Molecular, Cellular, and Regulatory Characterization of Cholesterol 7α-hydroxylase

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Dimmi se mai fu fatto alcuna cosa

-Leonardo da Vinci

This thesis has been composed by myself and all the work contained herein is my own unless otherwise indicated.

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List of Publications

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Abstract

This thesis is concerned with two aspects of bile acid synthesis: 1) the characterization of cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis and 2) the characterization of a human model for bile acid synthesis.

Using polyclonal antibody probes it has been possible to begin to detail the molecular nature of cholesterol 7α-hydroxylase. A polyclonal antibody raised against a male-specific cytochrome P450 (PB2a, P450h) from the P450 IIC gene subfamily was found to inhibit cholesterol 7α-hydroxylase activity in rat liver microsomes. This antibody was also found to inhibit 7α-hydroxylase activity in both hamster and mouse, indicating a common structural feature shared across species. Inhibition could be reversed by the addition of purified PB2a antigen to the assay. Using this antibody, a protein associated with 7α-hydroxylase activity was immunoprecipitated from liver microsomes of cholestyramine-treated female rats. The amino-terminal sequence from the immunoprecipitated protein was distinct from that of PB2a and other P450s. These data indicate that cholesterol 7α-hydroxylase is a unique cytochrome P450 which shares structural homology with enzymes from the P450 IIC gene subfamily.

The effect of substrate supply on bile acid synthesis by the human hepatoblastoma cell line HepG2 was also investigated. Incubation of HepG2 cells with the bile acid precursor 7α-hydroxycholesterol resulted in the production of bile acid, indicating that HepG2 cells have a viable bile acid synthetic pathway. Increasing the cholesterol content of the cells by the addition of the cholesterol precursor mevalonate did not stimulate bile acid synthesis. The addition of HDL to the cells also did not stimulate bile acid synthesis. Incubation of HepG2 cells with LDL, however, did increase the synthesis of bile acid. The addition of an ACAT inhibitor, 58-035, was shown to further enhance synthesis stimulated by LDL. These results are in contrast to those obtained from primary rat hepatocytes in which both mevalonate and HDL enhanced bile acid synthesis. The data presented here suggest that, in man, LDL-derived cholesterol may be preferentially utilized for bile acid synthesis.

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Abbreviations

ACAT: acyl coenzyme A:cholesterol O-acyltransferase (E.C. 2.3.1.26)

CA: cholic acid

CDCA: chenodeoxycholic acid

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

cholesterol: 5-cholesten-3β-ol

cholesterol 7α-hydroxylase: cholesterol 7α-monooxygenase (E.C. 1.14.13.17)

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

Ci: Curie

clofibrate: ethyl-2-(4-chlorophenoxy)-2-methylpropionate

CM: chylomicrons

DMEM: Dulbecco's modified Eagle's medium

EDTA: ethyldiaminetetraacetic acid

GCA: glycocholic acid

GCDC: glycochenodeoxycholic acid

HDL: high density lipoprotein

HMG-CoA: 3-hydroxy-3-methyl glutaryl coenzyme A

HMG-CoA reductase: 3-hydroxy-3-methyl glutaryl coenzyme A reductase (E.C. 1.1.1.34)

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

IDL: intermediate density lipoprotein

IgG: immunoglobulin G

isosafrole: 1,2-methylendioxy-4-propenyl benzol

LCA: lithocholic acid

LDL: low density lipoprotein

NADH: reduced nicotinamide

NADPH: reduced nicotinamide adenine dinucleotide

phenobarbital: 5-ethyl-5-phenyl-2,4,6(1H,3H,5H) pyrimidinetrione

PAGE: polyacrylamide gel electrophoresis

SDS: sodium dodecyl sulphate

SKF-525A: diethylaminoethyl-2,2-diphenyl valerate hydrochloride

TCA: taurocholic acid

TCDC: taurochenodexoycholic acid

TEMED: N,N,N',N'-tetramethylethyleme diamine

Tris:HCl: tris(hydroxymethyl)aminomethane hydrochloride

VLDL: very low density lipoprotein

Chapter 1

Introduction

1.1 General Introduction

Cholesterol is found in all animal cells and has both structural and metabolic functions. Its rigid ring configuration provides structural integrity in the plasma membrane. It is also the precursor for steroid hormones, vitamin D, and bile acids. Cholesterol can be obtained from two sources: diet and *de novo* synthesis. Dietary cholesterol is absorbed through the small intestine and transported to the liver. The liver and small intestine are also the major sites of cholesterol production, although synthesis can occur in every nucleated cell of the body. The body maintains a complex transport system of lipoproteins to shuttle cholesterol from the liver and small intestine to peripheral cells and back to the liver.

Mammalian cells do not contain enzymes capable of breaking down the sterol nucleus of cholesterol. Hepatocytes, however, can modify substituents of the ring structure and alter the hydrophobic side chain, producing bile acids. Bile acids are secreted into the small intestine and function in the absorption of dietary fats. Most of secreted bile acids are reabsorbed in the small intestine and transported back to the liver via the enterohepatic circulation. The loss of bile acids is the main route of cholesterol elimination from the body.

In many people, especially in developed countries, more cholesterol accumulates in the body than is utilized. This has potentially pathological consequences because high levels of plasma cholesterol have been implicated in atherosclerosis and ischemic

heart disease. Because the liver is the main site of both cholesterol synthesis and lipoprotein synthesis and degradation and is the main site of cholesterol removal in the form of bile acids, the study of the liver is important to the understanding of cholesterol flux in the body.

1.2 Cholesterol Synthesis

Cholesterol is formed *in vivo* from acetyl-CoA. The rate-limiting step in this pathway is the conversion of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) to mevalonate by HMG-CoA reductase, which is located in the endoplasmic reticulum. Mevalonate is condensed to squalene, which is cyclized to lanosterol before the final modifications of the sterol nucleus and hydrophobic side chain (Myant, 1981).

The regulation of cholesterol synthesis in the liver is quite complex. HMG-CoA reductase activity has been shown to be inhibited by increased levels of intracellular cholesterol. Much work has been done which indicates that the regulating sterol is not cholesterol, but endogenously generated oxysterols like 25-hydroxycholesterol and 24-25 epoxylanosterol (reviewed by Rudney & Sexton, 1986). Lipoprotein uptake also influences cholesterol production. Uptake of low density lipoprotein (LDL) has been shown to suppress cholesterogenesis by inhibiting HMG-CoA reductase synthesis and accelerating is degradation (Rudney & Sexton, 1986). The uptake of chylomicrom (CM) remnants has also been shown to supress cholesterol synthesis while the uptake of high density lipoprotein (HDL) does not (Andersen & Dietschy, 1977). HMG-CoA reductase is also subject to activation-inactivation by

phosphorylation (Beg *et al.*, 1978). The enzyme is most active in its dephosphorylated state while phosphorylation by AMP-activated protein kinase inactivates the enzyme (Carling *et al.*, 1987). There has been some evidence to suggest that the phosphorylated (inactive) form of the enzyme is more susceptible to degradation then the dephosphorylated (active) form (Rudney & Sexton, 1986).

1.3 The Plasma Lipoproteins

Lipoproteins are important in transporting cholesterol and triglycerides throughout the body (Fig 1.1). They are complex particles, composed of a hydrophilic coat containing apoproteins, phospholipids and free cholesterol surrounding a hydrophobic core of triglycerides and cholesteryl esters. Lipoproteins are held together by non-covalent forces, allowing the ready exchange of certain components, like cholesterol and triglycerides, among different lipoproteins and between lipoproteins and cellular plasma membranes. Other lipoprotein components, like apoproteins, are not interchanged indiscriminately, but are specifically tranferred between particular lipoprotein classes. Triglycerides and cholesteryl esters must be hydrolyzed to free fatty acids and free cholesterol before they can be used by cells. Free fatty acids are delivered to adipose and muscle tissues for storage and oxidation, respectively. Free cholesterol delivered to extrahepatic tissue is used by proliferating cells for membrane synthesis and by steroidogenic tissue for the production of steroid hormones.

There are five main classes of lipoproteins as determined by their flotation after ultracentrifugation (Deckelbaum, 1987): chylomicrons (CM), very low density lipoproteins (VLDL), intermediate

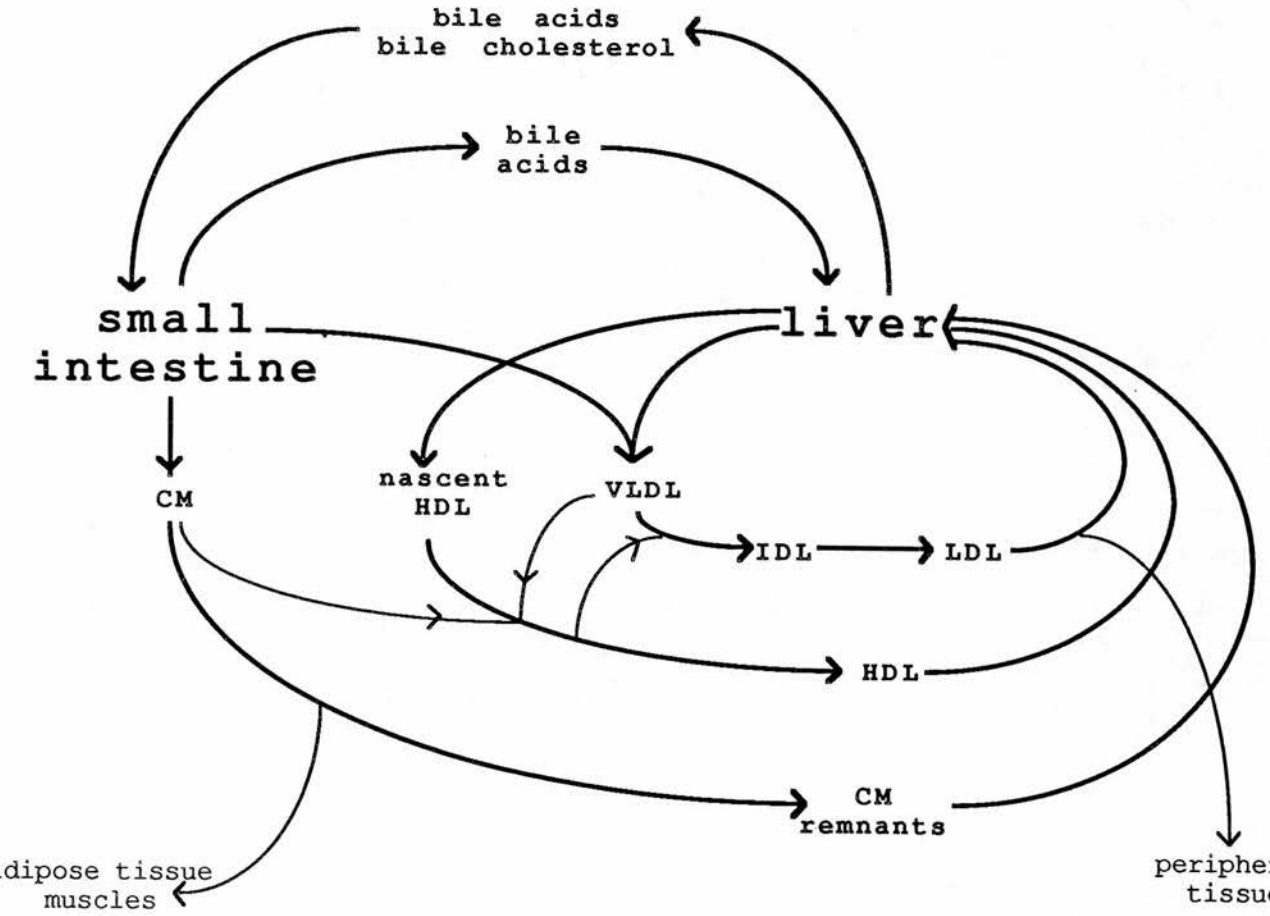


Figure 1.1. Lipoprotein Interactions in the Body

density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). The density range for classification is based on lipoproteins isolated from human plasma and does not apply to all species. The physical properties and chemical composition of human lipoproteins are summarized in Table 1.1.

Lipoprotein functions are directed by the apoprotein moiety of the particle (Table 1.2). These apoproteins facilitate the exchange of lipids between lipoprotein classes as well as direct lipoprotein hydrolysis and cholesterol esterification (Hoeg & Brewer, 1986). Apoproteins can both act as ligands for or inhibitors of receptor-mediated uptake of lipoproteins (Hoeg & Brewer, 1986). Some apoproteins are themselves transferred between lipoprotein classes. Apo B-100 is notably one apoprotein that is not transferred between lipoproteins. It is initially incorporated in VLDL and remains with the lipoprotein throughout its metabolism to LDL.

1.3.a Chylomicrons (CM)

CM are the largest and least dense of the lipoproteins. They are synthesized in the small intestine from the absorption of dietary fats and contain primarily triglycerides (Deckelbaum, 1987). The presence of large amounts of apo C-III relative to apo E in the particle prevents immediate uptake and catabolism of the lipoprotein by the liver (Hoeg & Brewer, 1986). CM therefore function mainly to transport triglycerides to extrahepatic tissue. The presence of apo C-II in the CM coat activates liprotein lipase, located in the of adipose tissue and skeletal muscle capillary endothelium. This enzyme hydrolyzes the lipoprotein triglycerides, releasing monoglycerides, diglycerides, and free fatty acids into the plasma. These molecules are then either delivered to

Table 1.1 Physical and Chemical Characteristics of the Human Plasma Lipoproteins.

Lipoprotein	Physical Properties		Chemical Composition* (%)				
class	Density (g/ml)	Diameter (nm)	Protein	PL	FC	CE	TG
CM	<0.95	>80	1-2	2-8	1	1-3	90-96
VLDL	0.95-1.006	30-80	6-10	12-18	5-8	8-14	50-65
IDL	1.006-1.019	25-30	12-16	15-22	7-11	20-35	25-40
LDL	1.019-1.063	19-25	20-25	20-25	6-10	35-45	6-12
HDL	1.063-1.210	6-11	35-55	25-40	1-6	10-20	3-8

*abbreviations: PL=phospholipids; FC=free cholesterol; CE=cholesteryl ester; TG=triglycerides.
(Deckelbaum, 1987)

Table 1.2 Summary of the Apoproteins and Their Probable Functions.

Apoprotein	Lipoprotein Class	Function
A-I	HDL	structural protein of HDL activates LCAT recognizes cellular receptor
A-II	HDL	structural protein of HDL recognizes cellular receptor cofactor for hepatic lipase in vitro
A-IV	CM	unknown
B-100	VLDL, IDL, LDL	essential ofr VLDL formation structural protein of LDL ligand for B/E receptor
B-48	CM, VLDL	essential for CM formation possibly facilitates CM remnant removal
C-I	CM, VLDL, IDL, LDL, HDL	activates LCAT
C-II	CM, VLDL, IDL, LDL, HDL	activates lipoprotein lipase
C-III	VLDL, IDL, LDL, HDL	possibly blocks hepatic uptake
D	HDL	possibly cholesteryl ester exchange protein
E	VLDL, IDL, LDL, HDL	ligand for E and B/E receptors inhibits lipoprotein lipase
F	HDL	unknown
G	VLDL	unknown
H	CM, VLDL	cofactor for lipoprotein lipase in vitro

muscle and adipose tissue for oxidation or storage or scavenged by albumin for delivery to the liver. After depletion of the core triglycerides, surface components containing apo A-I, A-IV, and C, plus free cholesterol and phospholipids 'bud off' and are transferred to nascent HDL particles (Tall & Small, 1980). The resultant CM remnants, enriched in cholesteryl ester and containing apo B-48 and E, are taken up by the liver via the hepatic apo E receptor (Havel *et al.*, 1987).

1.3.b Very Low Density Lipoproteins (VLDL)

VLDL are synthesized primarily in the liver, but also in the small intestine. They, too, contain primarily triglycerides, either from dietary fatty acids absorbed in the small intestine or from fatty acids produced in the liver. Consequently, VLDL function in much the same way as CM in transporting triglycerides to extrahepatic tissues and their metabolism follows a similar pattern. In plasma, VLDL transfer apo A-I and A-IV to nascent HDL and in return acquire apo C and E and cholesteryl ester (Green & Glickman, 1981). As with CM, the presence of apo C-III prevents immediate hepatic uptake while apo C-II activates lipoprotein lipase for the breakdown of the triglycerides. After the delivery of triglycerides, the remaining surface components apo A-I, A-IV, C, free cholesterol, and phospholipid are transferred to HDL (Tall & Small, 1980). The VLDL remnants, containing apo B and E, can be endocytosed by the apo B/E receptor, also known as the LDL receptor, in the liver (Havel *et al.* 1987). In humans, however, roughly half of VLDL remnants are further metabolized to LDL (Deckelbaum, 1987). VLDL remnants are reprocessed

to LDL by the loss of the remaining triglyceride and most apoproteins to HDL.

1.3.c Intermediate Density Lipoproteins (IDL)

IDL are found in only low concentrations in the plasma. They are formed from the catabolism of VLDL by lipoprotein lipase and probably represent an intermediate step between VLDL and LDL (Rifai, 1986). Their function is unknown at present, but they accumulate in certain lipid disorders (Deckelbaum, 1987).

1.3.d Low Density Lipoproteins (LDL)

LDL are formed as a result of the catabolism of VLDL. They are more homogeneous than other lipoproteins because the LDL core is almost exclusively composed of cholesteryl ester and the only apoprotein in the coat is apo B-100 (Deckelbaum, 1987). Despite the fact that the liver is largely responsible for LDL uptake, LDL function to transport cholesterol to peripheral cells (Dietschy *et al.*, 1988). As with VLDL, LDL are primarily taken up by receptor-mediated endocytosis through recognition of apo B-100 (Brown & Goldstein, 1986), although a receptor-independent process does exist (Spady *et al.*, 1985). The introduction of preformed cholesterol into a cell by LDL alters intracellular cholesterol homeostasis. Uptake of LDL results in the inhibition of HMG-CoA reductase and the stimulation of acyl coenzyme A:cholesterol O-acyl transferase (ACAT), effectively inhibiting cholesterol synthesis and increasing its storage as cholesteryl ester (Brown & Goldstein, 1976). LDL receptor synthesis is also inhibited in order to restrict further LDL uptake (Brown & Goldstein, 1976).

1.3.e High Density Lipoproteins (HDL)

As a class of lipoproteins, HDL are the smallest and consist of an heterogeneous group of particles. Nascent HDL* are synthesized as bilaminar discs containing phospholipid, free cholesterol, and apo E, which is secreted into plasma (Deckelbaum, 1987). They are rapidly remodeled by exchange of apoproteins and cholesteryl ester with other lipoproteins and become spherical in shape. HDL appear to act in the removal of free cholesterol from plasma membranes (Tall & Small, 1980). The presence of apo A-I in the hydrophilic coat activates lecithin:cholesterol acyl transferase, which esterifies this free cholesterol, causing it to be stored in the core of the lipoprotein. The cholesteryl ester is subsequently transfered to LDL via cholesterol ester transfer protein for pickup by the liver (Fielding & Fielding, 1981). This evidence suggests that HDL is responsible for the 'reverse cholesterol transport', that is the movement of cholesterol from the periphery to the liver (Roheim, 1986). Plasma HDL concentration has been reported to vary inversely with the development of atherosclerosis and is therefore considered to be a negative risk factor (Rifai, 1986). HDL itself has recently been shown to be taken up by the liver by a receptor-mediated process that requires apo E (Mackinnon *et al.*, 1987).

Cholesterol in the liver, either synthesized *de novo* or taken up from the plasma lipoproteins, can be metabolized by several routes (Fig 1.2) (Suckling & Stange, 1985). It can be used in the formation of membranes or esterified for storage or export in lipoproteins. It can also be catabolized to bile acid or secreted as bile cholesterol.

*produced predominantly in the liver, but also to a certain extent in the small intestine

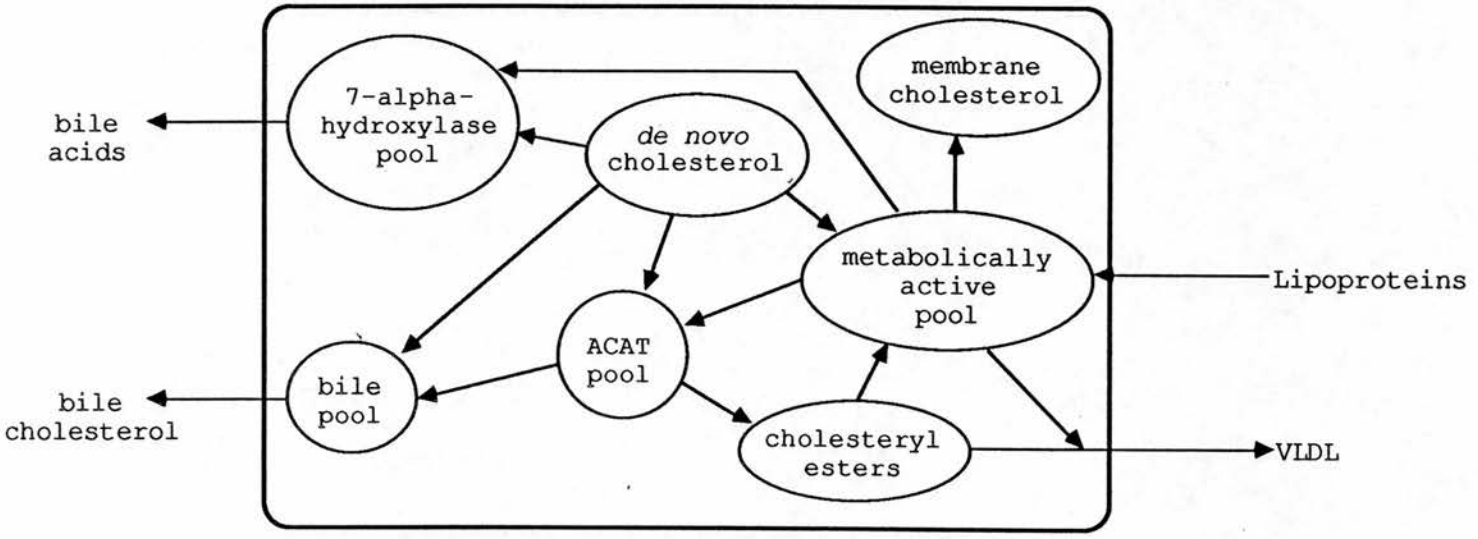
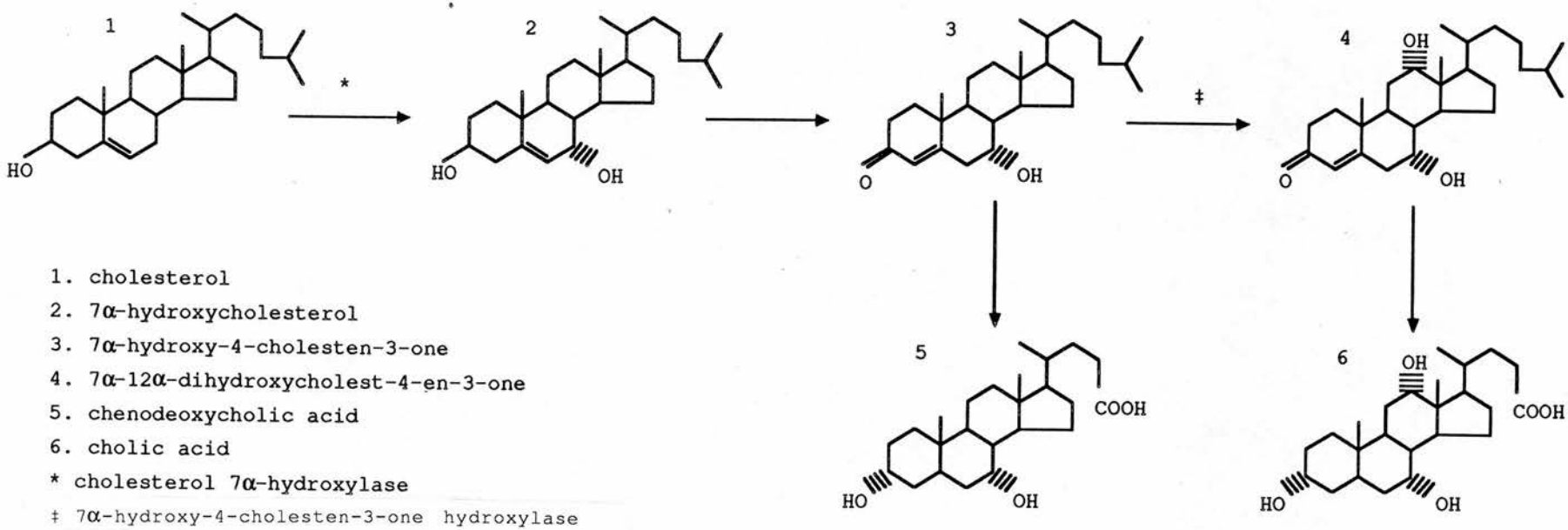


Figure 1.2 Cholesterol Metabolism in the Liver

The balance of cholesterol at this level is crucial to the overall homeostasis in the body. Of primary interest in this study was the catabolism of cholesterol to bile acids, as this is the major route by which cholesterol is eliminated from the body.

1.4 The Bile Acids

Bile acid synthesis occurs solely in the liver (Fig 1.3). Cholesterol is oxidized by cholesterol 7α-hydroxylase to form 7α-hydroxycholesterol. This step is considered to be rate-limiting and this enzyme will be considered in greater detail later in the chapter. 7α-hydroxycholesterol is then dehydrogenated to form 7α-hydroxy-4-cholesten-3-one, the last common precursor to the different bile acids. This conjugated ketone can be further hydroxylated at the 12α position by 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase to form 7α,12α-dihydroxy-4-cholesten-3-one. This second enzyme determines the ratio of chenodeoxycholic acid (CDCA) to cholic acid (CA). High rates of bile acid synthesis favor a larger CA:CDCA ratio while low rates favor a smaller CA:CDCA ratio (Miettinen, 1987). Both the mono- and di-hydroxylated sterols are then reduced at the C 4-5 double bond and at the C-3 ketone to yield di- and tri-hydroxylated compounds. Finally, the isooctane side chain is shortened by hydroxylation first at the C-26 and then at the C-24, resulting in the removal of propionic acid. The products of this pathway are the C24 primary bile acids CA and CDCA (Myant, 1981). These bile acids are the main ones produced in most mammalian species, but there do exist other end products such as the 5β and 5α bile acids (Danielsson and Sjövall, 1975). Bile acids are often



1. cholesterol
2. 7α-hydroxycholesterol
3. 7α-hydroxy-4-cholesten-3-one
4. 7α-12α-dihydroxycholest-4-en-3-one
5. chenodeoxycholic acid
6. cholic acid

* cholesterol 7α-hydroxylase

‡ 7α-hydroxy-4-cholesten-3-one hydroxylase

the C-5 H is in the β position

Figure 1.3 Bile Acid Synthesis

conjugated to taurine and glycine prior to secretion from the liver (Danielsson & Sjövall, 1975).

The pathway initiated by the 7α-hydroxylation of cholesterol is thought to be the main pathway for bile acid synthesis (Danielsson & Sjövall, 1975). Alternative routes which bypass this step have been proposed involving the synthesis of bile acids from 25-hydroxycholesterol and 26-hydroxycholesterol (Salen & Shefer, 1983). In practice, however, these routes contribute to only a minor portion of normal bile acid synthesis in man (Swell *et al.*, 1980, 1981).

Bile acids function as detergents to solubilize fats and other lipophilic compounds consumed in the diet. In man, bile acids are secreted into the duodenum during a meal. Within the intestinal lumen, they form mixed micelles with dietary fats. Fats and cholesterol are hydrolyzed before absorption by the gut. Once absorbed, fats and cholesterol are converted into triglycerides and cholesteryl esters, respectively, and packaged into lipoproteins for transport in the body as described earlier. Bile acids are taken up by a specific transport system in the terminal ileum and are also absorbed passively in the proximal ileum (Miettinen, 1987). Bile acids are then redirected to the liver via the enterohepatic circulation (Fig 1.1) (Danielsson & Sjövall, 1975). In fact, 95% of the bile acids secreted are reabsorbed in the gut and returned to the liver (Miettinen, 1987). The 5% loss of bile acids represents the main mechanism for cholesterol elimination from the body.

1.4.a Effect of Substrate Supply on Bile Acid Synthesis

Bile acid synthesis is very much dependent upon the supply of substrate cholesterol. *In vivo*, it is impossible to identify a specific cholesterol source for bile acid synthesis because cholesterol in all pools is exchangeable. Some workers report that *de novo* synthesized cholesterol is a 'preferred' source for bile acid synthesis in the rat (Balasubramanian *et al.*, 1973; Mitropoulos *et al.*, 1974). Indeed, cholesterol 7α-hydroxylase activity often varies in parallel with the activity of HMG-CoA reductase, indicating an obligatory response to a change in intracellular cholesterol (Table 1.3). While there is some evidence that newly formed cholesterol is also used in humans, (Einarsson *et al.*, 1979), other work has indicated that HDL free cholesterol is utilized for bile acid synthesis (Halloran *et al.*, 1978; Schwartz *et al.*, 1981). Experiments carried out in isolated rat hepatocytes indicate that increasing the cholesterol pool in the liver in general leads to an increase in bile acid synthesis (Davis *et al.*, 1983). This subject is considered in greater detail in Chapter 4.

1.4.b Negative Feedback Regulation on Bile Acid Synthesis

Bile acid synthesis has long been known to be increased by the interruption of the enterohepatic circulation. Administration of bile acid sequestrants, like colestipol and cholestyramine, dramatically enhances bile acid synthesis (Boyd *et al.*, 1969). Surgical procedures, which drain bile from the liver, divert the normal flow of bile from the gut, increasing bile acid synthesis several-fold (Danielsson *et al.*, 1967; Shefer *et al.*, 1968). Infusion of bile acids into the gut of bile fistula animals has been

Table 1.3 Effect of Various Modifications on the Activities of HMG-CoA reductase and Cholesterol 7α-hydroxylase.

Modification	HMG-CoA reductase	Cholesterol 7α-hydroxylase
bile acid sequestrant	+	+
bile fistula	+	+
portacaval anastamatosis	+	+
diurnal rhythm		
dark cycle	+	+
light cycle	-	-
thyroid hormone	+	+
adrenalectomy	-	-
fasting	-	-
glucose after starvation	+	+
estrogen	+	+
cholesterol feeding	-	+
phosphorylation	-	+

(Myant and Mitropoulos, 1977; Suckling and Stange, 1985)

shown to diminish this increase (Shefer *et al.*, 1969). These data indicated that synthesis was inhibited by the negative feedback of bile acids. Experiments in which bile acids were added to microsomal assays resulted in the inhibition of cholesterol 7α-hydroxylase activity. However, this inhibition may have been caused by a detergent effect on the enzyme (Boyd *et al.*, 1973). Moreover, recent findings have come to question the regulation of cholesterol 7α-hydroxylase by feedback inhibition. Work in cell culture systems has demonstrated that the uptake of bile acids by isolated hepatocytes does not influence bile acid synthesis (Davis *et al.*, 1983; Kubaska *et al.*, 1985). In addition, work *in vivo* has shown that the treatment of bile fistula animals with sodium bicarbonate prevents the inhibition of bile acid synthesis due to the infusion of bile acids (Davis *et al.*, 1988; Duane *et al.*, 1988). In light of these more recent observations, it may be that other factors in the recirculating bile are important for the regulation of bile acid synthesis.

1.5 Cholesterol 7α-hydroxylase

Cholesterol 7α-hydroxylase is the rate-limiting step in bile acid synthesis (Myant & Mitropoulos, 1977). Although most bile acids are conserved by the enterohepatic circulation, they must be continually synthesized to balance the loss. Because the loss of bile acids represents the major route of cholesterol elimination from the body and because the rate of bile acid synthesis is controlled by cholesterol 7α-hydroxylase, it is clearly important to know more about this enyme.

1.5.a Assay

In general, the incubation of cholesterol with microsomes under suitable conditions will result in the production of 7α-hydroxycholesterol. Activity requires the presence of NADPH and O2. The presence of a sulfhydryl reagent prevents autooxidation of cholesterol. Conversion of 7α-hydroxycholesterol to other bile acid intermediates only occurs in the presence of NADH, a cofactor for the oxidation of 7α-hydroxycholesterol to the more stable conjugated 3-ketone.

Experiments carried out during this study used the isotope incorporation method to determine cholesterol 7α-hydroxylase activity (Myant & Mitropoulos, 1977). This procedure, based on the conversion of [4-^{14}C]cholesterol to radiolabelled 7α-hydroxycholesterol, is the most straightforward and the least expensive of the many techniques available. Activity is expressed as the per cent conversion of cholesterol to its 7α-derivative. 7α-hydroxycholesterol cannot be quantified directly because the presence of endogenous cholesterol alters the specific activity of the substrate to an unknown extent. Providing internal controls for each assay permits comparison across experiments. It is important in enzyme assays to ensure that the supply of substrate is not rate-limiting. However, it has not been possible to demonstrate the saturation of cholesterol 7α-hydroxylase because cholesterol comes out of solution at high concentrations (Myant & Mitropoulos, 1977). Bosisio et al, (1983) showed that the addition of exogenous cholesterol at concentrations exceeding 60μM equilibrates fully with the microsomal pool destined for 7α-hydroxylase catabolism. In the experiments presented here, exogenous cholesterol is added to the assays in acetone because acetone does

not disperse membrane lipids in the same way as detergents (Myant & Mitropoulos, 1977).

1.5.b Characterization of Cholesterol 7α-hydroxylase

Despite its pivotal role in bile acid synthesis, little detailed molecular information is available on cholesterol 7α-hydroxylase. It is classified as a monooxygenase because of its requirement for NADPH and oxygen (Mitropoulos & Balasubramanian, 1972; Boyd *et al.*, 1973). It also requires the activity of NADPH-cytochrome P450 reductase to catalyze the transfer of electrons from NADPH to the terminal acceptor, cytochrome P450 (Wada *et al.*, 1969). Cholesterol 7α-hydroxylase undergoes reversible inhibition by carbon monoxide (Boyd *et al.*, 1969, 1973), a common feature of cytochromes P450. Cholesterol 7α-hydroxylase does not appear to require phospholipid for activity (Hansson & Wikvall, 1979). Cytochrome b_5 has been shown to increase activity two-fold, suggesting that it is a vital component of the enzyme complex *in vivo* (Miki *et al.*, 1988). Activity was found to be inhibited by the 7α-hydroxycholesterol product as well as the 7β-epimer (Boyd *et al.*, 1973). Studies in microsomes revealed that cholesterol 7α-hydroxylase expressed specificity for the flat planar sterol structure and isooctane side chain of cholesterol (Boyd *et al.*, 1980). These studies were later confirmed in purified preparations of the enzyme (Ozasa & Boyd, 1981; Ogishima *et al.*, 1987).

Cholesterol 7α-hydroxylase has been shown to be more sensitive to the effects of ionic detergents than nonionic ones (Hattersley & Boyd, 1975). The resulting inactivation may have explained the loss in activity in early purification attempts. Over the past decade,

there have been several reports on the purification of cholesterol 7α-hydroxylase (Table 1.4). The more recent purifications have demonstrated that cholesterol 7α-hydroxylase is a unique form of P450 based on the determination of substrate specificity. However, the specific content of P450 in the final preparation has been low.

1.5.c Regulation of Cholesterol 7α-hydroxylase

Cholesterol 7α-hydroxylase activity is known to exhibit a diurnal rhythm, with maximum activity expressed in mid-dark phase. This rhythm is apparently under the influence of both the adrenal cortex and the pituitary (Myant & Mitropoulos, 1977). Activity also varies with the age, gender, and species of an animal (Gielen *et al.*, 1976). In the rat, the activity of cholesterol 7α-hydroxylase has been shown to increase dramatically at weaning, at which time it reaches adult levels (Gielen *et al.*, 1976; Hahn & Innis, 1984). This increase is paralleled by an increase in HMG-CoA reductase activity (Carlson *et al.*, 1978). The activities of both 7α-hydroxylase and HMG-CoA reductase then slowly decline over the lifetime of the animal (Gielen *et al.*, 1976; Carlson *et al.*, 1978). Much work has been done to follow the ontogeny of bile acid synthesis and its significance in cholesterol homeostasis (Subbiah & Hassan, 1982). It has been found that the stimulation of bile acid synthesis early in life can result in an increased capacity of cholesterol catabolism in later years (Subbiah, 1986).

Cholesterol 7α-hydroxylase activity has been reported to be higher in female than male rats (Gielen *et al.*, 1976; Carlson *et al.*, 1978). This variation in activity may be caused by hormonal differences between the two genders. Estradiol administration to

Table 1.4 Summary of the Reports on the Purification of Cholesterol 7α-hydroxylase.

	Apparent Mr	Activity*	P450 content (nmol/ml)	Specificity‡
Hansson and Wikvall (1979)	50000	0.65	3.6	0.2
Danielsson and Wikvall (1981)	N.D	7.5	3.0	15
Ozasa and Boyd (1981)	N.D.	6	1.35	1.6
Andersson et al (1985)	51000	15	3.0	150
Miki et al (1987)	48000	500	7.0	N.D.
Ogishima et al (1987)	52000	500	9.0	∞

* fold increase of the purified activity over microsomal activity
‡ fold increase of the 7α-hydroxylation of cholesterol over the combined hydroxylation of cholesterol at the 12α, 25, and 26 positions
N.D.= not determined

female rats has been shown to enhance cholesterol 7α-hydroxylase activity three-fold (Ferreri *et al.*, 1977). When given to castrated male rats, this hormone can double enzyme activity (Deliconstantinos & Ramantanis, 1982).

1.5.d Induction of Cholesterol 7α-hydroxylase Activity

The activity of many cytochromes P450 is increased by treatment with xenobiotics (see section 3.1). While cholesterol 7α-hydroxylase has been shown to be a P450, evidence demonstrating its induction has been equivocal. Phenobarbital has been shown to both enhance 7α-hydroxylase activity up to three-fold (Brandau & Keup, 1976) and decrease it by 40% (Balasubramanian & Mitropoulos, 1975; Gielen *et al.*, 1976). 3-methylcholanthrene has been reported not to effect (Appleton *et al.*, 1981) or to decrease (Gielen *et al.*, 1976) 7α-hydroxylase activity. Treatment with clofibrate has been shown to decrease bile acid output in rats (Cohen *et al.*, 1974), but has been reported not to affect 7α-hydroxylase activity *in vitro* (Angelin *et al.*, 1976). Other cytochrome P450 inducers, like SKF-525A, have also exhibited inconsistencies as to their effect on cholesterol 7α-hydroxylase activity (Rush & Fears, 1986). These differences, however, may result from variations in dosage regimes and methods of administration. More convincing evidence about the non-inducibility of cholesterol 7α-hydroxylase is that its activity does not alter in parallel with microsomal P450 content (Brown & Boyd, 1974; Balasubramanian & Mitropoulos, 1975).

