled with either [4-^{14}C]cholesterol or [4-^{14}C]cholesteryl oleate. The degradation of the radiolabelled HDL_2-cholesterol to bile salts was subsequently determined. The results indicated that the increase in the synthesis of bile salts was due to the utilization of HDL_2-cholesteryl ester.

Finally, the effect of HDL_2 on the synthesis of cholesterol and the utilization of newly synthesised cholesterol for the synthesis of bile salts in hepatocyte monolayers was determined. The results showed that HDL_2 had no effect on either cholesterol synthesis or the utilization of newly synthesised cholesterol for the synthesis of bile salts.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Cholesterol is an essential component of all mammalian systems being a component of cell membranes and the precursor of bile acids, steroid hormones and some vitamins. The cholesterol that contributes to the total body pool is derived from two sources. Cholesterol can be synthesised de novo, primarily by the liver and small intestine, or it can be absorbed from the diet. It has long been established that high plasma cholesterol levels show a positive correlation with the incidence of ischaemic heart disease (Kannel et al., 1971; Carlson and Bottiger, 1972). It is therefore important that efficient regulatory mechanisms exist to prevent the accumulation of total body cholesterol. These findings prompted extensive research into possible mechanisms for the removal of cholesterol from the body. In particular there have been intensive investigations into the synthesis of bile acids and also the removal of plasma lipoproteins from the circulation. The liver plays a particularly important role in both of these processes being a major site for the uptake and degradation of plasma lipoproteins and also the only organ where cholesterol is degraded to bile salts. The synthesis of bile salts provides the major route by which cholesterol is degraded and eventually eliminated from the body.

The following account discusses the current knowledge of the complex metabolism of cholesterol whilst referring the reader to recent reviews that provide more detailed accounts of specialised areas.

1.2 The Plasma Lipoproteins

The plasma lipoproteins serve to transport lipid in a water soluble form from their sites of assembly to specific target sites. The plasma lipoproteins consist of a hydrophobic core of triglyceride and cholesteryl ester, surrounded by a hydrophilic coat of protein, phospholipid and free cholesterol. The binding of the inner lipid to the outer solubilising coat is non-covalent (primarily through hydrogen bonds and van der Waal's forces) and allows ready exchange of components between plasma lipoprotein classes and also between plasma and tissue lipoproteins.

There are four main classes of lipoproteins (Table 1.1) each floats within a different density range in the ultracentrifuge (Havel et al., 1955). These classes are chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). As well as their different physical characteristics the chemical composition of each of the lipoproteins is also quite distinct (Table 1.1). The conventional salt densities chosen for the isolation of the plasma lipoproteins are based on the density ranges of the human plasma lipoproteins. This does not necessarily apply to all species. In the rat the isolation of LDL in the conventional density range of 1.006 - 1.063 g ml^{-1} results in the contamination of LDL with apoprotein E (apo E). This has subsequently been shown to be due to the contamination of the LDL fraction with a subfraction of HDL rich in apo E (Innerarity et al., 1980). It is therefore important not only to characterise lipoproteins by their density but also by their apoprotein content. This is particularly important as the apoproteins not only act as detergents by solubilising the lipid

LIPOPROTEIN AND ABBREVIATION	DENSITY	DIAMETER A^{o}	ELECTRO-PHORETIC MOBILITY	TRIGLYCERIDES	PHOSPHOLIPID	CHOLESTEROL FREE	CHOLESTEROL ESTER	PROTEIN	SOURCE
Chylomicrons	<0.95	>700	ORIGIN	84	7	2	5	2	Carry dietary triglyceride from the intestine to non-hepatic tissues for utilization or storage
Very low density VLDL	0.95-1.006	250-700	β	50	18	7	12	8	Contain triglyceride made primarily in the liver
Low density LDL	1.006-1.063	180-250	pre-β	11	22	8	37	21	Derived from VLDL catabolism
High density HDL	1.063-1.210	40-100	α	5	25	5	15	50	Synthesised in the liver

Table 1.1 Physical and Chemical Characteristics of Human Plasma Lipoproteins

moiety but also act as recognition sites for cell surface receptors and as co-factors for the enzymes involved in lipoprotein metabolism (Table 1.2). The site of catabolism of the lipid component in each lipoprotein class is therefore dependent on the apoprotein content.

The ratio of the plasma lipoprotein classes varies according to diet (Callandra et al., 1977; Dolphin, 1981) and also from species to species (Mills and Taylaur, 1971; Johansson and Karlsson, 1976; Chapman, 1980; Oschry and Eisenberg, 1982). The differences between the plasma lipoprotein profiles in the rat and human are shown in Table 1.3.

The metabolism of the lipoproteins is extremely complex and each species have their own characteristics. The review that follows describes the metabolism of lipoproteins in the rat with occasional reference to the human system.

1.2.1 The origin and metabolism of the plasma lipoproteins

There is substantial evidence that plasma lipoproteins are synthesised only by the liver and small intestine and are secreted into the plasma as nascent particles (Havel, 1980). These particles on entry into the plasma compartment are subject to modification by enzymes, together with the physical transfer of components to yield the typical plasma lipoproteins.

(a) Chylomicrons

Chylomicrons are triglyceride-rich particles formed in the small intestine during the absorption of dietary lipids. The lipid and protein moieties are packaged into particles and secreted into the lymph where they are transported to the plasma compartment via the thoracic duct. On entry

APOPROTEIN	MOLECULAR WEIGHT	CHYLOMICRONS	LIPOPROTEIN CLASS			FUNCTION	ORIGIN
			VLDL	LDL	HDL		
			Mole %				
A-I	28K	3	-	-	43	Activation of LCAT Receptor mediated uptake of HDL?	INTESTINE/LIVER
A-II	17K	3	-	-	23	Activation of LPL?	INTESTINE/LIVER
A-IV	46K	2	-	-	-	?	INTESTINE
B-48	264K	0.8	-	-	-	?	INTESTINE
B-100	550K	trace	1	74	-	Uptake of LDL	LIVER
C-I	9.8K	20	8	-	18	Activation of LCAT	LIVER/INTESTINE
C-II	9.8K	22	20	-	2	Activation of LPL	LIVER/INTESTINE
C-III	8.7K	49	60	17	3	Inhibits uptake of apo E lipoproteins	LIVER
D	20K	trace	-	-	5	Transfer of cholesterol ester from HDL	LIVER
E	35K	trace	9	9	1	Uptake of lipoproteins	

Table 1.2 The Function, Origin and Distribution of Apoproteins

LIPOPROTEIN		TRIGLYCERIDES	PHOSPHOLIPID	CHOLESTEROL		PROTEIN	APOPROTEIN CONTENT
				FREE	ESTER		
		% COMPOSITION					
Very low density (VLDL)	RAT	75	11	4	4	6	B,E,C-I,C-II,C-III
	HUMAN	50	18	7	12	8	
Low Density (LDL)	RAT	18	21	9	27	25	B
	HUMAN	11	22	8	37	21	
High density (HDL)	RAT	2	28	4	33	33	A-I,A-II,C-I,C-II,C-III,E
	HUMAN	5	25	5	15	50	A-I,A-II,C-I,C-II,C-III (subclass possesses apo E)

Table 1.3 Comparison of the chemical composition of rat and human plasma lipoproteins

into the plasma the chylomicrons are subsequently catabolised (Green and Glickman, 1981). The major apoproteins 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 apo C family serves two major functions. Firstly, large amounts of apo C-III relative to apo E result in an inhibition of the uptake of the chylomicron particle by the liver allowing catabolism of dietary triglyceride (Redgrave, 1970). Secondly, apo C-II activates the enzyme lipoprotein lipase (LPL). LPL is bound to the capilliary endothelium and has been detected in most extrahepatic tissues. Hydrolysis of the core triglyceride by LPL results in the release of monoacylglycerol, diacylglycerol and fatty acids. The mono- and diacylglycerol are then either delivered to adipose tissue and muscle or further hydrolysed to fatty acids in the plasma. The fatty acids released by hydrolysis of triglycerides are scavenged by plasma albumin and are thence transported to the liver. As the core triglyceride is depleted the surface components of the chylomicron particle 'bud off' and form vesicles consisting of free cholesterol, phospholipid and the apoproteins A-I, A-IV and C. These components are then transferred to nascent HDL (Redgrave and Small, 1979; Tall et al., 1979; Tall and Small, 1980). Transfer of these components to HDL results in the formation of a chylomicron remnant particle which is relatively rich in cholesterol esters compared to the nascent chylomicron particle whilst the protein component mainly consists of apo B and apo E. The transfer of apo C to HDL no longer inhibits the uptake of the chylomicron remnant by the hepatic apo E receptor (Sherrill and Dietschy, 1978; Cooper et al., 1982; Kita et al., 1982).

This leads to an appreciable uptake of dietary cholesterol by the liver. It has further been shown that uptake of the chylomicron remnant particle results in the entry of the protein and cholesteryl ester moieties into the lysosomes where they are subsequently hydrolysed (Stein et al., 1969; Sherrill and Dietschy, 1978).

(b) Very low density lipoprotein (VLDL)

Very low density lipoproteins are synthesised by both the small intestine and the liver. The intestinal VLDL functions to transport dietary lipid into the plasma in a similar way to the chylomicrons. However, the chylomicrons are quantitatively more important than VLDL in the absorption of dietary lipid. On entry into the plasma apo A-I and apo A-IV are transferred from VLDL to HDL and apo E and apo C are transferred from HDL to VLDL (Green et al., 1979; Green and Glickman, 1981). This results in the VLDL assuming its normal apoprotein profile (Table 1.2). However, the major source of VLDL is the liver (Risser et al., 1978). The subsequent metabolism of the VLDL is quite similar to that of the chylomicrons. Uptake of VLDL is inhibited by apo C-III and LPL is activated by apo C-II. These apoproteins are situated on the surface of the VLDL particle. Hydrolysis of the core triglycerides results in the delivery of mono- and diacylgycerol to the peripheral tissues. Surface components are then transferred from VLDL to HDL in much the same way as occurs during the metabolism of chylomicrons (Tall and Small, 1980). The transfer of free cholesterol, phospholipid and apoproteins A-I, A-IV and C from VLDL to HDL results in the formation of VLDL remnants. In the rat,

VLDL remnants are rapidly removed from the circulation resulting in only 3% of the remnants being converted to LDL (Faergeman and Havel, 1975). In humans, VLDL remnants are not so efficiently removed resulting in the further metabolism of VLDL to yield LDL Cholesteryl esters are transferred from HDL to VLDL by means of a cholesteryl ester transfer protein (Barter and Lally, 1978, Barter et al., 1982). Further transfer of VLDL apoproteins to HDL results in the formation of a cholesterol-rich lipoprotein particle containing only apo B i.e. LDL. In rats the activity of the cholesteryl ester transfer protein is very weak. This results in rat VLDL having a lower cholesteryl ester content than human VLDL. Rat HDL has a correspondingly higher content of cholesteryl ester compared to human HDL (Table 1.3).

(c) Low density lipoprotein (LDL)

In chapter 1.2.1bthe catabolism of VLDL to LDL in plasma was described. LDL functions primarily to transport cholesterol to the extrahepatic tissues although the liver also provides a significant contribution to the catabolism of LDL (Pittman et al., 1979). Uptake of LDL by extrahepatic tissues is known to result in the inhibition of 3-hydroxy - 3 methylglutaryl coenzyme A reductase (HMG-Co A reductase) causing a reduction in the synthesis of cholesterol whilst also stimulating acyl Co A : cholesterol acyltransferase (ACAT) thereby stimulating esterification of the cholesterol taken up by the tissue (Goldstein et al., 1974; Brown et al., 1975). The uptake of LDL has been well characterised and can take place by both receptor and non-receptor pathways (see chapter 1.3).

(d) High density lipoprotein (HDL)

High density lipoprotein is synthesised by the liver and small intestine and enters the plasma compartment as a discoidal nascent particle. The metabolism of nascent HDL in the plasma results in the production of subfractions of HDL (Fig. 1.1). In the rat these subfractions are termed HDL_2 and HDL_1 (Oschry and Eisenberg, 1980) whilst in humans the subfractions are HDL_3 and HDL_2 (Patsch et al., 1974). The physical and chemical characteristics of rat and human HDL_2 are similar. The densities of the HDL subfractions decrease from $HDL_3 \rightarrow HDL_2 \rightarrow HDL_1$ as does their size. The chemical composition also varies, most importantly the relative content of cholesteryl ester and apo E increase from $HDL_3 \rightarrow HDL_2 \rightarrow HDL_1$.

The nascent HDL particle secreted into the plasma consists of a bilamellar disc of free cholesterol, phospholipid and apo E (Hamilton et al., 1976). Compared to normal plasma HDL the nascent particle is deficient in cholesteryl esters and apo A-I. However on entry into the plasma, cholesterol, phospholipid and apo A-I, apo A-IV and apo C are transferred from the surface of the chylomicrons and VLDL to HDL giving rise to the production of HDL_2 (HDL_3 in humans). The major apoprotein associated with the HDL_2 fraction is apo A-I and is the main activator of the enzyme lecithin cholesterol acyl transferase or LCAT (Fielding et al., 1972). The surface free cholesterol of the HDL_2 particle is esterified by the action of LCAT and involves the transfer of the fatty acid from the sn-2 carbon of lecithin

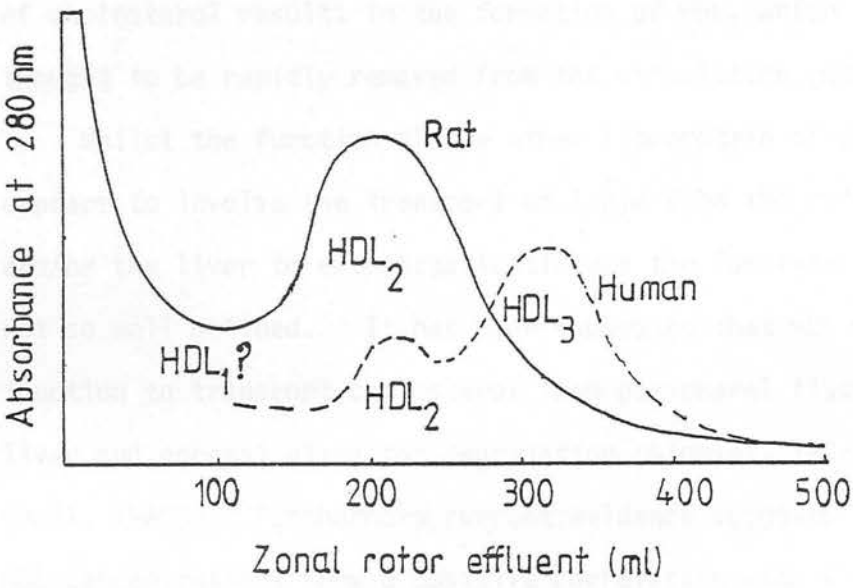


Fig. 1.1 Fractionation of HDL by rate zonal ultracentrifugation. Rat and human plasma lipoproteins were subjected to rate zonal ultracentrifugation as described by Oschry and Eisenberg (1982). Rat HDL was fractionated into HDL_1 and HDL_2 whilst human HDL was separated into HDL_2 and HDL_3.

to cholesterol. The lysolecithin so formed is scavenged by albumin. Esterification of the cholesterol is followed by movement of the non-polar ester into the core of the HDL_2 particle. Further esterification of the surface cholesterol would eventually lead to a depletion in the surface components of the HDL. However, transfer of cholesterol and phospholipid from chylomicrons and very low density lipoprotein replenishes the surface lipids of the HDL particle. Further esterification of cholesterol results in the formation of HDL_1 which is then thought to be rapidly removed from the circulation (Chapter 1.3).

Whilst the function of the other lipoprotein classes appears to involve the transport of lipid from the intestine and/or the liver to extrahepatic tissues the function of HDL is not so well defined. It has been suggested that HDL may function to transport cholesterol from peripheral tissues to the liver and adrenal gland for degradation (Glomset, 1968; Tall and Small, 1980). Furthermore current evidence suggests that plasma HDL concentrations show a positive correlation with a decrease in the risk of ischaemic heart disease (Miller and Miller, 1975). As was described earlier catabolism of HDL results in a depletion of surface free cholesterol and this cholesterol can be replaced by the transfer of surface components from chylomicrons and VLDL. However transfer of cholesterol from peripheral tissues to HDL may also occur (Glomset , 1968; Stein et al., 1977; Henriksson, 1979; Stoudemine, 1980). This may provide a mechanism by which cholesterol can be accepted from the peripheral tissues and transported to the liver for its subsequent catabolism (Fig. 1.2). In humans, the existence of the cholesteryl ester

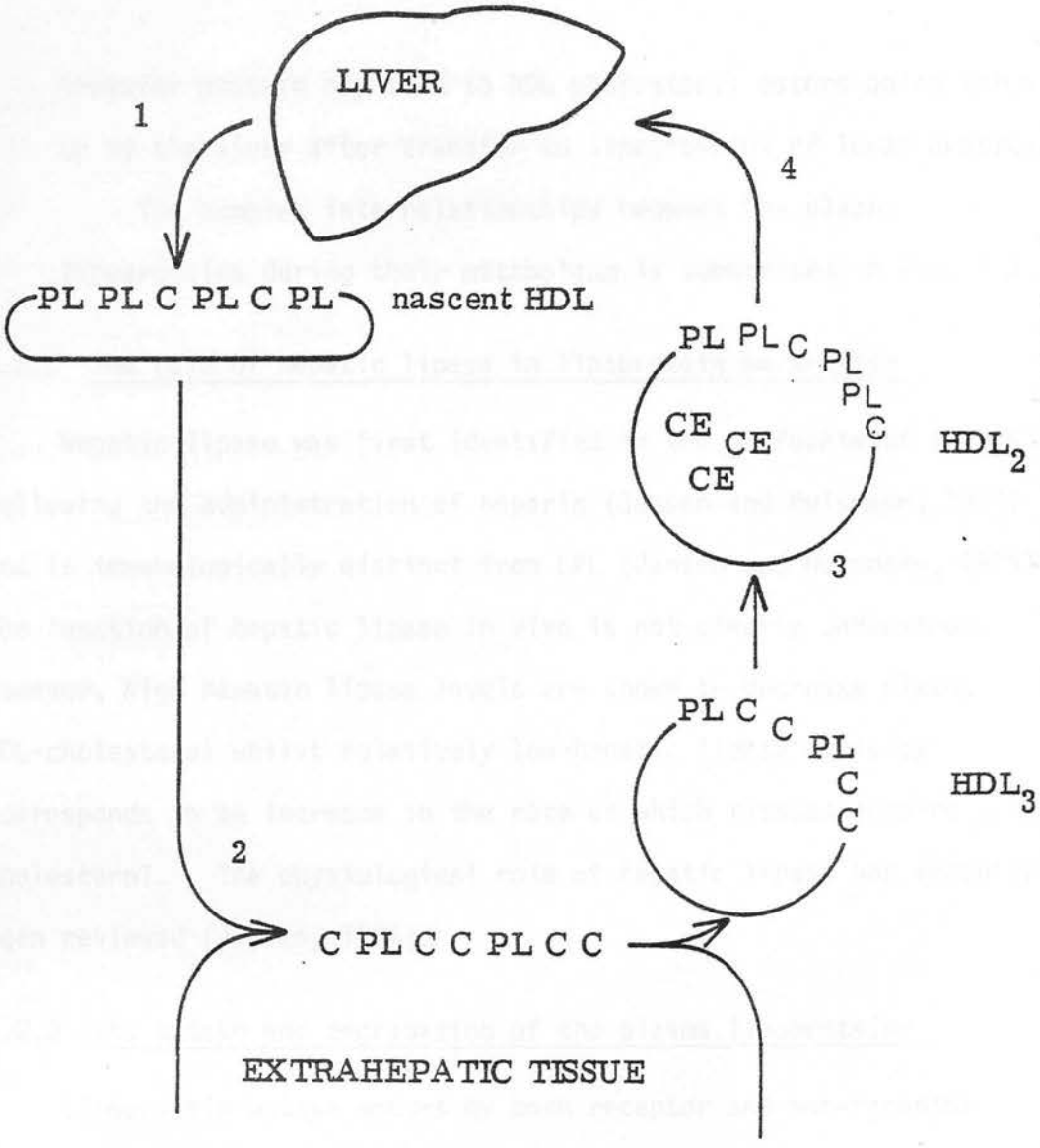


Fig. 1.2 Possible function of HDL in transporting cholesterol from extrahepatic tissue to the liver for its degradation.

1. Secretion of nascent HDL into the plasma by the liver.
2. Transfer of cholesterol from extrahepatic tissue to HDL.
3. Action of plasma lecithin : cholesterol acyl transferase (LCAT) on HDL_3 to give HDL_2.
4. Uptake of cholesterol ester rich, apo E rich HDL_2.

PL-phospholipid; C-cholesterol; CE-cholesteryl ester

transfer protein may lead to HDL cholesteryl esters being taken up by the liver after transfer to lipoproteins of lower density.

The complex interrelationships between the plasma lipoproteins during their metabolism is summarised in Fig. 1.3.

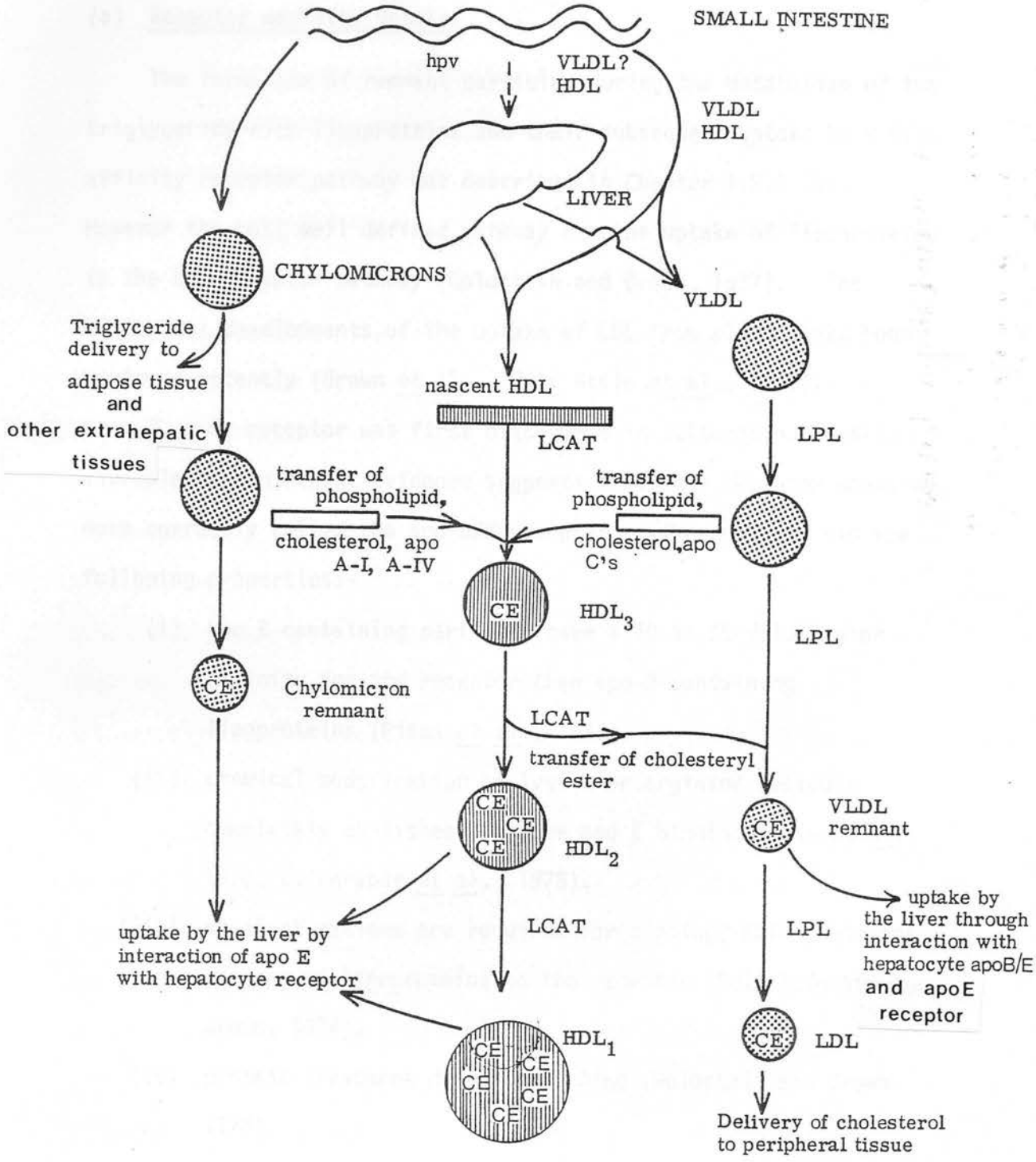
1.2.2 The role of hepatic lipase in lipoprotein metabolism

Hepatic lipase was first identified in the perfusate of livers following the administration of heparin (Jansen and Hulsmann, 1974) and is immunologically distinct from LPL (Jansen and Hulsmann, 1975). The function of hepatic lipase in vivo is not clearly understood. However, high hepatic lipase levels are known to decrease plasma HDL-cholesterol whilst relatively low-hepatic lipase activity corresponds to an increase in the rate at which tissues acquire cholesterol. The physiological role of hepatic lipase has recently been reviewed (Jansen, 1984).

1.2.3 The uptake and degradation of the plasma lipoproteins

Lipoprotein uptake occurs by both receptor and non-receptor pathways (Shepherd et al., 1979, Pittman et al., 1982b) and results in the degradation of the lipoprotein particle with the cholesterol portion remaining intact. In extrahepatic tissues the cholesterol is utilised for the synthesis of membrane whereas in the adrenal gland and gonads cholesterol also provides substrate for the synthesis of steroid hormones. In the liver cholesterol can be utilized for the assembly of lipoproteins, secreted into bile, or degraded to bile salts as well as providing cholesterol for cell membranes.

Fig. 1.3 Metabolic interrelationships of the plasma lipoproteins. The pathways shown are a general outline of the physical and chemical modifications of the plasma lipoproteins. Some of the important differences between human and rat plasma lipoproteins are as follows:- a) HDL is fractionated into HDL_3 and HDL_2 in humans and HDL_2 and HDL_1 in rats b) the cholesteryl ester transfer activity in rat plasma is not significant c) VLDL remnants are rapidly removed from rat plasma with only a small proportion being further metabolised to LDL. In humans appreciable quantities of VLDL remnants are converted to LDL.



CE-cholesteryl ester

(a) Receptor mediated uptake

The formation of remnant particles during the metabolism of the triglyceride-rich lipoproteins and their subsequent uptake by a high affinity receptor pathway was described in Chapter 1.2.1 (a). However the most well defined pathway for the uptake of lipoproteins is the LDL receptor pathway (Goldstein and Brown, 1977). The historical developments of the uptake of LDL from plasma have been reviewed recently (Brown et al., 1981; Attie et al., 1982).

The LDL receptor was first discovered in cultured human skin fibroblasts and recent evidence suggests that this receptor would be more correctly called the apo B/E receptor. The receptor has the following properties:-

(i) apo E containing particles have a 10 to 25 fold higher affinity for the receptor than apo B containing lipoproteins (Pitas et al., 1980).

(ii) chemical modification of lysine or arginine residues completely abolishes apo B or apo E binding (Basu et al., 1976; Weisgraber et al., 1978).

(iii) divalent cations are required for binding; EDTA abolishes binding of lipoproteins to the receptor (Goldstein and Brown, 1974).

(iv) pronase treatment destroys binding (Goldstein and Brown 1974).

(v) binding of LDL is abolished by treatment of cells with an antibody raised against the bovine LDL receptor (Beisiegel et al., 1981).

(vi) binding of an apo E rich HDL fraction isolated from the plasma of cholesterol fed dogs (apo E HDL_C) to the receptor, at saturation, was about a quarter of LDL

binding. This was explained by apo E binding to four LDL receptors whereas each LDL particle only binds to one receptor site (Pitas et al., 1980). Lipoproteins containing apo E have a high affinity for the receptor but the receptor has a low capacity for apo E containing lipoproteins.

Studies in vivo have enabled the quantitation of the uptake of LDL by the various tissues in the rat (Pittman et al., 1979; Carew et al., 1982; Pittman et al., 1982a).

Although the catabolism of LDL by the receptor mediated pathway is now well characterised, catabolism of HDL is not so well defined. However, the contribution of the liver to the uptake of HDL has been studied by a number of research groups. In vivo studies have shown that the liver is a major site for the catabolism of HDL but other tissues also play a significant role (Roheim et al., 1971; Stein et al., 1983). Furthermore, partial hepatectomy in rats, whilst not affecting the half-life of HDL-protein, increases the half-life of HDL-cholesteryl esters (Van Tol et al., 1978). However in studies using a recirculating system for perfusion of the liver it has been reported that the hepatic contribution to HDL catabolism was only 7% after 4h and it was therefore concluded that only a small fraction of HDL was degraded directly by the liver (Sigurdsson et al., 1979). In those studies using isolated hepatocytes it has been concluded that the liver does in fact play a major part in the catabolism of HDL (Nakai et al., 1976; Drevon et al., 1977; Ose et al., 1980; Ghiselli et al., 1981). If HDL does function to transport cholesterol from peripheral tissues for its ultimate degradation it would be expected that

the liver plays a significant role in the catabolism of HDL as the liver is the major organ which degrades cholesterol.

Further studies on the hepatic degradation of HDL are required. Most studies so far reported have used HDL isolated in a broad density range. The study by Ghiselli et al., (1981) indicated that uptake of HDL subfractions by hepatocyte monolayers could occur at different rates. The use of a defined HDL subfraction may lead to more conclusive evidence for the importance of the liver in the degradation of HDL. It is likely that hepatic uptake of the HDL subfractions would occur at rates which reflect their relative apo E content. Uptake of HDL_1 would therefore be expected to be greater than uptake of HDL_2. It is possible that the acceptance of cholesterol from the peripheral tissues by the HDL_2 subfraction followed by its catabolism to HDL_1 in the plasma, provides a mechanism whereby HDL could specifically deliver cholesterol to the liver.

At the present time the effect of HDL on hepatic cholesterol metabolism is not clear. Incubation of HDL with hepatocyte monolayers has been reported to stimulate the synthesis of cholesterol (Breslow et al., 1977). This was attributed to the ability of HDL to accept cholesterol from plasma membranes. However, it has since been reported that incubation of hepatocytes with LCAT-treated HDL inhibits the synthesis of cholesterol (Ray et al., 1980). The use of defined HDL subfractions may help to improve our understanding of the metabolic consequences of HDL uptake by cells.

Whilst receptor mediated uptake of LDL (and possibly HDL) occurs through recognition by the apo B/E receptor the uptake of chylomicron remnants occurs by a separate quite distinct receptor

called the apo E receptor. Although the existence of a saturable high affinity pathway for the uptake of chylomicron remnants was well established (Sherrill and Dietschy, 1978), the possibility of an alternative receptor site had only been suggested (Sherrill et al., 1980; Hui et al., 1981). Conclusive evidence was provided when uptake of chylomicron remnants was found to be unaffected in patients with familial hypercholesterolemia (FH) and in a Watanabe heritable hyperlipidemic (WHHL) rabbits (Brown et al., 1981; Kita et al., 1982). In FH patients and WHHL rabbits the number of apo B/E receptors expressed is either halved - heterozygous FH - or completely abolished - homozygous FH (Goldstein et al., 1983). In patients with homozygous FH the catabolism of LDL occurs entirely by the non-receptor pathway whilst uptake of chylomicron remnants is unaffected. This is due to expression of the apo E receptor being unaffected in these patients (Goldstein et al., 1983).

(b) Non-receptor mediated uptake

Although the receptor mediated pathway is important in the regulation of plasma cholesterol levels the non-receptor pathway also plays a significant role in lipoprotein catabolism. It has been suggested that approximately 33% of LDL catabolism normally occurs by means of the non-receptor pathway (Bilheimer et al., 1982) but in the WHHL rabbit the uptake of LDL by this pathway is significantly increased (Bilheimer et al., 1982; Pittman et al., 1982b). It has been suggested by Shepherd et al., (1979) that as much as 66% of LDL catabolism occurs by non-receptor mediated uptake. The mechanism involved in the non-receptor pathway is not fully understood and its importance in the catabolism of LDL has recently been reviewed (Attie et al., 1982).

1.3 The Synthesis of Cholesterol

The other major source of cholesterol to the total body pool is derived from de novo synthesis. In the rat the rate of sterol synthesis by the major organs has been investigated and it has been shown that the liver accounts for most of the total body sterol synthesis (Turley et al., 1981). The regulation of cholesterol synthesis in the rat is highly complex (Andersen and Dietschy, 1979a). Most importantly it has been shown that uptake of cholesterol in the form of lipoproteins influences the rate of hepatic cholesterol synthesis. Uptake of either chylomicron remnants or LDL was shown to suppress hepatic cholesterol synthesis whilst HDL had no effect (Andersen and Dietschy, 1977b). Furthermore, in the rat the rate of cholesterol synthesis is subject to wide variation depending on the stimuli (Andersen and Dietschy, 1977a, 1977b; Koelz et al., 1981).

The rates of cholesterol synthesis by the liver and its contribution to whole body cholesterol in a number of species has recently been reviewed (Turley and Dietschy, 1982).

1.4 The Bile Acids

Bile acids are formed from cholesterol in the liver via a series of reactions initiated by 7α-hydroxylation of cholesterol (Lindstedt, 1957; Bergstrom et al., 1958).

On leaving the biliary tract bile acids are almost entirely present in their conjugated form; conjugation occuring with either glycine or taurine, through amide linkage with the carboxyl group of the side chain. Within the intestinal lumen the bile acids facilitate formation of the micellar phase which appears to be obligatory for the absorption of cholesterol and plays an essential

role in the absorption of fat-soluble vitamins and the products of triglyceride hydrolysis during fat digestion. A decrease in bile salt concentration below the critical micellar concentration prevents the formation of the micellar phase, resulting in lipid malabsorption. The synthesis of bile acids also provides the major route for the degradation of cholesterol.

The synthesis of bile acids has been investigated in a number of mammalian species (Elliot and Hyde, 1971) but here particular attention will be paid to the rat.

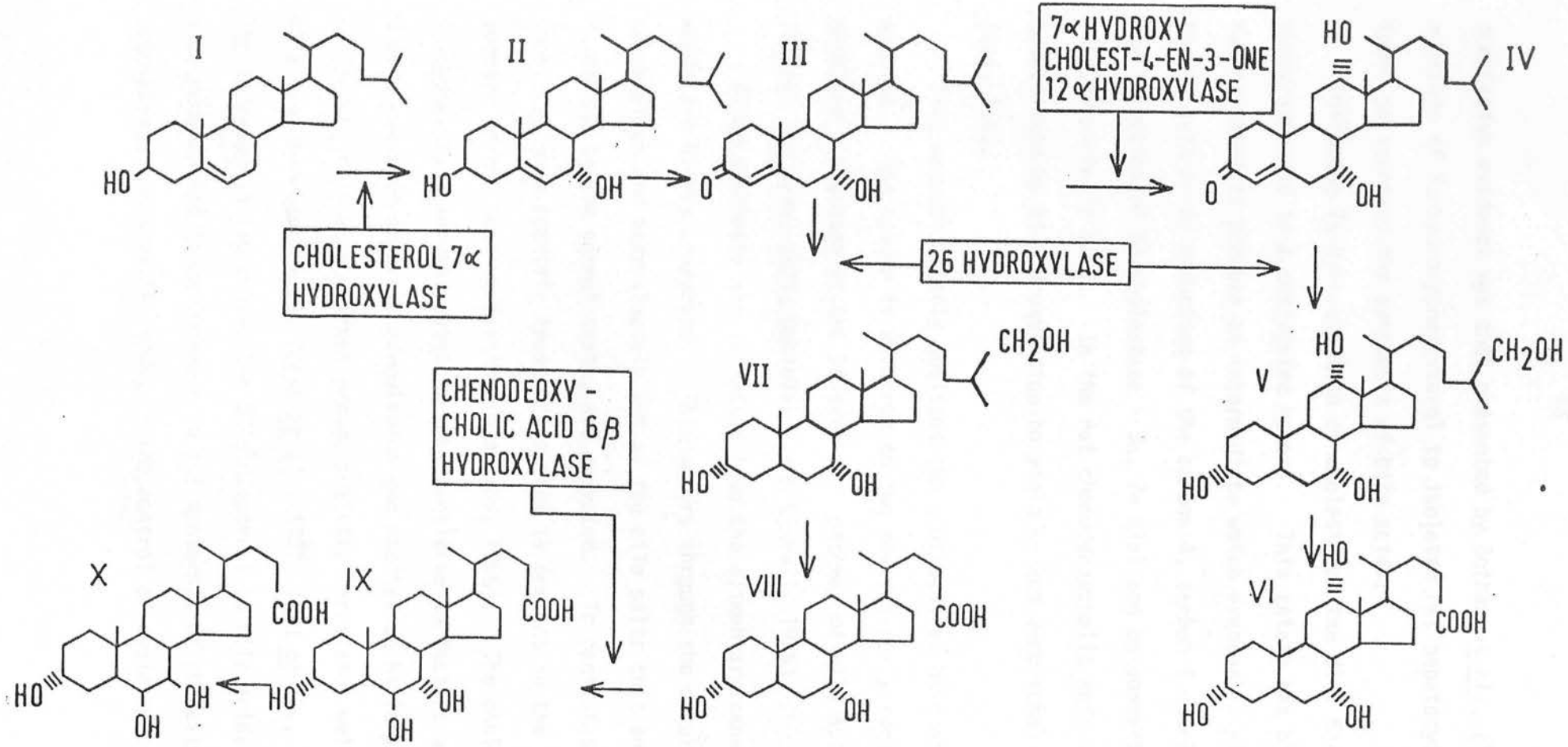
1.4.1 The synthesis of bile acids

Although the pathway of bile acid synthesis in mammalian species is essentially the same (Fig. 1.4) there are considerable variations in the end products produced (Elliot and Hyde, 1971). In most species the major bile acids produced are cholic and chenodeoxycholic acids but in rats chenodeoxycholic acid can be further metabolised to α- and β-muricholic acids (Mahawold et al., 1957; Matschiner et al., 1957; Voight et al., 1968).

The first step in the synthesis of all bile salts is the 7α-hydroxylation of cholesterol. The enzyme that catalyses this reaction, cholesterol 7α-hydroxylase, is located in the endoplasmic reticulum and requires a thermostable co-factor, oxygen and NADPH (Scholan and Boyd, 1968; Boyd et al., 1969). The reaction is known to be dependent on cytochrome P-450, the cytochrome P-450 species being a minor form (Danielsson and Wikrall, 1981). Considerable evidence has also been provided to show that this first step is also the rate limiting step in the overall conversion of cholesterol to bile salts (Danielsson et al., 1967, Boyd and Percy-Robb, 1971; Botham et al., 1980). The most

Fig. 1.4 The biosynthetic pathway of bile acids.

I - cholesterol; II - 7α-hydroxycholesterol; III - 7α-hydroxycholest-4en-3one; IV - 7α, 12α-dihydroxycholest-4en-3one; V - 5β-cholestane-3α, 7α, 12α, 26 tetrol; VI - cholic acid; VII - 5β-cholestane-3α, 7α-diol; VIII - chenodeoxycholic acid; IX - α-muricholic acid; X - β-muricholic acid.


I
II
III
IV
V
VI
VII
VIII
IX
X
HO
OH
O
COOH
CH2OH
CHOLESTEROL 7α HYDROXYLASE
7α HYDROXY CHOLEST-4-EN-3-ONE 12α HYDROXYLASE
26 HYDROXYLASE
CHENODEOXY - CHOLIC ACID 6β HYDROXYLASE

conclusive evidence was that presented by Botham et al., (1980) where addition of 7α-hydroxycholesterol to isolated rat hepatocytes was found to increase the synthesis of bile acids.

Following 7α-hydroxylation of cholesterol the diol formed is dehydrogenated to a conjugated ketone. This ketone can be 12α-hydroxylated to produce an intermediate which eventually yields cholic acid or by reduction of the carbon 4, carbon 5 double bond and formation of 5β-cholestane - 3α, 7α diol can be converted to chenodeoxycholic acid. In the rat chenodeoxycholic acid is further metabolised by 6β-hydroxylation to yield α- and β-muricholic acids (Fig. 1.4).

This account briefly outlines the synthesis of bile acids in the rat. The reader is referred to two reviews for a more detailed discussion of the biosynthetic pathway of bile acids (Elliot and Hyde, 1971; Danielsson and Sjovall, 1975).

After synthesis and secretion into the alimentary canal bile acids are highly conserved. This occurs through the enterohepatic circulation and approximately 95% of the bile salts that enter the duodenum, in the normal state, are recycled. To facilitate conservation a specific transport system is present in the terminal portion of the small intestine (Dietschy, 1968). The cholesterol 7α-hydroxylase enzyme system is very sensitive to factors which modify the enterohepatic circulation and studies on biliary drained rats have shown that enzyme activity correlates well with bile acid synthesis rates (Boyd et al., 1967; Boyd et al., 1969). The increase in the catabolism of cholesterol to bile acids is also accompanied by an increase in the synthesis of cholesterol. (Danielsson and Sjovall, 1975). The control of cholesterol 7α-

hydroxylase and hence bile acid production is not clearly understood. However, cholesterol 7α-hydroxylase activity exhibits diurnal rhythm in both fed a fasted animals, as do a number of other enzymes. It has been suggested that the fall and rise in the enzyme activity is due to alterations in the rates of enzyme synthesis and degradation (Schinike et al., 1965; Higgins et al., 1971, Mitropoulos et al., 1972). The effects of various drugs and hormones have also been studied (Boyd and Lawson, 1970; Abrams and Grundy, 1981; Cole et al., 1982). Removal of the gonads or thyroid gland has no effect on the stimulation of cholesterol 7α-hydroxylase by dietary cholestyramine, whereas removal of the adrenal glands were found to lower the response to cholestyramine. Further studies on the effect of the thyroid hormone, thyroxine have shown that the synthesis of chenodeoxycholic acid was stimulated so that equal amounts of chenodeoxycholic and cholic acids are formed (Abrams and Grundy, 1981). This is thought to be caused by low rates of 12α-hydroxylation (Mitropoulos et al., 1968). Recent evidence has also indicated that the synthesis of bile salts is stimulated by phosphorylation of cholesterol 7α -hydroxylase (Sanghvi et al., 1981). It was therefore suggested that the synthesis of bile acids may be under the control of cyclic AMP. It has since been shown in isolated hepatocytes that glucagon stimulates the synthesis of bile salts and that this effect is mediated through an increase in the intracellular cyclic AMP levels (Botham et al., 1983a, 1984).

The regulation of the synthesis of bile acids is complex and involves many factors. The possible mechanisms for the regulation of bile acid synthesis have recently been reviewed (Salen and Shefer, 1983; Botham, 1984).

1.4.2 The sources of substrate for the synthesis of bile acids

Bile acids can be synthesised from cholesterol obtained from either de novo synthesis or from uptake of the sterol from plasma in the form of lipoproteins. The contribution of each of these sources in providing substrate for the synthesis of bile acids is of great interest in determining the mechanisms of body cholesterol removal.

There are a number of reports which show that newly synthesised cholesterol can be degraded to bile acids and that this cholesterol is the preferred substrate for the synthesis of bile acids (Staple and Gurin, 1954; Mitropoulos et al., 1974; Norman and Norum, 1976; Bjorkhem and Lewenhaupt, 1979). Evidence for the compartmentation of cholesterol in rat liver microsomes has been presented by Balasubramaniam et al., (1973). It has also been shown that the preferred substrate for the synthesis of 7α-hydroxycholesterol is newly synthesised cholesterol (Bjorkhem and Danielsson, 1975). These findings have led to the suggestion that different pools exist within the hepatocyte which contribute different amounts of substrate to the synthesis of bile acids. However it is important to note that the term 'preferred substrate' actually means that newly synthesised cholesterol is converted to bile acids in preference to equilibrating with other cellular cholesterol and does not necessarily 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 synthesised are derived from newly synthesised cholesterol (Long et al., 1979). The remaining 75% is synthesised from pre-equilibrated cholesterol.

However, under certain conditions the contribution

of these two sources of substrate to the synthesis of bile acids can change. Introduction of a bile fistula was shown to increase the contribution of the newly synthesised sterol to the synthesis of bile acids, whilst the mass of the alternative substrate pool that was degraded to bile salts was unchanged (Long et al., 1979; Stange et al., 1984). In effect, introduction of a bile fistula interrupts the enterohepatic circulation and therefore results in the stimulation of bile acid synthesis. These results by Long et al., (1979) reflect the ability of the rat to alter the rate of cholesterol synthesis to meet the increase in the demand of substrate for the synthesis of bile acids (Koelz et al., 1982) without requiring an increase in the mass of plasma cholesterol degraded to bile salts. This data also supports the results by Koelz et al., (1982) that dietary cholestyramine does not increase the uptake of lipoproteins by the rat liver. (Cholestyramine interrupts the enterohepatic circulation by preventing reabsorbtion of bile acids from the small intestine). However, in rabbits and humans the administration of cholestyramine results in an increase in the uptake of plasma lipoproteins as well as an increase in the synthesis of cholesterol (Shepherd et al., 1980; Slater et al., 1980). More recently a similar effect on the contribution of newly synthesised cholesterol to the synthesis of bile acids was observed in isolated hepatocytes. The contribution of newly synthesised cholesterol to the synthesis of bile acids was found to increase with increasing incubation time (Kempen et al., 1983). Isolation and subsequent incubation of hepatocytes results in these hepatocytes being deprived of a supply of cholesterol from a lipoprotein source. This would therefore lead to the hepatocyte cholesterol pool that is

derived from plasma sources becoming depleted. As a result the contribution of newly synthesised cholesterol to the synthesis of bile acids will increase. Further evidence for this conclusion has been presented by Edwards (1975) who reported that cholesterol synthesis in isolated hepatocytes increases with increasing incubation time. These observations support the concept that rates cholesterol synthesis and bile acid synthesis are closely coupled (Danielsson and Sjovall, 1975).

A second approach in research to investigate the sources of substrate for the synthesis of bile acids has centred on the possibility of the preferential utilization of cholesterol from a particular lipoprotein fraction. There is now considerable evidence to suggest that HDL-cholesterol is used in preference to LDL-cholesterol for the synthesis of bile acids. This has been reported in humans (Halloran et al., 1978), squirrel monkeys (Portman et al., 1980) and rats (Miller et al., 1982). However, these studies have been beset with the problems of rapid exchange of sterol between lipoprotein classes. These studies have also indicated that the major source of substrate is HDL-free cholesterol as opposed to HDL-cholesteryl esters. In humans, only 10% of total biliary steroids were derived from lipoprotein cholesteryl ester (Schwartz et al., 1981). It was therefore concluded that cholesterol liberated from hydrolyzed esters undergoes recirculation into the free cholesterol pool rather than excretion into the bile. The preferential utilization of HDL-cholesterol for biliary steroids is consistent with the proposed greater metabolic activity of this sterol compared to the other lipoprotein classes.

Further studies into the investigation of the substrate pools for the synthesis of bile salts have also suggested that the individual bile acids themselves are 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., 1979) or cholic acid and β-muricholic acid (Ayaki et al., 1981; Kempen et al., 1983; Stange et al., 1984) were derived from separate substrate pools.

In summary, it is recognised that although newly synthesised cholesterol is the preferred substrate for the synthesis of bile acids, the major contribution of substrate is derived from a pre-equilibrated source. However, under certain conditions e.g. interruption of the enterohepatic circulation, the role of each of these sources of substrate can be reversed. The concept of separate substrate pools for the synthesis of the individual bile acids now appears to be well established. Further studies are required in this area as alteration in the contribution of these substrate pools to the synthesis of the individual bile acids may affect the lithogenicity of the bile and may therefore be important in gallstone disease.

1.5 Summary

Total body cholesterol is derived from either absorption from the diet or from de novo synthesis. The major organs that contribute to the source of newly synthesised cholesterol are the liver and small intestine. The cholesterol is transported between tissues as plasma lipoproteins. Each of the plasma lipoprotein

classes delivers lipid to a specific target site. It has been suggested that the function of one of the plasma lipoprotein classes is to transport cholesterol to the liver for subsequent degradation.

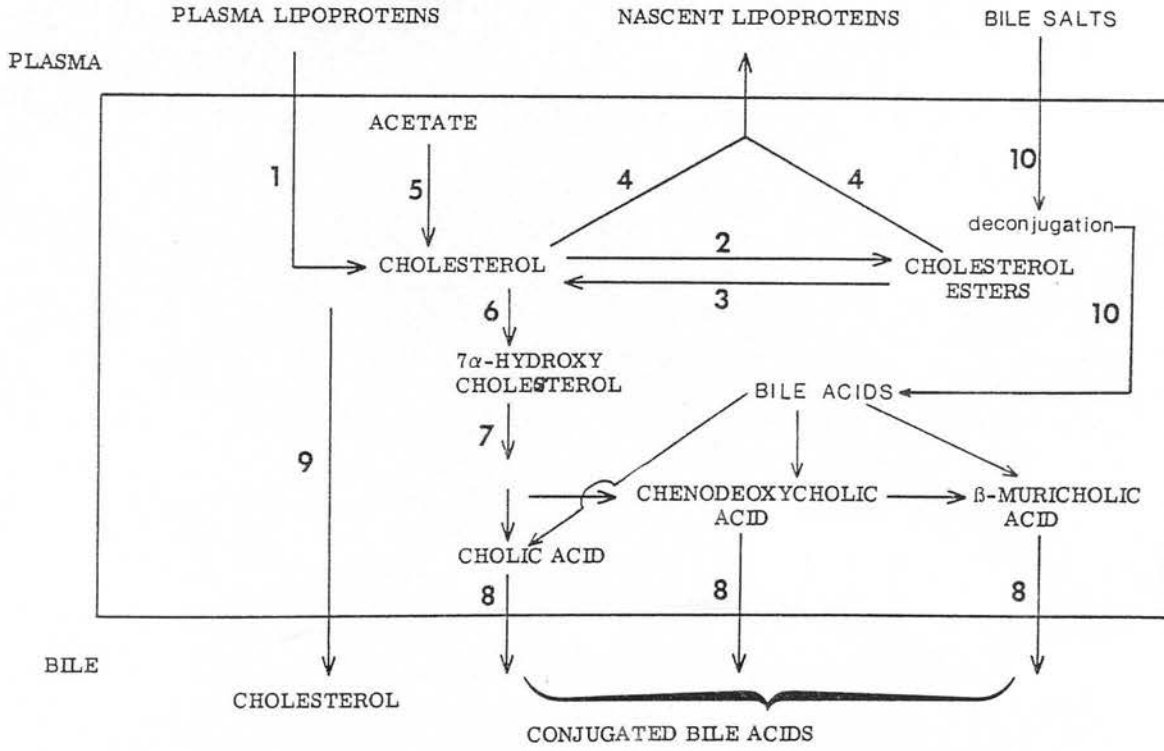
The major route for the degradation of cholesterol is via the synthesis of bile acids. Both newly synthesised cholesterol and plasma cholesterol serve as substrate for this pathway. It is likely that the contribution of each of these sources to the synthesis of bile acids varies from species to species. In the rat, an increase in the demand of cholesterol for the synthesis of bile acids as occurs during interruption of the enterohepatic circulation is entirely met by an increase in the synthesis of cholesterol. However, in the human and rabbit uptake of cholesterol from the plasma is also increased. The uptake and degradation of plasma cholesterol to bile acids is of great importance in the regulation of total body cholesterol.

HDL-cholesterol has been shown to be the preferred source of substrate for the synthesis of bile salts, from the plasma. This may explain why a high HDL/LDL ratio is beneficial to decreasing the risk of ischaemic heart disease. A summary of hepatocyte cholesterol metabolism is shown in Fig. 1.5.

1.6 Aims of this study

The study of the synthesis of bile acids and the source of substrate for this pathway has been confined to studies in vivo or shorter term studies in vitro (i.e. isolated hepatocytes in suspension and the perfused liver).

Fig. 1.5 Summary of cholesterol metabolism in the hepatocyte (1) uptake of lipoproteins from the plasma (2) esterification of cholesterol by ACAT (3) hydrolysis of cholesteryl esters by cytosolic cholesterol esterase (4) utilization of cholesterol and cholesteryl esters for the synthesis of lipoproteins (5) de novo synthesis of cholesterol (6) 7α-hydroxylation of cholesterol (7) synthesis of bile salts from 7α-hydroxycholesterol (8) conjugation and secretion of bile acids into the bile (9) secretion of biliary cholesterol (10) uptake of bile salts from the plasma which are recirculated in the enterohepatic circulation.



The major aims of this thesis are to develop a hepatocyte monolayer system which will enable longer term investigation into the synthesis of bile salts under more defined conditions than is allowed in vivo. In particular, the effect of plasma lipoproteins on the synthesis of bile salts will be investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Collagenase, gentamycin sulphate, insulin, bovine serum albumin (BSA) fraction V powder, ornithine hydrochloride, galactose, activated charcoal, reduced nicotinamide adenine dinucleotide, cholesterol, dipalmitoyl phosphatidylcholine, oleic acid, taurocholic acid, taurochenodeoxycholic acid, horseradish peroxidase, Coomassie brilliant blue G and PAGE Blue 83, were obtained from Sigma Chemical Company, Poole, Dorset, U.K. Bile acids were removed from BSA fraction V powder as described by Botham et al. (1980).

Trypan blue, Nonidet P.42, polyethylene glycol 10 000, glycerol trioleate, molecular sieve type 5A (calcium alumino-silicate), 2-mercaptoethanol, polyacrylamide, sodium lauryl sulphate (SDS) and dodeca - molybdophosphoric acid were from BDH Chemicals Ltd., Poole, Dorset, U.K.

Non-essential amino acids, foetal calf serum, newborn bovine serum and 50 mm plastic petri dishes were from Flow Laboratories Ltd., Irvine, Scotland, U.K. Foetal calf serum was heat inactivated by incubating for 30 min at 56^{o}C. Bile acids were removed from newborn bovine serum using activated charcoal as described by Botham et al. (1980).

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

Dri Film SC87 was obtained from Pierce Chemical Company, Rockford, Illinois, U.S.A.

2,5-diphenyl-oxazole (PPO) and 1,4-bis 5-phenyl oxazyl-2-benzene (POPOP), p-phenylhydroxyacetic acid, Triton-X-100, acrylamide, N N' methylene bis acrylamide, N, N, N', N' - tetramethylethylene diamine were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K.

Cholestyramine (Cuemid) was a product of Merck, Sharp and Dohme, West Point, Philadelphia, U.S.A.

Kieselgel 60H (silica gel)was from Merck, Darmstadt, Germany.

'Percoll' was obtained from Pharmacia (Great Britain) Ltd., Milton Keynes, Bucks, U.K.

Sodium pyruvate, cholesterol oxidase and cholesterol esterase were from Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K.

Nystatin was from E.R. Squibb and Sons Ltd., Hounslow, Middlesex, U.K.

Nylon Mesh (200 μm) was from Henry Simon Ltd., Stockport, Cheshire, U.K.

[1-^{14}C]acetic acid (57 mCi/mmol), L-[4,5-^{3}H]leucine (54 Ci/mmol), [4-^{14}C]cholesterol (> 50 mCi/mmol), [11,12(n)-^{3}H]chenodeoxycholic acid (104 Ci/mmol) and sodium [^{125}I]iodide (> 1375 Ci/mmol) 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 throughout. Animals were fed either a standard laboratory chow, a soft diet, consisting of 70% wholemeal flour, 25% skimmed milk powder and 5% yeast or the soft diet supplemented with 4% cholestyramine. Animals were allowed access to both food and water ad libitum.

2.3 Treatment of glassware used for preparation of hepatocytes

To prevent adhesion of hepatocytes to glassware all glass that came into contact with hepatocyte suspensions was treated as described below.

Glassware was washed with chromic acid (80 - 85ml saturated sodium chromate in 2.2l concentrated sulphuric acid) then thoroughly rinsed with double distilled water and allowed to dry. This was followed by treatment with Dri Film SC 87 (10% $^{v}/_{v}$ in petroleum ether). After allowing glassware to drain it was washed with hot water and dried in an oven at 60^{o}C for 2h.

2.4 Preparation and maintenance of isolated rat hepatocytes

Isolation of rat hepatocytes was carried out under sterile conditions. The perfusion apparatus and dissecting instruments were sterilised by exposure to ultra violet light for at least 12h. All other equipment was sterilised using a steam autoclave. Culture media and perfusates were sterilised by filtration through a 0.4 μm Millipore filter.

2.4.1 Solutions required for isolation of hepatocytes

Glucose- and calcium-free Locke's solution: sodium chloride (9g l^{-1}) and potassium chloride (0.24g l^{-1}) in double distilled water.

Glucose- and calcium-free Hank's solution: sodium chloride (8g l^{-1}), potassium chloride (0.4g l^{-1}), magnesium sulphate .7 H_2O (0.2g l^{-1}), potassium dihydrogen phosphate (0.06g l^{-1}) and phenol red (0.02% $^{w}/_{v}$) in 0.01M sodium phosphate buffer, pH 7.4. The phenol red was present to monitor the pH of the perfusate during the perfusion.

2.4.2 Preparation of isolated hepatocytes

Isolated hepatocytes were prepared by a method based on that described by Quirstorff et al. (1973) and Botham and Boyd (1979).

Female Wistar rats (200-250g) were anaesthetized with diethyl ether. A longitudinal incision in the abdomen was made and the alimentary canal displaced. The hepatic portal vein was canulated and the liver washed free of blood with calcium- and glucose-free Locke's solution. After blanching of the liver was complete, the perfusion was stopped and the liver excised. After placing the liver in the perfusion apparatus (Fig. 2.1) it was washed for a further two minutes prior to perfusion with glucose- and calcium-free Hank's solution supplemented with 0.5 mM calcium chloride and containing 0.5 - 1% ($^{w}/_{v}$) collagenase for 15 - 20 min. The amount of collagenase used depended on the commercial preparation obtained. The liver was finally perfused with calcium- and glucose-free Locke's solution containing 2 mM EDTA for 5 min.

All perfusates were oxygenated during the 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. Perfusion of the liver was performed at 30 - 35^{o}C.

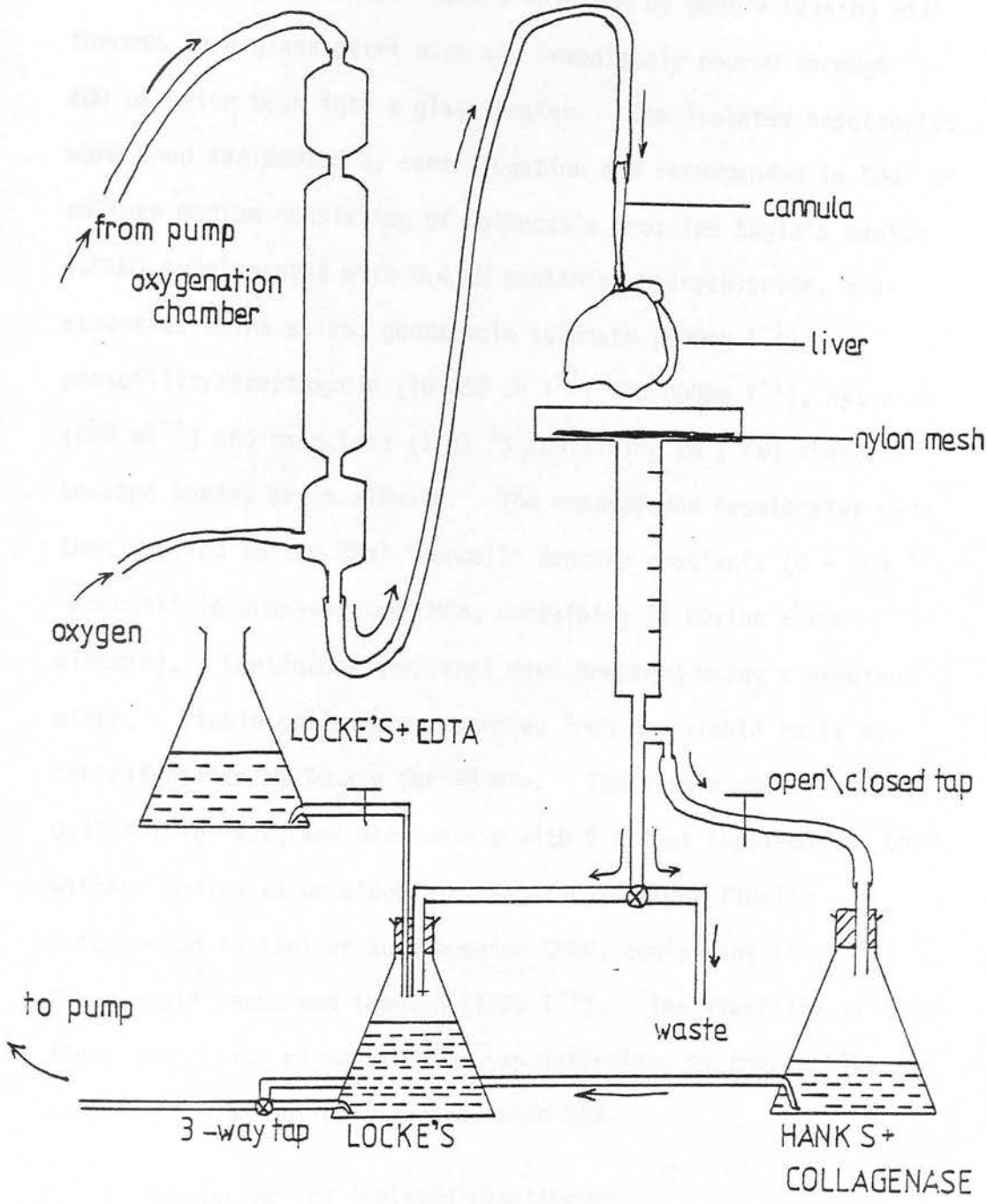


Fig. 2.1. Liver perfusion apparatus.

Following perfusion of the liver, cells were dispersed in calcium- and glucose-free Hank's solution by gentle teasing with forceps in a glass petri dish and immediately poured through 200 μm nylon mesh into a glass beaker. The isolated hepatocytes were then sedimented by centrifugation and resuspended in 10ml 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 (100mg l^{-1}), penicillin/streptomycin (10 000 IU l^{-1}; 10 000μg l^{-1}), nystatin (20U ml^{-1}) and galactose (1 gl^{-1}) containing 2% ($^{w}/_{v}$) charcoal treated bovine serum albumin. The resuspended hepatocytes were then applied to two 35ml 'Percoll' density gradients (0 - 70% $^{v}/_{v}$ 'Percoll' in supplemented DMEM, containing 2% bovine serum albumin). Continuous gradients were prepared using a gradient mixer. Viable cells were separated from non-viable cells by centrifugation at 50 x g for 15 min. The viable cells formed a pellet (Fig. 2.2) and were washed with 2 x 15ml supplemented DMEM, without bovine serum albumin. Hepatocytes were finally resuspended in 15ml of supplemented DMEM, containing 15% ($^{v}/_{v}$) foetal calf serum and insulin (100U l^{-1}). The viability of this final suspension of hepatocytes, as determined by trypan blue exclusion, was routinely greater than 95%.

2.4.3 Maintenance of isolated hepatocytes

Hepatocytes were diluted with supplemented DMEM containing 15% ($^{v}/_{v}$) foetal calf serum and insulin (100U l^{-1}), to give a final cell density of approximately 1 x 10^{6} cells ml^{-1}. Hepatocytes were then incubated for 2 - 3h in 50 mm plastic petri dishes (2.5 - 3.0 x 10^{6} cells $3ml^{-1}$ $dish^{-1}$) during which

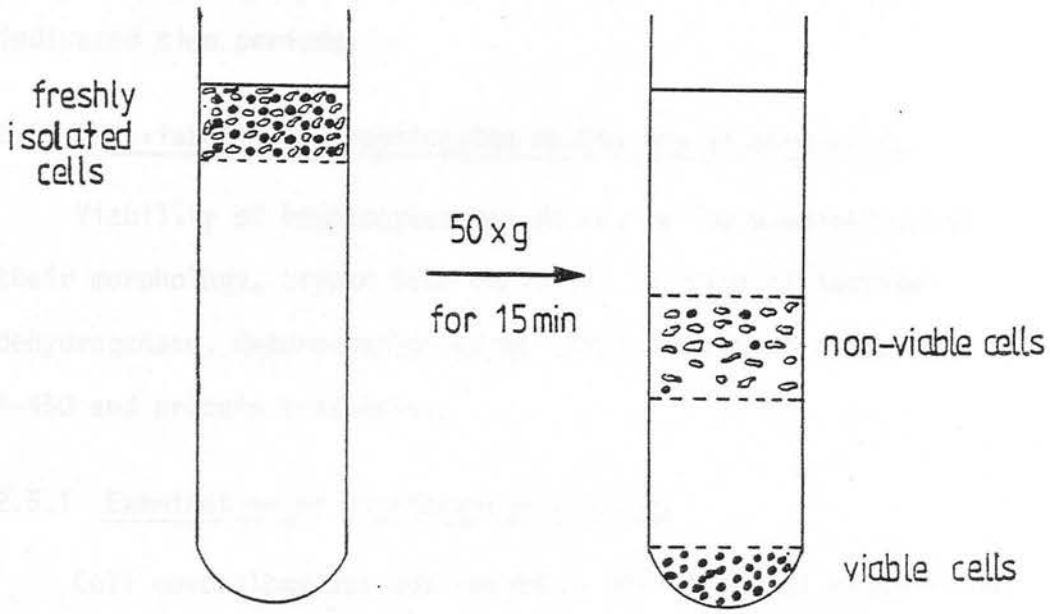


Fig. 2.2 Separation of non-viable hepatocytes from viable hepatocytes. The freshly isolated hepatocytes were applied to the top of a 0-70% ($^{v}/_{v}$) Percoll gradient and centrifuged at 50×g for 15 min. The viable cells formed a pellet at the bottom of the gradient.

time cell adhesion took place. The medium was then aspirated and replaced with 2ml of supplemented DMEM. This medium was designated the control medium. Additions to the control medium were made as indicated. Hepatocyte monolayers were incubated in an atmosphere of 5% carbon dioxide, 95% air at 37^{o}C for the indicated time periods.

2.5 The viability of hepatocytes maintained as monolayers

Viability of hepatocytes was determined by examination of their morphology, trypan blue exclusion, leakage of lactate dehydrogenase, determination of cellular content of cytochrome P-450 and protein synthesis.

2.5.1 Examination of hepatocyte morphology

Cell morphology was determined by microscopical examination. Viability and normal functioning of hepatocytes was dependent on cell adhesion and cell spreading.

Photomicrography of cells was performed with an Olympus 35mm camera back model PM-6 attached to an Olympus IMT model 213 light microscope. Exposure time was determined using an Olympus photomicrographic exposure meter model EMM-7.

2.5.2 Exclusion of trypan blue

Percentage viability of hepatocyte monolayers was determined by addition of 0.2ml 0.4% ($^{w}/_{v}$) trypan blue in 5ml of culture medium. Monolayers were incubated for 5 min at room temperature after which time the trypan blue was removed and percentage viability assessed. Viability was expressed as the percentage of cells excluding the dye.

2.5.3 Leakage of lactate dehydrogenase

Lactate dehydrogenase was determined spectrophotometrically with pyruvate and reduced nicotinamide adenine dinucleotide (NADH) according to Bergmeyer and Bernt (1974).

The equilibrium of reaction (1) lies far to the side of lactate and NAD

$$\text{Pyruvate} + \text{NADH} + H^+ \underset{\text{dehydrogenase}}{\overset{\text{Lactate}}{\rightleftharpoons}} \text{Lactate} + NAD^+ \quad \ldots \quad (1)$$

The activity of lactate dehydrogenase was therefore determined from the rate of oxidation of NADH, by measuring the decrease in absorbance at 340nm.

To determine the leakage of lactate dehydrogenase, the enzyme was assayed in both culture medium and the disrupted cells. Cells were disrupted by sonication of approximately 5mg of cellular protein in 2.5ml phosphate buffered saline, pH 7.4. An aliquot was assayed for lactate dehydrogenase activity. The culture medium was assayed directly. Percentage viability was expressed as the total units of lactate dehydrogenase in the medium divided by the total units in cells and medium x 100%.

2.5.4 Determination of cytochrome P-450 in rat hepatocyte monolayers

The intracellular content of cytochrome P-450 in hepatocyte monolayers was determined according to the method of Omura and Sato (1964) with the modifications described by Paine et al. (1979).

Hepatocytes were washed with 10ml of 0.15M sodium chloride and then scraped into 5 ml of ice cold 0.15M potassium chloride

in 50 mM tris buffer, pH 7.4. Samples were diluted to a concentration of 3 - 5mg cultured cell protein ml^{-1} and homogenised in 20% ($^{v}/v$) glycerol in 0.1M sodium phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.2% ($^{v}/v$) Nonidet P.42. Cytochrome P-450 was then determined by measuring the carbon monoxide difference spectra at 420nm using a Pye-Unicam SP8-200 UV/VIS spectrophotometer.

2.5.5 Determination of protein synthesis by rat hepatocyte monolayers

Protein synthesis was determined by incubation of hepatocytes in culture medium containing [^{3}H]leucine (ca. 2.5 µCi/ml). Incorporation of [^{3}H]leucine into cellular protein was determined according to the method of Dazord et al. (1977).

After the indicated time periods the medium was aspirated and 1ml of 0.4% ($^{w}/v$) sodium deoxycholate in 0.1M sodium hydroxide added. Cells were removed from the petri dish by scraping with a rubber policeman and transferred to a 75 x 13mm test tube. The cells were sonicated for 5 sec at 4^{o}C, 1ml of ice-cold 20% ($^{w}/v$) trichloracetic acid was added and the mixture centrifuged at 500 x g for 1 min to sediment precipitated protein. The precipitate was washed with 2 x 1ml of ice cold 10% ($^{w}/v$) trichloroacetic acid, followed by 1ml of ethanol.

The pellet obtained, was solubilized in 1ml of 0.4% ($^{w}/v$) sodium deoxycholate in 0.1M sodium hydroxide and incubated for 1h at 37^{o}C to hydrolyse amino-acyl transfer ribonucleic acids. An equal volume of ice cold 20% ($^{w}/v$) trichloroacetic acid was then added to precipitate the protein. After washing the precipitate with 2 x 1ml 10% ($^{w}/v$) trichloroacetic acid the

precipitate was solubilised by the addition of 1ml 0.4% ($^{w}/_{v}$) sodium deoxycholate in 0.1M sodium hydroxide. An aliquot was assayed for protein content and an aliquot used to determine radioactivity.

2.6 Determination of conjugated bile acids

Conjugated cholic, chenodeoxycholic and β-muricholic acids were determined by radioimmunoassay (Beckett et al. 1978; Beckett et al. 1979; Botham et al. 1983). Bile salt content of the medium was measured directly. Cellular bile salt content was determined after overnight extraction with 1M sodium hydroxide.[1] This treatment has been shown to extract 100% of conjugated bile acids from tissue samples (Strange, et al. 1979).

2.6.1 Preparation of immunogens

Immunogens in the following bile salt: BSA molar ratios, cholic acid (17:1), chenodeoxycholic acid (18:1) and β-muricholic acid (10:1), were prepared by the mixed anhydride method of Erlanger et al. (1957) as modified by Murphy et al. (1974).

2.6.2 Preparation of antiserum

1mg of each bile acid - BSA immunogen in 1ml of a 1:1 ($^{v}/_{v}$) emulsion of Freund's complete adjuvant in saline was injected subcutaneously into individual New Zealand white rabbits. A booster injection was given six weeks later using 1mg of each conjugate in Freund's incomplete adjuvant emulsion. Blood was obtained from the ear vein and the serum separated from blood cells by centrifugation. The antiserum was used from these animals without further treatment.

1. The total amount of the individual bile salts synthesised was determined by addition of the amount present in the medium to that in the cells.

2.6.3 Determination of the optimum dilution of the antiserum

The dilution of antiserum used in the assay of each bile acid was determined by incubation of ^{125}I-labelled bile acid-histamine with serial dilutions of the homologous antiserum, in the presence and absence of the highest concentration of standard used in each assay (Table 2.1). Binding of the ^{125}I-labelled ligand to the antiserum was determined as described in section 2.6.6. The antiserum dilutions which produced the largest differences in binding (Fig. 2.3) were subsequently used in each assay (section 2.6.6). This gave the most sensitive assay conditions (Beckett et al., 1978).

2.6.4 Preparation of bile acid-histamine conjugates

Bile acid-histamine conjugates were prepared as described by Beckett et al. (1979).

2.6.5 Iodination of bile acid-histamine conjugates

Iodination of conjugates was carried out by a method based on that described by Hunter et al. (1975).