1.5.e Modulation of Cholesterol 7α-hydroxylase Activity

There have been several reports in the past few years concerning the modulation of cholesterol 7α-hydroxylase activity by cytosolic factors. Cholesterol 7α-hydroxylase has been shown to have a very short half life (Brown & Boyd, 1974; Danielsson & Wikvall, 1981), which may reflect on the method of regulation. *In vitro*, cytosolic protein fractions have been shown to enhance activity (Danielsson & Wikvall, 1980; Sanghvi *et al.*, 1981; Kwok *et al.*, 1981a). Workers, however, do not agree on the specific cytosolic constituent that causes the activation of the enzyme. Several groups attribute the enhancement of 7α-hydroxylase activity to phosphorylation by cAMP-dependent kinase (Goodwin *et al.*, 1982; Kwok *et al.*, 1983; Tang & Chiang, 1986), but the relevance of this kinase *in vivo* has not been evaluated. cAMP-dependent kinase has been shown to transform a cytochrome P450 to its denatured P420 form, indicating that cAMP may regulate the biotransformation system through the degradation of P450s (Taniguchi *et al.*, 1985).

Cholesterol 7α-hydroxylase activity has been shown to be decreased by the action of alkaline phosphatase (Sanghvi *et al.*, 1981; Goodwin *et al.*, 1982; Tang & Chiang, 1986) as well as by that of specific microsomal phosphatases (Diven *et al.*, 1988). These studies, however, did not address the possibility that phosphatases can affect P450 activity by decreasing cytochrome P450-NADPH reductase activity (Pyerin *et al.*, 1986). Other work has indicated that secondary proteins, like sterol carrier protein2 (Seltman *et al.*, 1985; Lidström-Olsson & Wikvall, 1986), can affect cholesterol 7α-hydroxylase activity.

Other lipid metabolizing enzymes have been shown to be modulated by phosphorylation. Both acetyl-CoA carboxylase, the rate-limiting enzyme of fatty acid synthesis, and HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis, are inactivated by phosphorylation (Hardie et al, 1989). Hormone sensitive lipase, which catalyzes the breakdown of triacylglycerols and cholesterol esters, has been shown to be both activated and inactivated by two distinct kinases (Hardie *et al.*, 1989). Extensive work on these enzymes has delineated both the responsible kinases and the sites phosphorylated. There have also been reports that ACAT, the enzyme responsible for cholesterol esterification, is activated by phosphorylation in the liver (Suckling *et al.*, 1983).

Scallen and Sanghvi (1985) suggested that HMG-CoA reductase, cholesterol 7α-hydroxylase, and ACAT are coordinately regulated by phosphorylation. This proposal states that the utilization of cholesterol, by removal to bile acids or esterification to cholesteryl ester, is enhanced by kinase activity. The synthesis of cholesterol, on the other hand, is decreased (Fig 1.4).

1.6 Summary

Atherosclerosis and ischaemic heart disease are leading causes of death in the Western world. These diseases have been shown to be related to high levels of plasma cholesterol (Lipid Research Clinics Program, 1984). As outlined in this chapter, cholesterol is transported throughout the body in lipoproteins, primarily in LDL. The central role of the liver in systemic cholesterol homeostasis is emphasized by the fact that the liver is responsible for almost all (75%) LDL uptake in the body (Dietschy *et al.*, 1988). The liver is

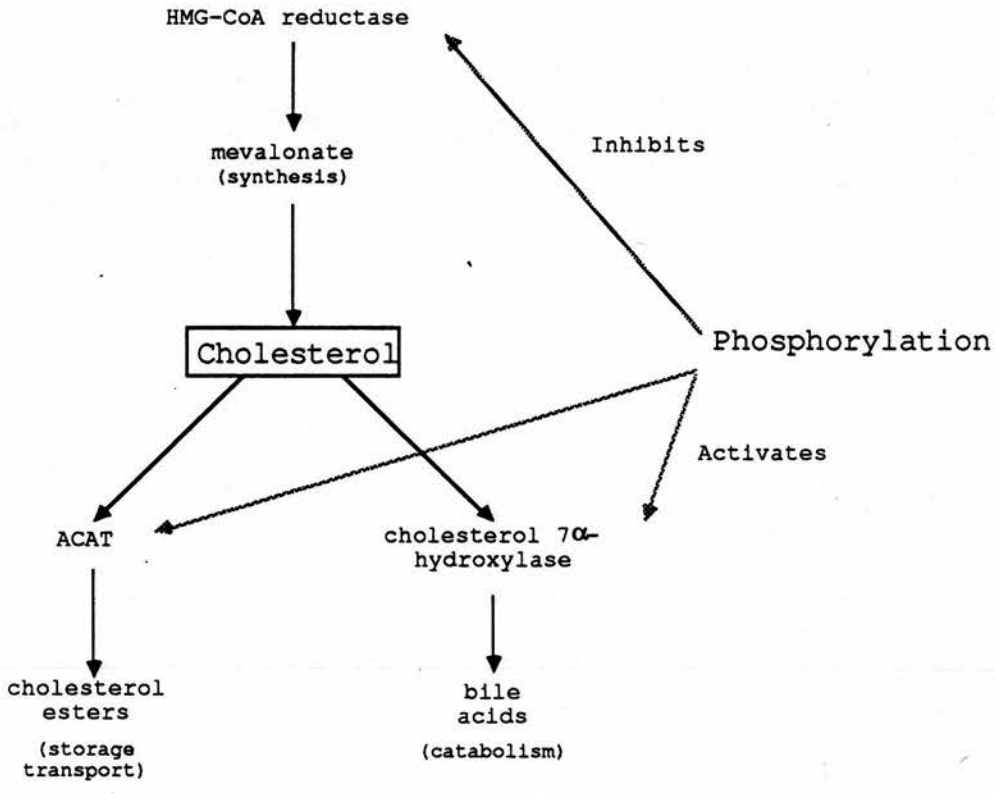


Figure 1.4 Coordinate Regulation
of Cholesterol Metabolism

also the sole site for cholesterol degradation to bile acids. As the rate-limiting enzyme in bile acid synthesis, cholesterol 7α-hydroxylase represents a powerful tool for the catabolism of cholesterol. The understanding of the factors affecting this enzyme would yield valuable information on how it is regulated and how it could be manipulated for the treatment of related diseases. For example, in the treatment of atherosclerosis, it is beneficial to increase bile acid removal from the body to lower serum cholesterol. It is therefore important to up-regulate cholesterol 7α-hydroxylase to increase cholesterol elimination from the body. The aim of the work presented in this thesis is two-fold: 1.) to examine cholesterol 7α-hydroxylase at a molecular level using antibody probes and to compare it to other, well-characterized cytochromes P450 and 2.) to investigate the regulatory mechanisms affecting bile acid synthesis in a human hepatocyte model.

Chapter 2

Materials and Methods

2.1 Animals

Adult female Wistar rats (200-250g) were used for most of the studies. Occasionally, both male and female rats were used. Syrian hamsters (120-130g) and CD-1 mice (25-30g) were also used. These animals are noted throughout.

Animals were maintained on either powdered rat diet [standard laboratory control diet]; 4% (w/w) cholestyramine in powdered diet [cholestyramine diet]; or 70% (w/w) wholemeal flour, 25% (w/w) dried skimmed milk, 5% (w/w) dried yeast [soft diet] for at least seven days prior to experiments.

Some animals were also treated with xenobiotics as follows: phenobarbitone (80mg/kg in saline); clofibric acid (400mg/kg in corn oil); isosafrole (400mg/kg in corn oil); SKF-525A (50mg/kg in saline). Animals were given daily i.p. injections for three days before use.

2.2.a Preparation of Microsomes

Microsomes* were prepared by the method of Boyd *et al.* (1973). The liver was partially perfused with 0.104M KCl containing 50mM NaF (KCl solution) through the portal vein before the vena cava was severed and was susequently fully perfused until it was completely pale. The liver was diced into small pieces, washed in 30ml KCl solution, and resuspended as a 25% (w/v) solution. Samples were homogenized with three passes of a glass-teflon homogenizer and centrifuged at 30000g for 20min at 4°C in a Beckman JA21 centrifuge. Supernatants were decanted and centrifuged at 100000g for 60min at

*Animals were maintained on a normal light cycle and sacrificed at 9am

5°C in a Beckman L8 ultracentrifuge. The resulting cytosolic supernatants were decanted and the inside of each centrifuge tube was carefully wiped clean of fat before the resuspension of microsomal pellets in potassium phosphate pH 7.4 containing 50mM NaF, 25mM sucrose, and 1mM EDTA. Microsomes were rehomogized in a microteflon homogenizer, assayed on the day for cholesterol 7α-hydroyxlase activity, and stored frozen at -70°C.

2.2.b Phosphorylation of Microsomal Proteins

Microsomes were phosphorylated following the methods of Carling *et al.* (1987). Microsomes (10-20mg/ml) were incubated in phosphorylation reaction buffer with protein kinases at 4°C for 5-60min. The reaction was stopped by the addition of 10mM EDTA. Microsomes were immediately assayed for either cholesterol 7α-hydroxylase activity or HMG-CoA reductase activity.

Phosphorylation reaction buffer:

Tris:HCl pH 7.4	100mM
AMP	1mM
ATP	2mM
$MgCl_2$	7.5mM

2.2.c Immunoprecipitation of Microsomal Proteins

Proteins were immunoprecipitated from microsomes following the method of Davies and Brown (1987). Microsomes (200µg) were solubilized in lysis buffer (450µl) and incubated with antisera (20µl) for 60min at room temperature. 10% (w/v) Protein A Sepharose beads in lysis buffer (50µl) were then added to the sample tubes and the tubes rotated for 60min at 4°C. Antibody:antigen complexes bound to the Protein A Sepharose beads were pelleted by centrifugation in a

microfuge at 10000g for 30sec. Supernatants were removed with a point style 3 blunt bore Hamilton syringe and the beads were rinsed sequentially in washes 1 (100µl), 2 (100µl), and 3 (100µl), each time pelleting the beads at 10000g for 30sec before the removal of the supernatant.

Lysis buffer:	10mM Tris:HCl pH 7.4, 1% (w/v) Nonidet P-40, 150mM NaCl, 1mM EDTA, 0.1% (w/v) NaN_3
Wash 1:	lysis buffer plus 0.5M NaCl
Wash 2:	lysis buffer plus 0.1% (w/v) SDS
Wash 3:	10mM Tris:HCl pH 7.4, 0.1% (w/v) Nonidet P-40

Precipitated antigens were analyzed using SDS-PAGE (see section 2.12). Non-reducing boiling mix (100µl) (see Table 2.1) was added to the precipitated beads, washes, and supernatants. Samples were heated at 100°C for 5min and 10µl was loaded directly onto SDS polyacrylamide gels. Protein bands were visualized by either silver staining or Western blotting the gels.

2.3 Purification of Cytochromes P450 and NADPH-cytochrome P450 Reductase

All purified cytochromes P450 used in work described here were prepared by Dr. Wolf and coworkers as reported previously (Wolf & Oesch, 1983; Wolf *et al.* 1986, 1988). Purified NADPH-cytochrome P450 reductase was prepared by Dr. Wolf as described by Yasukochi and Masters (1976). Purified proteins were stored at -40°C in 10mM potassium phosphate buffer pH 7.4 containing 20% (v/v) glycerol and 0.1mM EDTA. P450s were isolated from rat liver.

2.4 Antibody Generation

Antibodies to purified cytochromes P450 and NADPH-cytochrome P450 reductase were raised in New Zealand White Rabbits as reported by Wolf and Oesch (1983). IgG fractions were isolated from serum by the method of Good *et al.* (1980). Saturated ammonium sulfate pH 7.4 was added in a dropwise manner to serum at 4°C until the final concentration of ammonium sulfate was 40%. Samples were stirred for at least 4h or as long as 18h at 4°C. The resulting precipitates were pelleted by centrifugation at 10000g for 15min and the pellets resuspended in distilled H_2O before dialyzing against 200vol 10mM potassium phosphate buffer pH 7.4 for 72h at 4°C. Dialyzed solutions were were lyophilized overnight and stored at 4°C for future use.

2.5 Cytochrome P450 Concentration

The concentration of cytochrome P450 in microsomes was determined from the ferrous-cytochrome P450-CO absorption peak at 450nm (Wolf *et al.*, 1986). Microsomes (2mg/ml) were suspended in 100mM Tris:HCl, pH 7.4 and divided between two cuvettes. Sodium dithionite (microspatula tip) was added to both the sample and reference cuvettes to prevent absorbance due to cytochrome b5 and hemoglobin. CO was bubbled through the sample solution for 30sec and the difference spectra between 400 and 500nm was determined. An extinction coefficient of 91mM/cm was used for the calculation of concentration.

2.6 Reconstitution of Cytochrome P450 Activity

Cytochrome P450 monooxygenase systems were reconstituted by following the method of Wolf and Oesch (1983). Assay tubes were coated with dilaurophosphatidylcholine (50µg) before the addition of purified cytochrome P450 (0.1nmol) and NADPH-cytochrome P450 reductase (500U) in 0.1M potassium phosphate buffer pH 7.4 containing 20% glycerol and 0.1mM EDTA. Tubes were rapidly mixed and incubated for 15min at 37°C. Reconstituted monooxygenase systems were used immediately after preparation for either phenoxazone dealkylation assays or cholesterol 7α-hydroxylase assays.

2.7 Protein Determination

Protein concentration was determined by either the method of Lowry *et al.* (1951) or Bradford (1976). The difference between the two was not significant (Spearman correlation p=0.000). Standard curves were linear between 0 and 60µg BSA protein.

2.7.a Protein Assay According to Lowry

Reagent C (1 vol) was mixed with protein samples (5-200µg protein) and incubated for 10min at room temperature. Colour production was initiated by the addition of Reagent D (0.1 vol) and developed by 30min incubation at room temperature. Absorbance was read at 750nm on a Shimadzu UV-160.

Reagent A: 0.5% (w/v) $CuSO_4 \cdot 5H_2O$ in 0.1% (w/v) NaK tartrate

Reagent B: 2.0% (w/v) Na_2CO_3 in 0.1 N NaOH

Reagent C: A:B at a ratio of 1:50, made fresh daily

Reagent D: 1N Folin & Ciocalteau's Phenol Reagent

2.7.b Protein Assay According to Bradford

A 1:5 dilution of Biorad assay dye reagent concentrate (5ml) was rapidly mixed with protein samples (5-200µg protein) and the absorbance read at 595nm on a Pye Unicam SP6-550 UV/VIS spectrophotometer.

2.8 Cytochrome P450-mediated Dealkylation of Phenoxazone Analogues

Assays were carried out according to the method of Burke *et al.* (1985) based on the fluorescence of phenoxazone. Microsomes (20-200µg) or reconstituted monooxygenase systems (0.1nmol P450) were incubated in 0.1M Tris:HCl pH 7.4 containing 1µM of a phenoxazone analogue (1ml). Metabolism of phenoxazone analogues to phenoxazone was initiated by the addition of NADPH (final concentration 1mM) and was followed on a Perkin-Elmer LS-3 fluorescence spectrometer at an excitation wavelength of 530nm and an emission wavelength of 580nm. Activity was calculated as the nmol substrate metabolized/mg protein or nmol P450/min. Phenoxazone metabolism was calibrated by measuring the pen deflection resulting from the injection of 0.1nmol phenoxazone standard. Initial rates of enzyme activity were measured.

2.9 NADPH-cytochrome P450 Reductase Assay

NADPH-cytochrome P450 reductase activity was determined by the method of Yasukochi and Masters (1976) based on the NADPH-dependent reduction of cytochrome c. Reference and sample cuvettes contained microsomes (20µg) and cytochrome c (1.5mg) in 300mM potassium phosphate buffer pH 7.7 (1ml). The reaction was initiated in the sample cuvette by the addition of NADPH (final concentration 2mM) and

monitored at 550nm on a Shimadzu MPS 200 spectrophotometer. An extinction coefficient of 18.7μM/cm was used for the calculation of activity. One unit of activity is defined as the amount of reductase required to reduce 1nmol of cytochrome c/min at 30°C.

2.10 HMG-CoA Reductase Assay

HMG-CoA reductase assays were carried out according to the method of Ingebritsen and Gibson (1981). Microsomes (0.5mg) were preincubated in HMG-CoA reaction buffer (100μl) for 10min at 37°C. The reaction was initiated by the addition of [3-^{14}C]HMG-CoA (0.025μCi, final concentration 3mM) and NADPH (final concentration 1mM) and incubated for a further 15min at 37°C. DL-[2-^{3}H]mevalonic acid lactone (0.01μCi) was added as a recovery marker. The assay was terminated by the addition of 6N HCl (10μl) and the samples were incubated for a further 30min to lactonize mevalonic acid to mevalonolactone. Denatured protein was pelleted by centrifugation in a microfuge at 10000g for 5min and 100μl of the supernatant was spotted onto silica tlc plates. The plates were developed in acetone:toluene (1:1, v/v) and scanned on a Rita 68000 tlc scanner. The area of the plate corresponding to the mevalonic acid lactone standard was scraped into a scintillation vial and counted. Activity was calculated as the nmol mevalonic acid formed/min/mg.

HMG-CoA reaction buffer:

orthophosphate pH 7.4	50mM
NaCl	250mM
EDTA	1mM
DTT	5mM

2.11.a Cholesterol 7α-hydroxylase Assay

Microsomes (0.1 to 1.0mg) or reconstituted cytochrome P450 monooxygenases systems (0.1nmol P450) were preincubated in 7α-reaction buffer (1ml) and exogenous [4-^{14}C]cholesterol (0.1μCi, final concentration 2μM) for 5min at room temperature. The reaction was initiated upon the addition of NADPH (final concentration 1mM) and incubated at 37°C for 60min in a shaking water bath. The assay was terminated by the addition of 5ml chloroform:methanol (2:1, v/v). The assay was linear up to 1mg protein for 60min.

7α-reaction buffer:

Tris:HCl pH 7.4 or	0.1M
phosphate buffer pH 7.4	0.1M
NaF	50mM
2-mercaptoethylamine	26mM

2.11.b Antibody Inhibition of Cholesterol 7α-hydroxylase Activity

The effects of anti-cytochrome P450 antibodies on cholesterol 7α-hydroxylase activity were carried out as described above using 0.1mg microsomal protein and the Tris:HCl reaction buffer. IgG was made up to 20mg/ml in distilled water and up to 100μl was added to the reaction buffer along with the microsomes. The mixture was incubated for 20min at room temperature before the 5min preincubation with cholesterol. The reaction was initiated upon the addition of NADPH (final concentration 1mM) and incubated at 37°C for 60min in a shaking water bath. The assay was terminated by the addition of 5ml chloroform:methanol (2:1, v/v).

2.11.c Analysis of Reaction Products

Cholesterol and 7α-hydroxycholesterol were removed from the reaction mixture by two extractions with (5ml) chloroform:methanol (2:1, v/v). The combined organic layer was then dried under a stream of N_2 at 60°C. The residue was resuspended in 200µl chloroform:methanol (2:1, v/v), spotted onto silica gel tlc plates and the tubes were rinsed with a further 100µl chloroform:methanol. Neutral lipids were resolved by developing tlc plates in ethyl acetate:toluene (70:30, v/v) and scanning on a Rita 68000 tlc scanner. Per cent conversion of cholesterol to 7α-hydroxycholesterol was calculated by counting areas of the gel corresponding to the major peaks of radioactivity.

2.12.a Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) using a Biorad Protean II gel kit. All solutions used in for SDS-PAGE are listed in Table 2.1.

Separating gels ranging in concentration from 7.5% to 12.0% (w/v) acrylamide [acrylamide-bisacrylamide (30:0.8)] were prepared in separating gel buffer. Polymerization was begun by the addition of ammonium persulfate (final concentration 0.1% (w/v)) and TEMED (final concentration 0.001% (v/v)) and the mixture poured into the gel mold (16cm x 18cm x 1.5mm). Water was carefully laid over the polymerizing gel to form an even meniscus; this water was removed before the addition of the stacking gel.

Table 2.1 Solutions used for SDS-PAGE

Separating gel buffer: 385mM Tris:HCl pH 8.8 containing 0.125% (w/v) SDS

Stacking gel buffer: 125mM Tris:HCl pH 6.8 containing 0.125% (w/v) SDS

Boiling mix: 25mM Tris:HCl pH 6.8 containing 1% (w/v) SDS, 5% (v/v) glycerol, 2.5% (v/v) β-mercaptoethanol, and 0.0025% (w/v) bromophenol blue

Non-reducing boiling mix: 25mM Tris:HCl pH 6.8 containing 1% (w/v) SDS, 5% (v/v) glycerol, and 0.0025% (w/v) bromophenol blue

Running buffer: 52mM Tris:HCl pH 9.4 containing 53mM glycine, and 1% (w/v) SDS

Coomassie stain: 45% (v/v) methanol, 9% (v/v) acetic acid, and 0.25% (w/v) Coomassie Blue R-250

Coomassie destain: 5% (v/v) methanol and 7% (v/v) acetic acid

Solution A: 50% (v/v) methanol, 12% (v/v)acetic acid, and 0.5% (v/v) formaldehyde

Solution B: 0.02% (w/v) $AgNO_3$ in 0.075% (v/v) formaldehyde

Solution C: 6% (w/v) Na_2CO_3 and 0.0004% (w/v) $Na_2S_2O_3 \cdot 5H_2O$ in 0.05% (v/v) formaldehyde

Solution D: 50% (v/v) methanol in 12% (v/v) acetic acid

Transblot buffer: 20mM $Na_2HPO_4 \cdot 12H_2O$ in 20% (v/v) methanol

TBST: 50mM Tris:HCl pH 7.9 containing 154mM NaCl, and 0.05% (v/v) Tween 20

Chloronaphthol stain: 50mM Tris:HCl pH 7.9 containing, 154mM NaCl, 0.5mM 4-chloro-1-naphthol, and 0.01% (w/v) H_2O_2 in 16.6% (v/v) methanol

The stacking gel was prepared from 4.5% (w/v) acrylamide [acrylamide-bisacrylamide (30:0.8)] in stacking gel buffer. Ammonium persulfate (final concentration 0.1% (w/v)) and TEMED (final concentration 0.001% (v/v)) were added to intiate polymerization and the gel mixture poured on top of the separating gel. The sample well comb was inserted into the unpolymerized gel, making certain no bubbles were trapped between the gel mixture and the comb.

Protein samples (1mg/ml) were heated in boiling mix for 5min at 100°C. 5-15µl samples were loaded into wells. Gels were electrophoresed in running buffer at a constant current setting of 40mA through the stacking gel and 20mA through the separating gel. SDS-PAGE gels were either stained to examine the overall protein profile or transblotted for development as a Western blot.

2.12.b Coomassie Blue Staining of Sodium Dodecyl Sulfate Polyacrylamide Gels.

SDS polyacrylamide gels were incubated in Coomasie stain for 60min. Proteins were visualized upon destaining in Coomassie destain over several hours.

2.12.c Silver Staining of Sodium Dodecyl Sulfate Polyacrylamide Gels.

SDS polyacrylamide gels were silver-stained according to Blum *et al.* (1987). Gels were soaked in Solution A for a minimum of 1h and were then washed 3 x 20min in 50% EtOH. To reduce background staining, gels were pretreated with 0.02% (w/v) $Na_2S_2O_3 \cdot 5H_2O$ for 1min and washed 3 x 20sec in H_2O. Gels were impregnated with Solution B for 20min, rinsed in H_2O for 10min, and subsequently developed in

Solution C until the desired stain was produced. Staining was halted by 2 x 30sec washes in H_2O and a 10min incubation in Solution D.

2.12.d Western Blotting of Sodium Dodecyl Sulfate Polyacrylamide Gels.

Proteins from polyacrylamide gels were transferred onto nitrocellulose membranes by the method of Towbin *et al.* (1979) using a Biorad Transblot Apparatus and transblot buffer at a constant current of 250mA for 18h.

After transfer, gels were checked for protein by Coomassie staining and were routinely found to be blank. Nitrocellulose filters were washed twice in Tris-buffered saline and Tween (TBST) and extra protein binding sites blocked by a 60min incubation in 3% (w/v) dried skimmed milk in TBST. Filters were washed twice to remove blocking solution, incubated with the appropriate antibody at a dilution of 1:1000 for 60min, and washed four times. Filters were subsequently incubated with donkey anti-rabbit antibody conjugated to horseradish peroxidase at a dilution of 1:500 for 60min, and washed four times. All washes were carried out in TBST for 10min at room temperature. Antibody dilutions, prepared in TBST, were routinely stored at -20°C. Protein bands were visualized by incubating in chloronaphthol stain for 10 min at room temperature.

Following chloronaphthol staining, nitrocellulose filters were incubated with ^{125}I-Protein A (50µCi) in of TBST (50ml) for 60min. Radioactive TBST was discarded, and filters were washed repeatedly until no radioactivity was detected in the wash. Filters were dried completely and autoradiographed with Kodak X-AR film.

2.13 Cell culture

2.13.a Maintenance

HepG2 cells were obtained from the British Cell Culture Collection and routinely maintained in Dulbecco's Modification of Eagle's Medium (DMEM) containing 15% heat-inactivated calf serum, 0.3% L-glutamine, 100IU penicillin, and 100mg/ml streptomycin. Cells were incubated at 37°C, 5% CO_2:95% air, and 100% humidity. Culture media was changed three times a week and the cells were subcultured accordingly.

2.13.b Cell harvesting and Viability

Cells were washed three times with Dulbecco's 'A' phosphate buffered saline (PBS) and harvested from confluent cultures with 0.1% trypsin-0.001% EDTA. Cells were suspended in 4vol of 15% DMEM and viability was assessed through nigrosin dye exclusion by mixing the harvested cell suspension 1:1 with 0.5% (w/v) nigrosin in PBS. Cells were counted manually using a hemocytometer and were routinely found to be >95% viable.

2.13.c Storage of Cell Stocks

Cells were harvested, counted, and mixed with heat-inactivated newborn calf serum containing 10% (v/v) DMSO at a concentration of 5 x 10^6 cells/ml. 1ml aliquots were placed in cryotubes and stored at -70°C for 5h before being immersed in liquid N_2 for long-term storage.

Cells were retrieved from cryopreservation by rapid thawing at 37°C and immediate introduction into 20vol of 15% DMEM. Cells were washed once with 15% DMEM to remove DMSO before being transferred to

25cm^2 culture flasks. They were then incubated overnight and the culture medium was changed following day.

2.13.d Mycoplasma Testing

HepG2 cells were routinely screened for mycoplasma by the MRC Clinical and Population Cytogenetics Unit at the Western General Hospital, Edinburgh. Results were always negative.

2.14 Assay for Bile Acid Production

All experiments were carried out on cells within five passages of each other to control for long-term culturing changes. Culture flasks were seeded at a density of 8 x 10^4 cells/cm^2 and left 4 days to grow nearly confluent. The day prior to the start of the experiment (day 5), the cells were given fresh DMEM containing 10% (v/v) heat-inactivated calf serum. The day of the experiment (day 6), HepG2 cells were washed three times in PBS to remove serum-containing culture media before being given serum-free DMEM supplemented with 0.3% L-glutamine, 100IU penicillin, and 100mg/ml streptomycin, 50µg/ml gentamicin sulfate, and insulin (5µg/ml), transferrin (5µg/ml), and selenium (5ng/ml) (ITS). ITS was added to serum-free medium to help maintain cell viability (Barnes & Sato, 1980).

Cells were exposed to mevalonic acid (0-10mM), LDL (300µg/ml), and HDL (300µg/ml), 7α-hydroxycholesterol (0-100µM), and 58-035 (5µg/ml). At 24h time intervals, culture medium was removed and stored at -20°C. Cells were harvested, washed in PBS (10ml), and then resuspended in PBS (1ml) . Cells were then lysed by sonication with three 5sec bursts on a MSE Soniprep 150 and stored at -20°C.

2.15 Analysis of Bile Acids

2.15.a Radioimmunoassay

The radioimmunoassay (RIA) was carried out according to Beckett *et al.* (1978, 1979). Bile acid standards were prepared in RIA buffer and cell culture medium was assayed directly following the method of Botham and Boyd (1983).

RIA buffer:

sodium phosphate buffer pH 7.4	100mM
NaN_3	15mM
NaCl	15mM

Samples (10µl) and standards (10µl) were incubated in RIA buffer (200µl) containing 10% (v/v) bile acid-free newborn calf serum, and either anti-glycocholic (anti-GC) or anti-glycochenodeoxycholic (anti-GCDC) antisera at the appropriate dilution, with the homologous ^{125}I-labelled histamine-bile acid conjugate (Ford *et al.*, 1985). The reaction was incubated for a minimum of 3h at room temperature. Antibody-bile acid complexes were precipitated with 2.5% (w/v) $(NH_4)_2SO_4$ (3ml) and pelleted by centrifugation at 2500g for 15min at 4°C in a Beckman J6-B centrifuge. Supernatants were removed by aspiration and precipitated radioactivity was determined using a Nuclear Enterprise 1600 γ detector. Bile acid concentration was calculated from standard curves generated by the WHO Immunoassay Program designed by P.R. Edwards.

2.15.b 3α-hydroxysteroid Dehydrogenase Assay

Bile acid concentration was also determined using the Sterognost 3α Flu diagnostic kit (Mashige *et al.*, 1976) based on the oxidation of 3α-hydroxysteroids by 3α-hydroxysteroid dehydrogenase (3α-HSD).

Bile acid standards (0.1-20μM) were prepared in 100mM phosphate buffer pH 7.4. Bile acids were isolated from culture media following the method of Lawson *et al.* (1986) using Analylitichem Bond Elut columns. Columns containing C18 solid support (300-500mg) were attached to an Analyitichem Vac Elut vacuum apparatus, preconditioned with Methanol (3ml) and then equilibrated with H_2O (3ml). Culture media samples (3-10ml) were applied to the columns, which were subsequently washed with H_2O (3ml) and hexane (3ml). Bile acids were eluted from the columns with methanol (two x 3ml) and stored at 4°C. Solutions were allowed to drip through the columns by the force of gravity or <5mm pressure on the aspirator drawn vacuum apparatus. Prior to 3α-HSD assay, samples were dried under a stream of N_2 and resuspended in 0.1M Tris:HCl pH 9.0.

Samples (25μl) and standards (25μl) were incubated in the commercial 3α-HSD assay mixture (725μl) for 15min at room temperature as directed by manufacturers. 3α-HSD was omitted in control incubations. Fluorescence was determined within 15min at excitation and emission wavelengths of 565nm and 580nm, respectively, using a Perkin-Elmer LS-3 Fluorescence spectrometer.

2.15.c High Performance Thin Layer Chromatography

Bile acid standards (2.5-120μM) were prepared in HPLC grade methanol. Samples for HPTLC analysis were prepared from cell culture media using the Bond Elut method of Lawson *et al.* (1986) described earlier. Prior to loading onto HPTLC plates, samples were concentrated by drying down under N_2 and resuspended in HPLC grade methanol.

To reduce background flourescence, 10cm x 20cm HPTLC plates were preconditioned in Solvent 1 and Solvent 2. Plates were completely air-dried between washes and stored upright in a closed chromatography tank until needed. Plates were activated prior to use at 110°C for 60min and allowed to cool before spotting.

Solvent 1: ethyl acetate:methanol:acetic acid (7:2:1, v/v)

Solvent 2: cyclohexane:ethyl acetate:acetic acid (9:9:2, v/v)

Samples (10μl) and standards (10μl) were spotted onto HPTLC plates using a CAMAG Linomat IV Spotter. Plates were placed in Solvent 1 until the mobile phase had traveled 4cm up the plate, and in Solvent 2 until the mobile phase came within 1cm of the top of the plate. Plates were dried completely between solvents and kept in a dust-free area. Charring solution was then sprayed lightly and evenly over the surface of the plates until the silica gel was just saturated. Fluorescent bile acid products were visualized by heating plates at 110°C for 15min.

Charring solution:

methanol	150ml
H_2O	150ml
H_2SO_4	10ml
$MnCl_2 \cdot 4H_2O$	1g

Fluorescence was measured at 366nm on a CAMAG tlc scanner II and the raw data was manipulated using a CAMAG software package on a

Hewlett-Packard HP9216 computer. Peaks were integrated manually with the Video Integration Option to yield area values, which were subsequently processed to produce standard curves for each bile acid. These standard curves were used to calculate the unknown bile acid content of the culture media samples.

2.16 Cholesterol Determination

Total cholesterol content of cell lysates was determined using an Ames Sera Pak Cholesterol kit (Demacker *et al.*, 1983). Cholesterol standards and cell lysates (20-80µl) were incubated in the Sera Pak assay buffer for 5min at 37°C as described by the manufacturers. Absorbance was measured at 510nm on a Shimadzu UV-160. Standard curves were linear to 5mg/ml.

2.17 Statistical Analysis

Tests for statistical significance, the Spearman Correlation and the Student's t test, were calculated using the StatsWork software package on an Apple Macintosh computer.

2.18 Materials

All chemicals used throughout the work presented here were of analytical grade purity. The sources of special reagents are listed below. Cholestyramine was a gift of the Bristol-Myers Company (Cramlington, Northumberland, U.K.) and SKF-525A was a gift of Smith Kline & French Research, Ltd. (Welwyn Garden City, Herts., U.K.)

Aldrich Chemicals (Gillingham, Dorset, U.K.)

clofibrate

isosafrole

phenobarbital

Amersham International, plc (Amersham, Bucks., U.K.)

[γ-^{32}P]ATP

[4-^{14}C]cholesterol

[3-^{14}C]HMG-CoA

DL-[2-^{3}H]mevalonic acid lactone

[^{125}I]Protein A

[^{14}C]Rainbow Molecular Weight Markers

Bio-Rad Laboratories (Richmond, CA, U.S.A.)

acrylamide

ammonium persulfate

bisacrylamide

reagent dye concentrate

TEMED

BDH Ltd. (Poole, Dorset, U.K.)

ammonium sulfate

cholesterol

EDTA

glycine

glycerol

HPLC-grade solvents

Nonidet P40

potassium chloride

silver nitrate

sodium azide

sodium carbonate

sodium chloride

sodium dodecylsulfate

sodium fluoride

sodium hydroxide

Tris:HCl

Difco Laboratories (Detroit, MI, U.S.A.)

trypsin

GIBCO (Europe) Ltd. (Paisley, Scotland, U.K.)

fetal calf serum

newborn calf serum

Nuncalon tissue culture flasks

penicillin

streptomycin

Jones Chromatography (Hengoed, Mid-Glamorgan, U.K.)

Analytichem C18 Bond Eluts

Vac Elut vacuum apparatus

Kodak (Hemel Hemptstead, Herts., U.K.)

Kodak X-AR film

Miles Laboratories (Slough, England, U.K.)

Ames Serapak Cholesterol Kit

Molecular Probes Inc. (Junction City, OR, U.S.A.)

phenoxazone

methoxyphenoxazone

Northumbria Biologicals Ltd. (Cramlington, Northumbria, U.K.)

Dulbecco's modified Eagle's medium

Nycomed (U.K.) Ltd. (Birgingham, England, U.K.)

Sterognost 3α-Flu Kit

Oxoid Ltd. (Basingstoke, Hampshire, U.K.)

Dulbecco 'A' phosphate buffered saline

Pharmacia LKB Ltd. (Milton Keynes, Bucks., U.K.)

Protein A Sepharose CL-4B

Sigma Chemical Company (Poole, Dorset, U.K.)

bovine serum albumin (fatty acid free)

bovine serum albumin fraction IV

Coomassie Blue R-250

Coomassie brilliant blue G

chenodeoxycholic acid

chloronaphthol

cholic acid

copper sulfate

dilaurylphosphatidylcholine

Folin and Ciocalteau's Phenol Reagent

gentamicin sulfate

L-glutamine

glycochenodeoxycholic acid

glycocholic acid

hydrogen peroxide

insulin-transferrin-sodium selenite media supplement

lithocholic acid

2-mercaptoethanolamine

β-mercaptoethanol

mevalonid acid lactone

NADPH

nigrosin

sodium dithionate

taurochenodeoxycholic acid

taurocholic acid

Tween 20

Tween 80

Steraloids Inc. (Wilton, NH, U.S.A.)

7α-hydroxycholesterol

Chapter 3

Characterization of Cholesterol 7α-hydroxylase

3.1 Introduction

Cytochromes P450 are heme-containing proteins which vary in molecular weight between 48000 and 57000 (Åström & DePierre, 1986). The reduced P450-carbon monoxide complex has a characteristic absorbance maximum at 450nm, from which their name was derived (Whitlock, 1986). P450s, as a group of enzymes, catalyze many reactions summarized in Table 3.1 (Yang & Lu, 1987). They are active in several metabolic pathways like the synthesis and metabolism of steroid hormones, the synthesis of bile acids, prostaglandins, and thromboxanes, and the oxidation of fatty acids (Nebert & Gonzalez, 1987). P450s are also involved in drug oxidation and carcinogen activation (Wolf, 1986) as well as lanosterol metabolism.

Cytochromes P450 are dependent upon other membrane-bound proteins for activity (Yang & Lu, 1987). P450-dependent monooxygenase systems are embedded in both the endoplasmic reticulum and the mitochondria. In the endoplasmic reticulum, NADPH-cytochrome P450 reductase receives electrons from NADPH, transfering them to cytochrome P450 either directly or via cytochrome b_5 (Fig 3.1). The electron transport chain is somewhat different in the mitochondria, involving an NADPH-ferredoxin reductase and a ferredoxin (Whitlock, 1986). The ultimate electron acceptor is molecular oxygen, one atom of which is reduced to water while the other is incorporated into the substrate.