50 nmol of each bile acid-histamine conjugate was mixed with 1 mCi of sodium [^{125}I]iodide and 20 µl of 20 mM chloramine T in 0.5M phosphate buffer, pH 7.9. The mixture was vortexed for 20 sec and the reaction stopped by the addition of 10 µl 0.15M sodium metabisulphite, in 0.5M phosphate buffer, pH 7.9. The radio-labelled bile acid-histamine conjugate was then extracted by the addition of 0.1ml water followed by 0.3ml ethyl acetate. The ethyl acetate layer was subsequently collected and water removed by the addition of anhydrous sodium sulphate. The ethyl acetate fraction was transferred to a silica gel thin layer chromatography

Conjugated bile acid	Cholic	Chenodeoxycholic	β-muricholic
		pmol	
	0	0	0
	1	0.25	0.5
	2	0.5	1
	5	1	2
	10	2	3
	20	5	5
		10	10
Minimum detection limits (pmol)	0.5	0.5	0.5

Table 2.1 Bile salt standards used for the radioimmunoassays

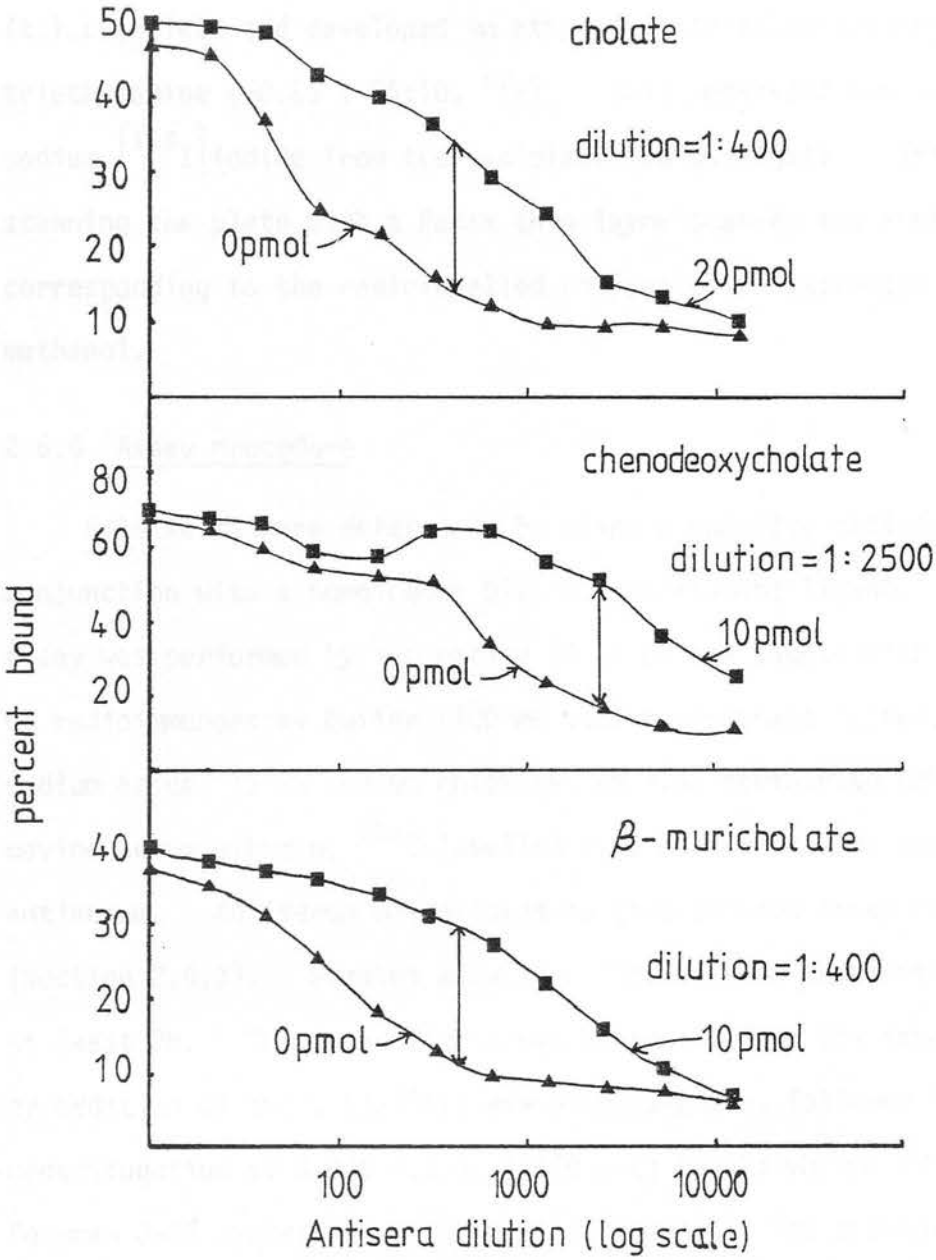


Fig. 2.3 Antibody dilution curves for the radioimmunoassay of bile salts. (A) conjugated cholic acid, (B) conjugated chenodeoxycholic acid, (C) conjugated β-muricholic acid. The dilution of antisera chosen was based on the largest difference between the highest concentration of standard and the control i.e. no standard.

(t.l.c.) plate and developed in ethyl acetate/toluene/ethanol/ triethylamine (50:25 : 25:10, $^{v}/_{v}$). This separated the unreacted sodium $[^{125}I]$iodide from the radiolabelled conjugate. After scanning the plate with a Panax thin layer scanner the area corresponding to the radiolabelled conjugate was extracted with methanol.

2.6.6 Assay procedure

Bile salts were determined by using a specific antiserum in conjunction with a homologous bile acid-histamine ligand. The assay was performed by incubating 10 µl of the sample with 200 µl of radioimmunoassay buffer (100 mM sodium phosphate buffer, 15 mM sodium azide, 15 mM sodium chloride, pH 7.4) containing newborn bovine serum albumin, ^{125}I-labelled bile acid-histamine and antiserum. Antiserum was diluted to give optimum assay conditions (section 2.6.3). Samples were incubated at room temperature for at least 2h. The bound ligand was separated from the free ligand by addition of 3ml 2.5% ($^{w}/_{v}$) ammonium sulphate, followed by centrifugation at 3,000 r.p.m. (2560 x g) for 15 min at $4^{o}C$ in a Beckman J-6B centrifuge using a JS-3.0 rotor. The precipitate was counted using a New England Enterprises NE 1600 Gamma counter. The bile salt content of samples was determined from standard curves. The concentration of standards used and the minimum detection limits are shown in Table 2.1.

2.7 Isolation of a rat plasma high density lipoprotein subfraction (HDL_2)

Rat HDL_2 was isolated essentially as described by Oschry and Eisenberg (1982).

Blood was obtained from female Wistar rats under ether anaesthesia by cardiac puncture, and collected in 10ml polypropylene tubes containing 40mg ethylenediaminetetraacetic acid (EDTA) to prevent clotting. Blood cells were separated from plasma by centrifugation at 2,000 r.p.m. (1140 x g) in a Beckman J-6-B centrifuge using a JS-3.0 rotor, at $4^{o}C$.

The rat plasma obtained was adjusted to a density of 1.23g ml^{-1} with solid sodium bromide and total plasma lipoproteins isolated by centrifugation at 35,000 r.p.m. (100000 x g) in a Beckman L8-5B ultracentrifuge using a 50.2 Ti rotor for 48h at $7^{o}C$. Using an MSE B XIV zonal rotor in an MSE Superspeed 75 ultracentrifuge the whole lipoprotein fraction obtained was subjected to rate zonal ultracentrifugation as described by Patsch et al. (1974) with the modifications of Shepherd et al. (1984).

The zonal rotor consists of a large cylindrical bowl, with the cavity divided into compartments by vanes attached to a central core. A removable rotating seal assembly was attached to the rotor at a speed of 3,500 r.p.m. Using a LKB Ultragrad gradient mixer and pump, a discontinuous gradient (Fig. 2.4) of density 1.00g ml^{-1} to 1.4g ml^{-1} was pumped in from the periphery, at a rate of 50ml min^{-1}. The solution of density 1.0g ml^{-1} consisted of 10 mM Tris-HCl 2 mM disodium EDTA, pH 7.6. The density of this solution was raised by the addition of solid sodium bromide to give a solution of density 1.4g ml^{-1}. The density of these solutions was checked with a hydrometer at $20^{o}C$.

After completion of the gradient the lightest density solution was present at the core and the heaviest density solution at the periphery of the rotor cavity. The whole lipoprotein fraction was then adjusted to a density of 1.4g ml^{-1}, using solid sodium bromide

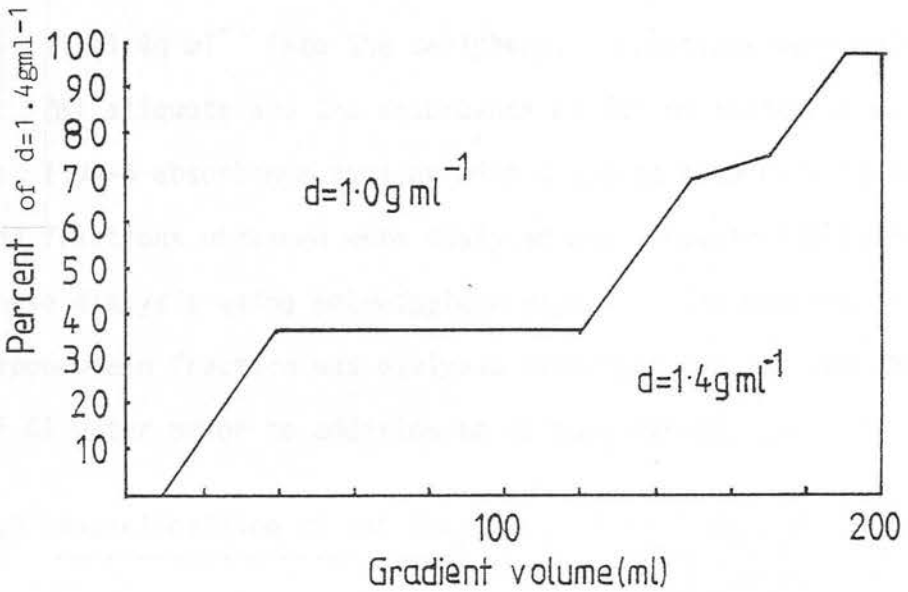


Fig. 2.4 Salt gradient for the isolation of HDL_2 by rate zonal ultracentrifugation.

and loaded at the periphery of the rotor. This was followed by a cushion of solution of density 1.4g ml^{-1} to ensure that all the sample was transferred to the rotor. The seal assembly was then removed and the rotor capped. The rotor chamber was sealed and brought down to vacuum. Lipoprotein subfractions were separated by centrifugation at 45,000 r.p.m. for 18h. The rotor was then decelerated to 3,500 r.p.m. The rotor cap was removed and the seal assembly attached to the rotor as before. The contents of the rotor were collected from the centre by pumping solution of density 1.4g ml^{-1} into the periphery. Fractions were collected in 12ml aliquots and the absorbance at 280 nm monitored using a model UA-5 absorbance monitor with a quartz flow cell (ISCO Ltd.). The fractions obtained were dialysed and concentrated by reverse phase dialysis using polyethylene glycol. The concentrated lipoprotein fraction was dialysed overnight against two changes of 4l water prior to addition to culture medium

2.8 Radiolabelling of rat HDL_2

Rat HDL_2 was labelled in either the free cholesterol or the cholesteryl ester moiety.

2.8.1 Labelling of HDL_2 with [4-^{14}C]cholesterol

25 μCi of [4-^{14}C]cholesterol was impregnated into Whatman No. 1 filter paper discs and incubated with isolated rat HDL_2, in saline, for 4h at 37°C. The filter paper discs were removed and the labelled rat HDL_2 added to culture medium prior to incubation with rat hepatocyte monolayers.

2.8.2 Labelling of HDL_2 with $[4\text{-}^{14}C]$cholesteryl oleate

Radiolabelling of HDL_2 cholesteryl ester was performed using a modification of the method described by Craig et al. (1982). $[4\text{-}^{14}C]$cholesteryl oleate was transferred from a lipid microemulsion to the lipoprotein fraction indicated by means of a cholesteryl ester transfer protein present in human plasma. Lipoproteins were first removed from human plasma by centrifugation at 35,000 r.p.m. (100,000 x g) in a Beckman L8-5B ultracentrifuge using a 50.2 Ti rotor for 48h after adjusting the density to 1.23g ml^{-1} by addition of solid potassium bromide. The lipoprotein-free plasma obtained was exhaustively dialysed against 0.1M sodium chloride and stored at $4^{o}C$ until required.

Method 1: A lipid microemulsion was prepared by injecting 0.21mg glycerol trioleate, 0.16mg cholesterol, 0.42mg dipalmitoyl phosphatidylcholine and approximately 10 µCi of $[4\text{-}^{14}C]$cholesteryl oleate in dry propan-2-ol, into a rapidly vortexing solution of 0.15M sodium chloride, containing 10 mM Tris-HCl, pH 7.4 and 0.3 mM EDTA. To this was added lipoprotein-free human plasma (final concentration, 35mg protein ml^{-1}) and total rat plasma lipoproteins (approximately 30mg lipoprotein protein). $[4\text{-}^{14}C]$cholesteryl oleate was transferred to lipoproteins by incubating for 24h at $37^{o}C$. The incubation mixture was then centrifuged at 35,000 r.p.m. (100,000 x g) in a Beckman L8-5B ultracentrifuge using a 50.2 Ti rotor for 90 min to remove the microemulsion. The microemulsion was aspirated from the tube and the infranatant adjusted to a density of 1.4g ml^{-1} with solid sodium bromide prior to isolation of HDL_2 by rate zonal ultracentrifugation.

Method 2: Plasma was obtained from rats, as described previously and a crude HDL fraction was prepared. The VLDL and LDL fractions were first removed by adjusting the density of the plasma to 1.06g ml^{-1} by addition of solid sodium bromide, followed by centrifugation at 35,000 r.p.m. (100,000 x g) for 18h, using a Beckman L8-5B ultracentrifuge and a 50.2 Ti rotor. HDL was then isolated after adjusting the density of the infranatant to 1.23g ml^{-1} with solid sodium bromide, followed by centrifugation for 48h using the same conditions as indicated above. The HDL fraction was then labelled with [4-^{14}C]cholesteryl ester. This was followed by isolation of HDL_2 from the incubation mixture using the procedure outlined in Method 1.

2.9 Synthesis of [4-^{14}C]cholesteryl oleate

30ml of toluene, dried by the addition of 2g molecular sieve 5A (calcium alumino-silicate), was added to 40mg oleic acid in a dry round bottomed flask. This was followed by the addition of 5 drops of thionyl chloride. The mixture was refluxed for 8h, allowed to cool and the solvent evaporated using a rotary evaporator. 25 μCi of [4-^{14}C]cholesterol was added to the resulting oleoyl chloride, followed by the addition of 10ml of dry toluene. The mixture was refluxed for 2h and allowed to cool prior to evaporation of the solvent. The residue was taken up in toluene and cholesterol and cholesteryl oleate separated by thin layer chromatography (t.l.c.) on silica gel t.l.c. plates. The chromatogram was developed in a solvent system consisting of petroleum ether/diethyl ether/acetic acid (95 : 5 : 1 $^{v}/_{v}$). After scanning of the t.l.c. plate with a thin-layer radioactive scanner (Panax equipment Ltd., Redhill, Surrey), the areas

corresponding to [4-^{14}C]cholesterol and [4-^{14}C]cholesteryl oleate were scraped into separate tubes. The radioisotopes were eluted from the silica gel by a two step procedure. Immediately after scraping the silica gel into tubes 15ml chloroform was added and allowed to stand for 30 min at 4^{o}C. The supernatant was collected and the silica gel extracted further with 2 x 10ml ethyl acetate. Following separation of the gel from the solvent by centrifugation the supernatants were pooled and the solvent evaporated. The residue was taken up in 2ml acetone. The percentage conversion of [4-^{14}C]cholesterol to [4-^{14}C]cholesteryl oleate was 86% and the total recovery of the radiolabel was greater than 90%. The purity of the cholesteryl oleate was determined by t.l.c. using petroleum ether/diethyl ether/acetic acid (95 : 5 : 1 $^{v}/_{v}$) and petroleum ether/diisopropyl ether/ acetic acid (30 : 70 : 2 $^{v}/_{v}$).

No obvious contaminant was observed.

2.10 Separation of cholesterol, cholesteryl ester and bile salts after incubation of hepatocyte monolayers with radioactive precursors

Following extraction of samples, cholesterol, cholesteryl ester and bile salts were separated by t.l.c. prior to the determination of radioactivity (section 2.11).

2.10.1 Preparation of samples for thin layer chromatography

Lipids and bile salts were extracted 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).

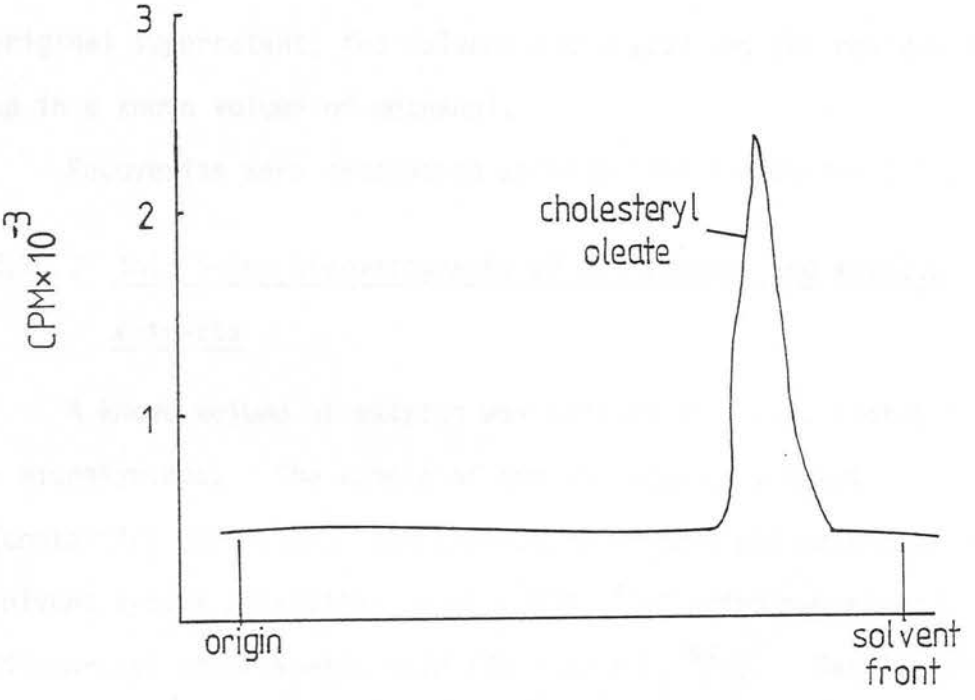


Fig. 2.5 Thin layer chromatogram of [4-^{14}C]cholesteryl oleate obtained by a thin-layer chromatogram scanner.

Cholesterol and cholesteryl ester partitioned in the non-aqueous phase. This was taken to dryness and the residue taken up in a known volume of chloroform.

The bile salts were extracted in the aqueous phase. Addition of sodium hydroxide prior to the extraction of samples improved the recovery of the bile salts. The protein precipitate present in the bile salt extract was removed by centrifugation and washed with 2 x 2ml methanol. The washes were pooled with the original supernatant, the solvent evaporated and the residue taken up in a known volume of methanol.

Recoveries were determined as indicated in Chapter 2.16.

2.10.2 Thin layer chromatography of non-aqueous and aqueous extracts

A known volume of extract was applied to t.l.c. plates using a microsyringe. The sample of the non-aqueous extract (containing cholesterol and cholesteryl ester) was developed in a solvent system consisting of 60 - 80% ($^{v}/_{v}$) petroleum ether/diisopropyl ether/acetic acid (30 : 70 : 2, $^{v}/_{v}$). Samples of the aqueous extract (containing bile salts) were developed in ethyl acetate/methanol/acetic acid/water (35 : 12 : 2 : 2, $^{v}/_{v}$).

Recovery of radioactivity from t.l.c. plates was improved by application of non-radioactive standards onto plates after application of samples. Identification of cholesterol, cholesteryl ester and bile salts was determined using non-radioactive standards adjacent to the samples being developed. The standards were visualised by spraying with 1% ($^{w}/_{v}$) phosphomolybdic acid in methanol, followed by incubating in an oven for 10 min at 70^{o}C.

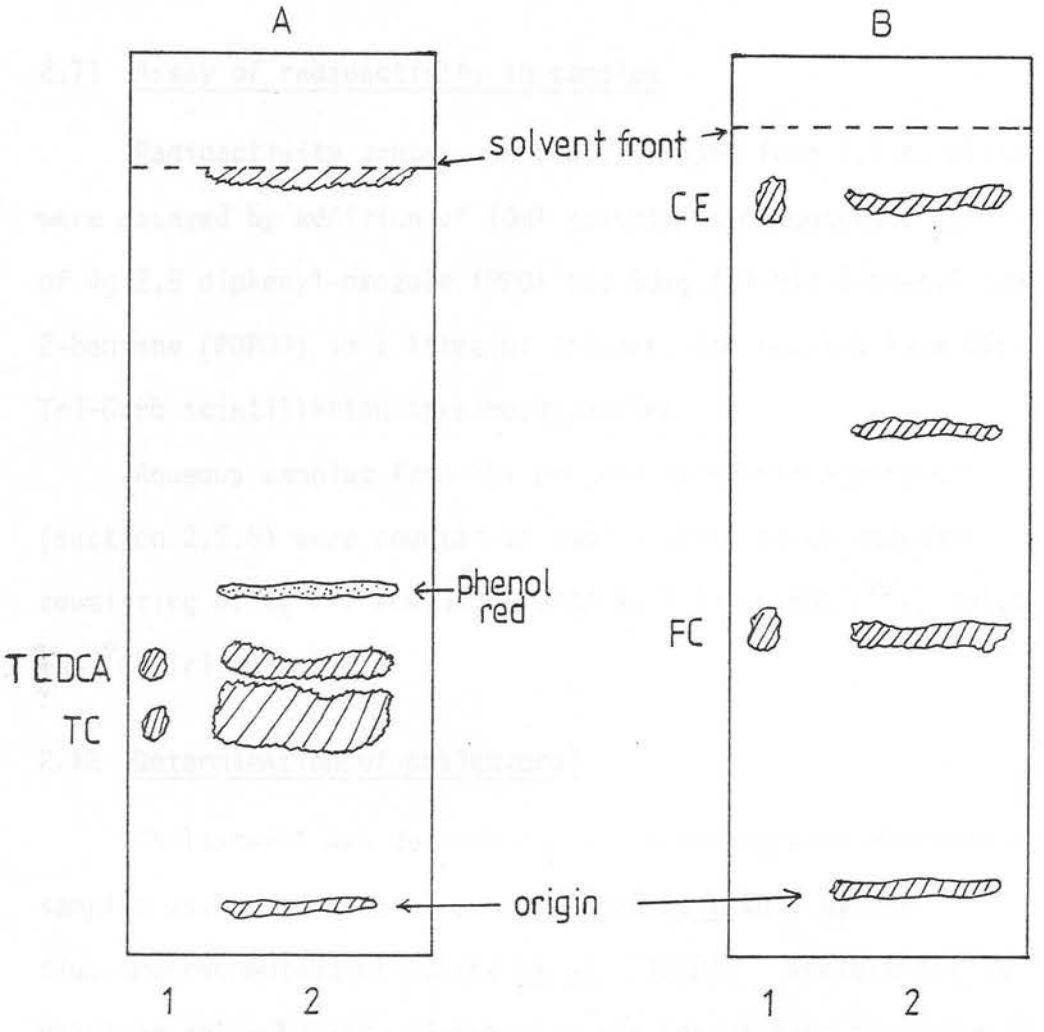


Fig. 2.6 Thin-layer chromatography of sample extracts (A) t.l.c. for determination of the radioactivity associated with bile salts. (1) standards, TC-taurocholate TCDCA - taurochenodeoxycholic (2) sample (B) t.l.c. for determination of the radioactivity associated with cholesterol and cholesteryl ester (1) standards FC-cholesterol; CE - cholesteryl ester (2) sample.

Cholesterol, cholesteryl ester and bile salts in the samples were visualised by iodine vapour (Fig. 2.6) and bands corresponding to the standards were scraped into phials and counted.

2.11 Assay of radioactivity in samples

Radioactivity present in bands scraped from t.l.c. plates were assayed by addition of 10ml scintillation cocktail consisting of 4g 2,5 diphenyl-oxazole (PPO) and 30mg 1,4-bis 5-phenyl oxazyl-2-benzene (POPOP) in 1 litre of toluene, and counted in a Packard Tri-Carb scintillation spectrophotometer.

Aqueous samples from the protein synthesis experiment (section 2.5.5) were counted in 10ml scintillation cocktail consisting of 4g PPO and 30mg POPOP in 1 litre 66% ($^{v}/_{v}$) toluene, 33%($^{v}/_{v}$)Triton-X-100.

2.12 Determination of cholesterol

Cholesterol was determined in the non-aqueous extracts of samples using cholesterol oxidase (*Nocardia* sp.) by the fluorimetric method of Gamble *et al*. (1978). Cholesterol is oxidised to 4-cholesten-3-one with the concomitant formation of hydrogen peroxide by the action of cholesterol oxidase. The hydrogen peroxide produced 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. Fluorescence was detected using a Perkin-Elmer 3000 fluorescence spectrophotometer.

For determination of total cholesterol, cholesteryl esters were hydrolyzed using cholesterol esterase from *Pseudomonas fluorescens* (Gamble *et al*. 1978).

2.13 Determination of protein

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

2.14 Sodium dodecyl sulphate polyacrylamide gel electrophoresis of HDL_2 apoproteins

Slab gel electrophoresis was performed according to the method of Laemmli (1970) with the modifications described by Douglas and Butow (1976).

2.14.1 Solutions required

A. Separating gel buffer: 1.5M Tris HCl, 8 mM EDTA, 0.4% ($^{w}/v$) SDS, 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 ay $4^{o}C$.

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

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

E. Concentrated sample buffer: 0.2M tris HCl buffer, pH 6.8, 8 mM EDTA, 8% ($^{w}/v$) SDS, 40% ($^{v}/v$) glycerol, 4% ($^{v}/v$) 2-mercaptoethanol, 0.0025% ($^{w}/v$) bromophenol blue. Samples were solubilised in the sample buffer and then diluted 4 times.

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

2.14.2 Preparation of separating gel

A gel cassette was assembled, which consisted of two 18 x 18 cm glass plates separated by two spacers smeared with high vacuum grease placed at the sides of the glass plates. The cassette was placed in a 'foil boat' and the bottom sealed by pouring 12ml of a solution consisting of 8.75ml B, 2.38 ml 2M Tris HCl, pH 8.8, 0.25 ml 10% ($^{w}/v$) SDS & 1.2ml water to which was added 250 µl 10% ($^{w}/v$) ammonium persulphate and 10 µl TEMED. TEMED was added to initiate polymerisation. After approximately 30 min 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 30ml of a separating gel mixture consisting of 7.5ml A, 15.0ml B, 5.0ml D, 2.2ml water and 0.3ml 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 approximately 1h (during which time the gel had set) the secondary butanol was removed and a stacking gel prepared. The stacking gel mixture consisted of 1.2ml B, 2.0ml C, 1.3ml D, 3.4ml water, 15 µl 10% ($^{w}/v$) ammonium persulphate and 5 µl TEMED. A toothed comb was inserted into the top of the cassette and the stacking gel allowed to set. When polymerisation was complete the toothed comb was carefully removed and samples placed into the sample holders.

2.14.3 Preparation of HDL_2 apoproteins prior to SDS-PAGE

HDL_2 apoproteins were delipidated essentially as described by Tocher and Boyd (1983).

10 volumes of ethanol/diethyl ether (1:4 $^{v}/v$) were added to the HDL_2 sample and left for 2h at -20^{o}C. After addition of

0.2 volumes of water the non-aqueous layer was removed. Lipid was further extracted by the addition of 5 ml cold ethanol/diethyl ether (1:4 $^{v}/_{v}$). The aqueous extract was freeze dried and protein solubilised in 6M urea containing 1 mM EDTA. Proteins were then precipitated by the addition of an equal volume of 20% ($^{w}/_{v}$) trichloroacetic acid. The precipitate obtained was washed with 10% ($^{w}/_{v}$) trichloroacetic acid, followed by ice cold acetone, before drying by vacuum desication. The apoproteins were finally solubilised in solution E and diluted 4 fold before being subjected to SDS - polyacrylamide slab electrophoresis. Approximately 100 µg of protein was applied to the gel.

Protein bands were visualised by staining with Coomassie PAGE Blue 83.

2.15 Statistical analysis

Results are expressed as ± standard deviation of the data presented. Statistical evaluation is by means of Student's t test for paired data.

2.16 Recoveries

Recovery of bile salts and of cholesterol in these experiments following extraction of samples was determined by the following method. After extraction of cells and medium the total radioactivity attributable to free cholesterol, cholesteryl ester and bile salts was determined. This enabled the determination of the total radioactivity recovered. Recovery of radioactivity was always greater than 95%. However as the bile salts only represented a small fraction of the total radioactivity recovery of bile salts was determined by radioimmunoassay of the extracts. The total bile salts detected were compared with the bile salts detected in the untreated sample. The total recovery of bile salts was always greater than 95%.

2.17 Counting efficiency

The efficiency of counting for ^{14}C disintegrations was 75% and for ^{3}H was 32%.

The efficiency of counting was determined using radioactive standards. No quenching effects were observed with silica gel scraped from t.l.c. plates. However it is important to note that no quench curves were obtained for addition of samples to the 'aqueous' scintillant (Chapter 2.11). This must be noted in the interpretation of those results obtained with the aqueous scintillant, namely those experiments where the incorporation of [^{3}H]leucine into protein was determined (Chapter 2.5.5.).

It is noted that results should be quoted in disintegrations per minute rather than counts per minute.

CHAPTER 3

THE VIABILITY OF RAT HEPATOCYTE MONOLAYERS

3.1 Introduction

In recent years isolated hepatocytes have been used to investigate an increasing number of problems in biochemical research. Investigations over short time periods of a few hours are facilitated by maintaining hepatocytes in suspension whilst longer term studies over 24h to a few days are achieved by maintenance of hepatocytes as monolayers. The use of isolated hepatocytes has proved to be particularly useful in studying certain aspects of cholesterol metabolism and has been the subject of a recent review (Forte, 1983).

In vitro techniques have distinct advantages over studies using the whole animal. A number of experiments on cells from the same animal can be performed whilst the use of a defined culture medium allows strict control of the cell's environment. Furthermore, when investigating the metabolism of lipoproteins, the use of isolated hepatocytes avoids the problem of in vivo exchange of the lipid and protein moieties between the different lipoprotein classes. This facilitates the investigation of the role of the liver in the catabolism of a defined lipoprotein class. However, there are some disadvantages in using in vitro techniques:-

a) some circulating hormones present in the plasma are known to influence the metabolism of cholesterol (Chapter 1). The culture medium used to maintain hepatocytes does not contain any of these hormones.

b) whilst the use of isolated hepatocytes enables the investigation of the catabolism of a defined lipoprotein

class, it is possible that transfer and exchange of lipids between lipoprotein classes provides a mechanism whereby these lipids are removed from the plasma (Chapter 1.2.1).

c) In vivo, hepatocytes are in contact with three compartments,

(i) the perisinusoidal space or space of Disse

(ii) the intercellular space

(iii) the bile canaliculi

The functional differentation of the hepatocyte plasma membrane has been demonstrated by studies on isolated plasma membrane fractions (Wisher and Evans, 1975). Bile acids are normally taken up on the sinusoidal surface and secreted on the bile canalicular side (Chapter 1.4). During isolation of hepatocytes this membrane polarity is lost. This may give rise to certain problems e.g. isolated hepatocytes synthesise and secrete bile salts into the medium from where they can be taken up (Iga and Klaassen, 1982). This could have an effect on the control mechanisms under study. It is important to be aware of these disadvantages, but providing they are taken into account much information can be obtained using viable isolated hepatocytes as an experimental system.

Before undertaking any detailed biochemical studies using rat hepatocytes a number of difficulties have to be considered. The liver is essentially a heterogeneous tissue. In normal rats hepatocytes account for 78% of the total liver volume with approximately 6% non-hepatocytes and 16% being intercellular spaces (Millward-Sadler and Jezequel, 1979). The procedure chosen for isolating hepatocytes is designed to obtain a homogeneous viable cell preparation. Many methods of hepatocyte isolation currently used involve perfusion of the liver with

collagenase followed by varying degrees of mechanical disruption. This procedure was first described in 1969 by Berry and Friend. However, modifications of this method vary widely (Quirstorff et al., 1973; Seglen, 1973; Berg and Inversen, 1976). This is partly due to the variation in the preparations of crude collagenase used in the cell isolation procedure. These enzyme preparations have varying powers of tissue dispersion and as a result the initial viability of hepatocytes obtained can vary from 40 - 95%.

The method of hepatocyte isolation used for this study (Chapter 2.4.2), included the use of a density gradient centrifugation step using 'Percoll'. ('Percoll' consists of colloidal silica particles of different size coated with polyvinylpyrrolidone which renders the particles non-toxic). This procedure facilitates the separation of non-viable cells from viable cells. Contamination from other cell types can also be avoided.

Following isolation of a viable preparation of hepatocytes it is essential that suitable methods for the maintenance of hepatocytes in monolayer culture are available. Just as the techniques for isolation of hepatocytes vary, so do the methods of hepatocyte maintenance. A variety of culture media ranging from basal salt solutions (Johansson et al., 1981; Paine et al., 1982) to more complex media (Williams et al., 1977; Davis et al., 1979; Horiuti et al., 1982; Paine et al., 1982) have been used.

The success of maintaining the viability of hepatocytes in monolayer culture is dependent on the adhesion of cells to a

suitable culture surface. Once adhesion has taken place the medium is removed and replaced with fresh medium prior to undertaking the desired experiments.

The experiments described in this thesis were all performed on hepatocytes maintained in monolayer culture. The results presented in this chapter illustrates the complexity of maintaining hepatocytes in monolayers and provide evidence of their biochemical integrity over the experimental periods used. The methods chosen for determination of hepatocyte viability are only a selection of the many criteria available (Crisp and Pogson, 1972; Baur et al. 1975).

3.2 Viability of freshly isolated hepatocytes

Initially rat hepatocytes were obtained in excellent yield ($1.2 - 3.4 \times 10^8$ cells per liver) and on average had a viability of 70% as assessed by exclusion of trypan blue. If viability was less than 65% the preparation was discarded. Following 'Percoll' density gradient centrifugation the viability of hepatocytes was routinely greater than 95%. The yield of hepatocytes decreased to $0.8 - 1.9 \times 10^8$ cells per liver.

3.3 Adhesion of hepatocytes to culture surfaces

Hepatocytes were maintained in monolayers as described in Chapter 2.4.3. Adhesion of hepatocytes to culture surfaces was achieved by incubating cells in culture medium supplemented with foetal calf serum and insulin. Donor calf serum, adult rat serum and foetal calf serum can all be used to promote cell adhesion. However, the efficiency of attachment of the

hepatocytes varies with the different sera used. Adhesion of cells to Petri dishes was found to be most efficient in DMEM supplemented with 15% foetal calf serum and insulin (results not shown). Incubation of cells for 2 - 3h was sufficient to allow adhesion of cells to occur. Following the attachment of the hepatocytes to the culture surface, the medium containing foetal calf serum and insulin was removed and hepatocytes were maintained in DMEM containing additions when required.

The viability of hepatocyte monolayers was then assessed prior to investigation of the major aims of the project (Chapters 4 - 6).

3.4 The viability of hepatocytes maintained in monolayers

Viability of hepatocytes was assessed by:-

a) examination of their morphology by light microscopy,
b) exclusion of trypan blue,
c) leakage of lactate dehydrogenase,
d) the ability to synthesise proteins,
e) the cellular content of cytochrome P-450.

3.4.1 Morphology of hepatocytes

Hepatocyte morphology was examined immediately after suspension in culture medium containing foetal calf serum and insulin. Initially hepatocytes were spherical and occured as either single cells or in aggregates of 4 or 5 (Fig. 3.1a). Following the adhesion of hepatocytes to the culture surfaces their morphology was again examined at 3h and 24h. After 3h cells had begun to flatten and reassociate (Fig. 3.1b). By 24h, hepatocytes had formed a nearly continuous monolayer (Fig. 3.1 c-f). Close

Fig. 3.1 Morphology of rat hepatocytes. Morphology was assessed as described in the text. Photographs were taken as described in Chapter 2.5.1. (a) freshly isolated hepatocytes; magnification x 100. (b) hepatocytes 3h after cell adhesion had taken place (approximately 5h after Fig. 3.1a); magnification x 200. (c-f) hepatocytes 24h after adhesion had taken place; (c) magnification x 10, (d) magnification x 100, (e) magnification x 200, (f) magnification x 400. Figs. 3.1 a-c and e were taken in bright field. Figs. 3.1d and 3.1e were taken under phase contrast.

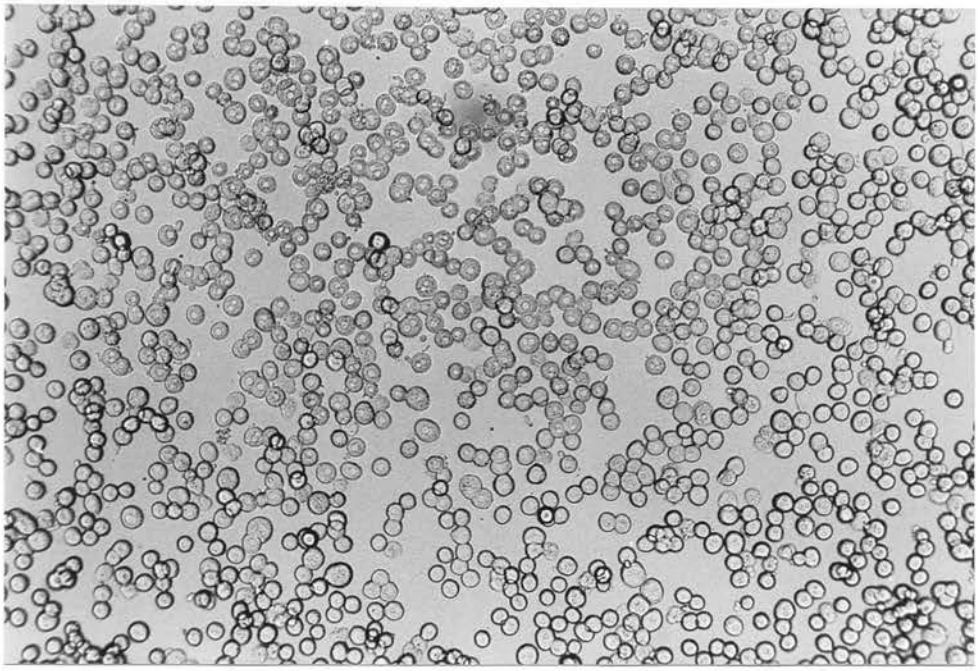


Fig. 3.1a

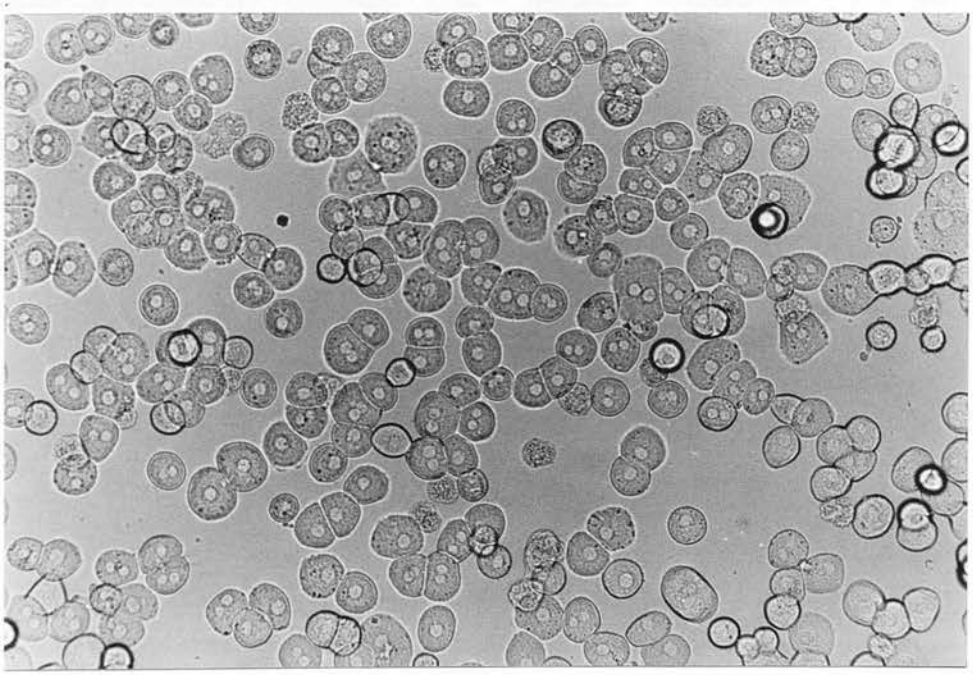


Fig. 3.1b

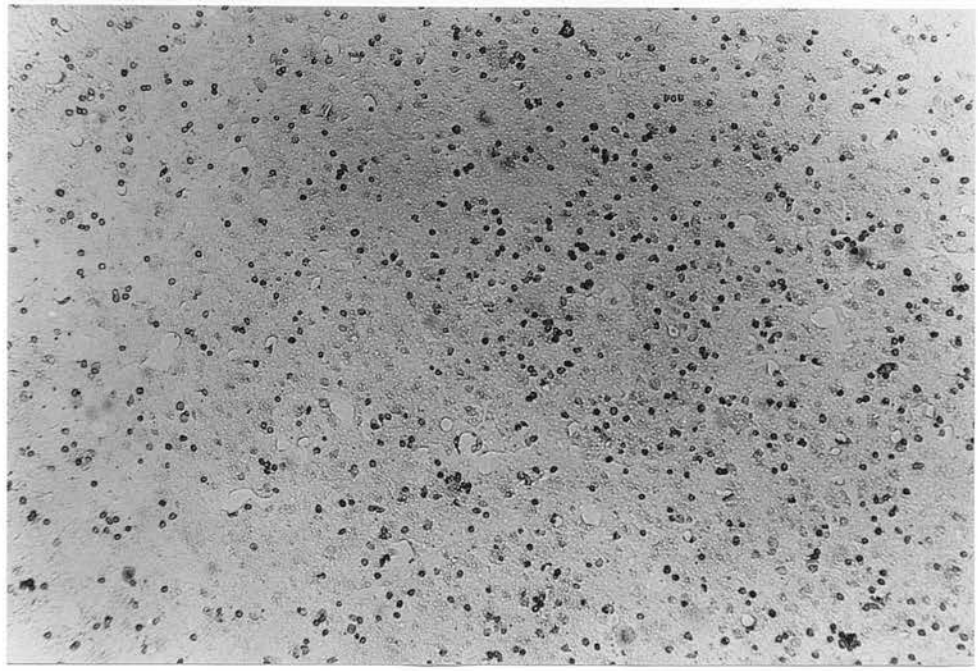


Fig. 3.1c

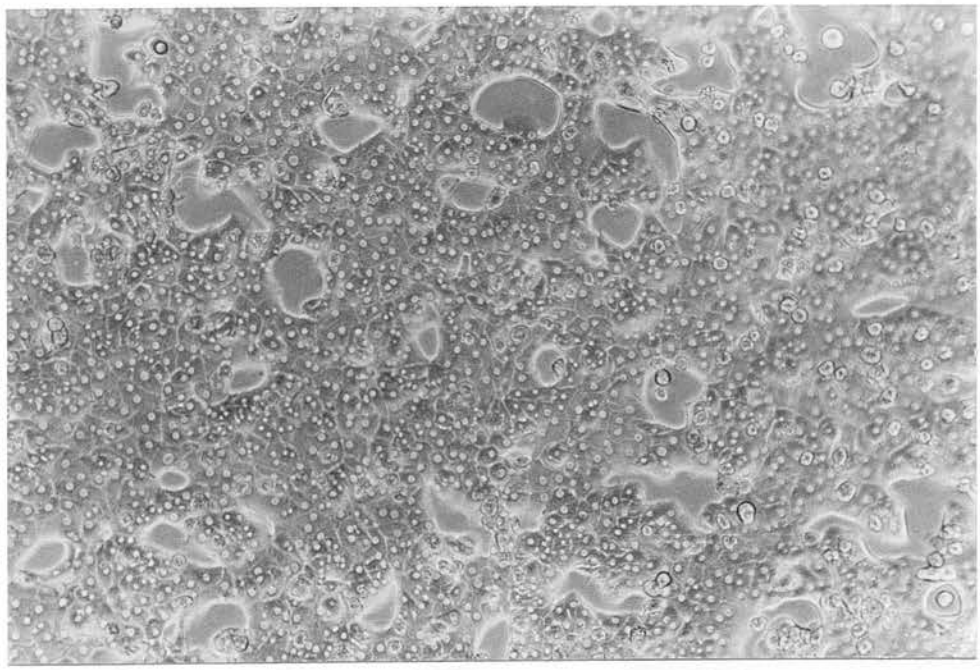


Fig. 3.1d

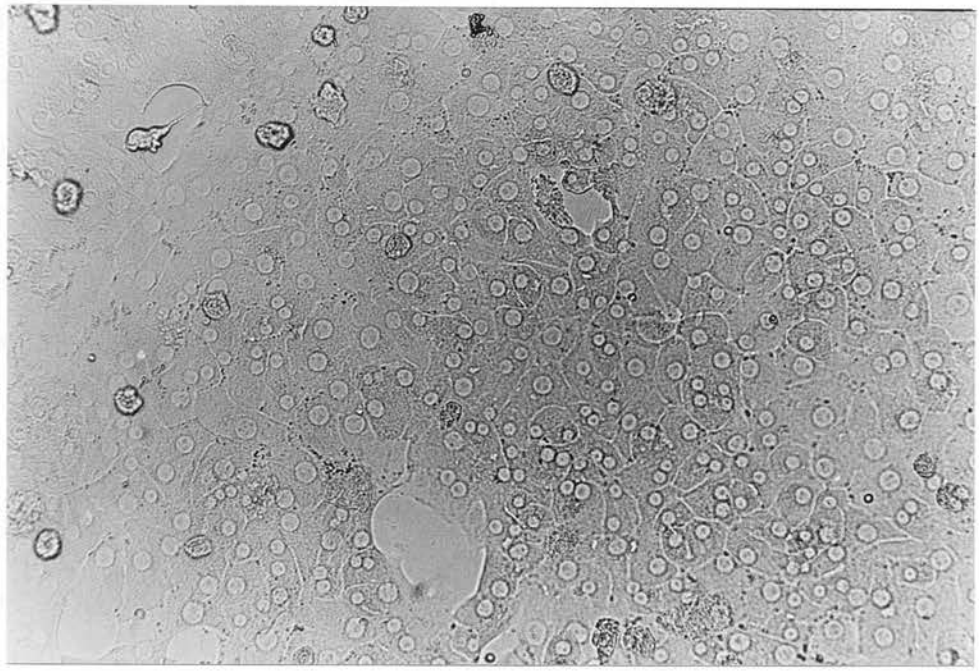


Fig. 3.1e

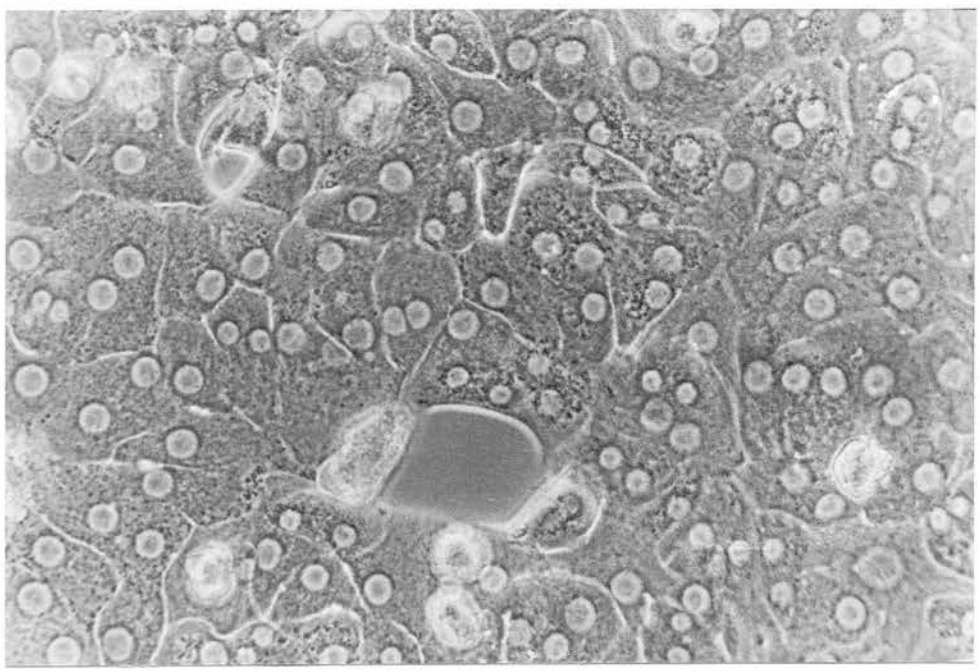


Fig. 3.1f

examination of Figs. 3.1 c-f reveals a number of spherical hepatocytes of granular appearance on the surface of the monolayer. These cells are dead. When cells adhere to culture plates in aggregates those cells not in contact with other cells die. The reason for this is unclear but it appears that cells are dependent on adhesion to the culture surface for their survival. This phenomenon has previously been observed by Bissel et al. (1973).

The sequence of events observed following the adhesion of hepatocytes to culture surfaces are consistent with the findings of others (Bissel et al., 1973; Boney, 1974; Seglen and Fossa, 1978).

3.4.2 Exclusion of trypan blue by rat hepatocyte monolayers

Exclusion of trypan blue provides a useful and rapid method for assessing cell viability. Viability was determined at 0, 3, 19 and 24h after the adhesion of hepatocytes to the Petri dishes and was greater than 85% over 24h (Fig. 3.2).

3.4.3 Leakage of lactate dehydrogenase by rat hepatocyte monolayers

Determination of viability by leakage of lactate dehydrogenase from the cells indicated that the viability fell to 60% after 24h (Fig. 3.2). However the addition of 1mg ml^{-1} galactose to the culture medium improved the survival of hepatocytes (Fig. 3.2). Subsequently in all experiments performed, galactose was added to the culture medium (Chapter 2.4.3). This increase in viability by the addition of galactose is in agreement with the findings of Williams et al. (1977). The basis for this improved survival is unknown.

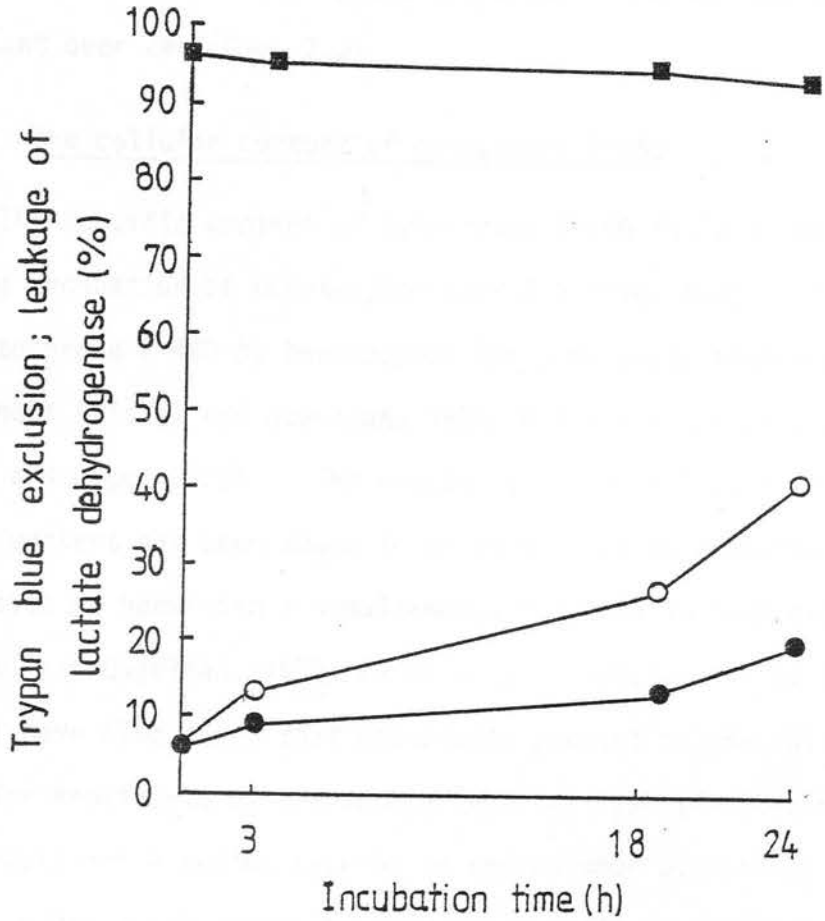


Fig. 3.2 Viability of rat hepatocyte monolayers as determined by trypan blue exclusion (■—■) and leakage of lactate dehydrogenase in the absence (○—○) and presence (●—●) of 1mg ml^{-1} galactose in the culture medium. Viability of hepatocytes was determined as described in Chapter 2. Results shown are representative of a typical hepatocyte preparation.

3.4.4 Protein synthesis by rat hepatocyte monolayers

Synthesis of protein by rat hepatocytes was determined by incubating cells with [^{3}H]leucine. Incorporation of [^{3}H]leucine into cellular protein and protein secreted into the medium was constant over 24h (Fig. 3.3).

3.4.5 The cellular content of cytochrome P-450

The specific content of cytochrome P-450 falls quite rapidly during incubation of hepatocytes over 24h (Fig. 3.4). The loss of cytochrome P-450 by hepatocytes has previously been reported by others (Bissel and Guzelian, 1979; Maslansky and Williams, 1982; Paine and Legg, 1978). The reason for this decline in cytochrome P-450 content has been shown to be partly due to a decrease in the synthesis of haem with a simultaneous increase in haem degradation (Bissel and Guzelian, 1979; Paine et al., 1982). Paine and Hockin (1980) have also shown that components present in the culture medium used for hepatocyte maintenance affect the loss of cytochrome P-450. By formulating a medium lacking in the sulphur containing amino acids, cystine and cysteine, they maintained cytochrome P-450 levels at 70% of the physiological concentration. Further, by the addition of 5-aminolaevulinic acid to culture medium and therefore bypassing the rate-limiting step in haem synthesis, cytochrome P-450 content was maintained at physiological concentrations.

3.5 Discussion

The procedure used for the isolation of rat hepatocytes has facilitated the preparation of a homogeneous cell type of generally excellent viability. The adhesion of hepatocytes to Petri dishes by preincubation in medium containing serum is consistent with the

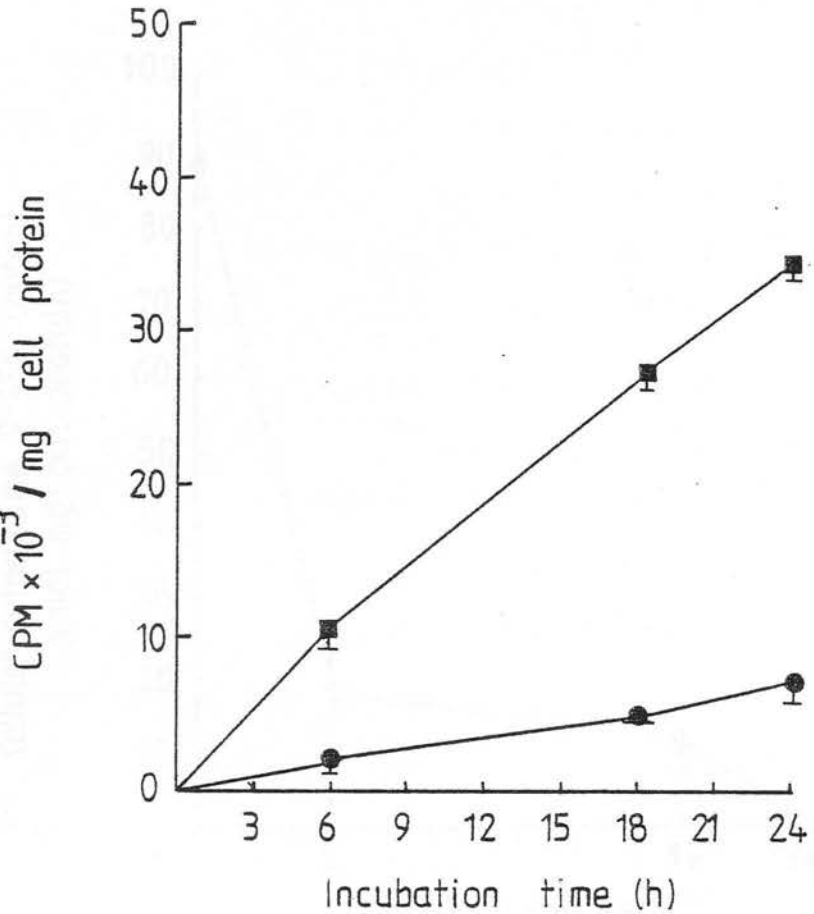


Fig. 3.3 Synthesis of protein by rat hepatocyte monolayers. Protein synthesis by rat hepatocyte monolayers was determined by measuring the incorporation of [^{3}H]leucine (Specific activity = 3.2μCi/μ into cellular protein (■—■) and protein secreted into the medium (●—●). The results are the mean of duplicate determinations from hepatocytes obtained from 2 rats. Error bars show the range of the values obtained.

reports of others (Horiuti et al., 1982; Laishes and Williams, 1976; Seglen and Fossa, 1978). The efficiency of attachment was also found to be largely dependent on the initial viability of the hepatocyte suspension obtained and was greatly improved by the addition of insulin. This is in agreement with the findings of Laishes and Williams (1976). Further studies have indicated that attachment of cells to the culture surface is also dependent on divalent cations and that the cell adhesion factor present in serum is fibronectin (Hook et al., 1977; Seglen and Fossa, 1978). The identification of a specific cell attachment protein has allowed cell adhesion to proceed without using serum and insulin. Durrington et al. (1982) have shown that the synthesis and secretion of VLDL by rat hepatocyte monolayers maintained on fibronectin-treated Petri dishes is increased by insulin. The effect of serum on the survival of hepatocytes has also been investigated. Serum was not required for hepatocyte survival up to 2 days after isolation. However, for maintenance of hepatocytes for periods in excess of 2 days viability was greatly improved by the addition of serum to the culture medium (Horiuti et al., 1982). The maintenance of hepatocytes on dishes coated with fibronectin and the effect on the synthesis of bile salts is discussed in Chapter 4.

The routine methods used for assessing hepatocyte viability were examination of morphology and the exclusion of trypan blue. The morphology of hepatocytes provides a quick and efficient means of assessing viability. The reaggregation and formation of flattened monolayers provides an excellent indication of hepatocyte function. Preparations in which such a change in morphology (Chapter 3.4.1) did not occur were discarded as were those

preparations in which viability as assessed by trypan blue exclusion fell to less than 85%.

A further possibly more sensitive method of assessing the integrity of cell membranes was provided by determination of the leakage of the cytoplasmic enzyme, lactate dehydrogenase. Non-viable cells have a tendency to detach from monolayers during incubation. Determination of lactate dehydrogenase activity in the medium not only assesses the leakage of this enzyme by hepatocytes adhered to culture dishes but also enzyme activity due to detached hepatocytes. The method for determination of viability by exclusion of trypan blue does not include detached cells in the viability count.

Many studies using isolated hepatocytes have confined tests of viability to the exclusion of vital dyes or leakage of cytoplasmic enzymes. These tests, whilst useful, only provide an indication of the permeability of the plasma membrane. In determining the biochemical integrity of an isolated cell preparation it is preferable to show not only the extent of damage to the plasma membrane but also that complex metabolic processes can take place. The method chosen for demonstrating that complex reactions can take place was incorporation of [^{3}H]leucine into protein. This provided an indication of the rate of protein synthesis. This is a useful method for assessing the biochemical integrity of hepatocytes as it is an indication of the interaction between different cell organelles. The incorporation of [^{3}H]leucine into protein was linear throughout the 24h incubation. The final criteria chosen for assessing the integrity of hepatocyte monolayers was determination of the cellular cytochrome P-450 content. This is particularly relevant to the work presented in

this thesis as synthesis of bile salts involves cytochrome P-450-dependent hydroxylations (Chapter 1.4). The results confirm previous reports that hepatocytes maintained in monolayers lose cytochrome P-450. The loss of cytochrome P-450 content would seem to indicate a loss in hepatocyte viability. However, it is clear from the results presented that the plasma membrane is intact and that hepatocytes synthesise protein at a constant rate over 24h. Other workers have also reported that isolated hepatocytes lose components such as glutamate (Cronell et al., 1974), methionine (Krebs et al., 1976) and glutathione (Vina et al., 1978). The current evidence would therefore seem to indicate that isolated hepatocytes, whilst useful, are only an approximation to the true in vivo state. It is therefore important to demonstrate that the metabolic pathway under investigation functions normally in isolated hepatocytes. The synthesis of bile salts by rat hepatocyte monolayers is discussed in detail in Chapter 4.

CHAPTER 4

THE SYNTHESIS OF BILE SALTS BY RAT HEPATOCYTE MONOLAYERS

4.1 Introduction

The synthesis of bile acids occurs only in the liver via a series of reactions initiated by 7α-hydroxylation of cholesterol. (Lindstredt, 1957; Bergstrom et al. 1958). The biosynthesis of bile acids is discussed in detail in Chapter 1.4.

The use of isolated hepatocytes to investigate the synthesis of bile acids has been reported by a number of research groups (Anwer et al., 1975; Botham et al., 1980; Gardner and Chenouda, 1978; Kempen et al., 1982; Yousef et al., 1978). These studies have all involved maintenance of hepatocytes in suspension. In this laboratory, the investigation of bile salt synthesis by hepatocytes has been facilitated by the use of radioimmunoassays for conjugated cholic, chenodeoxycholic and β-muricholic acids. This has allowed measurement of pmol quantities of these bile salts. Current evidence suggests that the measurement of these three bile salts should provide a reliable index of the total synthesis of bile acids by isolated hepatocyte preparations. Yousef et al. (1978) reported that 95% of the total bile salts secreted by isolated hepatocytes maintained in suspension consisted of trihydroxylated bile salts and chenodeoxycholic acid. More recently, however, Kempen et al. (1982) provided evidence that greater than 95% of the bile salts synthesised by hepatocytes in suspension, consisted of conjugated cholic, β-muricholic and chenodeoxycholic acids. Conjugated chenodeoxycholic acid was synthesised in relatively

low quantities and this was attributed to its further metabolism to β-muricholic acid. For clarity, 'total bile salt synthesis' is used to describe the sum of the three major bile salts determined.

The results in this chapter presents evidence for the synthesis of bile salts by rat hepatocytes maintained in monolayers. The most suitable conditions for maintenance of hepatocytes for studying synthesis of bile salts are also investigated. Whilst this work was in progress Davis et al. (1983a; 1983b), reported that rat hepatocytes in monolayer culture synthesise and secrete cholic and β-muricholic acids. They did not detect synthesis of chenodeoxycholic acid.

4.2 The effect of diet on the synthesis of bile salts by rat hepatocyte monolayers

Rat hepatocytes in monolayers were found to synthesise and secrete conjugated cholic, chenodeoxycholic and β-muricholic acids during 24h of incubation (Fig. 4.1). Conjugated cholic and conjugated β-muricholic acids were the major bile salts synthesised, conjugated chenodeoxycholic acid being synthesised in low quantities.

Alteration of the diets given to rats, resulted in alteration of the rates of synthesis of the three bile salts measured (Fig. 4.1). Hepatocytes isolated from rats fed the soft diet synthesised the lowest quantity of bile salts (Fig. 4.2). In the first 5h of incubation total bile salts were synthesised at a rate of 60 pmol . mg cell protein^{-1} . h^{-1} which fell to 8 pmol . mg cell protein^{-1} . h^{-1} between 5 - 24h. When rats were fed the pellet diet there was a significant increase in the synthesis

Fig. 4.1 The effect of diet on the synthesis of bile salts by rat hepatocyte monolayers. Hepatocyte monolayers were prepared as described in Materials and Methods. The effect of feeding the 'soft diet' (▼—▼), pellet diet (■—■) or the 'soft diet' + 4% cholestyramine (●—●) on the synthesis of (A) conjugated cholic acid (B) conjugated β-muricholic acid and (C) conjugated chenodeoxycholic acid, by isolated hepatocytes were determined. For the 'soft diet' and 'soft diet' + 4% cholestyramine each point represents duplicate determinations from hepatocytes obtained from four rats. For the pellet diet each point represents the mean of duplicate determinations from hepatocytes obtained from two rats. Statistical analysis is (a) a comparison of the 'soft diet' + 4% cholestyramine with the pellet diet and the 'soft diet' and (b) a comparison between the pellet diet and the 'soft diet'. Bile salts detected at 0h represent cell associated levels after adhesion of hepatocytes to the culture surface had taken place. Significance limits : a $P < 0.05$, b $P < 0.05$. Error bars show ± standard deviation.

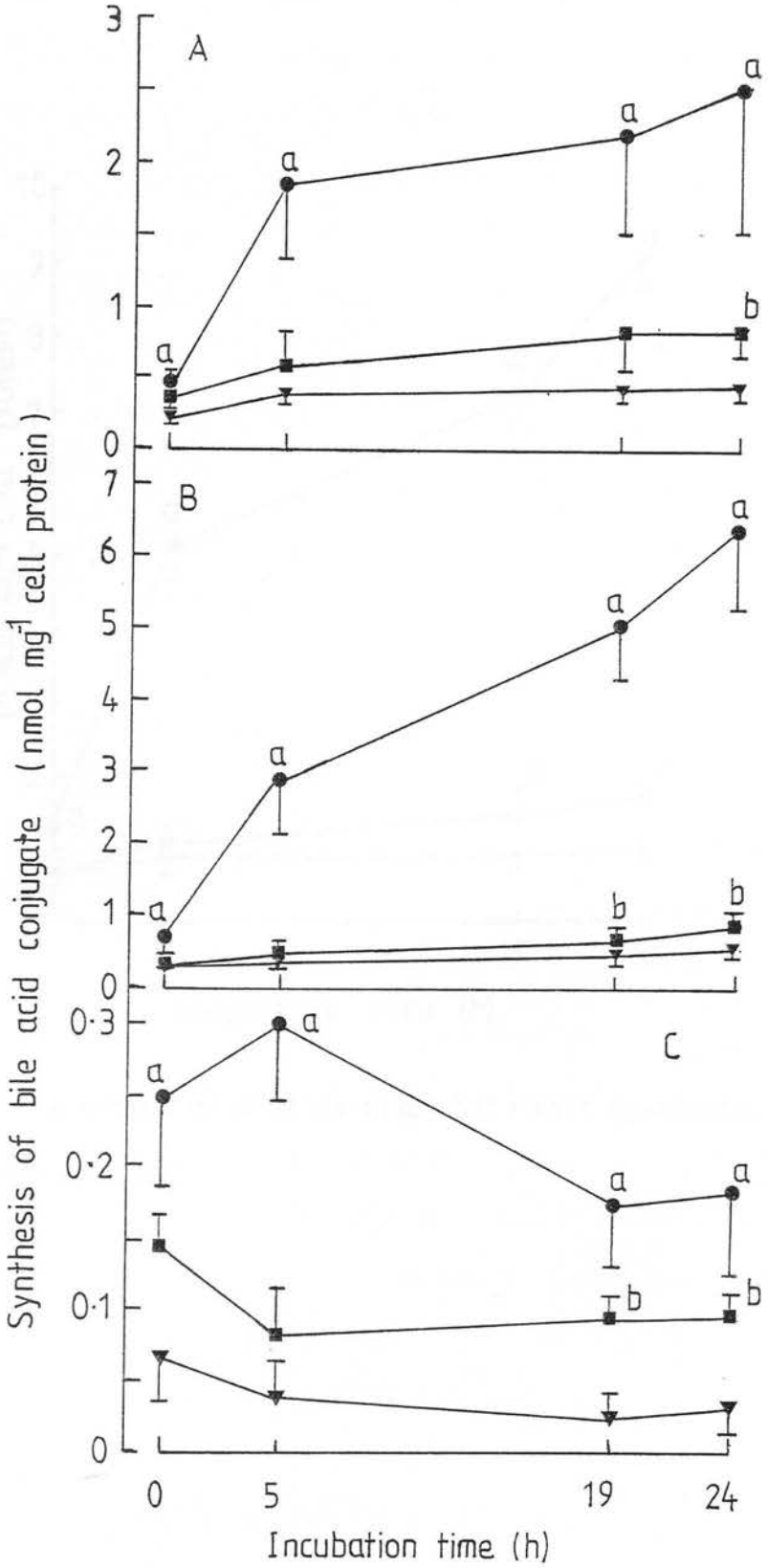

A
B
C
Synthesis of bile acid conjugate (nmol mg-1 cell protein)
Incubation time (h)
0
5
19
24

Fig. 4.1

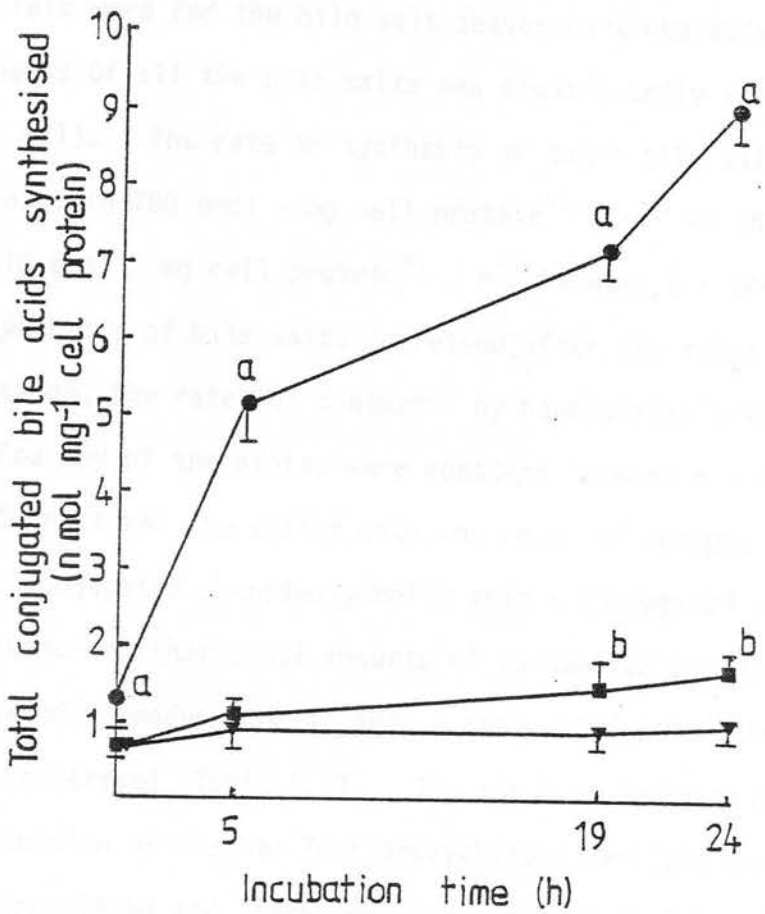


Fig. 4.2 The effect of diet on total bile salt synthesis.

Legend as for Fig. 4.1.

of total bile salts by hepatocytes. In the first 5h of incubation, synthesis was increased to a rate of 90 pmol mg cell protein^{-1} h^{-1} and was 30 pmol mg cell protein^{-1} h^{-1} between 5 - 24h. When rats were fed the bile salt sequestrant cholestyramine, synthesis of all the bile salts was significantly increased (Fig. 4.1). The rate of synthesis of total bile salts was increased to 780 pmol mg cell protein^{-1} h^{-1} in the first 5h and was 210 pmol mg cell protein^{-1} h^{-1} between 5 - 24h. Although the synthesis of bile salts decreased after the first 5h of incubation, the rates of synthesis by hepatocytes prepared from rats fed any of the diets, were constant between 5 - 24h.

In rats fed the pellet diet the ratio of conjugated cholic acid : conjugated chenodeoxycholic acid + conjugated β-muricholic acid indicated that equal amounts of conjugated cholic acid and conjugated chenodeoxycholic acid + conjugated β-muricholic acid were synthesised (Table 4.1). The ratio of these values provides an indication of whether 7α-hydroxycholest-5en-3one is first 12α-hydroxylated and therefore gives rise to cholic acid or is 26-hydroxylated and therefore produces chenodeoxycholic acid and its metabolite β-muricholic acid (Chapter 1.4). In hepatocytes isolated from rats fed the soft diet there was slightly more conjugated chenodeoxycholic acid + conjugated β-muricholic acid synthesised than conjugated cholic acid. Feeding cholestyramine resulted in a significant increase in the synthesis of all three bile salts; synthesis of conjugated chenodeoxycholic + conjugated β-muricholic acids was stimulated to a greater extent than conjugated cholic acid (Table 4.1). There was no change in the ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic

Ratio of conjugated cholic acid : conjugated chenodeoxycholic + conjugated β-muricholic acids

Diet	Pellet	Soft	Soft + 4% cholestyramine
Time (h)			
0	1.28 ± 0.39	0.66 ± 0.24*	0.42 ± 0.18*
5	1.10 ± 0.37	0.73 ± 0.30	0.56 ± 0.20*
19	1.10 ± 0.45	0.71 ± 0.28*	0.41 ± 0.20*
24	0.92 ± 0.37	0.61 ± 0.25*	0.37 ± 0.10*

Ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic acid

Diet	Pellet	Soft	Soft + 4% cholestyramine diet
Time (h)			
0	0.36 ± 0.10	0.63 ± 0.23	0.38 ± 0.10
5	0.09 ± 0.03	0.18 ± 0.10	0.11 ± 0.03
19	0.04 ± 0.02	0.20 ± 0.10*	0.04 ± 0.01
24	0.05 ± 0.03	0.17 ± 0.09*	0.03 ± 0.01

Table 4.1 The effect of diet on the ratio of conjugated cholic acid : conjugated chenodeoxycholic + conjugated β-muricholic acids and the ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic acid, as a measure of 12-hydroxylation and 6-hydroxylation respectively. Results are the mean ± standard deviation of duplicate determinations from hepatocytes obtained from 'soft diet' (4 rats), pellet diet (2 rats) & 'soft diet' + 4% cholestyramine (4 rats). Statistical analysis is a comparison with the pellet diet. Significance limits: * $P < 0.05$

acid in hepatocytes obtained from rats fed either of the diets indicating that 6β-hydroxylation of chenodeoxycholic acid to β-muricholic acid was not affected.