Cytochromes P450 comprise a large multigene family, which is thought to have evolved from a single ancestral gene (Nebert &

Table 3.1 Some Reactions Catalyzed by Cytochromes P450

aliphatic hydroxylation
aromatic oxidation
alkene epoxidation
N-dealkylation
O-dealkylation
S-dealkylation
oxidative deamination
N-oxidation
Oxidative desulfuration
Sulfoxidation
Oxidative dehalogenation
Oxidative denitrification
Oxidative denitrosation

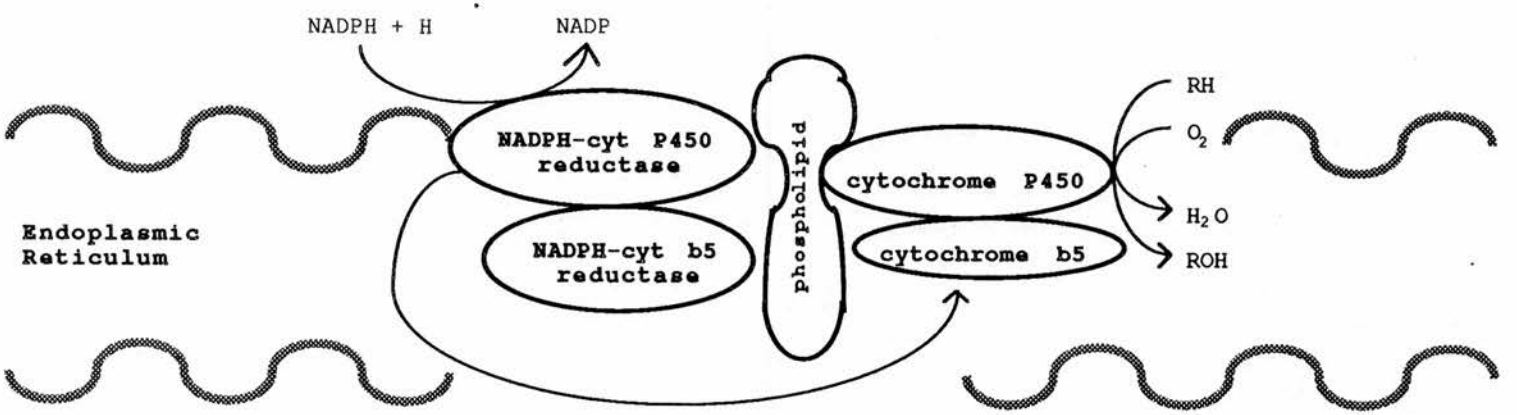


Figure 3.1 The Cytochrome P450-dependent Monooxygenase System

Gonzalez, 1987). The multiplicity of P450 activities makes P450s difficult to study in microsomes. Indeed, overlapping substrate specificities have been observed in purified P450s from different families (Åström & DePierre, 1986). P450s have been purified by many different groups and various systems of nomenclature have been developed based on the inducibility, chromatographic fraction, catalytic activity, or electrophoretic mobility of the purified preparation. An overview of this nomenclature is summarized in Table 3.2. The nomenclature system developed by Wolf (1986) will be used throughout this work.

Analysis of the primary structure of purified P450s has permitted the cloning and sequencing of P450 genes. It is therefore possible to classify P450s by their sequence homology at the DNA level. P450s are grouped as distinct proteins if they exhibit less than 36% homology, within the same family if they show greater than 36% homology, or within the same subfamily if they have greater than 68% homology (Nebert & Gonzalez, 1987). Cytochromes P450 are also highly conserved across species, with individual P450s having as much as 93% sequence homology between rat MC1a and mouse P1-P450 (Guengerich, 1987).

To date there are at least 10 cytochrome P450 families classified by sequence homology (Table 3.3). These families can be divided into three groups (Nebert & Gonzalez, 1987): 1.) non-mammalian P450s (families CI and LI) 2.) P450s involved in steroidogenesis (families XI, XVII, XIX, and XXI) 3.) P450s involved in xenobiotic metabolism (families I to IV). This classification is far from complete because P450s are still being cloned and sequenced.

P450s involved in steroidogenesis are considered to belong to

Table 3.2 Cytochrome P450 Nomenclature in the Rat*.

Family	Wolf	Levin	Guengerich	Waxman	Other
IA1	MC1b	c	BNF-B	-	P450-L, P448 L
IA2	MC1a	d	ISF-G	-	P450-H, P448 H
IIA1	UT1	a	UT-F	PB3	P452
IIB1	PB3a	b	PB-B	PB4	P450 PB1
IIB2	PB3b	e	PB-D	PB5	P450 III
IIC6	PB1a	k	PB-C	PB1	-
IIC7	-	f	-	-	-
IIC11	-	g	-	-	-
IIC12	PB2a	h	UT-A	PB2c	P450-male, RLM5
IIC13	-	i	UT-I	PB2d	P450-female
IID1	-	-	UT-H	-	-
IIE1	-	j	-	-	-
IIIA1	PB2c	-	PCN-E	PB2a	P450 PCN
IV	Clo	-	-	-	P452

*compiled from Wolf (1986), Åström and DePierre (1986), and Guengerich (1987)

Table 3.3 The Cytochrome P450 Gene Superfamily and Functions

Family		Function
Non-mammalian cytochromes P450		
P450LI		lanosterol metabolism
P450CI		camphor metabolism
Cytochromes P450 involved in steroidogenesis		
P450 XI		P450scc (steroid hormone synthesis)
		11β-hydroxylase (cortisol synthesis)
P450 XVII		17α-hydroxylase (androgen synthesis)
P450 XIX		aromatase (estrogen synthesis)
P450 XXI		21-hydroxylase (corticoid synthesis)
Cytochromes P450 involved in xenobiotic metabolism		
P450 I		benzo(a)pyrene hydroxylation (polycyclic aromatic hydrocarbon-inducible)
P450 II	A	testosterone 7α-hydroxylase, coumarin hydroxylation
	B	benzphetamine and aminopyrene demethylation (phenobarbital-inducible)
	C	hormone metabolism, amphetamine metabolism (constitutively expressed P450s)
	D	debrisoquine metabolism (constitutively expressed P450)
	E	nitrosamine metabolism (ethanol-inducible)
P450 III		testosterone 6β-hydroxylation (pregnenolone 16α-carbonitrile-inducible)
P450 IV		fatty acid omega oxidation (clofibrate-inducible)

the 'house-keeping' P450s (Nebert & Gonzalez, 1987). House-keeping P450s operate in important metabolic pathways and are constitutively expressed (Nebert & Gonzalez, 1987). They are not susceptible to the classic P450 inducers like phenobarbital and 3-methylcholanthrene, but do respond to hormones (Whitlock, 1986). These P450s exhibit strict stereo- and regio- specificity in the metabolism of substrates and do not metabolize foreign compounds (Åström & DePierre, 1986).

P450s in the last group metabolize xenobiotics and are the P450s involved in the pathways of drug oxidation and detoxification (Wolf, 1986). They are thought to have evolved from the house-keeping P450s, providing a selective advantage to those organisms exposed to harmful foreign compounds (Wolf, 1986). Many of these P450s are inducible by xenobiotics like polycyclic aromatic hydrocarbons (family I), phenobarbital (family IIB), pregnenolone 16α-carbonitrile (family III), and clofibrate (family IV). Each xenobiotic selectively induces certain P450 isozymes and can influence the expression of phase II detoxification enzymes like glutathione transferases, UDP-glucuronyl transferases, and epoxide hydrolase (Åström & DePierre, 1986). Induction of P450s often leads to increased metabolism of the inducer (Wolf 1986).

Some P450s in this last group also metabolize endogenous compounds (families IIA and IIC). They are often constitutively expressed, but are not considered to be house-keeping P450s because 1.) the compounds they produce have no known physiological function and 2.) they also metabolize some foreign compounds. These P450s are under the influence of steroid hormones and growth hormone and will be discussed in greater detail in a later section.

The subject of P450s is clearly a large and expanding field. Between 1980 and 1985 over 2700 publications dealing with cytochromes P450 were recorded on the MEDLINE database. In the past four years, over 5600 articles dealing with this subject have been listed there.

Antibodies have become very important in the study of P450s (Gelboin & Friedman, 1985; Åström & DePierre, 1986). As has already been pointed out, the multiplicity of cytochrome P450 isozymes and their overlapping substrate specificities have made P450s particularly difficult to study *in vitro*. Prior to the sequencing of P450 genes, P450s were grouped together according to their similarities (Gelboin & Friedman, 1985), including amino-terminal sequences and antibody cross-reactivity. However, even with the highly defined classification that now exists, it is difficult to determine which P450 isozymes are responsible for which reactions in microsomes. For example, four separate P450s have been shown to hydroxylate testosterone in the 16α position (Åström & DePierre, 1986). Monospecific antibodies have made possible the identification of the individual P450 isozymes responsible for the different catalytic activities (Waxman *et al.*, 1987). This technique is called reaction phenotyping. In concert with the sequence data, reaction phenotyping has helped to elucidate the complexities of P450 activities. Gelboin & Friedman (1985) have proposed the creation of an antibody library to be used as reference tools for the investigation of cytochromes P450.

Cholesterol 7α-hydroxylase is still an unclassified cytochrome P450. It has been purified, but not cloned, so there is no DNA sequence data available. An antibody capable of interacting with cholesterol 7α-hydroxylase has not yet been found (Waxman, 1986). Polyclonal antibodies have been raised against several P450s purified by Dr. C.R. Wolf in the ICRF Laboratory of Molecular Pharmacology and Drug Metabolism in Edinburgh. The work presented here was designed to see whether any of the available anti-P450 antibodies were capable of inhibiting this enzyme.

3.2 Initial Investigation of Anti-cytochrome P450 Antibody Inhibition of Cholesterol 7α-hydroxylase Activity

Eight anti-P450 antibodies were screened as inhibitors of cholesterol 7α-hydroxylase activity in rat liver microsomes from cholestyramine-treated animals. Microsomes were incubated with the IgG fraction isolated from anti-P450 antisera. Seven of the anti-P450 antibodies, anti-PB1a, anti-PB1b, anti-PB3a, anti-PB3b, anti-MC1a, anti-MC1b, and anti-UT1, did not significantly inhibit the reaction, a maximal inhibition of 25% being observed (Fig 3.2). Preimmune serum, used as a control for non-specific binding, did exhibit some inhibitory effects. Some anti-P450 antibodies appeared to enhance activity above preimmune levels, but none increased activity above control levels. All of these antibodies have been shown by workers in the ICRF-Edinburgh lab to be effective inhibitors of other P450-mediated reactions (C.R. Wolf, personal communication).

In marked contrast, the antibody to PB2a inhibited cholesterol 7α-hydroxylase activity more than 70% (Fig 3.2). As can be seen in Fig 3.3, the inclusion of this antibody in the cholesterol 7α-

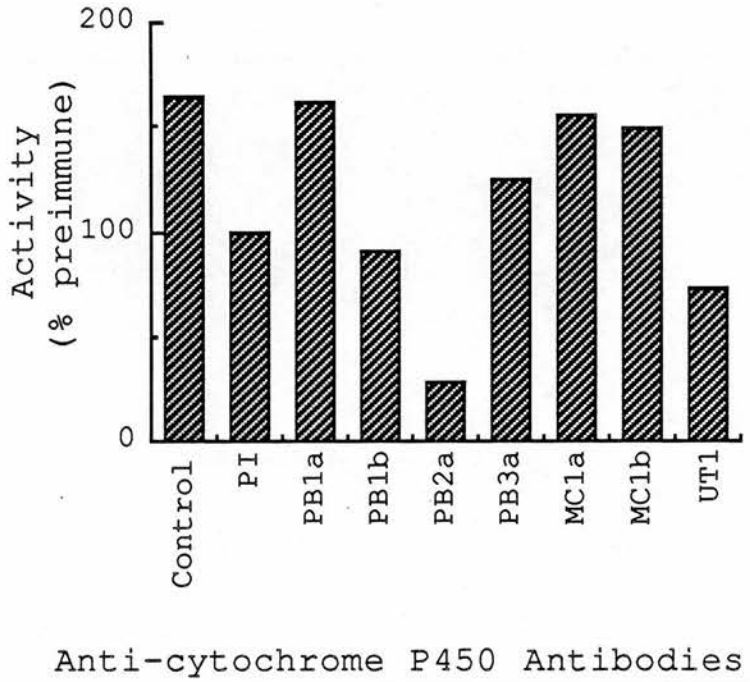


Figure 3.2. Initial Screening of Anti-cytochrome P450 Antibodies. Antibody inhibition of cholesterol 7α-hydroxylase activity was carried out as described in Materials and Methods. Antibody was added to the assay at a ratio of 10mg IgG/mg microsomal protein. Per cent conversion values of cholesterol to 7α-hydroxycholesterol have been normalized to those obtained with preimmune (PI) IgG (0.66% conversion) and are the result of single determinations. These are data from one of three identical experiments.

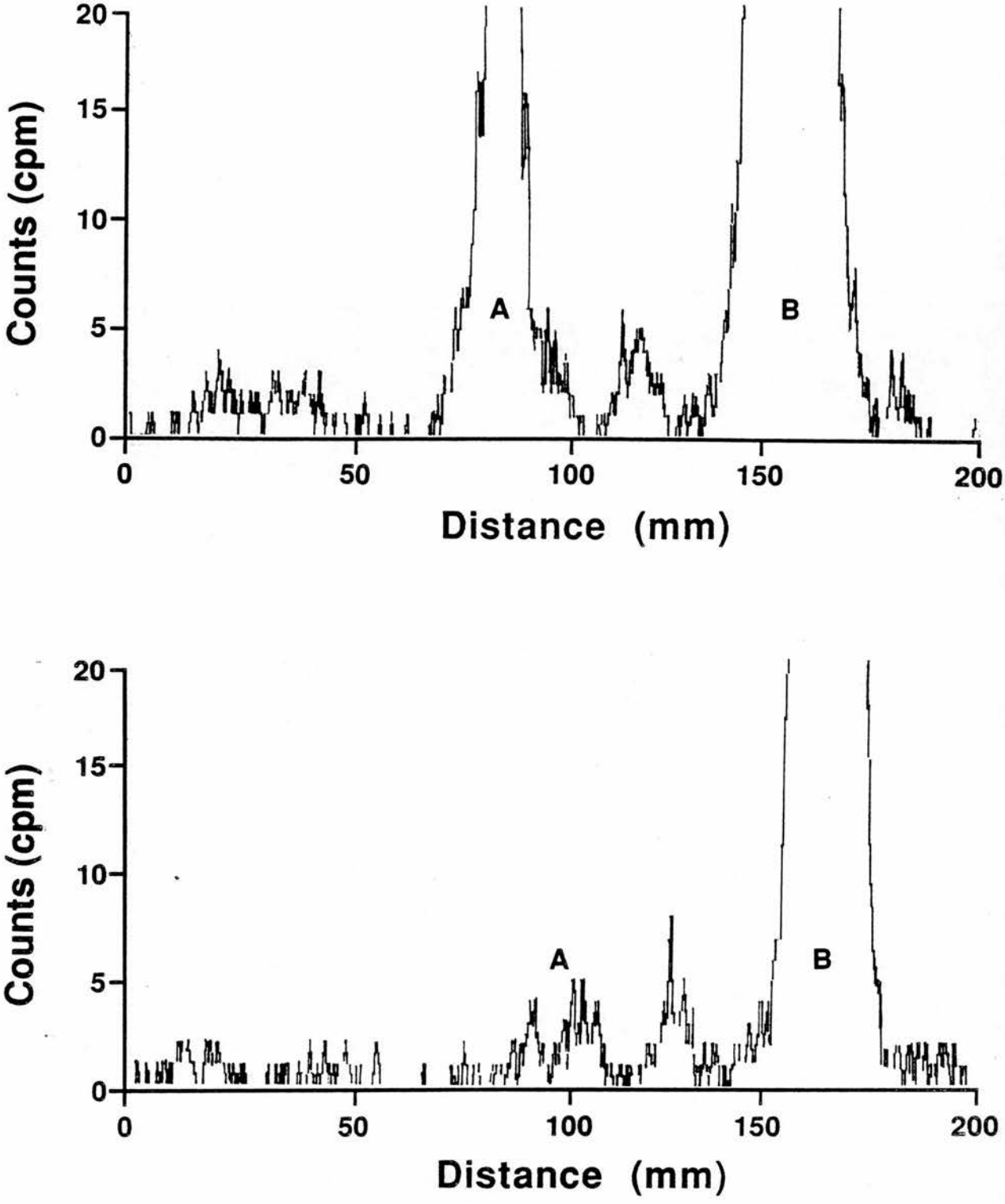


Figure 3.3. Effect of Anti-PB2a on Cholesterol 7α-hydroxylase Activity.

Antibody inhibition of cholesterol 7α-hydroxylase activity was carried out as described in Materials and Methods. Reaction products were extracted with chloroform:methanol (2:1, v/v) and resolved on tlc plates using ethyl acetate:toluene (70:30, v/v). The top tracing shows a typical control tlc scan. The lower chart shows a tracing obtained from an experiment containing anti-PB2a at a ratio of 20:1 mg IgG:mg microsomal protein. A and B indicate the positions of 7α-hydroxycholesterol and cholesterol, respectively.

hydroxylase assay at a ratio of 20mg IgG/mg microsomal protein effectively prevented the synthesis of 7α-hydroxycholesterol by microsomes. The level produced under these conditions is the same as that produced by the autooxidation controls.

In view of the fact that the anti-PB2a antibody inhibited cholesterol 7α-hydroxylase, it was of interest to determine whether the purified PB2a enzyme was capable of hydroxylating cholesterol at the 7α position. As shown in Table 3.2, PB2a is a well-characterized P450, classified in the P450IIC subfamily. It is known to hydroxylate testosterone in both the 16α and 2α positions (Cheng & Schenkman, 1982; Kamataki *et al.*, 1983). There have been no reports of its ability to hydroxylate cholesterol.

3.3 Hydroxylation of Cholesterol by Purified Cytochromes P450

Purified P450 preparations were reconstituted into monooxygenase systems with NADPH-cytochrome P450 reductase and dilaurylphosphatidylcholine as described in Materials and Methods. Radiolabelled cholesterol (0.2mM) was added to the reconstituted assay system either in acetone, as in the microsomal assay system, or solubilized in Triton X-100 (0.75mg) (Andersson *et al.*, 1985) or Tween 80 (0.3mg) (Waxman, 1986). Alternatively, cholesterol was included in the reconstitution event itself, coated onto the reaction vessel with the phospholipid. The reconstituted monooxygenase systems were shown to be active in the demethylation of methoxyphenoxazone, indicating that functional coupling of the cytochrome P450 with the NADPH reductase had been achieved.

Two different preparations of purified PB2a were not found to have any 7α-hydroxylase activity toward cholesterol (results not shown). Indeed, none of the purified P450 preparations available exhibited cholesterol 7α-hydroxylase activity. Two reasons may be put forward for this result:

1.) The reconstitution method might not have been sufficient to allow the substrate access to the enzyme. Reconstituted systems are micellar in nature. They consist of a single lipid layer and consequently do not represent the *in vivo* environment. Cholesterol may need directional presentation to the enzyme that is not possible under these conditions. This isolated system may also have been lacking other cofactors needed to express activity.

2.) Alternatively, none of the P450s examined is cholesterol 7α-hydroxylase. Each of these P450s has been characterized and assigned to a P450 family and subfamily (Table 3.2) (Wolf, 1986). It is highly unlikely that any one of them is cholesterol 7α-hydroxylase.

3.4 Effect of Anti-cytochrome P450 Antibodies on Cholesterol 7α-hydroxylase activity

To further investigate the nature of the antibody inhibition, three types of anti-P450 antibodies were selected. PB1a is a member of the same P450 gene subfamily as PB2a, sharing more than 68% sequence homology with PB2a at the DNA level (Nebert & Gonzalez, 1987). This homology results in the cross-reactivity of anti-PB1a and anti-PB2a antibodies with both antigens on Western blots. Anti-PB2a was chosen to examine the specificity of the inhibition. Anti-PB1a was chosen to see whether the cross-reactivity would affect 7α-

hydroxylase activity. PB2c is a member of a different P450 family, sharing less than 36% sequence homology with PB2a at the DNA level. Anti-PB2c was chosen to represent the effects of a non-related anti-P450 antibody.

Rat liver microsomes were incubated with increasing quantities of IgG fractionated from antisera prior to the cholesterol 7α-hydroxylase assay. The titration of antibody concentration against 7α-hydroxylase activity is shown in Fig 3.4. The preimmune IgG, included as a control for the effects of non-specific binding, did inhibit the reaction to some extent, and all values were normalized for this effect. Almost complete inhibition (96%) of 7α-hydroxylase activity by the anti-PB2a antibody was observed at 20mg IgG/mg microsomal protein. An inhibition greater than 50% was seen at 5mg IgG/mg microsomal protein. The anti-PB1a and anti-PB2c antibody preparations were not effective inhibitors relative to the preimmune control, with less than 25% inhibition at the maximum IgG ratio of 20mg IgG/mg microsomal protein. Anti-NADPH cytochrome P450 reductase was a potent inhibitor of cholesterol 7α-hydroxylase activity, as shown by the greater than 50% inhibition at 2mg IgG/mg microsomal protein and 85% inhibition at 20mg IgG/mg microsomal protein.

It was now clear that an antibody preparation raised against a well-characterized P450, PB2a, was capable of specifically inhibiting the activity of a second, apparently unrelated P450, cholesterol 7α-hydroxylase. Such an interesting observation required further investigation. Other experiments were undertaken to determine whether there was a definite connection between the two P450s.

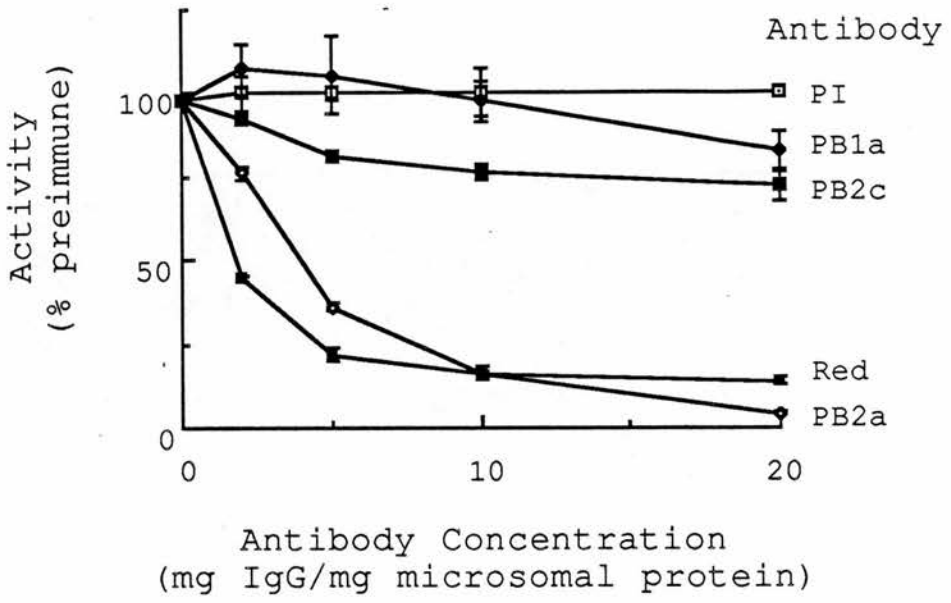


Figure 3.4. Effect of Anti-cytochrome P450 Antibodies on Cholesterol 7α-hydroxylase Activity.

The inhibition of cholesterol 7α-hydroxylase activity by antibodies was carried out as described in Materials and Methods. Microsomes were incubated with increasing amounts of IgG for 20min at room temperature prior to initiation of the assay. Production of 7α-hydroxycholesterol from samples containing preimmune IgG was 4.51, 3.70, 2.80, and 2.08 per cent conversion at 2:1, 5:1, 10:1, and 20:1 ratios of mg IgG/mg microsomal protein, respectively. Values have been normalized at each ratio to those obtained from preimmune IgG. Points are the mean ± s.d. of triplicate determinations. These are data from one of two identical experiments. Abbreviations: PI = preimmune; Red = NADPH-cytochrome P450 reductase antibody.

N.B. The standard deviations are so small that the error bars are contained within the symbols

3.5 Specificity of the Anti-PB2a Antibody

As with all P450s, cholesterol 7α-hydroxylase activity requires the activity of NADPH-cytochrome P450 reductase as an electron donor. The possibility existed that the anti-PB2a antibody could be interfering with the 7α-hydroxylase reaction by inhibiting the reductase activity. To explore this potential action, microsomes were incubated with different anti-P450 antibodies in the same manner as described for the cholesterol 7α-hydroxylase reaction. NADPH-cytochrome P450 reductase activity was then determined spectrophotometrically by its ability to reduce cytochrome c. Neither anti-PB2a nor anti-PB1a were able to inhibit this activity. Anti-NADPH reductase, however, caused greater than 85% inhibition of activity (Fig 3.5).

Another aspect that would explain the cross-reactivity of anti-PB2a with 7α-hydroxylase would be that the original purification of the PB2a protein contained a contaminant of cholesterol 7α-hydroxylase. Western blot analysis of purified PB2a with the anti-PB2a antibody revealed a single band at Mr 49500. This datum indicates purity of the enzyme preparation against which the antibody was raised (Fig 3.6).

These results suggested that the cross-reactivity of the antibody preparation with cholesterol 7α-hydroxylase activity was not due to the contaminating presence of either NADPH-cytochrome P450 reductase or cholesterol 7α-hydroxylase in the original purified PB2a preparation.

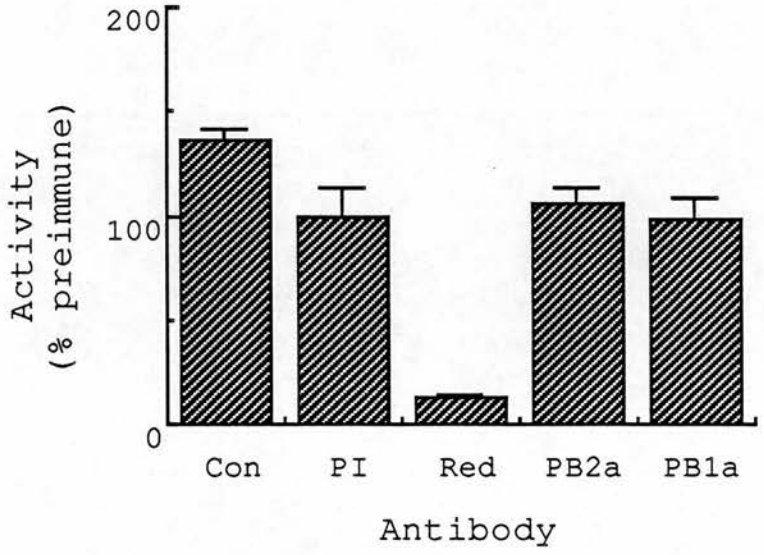


Figure 3.5. Effect of Anti-cytochrome P450 Antibodies on NADPH-cytochrome P450 Reductase Activity.

NADPH-cytochrome P450 reductase assays were carried out as described in Materials and Methods. Microsomes were incubated with antibody at a ratio of 10mg IgG/mg microsomal protein for 20min at room temperature prior to the start of the assay. Values have been normalized those obtained using preimmune serum (390U/mg microsomal protein) and are the mean ± s.d. of three determinations. Abbreviations: PI = preimmune; Con = control; Red = NADPH-cytochrome P450 reductase.

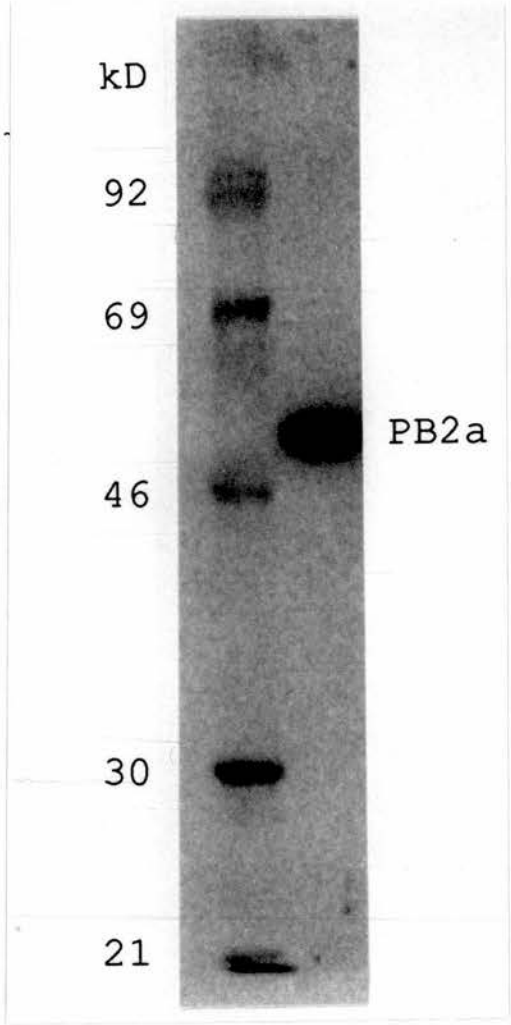


Figure 3.6. Western Blot Analysis of Purified Cytochrome P450 PB2a.

Purified PB2a was fractionated in a 9% SDS-polyacrylamide gel as described in Materials and Methods. Proteins were transblotted onto nitrocellulose and probed with anti-PB2a at a dilution of 1:500.

3.6 Reversal of the Anti-PB2a-Induced Inhibition of Cholesterol 7α-hydroxylase Activity by Purified PB2a.

It was now important to establish a direct link between PB2a and cholesterol 7α-hydroxylase. If the two proteins shared structural homology, then there would exist competition between the two proteins for recognition by the IgG. To investigate this, purified PB2a was added to the antibody inhibition assays of 7α-hydroxylase. Microsomes, IgG, and purified P450 were allowed to equilibrate before the cholesterol 7α-hydroxylase assay was initiated. The addition of highly purified PB2a to the cholesterol 7α-hydroxylase assay could completely reverse the antibody inhibition by anti-PB2a (Fig 3.7). Three different purified PB2a preparations brought activity levels to greater than 130% of the preimmune control. This large increase in activity over preimmune levels upon the addition of PB2a is probably due to the alleviation of non-specific binding by anti-PB2a in the presence of its antigen. The addition of a different purified P450 to the inhibition assay reaction did not have such an effect. Purified PB1a caused only a 50% reversal of inhibition. This partial reversal by purified PB1a may be in part due to the cross-reactivity of the anti-PB2a with PB1a due to the structural similarities between the two proteins.

The reversal of inhibition was most effective when the purified P450 antigens were left at room temperature for 18h prior to use. The reason for this is unclear, but may be due to partial protein denaturation or disaggregation. It is of interest to note that these purified P450 fractions still retained catalytic activity toward methoxyphenoxazone comparable to fractions stored continuously at -40°C (Fig 3.8). This evidence indicates that the epitopes of an

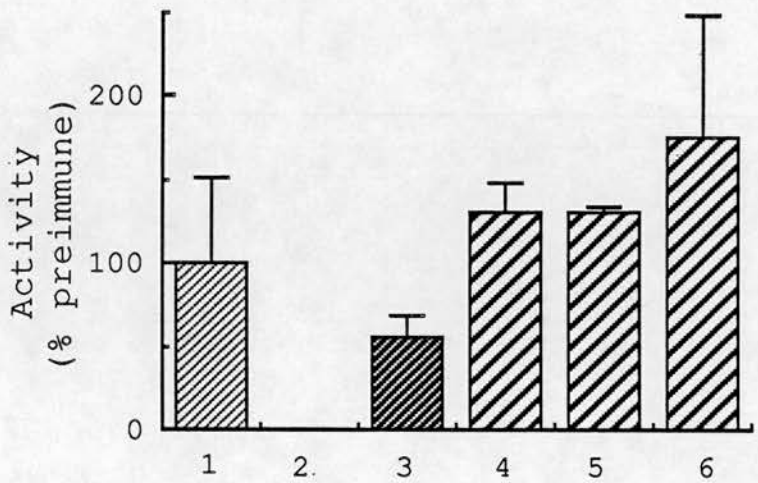


Figure 3.7. Reversal of Cholesterol 7α-hydroxylase Inhibition by the Addition of Purified Cytochrome P450 PB2a. Antibody inhibition assays were carried out as described in Materials and Methods. Antibody was added at a ratio of 10mg IgG/mg microsomal protein. Purified cytochromes P450 were left at room temperature overnight before use and added to the assay at a ratio of 10nmol P450/mg microsomal protein. Antibody, purified P450, and microsomes were incubated for 20min at room temperature prior to the assay. The additions to the assays are as follows: 1 = preimmune IgG; 2 = anti-PB2a IgG; 3 = anti-PB2a IgG + purified PB1a; 4 = anti-PB2a IgG + purified PB2a (fraction J4III); 5 = anti-PB2a IgG + purified PB2a (fraction J4I); 6 = anti-PB2a IgG + purified PB2a (fraction E1). The values shown have been normalized to those obtained with preimmune (PI) IgG (0.4% conversion) and are the means ± s.d. for triplicate determinations. These are data from one of three identical experiments. Purified fractions were obtained from a single purification procedure.

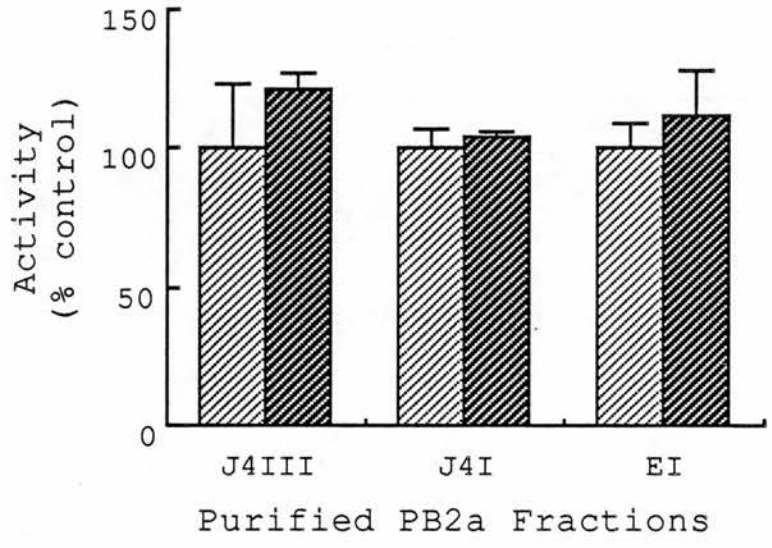


Figure 3.8. Activity of Different Purified PB2a Cytochromes P450.

Cytochrome P450 monooxygenase systems were reconstituted as described in Materials and Methods using purified PB2a fractions which have been stored continuously at -40°C (lightly shaded bars) or left at room temperature overnight (darkly shaded bars). Demethylation of methoxyphenoxazone was carried out as described in Materials and Methods. Values have been normalized to those obtained from the P450s stored continuously at -40°C: J4III (4.3nmol phenoxazone/min/nmol P450), J4I (6.1), EI (2.2). Values are the mean ± range of duplicate determinations.

active PB2a enzyme are competing with those of cholesterol 7α-hydroxylase for recognition by the anti-PB2a IgG.

3.7 Conservation of Cholesterol 7α-hydroxylase Activity Across Species.

Cytochromes P450 have been demonstrated to be highly conserved between species (Guengerich, 1987). Cholesterol 7α-hydroxylase has been studied in several animal models. It was therefore of interest to determine whether the anti-PB2a antibody that inhibited 7α-hydroxylase activity in rats was capable of recognizing the enzyme in other species.

Animals were maintained on a cholestyramine-supplemented diet for seven days before the experiment. Microsomes were incubated with anti-PB2a prior to being assayed. Cholesterol 7α-hydroxylase activity was shown to be very similar between rat and hamster and slightly lower in mouse (Fig 3.9). Anti-PB2a decreased activity most dramatically in the rat, resulting in 90% inhibition relative to the control level. Activity in the hamster was inhibited 75%. Inhibition was less marked in the mouse, activity being 50% of the control level. These results suggest that the anti-PB2a antibody had the greatest affinity for the rat enzyme. This is not surprising because the anti-PB2a antibody was raised against a P450 purified from rat. The general inhibition indicates that cholesterol 7α-hydroxylase shares common structural features between species.

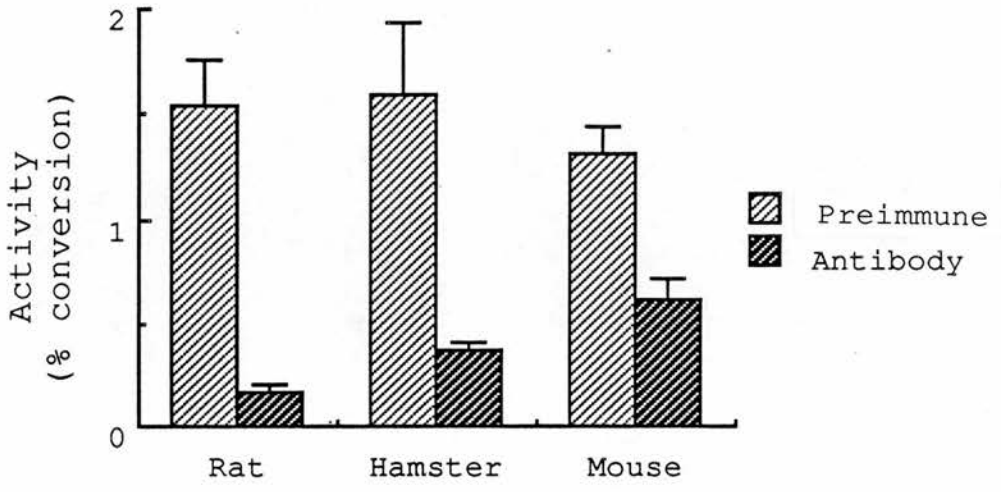


Figure 3.9. Conservation of Cholesterol 7α-hydroxylase Between Species.

Adult female animals, Wistar rat, Syrian hamster, and CD-1 mouse, were maintained on cholestyramine-supplemented diets for seven days prior to the experiment. Antibody inhibition of cholesterol 7α-hydroxylase activity was carried out as described in Materials and Methods using anti-PB2a antibody at a ratio of 10mg IgG/mg microsomal protein. Values are the mean ± s.d. of triplicate determinations. from one rat, one hamster, and the combined livers of two mice.

3.8 Western Blot Analysis of Rat Liver Microsomes with Anti-PB2a.

Much of the knowledge about P450s has been gained through Western blot analysis of microsomal proteins. Having obtained so much data demonstrating that PB2a and 7α-hydroxylase were structurally related, it was important to determine whether two P450s could be identified on a Western blot of rat liver microsomes. Cholesterol 7α-hydroxylase activity is known to be modulated by bile acid feedback (Myant & Mitropoulos, 1977). The addition of a bile acid sequestrant, such as cholestyramine, to a diet causes a marked increase in cholestrol 7α-hydroxylase activity. This increase has been associated with protein synthesis (Brown & Boyd, 1974). A semi-synthetic 'soft diet', consisting of wholemeal flour, powdered milk, and yeast, is associated with a decrease in 7α-hydroxylase activity. It was thought that a difference in band intensity would enable the identification of cholesterol 7α-hydroxylase on Western blots.