During the incubation of hepatocytes over 24h the ratio of conjugated chenodeoxycholic : β-muricholic acid fell significantly (Table 4.1). This was due to conversion of chenodeoxycholic acid to β-muricholic acid via 6β-hydroxylation by the hepatocytes.

The more efficient rates of synthesis of bile salts observed in hepatocytes isolated from rats fed the diet containing cholestyramine should facilitate the investigation of the major aims of this project (Chapter 1.6). As a result the following experiments to characterise the catabolism of cholesterol to bile salts by isolated hepatocytes, were performed using hepatocytes prepared from rats in which cholestyramine was included in the diet.

4.3 The effect of maintaining the cellular cytochrome P-450 content on the synthesis of bile salts by hepatocytes

As has been indicated in Chapter 3.4.4 the cellular content of cytochrome P-450 decreases during incubation of hepatocytes. However, Paine and Hockin (1980) have shown that the rate at which cytochrome P-450 content falls can be decreased by addition of 100 μM 5-aminolaevulinic acid to the culture medium. The addition of 100 μM 5-aminolaevulinic acid to the culture medium used in this study was also found to decrease the rate at which hepatocytes lost cytochrome P-450 (Fig. 4.3). However, it was not as effective in maintaining cellular cytochrome P-450 content as in the media used by Paine, et al. (1982). This may be due

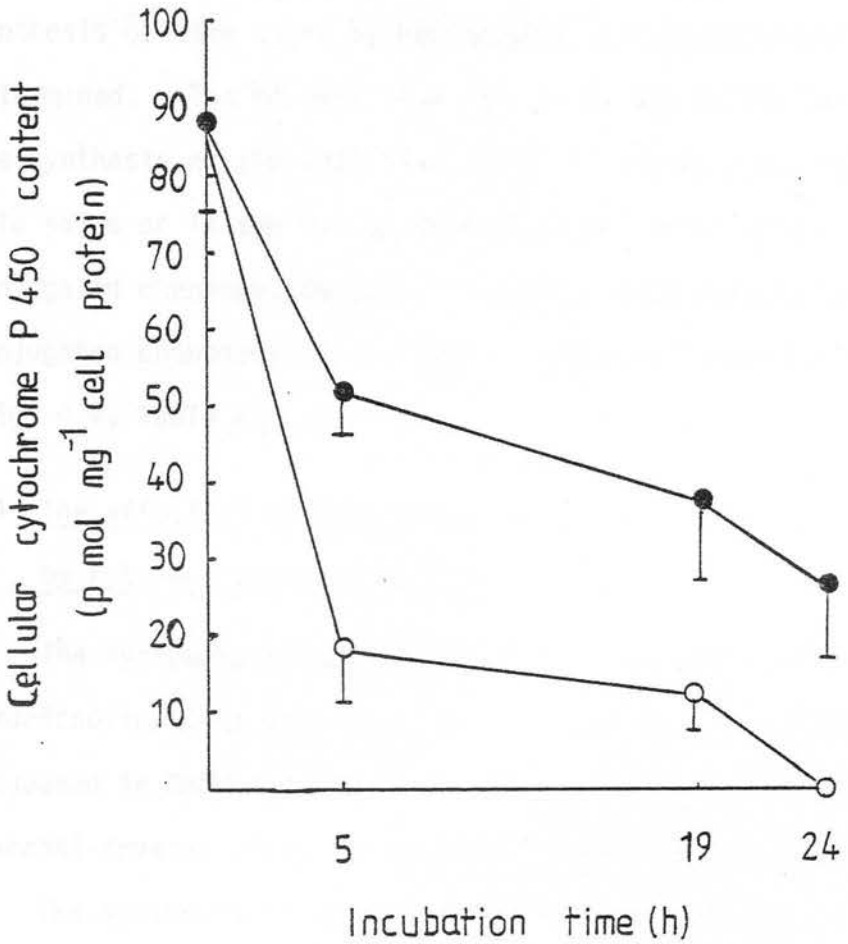


Fig. 4.3 The effect of 5-aminolaevulinic acid on the cellular cytochrome P-450 content of hepatocyte monolayers. Following adhesion of hepatocytes to plastic Petri dishes they were incubated in medium containing 100 µM aminolaevulinic acid (●—●) or in control medium (○—○). Cells were pooled from 5 - 8 plates and cytochrome P-450 content determined. Results are the mean of duplicate determinations from hepatocytes obtained from 2 rats. Error bars show the range of the values obtained.

to the higher cystine and cysteine content in DMEM compared to these media (see Chapter 3.4.4).

The effect of maintaining cytochrome P-450 content on the synthesis of bile salts by hepatocytes was subsequently determined. The results show that there was no difference in the synthesis of the individual bile salts measured, the total bile salts or in the ratios of conjugated cholic acid : conjugated chenodeoxycholic + conjugated β-muricholic acids or conjugated chenodeoxycholic acid : conjugated β-muricholic acid (Fig. 4.4, Table 4.2).

4.4 The effect of bovine serum albumin on bile salt synthesis by rat hepatocyte monolayers

The synthesis of conjugated cholic, chenodeoxycholic and β-muricholic acids were determined in hepatocyte monolayers incubated in DMEM containing 0% ($^{W}/_{v}$), 2% ($^{W}/_{v}$) and 4% ($^{W}/_{v}$) charcoal-treated bovine serum albumin fraction V powder (BSA).

The synthesis of conjugated cholic acid and conjugated β-muricholic acid were decreased in the presence of BSA, whilst synthesis of conjugated chenodeoxycholic acid was significantly increased (Fig. 4.5). To a certain degree the effect of BSA was dependent on the concentration used. Whilst synthesis of conjugated cholic acid was decreased in the presence of albumin this decrease was not significant. However, the decrease in the synthesis of conjugated β-muricholic acid although not significant in the presence of 2% ($^{W}/_{v}$) BSA was significant when hepatocytes were incubated in the presence of 4% ($^{W}/_{v}$) BSA. The increase in the synthesis of conjugated chenodeoxycholic acid was significant at both the concentrations of BSA used.

Fig. 4.4 The effect of 5-aminolaevulinic acid on the synthesis of bile salts by rat hepatocyte monolayers. Hepatocytes were incubated in the presence (●—●) or absence (○—○) of 100 μM 5-aminolaevulinic acid and the synthesis of (A) conjugated cholic acid (B) conjugated β-muricholic acid and (C) conjugated chenodeoxycholic acid were determined. Values shown represent the mean ± standard deviation of duplicate determinations in hepatocytes obtained from three rats. Bile salts detected at 0h represent cell associated levels following adhesion of hepatocytes to the culture surface.

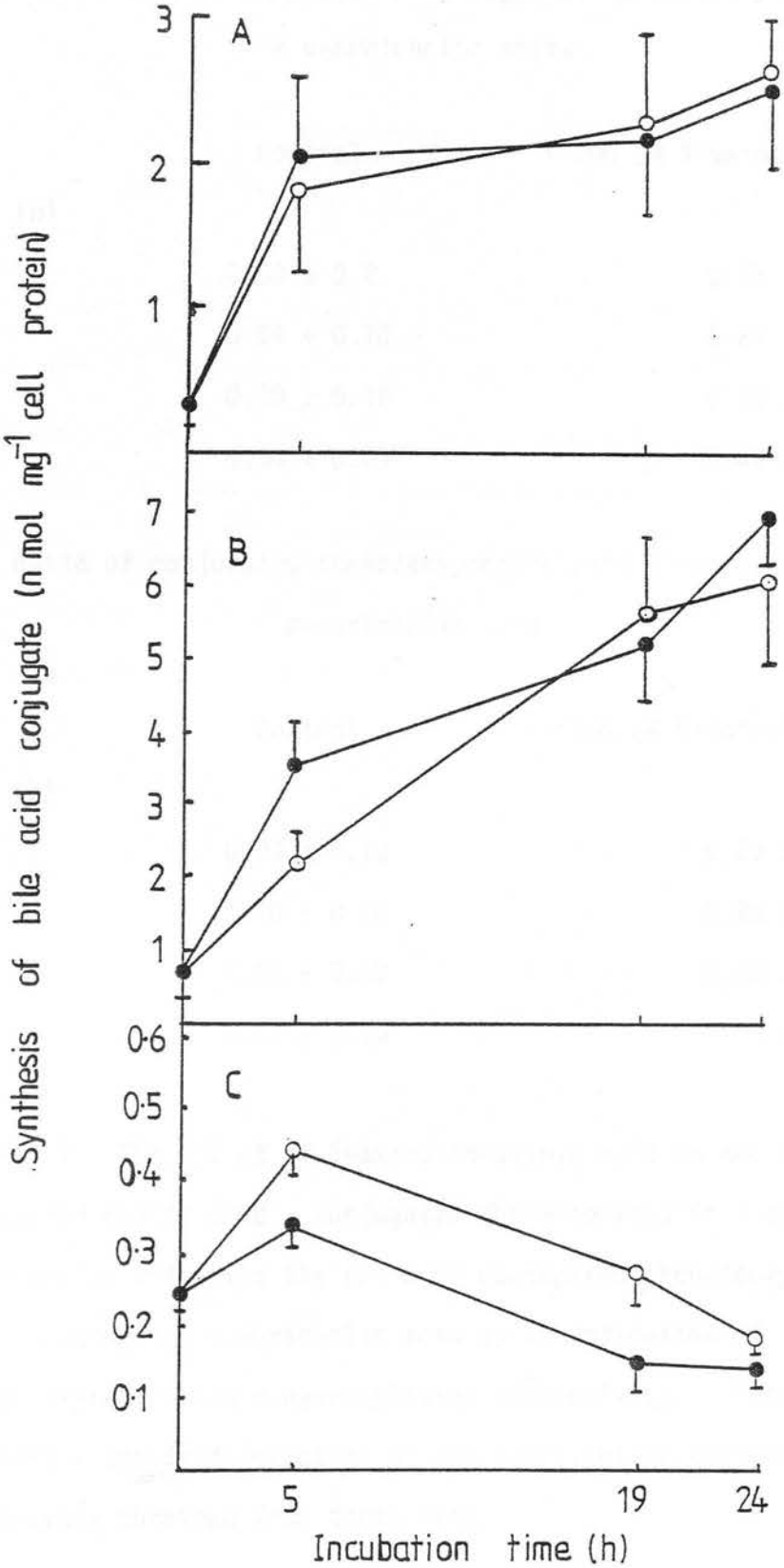

A
B
C
3
2
1
7
6
5
4
3
2
1
0·6
0·5
0·4
0·3
0·2
0·1
5
19
24
Incubation time (h)
Synthesis of bile acid conjugate (n mol mg⁻¹ cell protein)

Fig. 4.4

Ratio of conjugated cholic acid : conjugated chenodeoxycholic + β-muricholic acids

Time (h)	Control	+ 100 μM 5-aminolaevulinic acid
0	0.38 ± 0.2	0.38 ± 0.2
6	0.54 ± 0.10	0.67 ± 0.2
18	0.39 ± 0.10	0.38 ± 0.08
24	0.34 ± 0.09	0.42 ± 0.02

Ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic acid

Time (h)	Control	+ 100 μM 5-aminolaevulinic acid
0	0.29 ± 0.10	0.29 ± 0.10
6	0.10 ± 0.00	0.28 ± 0.04
18	0.03 ± 0.00	0.06 ± 0.02
24	0.02 ± 0.00	0.03 ± 0.01

Table 4.2 The effect of 5-aminolaevulinic acid on the ratio of conjugated cholic acid : conjugated chenodeoxycholic + conjugated β-muricholic acids and the ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic acid as an indication of 12-hydroxylation and 6-hydroxylation respectively. Results are the mean ± standard deviation of duplicate determinations from hepatocytes obtained from three rats.

Fig. 4.5 The effect of bovine serum albumin on the synthesis of bile salts by hepatocyte monolayers. The effect of 2% albumin (●—●) and 4% albumin (■—■) on (A) the synthesis of conjugated cholic acid (B) the synthesis of conjugated β-muricholic acid (C) the synthesis of conjugated chenodeoxycholic acid as compared to a control without albumin (○—○) were determined. Each point represents the mean of duplicate determinations from hepatocytes obtained from four rats. Bile salts detected at 0h represent cell-associated levels after adhesion of the hepatocytes to the culture surface had taken place. Statistical analysis is a comparison with the control. Significance limits : *$P < 0.05$. Error bars show ± standard deviation.

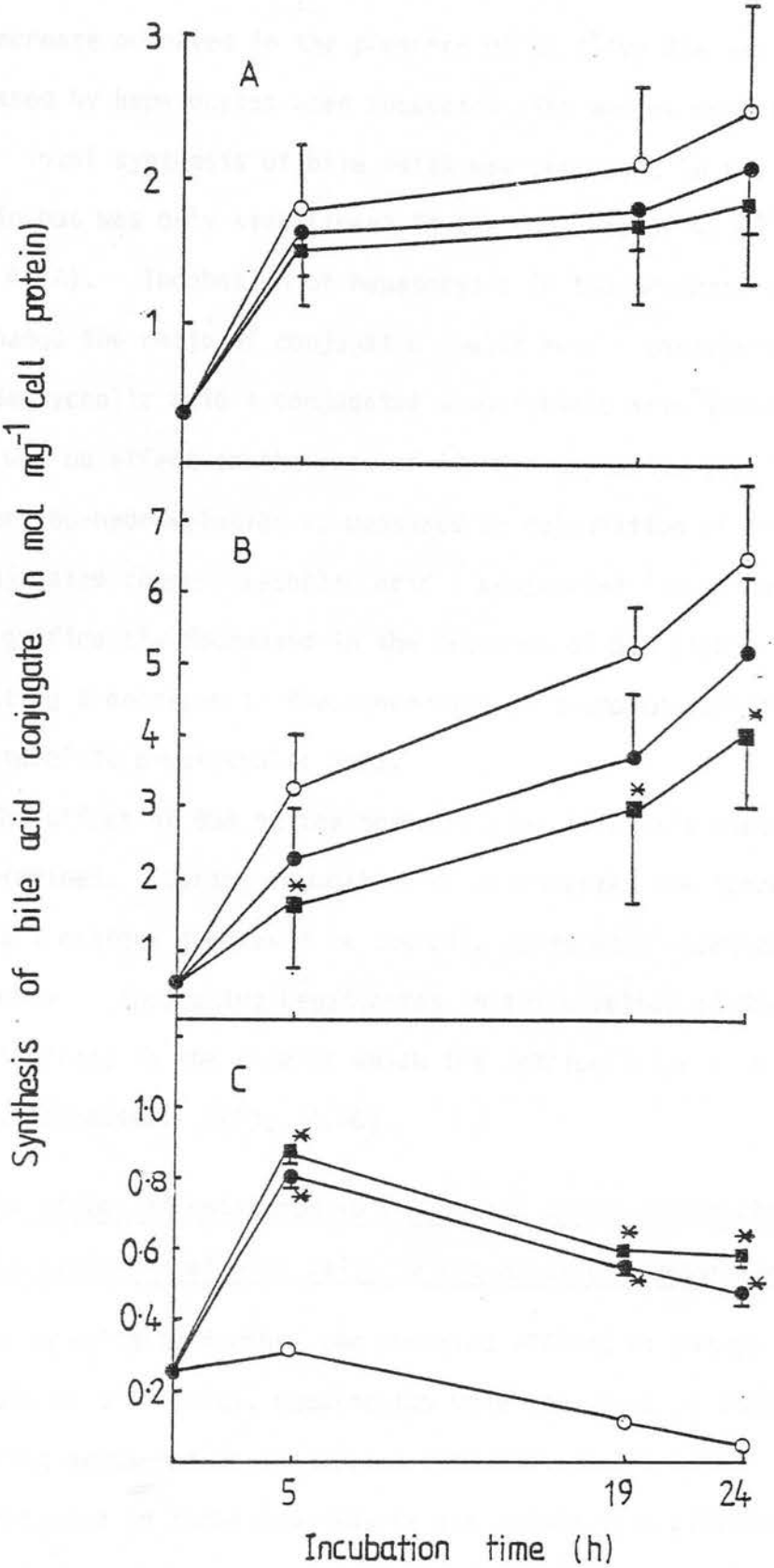

A
B
C
Synthesis of bile acid conjugate (n mol mg^{-1} cell protein)
Incubation time (h)
5
19
24

Fig. 4.5

The increase observed in the presence of 2% ($^{w}/_{v}$) BSA was not further increased by hepatocytes when incubated with medium containing 4% ($^{w}/_{v}$) BSA. Total synthesis of bile salts was decreased in the presence of albumin but was only significant in the presence of 4% ($^{w}/_{v}$) BSA (Fig. 4.6A). Incubation of hepatocytes in the presence of BSA did not change the ratio of conjugated cholic acid : conjugated chenodeoxycholic acid + conjugated β-muricholic acid indicating that there was no effect on the rate of 12α-hydroxylation (Table 4.3). However, 6β-hydroxylation as measured by calculation of the ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic acid was significantly decreased in the presence of BSA (Table 4.3), thus indicating a decrease in the conversion of chenodeoxycholic acid to its metabolite β-muricholic acid.

The effect of BSA on the intracellular bile salt content was also examined. During incubation of hepatocytes the intracellular bile salt content decreased as the bile salts were secreted into the medium. Incubating hepatocytes in the presence of BSA resulted in an increase in the rate at which the intracellular bile salt content decreased. (Fig. 4.6B).

4.5 The effect of antiserum to conjugated chenodeoxycholic acid on the synthesis of bile salts by rat hepatocyte monolayers

To investigate further the observed effects of BSA on the synthesis of bile salts, hepatocytes were incubated in DMEM containing antiserum to conjugated chenodeoxycholic acid. The antiserum used in these experiments was raised in a different rabbit to the antiserum used in the radioimmunoassay. This latter antiserum had a significantly higher binding capacity for conjugated chenodeoxycholic acid. However, at the concentrations

Fig. 4.6 The effect of bovine serum albumin on the synthesis of bile salts by rat hepatocyte monolayers. The effect of 2% albumin (●—●) and 4% albumin (■—■) on (A) total synthesis of conjugated bile salts and (B) intracellular content of total bile salts as compared to a control without albumin (○—○) were determined. Bile salts detected at 0h represent cell-associated levels after adhesion of hepatocytes to the culture surface had taken place. Each point represents the mean of duplicate determinations from hepatocytes obtained from four rats. Statistical analysis is a comparison with the control. Significance limits : *P < 0.05. Error bars show ± standard deviation.

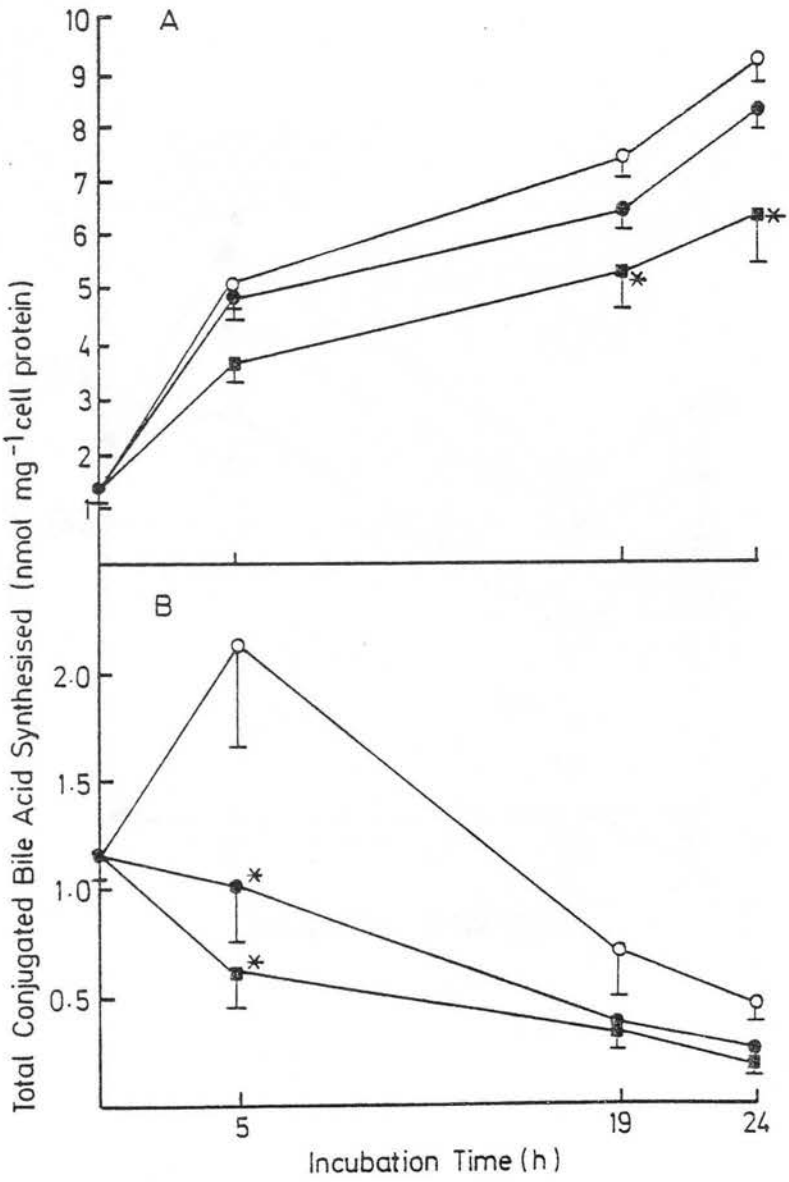
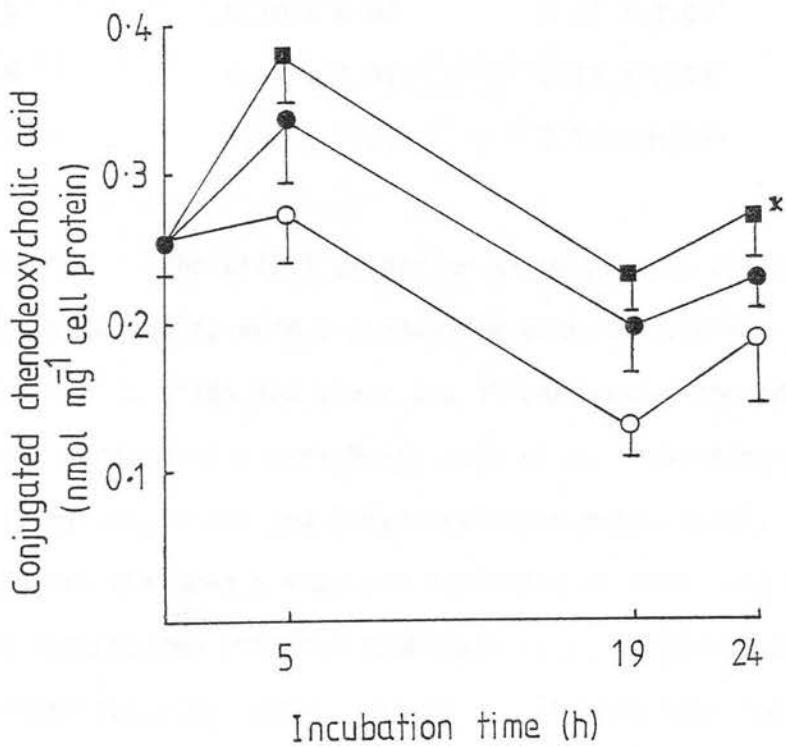


Fig. 4.7 The effect of a specific antiserum to conjugated chenodeoxycholic acid on the synthesis of conjugated chenodeoxycholic acid by rat hepatocyte monolayers. Hepatocytes were incubated in the absence (○—○) or presence of 0.25% ($^{v}/_{v}$) antiserum (●—●) and 0.5% ($^{v}/_{v}$) antiserum (■—■). The effect of the antiserum on the apparent synthesis of conjugated chenodeoxycholic acid was determined. Bile salts detected at 0h represent cell associated levels after adhesion of hepatocytes to Petri dishes had taken place. Each point represents the mean of duplicate determinations from hepatocytes obtained from four rats. Error bars show ± standard deviation. Significance limits : *P < 0.05.



Ratio of conjugated cholic acid : conjugated chenodeoxycholic + conjugated β-muricholic acids

Time (h)	Control	2%	4%
0	0.55 ± 0.20	0.55 ± 0.20	0.55 ± 0.20
6	0.54 ± 0.20	0.54 ± 0.20	0.61 ± 0.10
18	0.38 ± 0.09	0.41 ± 0.04	0.46 ± 0.08
24	0.36 ± 0.08	0.36 ± 0.01	0.39 ± 0.04

Ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic acid

Time (h)	Control	2%	4%
0	0.63 ± 0.30	0.63 ± 0.30	0.63 ± 0.30
6	0.10 ± 0.03	0.24 ± 0.03[a]	0.40 ± 0.10[a]
18	0.04 ± 0.01	0.15 ± 0.09[a]	0.21 ± 0.10[a]
24	0.03 ± 0.01	0.11 ± 0.06[a]	0.16 ± 0.08[a]

Table 4.3 The effect of bovine serum albumin on the ratios of conjugated cholic acid : conjugated chenodeoxycholic + conjugated β-muricholic acids and the ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic acid as an indication of the rates of 12-hydroxylation and 6-hydroxylation respectively. Values shown are the mean ± standard deviation of duplicate determinations from hepatocytes obtained from four rats. Statistical analysis is a comparison with control values. Significance limits : a $P < 0.05$.

of the antiserum used in the incubation with hepatocytes slight interference with the assay of conjugated chenodeoxycholic acid occurred. As a result in those samples containing the antiserum of lower binding capacity conjugated chenodeoxycholic acid was measured using a standard curve obtained with both antisera present in the incubation buffer used in the radioimmunoassay. There was no cross reactivity between the antiserum used in the incubation of hepatocytes with either conjugated cholic acid or conjugated β-muricholic acid.

Total synthesis of bile salts was unaffected but a significant increase in conjugated chenodeoxycholic acid was observed (Fig. 4.7). There was no change in the ratio of conjugated cholic acid : conjugated chenodeoxycholic acid + conjugated β-muricholic acid or the ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic acid (Table 4.4).

4.6 The effect of different methods used to promote cell adhesion on the subsequent synthesis of bile salts by hepatocyte monolayers

The effect of the procedure used to facilitate adhesion of hepatocytes to culture surfaces on the synthesis of bile salts by hepatocytes was investigated. Hepatocyte adhesion to culture surfaces was facilitated using either foetal calf serum and insulin as described in Chapter 2.4.3 or by incubation of hepatocytes in serum- and insulin-free medium, on Petri dishes pretreated with fibronectin. Petri dishes were coated with fibronectin by a procedure based on that used by Durrington et al. (1982). Culture medium (1ml) containing 20 µg fibronectin

Ratio of conjugated cholic acid : conjugated chenodeoxycholic + conjugated β-muricholic acids

Time (h)	Control	0.25%	0.5%
0	0.5 ± 0.2	0.5 ± 0.02	0.5 ± 0.2
6	0.46 ± 0.07	0.47 ± 0.09	0.61 ± 0.22
18	0.45 ± 0.03	0.44 ± 0.10	0.46 ± 0.12
24	0.48 ± 0.04	0.44 ± 0.10	0.49 ± 0.2

Ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic acid

Time (h)	Control	0.25%	0.5%
0	0.36 ± 0.10	0.36 ± 0.10	0.36 ± 0.10
6	0.07 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
18	0.03 ± 0.00	0.06 ± 0.01	0.04 ± 0.00
24	0.03 ± 0.00	0.05 ± 0.01	0.04 ± 0.01

Table 4.4 The effect of a specific antiserum to conjugated chenodeoxycholic acid on the ratio of conjugated cholic acid : conjugated chenodeoxycholic acid + β-muricholic acid and the ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic acid. The results are the mean ± standard deviation of duplicate determinations from hepatocytes obtained from 4 rats.

(Speywood Laboratories, Nottingham, U.K.) was added to Petri dishes and incubated for 2h at 37°C, after which time the medium was removed. After incubation of hepatocytes in treated dishes for 1h, adhesion of hepatocytes to the culture surface was essentially complete. Following adhesion of hepatocytes to Petri dishes the medium was removed and replaced with serum- and insulin-free medium. Hepatocytes were incubated for 24h after which time the synthesis of conjugated cholic, chenodeoxycholic and β-muricholic acids was determined (Table 4.5). No difference in the synthesis of conjugated cholic or chenodeoxycholic acid was observed. However, the synthesis of conjugated β-muricholic acid appeared to be greater in those hepatocytes in which adhesion had been facilitated by foetal calf serum and insulin. As a result of this increase in synthesis of conjugated β-muricholic acid, total synthesis was also increased in these hepatocytes. Viability as assessed by examination of hepatocyte morphology and exclusion of trypan blue was similar for hepatocytes maintained on fibronectin-coated dishes as for those preincubated in medium containing foetal calf serum and insulin.

4.7 The effect of insulin on the synthesis of bile salts by rat hepatocyte monolayers

In an attempt to establish the basis for the greater rates of bile salt synthesis by hepatocytes in which adhesion was facilitated by foetal calf serum and insulin, the effect of insulin on the synthesis of bile salts was investigated.

Following adhesion of hepatocytes to Petri dishes coated with fibronectin, hepatocytes were incubated for 24h in the absence or presence of insulin. As well as investigating the effect of physiological concentrations of insulin (10^{-8} - 10^{-12} M), hepatocytes

	Fibronectin	Foetal calf serum and insulin
Conjugated cholic acid (nmol mg^{-1} cell protein)	2.41 ± 0.90	2.24 ± 0.40
Conjugated chenodeoxycholic acid (nmol mg^{-1} cell protein)	0.18 ± 0.05	0.13 ± 0.02
Conjugated β-muricholic acid (nmol mg^{-1} cell protein)	2.95 ± 0.60	4.33 ± 1.50
Total (nmol mg^{-1} cell protein)	5.56 ± 1.60	6.70 ± 1.60
$\frac{\text{conjugated cholic acid}}{\text{conjugated chenodeoxycholic + β-muricholic acids}}$	0.73 ± 0.20	0.59 ± 0.20
$\frac{\text{conjugated chenodeoxycholic acid}}{\text{conjugated β-muricholic acid}}$	0.06 ± 0.00	0.04 ± 0.00

Table 4.5 The synthesis of bile salts by hepatocyte monolayers. Attachment of hepatocytes to Petri dishes was achieved by either incubation in DMEM on fibronectin-treated Petri dishes or by incubation in DMEM containing foetal calf serum and insulin. After attachment of hepatocytes to the culture surface the medium was replaced with serum- and insulin- free DMEM. Hepatocytes were incubated for 24h and the synthesis of bile salts determined. Results are the mean ± range of duplicate determinations from hepatocytes obtained from 2 rats.

were exposed to the same concentration of insulin as used in the promotion of cell adhesion, i.e. 100U l^{-1}. Insulin was found to have no effect on the synthesis of any of the bile salts determined (results not shown).

4.8 Discussion

The results show that isolated hepatocytes maintained in monolayers synthesise conjugated cholic chenodeoxycholic and β-muricholic acids. Hepatocytes isolated from rats fed the 'soft diet' synthesised bile salts at the lowest rates, whilst feeding the pellet diet to rats resulted in higher rates of synthesis. Brydon et al. (1980) have reported that the activity of the enzyme cholesterol 7α-hydroxylase was lower in animals fed a low-fibre diet. It is possible that the higher rate of synthesis observed in hepatocytes isolated from rats fed the pellet diet is due to the higher fibre content of that diet.

The rate of synthesis of total bile salts in these hepatocyte preparations are comparable with the rate reported by Davis et al. (1983a, 1983b). Feeding cholestyramine resulted in a 5 - 10 fold increase in the synthesis of bile salts by hepatocyte monolayers, a finding consistent with the known effects of cholestyramine (Huff et al., 1963; Botham et al. 1980; Kempen et al. 1982). The observation that synthesis of conjugated β-muricholic acid is stimulated to a greater extent than conjugated cholic or chenodeoxycholic acids, confirms the results obtained with hepatocytes maintained in suspension (Botham et al., 1980; Botham and Boyd, 1983). It is likely that the synthesis of bile salts by rat hepatocytes in monolayer culture reflect the in vivo activity of cholesterol 7α-hydroxylase.

Maintenance of the total cytochrome P-450 content by supplementing the culture medium with 5-aminolaevulinic acid had no effect on the synthesis of bile salts by rat hepatocytes. The regulation of cholesterol 7α-hydroxylase, the enzyme catalyzing the rate limiting step in bile salt synthesis, is complex. Although cholesterol 7α-hydroxylase is a cytochrome P-450-dependent enzyme, current evidence suggests that the activity of cholesterol 7α-hydroxylase is not dependent on total cytochrome P-450 content. Administration of phenobarbitone results in an increase in cytochrome P-450 but does not affect cholesterol 7α-hydroxylase activity (Boyd et al., 1969; Botham and Boyd, 1979). However, interruption of the enterohepatic circulation whilst increasing the activity of cholesterol 7α-hydroxylase has no effect on total cytochrome P-450 (Boyd et al., 1969). Danielsson and Wikvall (1981) have reported that the cytochrome P-450 species associated with cholesterol 7α-hydroxylase is a minor constituent of total cellular cytochrome P-450. It is therefore possible that when total cytochrome P-450 is induced by drugs such as phenobarbitone, no significant change in the cytochrome P-450 form specific for 7α-hydroxylation occurs. Conversely the increase in cholesterol 7α-hydroxylase observed on diversion of the enterohepatic circulation, could be due to an increase in the specific cytochrome P-450 form associated with this enzyme, which may only produce a negligible affect on total cytochrome P-450. In the experiments reported here total cytochrome P-450 content seems to be defective but there is no indication of the hepatocyte content of the cytochrome P-450 specific for 7α-hydroxylation. It is likely that incubation of hepatocytes with medium containing

5-aminolaevulinic acid whilst decreasing the rate at which cellular cytochrome P-450 content decreases does not have a significant effect on the cytochrome P-450 specific for cholesterol 7α-hydroxylase.

Incubating hepatocytes in culture medium containing BSA led to an overall decrease in the synthesis of total bile salts. However, a significant increase in the amount of chenodeoxycholic acid was observed. In rats, chenodeoxycholic acid is known to be metabolised to α- and β- muricholic acids (Mahawold et al., 1957; Matschiner et al., 1957; Voight et al., 1968). In isolated hepatocytes maintained in suspension β-muricholic acid has been shown to be a major product of bile salt synthesis (Kempen et al., 1982; Botham and Boyd, 1983). More recently conversion of chenodeoxycholic acid to β-muricholic acid has been demonstrated in both hepatocyte suspensions (Botham and Boyd, 1983) and hepatocyte monolayers (Davis et al., 1983b). The apparent increase in the synthesis of conjugated chenodeoxycholic acid may be due to the formation of a conjugated chenodeoxycholic acid - BSA complex. Binding of bile salts to serum albumin has been reported to inhibit their re-uptake by hepatocytes maintained in suspension (Anwer et al., 1976; Iga and Klaassen, 1982). Inhibition of the re-uptake of chenodeoxycholic acid would prevent its further metabolism to β-muricholic acid. This would result in the observed apparent increase in the synthesis of conjugated chenodeoxycholic acid.

The uptake of bile acids by hepatocytes has been shown to be inversely correlated with the affinity of the bile acid for albumin (Hoffman et al., 1975). The formation of bile salt - BSA complexes would also provide an explanation for the increase in the

rate at which intracellular bile salt concentrations fall as re-uptake of secreted bile acids by hepatocytes would be inhibited.

In an attempt to mimic the effect of BSA on the apparent increase in the synthesis of conjugated chenodeoxycholic acid, hepatocyte monolayers were incubated in the presence of an antiserum specific to conjugated chenodeoxycholic acid. The increase in conjugated chenodeoxycholic acid observed (Fig. 4.7) further supports the conclusion that the increase in the synthesis of conjugated chenodeoxycholic acid in the presence of BSA is caused by binding thereby preventing its re-uptake and further metabolism to β-muricholic acid. No other effects on the synthesis of bile salts were observed. This also provides further evidence of the specificity of the antiserum used.

The reason for the overall decrease in the synthesis of bile salts by hepatocytes in the presence of albumin is unclear. Studies on hepatocyte monolayers have shown that alteration of the viscosity of the culture medium causes a decrease in the synthesis and secretion of plasma proteins (Davis et al., 1980) and also VLDL (Yedgar et al., 1982). It is possible that slight changes in the viscosity of the medium, due to the addition of albumin, may cause the observed decrease in the total synthesis of bile acids.