Rats were treated with both of these diets as well as the corresponding control diet containing normal rat chow for seven days prior to the experiment. Cholesterol 7α-hydroxylase assays were carried out as described in Material and Methods. Microsomal samples were fractionated on SDS-PAGE gels and transferred onto nitrocellulose membranes. Multiple bands were identified when using anti-PB2a as a probe on Western blots (Fig 3.10). It was found that rats treated with cholestyramine, expressing enhanced cholesterol 7α-hydroxylase activity, exhibited a significant elevation in the higher molecular weight band (Mr 54000). In contrast, when 7α-hydroxylase activity was suppressed by feeding a soft diet, a significant decrease in this band was observed. A direct correlation between

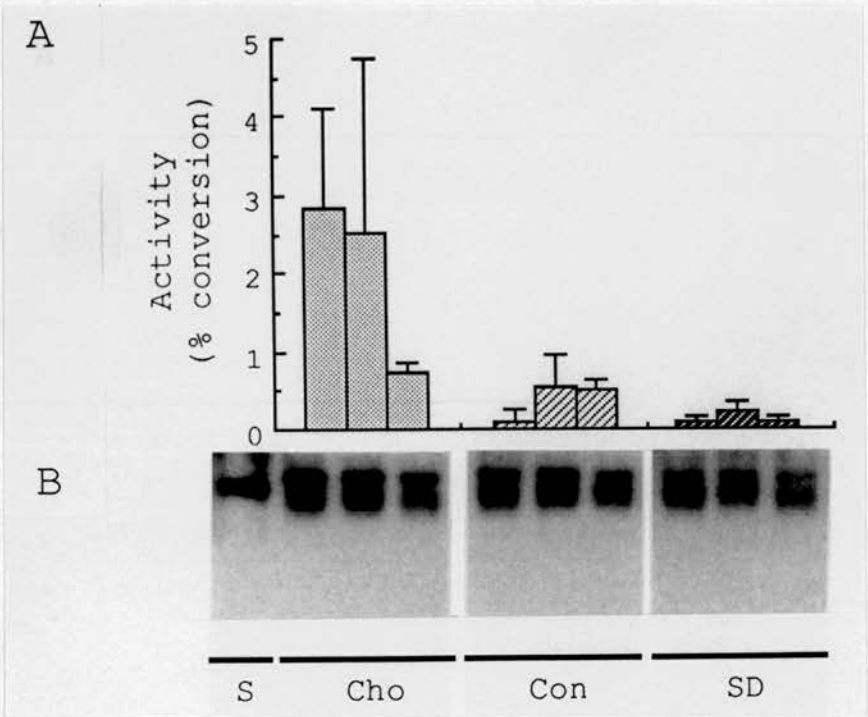


Figure 3.10. Correlation Between PB2a-related Proteins and Cholesterol 7α-hydroxylase Activity.

(A) Cholesterol 7α-hydroxylase assays were carried out as described in Materials and Methods using the phosphate reaction buffer and 1.0mg microsomal protein. Values are the mean ± s.d. of triplicate determinations.

(B) Microsomes (10μg) were fractionated in 9% SDS-polyacrylamide gels and transblotted onto nitrocellulose as described in Materials and Methods. Western blots were probed with anti-PB2a antibody at a dilution of 1:500. Each group contained three rats.

Samples are in the same order in both the graph and the Western blot. S = PB2a standard; Cho = cholestyramine-fed rats; Con = control-fed rats; SD = soft diet-fed rats.

band intensity and 7α-hydroxylase activity was obtained (Spearman correlation 0.800, p=0.010, n=9). It is worthy to note that the purified PB2a antigen did not contain a band of Mr 54000.

3.9 Summary

The data presented so far provide evidence that is consistent with the hypothesis that cholesterol 7α-hydroxylase may be structurally related to PB2a.

1. The initial observation that cholesterol 7α-hydroxylase activity was inhibited by an anti-PB2a antibody indicated that the anti-PB2a antibody recognized cholesterol 7α-hydroxylase (Fig 3.2).

2. The antibody titration experiments confirmed that the inhibition of 7α-hydroxylase activity was specific for the anti-PB2a antibody (Fig 3.4).

3. The fact that the anti-PB2a antibody recognized a single band when used to probe purified PB2a on Western blots indicates that the antigen was pure when used to raise antibody (Fig 3.6).

4. The reversal of 7α-hydroxylase inhibition caused by the addition of purified PB2a demonstrates that the two proteins compete for recognition by the inhibitory antibody (Fig 3.7).

5. The presence of multiple bands on Western blots probed with anti-PB2a coupled with the correlation of band intensity and 7α-hydroxylase activity reinforces the concept that anti-PB2a recognizes a separate protein associated with 7α-hydroxylase activity (Fig 3.10).

3.10 Investigation of Other Anti-PB2a Antibodies.

The anti-PB2a preparation used for all studies thus far was precipitated from an antiserum known as 34. It was of interest to determine whether other antibody preparations raised against PB2a had inhibitory activity toward cholesterol 7α-hydroxylase. IgG was fractionated from several other anti-PB2a antisera, including another precipitation from the original anti-PB2a antiserum 34. These fractions, known as 27, 31, 34n, and 86, were added to the inhibition assays as before. None of these IgG preparations was capable of inhibiting 7α-hydroxylase activity (Fig 3.11). Surprisingly, even the second precipitation of IgG from antiserum 34, 34n, did not inhibit activity. It is not clear why this is so.

These IgG preparations were subsequently analyzed for their ability to inhibit the demethylation of methoxyphenoxazone by the purified PB2a enzyme. Purified PB2a was reconstituted into monooxygenase systems and incubated with the antibodies prior to the assay. IgG 31 and the original IgG 34 were found to inhibit the reaction while IgG 27, 86, and the new IgG 34 did not (Fig 3.12). These results demonstrate that different anti-PB2a preparations exhibit different specificities towards both the purified PB2a antigen and cholesterol 7α-hydroxylase.

Antiserum 34 had been shown to recognize multiple bands on Western blots of rat liver microsomes as well as a difference in band intensities between microsomes from cholestyramine-treated animals and those from animals maintained on a soft diet. The remaining anti-PB2a antisera were tested for their ability to recognize similar patterns on Western blots. Microsomes from cholestyramine-treated and soft diet-treated rats were fractionated using SDS-PAGE,

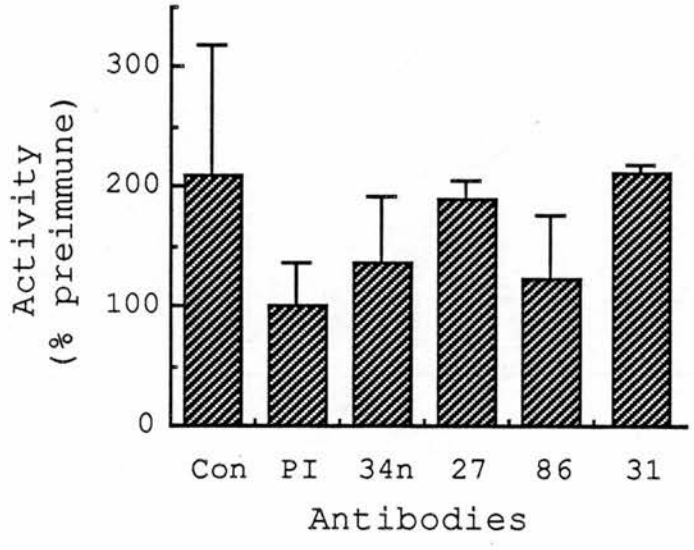


Figure 3.11. Effect of Different Anti-PB2a Antibodies on Cholesterol 7α-hydroxylase Activity.

Antibody inhibition assays were carried out as described in Materials and Methods. Different anti-PB2a antibodies (34n, 27, 86, and 31) were added to the assays at a ratio of 10mg IgG/mg microsomal protein. Values have been normalized to those obtained from preimmune IgG (PI) (2.28% conversion). Bars represent the mean ± s.d. of triplicate determinations. Abbreviations: Con = control, PI = preimmune serum.

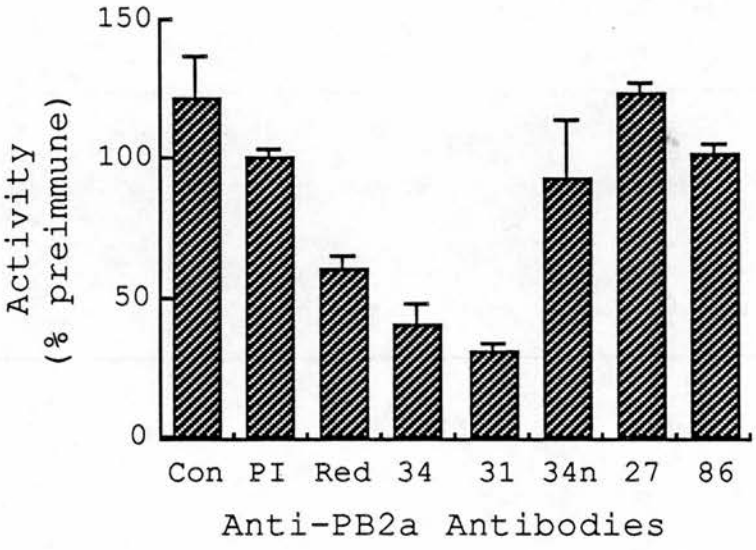


Fig 3.12. Effect of Different Anti-PB2a Antibodies on the Demethylation of Methoxyphenoxazone by Purified PB2a.

Cytochrome P450 monooxygenase systems were reconstituted as described in Materials and Methods using 0.01nmol of purified PB2a. Different anti-PB2a antibodies (34, 31, 34n, 27, and 86) were added at a ratio of 10mg IgG/0.1nmol purified P450. Dealkylation of methoxyphenoxazone was carried out as described in Materials and Methods. Values have been normalized to those obtained from preimmune IgG (PI) (4.21nmol phenoxazone/min/nmol P450) and are the mean ± range of duplicate determinations. Abbreviations: Con = control, Red = NADPH-cytochrome P450 reductase.

transferred onto nitrocellulose membranes, and probed with several anti-PB2a antibodies. Two of these antisera, numbers 27 and 86, identified the same protein profile as antiserum 34 when used as probes (Fig 3.13). Antiserum 31 did not yield this pattern. These results indicate that some anti-PB2a antibodies recognize the tentatively identified cholesterol 7α-hydroxylase protein while others do not.

It is accepted that specificity of an antibody preparation for its antigen changes with each immunization (Gelboin & Friedman, 1985). Such differences arise from the varying antigenicity of the epitopes present on the antigen. Antibodies can be divided into three categories (Gelboin & Friedman, 1985): 1.) those that can recognize antigens 2.) those that can recognize and precipitate antigens 3.) those that can recognize and precipitate antigens as well as inhibit antigen activity. Thus, the failure of a different IgG preparation to inhibit cholesterol 7α-hydroxylase activity does not lessen earlier conclusions that 7α-hydroxylase may be structurally similar to PB2a. With regards to 7α-hydroxylase, antiserum 34 appears to be a member of the last category. Antisera 27 and 86, which produce similar blots, appear to be members of the first one. As far as PB2a is concerned, antisera 34 and 31 appear to be members of the last categories while the other anti-PB2a antisera appear to belong to the first category.

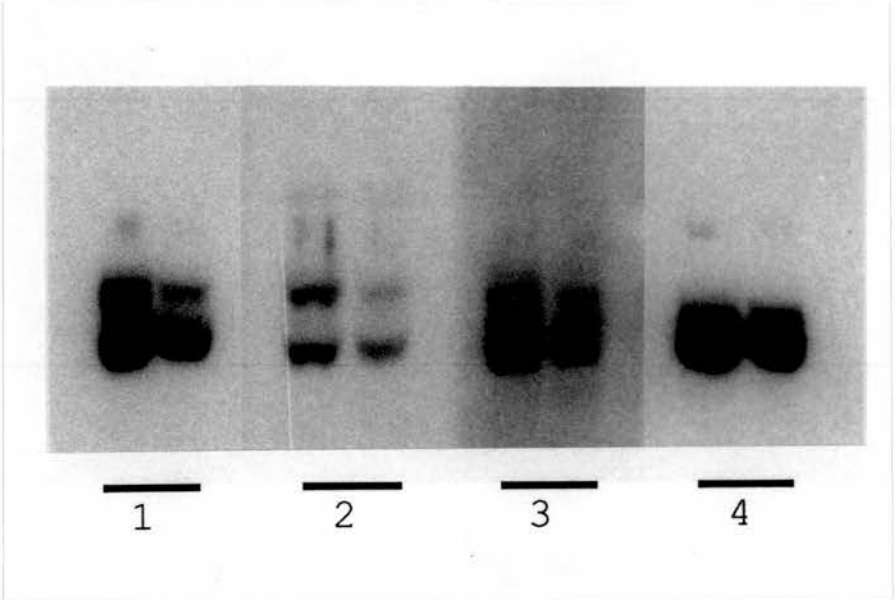


Fig 3.13. Western Blot Analysis of Microsomes with Different Anti-PB2a Antibodies.

Microsomes (7.5 µg) from cholestyramine- and soft diet-fed rats were fractionated in 7% SDS-polyacrylamide gels and transblotted onto nitrocellulose as described in Materials and Methods. Western blots were probed with different anti-PB2a antisera at a dilution of 1:500 as follows: Set 1 was probed with antiserum 34; Set 2 was probed with antiserum 27; Set 3 was probed with antiserum 86; Set 4 was probed with antiserum 31. The first track of each set contains microsomes from cholestyramine-fed rats, and the second track of each set contains microsomes from rats fed a soft diet.

3.11 Immunoprecipitation of Proteins that Cross-react with Anti-PB2a Antiserum.

The immunoprecipitation of antigens represents a powerful tool for the study of proteins. It allows for the immediate purification of proteins and subsequent sequence analysis (Cheng *et al.*, 1986). The band in the Western blot analysis of cholestyramine-treated rats which varied with cholesterol 7α-hydroxylase activity was the one with the highest molecular weight. It was decided to immunoprecipitate all proteins recognized by anti-PB2a antisera for further investigation. Microsomes were solubilized and incubated with either the antisera or the corresponding IgG fractions before immunoprecipitation. Preimmune IgG was used as a control for non-specific binding. Proteins recognized by the IgG component of the serum were precipitated using Protein A-Sepharose beads. Immunoprecipitated proteins were fractionated in non-reducing SDS polyacrylamide gels and analyzed by both silver stain and Western blotting techniques.

Several protein bands were resolved after SDS-PAGE fractionation of immunoprecipitates. The antiserum 34 and both IgG 34 and IgG 34n were able to immunoprecipitate a protein that was not immunoprecipitated by either the antisera or IgG fractions of 27, 31, 86, or preimmune IgG (Fig 3.14A). The band was dependent upon the presence of microsomes in the immunoprecipitation reaction (Fig 3.14B). The immunoprecipitated protein corresponded to the upper band seen in Western blots of cholestyramine-treated rats. Other bands present in the Western blot analysis were shown to react with anti-rabbit IgG antibodies and Protein A (Fig 3.14B). These bands probably resulted from IgG breakdown products in the serum that

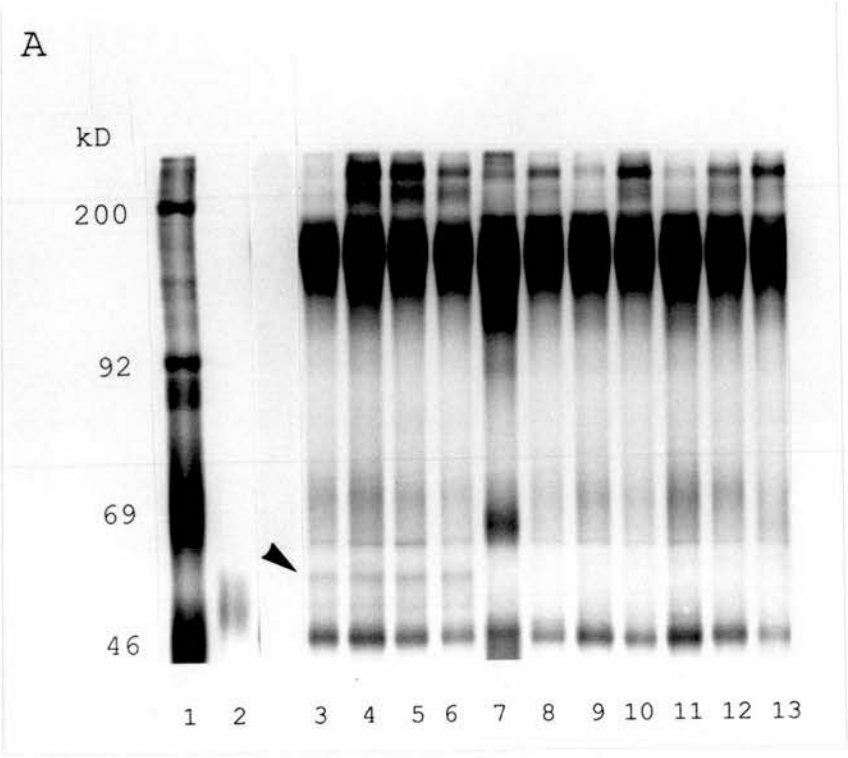


Figure 3.14. Immunoprecipitation of PB2a-related Proteins.

(A) Proteins were immunoprecipitated from microsomes using both serum and IgG fractions as described in Materials and Methods. Immunoprecipitates were fractionated in a 7.5% SDS-polyacrylamide gel under non-reducing conditions and proteins were visualized with silver stain. Five different anti-PB2a antibody preparations (27, 31, 34, 34n, and 86) were used for the immunoprecipitation reaction: track 3 = antiserum 34; track 4 = IgG 34; tracks 5 and 6 = IgG 34n; track 7 = antiserum 27; track 8 = IgG 27; track 9 = antiserum 31; track 10 = IgG 31; track 11 = antiserum 86; track 12 = IgG 86; track 13 = preimmune IgG. Track 1 contains molecular weight markers, and track 2 contains the PB2a standard. The arrow points to the immunoprecipitated protein.

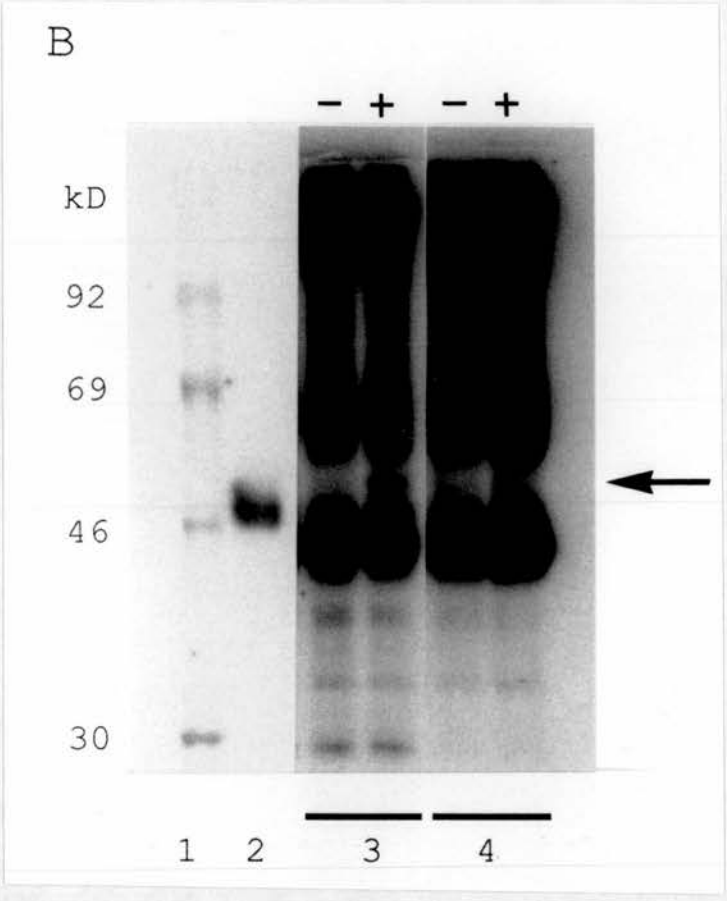


Figure 3.14. Immunoprecipitation of PB2a-related Proteins.

(B) Immunoprecipitaion reactions were carried out in the presence (+) and absence (-) of rat liver microsomes. Immunoprecipitates were fractionated in a 9% SDS-polyacrylamide gel under non-reducing conditions and transblotted as described in Materials and Methods. Western blots were probed either with (tracks 3) or without (tracks 4) anti-PB2a 34 prior to development with anti-rabbit antibody and labelling with [^{125}I]Protein A. Track 1 contains molecular weight markers and track 2 contains the PB2a standard. The arrow points to the immunoprecipitated protein.

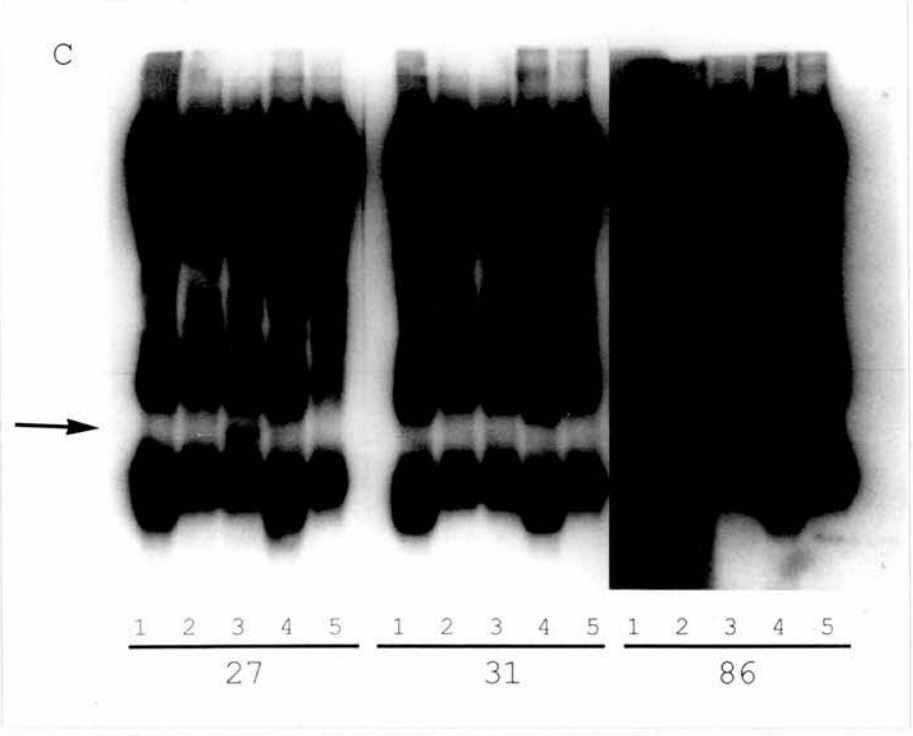


Figure 3.14. Immunoprecipitation of PB2a-related Proteins.

(C) Five different anti-PB2a antibody preparations were used for the immunoprecipitation reaction: track 1 = antiserum 27; track 2 = antiserum 31; track 3 = antiserum 34; track 4 = antiserum 74; track 5 = antiserum 86. Immunoprecipitates were fractionated in 7.5% SDS-polyacrylamide gels under non-reducing conditions, and transblotted as described in Materials and Methods. Western blots were probed with three different anti-PB2a antisera at a dilution of 1:500: antiserum 27, antiserum 86, and antiserum 31. The arrow points to the immunoprecipitated protein.

consequently bound to the Protein A-Sepharose beads during the immunoprecipitation.

This result demonstrates that antiserum 34 belongs to the third class of antibodies: it is able to inhibit cholesterol 7α-hydroxylase and recognize and precipitate the band associated with that activity. It is worthy of note that the IgG fraction prepared from antiserum 34 which did not inhibit the hydroxylase reaction did immunoprecipitate this protein (Fig 3.14A).

Western blot analysis of the same immunoprecipitates probed with other anti-PB2a antibodies shows that antisera 27 and 86 both recognize the protein immunoprecipitated by antiserum 34. Antiserum 31 fails to recognize this band (Fig 3.14C). These data support the idea that antisera 27 and 86 are capable only of recognizing the band that may be cholesterol 7α-hydroxylase. Antiserum 31 does not even recognize this band. These data also support the idea that the recognition of 7α-hydroxylase by antiserum 34 is not intrinsic to that anti-PB2a antibody preparation alone. A collection of antisera therefore exists that may recognize cholesterol 7α-hydroxylase and one IgG preparation exists that not only inhibits its activity, but also immunoprecipitates a protein band that appears to correspond to that activity.

3.12 Amino-terminal Sequence Analysis of the Immunoprecipitated Protein.

Although there have been several reports on the purification of cholesterol 7α-hydroxylase in the past decade, there has only been a single report relating any protein sequence information (Ogishima *et al.*, 1987). This publication gives the sequence of just six amino-

terminal amino acids. The immunoprecipitation technique used in the present work had been shown to select the protein associated with cholesterol 7α-hydroxylase activity. This technique has been used by Cheng and coworkers to obtain enough protein for amino-terminal sequence analysis (Cheng *et al.*, 1986). It was therefore of interest to sequence the immunopurified protein associated with cholesterol 7α-hydroxylase activity. This work was carried out in conjunction with Mr. Bryan Dunbar of the SERC Protein Sequencing Unit at the Department of Biochemistry, Aberdeen University, Scotland.

The protein was immunoprecipitated from cholestyramine-treated female rats using antiserum IgG 34. Immunoprecipitates were fractionated on a 10% (w/v) SDS polyacrylamide [acrylamide:bisacrylamide (30:0.8)] gel according to Yuen *et al.* (1986). The gel was pre-electrophoresed for 2h at 3mA in 200mM Tris:HCl pH 6.8 containing 25mM glycine, 0.1% SDS, and 50μM glutathione [electrophoresis buffer] in order to absorb any free radicals. Glutathione was removed by running the gel for 1h in fresh electrophoresis buffer minus glutathione.

Immunoprecipitates were loaded onto the gel and electrophoresed for 5h at 10mA in electrophoresis buffer minus glutathione. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane according to Matsudaira (1987). Both the gel and the PVDF membrane were soaked in 10mM 3-cyclohexylamino-1-propanesulphonic acid pH 11.0 containing 10% methanol (PVDF transblot buffer) for 5min. The gel was assembled into a blotting sandwich and electrophoresed for 2h at 100mA in PVDF transblot buffer. The PVDF membrane was rinsed in water for 5min and stained in 50% methanol containing 0.1% Coomassie Blue R250 for 5min. Protein bands were visualized after destaining

in 50% methanol and 10% acetic acid for 10min. The area of the PVDF membrane containing the immunoprecipitated band was excised with a scalpel.

The protein was sequenced on an Applied Biosystems protein sequencer (model 470A) using a standard 03R PTH program and a 120A on-line PTH analyzer. The protein sequence was deciphered by Mr. Bryan Dunbar: X-Lys-Leu-Leu-Val-Ile-Pro-Ile-Asp-Gly-Ser-(Thr)-X-Leu. While this sequence does not match the previously published sequence of cholesterol 7α-hydroxylase from Ogishima* *et al.* (1987) it is distinct from that of PB2a and its female homologue P450-female.

3.13 Comparison of PB2a Expression and Cholesterol 7α-hydroxylase Activity.

Data presented thus far all support the hypothesis that cholesterol 7α-hydroxylase is structurally related to PB2a. As yet, no evidence has been presented to show that PB2a does not itself hydroxylate cholesterol. The reconstitution assays performed were inconclusive on this point. In order to show the divergence in the expression of PB2a and cholesterol 7α-hydroxylase, experiments were undertaken that would demonstrate the continued expression of 7α-hydroxylase activity in the absence of PB2a expression.

PB2a is sexually dimorphic, expressed exclusively in the livers of mature male rats (Waxman, 1984). It has been shown *in vitro* to hydroxylate testosterone in the 16α and 2α positions (Cheng & Schenkman, 1982; Kamataki *et al.*, 1983). There exists a female homologue of PB2a, P450 female, expressed in the livers of mature females. This P450 is known to hydroxylate steroid sulfates in the 15β position (MacGeoch *et al.*, 1984). All the work on cholesterol

*Met-Phe-Glu-Val(Ile)-Ser-Leu

7α-hydroxylase presented so far was carried out on mature female rats and was therefore performed in the absence of PB2a. Cholesterol 7α-hydroxylase activity is not sexually dimorphic (Gielen *et al.*, 1976; Carlson *et al.*, 1978). Another test of antibody specificity would be to see whether the inhibitory antibody were capable of interacting with cholesterol 7α-hydroxylase from mature and immature rats of either gender.

Male and female rats aged 4-5 weeks and 8-10 weeks were fed a 4% (w/w) cholestyramine diet for seven days prior to experiments. Cholesterol 7α-hydroxylase activity in these microsomal preparations did not vary significantly (Fig 3.15A). In addition, anti-PB2a was capable of inhibiting activity dramatically in each case. Inhibition of activity in each group indicates that the antibody recognizes the enzyme in both females and males. Microsomes were subsequently fractionated on SDS-PAGE, transferred onto nitrocellulose and probed with the anti-PB2a antibody. The resulting Western blot reveals the series of multiple bands seen earlier (Fig 3.15B). The central band in the male samples corresponds to PB2a and is significantly elevated in the mature male rats with respect to the female rats. The fact that the immature males also had an increased level of this protein indicated that the factors regulating PB2a expression had to some degree already developed. The central band in the female samples may be related to the female homologue to PB2a, P450-female. This band is seen to increase slightly in the mature females. The upper band, tentatively identified as cholesterol 7α-hydroxylase, does not vary significantly in the samples. This band was, however, difficult to discern from the male-predominant PB2a band in the mature male rats.

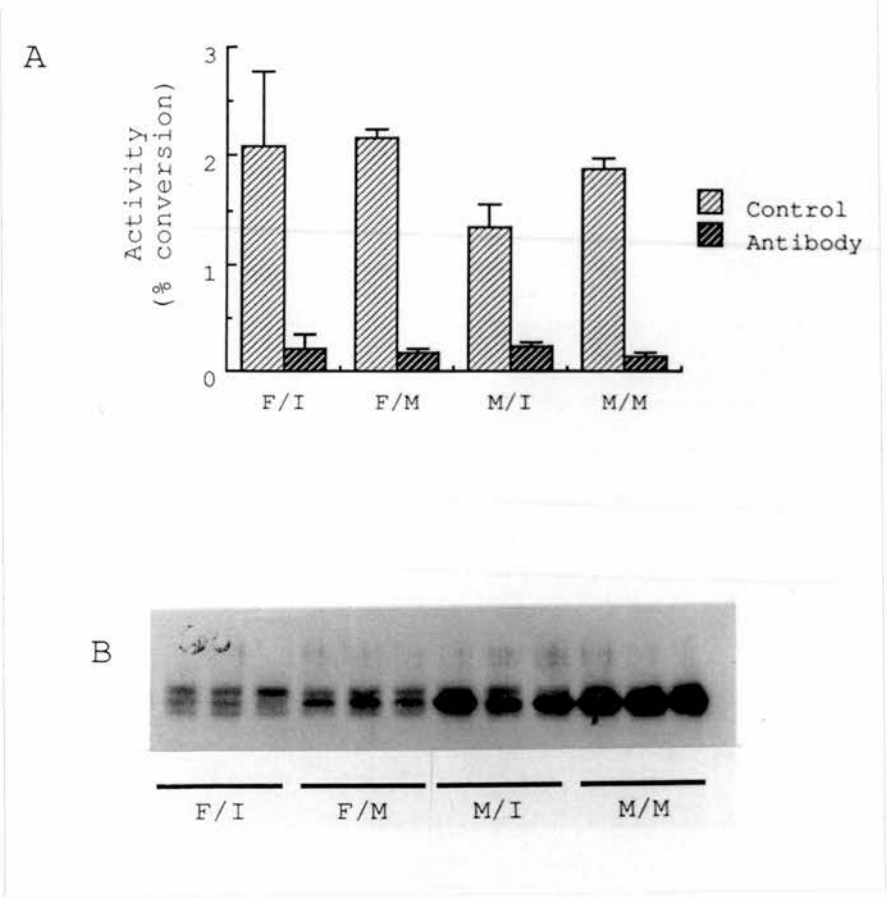


Figure 3.15. The Divergence of Cholesterol 7α-hydroxylase Activity and Cytochrome P450 PB2a Expression in Immature and Mature Rats.

(A) Sexually immature female rats (100 ± 10g) (F/I), sexually mature female rats (180 ± 10g) (F/M), sexually immature male rats (125 ± 10g) (M/I), and sexually mature male rats (190 ± 10g) (M/M) were maintained on a 4% (w/w) cholestyramine diet for seven days prior to the experiment. Antibody inhibition of cholesterol 7α-hydroxylase activity was carried out as described in Materials and Methods using anti-PB2a antibody at a ratio of 10mg IgG/mg microsomal protein. Values are the mean ± s.d. of triplicate determinations.

(B) Microsomes (10μg) were fractionated in 9% SDS-polyacrylamide gels and transblotted onto nitrocellulose as described in Materials and Methods. Western blots were probed with anti-PB2a at a dilution of 1:500. Each group contained three rats.

PB2a expression is initiated by the neonatal imprinting of androgens and maintained by pituitary-directed hormones (Waxman *et al.*, 1985. Removal of the pituitary is known to halt PB2a expression in mature males (Morgan *et al.*, 1985b). Hypophysectomized animals are known to retain the ability to produce bile acids, albeit to a lesser extent (Mayer, 1986). A comparison of the levels of PB2a and cholesterol 7α-hydroxylase in hyphophysectomized male rats would show whether their expression might be related.

Hypophysectomized and sham-operated male rats were maintained on the normal rat diet for two weeks until the experiment to allow them to recover from surgery. Microsomes were assayed for cholesterol 7α-hydroxylase activity and probed on Western blots with anti-PB2a to examine PB2a expression. Cholesterol 7α-hydroxylase activity in the hypophysectomized rats was shown to be about half that of the sham-operated controls and intact animals (Fig 3.16A). Analysis of Western blot data illustrates that the PB2a band diminishes and in some cases completely disappears in the hypophysectomized animals (Fig 3.16B). The upper band, however, is clearly present to roughly the same extent in all hypophysectomized animals. This upper band is difficult to detect in intact males and in sham-operated controls due to the intensity of the PB2a band. Nonetheless, it is evident that the variations in the expression of PB2a does not reflect the activity of cholesterol 7α-hydroxylase.

3.14 Summary

The fact that cholesterol 7α-hydroxylase activity does not alter in parallel with the expression of PB2a provides further evidence that the two proteins are distinct forms of cytochrome P450.

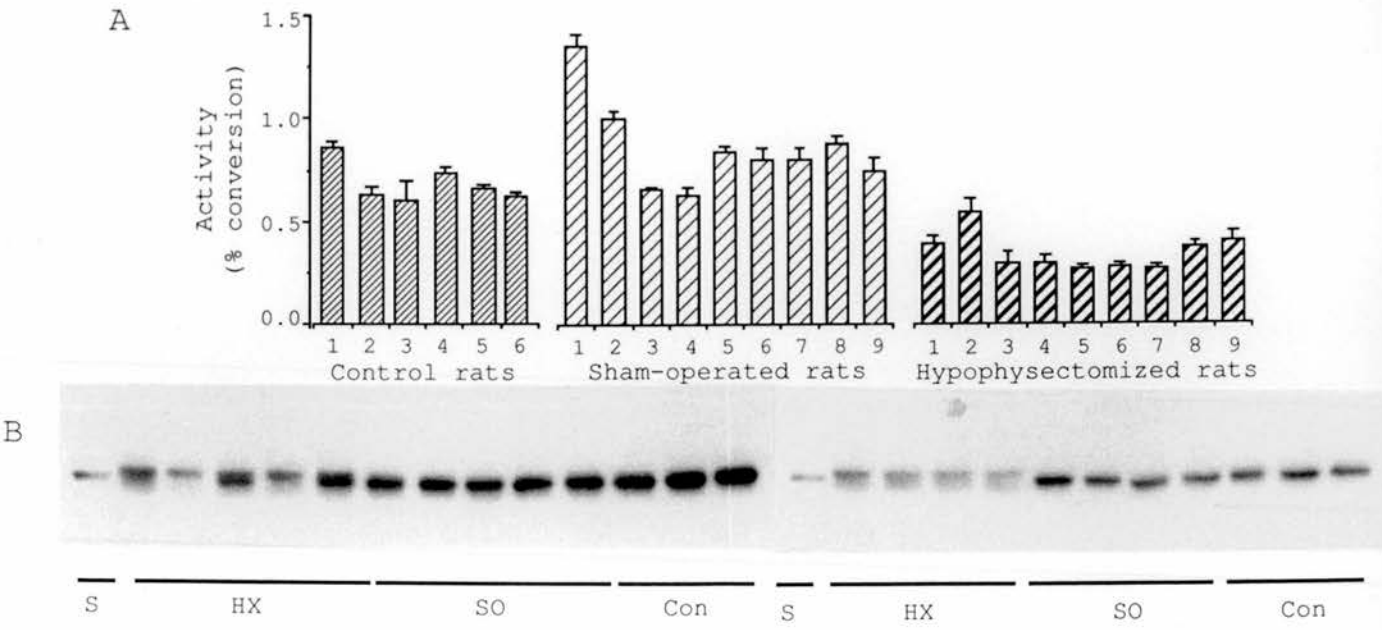


Figure 3.16. The Divergence of Cholesterol 7α-hydroxylase Activity and Cytochrome P450 PB2a Expression in Hypophysectomized Male Rats.

(A) Cholesterol 7α-hydroxylase assays were carried out as described in Materials and Methods using the phosphate reaction buffer and 1.0mg microsomal protein. Values are the mean ± s.d. of triplicate determinations.

(B) Microsomes (10μg) were fractionated in 9% SDS-polyacrylamide gels and transblotted onto nitrocellulose as described in Materials and Methods. Western blots were probed with antiserum 34 at a dilution of 1:500. Abbreviations: Con = control animals, HX = hypophysectomized animals, SO = sham-operated animals. Samples are in the same order from left to right in both the graph and the Western blots.

1.) Cholesterol 7α-hydroxylase activity is not sexually dimorphic and is detected in both male and female rats (Fig 3.15).

2.) In hypophysectomized males, cholesterol 7α-hydroxylase activity is present in the absence of PB2a expression (Fig 3.16).

3.15 Induction of Cholesterol 7α-hydroxylase Activity

Although PB2a is classified with P450s that are constitutively expressed (Table 3.3), it does not belong to the house-keeping P450s, to which cholesterol 7α-hydroxylase is thought to belong. House-keeping cytochromes P450 catalyze essential metabolic reactions and are not induced by xenobiotics. As pointed out in the Introduction, there have been conflicting reports in the literature as to the inducibility of cholesterol 7α-hydroxylase. To clarify this matter, rats were dosed with several inducing agents including phenobarbital (family IIB-inducer), isosafrole (family I-inducer), clofibrate (family IV-inducer), and SKF-525A (general P450 inducer). These drugs are known to enhance xenobiotic metabolism several fold.

Female rats were maintained on the control diet and xenobiotics were injected i.p. for three days prior to the experiment. These classic P450 inducers were not shown to cause an consistent increase in 7α-hydroxylase activity (Fig 3.17A). The response between animals treated with the same drug is quite variable. Phenobarbital appears to either not affect or decrease activity. Treatment with isosafrole does not appear to alter activity dramatically. The P450 inhibitor, SKF-525A, seems to enhance activity slightly. The response to treatment with clofibrate is anomalous. In two instances, there is no trace of activity, while in the third, activity is increased over control levels.