The results presented confirm that conjugated cholic acid and conjugated β-muricholic acid are the major bile salts synthesised by isolated rat hepatocytes. The rat hepatocyte monolayers also synthesise and secrete conjugated chenodeoxycholic acid. In related work, it has been reported that cholic and β-muricholic acids were synthesised by rat hepatocyte monolayers but the synthesis of chenodeoxycholic acid was not detected (Davis et al.

1983a, 1983b). However, a gas liquid chromatographic method was used for the detection of bile acid derivatives and the presence of an unidentified peak was reported (Davis et al., 1983a).

The investigation of the effect of various additions to the culture medium has enabled the optimum conditions for hepatocyte maintenance to be chosen. These conditions are based firstly on maintaining rates of bile salt synthesis over 24h and secondly, on keeping additions to the culture medium to a minimum. Whilst addition of 5-aminolaevulinic acid decreased the rate at which cellular cytochrome P-450 content declined, no effect on the synthesis of bile salts was observed. Addition of BSA led to a decrease in the rate of synthesis of bile salts and inhibited the metabolism of chenodeoxycholic acid to β-muricholic acid. The optimum conditions of hepatocyte maintenance were therefore provided by using serum- and insulin-free DMEM.

The results of a preliminary investigation undertaken indicate that fibronectin-treated Petri dishes can be used to study the synthesis of bile salts by hepatocyte monolayers. The results also suggest that insulin has no effect on the synthesis of bile salts over a 24h incubation. However, before undertaking the routine use of fibronectin-coated Petri dishes, it is desirable to first investigate the metabolic integrity of hepatocytes maintained on these dishes in more detail. It is hoped that in the future the use of Petri dishes coated with fibronectin will allow more defined conditions of hepatocyte maintenance.

In conclusion, all the experiments described in this thesis were performed using hepatocytes obtained from rats fed the soft

diet supplemented with 4% cholestyramine, unless otherwise indicated. Following the isolation of hepatocytes, adhesion to Petri dishes was facilitated by preincubation of cells in culture medium supplemented with foetal calf serum and insulin. After cell attachment was complete, hepatocytes were maintained in serum- and insulin-free DMEM, with additions as indicated e.g. lipoproteins, radioactive precursors.

CHAPTER 5

THE EFFECT OF A RAT PLASMA HIGH-DENSITY LIPOPROTEIN SUBFRACTION ON THE SYNTHESIS OF BILE SALTS BY RAT HEPATOCYTE MONOLAYERS

5.1 Introduction

The catabolism of cholesterol to bile salts provides the major route for the degradation of cholesterol (Bergstrom and Norman, 1953). The cholesterol that is degraded to bile salts can be derived from either de novo synthesis or from the plasma lipoproteins (Chapter 1.4.2). Current evidence suggests that the preferred substrate for the synthesis of bile salts is newly synthesised cholesterol (Mitropoulos et al., 1974; Bjorkhem and Danielsson, 1975; Norman and Norum, 1976; Bjorkhem and Lewenhaupt, 1979). However, it has been suggested that of the plasma lipoproteins HDL may function to regulate the cholesterol content of extrahepatic tissues by transporting cholesterol from these tissues to the liver for its ultimate removal from the body (Glomset, 1968; Tall and Small, 1980). This can be achieved by either degradation of cholesterol to bile salts or by direct secretion of the unmodified sterol into bile. Although the liver has been shown to be a major site for the uptake of HDL (Stein et al., 1983) there has been no evidence to suggest that HDL stimulates the synthesis of bile acids. The experiments described in this chapter were designed to investigate the effect of HDL_2 on the synthesis of bile salts by rat hepatocyte monolayers. The effect of HDL_2 on the cholesterol content of hepatocytes was also determined.

5.2 Isolation and characterisation of rat HDL_2

Rat HDL_2 was isolated by rate zonal ultracentrifugation as described in Chapter 2.7. The lipoprotein fraction obtained was characterised by its zonal elution profile and its apoprotein content. The protein : cholesterol ratio of the HDL_2 obtained was also determined.

5.2.1 Rate zonal ultracentrifugation

Fig. 5.1 shows the zonal elution profile obtained from rat plasma lipoproteins. The absorbance at 280nm of the first 50ml of eluate is high. This is due to the presence of VLDL which is colloidal in nature. This fraction also contains LDL. The peak obtained at an elution volume of 140 - 250ml represents the HDL_2 fraction. This fraction was taken and subsequently concentrated for use in the experiments to be undertaken.

5.2.2 SDS-PAGE of HDL_2 apoproteins

Following delipidation, HDL_2 apoproteins were subjected to SDS-PAGE as described in Chapter 2.14. The major protein identified was apo A-I whilst apo A-IV and apo E were also present (Fig. 5.2). These are the major apoproteins reported to be associated with HDL_2 (Oschry and Eisenberg, 1982). Apo C was also found to be present.

5.2.3 The protein and cholesterol content of the HDL_2 fraction

The HDL_2 fraction obtained was diluted with culture medium to provide a physiological concentration of 500 $\mu g\ ml^{-1}$ of HDL_2 protein. Following dilution of the HDL_2 the cholesterol

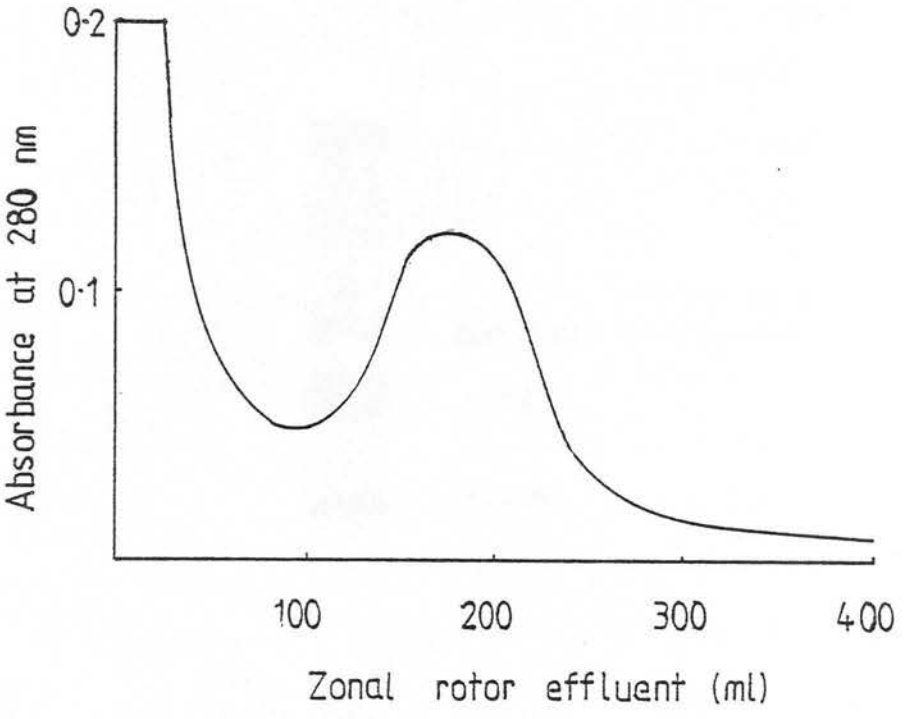


Fig. 5.1 Rate zonal ultracentrifugation of rat plasma lipoproteins. Rat lipoproteins were subjected to rate zonal ultracentrifugation in a 1.0 - 1.4g ml^{-1} sodium bromide gradient according to the method of Oschry and Eisenberg (1982).

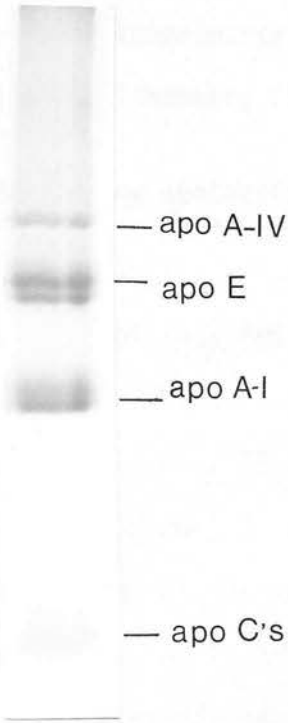


Fig. 5.2 SDS-PAGE of the apoproteins of rat plasma HDL_2. Following delipidation of the HDL_2-apoproteins approximately 100 µg of HDL_2-protein was loaded onto gels (15% acrylamide).

content due to HDL_2 in the culture medium was determined (Table 5.1). The results show that for every 500 µg of HDL_2 protein, there is 360 µg of cholesterol, with 35.9 µg of this cholesterol being in the unesterified form. The ratios of cholesterol : protein and unesterified cholesterol : total cholesterol were also calculated so that the HDL_2 used in these experiments could be compared with the HDL_2 prepared by Oschry and Eisenberg (1982). (Table 5.1).

5.3 The effect of HDL_2 on the synthesis of bile salts by rat hepatocyte monolayers

Hepatocytes prepared from rats fed a pellet diet were maintained in monolayers for up to 24h. Hepatocytes were incubated in the presence or absence of HDL_2 (500 µg HDL_2 protein ml^{-1}) and the effect on the synthesis of conjugated cholic, conjugated chenodeoxycholic and conjugated β-muricholic acids was determined. The synthesis of these bile acid conjugates was not affected by incubating hepatocytes with HDL_2 (Fig. 5.3). However the experiment was repeated using hepatocytes obtained from rats fed the bile salt sequestrant, cholestyramine. Incubation of these hepatocytes with HDL_2 increased the synthesis of conjugated cholic and conjugated β-muricholic acids but this increase was not significant at all of the time points (Fig. 5.4). Synthesis of conjugated chenodeoxycholic acid was significantly increased in the first 3h of incubation but no change was observed when compared to the control at 19h and 24h. Total synthesis of bile salts (i.e. conjugated cholic acid + conjugated chenodeoxycholic acid + conjugated β-muricholic acid) was significantly increased by hepatocytes incubated in the presence of HDL_2 (Fig. 5.5).

	Protein	Free cholesterol (FC)	Total cholesterol (TC)	FC / TC	TC / Protein
This study	500	35.9	360	0.10	0.72
μg/ml culture medium					
Oschry and Eisenberg (1982)	40.5	3.6	25.5	0.12	0.73
mg/100mg lipoprotein					

Table 5.1 Protein and cholesterol content of rat HDL_2. The protein and cholesterol content of rat HDL_2 prepared for use in this study was compared to the HDL_2 prepared by Oschry and Eisenberg.

Fig. 5.3 The effect of HDL_2 on the synthesis of bile salts by rat hepatocytes prepared from rats fed the pellet diet. The effect of HDL_2 (●—●) on (A) the synthesis of conjugated cholic acid (B) the synthesis of conjugated β-muricholic acid (C) the synthesis of conjugated chenodeoxycholic acid as compared to a control without HDL_2 (○—○) were determined. Each point represents the mean of duplicate determinations from hepatocytes obtained from two rats. Bile salts detected at 0h represent cell-associated levels after adhesion of the hepatocytes to the culture surface had taken place. Error bars show ± range of the values obtained.

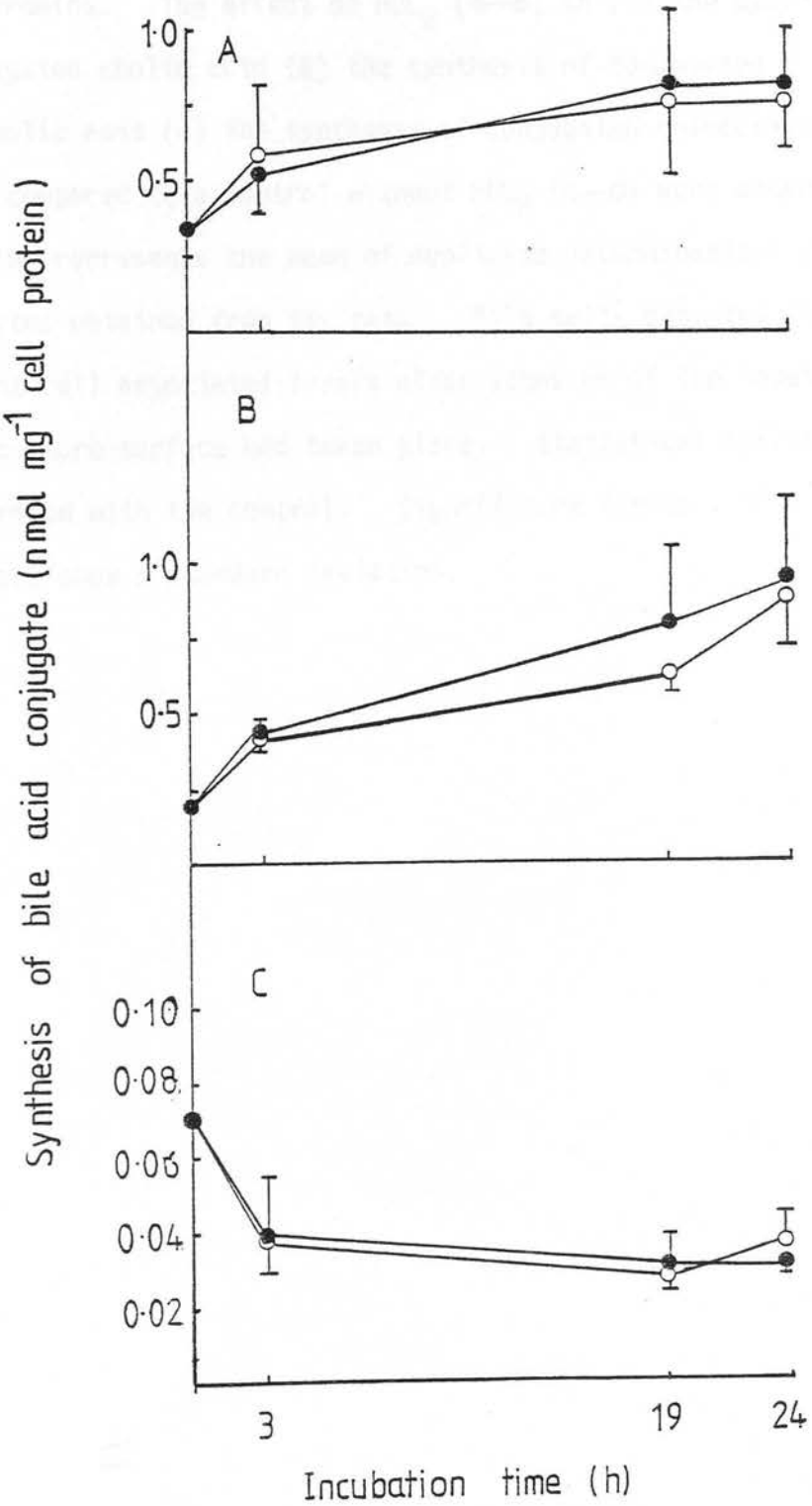

A
B
C
1·0
0·5
1·0
0·5
0·10
0·08
0·06
0·04
0·02
3
19
24
Incubation time (h)
Synthesis of bile acid conjugate (n mol mg^{-1} cell protein)

Fig. 5.3

Fig. 5.4 The effect of HDL_2 on the synthesis of bile salts by rat hepatocytes obtained from rats fed the 'soft diet' + 4% cholestyramine. The effect of HDL_2 (●—●) on (A) the synthesis of conjugated cholic acid (B) the synthesis of conjugated β-muricholic acid (C) the synthesis of conjugated chenodeoxycholic acid as compared to a control without HDL_2 (○—○) were determined. Each point represents the mean of duplicate determinations from hepatocytes obtained from six rats. Bile salts detected at 0h represent cell associated levels after adhesion of the hepatocytes to the culture surface had taken place. Statistical analysis is a comparison with the control. Significance limits : $*P < 0.05$. Error bars show ± standard deviation.

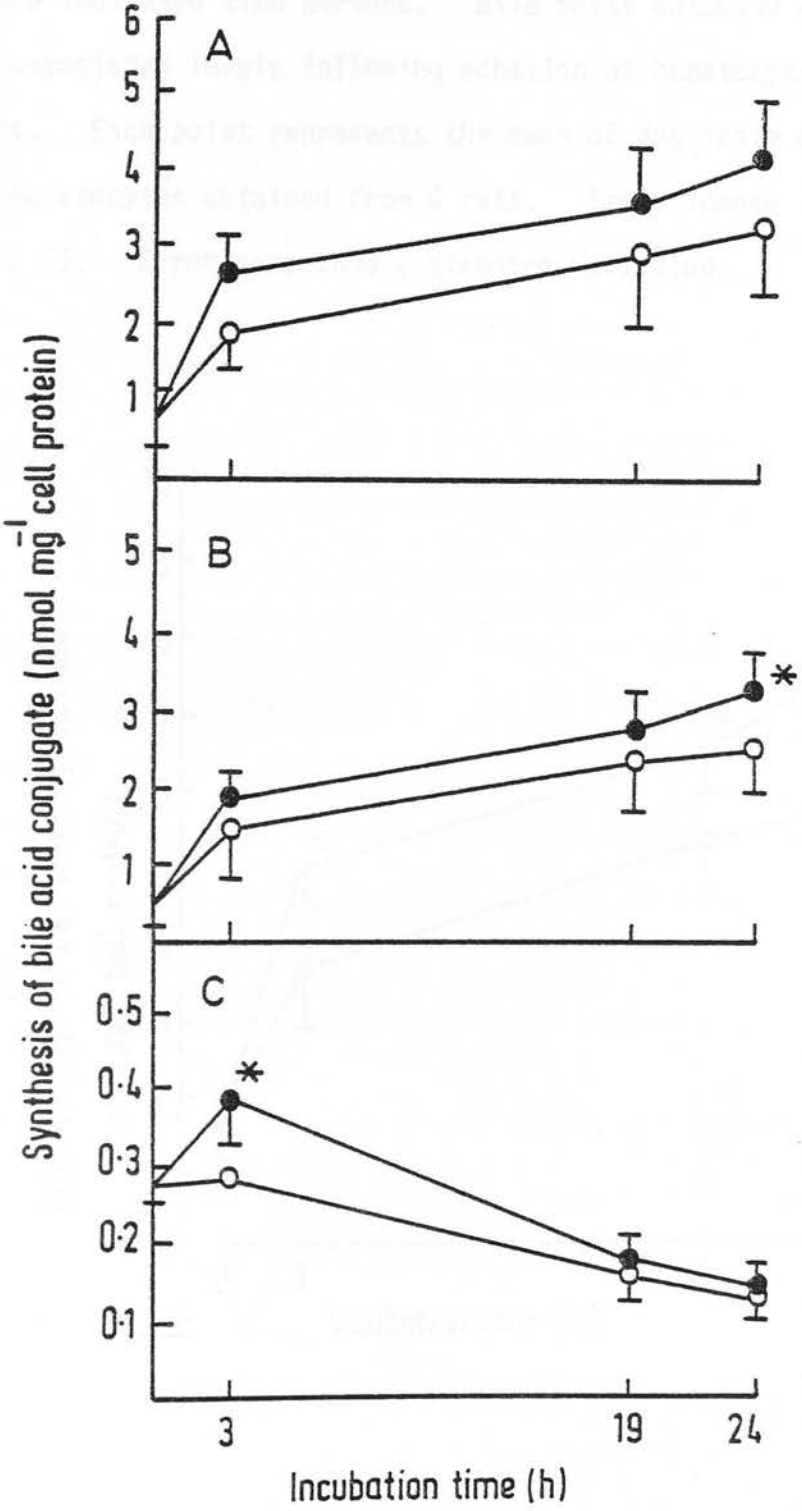
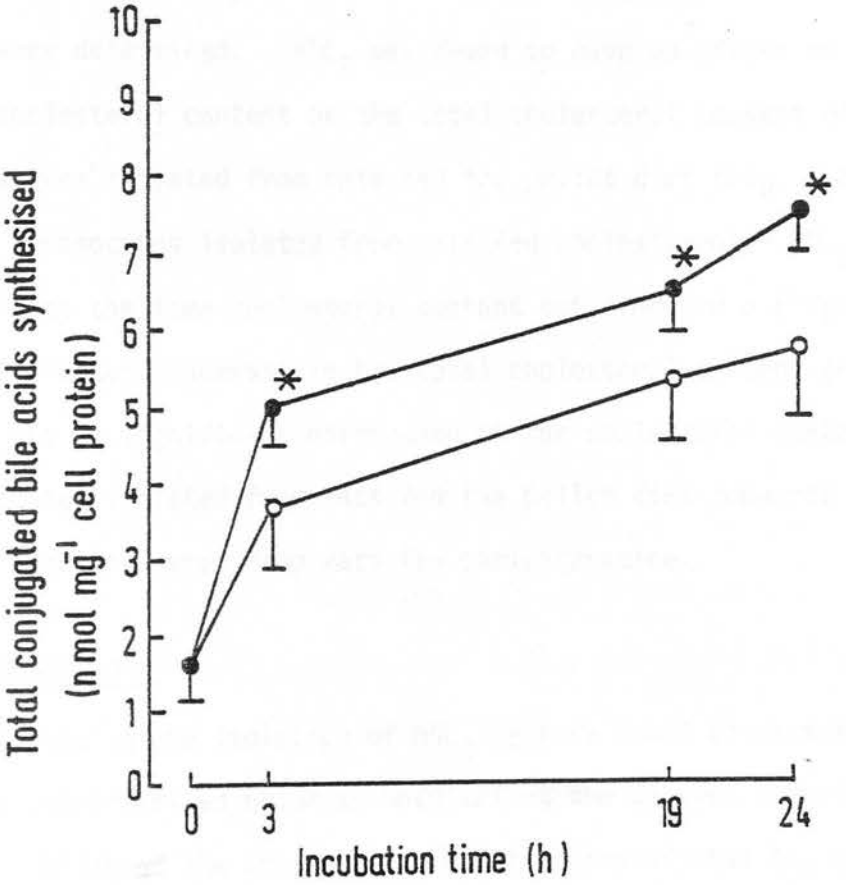

A
B
C
Synthesis of bile acid conjugate (nmol mg^{-1} cell protein)
Incubation time (h)
3
19
24
*
*

Fig.5.4.

Fig. 5.5 The effect of HDL_2 on the total synthesis of bile salts by rat hepatocyte monolayers prepared from rats fed a diet containing cholestyramine. Hepatocytes were maintained in monolayers in the absence (o—o) or presence (●—●) of HDL_2 (500 µg HDL_2 protein ml^{-1}) for the indicated time periods. Bile salts detected at 0h represent cell associated levels following adhesion of hepatocytes to Petri dishes. Each point represents the mean of duplicate determinations from hepatocytes obtained from 6 rats. Significance limits : *P < 0.05. Error bars show ± standard deviation.



The ratio of conjugated cholic acid : conjugated chenodeoxycholic acid + conjugated β-muricholic acid and the ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic acid were also determined. These ratios provide good indications of the rates of 12α-hydroxylation and 6β-hydroxylation respectively (Chapter 4.2). HDL_2 did not affect these ratios in hepatocytes isolated from rats fed the pellet diet or the diet supplemented with cholestyramine.

5.4 The effect of HDL_2 on the cellular cholesterol content of rat hepatocytes

The free cholesterol content and the total cholesterol content of hepatocyte monolayers incubated in the presence or absence of HDL_2 were determined. HDL_2 was found to have no effect on the free cholesterol content or the total cholesterol content of hepatocytes isolated from rats fed the pellet diet (Fig. 5.6). In those hepatocytes isolated from rats fed cholestyramine HDL_2 did not affect the free cholesterol content but produced a slight but not significant increase in the total cholesterol content (Fig. 5.7). There was no significant difference in the cholesterol content of hepatocytes isolated from rats fed the pellet diet compared to those hepatocytes prepared from rats fed cholestyramine.

5.5 Discussion

Following the isolation of HDL_2 by rate zonal ultracentrifugation it was characterised prior to undertaking the desired experiments. Determination of the apoprotein content indicated that the major apoproteins reported to be associated with HDL_2 were present. Apo A-I was the major apoprotein present and it is known to be involved in the activation of LCAT. The presence of apo E was also

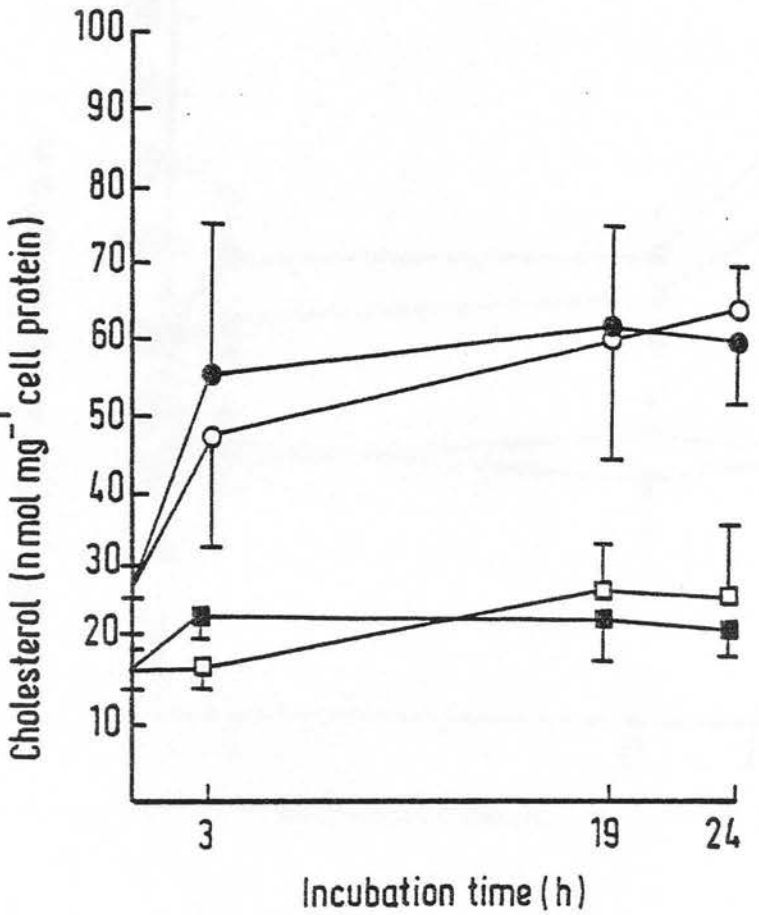


Fig. 5.6 Effect of HDL_2 on the cellular cholesterol content of rat hepatocytes. Hepatocytes isolated from rats fed the pellet diet were maintained in monolayers for the indicated time periods. The free cholesterol (□—□ control ; ■—■ + HDL_2) and the total cholesterol (○—○ control ; ●—● + HDL_2) content of hepatocytes were determined. Each point represents the mean of duplicate determinations from hepatocytes obtained from 2 rats. Error bars show ± standard deviation of the results obtained.

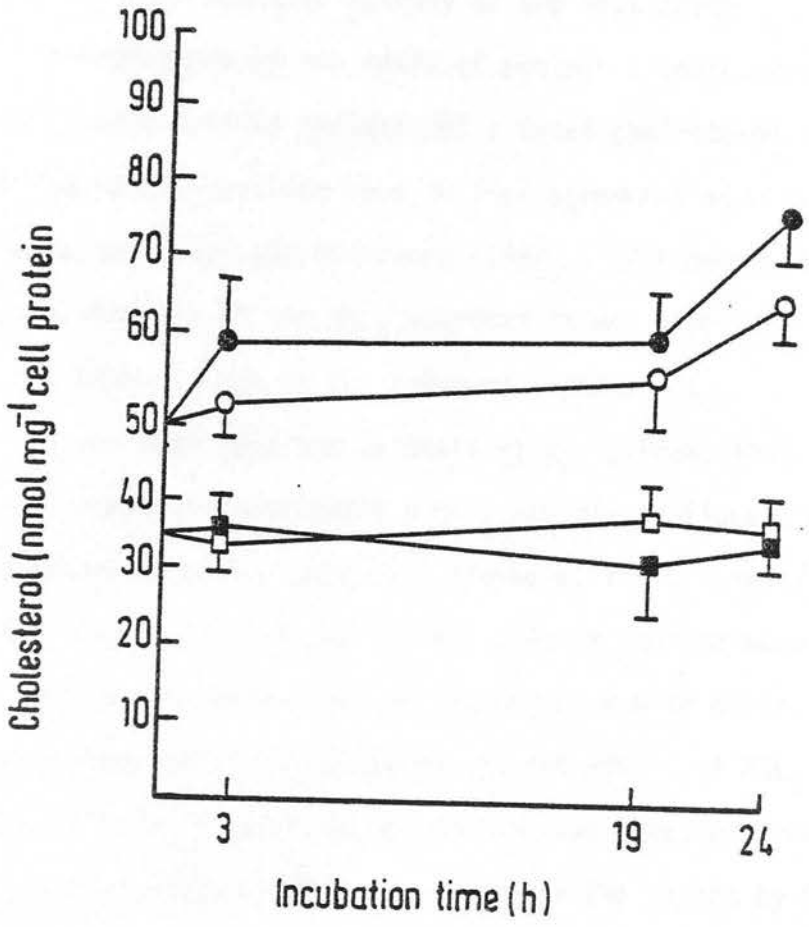


Fig. 5.7 Effect of HDL_2 on the cellular cholesterol content of rat hepatocytes. Hepatocytes isolated from rats fed the soft diet + 4% cholestyramine were maintained in monolayers for the indicated time periods. The free cholesterol (□—□ control ; ■—■ + HDL_2) and the total cholesterol (○—○ control ; ●—● + HDL_2) content of hepatocytes was determined. Each point represents the mean of duplicate determinations from hepatocytes obtained from six rats.

Error bars show ± standard deviation.

confirmed. This apoprotein is essential for the uptake of HDL_2 by the receptor-mediated pathway of the hepatocyte.

Determination of the ratio of protein : cholesterol and the ratio of unesterified cholesterol : total cholesterol indicated that the values obtained were in good agreement with those reported by Oschry and Eisenberg (1982). Following the characterisation of the HDL_2 obtained it was concluded that it was suitable for use in the intended experiments.

It has been reported by Davis et al. (1983a) that incubation of rat hepatocyte monolayers with a rat plasma lipoprotein fraction of density < 1.02g ml^{-1} increases the synthesis of bile acids. Incubation of LDL and HDL with hepatocyte monolayers was found to have no effect on the synthesis of bile salts. The results presented in this chapter on the effect of HDL_2 on the synthesis of bile salts by rat hepatocytes obtained from rats fed a pellet diet, confirm one aspect of the report by Davis et al. (1983a). However, the results obtained with hepatocytes prepared from rats cholestyramine indicate for the first time that HDL can increase the synthesis of bile salts. These results appear to indicate that under certain conditions HDL can deliver cholesterol to the liver for degradation to bile salts. However, this does not necessarily mean that HDL functions in vivo to transport cholesterol from extrahepatic tissues to the liver for its degradation. It is important to note that cholestyramine has a number of effects on the metabolism of cholesterol in the liver. It has been shown in Chapter 4 that cholestyramine stimulates the synthesis of bile salts. The mechanism by which this occurs is not fully understood.

However, dietary cholestyramine also results in both an increase in the synthesis of cholesterol by the liver (Huff et al., 1963; Fears and Morgan, 1976) and an increase in the uptake of cholesterol from the plasma. In humans (Shepherd et al., 1980) and rabbits (Slater et al., 1980) feeding cholestyramine results in an increase in the clearance of LDL from plasma. Slater et al. (1980) showed that, whilst other tissues contribute, the major organ responsible for the increase in the catabolism of LDL was the liver. Further studies in dogs indicated that the increase in the catabolism of LDL was probably due to an increase in the expression of the apo B/E receptor on the hepatocyte plasma membrane (Hui et al., 1981; Chao et al., 1982). Uptake of LDL by the liver is mediated through recognition of apo B on the surface of the LDL particle, by the hepatocyte B/E receptor. Although these studies were performed in different species, feeding cholestyramine appears to involve an increase in the availability of cholesterol to the hepatocyte due to an increase in the uptake of plasma cholesterol and a stimulation in the synthesis of cholesterol. It is known that a proportion of the increase in the cholesterol delivered to the hepatocyte is utilized for the increased synthesis of bile salts. However, the contribution of cholesterol derived from de novo synthesis versus cholesterol from plasma, in supplying the substrate for the degradation to bile salts is unknown.

The results indicate that HDL_2 can increase the synthesis of bile salts by hepatocytes maintained in monolayers, but as yet there is no indication as to the mechanism by which this occurs. There are however two possibilities. As has been

suggested, HDL_2 may function to deliver cholesterol to the liver for degradation. This may also involve an increase in the synthesis of bile salts by stimulation of cholesterol 7α-hydroxylase activity. Alternatively the presence of HDL_2 in the hepatocyte culture medium may serve to provide an additional source of substrate to satisfy the increase in the activity of cholesterol 7α-hydroxylase as a result of feeding cholestyramine. It is possible that in hepatocytes isolated from rats fed cholestyramine there is a deficiency in the cholesterol available for degradation to bile salts.

In vivo, the increase in the supply of cholesterol required for the synthesis of bile salts as a result of feeding cholestyramine can be met by both an increase in de novo synthesis of cholesterol and an increase in the uptake of cholesterol from the plasma. However, in those hepatocytes maintained in lipoprotein-free medium all of the cholesterol utilized for the synthesis of bile salts has to be derived from newly synthesised cholesterol. (In the first few hours of incubation some of the cholesterol used by the hepatocyte for the synthesis of bile salts may be derived from a preformed pool within the cell. However, this is likely to be utilized within the first few hours of incubation (Kempen et al., 1982)). In those hepatocytes incubated in lipoprotein-free medium the newly synthesised cholesterol supplied to the substrate pool for the synthesis of bile salts may not be sufficient to meet the increase in the supply of substrate necessary as a result of feeding cholestyramine. The addition of HDL_2 to the culture medium would therefore serve to provide the additional cholesterol required for the synthesis of

bile salts. The observation that the presence of HDL_2 in the hepatocyte culture medium did not increase the synthesis of bile salts by hepatocytes prepared from rats fed the pellet diet further suggests that the availability of cholesterol for the synthesis of bile salts may be limiting in hepatocytes isolated from rats fed cholestyramine. If this were the case the increase in the synthesis of bile salts observed in the presence of HDL_2 would not be due to the stimulation of cholesterol 7α-hydroxylase by HDL_2. This possibility will require further investigation.

If the function of HDL_2 is to deliver cholesterol to the liver for its degradation it might be expected that the incubation of HDL_2 with hepatocytes obtained from rats fed the pellet diet would also result in an increase in the synthesis of bile salts. However, it is important to stress that this conclusion may not be valid. If incubation of hepatocytes with HDL_2 results in the inhibition of the synthesis of cholesterol, the total amount of cholesterol available within the hepatocyte may not change. HDL_2 could, by decreasing the contribution of newly synthesised cholesterol to the synthesis of bile salts, supply cholesterol for degradation without producing an increase in the total bile salts synthesised. It is clear that the effects of HDL_2 observed on the synthesis of bile salts by hepatocytes require further investigation.

Determination of the ratio of conjugated cholic acid : conjugated chenodeoxycholic acid + conjugated β-muricholic acid and the ratio of conjugated chenodeoxycholic acid : conjugated β-muricholic acid in the presence of HDL_2 showed that this

lipoprotein fraction did not affect these ratios when compared to the control (data not shown). As these ratios were unaffected it appears that the HDL_2-cholesterol utilized for the synthesis of bile salts enters a common precursor pool for the synthesis of the major bile acid conjugates measured.

In an attempt to establish whether the increase in the catabolism of cholesterol to bile salts by hepatocytes prepared from rats fed cholestyramine, was as a direct result on an increase in the availability of cholesterol, the free and total cholesterol content of hepatocytes was determined. HDL_2 did not affect these parameters in hepatocytes isolated from rats fed the control diet or the diet supplemented with cholestyramine. It therefore appears likely that uptake of HDL_2 by hepatocytes results in the cholesterol being utilized for either the assembly of lipoproteins, secretion as biliary cholesterol or the degradation to bile salts. Utilization of HDL_2-cholesterol in this way would result in the hepatocyte maintaining the intracellular cholesterol content at a constant level.

In conclusion, interruption of the enterohepatic circulation by feeding dietary cholestyramine results in an increase in the catabolism of cholesterol to bile salts which is not evident in hepatocytes isolated from rats fed the control diet. The possible mechanisms involved in the increase in the synthesis of bile salts by hepatocytes are discussed in Chapter 6.