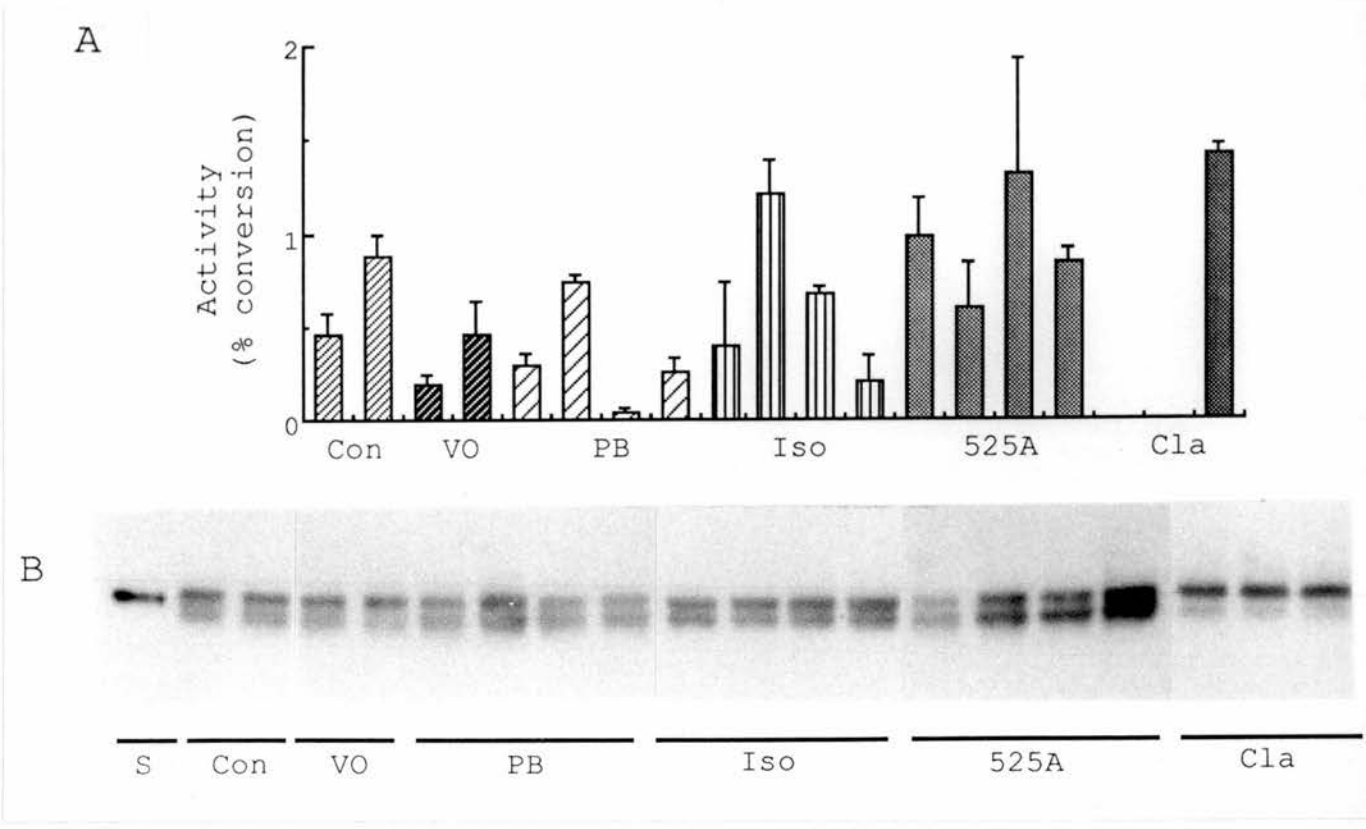


Figure 3.17. The Inducibility of Cholesterol 7α-hydroxylase.

(A) Female rats were injected with xenobiotics i.p. as described in Materials and Methods for three days prior to the experiment. Cholesterol 7α-hydroxylase assays were carried out as described in Materials and Methods using the phosphate reaction buffer and 0.5mg microsomal protein. Values are the mean ± s.d. of triplicate determinations.

(B) Microsomes (10μg) were fractionated in SDS-polyacrylamide gels and transblotted as described in Materials and Methods. Western blots were probed with antiserum 34 at a dilution of 1:500. Samples are in the same order in both the graph on the Western blots. Abbreviations: Con = control animals, VO = vehicle control animals, PB = phenobarbital-treated animals, Iso = isosafrole-treated animals, 525 = SKF-525A-treated animals, Cla = clofibrate-treated animals.

Microsomal proteins were also subjected to Western blot analysis to investigate the induction of different families of cytochrome P450. Proteins were transferred onto nitrocellulose and probed with anti-PB2a, anti-MC1a, and anti-PB3a. The band tentatively identified as cholesterol 7α-hydroxylase was not induced (Fig 3.17B). Induced proteins can be seen in the Western blot analysis of the microsomes (Fig 3.18). The P450 MC1a was induced by the administration of isosafrole while the P450 PB3a was induced by the administration of phenobarbital. SKF-525A and clofibrate appear to have induced phenobarbital-related, but not polycyclic aromatic hydrocarbon-related, cytochromes P450. These results are in accord with the classification of cholesterol 7α-hydroxylase as a house-keeping P450.

3.16 Phosphorylation of Cholesterol 7α-hydroxylase

PB2a has been shown to be phosphorylated specifically by the Ca^{+2} phospholipid-dependent kinase (Pyerin et al, 1987). The effect of phosphorylation on enzyme activity was not determined. The significance of phosphorylation on the physiological role of the enzyme also remains to be established. Previous studies on P450s revealed that phosphorylation converted the enzyme to its inactive P420 state (Taniguchi *et al.*, 1985). As outlined in the Introduction, there are conflicting views as to whether cholesterol 7α-hydroxylase activity is regulated by phosphorylation. In light of the apparent similarity between cholesterol 7α-hydroxylase and PB2a as well as the confusion in the literature, it was decided to investigate the effect of phosphorylation of 7α-hydroxylase activity. This work was done in collaboration with Dr. Dave Carling at the

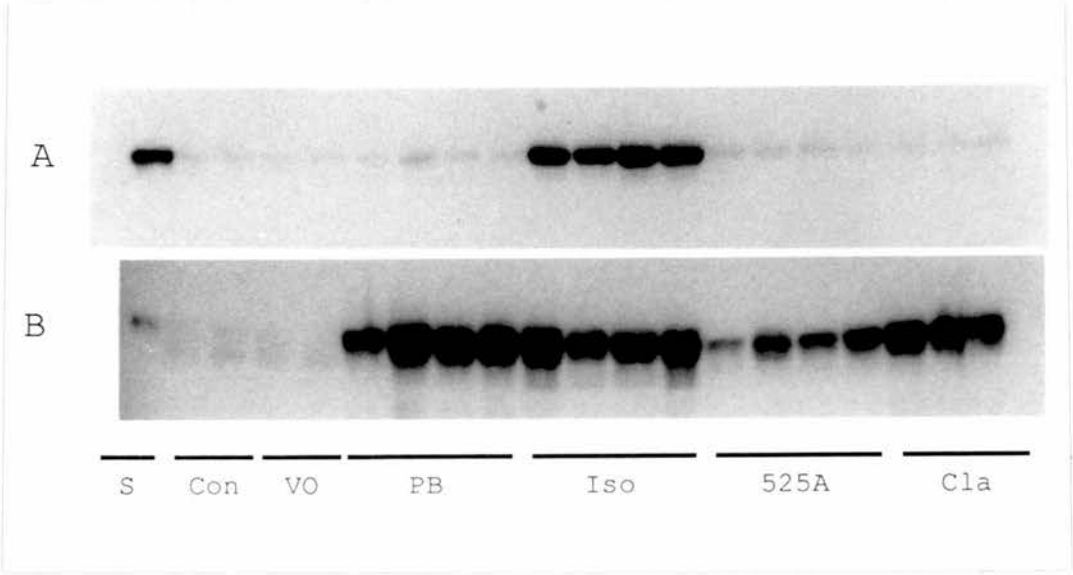


Figure 3.18. The Inducibility of MC1a and PB3a.

Female rats were injected with xenobiotics i.p. as described in Materials and Methods for three days prior to the experiment. Microsomes (10μg) were fractionated in SDS-polyacrylamide gels and transblotted as described in Materials and Methods. Western blots were probed with anti-MC1a (A) and anti-PB3a (B) at a dilution of 1:500. Abbreviations: Con = control animals, VO = vehicle control animals, PB = phenobarbital-treated animals, Iso = isosafrole-treated animals, 525 = SKF-525A-treated animals, Cla = clofibrate-treated animals.

M.R.C. Protein Phosphorylation Group at the University of Dundee. Purified cAMP-dependent protein kinase and AMP-activated protein kinase were kindly donated by Dr. Dave Carling. Purified protein kinase C was the generous gift of Dr. Peter Parker.

One of the first aspects of phosphorylation investigated was the effect of NaF on 7α-hydroxylase activity. This phosphatase inhibitor was routinely included in the assay reaction mixture because it had been reported to stabilize activity in microsomes (Sanghvi *et al.*, 1981). Microsomes from rats maintained on cholestyramine diet were prepared in the presence and absence of NaF. Microsomes were incubated at 37°C for 30min to allow endogenous phosphatase activity to dephosphorylate proteins prior to the assay. The presence of NaF was found to have no effect on cholesterol 7α-hydroxylase activity (Fig 3.19).

It was then decided to investigate the direct effect of phosphorylation on cholesterol 7α-hydroxylase activity using purified kinases. Kinases selected included the cAMP kinase and AMP-dependent kinase as these have already been implicated in the phosphorylation of other cholesterol metabolizing enzymes (Hardie *et al.*, 1989). Cytosol was used to ascertain whether there were any effects from other proteins (Danielsson *et al.*, 1980).

Microsomes were prepared in the absence of NaF and incubated at 37°C for 30min to allow endogenous phosphatase activity to act on protein. Phophorylation reactions were carried out as described in Fig 3.20. Microsomes were immediately assayed for both cholesterol 7α-hydroxylase and HMG-CoA reductase activities. HMG-CoA reductase activity was shown to be effectively decreased after a 5min incubation with the AMP-dependent kinase (Fig 3.20A), indicating that

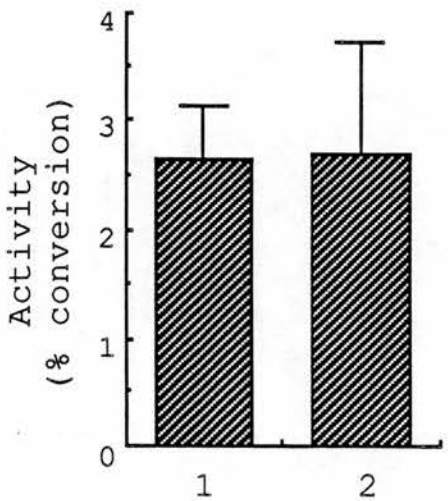


Figure 3.19. Effect of NaF on Cholesterol 7α-hydroxylase Activity.

Female rats were maintained on a 4% cholestyramine diet for seven days prior to the experiment. Microsomes were prepared in the presence (1) and absence (2) of NaF. NaCl (50mM) was included in the absence of NaF to maintain the ionic status of the solutions used. Microsomes were incubated at 37°C for 30min to allow endogenous phosphatase activity to dephosphorylate protein prior to the assay. Cholesterol 7α-hydroxylase assays were carried out as described in Materials and Methods using the phosphate reaction buffer and 0.5mg microsomal protein. Values are the mean ± s.d. for triplicate determinations.

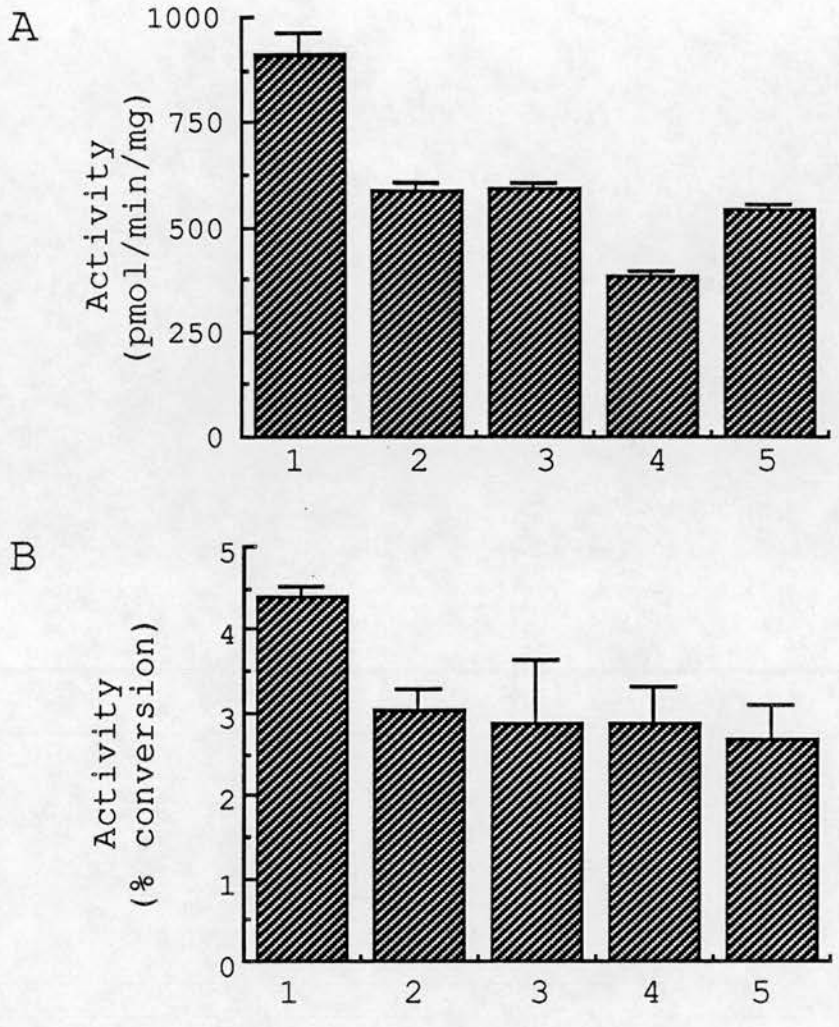


Figure 3.20. Effect of Phosphorylation on HMG-CoA Reductase and Cholesterol 7α-hydroxylase.

Two Female rats were maintained on a cholestyramine-supplemented diet for one week prior to the experiment. Microsomes were prepared in the absence of NaF and incubated at 37°C for 30min to allow endogenous phosphatase activity to act on microsomal protein. NaF (50mM) was then added to prevent further dephosphorylation. Microsomes were incubated in the phosphorylation reaction buffer for 5-60min as follows: 1 = no addition; 2 = ATP/$MgCl_2$ (2mM/7.5mM); 3 = cAMP-dependent protein kinase (260U/ml) + ATP/$MgCl_2$ (2mM/7.5mM); 4 = AMP-activated protein kinase (1.5U/ml) + ATP/$MgCl_2$ (2mM/7.5mM); 5 = cytosol (2.4mg/ml) + ATP/$MgCl_2$ (2mM/7.5mM).

(A) HMG-CoA Reductase Activity. Phosphorylation reactions were terminated after 5min. HMG-CoA reductase assays were carried out as described in Materials and Methods using 0.5mg microsomal protein. Values are the mean ± range for duplicate determination.

(B) Cholesterol 7α-hydroxylase Activity. Phosphorylation reactions were terminated after 60min. 7α-hydroxylase assays were carried out as described in Materials and Methods using the phosphate reaction buffer and 0.5mg microsomal protein. Values are the mean ± s.d. for triplicate determinations.

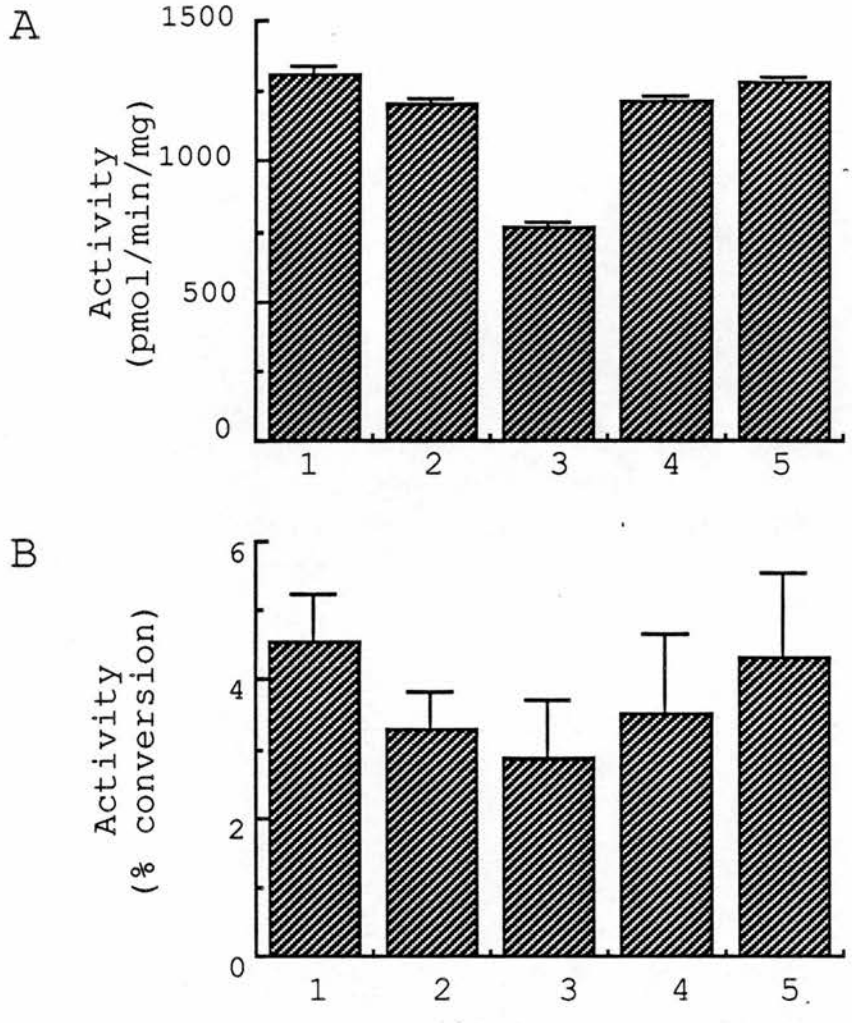
all the necessary components were present for phosphorylation. No increase in 7α-hydroxylase activity was observed after a 60min incubation with either of the two purified kinases or the cytosolic fraction (Fig 3.20B). These results suggest that cholesterol 7α-hydroxylase activity is not increased.

3.17 Immunoprecipitation of Phosphorylated Microsomal Proteins

Further studies were undertaken in order to identify a phosphorylated protein. Evidence presented earlier showed that an anti-PB2a antibody was capable of immunoprecipitating a protein associated with cholesterol 7α-hydroxylase activity. This procedure could therefore be employed to detect a phosphorylated enzyme.

Microsomes were prepared in the absence of NaF and dephosphorylated with a specific phosphatase. They were subsequently washed in 0.25M NaCl to dissociate endogenous phosphatases and kinases and phosphorylated as described in Fig 3.21. Cholesterol 7α-hydroxylase and HMG-CoA reductase were immediately assayed for activity. In agreement with earlier results, 7α-hydroxylase was unaffected by any kinase activity while HMG-CoA reductase activity was specifically inactivated by the AMP-dependent kinase (Fig 3.21).

Microsomes from each phosphorylation reaction were subsequently immunoprecipitated with anti-PB2a as described earlier. Immunoprecipitated proteins and whole microsomal samples were fractionated using SDS-PAGE and silver-stained to visualize proteins (Fig 3.22A). Gels were dried down and exposed to X-ray film for 3weeks to detect ^{32}P incorporation into protein. Microsomal samples were shown to contain some radiolabelled products (Fig 3.22B). The



Figures 3.21. Effect of Phosphorylation on HMG-CoA Reductase and Cholesterol 7α-hydroxylase.

Microsomes were prepared from female rats fed a cholestyramine-supplemented diet in the absence of NaF. Microsomal proteins were dephosphorylated with the catalytic subunit of protein phosphatase type I from rabbit skeletal muscle. Microsomes were subsequently washed in 0.25M NaCl and NaF was included to prevent further phosphatase activity. Microsomes were incubated in 50mM Tris:HCl, pH 7.4 containing 1mM AMP with the following additions: 1 = no addition; 2 = [γ-^{32}P]ATP (0.5mM, final specific activity of 180cpm/mmol) + $MgCl_2$ (10mM); 4 = cAMP-dependent protein kinase (10U/ml) + [γ-^{32}P]ATP/$MgCl_2$ (0.5mM/10mM); 3 = AMP-activated protein kinase (25U/ml) + [γ-^{32}P]ATP/$MgCl_2$ (0.5mM/10mM); 5 = protein kinase C (3U/ml) + phosphatidyl serine (1.25mg/ml in 2.5% Triton X-100) + 12-O-tetradecanoyl phorbol-13-acetate (1.25μg/ml) + [γ-^{32}P]ATP/$MgCl_2$ (0.5mM/10mM). Phophorylation reactions were carried out at 30°C for 30min and terminated by the addition of 10mM EDTA.

(A) HMG-CoA reductase assays were carried out as described in Materials and Methods using 0.5mg microsomal protein. Values are the mean ± range for duplicate determination.

(B) Cholesterol 7α-hydroxylase assays were carried out as described in Materials and Methods using the phosphate reaction buffer and 0.5mg microsomal protein. Values are the mean ± s.d. for triplicate determinations.

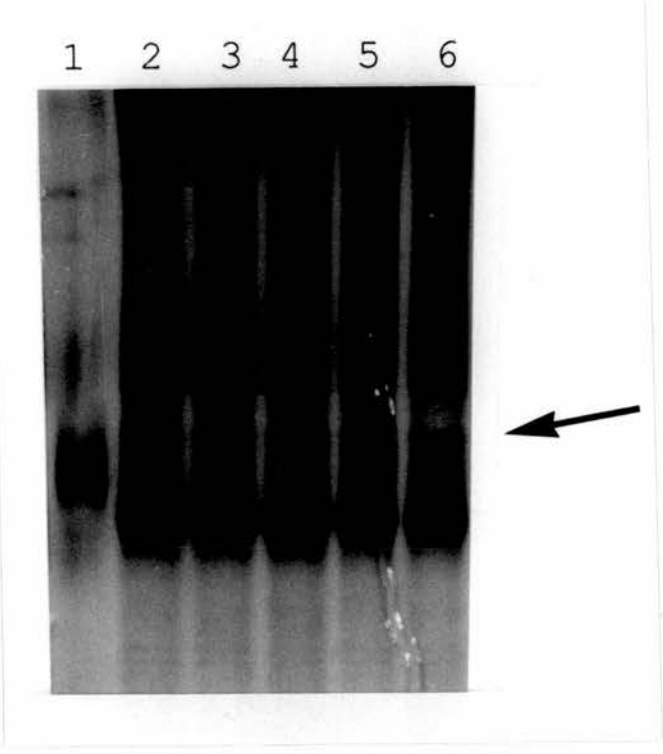


Figure 3.22. Immunoprecipitation of Phosphorylated Proteins.

(A) PB2a-related proteins were immunoprecipitated with antiserum 34 as described in Materials and Methods. Immunoprecipitated proteins were fractionated in 9% SDS-polyacrylamide gels under non-reducing conditions and proteins were visualized with silver stain. Track 1 = PB2a standard; track 2 = immunoprecipitates from control microsomes; track 3 = immunoprecipitates from microsomes treated with [γ-^{32}P]ATP; track 4 = immunoprecipitates from microsomes treated with AMP-activated protein kinase; track 5 = immunoprecipitates from microsomes treated with cAMP-dependent protein kinase; track 6 = immunoprecipitates from microsomes treated with protein kinase C. The arrow points to the immunoprecipitated protein.

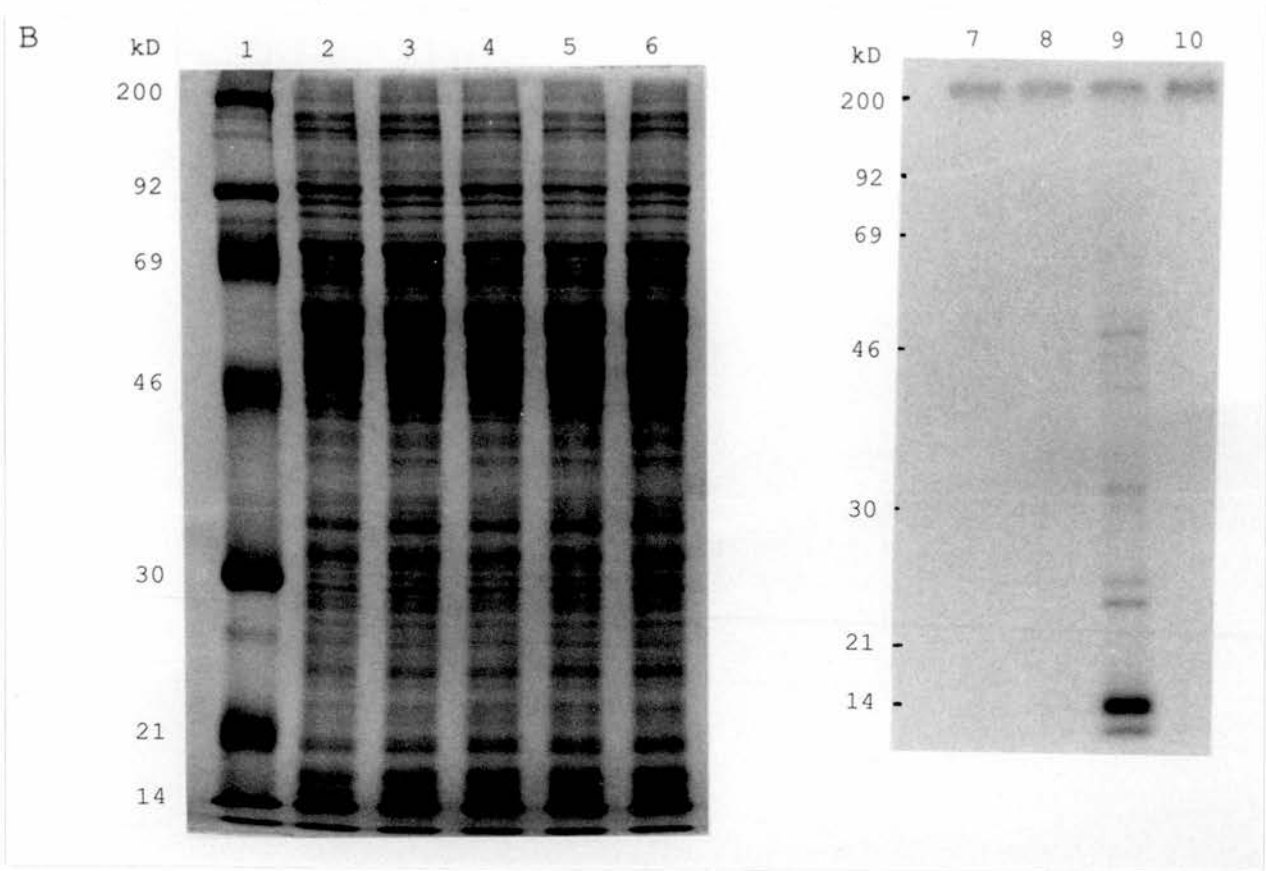


Figure 3.22. Immunoprecipitation of Phosphorylated Proteins.

(B) Whole microsomal samples were fractionated in 9% SDS-polyacrylamide gels under non-reducing conditions. The gel was stained, dried down and exposed to X-ray film for 3 weeks to detect ^{32}P incorporation into protein. Track 1 = molecular weight markers; track 2 = control microsomes; track 3 = microsomes treated with [γ-^{32}P]ATP; track 4 = microsomes treated with AMP-activated protein kinase; track 5 = microsomes treated with cAMP-dependent protein kinase; track 6 = microsomes treated with protein kinase C; tracks 7-10 are the autoradiographs of tracks 3-6.

most activity was in the fraction phosphorylated by cAMP-dependent protein kinase. No trace of radiolabel was found in the immunoprecipitate samples (results not shown).

These data indicate that cholesterol 7α-hydroxylase does not appear to be phosphorylated by cAMP-dependent protein kinase, AMP-activated protein kinase, or protein kinase C under the conditions used here. The selective decrease in HMG-CoA reductase activity and the incorporation of ^{32}P into microsomal proteins indicates a degree of phosphorylation was attained. The lack of effect on cholesterol 7α-hydroxylase by these kinases would suggest that these kinases do not interact directly and specifically with 7α-hydroxylase.

Chapter 4

Bile Acid Synthesis in HepG2 Cells

4.1 Introduction

Cholesterol metabolism can follow many pathways in the liver (Stange, 1987) (see Fig 1.2). The liver can create an endogenous supply of cholesterol from *de novo* synthesis. Alternatively, it can take up exogenous sources of cholesterol in the form of lipoproteins. Cholesterol can be used as an integral component of the cellular membrane or esterified for storage within the cell or packaged in lipoproteins for delivery to peripheral tissues. Cholesterol can also be secreted in bile or catabolized to bile acids for use in the digestion of fats. The flux of cholesterol within the hepatocyte is influenced by both its rate of synthesis and degradation as well as its input and output from the cell. Evidence has accumulated indicating that certain intracellular cholesterol pools serve individual functions (Stange, 1987).

Bile acids are of major importance in the flow of cholesterol. With biliary cholesterol, the secretion of bile acids from the liver represents the sole method for the elimination of cholesterol from the body (see Introduction). It is therefore important to determine which pools of cholesterol are available for bile acid synthesis and how these pools can be influenced.

Some of the first work to suggest that bile acid synthesis was affected by the availability of cholesterol showed that cholesterol 7α-hydroxylase activity was enhanced in rats maintained on a high cholesterol diet (Boyd *et al.*, 1969). These results indicated that bile acid synthesis increased with an increasing substrate pool. Much of the following work investigating the compartmentalization of

cholesterol used radiolabelled cholesterol and radiolabelled mevalonate *in vivo* to determine which source was favored for bile acid synthesis. The radiolabelled compound which resulted in a higher specific activity in the bile acid pool was determined to be the preferred source. In these experiments, *de novo* synthesized cholesterol was proposed to be the preferred substrate for bile acid synthesis in the rat (Balasubramanian *et al.*, 1973; Mitropoulos *et al.*, 1974). Other workers came to the same conclusion using different methods. Using radiolabelled mevalonate, Ogura *et al.* (1976) showed that *de novo* synthesized cholesterol was esterified more slowly than that of exogenous cholesterol. In addition, Ayaki *et al.* (1983) found that exogenous radiolabelled cholesterol has a greater propensity to partition into serum rather than bile acids. Takeuchi *et al.* (1974) showed that, in the rat, the inhibition of glucose-induced cholesterogenesis by the administration of triparanol resulted in a decrease in bile acid synthesis. Mitropoulos and coworkers (1978) later demonstrated that increasing *de novo* synthesis by the administration of mevalonate resulted in an increase in cholesterol 7α-hydroxylase activity.

Research in human systems is understandably less sophisticated. Most of the work in man has relied on turnover studies of radiolabelled lipoproteins. These experiments are very similar to the early studies on rats, examining the specific activity of the bile acid pool in reference to different sources of cholesterol. Present information indicates that HDL free cholesterol is utilized in preference to LDL free cholesterol for bile acid synthesis (Halloran *et al.*, 1978; Schwartz *et al.*, 1981). Cholesteryl ester has been shown not to be an important source for bile acid synthesis

in man (Schwartz *et al.*, 1981). There is some evidence in humans to support the finding in rats that *de novo* synthesized cholesterol is the preferred substrate for bile acid synthesis. Following the fate of tritiated mevalonate, Einarsson *et al.* (1979) showed that *de novo* synthesized cholesterol is found mostly in bile acid and to a lesser extent in bile cholesterol and serum free cholesterol.

Studies using rat hepatocytes in primary culture have been more readily able to define specific cholesterol sources for bile acid synthesis. The absence of both the enterohepatic circulation as well as the systemic circulation of lipoproteins simplifies the interpretation of results. Cell culture systems consequently provide a controlled environment with regards to lipoproteins. Davis *et al.* (1983) reported that primary rat hepatocytes were able to maintain cholesterol homeostasis by adjusting bile acid synthesis according to cholesterol availability. They demonstrated that bile acid synthesis was enhanced by the addition of either mevalonate or d<1.02 lipoproteins or by feeding donor rats a high cholesterol diet. In contrast, mevinolin, an HMG-CoA reductase inhibitor, caused a decrease in bile acid synthesis (Davis *et al.*, 1983). Sampson and coworkers showed that the increase in bile acid synthesis due to mevalonate was associated with an increase in cholesteryl ester synthesis (Sampson *et al.*, 1987). The addition of 58-035, an ACAT inhibitor, to cells given mevalonate was found to further increase the synthesis of bile acids (Sampson *et al.*, 1987). The addition of rat HDL_2 to rat hepatocytes maintained in primary culture was also shown to increase bile acid synthesis (Ford *et al.*, 1985). Moreover, it was the cholesterol ester content of HDL that was shown to be responsible for this increase (MacKinnon *et al.*, 1987). Further

studies indicated that the free cholesterol component of HDL must be esterified before it can enter the pool of cholesterol destined for bile acid synthesis and that cholesterol, once destined for bile acid synthesis, cannot then be esterified (Sampson *et al.*, 1988a, 1988b). While these studies have not established that specific pools of cholesterol are destined to become bile acids, they do indicate that bile acid synthesis can be driven from many sources of cholesterol within the cell.

Primary rat hepatocytes, however, may not accurately reflect the regulation of bile acid synthesis in humans for several reasons: 1.) rats are known to catabolize cholesterol at a much faster rate than man (Dietschy *et al.*, 1988) 2.) rats synthesize a different pattern of lipoproteins with different functions than man (Oschry & Eisenberg, 1982) 3.) rats synthesize and secrete different bile acids from man (Elliot & Hyde, 1971). The limited lifespan of primary hepatocytes also makes them unsuitable for long-term studies.

There have been a few studies on bile acid synthesis in established hepatocyte cell lines. Lambiotte and Thiery (1980) demonstrated that a well-differentiated rat hepatoma cell line, Reuber H35, could hydroxylate and conjugate bile acids added to culture medium, but they did not show *de novo* synthesis of bile acids. In fact, hepatocyte cell lines which actively synthesize and secrete bile acids have been difficult to establish. One group produced such a line by hybridizing primary rat hepatocytes to the Reuber H35 rat hepatoma cell line (Everson & Polokoff, 1984). In addition, one human hepatoblastoma has been reported to synthesize bile acids (Amuro *et al.*, 1982).

Another human hepatoblastoma cell line, HepG2, has been extensively studied and found to manifest several characteristics of a well-differentiated hepatocyte (Knowles *et al.*, 1980). HepG2 cells have been shown to produce liver-specific apoproteins (Zannis *et al.*, 1981; Rash *et al.*, 1981) as well as synthesize and secrete VLDL, HDL, and LDL-like lipoproteins (Ellsworth *et al.*, 1986; Dashti & Wolfbauer, 1987). HepG2 cells have also been shown to take up, bind, and degrade LDL (Havekes *et al.*, 1983; Dashti *et al.*, 1984; Illingworth *et al.*, 1984; Wu *et al.*, 1984; Leichtner *et al.*, 1984), HDL (Dashti *et al.*, 1985), and CM remnants (Lenich & Ross, 1987).

At the onset of this work, an abstract had been published indicating that HepG2 cells synthesized and secreted bile acids (Craig & Cooper, 1985). However, no documentation of regulatory mechanisms was provided. The present work was designed to evaluate the regulatory controls affecting bile acid synthesis in HepG2 cells. The effect of substrate supply on synthesis was of primary interest because it was not known which pool of cholesterol contributes directly to the bile acid synthesis (see Introduction).

4.2 Initial Investigation of Bile Acid Production by HepG2 Cells

Preliminary experiments carried out using HepG2 cells investigated whether this particular clone of cells had retained the capacity to synthesize bile acids. Cells maintained in culture for an extended length of time often de-differentiate as they adapt to expressing only those functions necessary for survival. Bile acid production is a function of a differentiated hepatocyte and is not

necessary for the maintainance of the viability of the cell or its growth. All experiments were carried out on cells within five passages of each other to control for any long-term culturing changes.

4.2.1 Radioimmunoassay Analysis

Bile acids were initially analyzed using a radioimmunoassay (RIA) (Beckett *et al.*, 1978, 1979). RIA is a commonly used method of detection. It is simple, quick, and can be highly sensitive, measuring as little as 2pmol of a compound. The RIA method employed here measured 100% of the glyco- and tauro-conjugates of chenodeoxycholic acid (CDCA) and cholic acid (CA) (Ford *et al.*, 1985). However, during the course of this work, it was reported that HepG2 cells exhibited abnormalities in the synthesis and conjugation of bile acids (Everson & Polokoff, 1986). Using the highly specific method of gas chromatography-mass spectrometry for the separation and identification of bile acids, they demonstrated that HepG2 cells produced mainly unconjugated CDCA, some unconjugated CA, and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) and 3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA) (Everson & Polokoff, 1986). The latter two compounds are precursors of CA and CDCA, respectively (Fig 4.1). Antisera used in the RIA measured the sum total of glyco- and tauro- bile acid conjugates. The RIA could differentiate between CA and CDCA, but not between glyco- to tauro-conjugates. The antisera showed less than 10% cross-reactivity with the unconjugated bile acids. It was not possible to examine the cross-reactivity of the antisera with THCA and DHCA because these standards were not

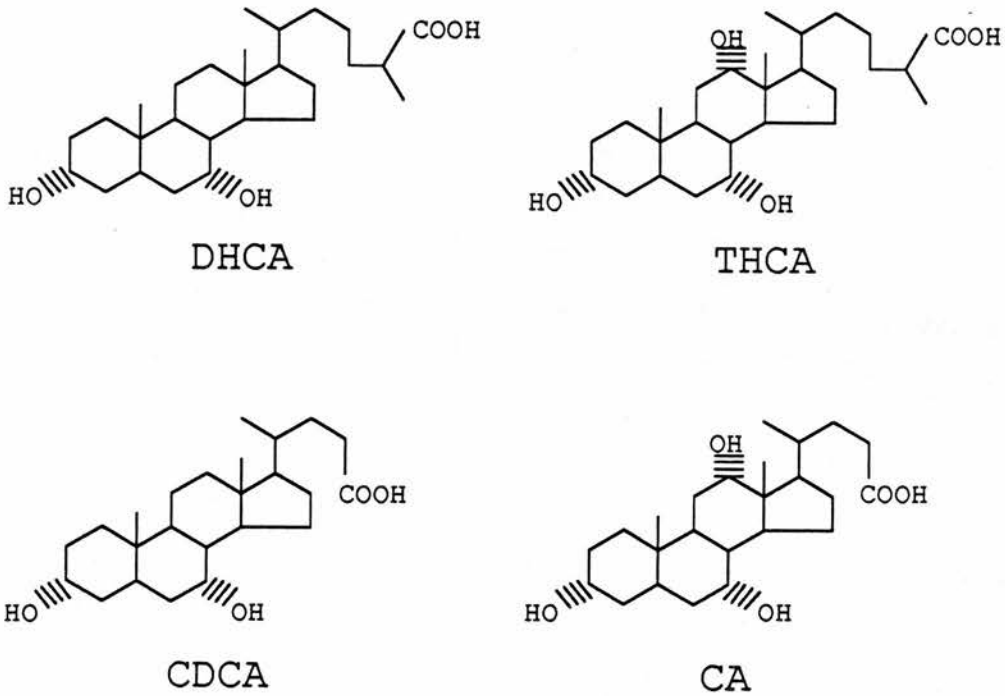


Figure 4.1. Major Bile Acids Produced by HepG2 Cells. Abbreviations: DHCA = 3α,7α-dihydroxy-5β-cholestan-26-oic acid, THCA = 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid, CDCA = chenodeoxycholic acid, CA = cholic acid

available. For these reasons, this RIA with the antisera available was deemed unsuitable to study bile acid synthesis by HepG2 cells.

4.2.3 3α-hydroxysteroid Dehydrogenase Analysis

A diagnostic kit was then used which identified bile acids through a reaction with the 3α-hydroxyl group. Cholesterol and other steroid products do not interfere with this assay method as they contain a 3β-hydroxyl group. The Sterognost 3α-Flu kit, developed for clinical use, was designed to detect bile acids in serum samples. Determination of bile acid content is done indirectly via the oxidation of the 3α-hydroxyl group of the bile acid to a 3-keto group by 3α-hydroxysteroid dehydrogenase (3α-HSD). This reaction is coupled to the reduction of resazurine to resorufin, which fluoresces at 585nm when excited at 530nm. The reaction is driven by the cycling of NADH (Fig 4.2).

To establish whether the kit was suitable for the determination of bile acids in culture media, standard curves of six bile acids, CA, GCA, TCA, CDCA, GCDCA, TCDCA, were prepared in DMEM. Detection was shown to be linear between 12.5 and 1250pmol (Fig 4.3).

Malate dehydroxygenase (MDH) has been reported to copurify with the 3α-HSD used in this assay kit (Whiting & Edwards, 1979; Barth & Wirthesohn, 1981). MDH could cause false high readings of bile acid content by producing NADH from the oxidation of malate in the culture media. To avoid this possible complication, the bile acids were isolated from the samples through an extraction procedure using C18 Bond Elut columns. These columns bind negatively charged hydrophobic compounds, allowing hydrophilic compounds like malate to pass through.

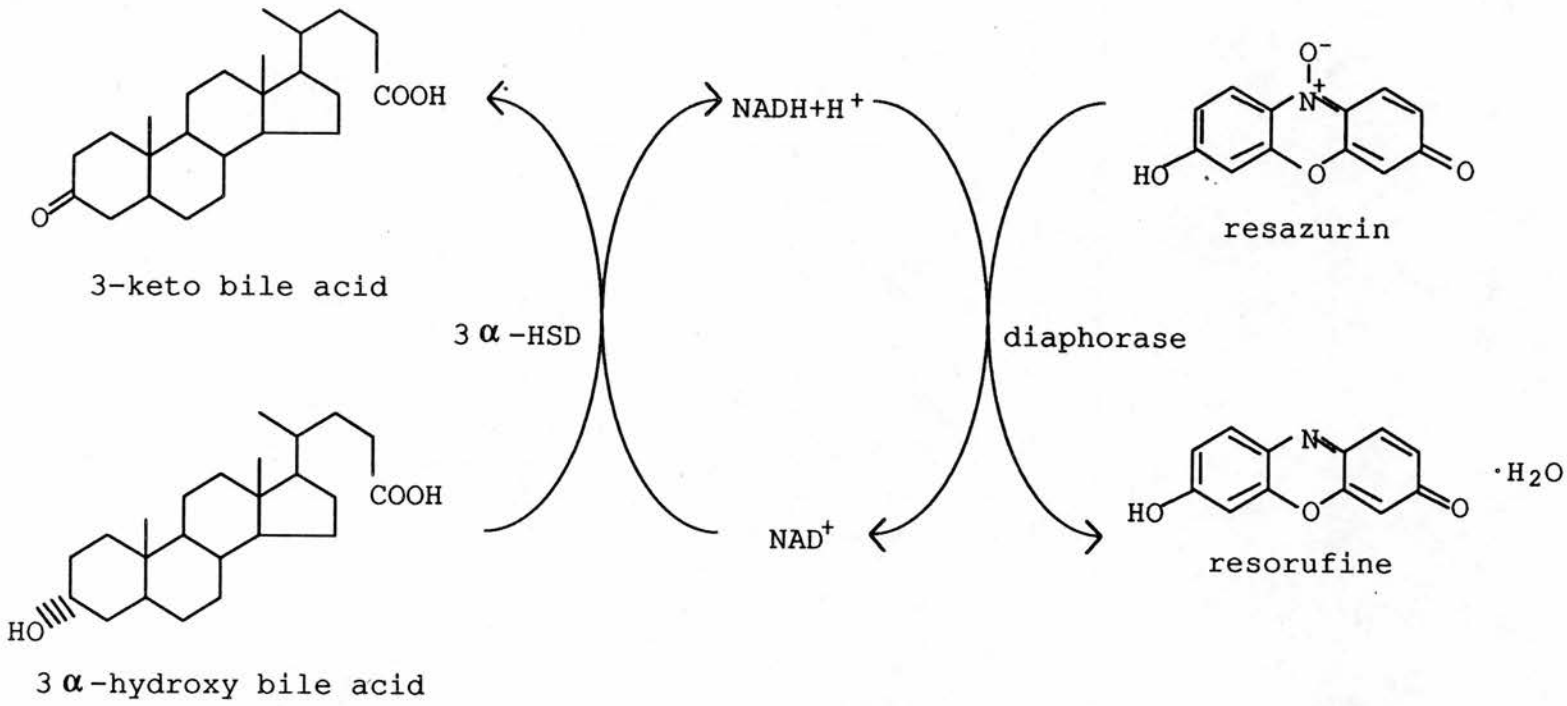


Figure 4.2. Schematic Diagram of the 3α-hydroxysteroid Dehydrogenase (3α-HSD) Assay.

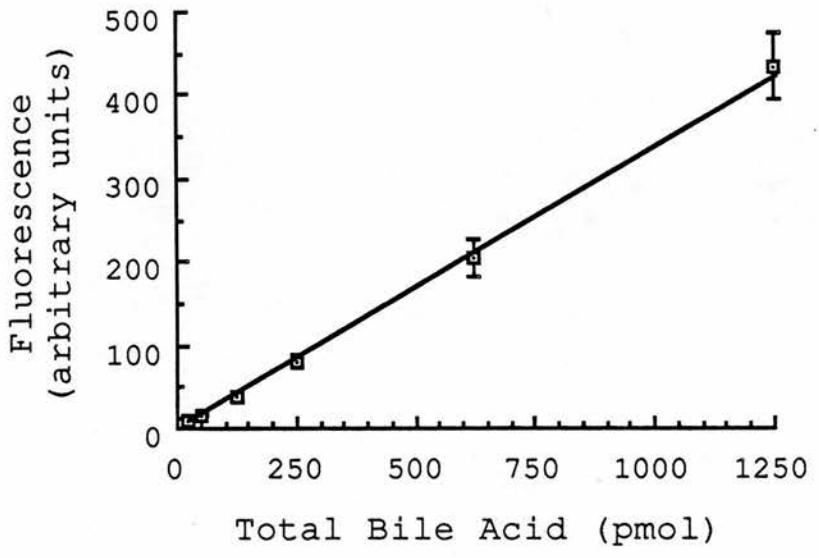


Figure 4.3. Calibration of 3α-HSD Assay with Six Bile Acids.

Standard curves of CA, GCA, TCA, CDCA, GCDC, and TCDC were prepared in ITS DMEM. 3α-HSD assays were carried out as described in Materials and Methods. Values are the mean ± s.d. (n=6).

The recovery of bile acids from samples extracted with Bond Eluts was determined by supplementing spent ITS DMEM culture media with radiolabelled TCA. Culture samples were then applied to the preconditioned Bond Elut columns and bile acids were eluted with methanol as described in Materials and Methods. This extraction procedure was shown to contain 97.8% ± 0.8% (n=12) of the TCA in the eluate.

No bile acid was detected in eluates from media exposed to cells treated with 7α-hydroxycholesterol for 72h. However, the simultaneous concentration of phenol red during the isolation of bile acids, caused samples to become very dark red. Phenol red is a pH indicator contained in DMEM. Standard curves subsequently prepared in neat DMEM and DMEM concentrated five-fold through the Bond Elut columns clearly demonstrate that the presence of concentrated phenol red caused quenching of fluorescence (Fig 4.4). Because no bile acids could be detected in dilute eluates and the further concentration of the eluates resulted in levels of phenol red that quenched fluorescence, this method was found to be unsuitable for bile acid detection in HepG2 cells.

4.2.4 High Performance Thin Layer Chromatography Analysis

High Performance Thin Layer Chromatography (HPTLC) was the third method of bile acid detection selected. This method is based on the separation of individual bile acids by thin layer chromatography. Bile acids are then converted to fluorescent derivatives by spraying the tlc plates with a charring solution and heating to 110°C for 15min. An ultraviolet scanner connected to a microprocessor is used to detect the bile acid derivatives by

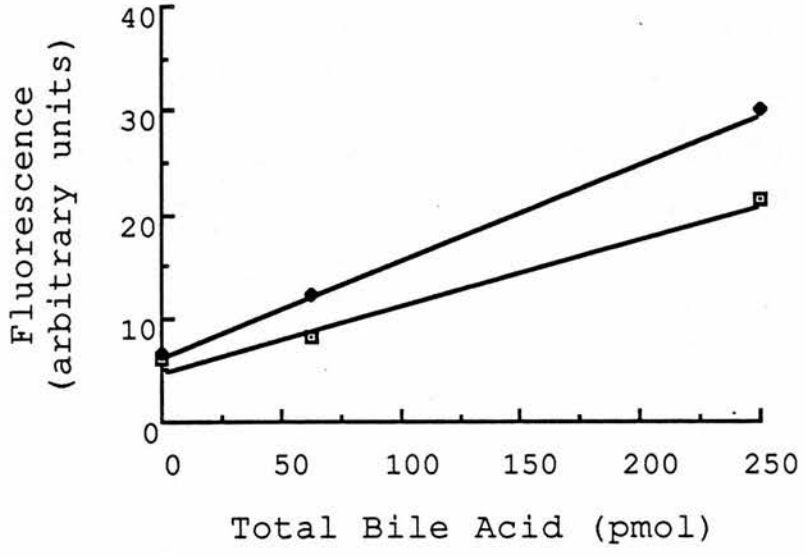


Figure 4.4. Quenching of Fluorescence by Concentrated DMEM.

Standard curves containing a mixture of CA and CDCA were prepared in neat DMEM (◆) and DMEM concentrated fivefold by C18 Bond Elut extraction (■). Bile acid content was determined using the 3α-HSD assay as described in Materials and Methods.

measuring the fluorescence emitted from the plate. The resulting trace of relative band intensities is used to construct standard curves by graphing peak area against known bile acid concentration. Unknown bile acid concentrations in samples are extrapolated from these curves. Standards and samples are spotted onto plates with a special HPTLC loading device. This instrument accurately applies 10µl of a sample over a 5mm line.

The fluorescence of the derivative is intrinsic to the bile acid, so standard curves must be prepared for each one. To conserve space on plates, standard curve cocktails are prepared containing several bile acids. In such a manner, four tracks on a plate can generate seven standard curves. Standard curves must be included on each plate because fluorescence varies with individual plate development. Standards and samples are spotted in duplicate across the plate to control for the variation of spraying the charring solution. For example, one plate has 20 tracks spotted in the following way:

tracks 1,11	Standard 1	tracks 6,16	Sample 4
tracks 2,12	Sample 1	tracks 7,17	Standard 2
tracks 3,13	Sample 2	tracks 8,18	Sample 5
tracks 4,14	Standard 3	tracks 9,19	Sample 6
tracks 5,15	Sample 3	tracks 10,12	Standard 4

Adapting the procedure routinely used at Smith Kline & French Research, the HPTLC technique was shown to detect as little as 2.5pmol of bile acid. This level of detection is comparable to the original RIA technique employed, with the added advantage that individual bile acids could be identified.

Bile acids were isolated from culture media using the Bond Elut procedure described above. It should be mentioned that the isolation of bile acids does not purify them from other media components with similar chemical characteristics. The profile from the HPTLC separation of unused culture media revealed several inherent fluorescent bands (Fig 4.5). The only bile acid peak that could be clearly observed was CDCA. This bile acid has been reported to be the main bile acid produced by HepG2 cells (Everson & Polokoff, 1986). Identification of this peak as a C24 dihydroxy-bile acid was confirmed by fast atom bombardment mass spectrometry analysis (Fig 4.6). For these reasons, this peak was used as the index of bile acid synthesis throughout the course of the following experiments.

4.3 Effect of 7α-hydroxycholesterol on Bile Acid Synthesis by HepG2 Cells

The goal of this work was to examine the regulatory mechanisms affecting bile acid synthesis in HepG2 cells. The initial aim was ensure that these cells had the capacity to produce bile acids. Cholesterol 7α-hydroxylase is the rate-limiting step in bile acid synthesis (see Introduction), producing 7α-hydroxycholesterol. In order to evaluate the entire synthetic pathway, it is necessary to circumvent restrictions placed on the system by the rate-limiting steps. Exogenous 7α-hydroxycholesterol has been shown to enter the biosynthetic pathway in isolated rat hepatocytes (Botham *et al.*, 1980). In this instance, the addition of 7α-hydroxycholesterol directly to HepG2 cells would demonstrate the ability of the cells to synthesize bile acids.

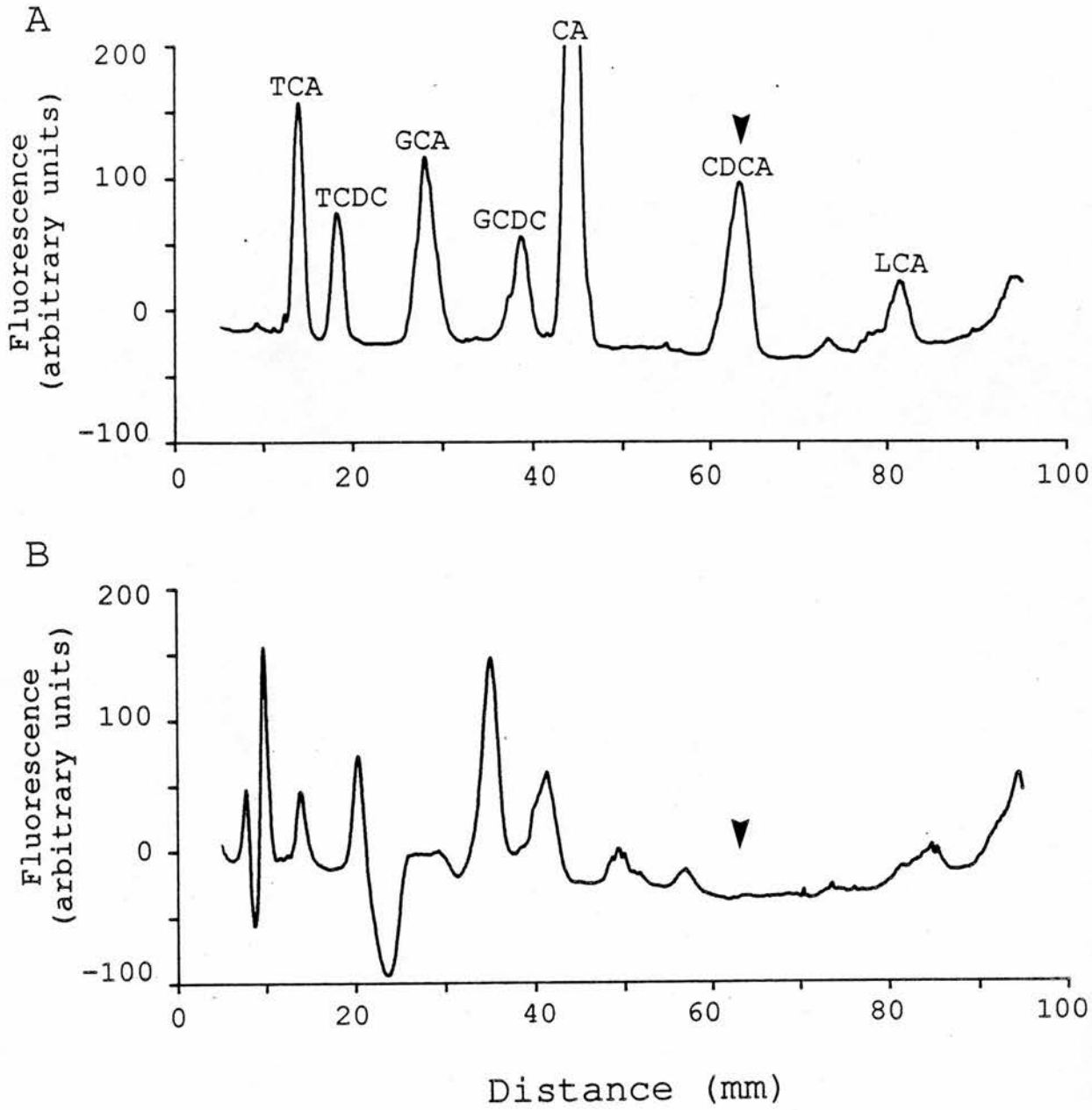


Figure 4.5. HPTLC Analysis of Bile Acid Standards and DMEM.

DMEM was extracted using C18 Bond Eluts as described in Materials and Methods. A standard cocktail was prepared containing TCA, TCDC, GCA, GCDC, CA, CDCA, AND LCA. Both the standard coctail (A) and the extracted DMEM (B) were fractionated on HPTLC plates as described in Materials and Methods. The arrow points to the position of the CDCA standard.

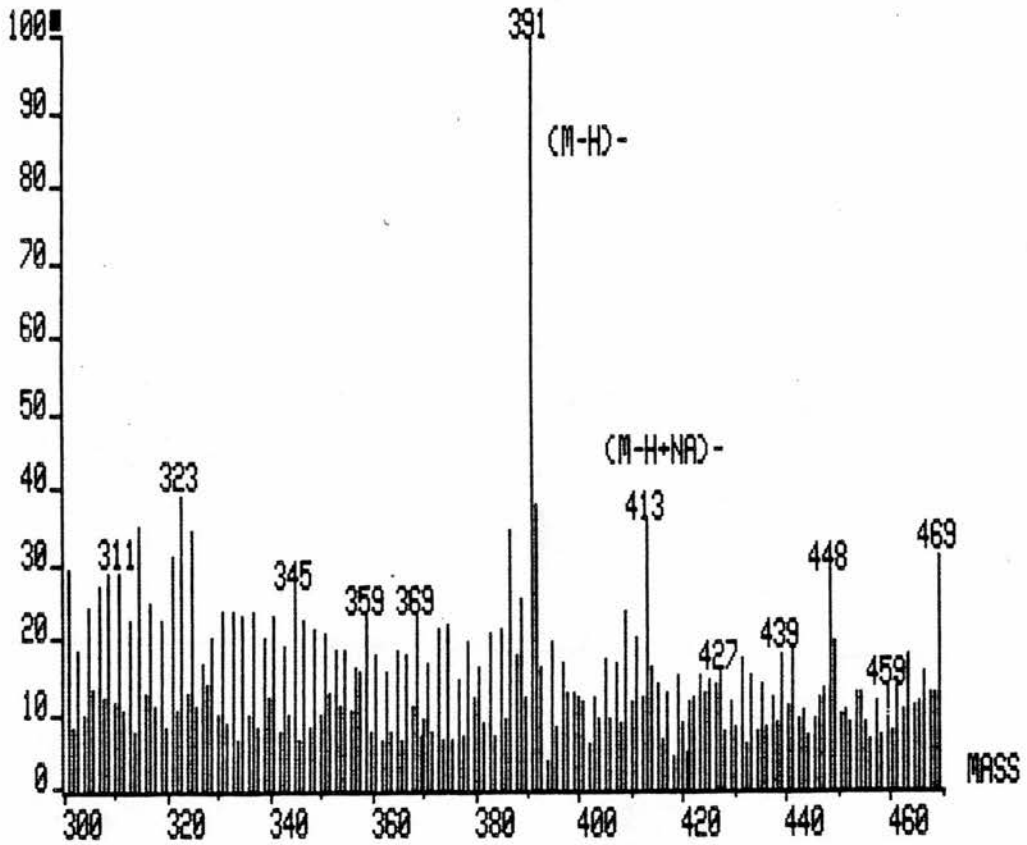


Figure 4.6. Identification of CDCA by Mass Spectrometry.

Bile acids were isolated from culture medium supplemented with 50μM 7α-hydroxycholesterol which had been left on HepG2 cells for 72h. Samples were fractionated using HPTLC as described. The area of the plate containing the peak corresponding to the CDCA standard was scraped off and the bile acid was eluted from the silica gel with methanol. The sample was filtered to remove particulate matter and concentrated under a stream of nitrogen. The sample was analyzed by fast atom bombardment mass spectrometry on a VG70250SEQ mass spectrometer. The M-H peak at 391 and (M-H)Na peak at 413 identify the compound as a C24 dihydroxy bile acid. This work was done by Dr. Christine Eckers at Smith Kline & French Research, Ltd.

HepG2 cells were routinely maintained in 15% DMEM and prepared for assay as described in Materials and Methods. Cells were incubated in ITS DMEM containing 7α-hydroxycholesterol for up to 72h. At 24h intervals, the culture media was removed and the cells harvested. Media samples were analyzed for bile acid as described in earlier.

No CDCA was seen to be produced by cells exposed to less than 5μM 7α-hydroxycholesterol (Fig 4.7). The addition of 5μM 7α-hydroxycholesterol to HepG2 cells resulted in substantial synthesis and secretion of CDCA (Fig 4.8). CDCA synthesis increased with time, being produced at a steady rate for 72h. The addition of 50μM 7α-hydroxycholesterol increased the levels of CDCA further, approximately doubling the rate of synthesis after 72h. These data indicate that HepG2 cells have retained the ability to produce bile acids. The fact that the cells do not synthesize CDCA under basal conditions is an unabiguous demonstration of the limitation of substrate. These cells do not channel cholesterol to cholesterol 7α-hydroxylase even though the capacity of the system is there. There has been some question as to the importance of 7α-hydroxylase in man (Swell *et al.*, 1980; 1981). These results suggest that this enzyme may be significant *in vivo*.

4.4 Effect of Increasing Substrate Supply on Bile Acid Synthesis by HepG2 Cells

As mentioned previously, cell culture systems are ideal for investigating sources of cholesterol destined for bile acid synthesis. Increasing the cholesterol supply in primary rat hepatocytes through the addition of either mevalonic acid (Davis *et*

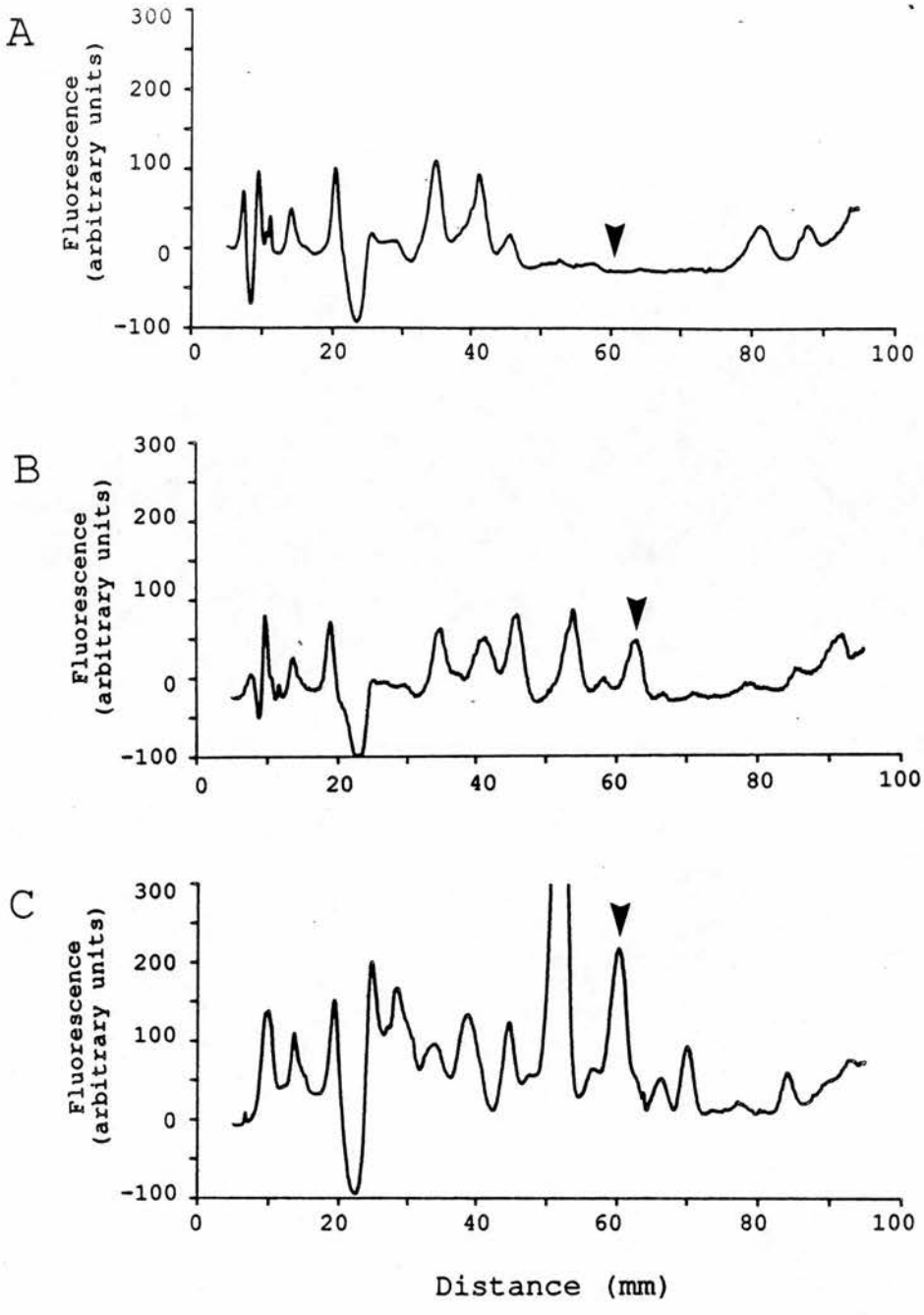


Figure 4.7. HPTLC Analysis of Culture Medium from HepG2 Cells Exposed to 7α-hydroxycholesterol.

HepG2 cells were routinely maintained in 15% DMEM and prepared for assay as described in Materials and Methods. 7α-hydroxycholesterol was dissolved in DMSO at a concentration of 10mM and added to ITS DMEM so that the final concentration ranged between 0 and 50μM. Cells were not exposed to concentrations of DMSO exceeding 1% (v/v). At 24h intervals, culture media was removed and cells harvested. Media samples were analyzed for bile acid as described earlier. The HPTLC traces shown are of Bond Elut extracts from media collected after incubation with cells for 72h. Media originally contained 0.5% (v/v) DMSO (A), 5μM 7α-hydroxycholesterol (B), and 50μM 7α-hydroxycholesterol (C). The arrow points to the position of the CDCA standard.

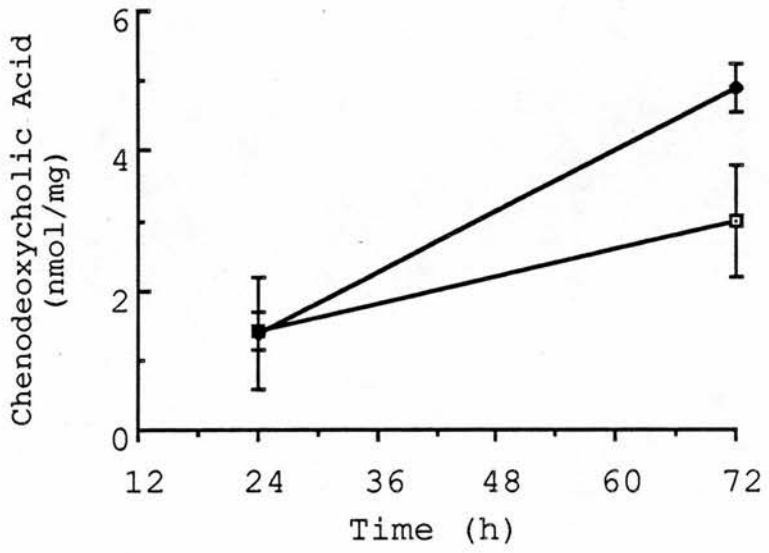


Figure 4.8. CDCA Production from HepG2 Cells Treated with 7α-hydroxycholesterol.

HepG2 cells were assayed as described in Fig 33. Bile acids were isolated from culture media and fractionated on HPTLC as described earlier. Culture medium originally contained 5μM (□) and 50μM (◆) 7α-hydroxycholesterol. Points represent the mean ± s.d. of two independent experiments each performed in triplicate.

al., 1983; Sampson *et al.*, 1987) or rat HDL_2 (Ford *et al.*, 1985; Mackinnon *et al.*, 1987) has been shown to increase bile acid production. At the start of this work, there had been no report on the effects of manipulating the cholesterol supply in HepG2 cells. It was therefore of interest to determine whether the addition of mevalonate or lipoproteins to HepG2 cells would enhance their rate of bile acid synthesis.

Cells were maintained in 15% DMEM and prepared for assay as described earlier. Cells were incubated with mevalonate, LDL, and HDL for up to 72h. Media samples were removed at 24h intervals and analyzed for bile acid content. The total cholesterol content of the cells was determined to check whether the cells had utilized the exogenous mevalonate.

As before, CDCA was not found to be produced by control cells. The addition of up to 10mM mevalonate to the culture media did not enhance CDCA synthesis (Fig 4.9). Total cholesterol synthesis, however, was seen to be increased 1.7-fold in cells receiving 5mM mevalonate and 2.7-fold in cells exposed to 10mM mevalonate (Fig 4.10). These data suggest that *de novo* synthesized cholesterol is not channeled directly into bile acid synthesis.

The addition of HDL to the medium did not result in the production of CDCA (Fig 4.11). The cholesterol content of these cells was stable over the 72h assay (Fig 4.12). These results suggest that HepG2 cells do not utilize HDL-derived cholesterol for bile acid synthesis. HepG2 cells have been shown to take up and degrade HDL (Dashti *et al.*, 1984). The fact that the amount of cholesterol in the cells does not change indicates that the cells

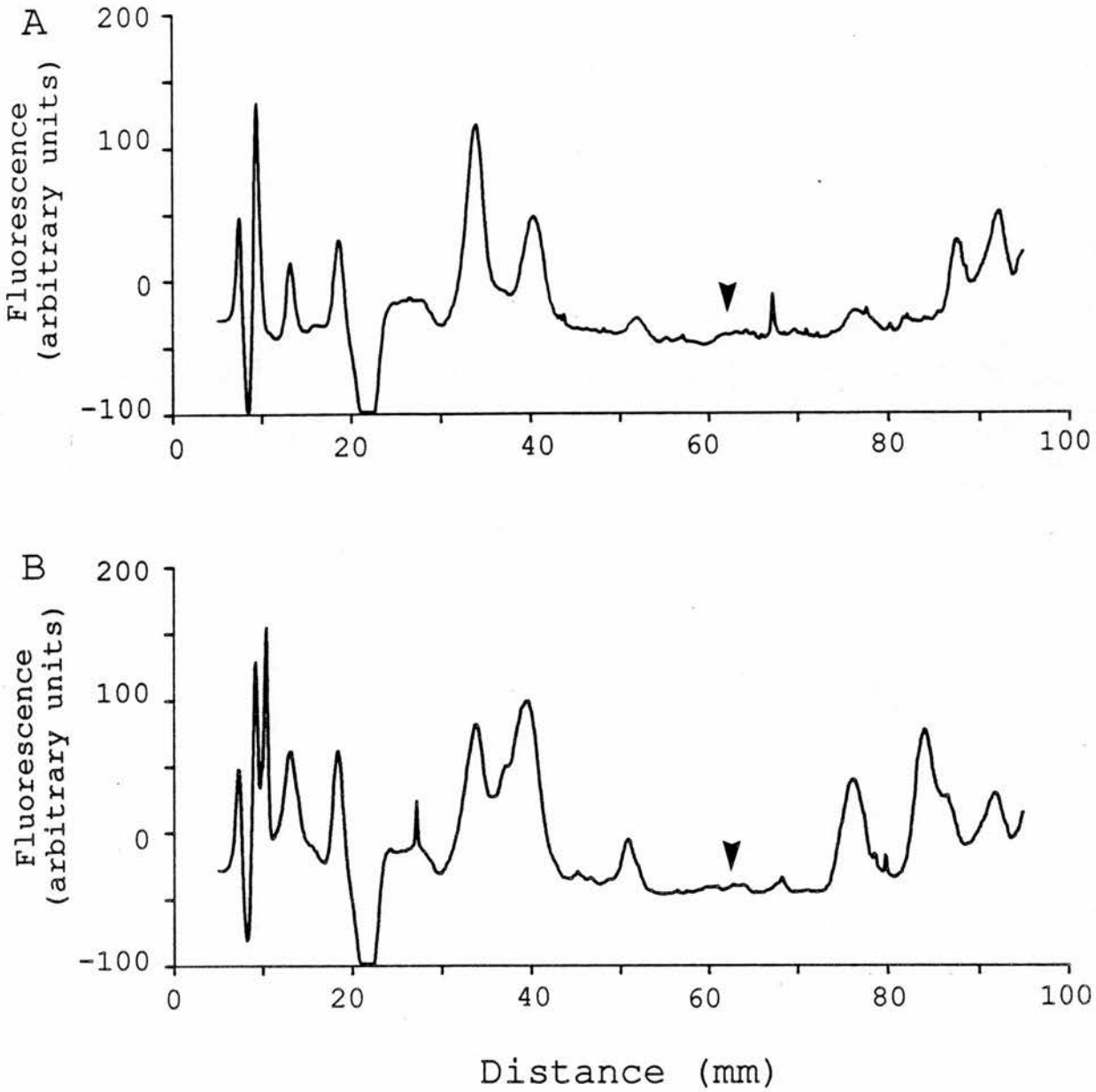


Figure 4.9. HPTLC Analysis of Culture Medium from HepG2 Cells Exposed to Mevalonate.

HepG2 cells were prepared for assay as described earlier. Mevalonic acid lactone was dissolved in water at a concentration of 2M and added to ITS DMEM so that the final concentration ranged between 0 and 10mM. Medium was removed at 24h intervals and cells harvested. Media samples were analyzed for bile acid as described earlier. The HPTLC traces shown are of Bond Elut extracts from medium containing 10mM mevalonate collected at the beginning of the assay (A) and after incubation with cells for 72h (B). The arrow points to the position of the CDCA standard.

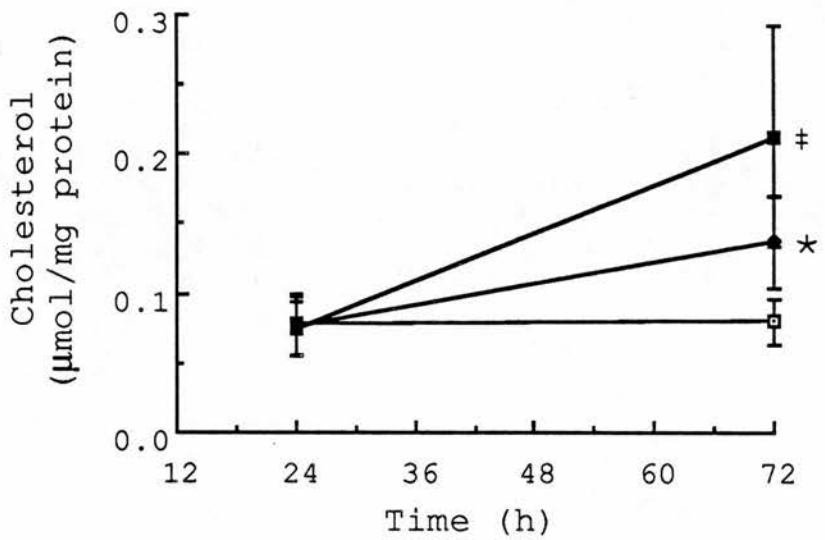


Figure 4.10. Cholesterol Content of HepG2 Cells Exposed to Mevalonate.