CHAPTER 6

THE CATABOLISM OF HDL_2-CHOLESTEROL BY RAT HEPATOCYTE MONOLAYERS

6.1 Introduction

The results in Chapter 5 have shown that under certain conditions HDL_2 can stimulate the synthesis of bile salts by rat hepatocyte monolayers. This stimulation in the synthesis of bile salts may require an increase in the supply of cholesterol which can be obtained from either a stimulation in the synthesis of cholesterol or through uptake of HDL_2-cholesterol. The contribution of each of these sources in providing substrate for the synthesis of bile salts by hepatocytes is unknown. However, in those hepatocytes incubated with control medium, the cholesterol used for the synthesis of bile salts has to be obtained entirely from newly-synthesised cholesterol. Addition of HDL_2 to the culture medium provides an alternative source of cholesterol for degradation to bile salts. It would therefore seem likely that the increase in the synthesis of bile salts observed on addition of HDL_2 to the culture medium was partly due to uptake and subsequent degradation of HDL_2-cholesterol. However, incubation of HDL with some cell types from extrahepatic tissues has been reported to facilitate the removal of cellular cholesterol and result in a stimulation of cholesterol synthesis (Stein *et al.*, 1977; Henriksson *et al.*, 1979; Stoudemire *et al.*, 1980). It is therefore possible that a stimulation in cholesterol synthesis by hepatocytes incubated in the presence of HDL_2 could result in newly synthesised cholesterol supplying the increase in the substrate required for the synthesis of bile salts.

To determine the source of cholesterol for synthesis of bile salts rat hepatocyte monolayers were incubated in medium containing HDL_2 labelled with either [4-^{14}C]cholesterol or [4-^{14}C]cholesteryl oleate. This enabled the determination of the contribution of HDL_2-free cholesterol and HDL_2-cholesteryl oleate to the synthesis of bile salts. The effect of HDL_2 on the synthesis of cholesterol and the utilization of newly-synthesised cholesterol for the synthesis of bile salts by rat hepatocytes was also determined. This was achieved by incubating hepatocyte monolayers in medium containing [^{14}C]acetate in the presence and absence of HDL_2.

6.2 Radiolabelling of HDL_2

The HDL_2 fraction of rat plasma was radiolabelled with either [4-^{14}C]cholesterol or [4-^{14}C]cholesteryl oleate as described in Chapter 2.8.

6.2.1 HDL_2 radiolabelled with [4-^{14}C]cholesterol

Following the radiolabelling of HDL_2 with [4-^{14}C]cholesterol the specific activity of the HDL_2-free cholesterol was determined. The specific activity of the free cholesterol was 68452 CPM nmol^{-1} of free cholesterol.

6.2.2 HDL_2 radiolabelled with [4-^{14}C]cholesteryl oleate

HDL_2 was radiolabelled in the cholesteryl ester moiety by transfer of [4-^{14}C]cholesteryl oleate from a lipid microemulsion to the HDL_2. Transfer of the [4-^{14}C]cholesteryl oleate was facilitated by a cholesteryl ester exchange protein present in human plasma (Barter and Lally, 1978).

Labelling of the whole lipoprotein fraction, followed by isolation of HDL_2 by rate zonal ultracentrifugation gave poor results. The percentage transfer of [4-^{14}C]cholesteryl oleate to HDL_2 was approximately 5%. However by radiolabelling the crude HDL fraction after removal of the VLDL and LDL fraction by density gradient ultracentrifugation (Chapter 2.8) 18% of the [4-^{14}C]cholesteryl oleate was transferred to the HDL_2 fraction. The specific activity of the esterified cholesterol was found to be 7.38 CPM $nmol^{-1}$. If the labelling procedure was repeated using rat lipoprotein-free plasma the transfer of [^{14}C]cholesteryl oleate was less than 1%. This would appear to confirm a previous report that the activity of the cholesteryl ester transfer protein in rat plasma is very low (Oschry and Eisenberg, 1982).

Following the isolation of HDL_2 labelled in the cholesteryl ester moiety it was characterised by its apoprotein content. Following delipidation HDL_2 apoproteins were subjected to SDS-PAGE (Fig. 6.1). The gels obtained indicated that the HDL_2 fraction was contaminated with albumin and had proportionately less apo A-IV and apo E than was found in the untreated HDL_2 (Chapter 5, Fig. 5.2). This serious loss of apoproteins may be due to the relatively harsh conditions of radiolabelling used. The abnormal protein content of this HDL_2 fraction will be discussed in Chapter 6.8.

6.3 The entry of HDL_2-cholesterol into rat hepatocyte monolayers following incubation in culture medium containing HDL_2-[4-^{14}C]cholesterol or HDL_2-[4-^{14}C]cholesteryl oleate

Hepatocyte monolayers were incubated in the presence of HDL_2 labelled in either the free cholesterol or cholesteryl ester moiety. The entry of the radiolabelled cholesterol into hepatocytes was

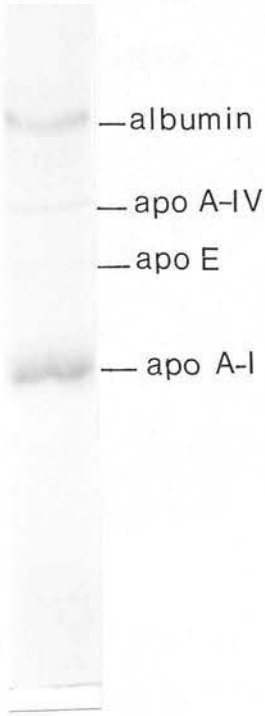


Fig. 6.1 Apoprotein content of HDL_2 radiolabelled with [4-^{14}C]cholesteryl oleate.

subsequently determined.

6.3.1 Entry of HDL_2-free cholesterol into hepatocytes

Hepatocyte monolayers prepared from rats fed cholestyramine were incubated with HDL_2 radiolabelled with [4-^{14}C]cholesterol for 3, 19 and 24h. After the indicated time periods the medium was aspirated and stored at -20^{o}C. The cells were washed with 3 x 1ml of phosphate buffered saline, 1M sodium hydroxide was added and the cells were scraped from the Petri dishes. The radioactivity attributable to cholesterol and cholesteryl esters was determined as described in Chapter 2.10. Uptake of HDL_2-free cholesterol into the hepatocyte was rapid during the first 3h of incubation but increased at a slower rate between 3h and 24h. (Fig. 6.2). The results also showed that uptake of HDL_2-free cholesterol into hepatocytes resulted in a proportion of this cholesterol being esterified.

Incubation of HDL_2 with hepatocyte monolayers therefore results in the uptake and subsequent entry of HDL_2-cholesterol into the hepatocyte cholesterol pool. However, these experiments do not indicate whether this cholesterol enters the hepatocyte via the non-receptor - or receptor-mediated pathway (Chapter 1.2.3). However it is likely that some of the HDL_2-free cholesterol enters the hepatocyte through exchange of cholesterol with the hepatocyte plasma membrane.

6.3.2 The entry of HDL_2-[4-^{14}C]cholesteryl oleate into hepatocytes

Entry of HDL_2-cholesteryl ester into hepatocytes was determined in the same way as was described for determination of the entry of HDL_2-free cholesterol.

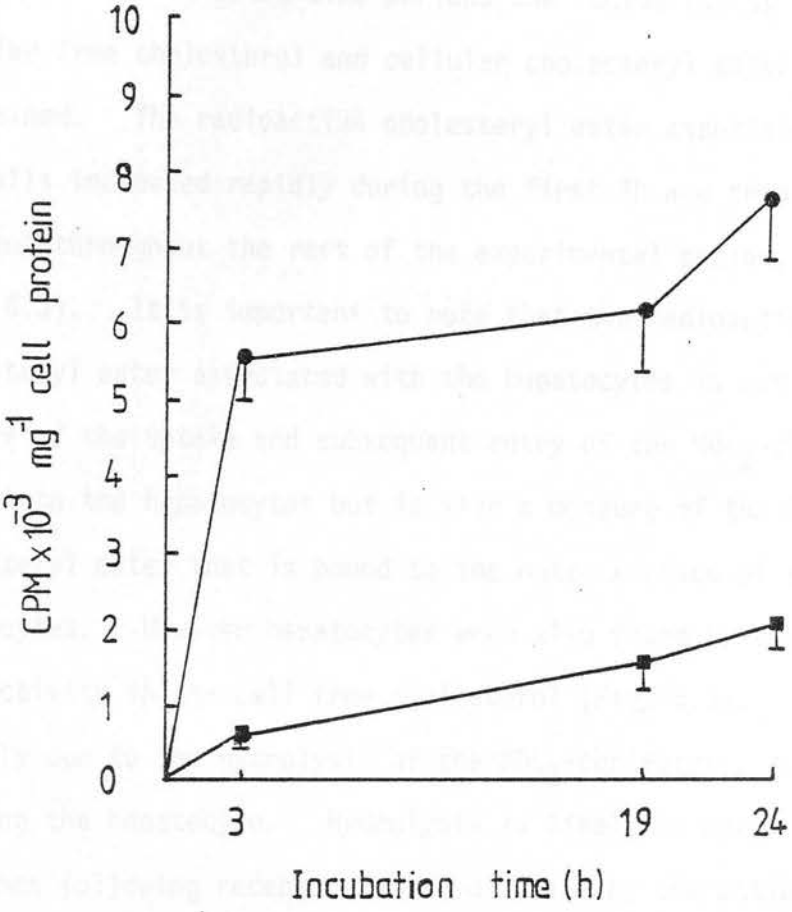


Fig. 6.2 Entry of HDL_2-free cholesterol into hepatocytes. The entry of HDL_2-free cholesterol into rat hepatocytes was determined as described in the text. The radioactivity attributable to free cholesterol (●—●) and cholesteryl ester (■—■) was determined as described in Chapter 2.10 and 2.11. The results are the mean ± the range of duplicate determinations from hepatocytes obtained from 2 rats.

After the indicated time periods the radioactivity due to cellular free cholesterol and cellular cholesteryl ester was determined. The radioactive cholesteryl ester associated with the cells increased rapidly during the first 3h and then remained constant throughout the rest of the experimental period. (Fig. 6.3). It is important to note that the radioactive cholesteryl ester associated with the hepatocytes is not only a measure of the uptake and subsequent entry of the HDL_2-cholesteryl ester into the hepatocytes but is also a measure of the HDL_2-cholesteryl ester that is bound to the outer surface of the hepatocytes. However hepatocytes were also found to contain radioactivity in the cell free cholesterol (Fig. 6.3). This is probably due to the hydrolysis of the HDL_2-cholesteryl ester on entering the hepatocyte. Hydrolysis is likely to occur in the lysosomes following receptor-mediated uptake by the action of the lysosomal cholesterol esterase (Chapter 1.2.1). The appearance of radioactive free cholesterol in the hepatocytes occurs very rapidly in the first 3h and remains fairly constant up to 19h. However, between 19 - 24h the radioactivity attributable to free cholesterol falls quite markedly (Fig. 6.3). The reason for this is unclear. However, it would seem reasonable to suggest that between 3 - 19h the supply of cholesterol to the hepatocyte free cholesterol pool from HDL_2-cholesteryl ester is equal to the utilization of that free cholesterol. During the 19h - 24h time points the output of the cholesterol from hepatocytes that is derived from HDL_2-cholesteryl ester exceeds the input resulting in a decrease in the intracellular free cholesterol derived from uptake of HDL_2-cholesteryl ester.

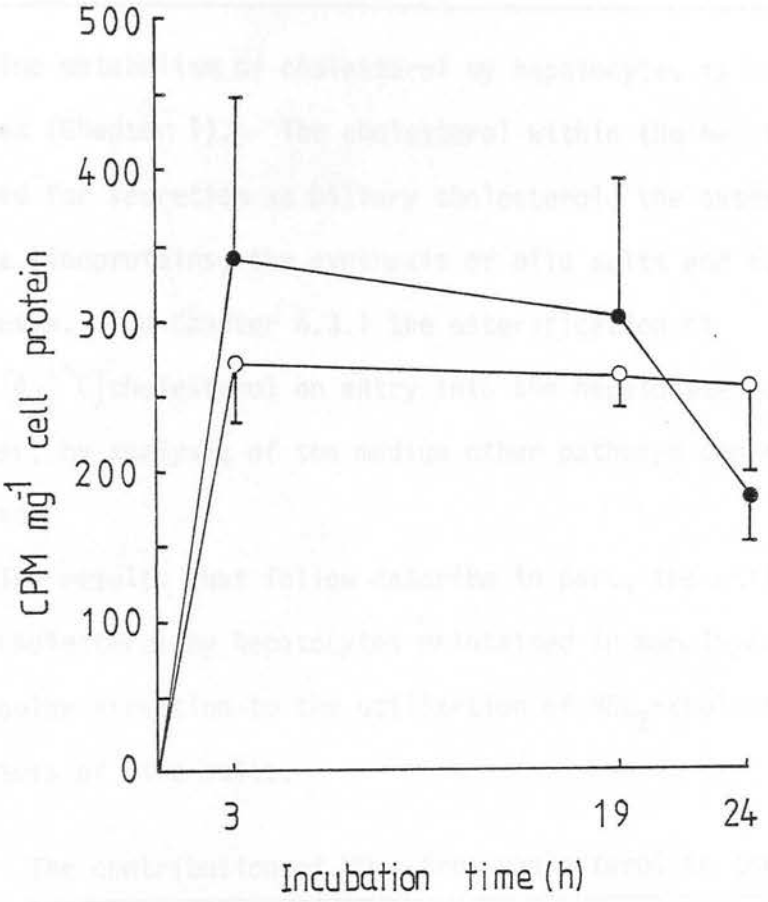


Fig. 6.3 Entry of HDL_2-cholesteryl ester into hepatocytes. The entry of HDL_2-cholesteryl ester into the hepatocyte was determined as described in the text. The results show the cellular radioactivity due to free cholesterol (●—●) and cholesteryl ester (○—○). The results are the mean ± the range of duplicate determinations from hepatocytes obtained from 2 rats.

6.4 The utilization of HDL_2-cholesterol by hepatocyte monolayers

The metabolism of cholesterol by hepatocytes is extremely complex (Chapter 1). The cholesterol within the hepatocyte can be used for secretion as biliary cholesterol, the assembly of plasma lipoproteins, the synthesis of bile salts and also membrane synthesis. In Chapter 6.3.1 the esterification of HDL_2-[4-^{14}C]cholesterol on entry into the hepatocyte was described. However, by analysis of the medium other pathways can also be studied.

The results that follow describe in part, the utilization of HDL_2-cholesterol by hepatocytes maintained in monolayers with particular attention to the utilization of HDL_2-cholesterol for the synthesis of bile salts.

6.4.1 The contribution of HDL_2-free cholesterol to the synthesis of bile salts

Following the incubation of hepatocyte monolayers for the indicated time periods the radioactivity due to bile salts was determined as described in Chapter 2.10. The results indicate that HDL_2 can supply free cholesterol for degradation to bile salts (Fig. 6.4). The degradation of HDL_2-free cholesterol to bile salts is constant throughout the duration of the experiment (Fig. 6.4).

A comparison of the specific activities of the cell free-cholesterol with the specific activity of the bile salts indicated that the cell free-cholesterol specific activity is significantly lower than the specific activity of the bile salts (Fig. 6.5). It would seem unlikely that all of the free cholesterol within the hepatocyte is available for the synthesis of bile salts,

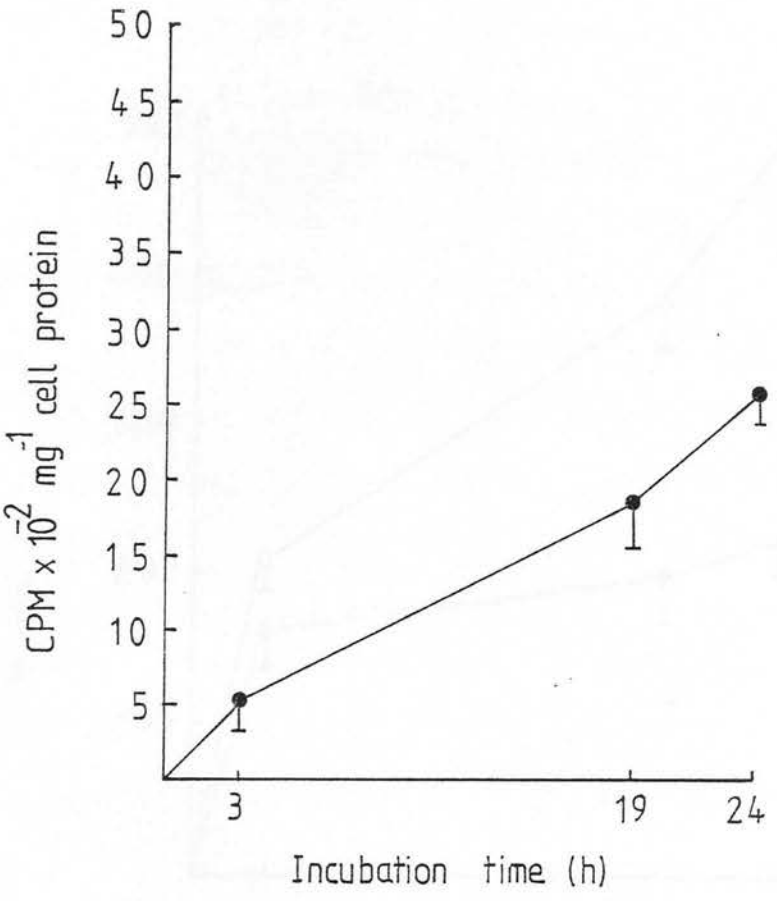


Fig. 6.4 The utilization of HDL_2-free cholesterol for the synthesis of bile salts by rat hepatocytes. The secretion of bile salts derived from HDL_2-free cholesterol into the hepatocyte culture medium was determined as described in the text. The results shown are the mean ± the range of duplicate determinations from hepatocytes obtained from 2 rats.

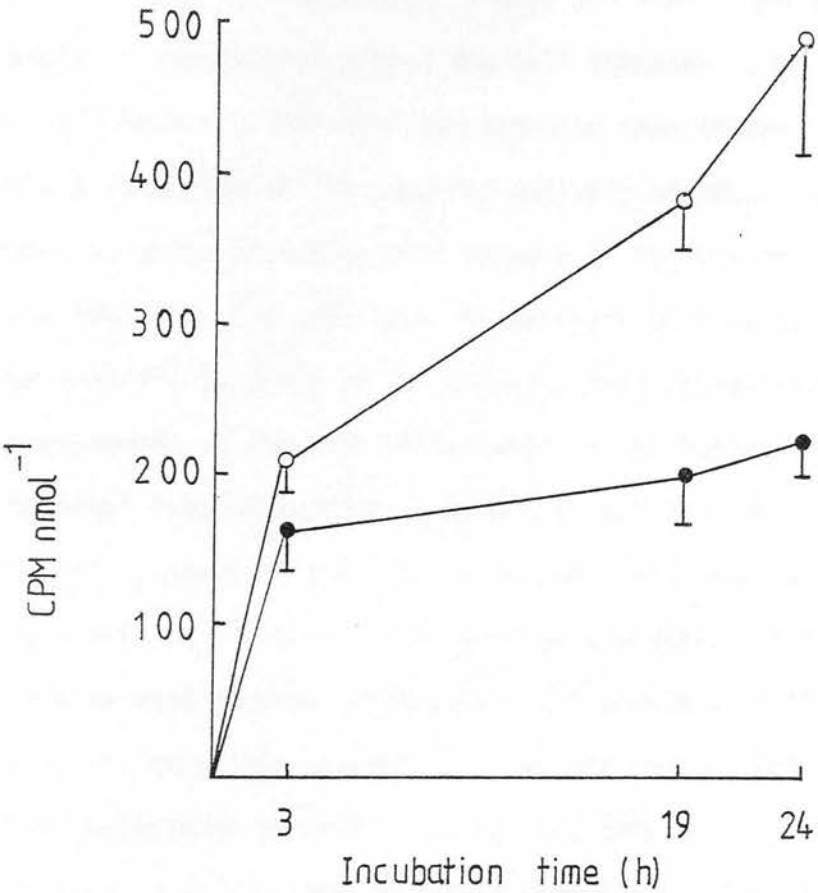


Fig. 6.5 Comparison of the specific activity of the cell free cholesterol with the specific activity of the bile salts. Following incubation of hepatocytes with HDL_2-[4-^{14}C]free cholesterol the specific activity of the hepatocyte free cholesterol (●—●) and the specific activity of the bile salts secreted into the medium (○—○) was determined. The results are the mean ± the range of duplicate determinations from hepatocytes obtained from 2 rats.

particularly as a proportion of the free cholesterol within the cell serves a structural function. If after entry of HDL_2-free cholesterol into the hepatocyte it does not freely equilibrate with the pools of cholesterol within the cell (Chapter 1.6) then some pools will have a higher specific activity than others. Evidence for the possibility of the specific delivery of HDL_2-free cholesterol to specific pools of cholesterol appears to be provided by the results that show the high specific activity of bile salts compared to the specific activity of the cellular free cholesterol (Fig. 6.5). The measurement of the free cholesterol in the hepatocytes is limited to the total free cholesterol within the cell and does not measure the cholesterol content of the individual pools of cholesterol. The specific activity of the hepatocyte free cholesterol therefore shows the average distribution of HDL_2-[4-^{14}C]cholesterol within the cell and does not take into account the possible non-uniform distribution of this cholesterol on entry into the hepatocyte.

Having shown that the HDL_2-free cholesterol could provide substrate for the synthesis of bile salts the proportion of bile salts synthesised from cholesterol derived from this source was determined. Calculations were performed by comparing the specific activity of the lipoprotein-free cholesterol with the specific activity of the bile salts. If the specific activity of the bile salts was found to be the same as the specific activity of the HDL_2-free cholesterol then all of the substrate for the synthesis of bile salts would be derived from HDL_2-free cholesterol. This, of course, would be an extreme situation! The results show that the contribution of HDL_2-free cholesterol to the synthesis of bile salts steadily increases throughout the incubation but does not exceed 1%. (Table 6.1). It was therefore concluded that HDL_2-free cholesterol

Incubation time (h)	3	19	24
1. Specific activity of bile salts (CPM $nmol^{-1}$) (Fig. 6.5).	210 ± 43	381 ± 54	488 ± 85
2. pmol of HDL_2-free cholesterol degraded to bile salts $nmol^{-1}$ of bile salts in the medium (Specific activity of HDL_2-free cholesterol ÷ line 1)	3.06 ± 0.63	5.57 ± 0.69	7.12 ± 1.25
3. Bile salts secreted into the medium (nmol mg^{-1} cell protein)	2.66 ± 0.57	4.86 ± 0.36	5.54 ± 0.9
4. Total HDL_2-free cholesterol degraded to bile salts i.e. line 2 x line 3 (pmol mg^{-1} of cell protein).	8.14 ± 2.83	27.07 ± 4.06	39.44 ± 2.98
5. % of bile salts synthesised from HDL_2-free cholesterol (4 ÷ 3 x 100).	0.31	0.56	0.71

Table 6.1 The degradation of HDL_2-free cholesterol to bile salts by rat hepatocyte monolayers. The proportion of bile-salts synthesised from HDL_2-free cholesterol by hepatocytes was calculated by comparing the specific activity of the bile salts with the specific activity of HDL_2-free cholesterol. The specific activity of the HDL_2-free cholesterol was calculated to be 68452 CPM $nmol^{-1}$. Results show the mean of duplicate determinations from hepatocytes obtained from two rats.

is not a major source of cholesterol for degradation to bile salts. It therefore appears that the major source of cholesterol for the synthesis of bile salts in these incubations is cholesterol derived from HDL_2-cholesteryl esters or from newly synthesised cholesterol.

6.4.2 The contribution of HDL_2-cholesteryl ester to the synthesis of bile salts

The contribution of HDL_2-cholesteryl ester in providing substrate for the synthesis of bile salts was determined by analysis of the culture medium after 3, 19 and 24h. The radioactivity associated with the bile salt fraction and the specific activity of the bile salts was determined in the same way as was described in section 6.4.1. The radioactivity associated with the bile salts steadily increased throughout the incubation (Fig. 6.6).

A comparison of the specific activity of the cellular free cholesterol pool in hepatocytes incubated with HDL_2-[4-^{14}C]cholesteryl oleate with the specific activity of the bile salts indicated that the specific activity of the free cholesterol was higher than the specific activity of the bile salts (Fig. 6.7). This is the opposite to what was observed when cells were incubated with HDL_2-[4^{-14}C]cholesterol. This probably indicates that whilst HDL_2-cholesteryl esters can supply cholesterol for the synthesis of bile salts the preferred fate for this esterified cholesterol is into pathways other than the synthesis of bile salts i.e. lipoprotein cholesterol and biliary cholesterol. Having established that HDL_2-cholesteryl esters supply cholesterol for degradation to bile salts the contribution of cholesterol from this source to the synthesis of bile salts was determined. The results are shown in Table 6.2. The results show that all of the increase in the cholesterol required for the synthesis of bile salts can be

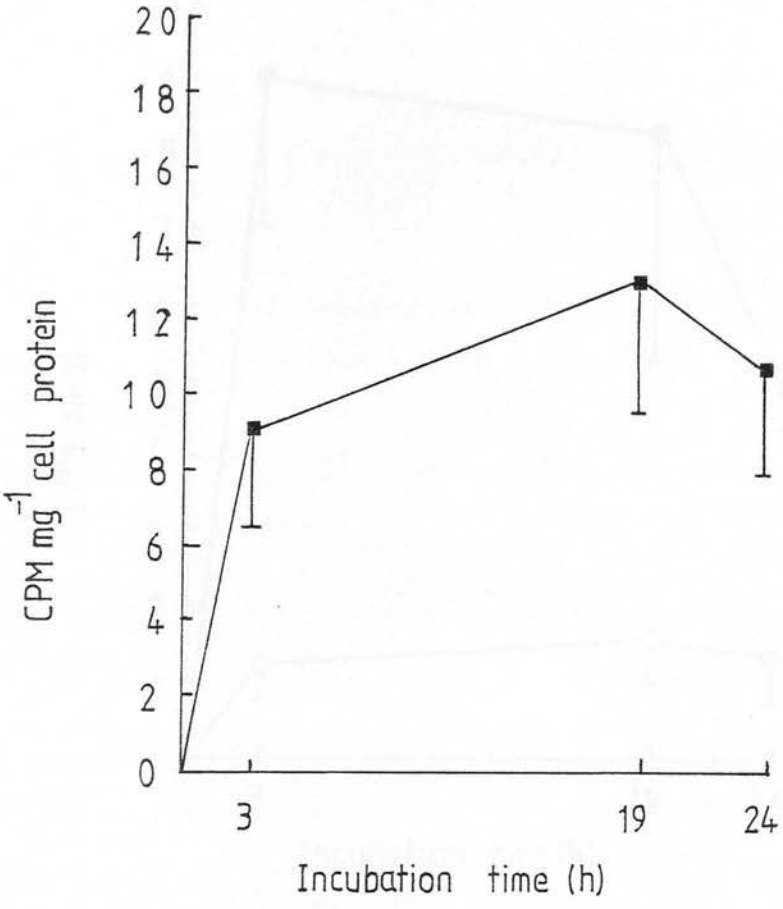


Fig. 6.6 The degradation of HDL_2-cholesteryl esters to bile salts by rat hepatocyte monolayers. The secretion of bile salts derived from HDL_2-cholesteryl ester into the hepatocyte culture medium was determined as described in the text. The results shown are the mean ± the range of duplicate determinations from hepatocytes obtained from 2 rats.

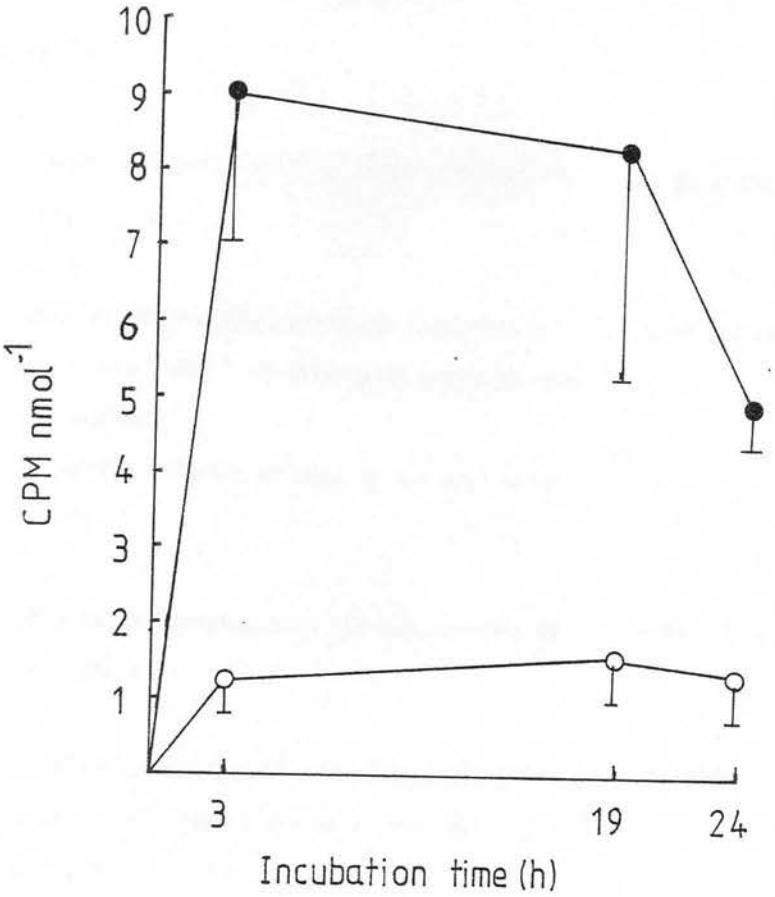


Fig. 6.7 Comparison of the specific activity of the cell free cholesterol with the specific activity of the bile salts. Following incubation of hepatocytes with HDL_2-[4-^{14}C]cholesteryl ester the specific activity of the hepatocyte free cholesterol (●—●) and the specific activity of the bile salts secreted into medium (○—○) was determined. The results are the mean ± the range of duplicate determinations from hepatocytes obtained from 2 rats.

Incubation	3	19	24
1. Specific activity of bile salts (CPM $nmol^{-1}$) (Fig. 6.7).	1.22 ± 0.42	1.76 ± 0.48	1.42 ± 0.60
2. nmol of HDL_2-cholesteryl ester degraded to bile salts $nmol^{-1}$ of bile salts secreted into the medium. (Specific activity of HDL_2-cholesteryl ester ÷ line 1).	0.17 ± 0.04	0.24 ± 0.09	0.19 ± 0.08
3. Bile salts secreted into the medium (nmol mg^{-1} cell protein).	4.92 ± 0.92	6.76 ± 0.75	7.44 ± 1.30
4. Total HDL_2-cholesteryl ester degraded to bile salts i.e. line 2 x line 3 (nmol mg^{-1} of cell protein).	0.81	1.62	1.43
5. % of bile salts synthesised from HDL_2-cholesteryl esters.	16.5	23.9	19.2

Table 6.2 The degradation of HDL_2-cholesteryl esters to bile salts by hepatocyte monolayers. The proportion of bile salts synthesised from HDL_2-cholesteryl esters was determined by comparing the specific activity of the HDL_2-cholesteryl ester with the specific activity of the bile salts. The specific activity of the HDL_2-cholesterol that was esterified was 7.38 CPM $nmol^{-1}$. The results shown are the mean ± the range of duplicate determinations from hepatocytes obtained from 2 rats.

accounted for by degradation of HDL_2-cholesteryl esters (Table 6.2).

6.4.3 The secretion of cholesteryl esters by hepatocytes derived from HDL_2-free cholesterol

In Chapter 6.3.1 we saw that on entry of HDL_2-free cholesterol into the hepatocyte a proportion is esterified. The cholesteryl ester pool within the hepatocyte primarily functions as a precursor site for the secretion of cholesteryl ester as a component of lipoproteins (Drevon et al., 1980). Following the determination of radioactivity attributable to cholesteryl esters in the hepatocyte culture medium, it was found that cholesteryl ester derived from HDL_2-free cholesterol was secreted into the medium at a steady rate (Fig. 6.8). A comparison of the CPM mg^{-1} of cell protein in the secreted cholesteryl esters with the CPM due to the HDL_2-free cholesterol provided an indication of the amount of HDL_2-free cholesterol used for the cholesteryl esters secreted as newly synthesised lipoproteins (Table 6.3). The results indicate that between 7 - 15 pmol of HDL_2-free cholesterol is utilized for secretion as cholesteryl esters per mg of hepatocyte cell protein. Whilst the utilization of hepatocyte cholesteryl ester by other pathways is possible, the contribution of this source of cholesterol to these pathways is probably negligible. The use of stored esterified cholesterol by the hepatocyte for pathways other than for secretion in lipoproteins requires hydrolysis by the cytosolic cholesterol esterase. It has been reported that in rat liver the activity of cytosolic cholesterol esterase does not provide a significant contribution to the degradation of hepatocyte cholesteryl esters (Gorban, 1980; Mathe et al. 1984).

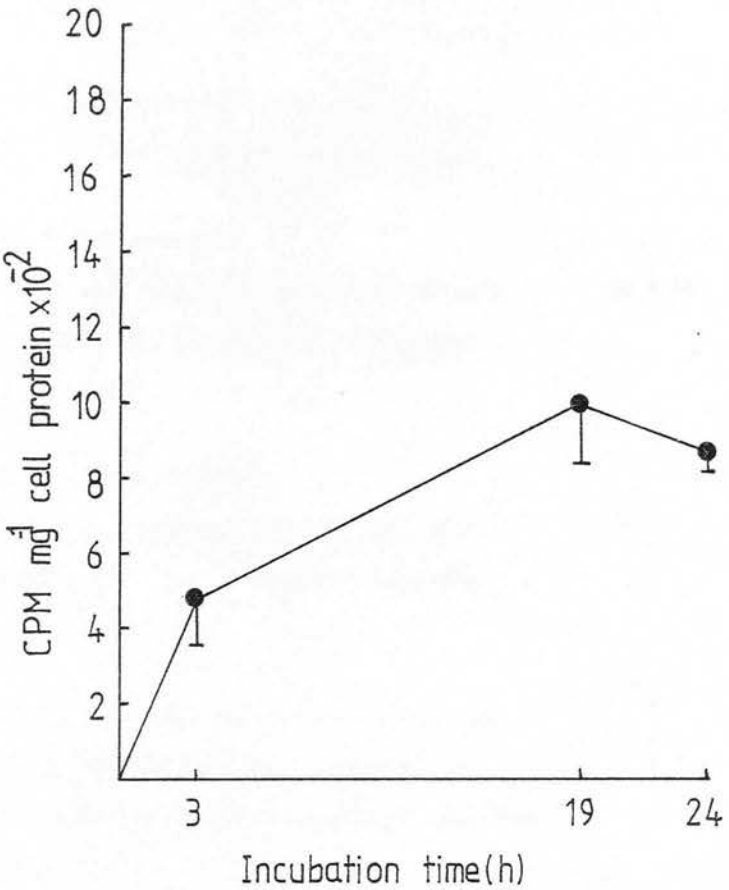


Fig. 6.8 The utilization of HDL_2-free cholesterol by hepatocyte monolayers for the secretion of cholesteryl esters. The secretion of cholesteryl esters into the hepatocyte culture medium that were derived from HDL_2-free cholesterol was determined as described in the text. The results show the mean ± the range of duplicate determinations from hepatocytes obtained from 2 rats.

Incubation time (h)	3	19	24
HDL_2-free cholesterol in (pmol mg^{-1} of cell protein)			
1. Cell free cholesterol (CPM of cell free cholesterol mg^{-1} of cell protein ÷ specific activity of HDL_2-free cholesterol).	80 ± 12	91 ± 15	112 ± 8
2. Cell cholesteryl esters (CPM of cell cholesteryl ester mg^{-1} of cell protein ÷ specific activity of HDL_2-free cholesterol).	9 ± 2	23 ± 4	30 ± 7
3. Cholesteryl ester secreted into the medium (CPM of medium cholesteryl ester mg^{-1} of cell protein ÷ specific activity of HDL_2-free cholesterol).	7 ± 3	16 ± 5	13 ± 2
4. Bile salts secreted into the medium (CPM due to bile salts in the medium mg^{-1} of cell protein ÷ specific activity of HDL_2-free cholesterol).	8 ± 3	27 ± 5	39 ± 8
5. Total.	104 ± 14	157 ± 16	194 ± 20
6. nmol mg^{-1} of cellular protein of HDL_2-free cholesterol present at 0h.	1.06	0.95	0.97
7. % uptake i.e. line 5 ÷ line 6 x 100.	9.8	16.5	20

Table 6.3 Estimation of the uptake of HDL_2-free cholesterol by hepatocytes. Uptake of HDL_2-free cholesterol was calculated by addition of the pmol of HDL_2-free cholesterol present in the cells as free and esterified cholesterol with the pmol of HDL_2-free cholesterol secreted into the medium as bile salts and cholesteryl ester. The results shown are the mean ± range of duplicate determinations from hepatocytes obtained from 2 rats.