HepG2 cells were treated as described in Fig 35. Cells were lysed by 3 x 5sec bursts on a sonicator. Cholesterol was determined using the Ames cholesterol kit as described in Materials and Methods. Culture medium originally contained 0mM (-□-), 5mM (-◆-), and 10mM (-■-) mevalonate. Points are the mean ± s.d. of three independent experiments each performed in triplicate. *$p<0.005$ ‡$p<0.001$

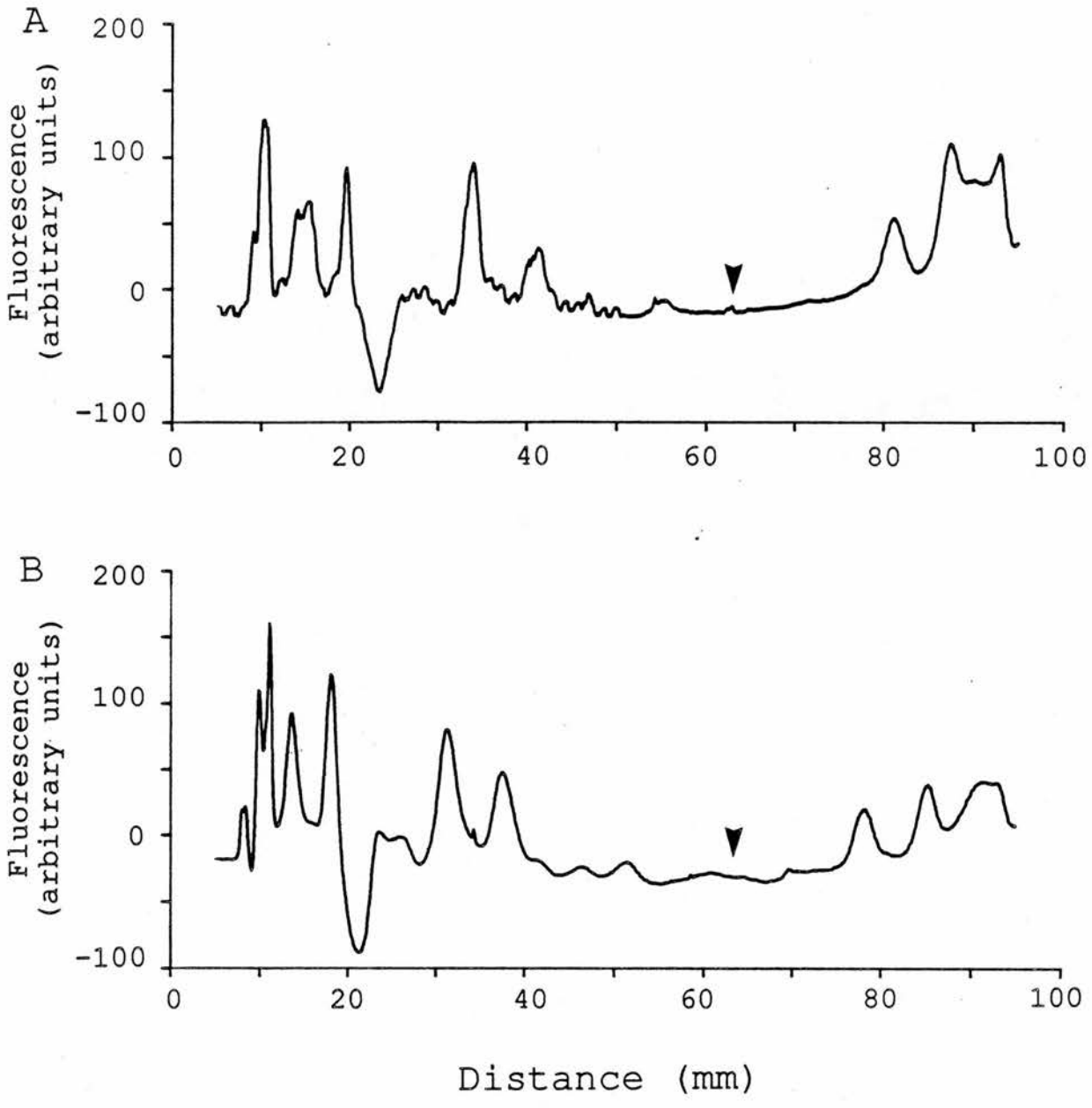


Figure 4.11. HPTLC Analysis of Culture Medium from HepG2 Cells Exposed to HDL.

HepG2 cells were prepared for assay as described earlier. Lipoproteins were isolated from human plasma by fractional ultracentrifugation. HDL was added to ITS DMEM to a final concentration of 300µg protein/ml. Medium was removed at 24h intervals and cells harvested. Media samples were analyzed for bile acid as described earlier. The HPTLC traces shown are of C18 Bond Elut extracts from media collected at the beginning of the assay (A) and after incubation with cells for 72h (B). The arrow points to the position of the CDCA standard.

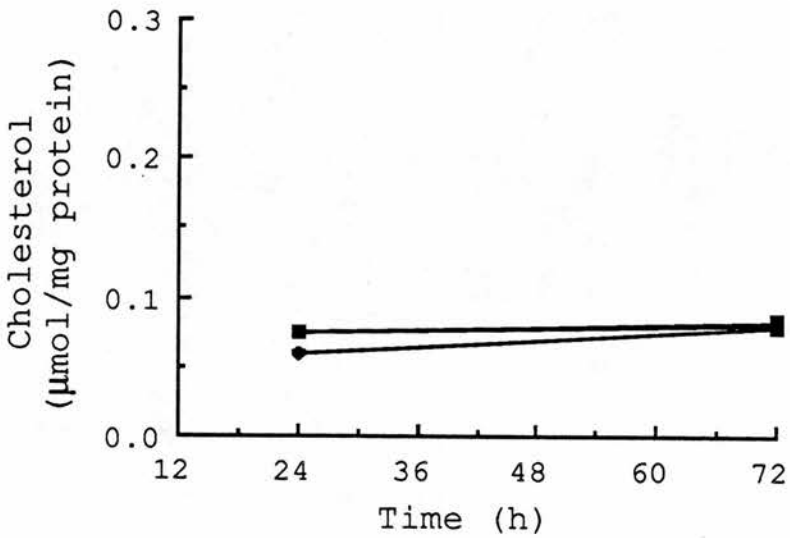


Figure 4.12. Cholesterol Content of HepG2 Cells Exposed to Lipoproteins.

HepG2 cells were treated as described in Fig 4.11 and 4.13. Cells were lysed by 3 x 5sec bursts on a sonicator. Cholesterol was determined using the Ames cholesterol kit as described in Materials and Methods. Culture medium originally contained no lipoprotein (⊟), 300µg/ml HDL (◆), or 300µg/ml LDL (■). Points are the mean ± range of duplicate determinations.

actively maintain homeostasis by either secreting cholesterol or stopping cholesterol production.

In contrast, the addition of LDL to HepG2 cells did result in bile acid production (Fig 4.13). When bile acid synthesis was stimulated by LDL, the area corresponding to the CDCA standards was seen to contain a doublet. Both peaks were combined during the HPTLC analysis because 1) the peaks were inseparable with different mobile phases and 2) they varied equally with time. Synthesis was seen to triple with time up to 72h (Fig 4.14A). The addition of the ACAT inhibitor 58-035 alone did not result in bile acid production. However, when 58-035 was included with LDL, an increase in synthesis above that of LDL alone was seen (Fig 4.14B). As with control cells and those exposed to HDL, the cholesterol content of HepG2 cells exposed to LDL remained steady over 72h (Fig 4.12).

4.5 Summary

To sum up, previous reports indicating that *de novo* synthesized cholesterol is the favored source of substrate for cholesterol 7α-hydroxylase have used rat models. The data presented here do not agree with those findings. The addition of mevalonate was not shown to enhance CDCA synthesis above basal levels. The addition of HDL was also not shown to increase CDCA synthesis. Cholesterol derived from LDL however, was found to increase CDCA synthesis. During the course of this work, Kempen *et al.* (1988) reported that the addition of LDL but not HDL amplified bile acid synthesis in HepG2 cells. The data recorded here are in agreement with this report.

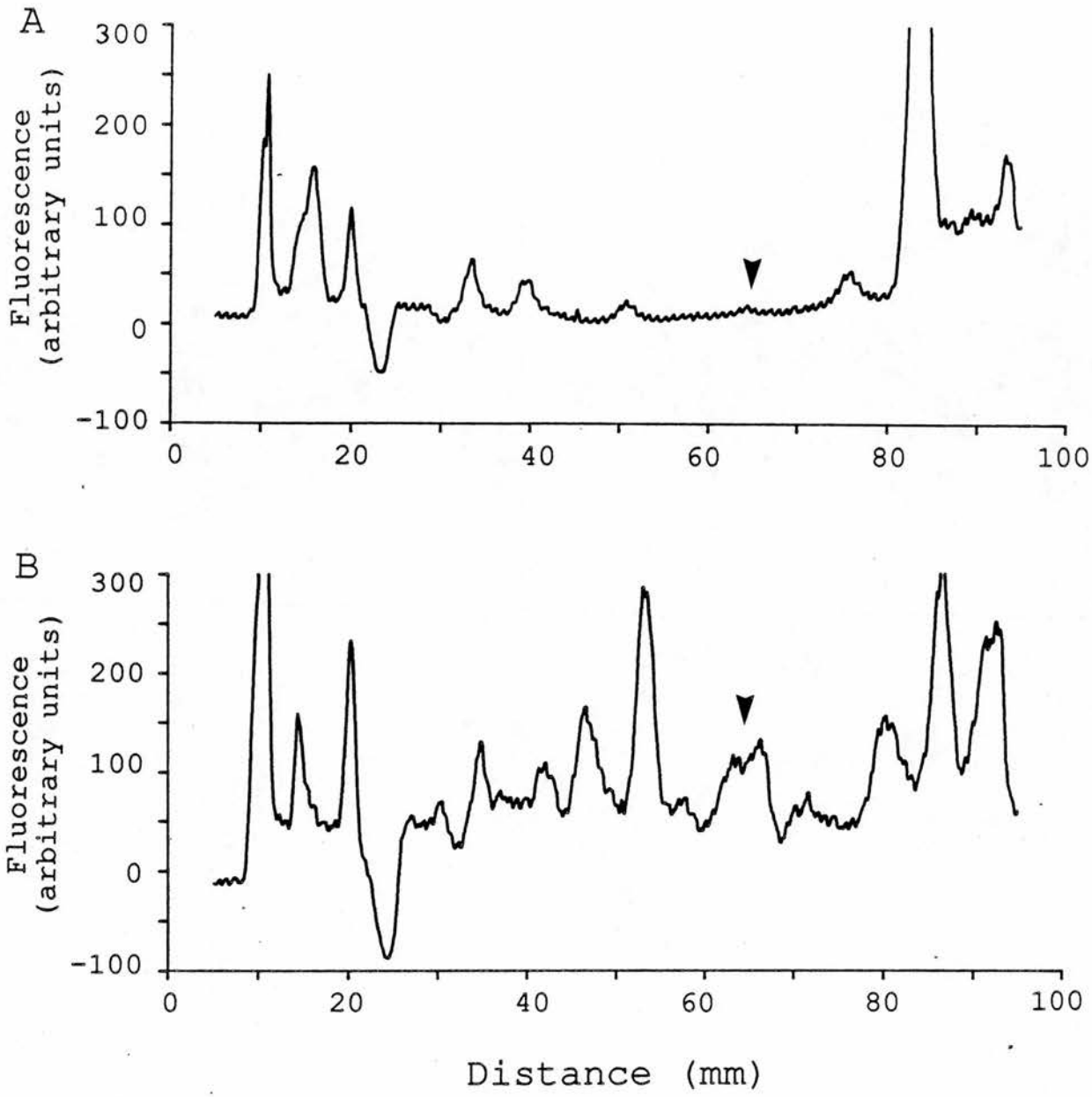


Figure 4.13. HPTLC Analysis of Culture Medium from HepG2 Cells Exposed to LDL.

HepG2 cells were prepared for assay as described earlier. Lipoproteins were isolated from human plasma by fractional ultracentrifugation. LDL was added to ITS DMEM to a final concentration of 300µg protein/ml. 58-035 was dissolved in DMSO at a concentration of 10mg/ml and added to ITS DMEM at a concentration of 5µg/ml. Medium was removed at 24h intervals and cells harvested. Media samples were analyzed for bile acid as described earlier. The HPTLC traces shown are of C18 Bond Elut extracts from media collected at the beginning of the assay (A) and after incubation with cells for 72h (B). The arrow points to the position of the CDCA standard.

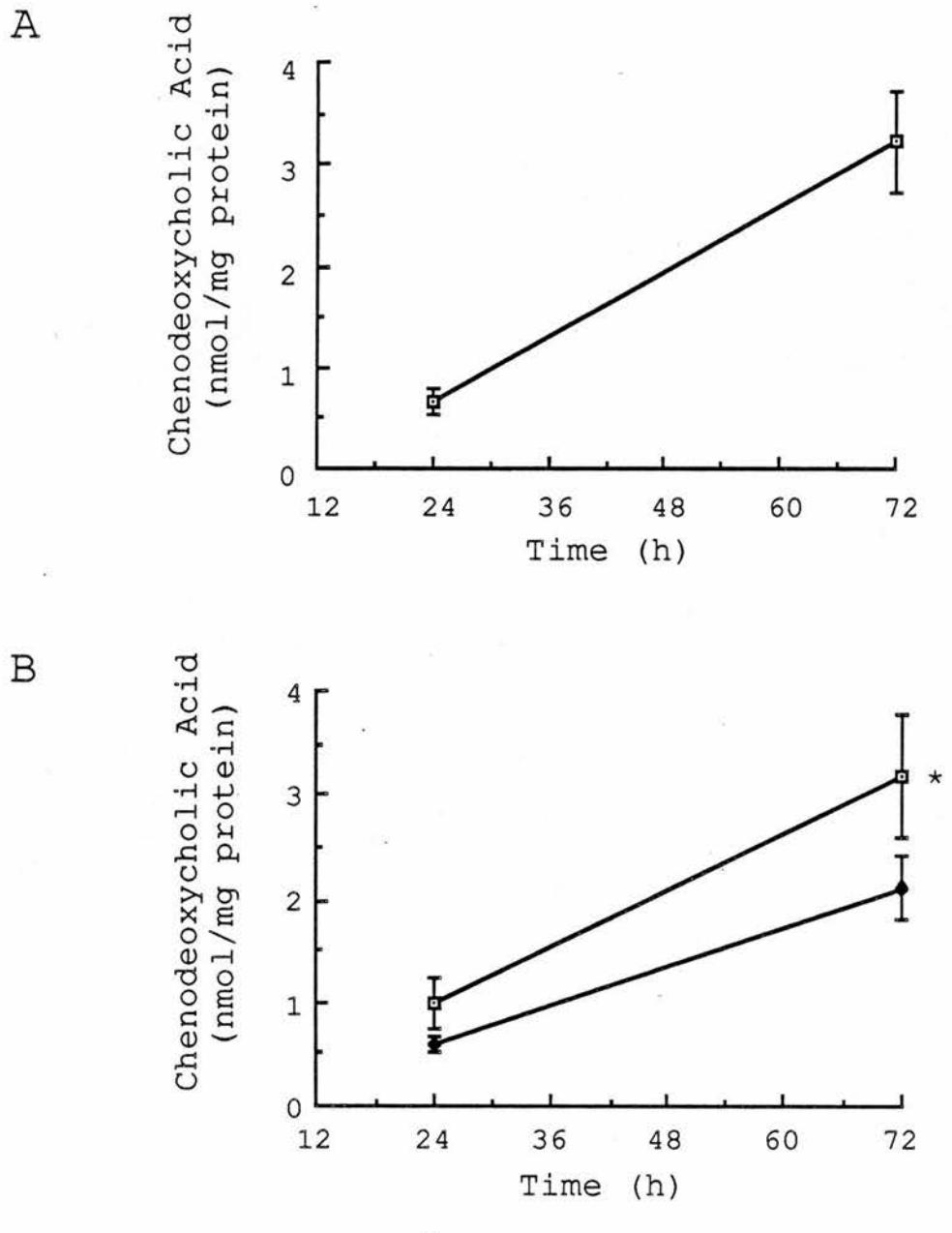


Figure 4.14. CDCA Production from HepG2 Cells Treated with LDL and 58-035.

HepG2 cells were assayed as described in Fig 4.13. Bile acids were isolated from culture media and fractionated on HPTLC as described earlier. Culture medium originally contained (A) 300µg/ml LDL and (B) 0.05% DMSO (◆) and 5µg/ml 58-035 (⊡). Points represent the mean ± s.d. of triplicate determinations. *$p<0.050$

Chapter 5

Discussion

In order to consider the two distinct themes concerning bile acid synthesis contained within this thesis, the discussion has been divided into two separate sections.

5.1 The Characterization of Cholesterol 7α-hydroxylase

5.1.a Anti-PB2a Antibody

The identification of a polyclonal anti-P450 antibody, anti-PB2a, that inhibits cholesterol 7α-hydroxylase activity in rat liver microsomes is reported in chapter 3. This anti-PB2a antibody, known as antiserum 34, was shown to recognize a protein band on Western blots that was associated with 7α-hydroxylase activity. Based on these results, it was proposed that PB2a and cholesterol 7α-hydroxylase shared some structural similarity. Other polyclonal anti-PB2a antibodies, 27 and 86, did not inhibit 7α-hydroxylase activity, but did recognize the same band on Western blots. A fourth anti-PB2a antibody, 31, neither recognized the band nor inhibited the activity.

Different antibody cross-reactivities against the same antigen are not unprecedented. Waxman found that his anti-PB1 (Wolf PB1a) antibody cross-reacted with his PB2c (Wolf PB2a) (Waxman, 1984). In contrast, Guengerich and coworkers found no cross-reactivity between antibodies raised against these proteins (Guengerich *et al.*, 1982). Thus, the differences in cross-reactivities of the various anti-PB2a antibodies reported here may simply be due to variations in the specificity of the different preparations. These differences may also

be indicative of a relatively low degree of structural homology between PB2a and cholesterol 7α-hydroxylase.

The polyclonal antibodies used in this study undoubtedly recognized more than one epitope on PB2a. However, the reversal of inhibition observed when purified PB2a was included in the antibody inhibition experiments demonstrates that both PB2a and cholesterol 7α-hydroxylase compete for recogition by the inhibitory IgG. This competitive binding indicates that they do share a common antigenic site or sites.

Similarities between distinct P450s is also not unprecedented. Boobis *et al.* (1985) reported the generation of a monoclonal antibody raised against a partially purified P450 that cross-reacted with P450 isozymes from different P450 families as well as other heme proteins like cytochrome c. Such cross-reactivity indicates that different P450s share common epitopes. Nevertheless, meaningful data can be obtained through the use of these antibodies.

The work presented here demonstrates that cholesterol 7α-hydroxylase activity does not vary markedly between the two sexes. The fact that the anti-PB2a antibody inhibited activity in both male and female rats also indicates that the enzyme responsible for this activity is conserved across sexes. The demonstration that the anti-PB2a antibody also inhibited activity in the mouse and hamster in addition to the rat indicates that cholesterol 7α-hydroxylase is also conserved across species. Other anti-P450 antibodies have been shown to recognize isozymes from different species. Boobis *et al.* (1985) demonstrated that an antibody raised against a rat P450 could cross-react with P450s from rabbit and human. Cheng *et al.* (1986) used a

monoclonal antibody to immunopurify P450s from guinea pig, mouse, and rat.

5.1.b Comparison of Cholesterol 7α-hydroxylase and PB2a

Having established a link between cholesterol 7α-hydroxylase and PB2a, it is now important to compare the two proteins. PB2a has been purified by several groups, the nomenclature being summarized in Table 3.3. PB2a has a unique amino-terminus (Waxman, 1984; Wolf *et al.*, 1986) and has been shown to be immunologically distinct from several other P450s (Waxman, 1984; Ryan *et al.*, 1984). Estimates of its molecular weight range between 50000 and 54000.

Unlike cholesterol 7α-hydroxylase, which expresses strict substrate specificity for cholesterol, PB2a has been shown to catalyze the hydroxylation of many substrates, expressing greater specificity toward steroids than xenobiotics (Guengerich, 1987). It is, perhaps, best known for its activity toward testosterone, which it hydroxylates in the 16α and 2α positions. Although there are four P450s that catalyze this reaction (Åström & DePierre, 1986), PB2a has been shown to be responsible for up to 85% of this activity in microsomes from untreated animals (Waxman *et al.*, 1987). 2α and 16α-hydroxytestosterone are the major metabolites produced upon the incubation of microsomes with testosterone (van der Hoeven, 1981). However, their function is unclear. It has been suggested that the physiological significance of PB2a is to regulate circulating levels of androgens by catalyzing the formation of less active, more easily eliminated products (Waxman, 1984). However, there have been no reports that PB2a expresses any activity toward the oxidation of the sterol nucleus in the 7α position, an activity associated with

members of the P450IIA subfamily. Indeed, experiments performed here found no evidence that purified PB2a expressed catalytic activity toward cholesterol.

PB2a was orginally purified from the livers of mature male rats (Cheng & Schenkman, 1982; Kamataki *et al.*, 1983; Ryan *et al.*, 1984), but was not detected in the livers of immature males (Waxman *et al.*, 1985) or mature females (Kamataki *et al.*, 1983; Waxman, 1984; Morgan *et al.*, 1985a). It therefore appeared to be a sexually dimorphic P450. Using gonadalectomized males and females, it became clear that the expression of PB2a was dependent upon the neonatal imprinting of androgens (Kamataki *et al.*, 1983; Morgan *et al.*, 1985a, 1985b; Waxman *et al.*, 1985). In contrast, cholesterol 7α-hydroxylase is neither sexually dimorphic nor developmentally regulated (see section 1.5.c)

It was important to demonstrate that the 7α-hydroxylation of cholesterol was not related to the expression of PB2a in order to prove that the two enzymes were indeed individual forms of cytochrome P450. Interestingly, the pituitary regulates the activity of both cholesterol 7α-hydroxylase and PB2a. Hypophysectomy has been shown to abolish PB2a expression in male rats, indicating that expression is maintained by the pituitary directed hormones (Morgan *et al.*, 1985b). In those studies, and also in studies with females, PB2a expression was found to be masculinized by episodic exposure to growth hormone and feminized by continous infusion of growth hormone (Morgan *et al.*, 1985b). The pituitary is also thought to regulate the diurnal rhythm of cholesterol 7α-hydroxylase. Hypophysectomy disrupts this rhythm and decreases the activity of cholesterol 7α-hydroxylase (Mayer, 1976). Experiments performed here show that the expression of PB2a and the activity of cholesterol 7α-hydroxylase do not vary in

parallel in hypophysectomized male rats. The enzymes must therefore be distinct forms of P450s.

5.1.c Comparison of Cholesterol 7α-hydroxylase and Other Cytochromes P450 belonging to the P450IIC Gene Family

The P450IIC gene family, which encodes PB2a, is very extensive and there exist a variety of other members which could express 7α-hydroxylase activity. All the antibody inhibition studies carried out here were performed on female rats. It is therefore of interest to be aware of the female counterpart to PB2a, P450-female, which also belongs to the P450IIC gene subfamily. This female homologue was discovered during the attempts at purifying PB2a from mature female rats (Kamataki *et al.*, 1983; Waxman, 1984; Morgan *et al.*, 1985a). It has several properties in common with PB2a: 1) it has similar chromatographic characteristics (Kamataki *et al.*, 1983; Waxman 1984, Waxman *et al.*, 1985; Morgan *et al.*, 1985a) 2) it exhibits antibody cross-reactivity with PB2a (Kamataki *et al.*, 1983; MacGeoch *et al.*, 1984) 3) it has similar chymotryptic maps to PB2a (Waxman *et al.*, 1985). The genes for both PB2a and P450-female have recently been cloned and sequenced, showing a high degree of homology (66%) (Zaphiropoulos *et al.*, 1988). In contrast to PB2a, P450-female has very differrent catalytic capabilities. P450-female catalyzes the 15β-hydroxylation of water soluble steroid sufates and has no 16α hydroxylating activity (MacGeoch *et al.*, 1984). Its expression is regulated in an opposite fashion to PB2a. P450-female is controlled by the continous exposure to growth hormone and expression is abolished by exposure to testosterone (MacGeoch *et al.*, 1984; Waxman *et al.*, 1985). P450-female has not been reported to hydroxylate

cholesterol. In view of the fact that P450-female is also a sexually dimorphic P450, it, too, is an unlikely candidate for the 7α-hydroxylation of cholesterol.

Interestingly, like cholesterol 7α-hydroxylase, members of the P450IIC gene subfamily are constitutively expressed P450s (Zaphiropoulos *et al.*, 1989). PB2a is not induced by treatment with xenobiotics (Guengerich *et al.*, 1982; Waxman, 1984). Yeowell *et al.* (1987) have demonstrated that the expression of PB2a mRNA is suppressed by the treatment of rats with 3-methylcholanthrene and hexachlorobiphenyl. As summarized in section 1.5.d, there has been much confusion in the literature about the inducibility of cholesterol 7α-hydroxylase by xenobiotics. The inconsistencies seem to arise from the many different assay systems used as well as differing regimes for dosing animals. Indeed, very little work has been done to directly compare the effects of the different classes of xenobiotics on 7α-hydroxylase activity. The most complete study published recently was carried out by Rush and Fears (1986). They examined the effects of polycyclic aromatic hydrocarbons, phenobarbital, and pregnenolone 16α-carbonitrile on cholesterol 7α-hydroxylase activity. The results presented here agree with the general trend they reported: 1) the administration of phenobarbital decreased cholesterol 7α-hydroxylase activity 2) treatment with isosafrole did not affect activity 3) treatment of rats with SKF-525A slightly increased activity. The activity of an induced enzyme is dramatically increased, often up to 10-20 fold. The lack of induction of cholesterol 7α-hydroxylase activity in response to any of these drugs indicates that 7α-hydroxylase does not belong to the cytochrome P450s involved in xenobiotic metabolism (P450 gene

families I, IIA, III, and IV). This conclusion supports the hypothesis that enzymes involved in endobiotic metabolism, like the synthesis of bile acids, belong to the house-keeping P450s (Nebert & Gonzalez, 1987). The fact that 7α-hydroxylase activity is markedly increased by treatment of rats with cholestyramine does not contradict this statement. Cholestyramine interferes with endogenous control mechanisms by interrupting the enterohepatic circulation. The resulting increase in 7α-hydroxylase activity is in keeping with house-keeping regulatory mechanisms.

Cholesterol 7α-hydroxylase is thought to be a house-keeping P450 because it synthesizes bile acids, necessary in the digestion of fats. PB2a also shares some characteristics with another protein thought to be a house-keeping P450, microsomal vitamin D3 hydroxylase: 1) Both are expressed only in mature males (Andersson *et al.*, 1983) 2) Vitamin D3 hydroxylase also exhibits constitutive expression that is not enhanced by treatment with xenobiotics (Andersson *et al.*, 1983) 3) purified vitamin D3 hydroxylase was shown to 16α-hydroxylate testosterone as well as hydroxylate both vitamin D3 and 1α-hydroxyvitamin D3 in position 25 (Andersson *et al.*, 1983) 4) 16α hydroxylase and vitamin D3 hydroxylase activities were found to be immunologically indistinguishable (Hayashi *et al.*, 1988a; Andersson *et al.*, 1986) 5) PB2a and vitamin D3 hydroxylase were found to have matching amino-terminal sequences (Andersson *et al.*, 1986).

Unlike PB2a, vitamin D3 hydroxylase has a known physiological function. It hydroxylates vitamin D3, 1α-hydroxyvitamin D3, and 5β-cholestane-3α,7α,12α-triol (Andersson *et al.*, 1983). It is therefore responsible for the bioactivation of vitamin D3, which is an anti-

rachitic factor and functions as a hormone to control calcium homeostasis in vertebrates (Matsumoto *et al.*, 1988). A role for it in the formation of bile acids has been suggested (Swell *et al.*, 1980). It seems unlikely that such a vital enzyme would be sexually differentiated. More recent work has indicated that microsomal vitamin D3 hydroxylase and PB2a arise from different genes and are thus distinct forms of cytochrome P450 (Hayashi *et al.*, 1988a). The recent demonstration that female rat liver microsomes contain a species of vitamin D3 hydroxylase immunochemically distinct from the male species indicates that this enzyme is not sexually dimorphic (Hayashi *et al.*, 1988b). There also exists a mitochondrial vitamin D3 hydroxylase, which, unlike its microsomal counterpart, does not show any activity toward foreign compound metabolism and appears to be more important in the conversion of vitamin D3 to its active form (Matsumoto *et al.*, 1988).

5.1.d Phosphorylation of Cholesterol 7α-hydroxylase

PB2a can be phosphorylated by a Ca^{2+}-phospholipid dependent kinase, but not by the cAMP-dependent kinase (Pyerin *et al.*, 1987). The effect of phosphorylation on activity, however, has not been demonstrated. There is some evidence to suggest that cholesterol 7α-hydroxylase is activated by phosphorylation (Sanghvi et al., 1981, Goodwin *et al.*, 1982). Initial reports showed that a crude cytosolic fraction enhanced activity in the presence of ATP and Mg^{2+} (Sanghvi *et al.*, 1981; Kwok *et al.*, 1981a). In one instance, the cytosolic activator was reported to be heat-stable (Kwok *et al.*, 1981a), which is not charactaristic of a protein kinase. Activity was subsequently demonstrated to be inhibited by ATP (Kwok *et al.*, 1981b) and bivalent

cations like Mg^{2+} (Sanghvi *et al.*, 1983). These latter effects were attributed to the ion activation of an endogenous microsomal phosphatase. NaF, a phosphatase inhibitor, has been reported not to affect (Danielsson *et al.*, 1980; Berglund *et al.*, 1986) and to enhance (Kwok *et al.*, 1981a; Goodwin *et al.*, 1982) cholesterol 7α-hydroxylase activity.

Goodwin *et al.* (1982) were the first to demonstrate that cholesterol 7α- hydroxylase could be activated *in vitro* by a specific kinase. They showed that cAMP-dependent kinase stimulated activity 22%. Tang and Chiang (1986) subsequently reported that cAMP-dependent kinase also activated purified cholesterol 7α-hydroxylase. This was the first report that a kinase activated a P450 in a reconstituted monooxygenase system. Previous work had shown that phosphorylation of P450s resulted in inactivation (Taniguchi *et al.*, 1985). In fact, Scallen and Sanghvi (1985) have proposed that there is an integrated response to phosphorylation by the enzymes directly involved in cholesterol metabolism (see Fig 1.4).

In recent years, however, there have been some questions raised as to the relevance of phosphorylation as a control mechanism for cholesterol 7α-hydroxylase (Lidström-Olsson, 1985; Berglund, *et al.*, 1986). The data presented indicate that cholesterol 7α-hydroxylase is not phosphorylated under the conditions used here. These experiments used three purified protein kinases: AMP-activated protein kinase because it is known to interact with HMG-CoA reductase (Carling *et al.*, 1987), cAMP-dependent protein kinase because it has been reported to enhance cholesterol 7α-hydroxylase activity (Goodwin *et al.*, 1982), and protein kinase C because its activity is dependent upon Ca^{2+}, which influences bile acid synthesis in isolated rat

hepatocytes (Botham, 1987). The selective decrease in HMG-CoA reductase activity by AMP-activated protein kinse and the incorporation of ^{32}P into microsomal proteins indicates that a degree of phosphorylation was attained. The lack of effect on cholesterol 7α-hydroxylase activity by these kinases would suggest that they do not interact directly and specifically with this enzyme.

Some experiments supporting the role of phosphorylation in the activation of cholesterol 7α-hydroxylase has been carried out in isolated rat hepatocytes. Hepatocytes treated with dibutyryl cAMP and glucagon were seen to increase bile acid output (Botham & Boyd 1983; Botham *et al.*, 1984). Both compounds are known to increase the intracellular level of cAMP, which stimulates cAMP-dependent protein kinase. The stimulation of activity caused by dibutyryl cAMP has been shown to be dependent upon the mobilization of Ca^{2+} from intracellular stores (Botham, 1987). All experiments were performed on hepatocytes from rats maintained on a soft diet. Increases in bile acid synthesis were not found in hepatocytes from cholestyramine-fed rats, suggesting that under these conditions, proteins were already phosphorylated (Botham & Boyd, 1983).

The precise mechanism by which cholestyramine induces cholesterol 7α-hydroxylase activity in unknown. The resin binds bile acids in the gut, removing them from the enterohepatic circulation and relieving the feedback inhibition on bile acid synthesis. The resulting increase in 7α-hydroxylase activity has been associated with protein synthesis (Brown & Boyd, 1974). Indeed, Western blot analysis of microsomes indicated that a protein band varied in intensity with cholesterol 7α-hydroxylase activity (see section 3.8). This was interpreted to mean that 7α-hydroxylase expression was

elevated in response to cholestyramine. Cholesterol 7α-hydroxylase activity in microsomes from rats maintained on the soft diet was often too low to detect. In order to maximize the response in phosphorylation assays, rats were fed a 4% (w/w) cholestyramine diet.

Microsomes were treated with phosphatases before treatment with kinases, so phosphate groups should have been cleaved from proteins. HMG-CoA reductase did respond to phosphorylation by AMP-dependent kinase, indicating that it was dephosphorylated prior to the assay. The fact that cholesterol 7α-hydroxylase from cholestyramine-fed animals did not respond to phosphorylation is therefore puzzling. If the increase in 7α-hydroxylase activity in response to cholestyramine is due a change in the phosphorylation state of the protein, then treatment of the microsomes with phosphatases should decrease this activity. Subsequent phosphorylation would result in an increase in activity as demonstrated by Goodwin *et al.* (1982). This does not appear to be the case here.

In order to demonstrate that the phosphorylation of a protein has physiological significance, it is necessary to show that an extracellular signal associated with kinase activity produces a change in activity *in vivo*. That effect must be demonstrated *in vitro* at a rate consistent with the effect *in vivo*. The site of phosphorylation must be proven to be the same both *in vivo* and *in vitro* (Hardie & Guy, 1982). To prove that a protein is phosphorylated *in vivo* is a difficult task. The phosphorylation of proteins involved in lipid metabolism has been shown to be of physiological significance (reviewed by Hardie et al., 1989). As indicated in section 1.5.e, acetyl-CoA carboxylase and HMG-CoA reductase are both inactivated *in vitro* by phosphorylation with AMP-

activated protein kinase. Direct sequencing of acetyl-CoA carboxylase has shown that it is phosphorylated at the AMP-activated protein kinase site in isolated hepatocytes treated with glucagon. The activation of AMP-activated protein kinase by long chain acyl-CoA esters suggests that the feedback mechanism which down-regulates acetyl-CoA carboxylase acts via the kinase. Measurement of the expressed:total activity ratio of HMG-CoA reductase also suggests that it is phosphorylated by this AMP-activated protein kinase both *in vivo* and in isolated hepatocytes treated with glucagon. The activation of one kinase (AMP-activated protein kinase) that controls two distinct enzymes involved in lipid metabolism (acetyl-CoA carboxylase and HMG-CoA reductase) is prudent because it prevents the shunting of substrate (acetyl-CoA) directly from one pathway to another. Botham and coworkers have shown that glucagon increases the synthesis of bile acids in isolated hepatocytes. They attributed this increase to an increase in cholesterol 7α-hydroxylase activity (Botham & Boyd, 1983). However, decisive evidence that cholesterol 7α-hydroxylase is phosphorylated *in vitro* has not yet been obtained.

The role of phosphorylation is to allow an extracellular signal to modulate or even override the intracellular control system (Hardie & Guy, 1982). If the treatment of animals with bile acid sequestrants so greatly enhances 7α-hydroxylase activity, then perhaps activity cannot be increased over that level by phosphorylation. This would explain why there is no increase in bile acid synthesis when isolated hepatocytes from animals maintained on a cholestyramine diet are stimulated with glucagon. However, it does not explain why activity is not increased by the phosphorylation of phosphatase-treated microsomes.

5.1.e Immunoprecipitation and Amino-terminal Analysis

A powerful technique that has developed from the use of antibodies is the immunoprecipitation of antigens (Davies & Brown, 1987). This technique can be used whether the epitope recognized by the antibody is in its native state on the surface of the protein or denatured, solubilized by detergents. The antigen can be isolated quickly and from small amounts of tissue. The resulting purified protein can then be further characterized. P450s, unfortunately, cannot be purified in their active state (Gelboin & Friedman, 1985), but they can be amino-terminal sequenced (Cheng *et al.*, 1986; Brown *et al.*, 1987).

Modifying this technique, antiserum 34 was shown to immunoprecipitate the band that corresponded to cholesterol 7α-hydroxylase activity. The amino-terminal sequence of the immunoprecipitated protein is distinct from the amino-termini of PB2a (Wolf *et al.*, 1986) and P450-female (Zaphiropoulos *et al.*, 1988) (Table 5.1A). It is also not homologous with the previously published sequence of cholesterol 7α-hydroxylase (Ogishima *et al.*, 1987). The amino-terminal sequence does not match any amino-terminal sequences contained within the National Biomedical Research Foundation protofile version January 1989 (accessed via the Protein Information Resource Package at SERC Daresbury, Warrington, England).

The amino-terminal sequence of the immunoprecipitated protein was also compared with amino-terminal sequences of several cytochromes P450 (Table 5.1A and B). Although cholesterol 7α-hydroxylase activity is markedly inhibited by an anti-PB2a antibody, the amino terminus of the immunoprecipitated protein bears little resemblance to members of the P450IIC gene family (Table 5.1B).

Table 5.1A Comparison of the Amino-terminal Sequence of the Immunoprecipitated Protein with Rat Cytochromes P450.

Immunoprecipitated protein		X K L L V I P I D G S(T)X L X
Family/ Subfamily	**P450**	**Sequence*** 1 5 10 15
IA1	MC1b	P S V Y G F P A F T S A T E L
IA2	MC1a	A S F G Y I S L A P E L L L A
IIA1	UT1	M L D T G L L L V V I I A S L
IIB1	PB3a	M L P S I L L L L A L L V G F
IIB2	PB3b	M L P S I L L L L A L L V G F
IIC6	PB1a	M D L V M L L V L T L T X L I
IIC7	f	M D L V T F L V L T L S S L I
IIC11	g	M D P V V V L L L S L F F L L
IIC12	PB2a	M D P V L V L V L T L S S L L
IIC13	P450-female	M D P F V V L V L S L S F L L
IIIA1	PCN-E	M D L L S A L T L E T W V L L
	PB2b	M K L L V A L P L E T W V L L
IV	Clo	M S V S A L S S T R F T G S I
		(I)
Cholesterol 7α-hydroxylase		M F E V S L

*Sequences from references listed in Nebert *et al.* (1989)

Table 5.1B Comparison of the Amino-terminal Sequence of the Immunoprecipitated Protein with Other Members of the P450IIC and P450IIIA Gene Families.

Immunoprecipitated protein		X K L L V I P I D G S(T)X L X
Family/ Subfamily	**P450**	**Sequence*** 1 5 10 15
IIC1	rabbit PBc1	L S C L L
IIC2	rabbit PBc2	M D L V V V L G L C L S C L L
IIC3	rabbit 3b	M D L L I I L G I C L S C V V
IIC4	rabbit 1-88	M D P V A G L V L G L C C L L
IIC5	rabbit 1	M D P V V V L V L G L C C L L
IIC8	human IIC2	M D S I V S L V C L S C L L L
IIC9	human IIC1	M E P F V V L V C L S F M L L
IIC10	human MP8	M D S L V V L V L C L S C L L
IIC14	rabbit pHP3	M D P V V V L V L C L S C L L
	human MP3	M E P F V V L V L L S F M L L
IIIA3	human HLp	M A L I P D L A M Q T W L L L
IIIA4	human nf	M A L I P D L A M E T W L L L
IIIA6	Rabbit 3c	M D L I F S L E T W V L L A A

*Sequences from references listed in Nebert *et al.* (1989)

There is some homology with PB2b, a member of the P450 IIIA gene family. However, it should be pointed out that the global homology found between similar cytochromes P450 is not necessarily reflected in sequence homology at the amino-terminus. For example, two P450s from the P450IIB gene family, human IIB6 and mouse testosterone 15α-hydroxylase, exhibit 83% sequence homology overall, but only 40% sequence homology at the amino-terminus (Miles *et al.*, 1989). The amino-terminal sequence may not be as highly conserved as other regions of the P450 protein because constraints for the membrane bound region are not critical. Several hydrophobic residues may be all that is required to anchor the protein into the membrane.