6.4.4 The contribution of HDL_2-cholesteryl ester to the secretion of free cholesterol by hepatocytes

The radioactivity attributable to free cholesterol that was present in the medium was determined as described previously (Chapter 2.10). The results indicate that between 2 - 4 times as much HDL_2-cholesteryl ester is utilized for secretion as free cholesterol into the medium than is utilized for the synthesis of bile salts (Fig. 6.9). The radioactive free cholesterol that is secreted into the medium represents the secretion of biliary cholesterol and lipoprotein-free cholesterol by hepatocyte monolayers. Calculation of the nmol of HDL_2-cholesteryl ester that provides cholesterol for secretion as free cholesterol indicates that between 4 - 6 nmol of HDL_2-cholesteryl ester per mg of hepatocyte cell protein was utilized for this pathway. This compares with approximately 1.5 nmol of HDL_2-cholesteryl ester being utilized for the synthesis of bile salts (Table 6.4).

6.5 The estimation of the uptake of HDL_2-cholesterol by hepatocyte monolayers

In Chapter 6.4 the fate of HDL_2-cholesterol after entry into hepatocytes was described. These results can also provide some indication as to the uptake of HDL_2-cholesterol throughout the period of study. This will indicate the significance of the degradation of HDL_2-cholesterol by these hepatocytes monolayers.

6.5.1 Estimation of the uptake of HDL_2-free cholesterol

The uptake of HDL_2-free cholesterol was determined by calculation of the mass of HDL_2-free cholesterol that entered hepatocytes and was utilized for secretion as cholesteryl esters

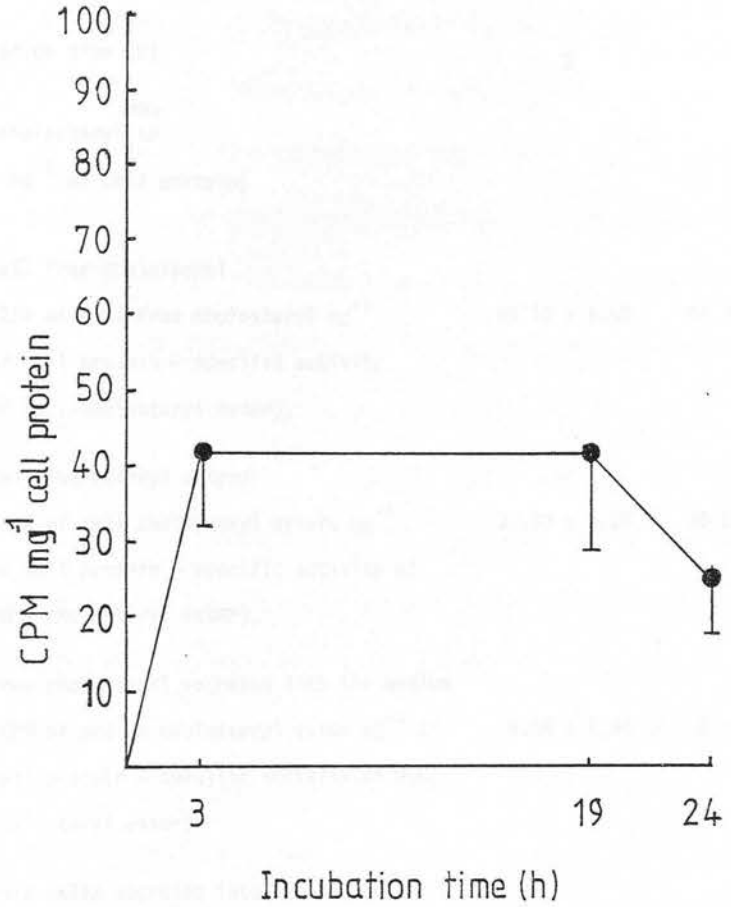


Fig. 6.9 Secretion of free cholesterol derived from HDL_2-cholesteryl esters by hepatocyte monolayers. The utilization of HDL_2-cholesteryl ester for secretion as free cholesterol by hepatocytes was determined as described in the text. The results show the mean ± the range of duplicate determinations from hepatocytes obtained from 2 rats.

Incubation time (h)	3	19	24
HDL_2-cholesteryl esters in (nmol mg^{-1} of cell protein)			
1. Cell free cholesterol (CPM of cell free cholesterol mg^{-1} of cell protein ÷ specific activity of HDL_2-cholesteryl ester).	46.10 ± 5.60	44.30 ± 6.20	24.90 ± 3.70
2. Cell cholesteryl esters (CPM of cell cholesteryl esters mg^{-1} of cell protein ÷ specific activity of HDL_2-cholesteryl ester).	37.30 ± 6.20	35.20 ± 4.30	34.60 ± 5.75
3. Free cholesterol secreted into the medium (CPM of medium cholesteryl ester mg^{-1} of cell protein ÷ specific activity of HDL_2-cholesteryl ester).	5.56 ± 2.40	5.69 ± 3.2	3.39 ± 1.60
4. Bile salts secreted into the medium (CPM due to bile salts in the medium mg^{-1} of cell protein ÷ specific activity of HDL_2-cholesteryl ester.	1.21 ± 0.63	1.76 ± 0.73	1.44 ± 0.54
5. Total.	90.17	86.95	64.33
6. nmol mg^{-1} of cellular protein of HDL_2-cholesteryl ester present at 0h.	512.1	480	442
7. % uptake i.e. line 5 ÷ line 6 x 100.	17.6	18.1	14.5

Table 6.4 Estimation of the uptake of HDL_2-cholesteryl esters by hepatocytes. Uptake of HDL_2-cholesteryl esters was calculated by addition of the nmol of HDL_2-cholesteryl ester present in the cells as free and esterified cholesterol with the nmol of HDL_2-cholesteryl ester secreted into the medium as bile salts and free cholesterol. The results shown are the mean ± range of duplicate determinations from hepatocytes obtained from 2 rats.

and bile salts. It is important to note that the radioactivity present within the hepatocyte at any given time is not representative of the uptake of HDL_2-free cholesterol. It has already been shown that uptake of HDL_2-free cholesterol results in its utilization and subsequent secretion into the medium. It is therefore important that in obtaining an estimate of the uptake of HDL_2-free cholesterol by hepatocytes the appearance of this lipoprotein free cholesterol into the medium is also taken into account. A comparison of the pmol of HDL_2-free cholesterol present in the hepatocyte culture medium at 0h with the pmol of HDL_2-free cholesterol utilized by the hepatocytes provides an indication of the uptake of HDL_2-free cholesterol (Table 6.3). After 3h approximately 10% of HDL_2-free cholesterol had been taken up by hepatocytes and this increased to 20% after 24h. However, this estimate of uptake is less than the actual uptake of HDL_2-free cholesterol. The experiments undertaken do not allow determination of that HDL_2-free cholesterol in the form of biliary cholesterol or newly synthesised lipoproteins. However the values obtained at the very least provide a minimum estimate of the uptake of HDL_2-free cholesterol by hepatocyte monolayers.

6.5.2 Estimation of the uptake of HDL_2-cholesteryl ester

In Chapter 6.5.1 the uptake of HDL_2-free cholesterol was estimated. Using the same reasoning as was used for estimating the uptake of HDL_2-free cholesterol, the uptake of HDL_2-cholesteryl ester can be determined. In this case the nmol of HDL_2-cholesteryl ester that enters the hepatocyte and is utilized by the hepatocytes was determined (Table 6.4). The results indicate that after 3h of incubation of hepatocytes with HDL_2-cholesteryl ester approximately

16% of the HDL_2-cholesteryl ester had been taken up. However at later time points the apparent uptake of HDL_2-cholesteryl ester by hepatocytes fell (Table 6.4). This may have been due to the utilization of HDL_2-cholesteryl ester for resecretion by hepatocytes as cholesteryl esters as a component of newly synthesised lipoproteins. This may explain the apparent decrease in the uptake of HDL_2-cholesteryl esters by hepatocytes during the incubation. As was indicated in Chapter 6.5.1 these values only provide a minimum estimate of the uptake of HDL_2-cholesterol by hepatocyte monolayers.

6.6 The effect of HDL_2 on the synthesis of cholesterol by hepatocyte monolayers

The results presented in Chapter 6.4 indicated that the increase in the synthesis of bile salts in the presence of HDL_2 was all due to the catabolism of HDL_2-cholesteryl esters. This would result in there being no alteration in the contribution of newly-synthesised cholesterol to the synthesis of bile salts. To confirm this conclusion the effect of HDL_2 on the synthesis of cholesterol by hepatocytes was determined. Synthesis of cholesterol was assessed by determination of the incorporation of [1-^{14}C]acetate into cholesterol. Following the incubation of hepatocytes with [1-^{14}C]acetate in the presence and absence of HDL_2 the cells and medium were extracted as described previously (Chapter 2.10). The radioactivity attributable to free cholesterol in both the cells and medium was subsequently determined by t.l.c. as described in Chapter 2.10.2. The newly-synthesised cholesterol that had been esterified was estimated after first hydrolysing the cholesteryl esters with alcoholic potassium hydroxide (0.5M potassium hydroxide in 90% ($^{v}/_{v}$)

ethanol) at 70°C for 30 min. The lipids were then extracted from the hydrolysate and the radioactivity attributable to total free cholesterol determined (Chapter 2.10).

The hydrolysis of the cholesteryl esters prior to determination of the radioactivity due to cholesterol was necessary as acetate is also a substrate for the synthesis of fatty acids following its activation by acetyl thiokinase. Determination of the radioactivity attributable to cholesteryl esters without prior hydrolysis would therefore result in an overestimate of the synthesis of cholesterol from [1-^{14}C]acetate.

The results indicated that whilst the incorporation of [1-^{14}C]acetate into cholesterol in the presence of HDL_2 is slightly increased this increase is not significant (Fig. 6.10). Furthermore, HDL_2 did not affect that proportion of newly synthesised cholesterol that was esterified (Fig. 6.11).

6.7 The effect of HDL_2 on the utilization of newly-synthesised cholesterol for the synthesis of bile salts

The utilization of newly-synthesised cholesterol for the synthesis of bile salts was estimated by measuring the incorporation of [1-^{14}C]acetate into bile salts. The radioactivity attributable to bile salts was determined as described previously (Chapter 2.10). The results show that HDL_2 has no significant effect on the utilization of newly-synthesised cholesterol for the synthesis of bile salts (Fig. 6.12).

The use of [^{14}C]acetate for determination of the synthesis of cholesterol has been criticised by a number of workers (Dietschy and Brown, 1974; Dietschy and McGarry, 1974, Gibbons and Pullinger, 1977) and it has been shown that the use of this substrate resulted

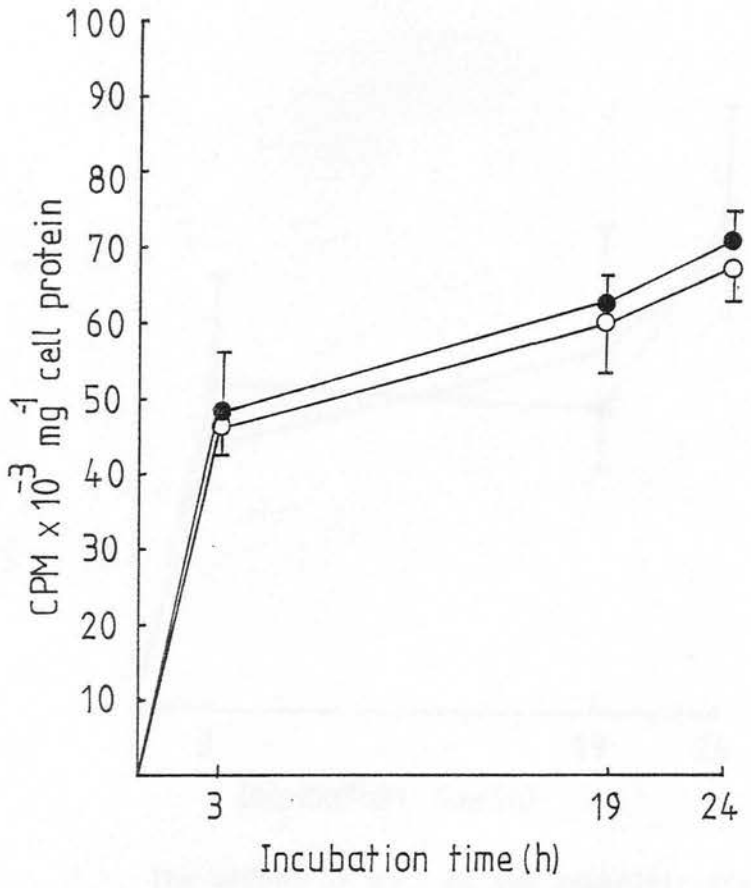


Fig. 6.10 The effect of HDL_2 on the synthesis of cholesterol by hepatocyte monolayers was determined by incubating hepatocytes in medium containing [^{14}C]acetate in the absence (○—○) or presence (●—●) of HDL_2. The results shown are the mean ± range of duplicate determinations from hepatocytes obtained from 2 rats. 2.5μCi of [^{14}C]acetate (specific activity 57mCi/mmol) were used in each incubation.

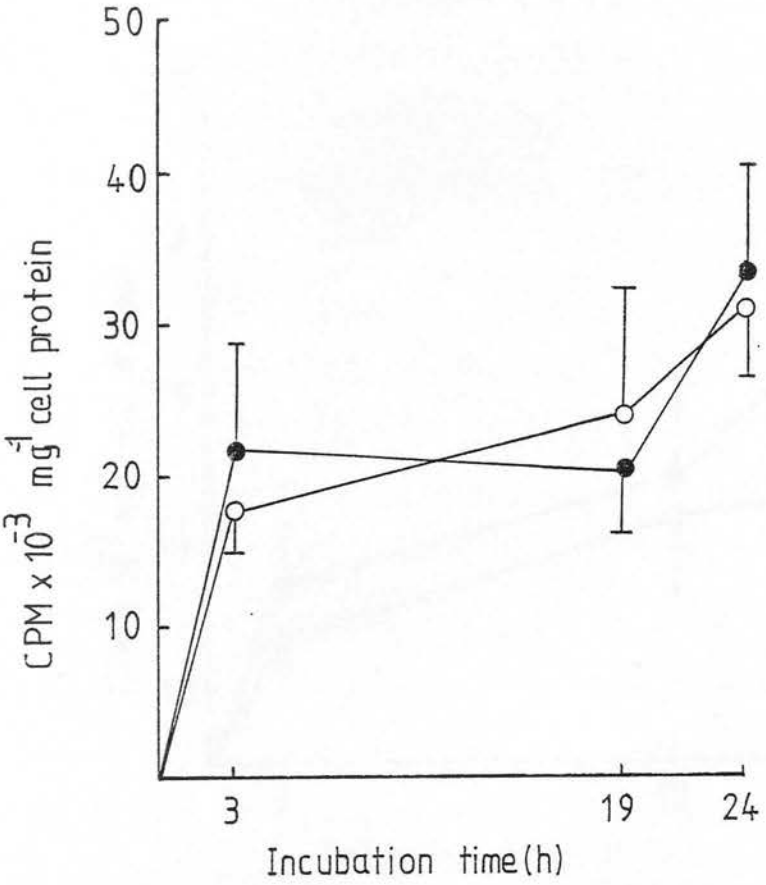


Fig. 6.11 The effect of HDL_2 on the esterification of newly synthesised cholesterol by hepatocyte monolayers. The esterification of newly synthesised cholesterol was determined by the difference in the radioactivity due to free cholesterol before and after hydrolysis of the cholesteryl esters with alcholic potassium hydroxide. The esterification of newly synthesised cholesterol in the presence (●—●) and absence (○—○) of HDL_2 was thus determined. The results are the mean ± range of duplicate determinations from hepatocytes obtained from 2 rats.
2.5μCi of [^{14}C]acetate (specific activity 57mCi/mmol) were used in each incubation.

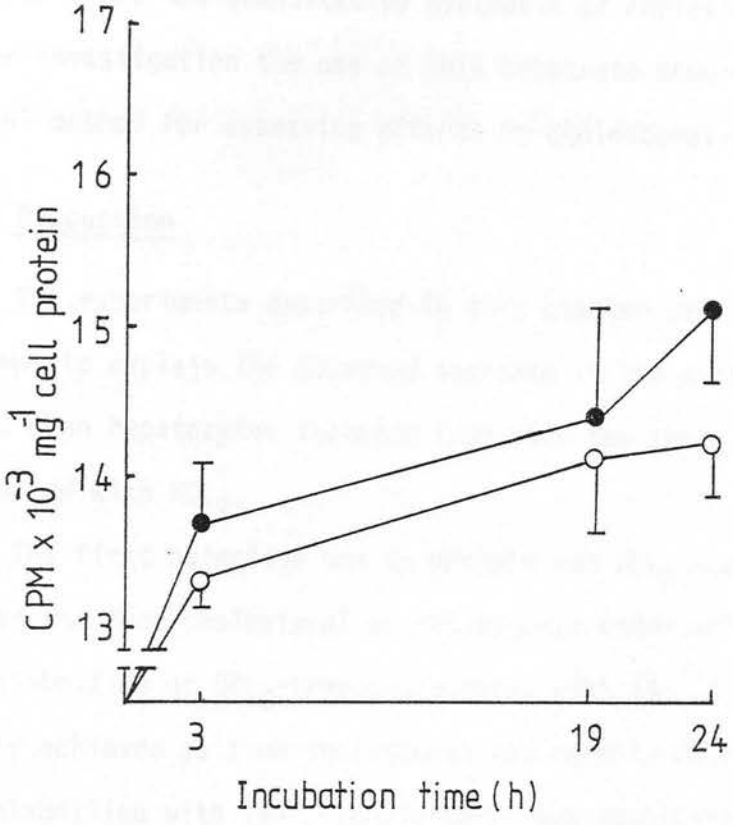


Fig. 6.12 The effect of HDL_2 on the utilization of newly synthesised cholesterol for the synthesis of bile salts by rat hepatocyte monolayers. The utilization of newly synthesised cholesterol for the synthesis of bile salts by hepatocyte monolayers in the presence (●—●) and absence (○—○) of HDL_2 was determined as described in the text. Results are mean ± range of duplicate determinations from hepatocytes obtained from 2 rats.

in an underestimate of sterol synthesis. However, in comparative studies where the quantitative synthesis of cholesterol is not under investigation the use of this substrate should provide a useful method for assessing effects on cholesterol synthesis.

6.8 Discussion

The experiments described in this chapter were designed in an attempt to explain the observed increase in the synthesis of bile salts when hepatocytes isolated from rats fed cholestyramine were incubated with HDL_2.

The first objective was to prepare rat HDL_2 radiolabelled in either the free cholesterol or cholesteryl ester moiety. Radiolabelling of HDL_2-free cholesterol with [4-^{14}C]cholesterol was easily achieved as free cholesterol can readily exchange. Radiolabelling with [4-^{14}C]cholesterol was facilitated by incubating HDL_2 with filter paper discs impregnated with [4-^{14}C]cholesterol. However radiolabelling of HDL_2-cholesterol esters presents certain problems. HDL_2-cholesteryl ester can be labelled by:-

1) injection of radioactive free cholesterol into rats followed by isolation of HDL from the plasma (Sigurdsson et al., 1979).

2) incubation of plasma with radioactive free cholesterol followed by isolation of HDL (Drevon et al., 1977). In this method the radioactive free cholesterol is incorporated into the free cholesterol on the surface of the lipoprotein particles. The HDL-free cholesterol is then esterified by the action of LCAT. Following esterification the cholesteryl esters are internalised.

3) HDL_2-cholesteryl esters can be labelled by transfer of radioactive cholesteryl ester from a lipid microemulsion to the HDL (Craig et al., 1983). The transfer of the cholesteryl ester is facilitated by a cholesteryl ester transfer protein that is present in the plasma of certain species (Chapter 1.2.4).

Whilst methods 1 and 2 allow radiolabelling of the HDL_2-cholesteryl ester the HDL_2-free cholesterol is also radiolabelled. However it has been reported that most of the radioactive free cholesterol can be removed by exchange with hepatocytes (Drevon et al., 1977). However, for the experiments described in this chapter HDL_2 was labelled in the cholesteryl ester moiety using method 3 (Chapter 2.8.2). This enabled only the cholesteryl ester moiety to be labelled. However there were some disadvantages. Firstly the specific activity of the HDL_2-cholesteryl ester is relatively low compared with the other methods and secondly the labelling procedure is harsh. This resulted in the loss of certain apoproteins (Fig. 6.1). It is possible that the loss of these apoproteins from the HDL_2 may result in poorer uptake of the HDL_2 particle by hepatocyte monolayers. The HDL_2 radiolabelled with [4-^{14}C]cholesteryl oleate was also found to be contaminated with albumin. It is probable that the source of this contamination is the human plasma used as a source of the cholesteryl ester transfer protein. However, the anomalies in the protein content of this HDL_2 did not affect the increase in the synthesis of bile salts by rat hepatocytes incubated in the presence of HDL_2, that was previously described in Chapter 5.

The results have shown that HDL_2-cholesterol enters the hepatocyte from which it can be utilized by numerous pathways. Estimation of the uptake of HDL_2-cholesteryl ester and HDL_2-free cholesterol by hepatocytes indicated that uptake of HDL_2 after 24h was 14% and 20% respectively. However, as was stated previously (Chapter 6.5) these values are the minimum estimates for the uptake of the HDL_2-cholesterol fractions. In those hepatocytes incubated in the presence of HDL_2 labelled with [4-^{14}C]cholesterol, analysis of the medium enabled the determination of the role of HDL_2-free cholesterol in supplying cholesterol for degradation to bile salts and for secretion as cholesteryl esters. The secretion of cholesteryl esters into the medium by hepatocytes reflects the secretion of newly-synthesised lipoproteins by hepatocytes (Drevon et al., 1980). However, the presence of the HDL_2 in the culture medium prevented the determination of mass measurements of cholesterol and cholesteryl esters secreted into the medium. The presence of HDL_2-[4-^{14}C]cholesterol also prevented the assessment of the utilization of HDL_2-free cholesterol for secretion as biliary cholesterol and lipoprotein-free cholesterol by hepatocytes. When hepatocytes were incubated with HDL_2 labelled with [4-^{14}C]cholesteryl oleate, analysis of the medium enabled the contribution of HDL_2-cholesteryl ester in supplying cholesterol for the synthesis of bile salts to be determined. Whereas in those incubations of hepatocytes with HDL_2 labelled with [4-^{14}C]cholesterol the secretion of radiolabelled cholesteryl esters could be detected, this pathway could not be assessed in those experiments where hepatocytes were incubated with HDL_2 labelled in the cholesteryl ester moiety. However, the

contribution of HDL_2-cholesteryl esters to the secretion of biliary cholesterol and lipoprotein-free cholesterol was determined. In these experiments differentiation between the biliary free cholesterol and lipoprotein-free cholesterol secreted into the medium by hepatocytes was not attempted. Although these experiments allowed estimation of the uptake of HDL_2-cholesterol it is important to understand that the limitations of these experiments do not allow an absolute determination.

The major objective of these studies was to determine the contribution of HDL_2-cholesterol to the synthesis of bile salts and to explain the observed increase in the synthesis of bile salts by hepatocyte monolayers incubated with HDL_2. The experiments using HDL_2 labelled with either [4-^{14}C]free cholesterol or [4-^{14}C]cholesteryl oleate indicated that the increase in the synthesis of bile salts was due to the degradation of HDL_2-cholesteryl esters to bile salts (Table 6.2). The mass of HDL_2-cholesteryl esters degraded to bile salts also indicated that the contribution of newly synthesised cholesterol to the synthesis of bile salts might be decreased when hepatocytes were incubated in the presence of HDL_2. However the experiments with [^{14}C]acetate indicated that this was not the case. The apparent overestimate of the contribution of HDL_2-cholesteryl ester to the synthesis of bile salts is not significant (see Table 6.2). The results in this chapter, together with the current evidence that is available, suggest that the increase in the synthesis of bile salts by hepatocytes in the presence of HDL_2, reflects a deficiency in the cholesterol available for degradation to bile salts, in those hepatocyte incubations which do not have an exogenous source of cholesterol. In vivo, the supply of cholesterol for the synthesis

of bile salts is derived from both the plasma and from endogenous synthesis in the liver. Feeding cholestyramine results in an increase in the synthesis of bile salts (see Chapter 4) which therefore results in an increase in the demand for substrate i.e. cholesterol. In the rat it is likely that the increased cholesterol required as a result of stimulating bile salt synthesis is provided by an increase in hepatic cholesterol synthesis (Koelz et al., 1982). Interruption of the enterohepatic circulation therefore results in a greater percentage of the bile salts synthesised being derived from newly-synthesised cholesterol (Long et al., 1978). However, in vivo a proportion of the bile salts would still be synthesised from cholesterol derived from the plasma. In an isolated cell system such as the hepatocyte monolayers used in this study, the plasma cholesterol no longer provides a source of substrate for the synthesis of bile salts. If in these hepatocytes the synthesis of cholesterol was maximally stimulated due to feeding cholestyramine, removal of the source of exogenous cholesterol would result in the hepatocytes becoming deficient in metabolically active cholesterol i.e. that cholesterol used to supply substrate for the synthetic and degradative pathways within the hepatocyte and not that cholesterol performing a structural role. This deficiency of cholesterol in hepatocytes isolated from rats fed cholestyramine would result in the synthesis of bile salts being limited by the supply of cholesterol. Addition of lipoprotein-cholesterol to the medium, in this case HDL_2, restored the supply of cholesterol thereby resulting in an increase in the synthesis of bile salts. There are numerous reports which would seem to support this explanation. In

Chapter 5 it was suggested that one reason for the increase in the synthesis of bile salts, in the presence of HDL_2 by hepatocytes obtained from animals fed cholestyramine, was an increase in the uptake of HDL_2. However evidence by Koelz et al., (1982) has shown that cholestyramine-feeding does not affect the in vivo uptake of ^{125}I-LDL or ^{125}I-HDL. The synthesis of cholesterol by the liver in the rat has been shown to be quantitatively more important than in other species (Koelz et al., 1982; Turley et al., 1981; Spady and Dietschy, 1983). In particular synthesis of cholesterol in the rat is subject to greater fluctuations under the influence of external stimuli than is known to occur in other species. Koelz et al., (1982) therefore suggested that the increase in the synthesis of cholesterol on feeding cholestyramine is sufficient to meet the requirements for cholesterol by the liver and it is therefore unecessary for an increase in the uptake of plasma cholesterol by the liver in rats. In other species such as the rabbit and human where cholesterol synthesis cannot be induced to the same extent as in the rat, feeding cholestyramine will result in an increase in the uptake of plasma lipoproteins by the liver (Slater et al., 1980; Shepherd et al., 1980).

In Chapter 5, it was also reported, that HDL_2 did not affect the synthesis of bile salts by hepatocytes obtained from animals fed a pellet diet. This observation may also reflect the efficient regulation of cholesterol synthesis by the rat. In isolated hepatocytes in suspension the synthesis of cholesterol increases with incubation time (Edwards, 1975), as does the contribution of newly-synthesised cholesterol to the synthesis of bile salts (Kempen et al., 1983). The addition of

HDL_2 to the culture medium may result in HDL_2-cholesterol being utilized for the synthesis of bile salts and an inhibition of the stimulation of cholesterol synthesis that is observed in the absence of exogenous cholesterol. This may then result in no alteration in the synthesis of bile salts but produce a decrease in the contribution of newly-synthesised cholesterol to the synthesis of bile salts. At the present time the effect of HDL_2 on cholesterol metabolism by hepatocytes prepared from rats fed a standard pellet diet is not well defined and will require further investigation (Chapter 7).

In conclusion, the effect of HDL_2 on the synthesis of bile salts by hepatocyte monolayers appears to reflect the nutritional status of the rats used for the preparation of hepatocytes. Although hepatocytes obtained from rats fed cholestyramine appear to be deficient in cholesterol, this system should still prove useful in further experiments (Chapter 7).

Footnote.

It must be pointed out that the large amount of cholesteryl ester transferred to the hepatocytes from HDL_2 (Table 6.4), is inconsistent with the effect of HDL_2 on the total cellular cholesterol content of hepatocytes described earlier (Chapter 5.4, Fig. 5.7). This inconsistency is most likely due to the errors involved in calculating the specific activity of HDL_2 cholesteryl ester. However, it is also possible that uptake of large amounts of HDL_2-cholesteryl ester by hepatocytes results in the secretion of cholesteryl esters in the form of lipoproteins. This would result in no nett change in the cellular cholesterol content of hepatocytes.

CHAPTER 7

FINAL CONCLUSIONS AND FURTHER EXPERIMENTS

7.1 Overall Summary

The major aims of this project were to characterise an in vitro system suitable for the long term study of bile salt synthesis and then to use this system to investigate the utilization of plasma cholesterol for the synthesis of bile salts.

The system used was isolated rat hepatocytes maintained in monolayers. The integrity of this system was assessed by the viability of the hepatocytes, together with their ability to synthesise bile salts (Chapters 3 and 4). After the determination of suitable conditions for the maintenance of hepatocytes the effect of a HDL subfraction (HDL_2) on the synthesis of bile salts by hepatocyte monolayers was investigated. The results in this thesis are summarised below:-

(1) rat hepatocytes of excellent viability were isolated using a recirculating perfusion system. Many of the problems associated with obtaining a viable cell preparation were overcome by centrifugation of the freshly isolated cells on a 'Percoll' density gradient,

(2) hepatocytes were maintained in monolayers for up to 24h during which time their metabolic integrity remained intact (Chapter 3).

(3) hepatocyte monolayers were shown to synthesise and secrete the major bile acid conjugates synthesised by the rat i.e. conjugated cholic, chenodeoxycholic and β-muricholic acids,

(4) alteration of the rates of bile salt synthesis in vivo by dietary manipulation was reflected in the rate of bile salt synthesis by the hepatocyte monolayers prepared from these rats.

(5) incubation of hepatocytes isolated from rats fed the pellet diet with a defined HDL subfraction (HDL_2) had no effect on the total synthesis of conjugated cholic, chenodeoxycholic and β-muricholic acids (Chapter 5).

(6) however, incubation of hepatocyte monolayers with HDL_2 was found to increase the synthesis of total bile salts i.e. conjugated cholic + chenodeoxycholic + β-muricholic acids when hepatocytes were prepared from rats previously fed cholestyramine (Chapter 5).

(7)[1] the increase in the synthesis of bile salts by these hepatocytes is tentatively attributed to the utilization of HDL_2-esterified cholesterol for the synthesis of bile salts (Chapter 6).

(8) HDL_2 did not affect the synthesis of cholesterol or the utilization of newly synthesised cholesterol for the synthesis of bile salts (Chapter 6).

The results in this thesis have shown that rat hepatocytes maintained in monolayers are suitable for the investigation of various aspects of bile salt synthesis. In particular, the system has proved to be suitable for the investigation of the degradation of lipoprotein cholesterol to bile salts. However, the experiments described have certain limitations:-

1. The possible errors involved in calculating the specific activity of HDL_2-cholesteryl ester may result in this conclusion being invalid.

(a) The detection of the bile salts synthesised by hepatocyte monolayers by using specific radioimmunoassays is confined to the measurement of conjugated cholic, chenodeoxycholic and β-muricholic acids. Although these are the major primary bile acids synthesised by the rat it is possible that other bile salts may be synthesised (Chapter 7.2). However, in rat hepatocytes maintained in suspension it has been reported that these three bile salts account for at least 95% of the total bile salts synthesised (Kempen et al., 1982). Current evidence would seem to indicate that this is also the case in hepatocytes maintained in monolayers (Davis et al., 1983a; R.A. Davis, personal communication).

(b) In those experiments in which degradation of HDL_2-[4-^{14}C]cholesterol and HDL_2-[4-^{14}C]cholesteryl oleate to bile salts was determined, the method for determination of the radioactivity associated with the bile acids had certain limitations. To measure the radioactivity associated with the bile salts samples were first subjected to t.l.c. The solvent system used resulted in poor resolution of the individual bile salts and only took into account the taurine conjugates. It is of course possible that glycine conjugates are also synthesised by hepatocytes. In rat hepatocytes maintained in suspension the synthesis of both taurine and glycine conjugates of bile acids has been reported (Kempen et al., 1982). However, in this report addition of taurine to the incubation medium resulted in the synthesis of only taurine

conjugated - bile acids, indicating that hepatocytes were initially deficient in taurine (Kempen et al., 1982). The results, therefore, indicated that taurine conjugates were the preferred bile acid conjugates synthesised and glycine conjugates were only synthesised when there was a deficiency of taurine. Whilst it is possible that in this present study glycine conjugated bile acids are synthesised it is probably unlikely due to the complex culture medium used. This conclusion requires to be substantiated. A further disadvantage of the separating system used is the poor resolution. This prevents the possible investigation of the individual bile acids being synthesised from separate substrate pools. (Chapter 1.4.2).

(c) A further limitation of the experiments with HDL_2 is that these studies do not indicate the mechanism of HDL_2 uptake into the hepatocytes. Preliminary studies do however suggest that a specific uptake mechanism is required. Incubation of hepatocytes with human VLDL, LDL and HDL at comparable concentrations to those used with rat HDL_2, were found not to affect the synthesis of bile salts (results not shown). It is now well established that heterologous lipoproteins are not recognised by rat hepatocytes with the same efficiency as homologous rat lipoproteins (Koelz et al., 1982). The finding that the human lipoproteins do not affect the synthesis of bile salts by rat hepatocytes would seem to indicate that specific receptor-mediated uptake of HDL_2 is required for degradation of the HDL_2-cholesterol to bile salts. However, it is important to

note that these human lipoprotein fractions were only characterised by their flotation in salt solutions following ultracentrifugation.

7.2 Further experiments

The results presented in this thesis whilst increasing our knowledge of certain aspects of the degradation of cholesterol to bile salts, have left a number of questions unanswered. For example:-

(i) do conjugated cholic, chenodeoxycholic and β-muricholic acids actually represent greater than 90% of the bile salts synthesised by rat hepatocyte monolayers?

(ii) What effect does HDL_2 have on cholesterol synthesis in hepatocytes isolated from rats fed the pellet diet?

(iii) Is HDL_2 used for the synthesis of bile salts by hepatocytes isolated from rats fed the pellet diet?

(iv) Do the individual bile acids arise from separate precursor pools?

(v) Is there any difference in the utilization of cholesterol from other lipoprotein classes for the synthesis of bile salts.

There follows some suggestions for ways of obtaining answers to these questions.

The major problem of using radioimmunoassays for the detection of bile salts is that a profile of the possible bile salts synthesised cannot be obtained. The use of high performance liquid chromatography (HPLC) should enable the determination of the profile of bile salts synthesised by hepatocytes. This method has a distinct

advantage over methods such as gas-liquid chromatography as lengthy extraction procedures and the derivitisation of bile acids are not required. HPLC has already been used successfully in obtaining a profile of bile salts in the bile of humans and rats (Bloch and Watkins, 1978; Shaw et al., 1978; Mingrone & Greco, 1980). The use of HPLC should refute or substantiate the assumption that greater than 90% of the bile salts synthesised by hepatocyte monolayers consists of conjugated cholic, chenodeoxycholic and β-muricholic acids. Further advantages of using HPLC for the separation of bile salts include the analysis of the radioactivity in individual bile salts that are produced by hepatocytes incubated in the presence of radioactive precursors.

The questions raised with respect to the effects of HDL_2 on the synthesis of cholesterol and the utilization of HDL_2-cholesterol for the synthesis of bile salts, in hepatocytes isolated from rats fed the pellet diet can be answered by using the methods already described with hepatocytes isolated from rats fed cholestyramine. However, perhaps the most intriguing question yet to be answered is whether a particular lipoprotein class is preferentially utilised for the synthesis of bile salts or indeed contributes greater amounts of substrate for degradation to bile salts. It would be of particular interest to determine the effect of the delivery to the liver of dietary cholesterol in the form of chylomicron remnants and cholesterol from peripheral tissues by HDL_1 following the plasma catabolism of HDL_2. Using the protocol already described for the HDL_2 studies in this thesis the utilization of cholesterol from these sources for the synthesis of bile salts could be determined.

There is one final question that has not yet been considered. In Chapter 1 the complex metabolism of the plasma lipoproteins

and the importance of certain plasma enzymes was discussed. There is considerable evidence to indicate that two of these enzymes, LCAT and hepatic lipase are synthesised by the liver from where they are secreted into the plasma (Jansen & Hulsmann, 1974; Nordby et al., 1976). It is possible that the synthesis of these enzymes by hepatocyte monolayers and their subsequent secretion into the culture medium could affect the metabolism of added lipoproteins. It remains to be seen whether these enzymes are actually synthesised and secreted by hepatocyte monolayers in significant quantities that would alter the metabolism of lipoproteins by hepatocytes.

In conclusion, the hepatocyte monolayer system described in this thesis is an important addition to the methods available to study the catabolism of cholesterol to bile acids. The system has the obvious advantage that the contribution of specific lipoprotein classes to the delivery of cholesterol to the liver for degradation to bile acids can be studied without the inherent disadvantages of the processes of transfer and exchange of components between lipoprotein classes that occur in vivo. The apparent deficiency of cholesterol available for degradation to bile acids in hepatocytes isolated from rats fed cholestyramine appears to provide a system where the requirement for exogenous cholesterol is at a premium. This system may therefore provide a unique means of assessing the utilization of cholesterol for the synthesis of bile acids from the individual lipoprotein classes.

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