5.1.f Summary

This section of the discussion has been concerned with the structural similarities of cholesterol 7α-hydroxylase and PB2a. However, structural homology between cytochromes P450 does not necessarily reflect functional homology. By examining the characteristics of P450-female and the male microsomal vitamin D3 hydroxylase, it can be seen that these P450s, while exhibiting sequence and structural homology with PB2a, have distinct functions. It is therefore quite feasible to propose that PB2a and cholesterol 7α-hydroxylase, while expressing disparate functions, have common antigenic sites. The protein sequence data shows that the immunoprecipitated protein, which is associated with cholesterol 7α-hydroxylase activity, has a distinct amino terminus. However, based on the present knowledge, the immunoprecipitated protein can neither be positively identified as cholesterol 7α-hydroxylase nor classified into a P450 gene family.

There do exist similarities between the PB2a and cholesterol 7α-hydroxylase. Both are constitutively expressed and do not appear to be induced by xenobiotics. They also may both be modulated by phosphorylation. There has been direct chemical evidence that PB2a is phosphorylated (Pyerin et al., 1987). However, a definitive answer to whether cholesterol 7α-hydroxylase is a phosphorylatable protein requires chemical evidence that the purified enzyme is phosphorylated.

5.2 Bile Acid Synthesis by HepG2 Cells.

The literature reports of bile acid production by HepG2 cells are in complete disagreement (Table 5.2). At the outset of this work, scant information was available concerning bile acid synthesis. The first report that HepG2 cells synthesized bile acids was an abstract that identified bile acids by their Rf on a tlc plate in reference to known standards (Craig & Cooper, 1985). Bile acid synthesis was quantified solely through the incorporation of [^{3}H]mevalonate. This report did not give total bile acid synthesis of HepG2 cells and did not address any regulatory mechanisms influencing the size of the bile acid pool. While work presented here was in progress, other reports were published concerning bile acid production by HepG2 cells.

Everson and Polokoff (1986) analyzed bile acids synthesized and secreted by HepG2 cells with the highly specific method of gas chromatography-mass spectrometry. The cells were seen to secrete predominantly unconjugated CDCA, but also the bile acid precursors THCA and DHCA (Fig 4.1). Unconjugated CA was reported to be secreted in very minor amounts. Less than 1% of the bile acids remained in

Table 5.2 Summary of Bile Acid Production in HepG2 cells.

	Craig & Cooper (1985)	Everson & Polokoff (1986)	Stephan et al. (1987)	Kempen et al. (1988)
bile acid	nmol/mg/day	nmol/mg/day	nmol/mg/day	nmol/mg/day
CDCA	0.42	2.17	--	0.125
GCDC	0.59	--	--	--
TCDC	0.30	--	--	--
DHCA	--	0.07	--	--
CA	1.74	0.05	--	0.130
GCA	0.59	--	--	--
TCA	0.13	--	--	--
THCA	--	0.46	--	--
TOTAL	3.77	2.75	268	0.255
method	tlc	gc-ms	HPLC	RIA

the cells. Using [7β-^{2}H]7α-hydroxycholesterol, they showed that CDCA, DHCA, and THCA, but not CA, were formed from this compound as an intermediate during synthesis. It was suggested that CA was derived from an alternative bile acid synthetic pathway.

In a study examining LDL binding, Stephan *et al.* (1987) showed that total bile acid synthesis was enhanced by the addition of taurine to HepG2 cells. The stimulation of LDL binding caused by the addition of taurine to the cells was seen to be dependent upon bile acid production by these cells. Control fibroblasts did not show an increase in LDL binding when exposed to taurine. The addition of taurine did not, however, increase bile acid conjugation. They used an HPLC detection system and reported total bile acid synthesis, but failed to report individual species.

Kempen *et al.* (1988) examined the regulation of bile acid synthesis in HepG2 cells. However, they used RIA kits (GC and GCDC) for bile acid quantification and made assumptions to account for the different species of bile acid that were reported to be synthesized. The kits showed only 50% crossreactivity with the unconjugated forms of bile acid as well as with the precursor bile acids. They assumed that the additive response of both kits would be representative of total bile acid production. Their figures cannot be taken as absolute, but a general trend can be discerned.

The RIA procedure originally used here for the detection of bile acids was discontinued when it became evident that the unconjugated bile acids and the precursor compounds could not be sufficiently measured. Anything less than 100% crossreactivity presents difficulties for quantification and identification. Commercial RIA kits for bile acid detection were investigated, but

manufacturers also reported only 10% cross-reactivity with unconjugates. The lack of crossreactivity with unconjugates is apparently because the peptide bond formed when the unconjugated bile acid is haptenized to the BSA carrier protein is the most immunogenic portion of the molecule. The peptide bond is present only in conjugated bile acids and is the reason why polyclonal antisera can recognize 100% of both taurine and glycine conjugates and only 10% of unconjugates. This lack of cross-reactivity is the reason why the results of Kempen *et al.* can only be used as an indicator for bile acid synthesis.

In spite of this, Kempen *et al.* (1988) estimated total bile acid synthesis to be two-fold lower than that reported by Everson and Polokoff. They attributed the difference to sulfated bile acids found in the tlc fractionation, but not detected by the RIA. They also reported almost equimolar ratios of CDCA and CA being produced, in contrast to the ratios reported by Everson and Polokoff. The addition of LDL to HepG2 cells was shown to double basal levels of bile acid production. HDL, however, did not enhance bile acid synthesis. 58-035, when added with LDL, increased the pool of free cholesterol in the cell, but did not further the increase in bile acid synthesis caused by LDL. Compounds influencing the *de novo* synthesized cholesterol pool, mevalonate and compactin, neither increased nor decreased synthesis.

In work presented here, the HPTLC method was chosen for bile acid detection because it was specific both for the identification and quantification of bile acids. CDCA was chosen as the index of bile acid production because it had been reported to the the major one synthesized (Everson & Polokoff, 1986). No bile acids were

produced under basal conditions. This result is at odds with the the data of others (Table 5.2). However, the literature reports vary in as much as four orders of magnitude. Bile acid synthesis is not a vital function of an hepatocyte and the clone of cells used in these experiments may be slightly different to those used in other labs.

The cells have retained, however, the ability to synthesize bile acids as shown by the metabolism of 7α-hydroxycholesterol to CDCA. Although the HPTLC traces are quite complex, it does not appear that any CA is produced by the addition of 7α-hydroxycholesterol. This result agrees with the report by Everson and Polokoff that CA may be produced via a different pathway in the HepG2 cells.

In principle, the addition of 5μM 7α-hydroxycholesterol should result in the production of 5μM bile acid. In 10ml of medium, this would be a total of 50nmol of bile acid or approximately 10nmol bile acid/mg cell protein. In practice, of course, there are the realistic considerations of the solubility of 7α-hydroxycholesterol in the medium, its binding affinity to proteins, its transport into the cells, and ultimately its presentation to the enzymes involved in its catabolism. After 72h, 3nmol CDCA/mg cell protein was produced. Bearing in mind that CDCA is probably not the sole bile acid product, production of CDCA is in the same order of magnitude as the amount of 7α-hydroxycholesterol added. This degree of CDCA production is therefore consistent with precursor supply.

Assuming that cholesterol 7α-hydroxylase is not saturated, the addition of 50μM 7α-hydroxycholesterol should increase the production of CDCA ten-fold. In fact, it increased it by only 1.6-fold. This would suggest that HepG2 cells become saturated with 7α-

hydroxycholesterol. Similar experiments using isolated rat hepatocytes showed that CDCA production could be stimulated by the addition of 7α-hydroxycholesterol (Botham *et al.*, 1980). Doubling the amount of 7α-hydroxycholesterol enhanced CDCA synthesis two-fold. Increasing the concentration of 7α-hydroxycholesterol ten-fold, however, did not further increase CDCA output (Botham *et al.*, 1980). The very fact that the addition of 7α-hydroxycholesterol to HepG2 cells results in bile acid synthesis suggests that cholesterol 7α-hydroxylase is the rate limiting step.

The original goal of this work was to delineate the regulatory mechanisms controlling bile acid synthesis in HepG2 cells. The method chosen for study was to perturb the various pools of cholesterol by adding mevalonate, LDL, and HDL to the cells. In summary, neither mevalonate nor HDL was able to increase CDCA production. LDL, however, did enhance CDCA synthesis. These results agree with the general trend reported by Kempen *et al.* (1988). Moreover, these are the first quantifiable results of the effect of altering the intracellular cholesterol concentration and eliciting a response from HepG2 cells.

These results do not agree with results obtained from experiments carried out in rats and humans. Those experiments indicated that *de novo* cholesterol is the preferred source of substrate for bile acid synthesis (Balasubramanian *et al.*, 1973; Mitropoulos *et al.*, 1974; Einarsson *et al.*, 1979). This hypothesis is attractive because of the juxtaposition of cholesterol 7α-hydroxylase and HMG-CoA reductase in the endoplasmic reticulum. Both enzymes are closely regulated and often vary in parallel (see Table 1.4). However, the term 'preferred substrate' is deceptive. It does

not mean that bile acids are synthesized exclusively from *de novo* cholesterol, rather that *de novo* synthesized cholesterol is converted to bile acid in preference to equilibration with the total cellular cholesterol pool. In the work presented here, mevalonate dramatically increased the synthesis of intracellular cholesterol, but did not augment bile acid synthesis. These data presented here are direct evidence that *de novo* synthesized cholesterol is not the preferred substrate for cholesterol 7α-hydroxylase in HepG2 cells.

The work presented here also does not agree with work carried out in primary rat hepatocytes. Those experiments demonstrated that bile acid synthesis could be driven from both *de novo* cholesterol and HDL-derived cholesterol (Davis *et al.*, 1983; Ford *et al.*, 1985; Mackinnon *et al.*, 1987). The results presented here suggest that, in man, bile acid synthesis is stimulated by LDL-derived cholesterol. The discrepancy in results probably reflects a species difference in cholesterol homeostasis. In rats, HDL is used to deliver cholesteryl esters to the liver (Mackinnon *et al.*, 1987). As outlined in section 4.1, it is the cholesteryl ester moiety of HDL that has been shown to increase bile acid synthesis in primary rat hepatocytes. In humans, however, a cholesteryl ester transfer protein moves cholesteryl ester from HDL to LDL for delivery to the liver (Barter & Lally, 1978; Fielding & Fielding, 1980). LDL consequently carries more cholesterol, both esterified and free, than HDL. This may be the reason why LDL, and not HDL, stimulates bile acid synthesis in HepG2 cells.

The findings of these experiments do concur with the results of Kempen et al (1988), which were published while work here was in progress. They, too, reported that LDL, not HDL or mevalonate,

increased bile acid synthesis. However, in their hands, the ACAT inhibitor 58-035 did not enhance bile acid synthesis stimulated by LDL. In work presented here, 58-035 was seen to further increase bile acid synthesis stimulated by LDL. These data indicate that free cholesterol derived from LDL is readily available for bile acid synthesis. This phenomenon is in contrast to that observed in primary rat hepatocytes, where exogenous cholesterol from HDL must first be esterified prior to its use in bile acid synthesis (Sampson *et al.*, 1988a).

There is not yet enough consistent data from HepG2 cells to propose a hypothesis of cholesterol flow and its effect on bile acid synthesis. At the moment, it appears that HepG2 cells preferentially utilize exogenous cholesterol, and not newly synthesized cholesterol, for bile acid synthesis. The turnover of preformed cholesterol to bile acid would permit the maintainance of homeostasis with the least expenditure of energy from the cell. It is doubtful, though, that the microsomal enzymes involved in cholesterol catabolism can discriminate between newly synthesized and preformed cholesterol. The two different pools are more likely to be distinguished by their method of presentation within the cell.

5.3 Conclusions

Understanding systemic cholesterol flux would greatly advance the understanding of diseases associated with high concentrations of cholesterol. In order to do so, first we must appreciate the regulatory mechanisms of cholesterol metabolism at the level of the liver and other cholesterol-utilizing tissues like the small intestine, steroidogenic tissue, and macrophages. Being able to

manipulate this flux would help in the treatment of atherosclerosis, cholesterol cholelithiasis, and cerebrotendinous xanthamatosis.

This thesis has examined some of the factors involved in bile acid synthesis, which, as we have seen, is central to cholesterol homeostasis. Firstly, we have begun to define the molecular nature of the rate-limiting enzyme of bile acid synthesis, cholesterol 7α-hydroxylase. This enzyme has been shown to share some structural homology with another P450 from the P450 IIC gene subfamily. This is the first report that cholesterol 7α-hydroxylase is homologous with any other P450 (Eldredge *et al.*, Biochem. J., in press see appendix). Secondly, we have examined the effect of substrate supply on bile acid synthesis in the human hepatoblastoma, HepG2. This cell line is a poorly characterized model for the study of bile acid synthesis. It has been shown that cholesterol is compartmentalized in HepG2 cells, albeit in a different fashion from primary rat hepatocytes. The data presented in this thesis are the first quantifiable results of the effect of altering the intracellular cholesterol concentration and eliciting a response from HepG2 cells.

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Appendix

Inhibition of Cholesterol 7α-hydroxylase by an Antibody to a Male-Specific Form of Cytochrome P450 from Subfamily P450IIC.

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ABSTRACT

The absence of antibodies to cholesterol 7α-hydroxylase (E.C. 1.14.13.17), the rate-determining enzyme for bile acid synthesis, has significantly compromised studies on this protein. Nine antibodies raised against proteins from the cytochrome P450 gene families (P450I, P450IIA, P450IIB, P450IIC and P450III) were tested for inhibitory activity against this protein. An antibody raised against a male-predominant P450 (PB_{2a}, P450h) from the P450IIC gene subfamily was an effective inhibitor of activity in liver microsomal fractions from rat, mouse, and hamster. The inhibition could be reversed by the addition of PB_{2a} antigen, indicating structural homology between cholesterol 7α-hydroxylase and proteins within the P450IIC subfamily. Western blot analysis of hepatic microsomal fractions with the PB_{2a} antibody gave three bands, two of which, like cholesterol 7α-hydroxylase, did not exhibit sexual dimorphism. The intensity of one of the bands (apparent m.w. 54000) correlated with changes observed in activity due to diet [Spearman correlation of 0.800 ($p = 0.01$)]. These findings suggest that cholesterol 7α-hydroxylase is a form of P450 which shares structural homology with cytochromes P450 in the P450IIC gene subfamily and that its regulation by bile acid feedback involves protein induction rather than simply post-translational modification.

INTRODUCTION

The 7α-hydroxylation of cholesterol, catalzyed by cholesterol 7α-hydroxylase, is considered to be the rate-limiting step in bile acid synthesis (1). Despite its pivotal role in this process, there is little detailed molecular information on the properties of this protein. Cholesterol 7α-hydroxylase was originally identified as a cytochrome P450 because of the requirement for molecular oxygen and NADPH as cofactors and the inhibitory effects of carbon monoxide (2,3). These early findings have been substantiated by the isolation of a cytochrome P450 with this activity (4-6). The isolation of this protein in an active form has proven to be very difficult, and studies on properties of the purified enzyme are still limited, due to the lack of a suitable antibody for immunochemical analysis (7) and molecular cloning.

Cytochromes P450 can be broadly grouped into two categories. Those involved in hormone biosynthesis, which appear to have restricted substrate specificities, and those involved in foreign compound metabolism (8). The latter family of proteins have diversified into a series of multigene families, encompassing proteins which, in addition to oxidizing foreign compounds, can also oxidize the sterol nucleus in a variety of positions (9).

In order to establish whether cholesterol 7α-hydroxylase may be a part of one of these latter gene families, we tested a variety of antibodies raised against proteins from families P450I, P450IIA, P450IIB, P450IIC, and P450III as inhibitors of this enzyme.

MATERIALS AND METHODS

Preparation of Microsomal Fractions. Adult male or female Wistar rats (100-200 g) were fed a diet containing either 4% (w/w) cholestyramine (Bristol Meyers Pharmeceuticals, p.l.c., Uxbridge, UK) in Purina PRD powdered rat chow, or a soft diet consisting of 75% whole wheat flour, 25% powdered milk and 5% yeast for seven days prior to the experiments. Livers were perfused with 0.105 M KCl with 50 mM NaF and microsomes prepared by fractional centrifugation (3). The microsomal fraction was resuspended in storage buffer [10 mM potassium phosphate, pH 7.4, containing 0.25 M sucrose, 50mM NaF, 1 mM EDTA] and either used immediately for activity measurements or stored at -70°C for Western blot analysis. Protein determinations were by the method of Bradford (10).

Preparation of Antibodies. Purified cytochromes P450 (11-13) were used to immunize New Zealand White rabbits as reported by Wolf & Oesch (12)[1]. Each antibody reacted strongly with its homologous antigen and in some cases weakly with other proteins from the same gene subfamily. The antibodies did not react significantly with proteins from other gene families. IgG fractions were prepared from serum by ammonium sulphate precipitation using the method of Heide & Schwick (14) and were reconstituted at a concentration of 20 mg/ml with distilled water before use.

Cholesterol 7α-hydroxylase Assay. Microsomes (100 µg), cysteamine (3 mg/ml) and antibody (at various antibody protein: microsomal protein ratios) were brought to a final volume of 1 ml with reaction buffer [0.1 M Tris:HCl, pH 7.4, containing 50 mM NaF], and allowed to stand at 22°C for 20 min. [4-^{14}C]-Cholesterol (0.1 µCi, 50 Ci/mol, Amersham International, p.l.c., Amersham, UK) was added (final concentration 2 mM) and the reaction started by the addition of NADPH (final concentration 1 mM). Following incubation for 60 min at 37°C reactions were stopped and extracted with 5 ml $CHCl_3$:MeOH (2:1, v/v). The aqueous phase was re-extracted with 5 ml $CHCl_3$:MeOH, the combined organic fractions dried under N_2, and redissolved in 200 µl of solvent. Samples were run on silica gel tlc plates (0.25 mm, Merck, BDH, Poole, UK) in ethyl acetate:toluene (70:30, v/v) and scanned by a Rita 68000 tlc scanner (Ray Test Instruments, Ltd., Sheffield, UK).

The conversion of cholesterol to 7α-hydroxycholesterol was calculated by scintillation counting of the peaks corresponding to cholesterol (0.82) and 7α-hydroxycholesterol (0.35).

Western Blot Analysis. SDS polyacrylamide gel electrophoresis was by the method of Laemmli (15) and proteins transferred to nitrocellulose by the method of Towbin et al. (16) using a Biorad transblot apparatus (Biorad UK Ltd, UK) and transblotting buffer [20 mM Na_2HPO_4 in 20% MeOH (v/v)] for 18 h at 250 mA. Following the transfer, nitrocellulose filters were washed twice in Tris-buffered saline containing Tween (TBST) [154 mM NaCl, 50 mM Tris, 0.05% Tween 20, pH 7.9] and blocked with 3% dried skimmed milk. Filters were then washed in TBST and incubated in cytochrome P450 antibody, diluted 1:1000, in TBST for 60 min. Following further washing in TBST, the filters were incubated in donkey anti-rabbit horseraddish peroxidase (Scottish Antibody Product Unit, Glasgow, UK) at a dilution of 1:500 in TBST for 60 min. After washing in TBST, filters were incubated with 0.19 MBq ^{125}I-Protein A (Amersham International. p.l.c., Amersham, UK) in TBST for 60 min and then washed until no more radioactivity was removed. Proteins were visualized following exposure to X-ray film (X-AR, Kodak). Bands were quantified by laser densitometry using a Joyce Loebl gel scanner. Linearity was checked by scanning at several sample dilutions.

RESULTS

Nine antibodies were screened as inhibitors of cholesterol 7α-hydroxylase in liver microsomes from cholestyramine-treated female rats. Eight of these antibodies, to PB_{1a}, PB_{1b}, PB_{2c}, MC_{1a}, MC_{1b}, UT_1, did not significantly inhibit the reaction, a maximal inhibition of 20% being observed. All these antibodies have been shown to be effective inhibitors of other P450 mediated reactions. In contrast, an antibody to PB_{2a} almost completely inhibited cholesterol 7α-hydroxylase activitiy (Figure 1).

A titration of antibody concentration against percent inhibition is shown in Figure 2. Almost complete (96%) inhibition of cholesterol 7α-hydroxylase with the PB_{2a} antibody (rabbit 34) was observed at 20 mg IgG/mg microsomal protein. An inhibition greater than 50% was observed at 5 mg IgG/mg of microsomal protein. An antibody to a different PB_{2a} preparation (rabbit 31) also partially inhibited the activity (53%). Antibodies to PB_{1a}, a protein with approximately 70% amino acid sequence homology to PB_{2a}, and an antibody to a pregnenolone 16α-carbonitrile-inducible P450 (PB_{2c}) were not effective inhibitors relative to the controls. The antibody to NADPH cytochrome P450 reductase was a potent inhibitor of cholesterol 7α-hydroxylase activity. Preimmune serum, included as a control for the effects of non-specific binding, did inhibit the reaction to some extent (Figure 2) and the results were normalized for this effect.

The addition of highly purified PB_{2a} could completely reverse the antibody inhibition (Figure 3). This was not the case when PB_{1a} was used. The reversal of inhibition was most effective if the antigen was left at room temperature for 18 hr before use. The reason for this in unclear, but may be because partial protein denaturation or disaggragation is required. These data indicate that either PB_{2a} or a protein structurally related to it, catalyzes the hydroxylase reaction.

Cytochrome PB_{2a} is sexually dimorphic and is expressed in much higher levels in the livers of mature male rats (17). In the samples tested here, the cholesterol 7α-hydroxylase activity in sexually immature males and females and in mature males and females treated

with cholestyramine were not significantly different (Figure 4). It therefore appears that it is a protein structurally related to PB_{2a} which is responsible for cholesterol 7α-hydroxylase activity. Western blot analysis of the microsomal samples used in the above experiment are shown in Figure 5. Three clearly distinguishable bands were observed. The central band corresponds to PB_{2a} and is significantly elevated in mature male rats. Indeed the "immature rats" which were 4-5 weeks old also had an increased concentration of this protein, indicating that the factors regulating the PB_{2a} gene had to some degree already developed. The upper and lower bands of molecular weight 54000 and 49500, respectively, did not vary significantly in the samples. These bands could not be discerned from the male-predominant P450 form.

It was interesting that in the animals treated with cholestyramine, an inducer of cholesterol 7α-hydroxylase activity, a significant elevation in the higher molecular weight band was seen (Figure 6). In order to pursue this change further, cholesterol 7α-hydroxylase activity was suppressed by feeding female rats a soft diet (18). Western blot analysis of these microsomes demonstrated that, in contrast to the elevation of this band in samples from cholestyramine-treated animals, a significant suppression was observed in animals on the soft diet (Figure 6). A direct correlation between band intensity and cholesterol 7α-hydroxylase activity was obtained (Spearman correlation of 0.800, $p < 0.01$). It is worthy of note that the purified PB_{2a} antigen did not contain a band of molecular weight 54000, indicating that recognition of the high molecular weight protein, as well as the reversal of cholesterol 7α-hydroxylase inhibition (Figure 1), was not the result of an impurity in the original antigen preparation.

In order to establish whether the protein catalyzing the cholesterol 7α-hydroxylase reaction was conserved between species the antibody was tested against mouse and hamster microsomal preparations. The cholesterol 7α-hydroxylase activity was similar in all three species following cholestyramine treatment and in all cases was inhibited by the PB_{2a} antibody (Figure 7). The antibody was not quite as effective in the mouse, inhibition being approximately 50%.

DISCUSSION

A significant limitation in the study of the cholesterol 7α-hydroxylase has been the inability to carry out immunological studies because of the absence of a suitable antibody. In this report we describe an antibody, derived from a protein within the P450IIC subfamily, which is a potent inhibitor of the cholesterol 7α-hydroxylase reaction. Based on a number of criteria, particularly that cholesterol 7α-hydroxylase activity is not sexually differentiated (19,20) it appears that the protein concerned is not the male-specific cytochrome PB_{2a}, to which the antibody was raised, but is structurally related to it. The inability of antibodies to PB_{1a}, which is approximately 70% homologous to PB_{2a} and from the same gene family, to inhibit cholesterol 7α-hydroxylase or of the antigen to reverse inhibition indicates that this enzyme is structurally more similar to PB_{2a}. This possibility is intriguing in view of the apparent structural homology of PB_{2a} with the hepatic microsomal vitamin D_3 25-hydroxylase (21). Similar to cholesterol 7α-hydroxylase, the 25-hydroxylase reaction is an important 'house-keeping' function in mammals. In other instances where this is the case, for example the P450 proteins involved in hormone biosynthesis, the reactions are catalyzed by distinct P450 forms which are not associated with gene families capable of foreign compound metabolism (22).

In a previous report, an antibody to a protein apparently identical to PB_{2a} did not inhibit the reaction (7). This may be due to the different assay procedures, i.e. the use of Tween 80 vs acetone for the solubilization of the substrate, or to the immunopurification of the antibodies.

Anti-PB_{2a} was capable of inhibiting cholesterol 7α-hydroxylase from several species: mature and immature, male and female rats, hamster, and mice. This general inhibition would indicate that cholesterol 7α-hydroxylase shares a common feature in these animals and supports the idea of the highly conserved structure of cholesterol 7α-hydroxylase. The inhibition by anti P450 reductase proves the expected involvement of this protein in the reaction pathway, as was shown earlier by Waxman (7).

The activity of cholesterol 7α-hydroxylase can be increased by the administration of cholestyramine (23) and suppressed by feeding with a 'soft diet' (18). The mechanism of this alteration in activity has been attributed to either an increase in the cholesterol 7α-hydroxylase protein (24), a change in substrate supply (25,26) or by activation through phosphorylation (27-29), but evidence that phosphorylation state is altered on cholestyramine treatment is equivocal. It has recently been shown that PB_{2a} is a protein which can be phosphorylated (30), and it will be interesting to determine whether this site is conserved in cholesterol 7α-hydroxylase.

In these studies, the inhibitory antibody to cholesterol 7α-hydroxylase identified a band on Western blots whose intensity was clearly influenced by both cholestyramine administration and by the use of a soft diet. Indeed the intensity of the band correlated with cholesterol 7α-hydroxylase activity. Whether this band comigrates with the reported purified cholesterol 7α-hydroxylase protein remains to be determined. However, the above data indicates that altered protein concentrations are at least in part responsible for the effects of diet on cholesterol 7α-hydroxylase activity.

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FOOTNOTE

[1]From NH_2-terminal sequence analysis the proteins used in this study were equivalent to the following proteins described by Ryan et al. (9). MC1a = form d, MC1b = form c (family P450I); PB1a = form k, PB2a = form h (family P450IIC); PB3a = form b, PB3b = form e (family P450IIB); UT1 = form a (family P450IIA). PB2c is a protein from subfamily P450III (11) and is characterized by its induction by dexamethasone and pregnenolone-16α-carbonitrile. The designation of cytochrome P450 into families and subfamilies is based on the recommended nomenclature of Nebert et al. (31).

FIGURE LEGENDS

Figure 1: Effect of Anti-PB_{2a} on Cholesterol 7α-hydroxylase Activity.

Cholesterol 7α-hydroxylase activity was determined in liver microsomes from cholestyramine-treated female rats by tlc as described in the Materials and Methods. The top chart shows a typical control tlc scan. The lower chart shows a typical trace obtained from an experiment carried out in the presence of anti-PB_{2a}. The IgG:microsomal protein ratio was 20:1. A and B indicate the positions of 7α-hydroxycholesterol and cholesterol, respectively.

Figure 2: Effect of Antibodies on Cholesterol 7α-hydroxylase Activity.

Assays using liver microsomes from cholestyramine-treated female rats were as described in Materials and Methods. Values have been normalized to those obtained with preimmune serum (PI). Preimmune serum values were 4.51, 3.70, 2.80, and 2.08 % conversion at 2:1, 5:1, 10:1, and 20:1 ratios IgG:microsomal protein, respectively. Values shown are the means of triplicate determinations, the mean error being not greater than 3.0 %.

Figure 3: Reversal of Cholesterol 7α-hydroxylase Inhibition by the Addition of Purified Rat Liver PB2a.

Assays were carried out in liver microsomal samples from cholestyramine-treated female rats as described in Materials and Methods with the addition of anti-PB_{2a} at a ratio of 10:1 IgG/mg microsomal protein for the inhibition of the enzyme. Samples were mixed and preincubated with the reaction mixture for 20 min at 22°C. Purified cytochrome P450's, left 18 h at room temperature, were added at a concentration of 10 nmol/mg microsomal protein. The incubations contained (1) preimmune serum; (2) anti-PB_{2a}; (3) anti-PB_{2a} and PB_{2a} protein; (4) anti-PB_{2a} and PB_{1a} protein. The values shown are the means of 3 determinations ± standard deviation.

Figure 4: The Effect of Age and Sex on Cholesterol 7α-hydroxylase Activity in the Rat.

Cholesterol 7α-hydroxylase activity in microsomes from sexually immature females (100 ± 10 g) (F/I), sexually mature females (180 ± 10 g) (F/M), sexually immature males (125 ± 10 g) (M/I), and sexually mature males (190 ± 10 g) (M/M). All animals were maintained on a 4% (w/w) cholestyramine diet for seven days prior to the experiment. The assay was carried out as described in Materials and Methods with the addition of anti-PB_{2a} at a ratio of 10:1 IgG/mg microsomal protein. Activitiy is expressed as % conversion of cholesterol to 7α-hydroxycholesterol and the values are the means of 3 determinations ± standard deviation.

Figure 5 : Western Blot Analysis of Rat Liver Microsomes with Anti-PB_{2a}.

Western blots were carried out as described in Materials and Methods using 7.5 µg of microsomal protein. F/I = immature female, F/M = mature female, M/I = immature male, M/M = mature males, as described in Figure 4.

Figure 6 : Correlation Between PB_{2a}-related Proteins and Cholesterol 7α-hydroxylase Activity.

a) Western blot analysis was carried out on mature female rat liver microsomes as described in the Materials and Methods section. 7.5 µg of microsomal protein was run per track. S = PB_{2a} standard, Cho = cholestyramine-fed rats, SD = rats maintained on a soft diet, Con = rats fed the control diet.

b) Cholesterol 7α-hydroxylase activity is expressed as % conversion from cholesterol. Samples are in the same order as on the Western blot. Microsomes from the fourth sample of each series were not assayed for enzyme activity.

Figure 7 : Effect of anti-PB2a on Cholesterol 7α-hydroxylase Activity in Different Species.

Incubations on female liver microsomal samples were carried out as described in the Materials and Methods with the addition of anti-PB_{2a} at a ratios of 10:1 IgG/mg microsomal protein. Activity is expressed as % conversion of cholesterol to 7α-hydroxycholesterol and the values are means of 3 determinations ± standard deviation

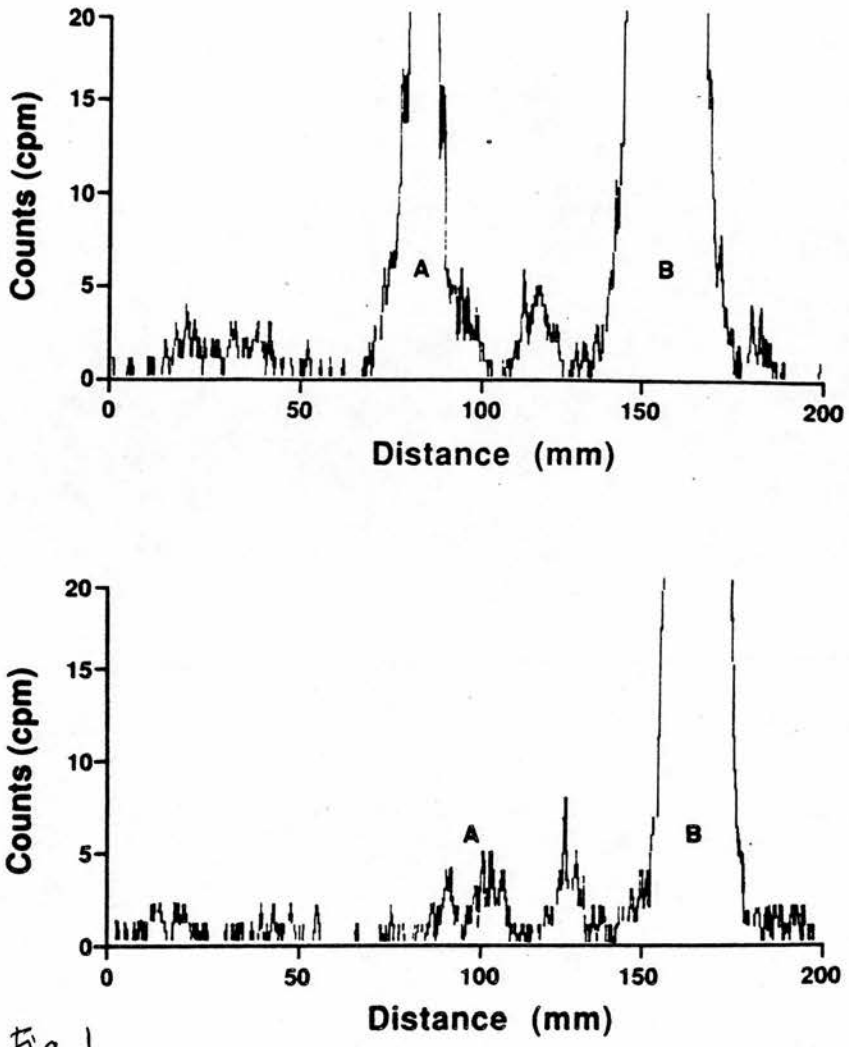
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Fig 1

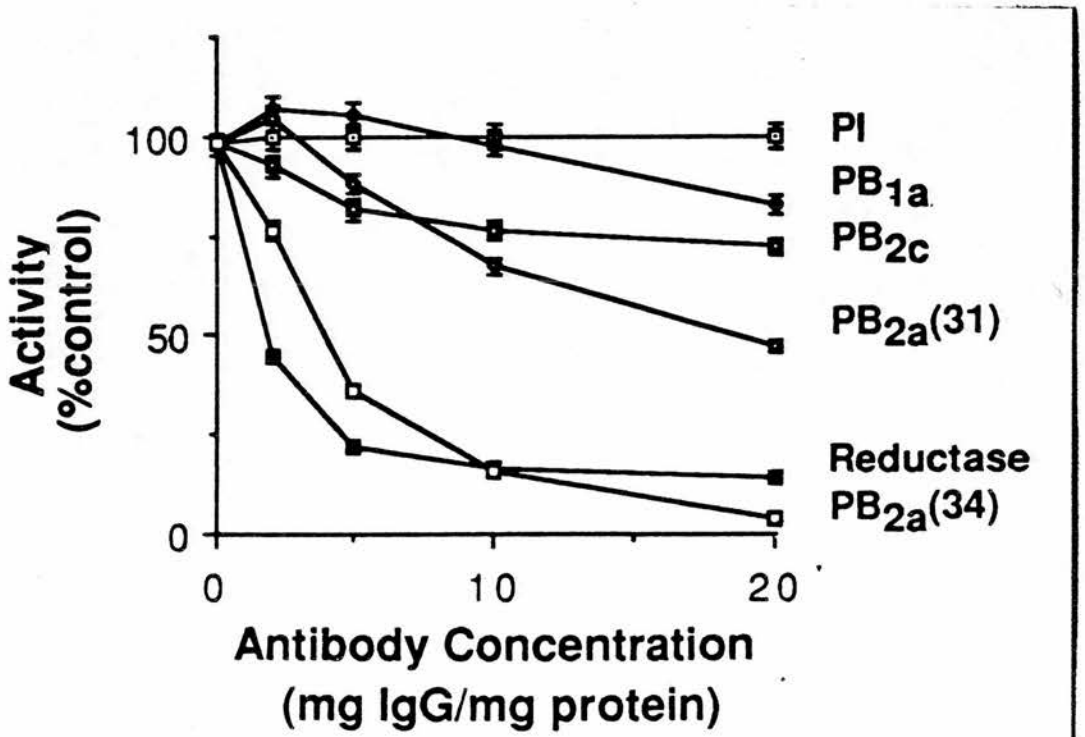
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Fig 2

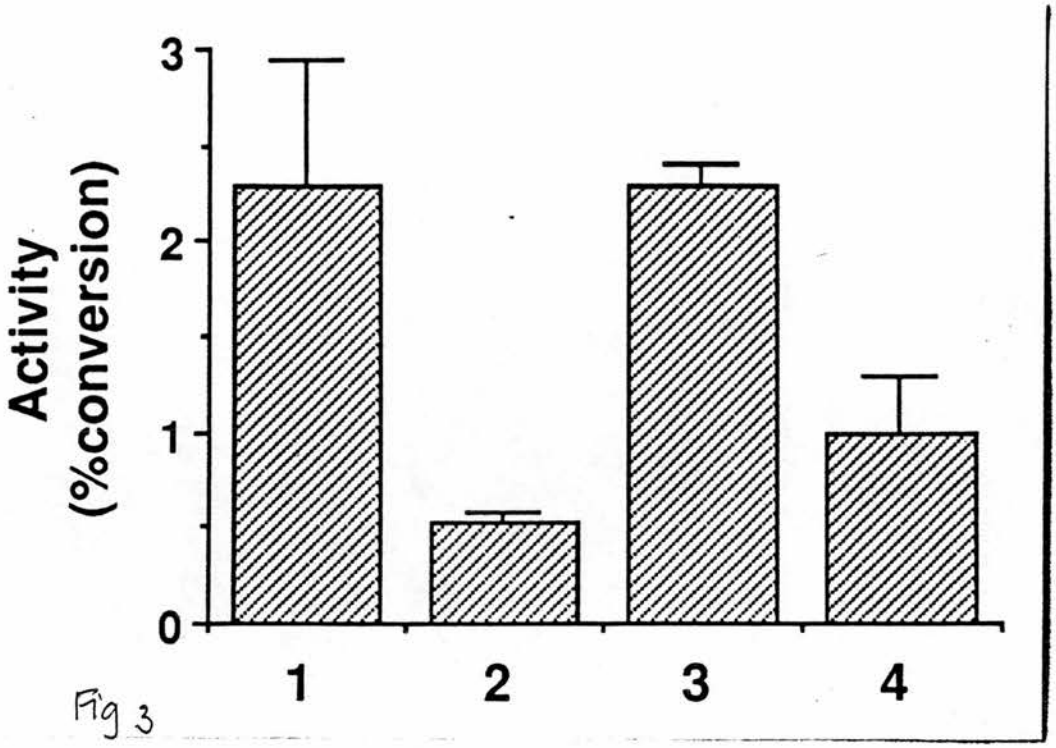
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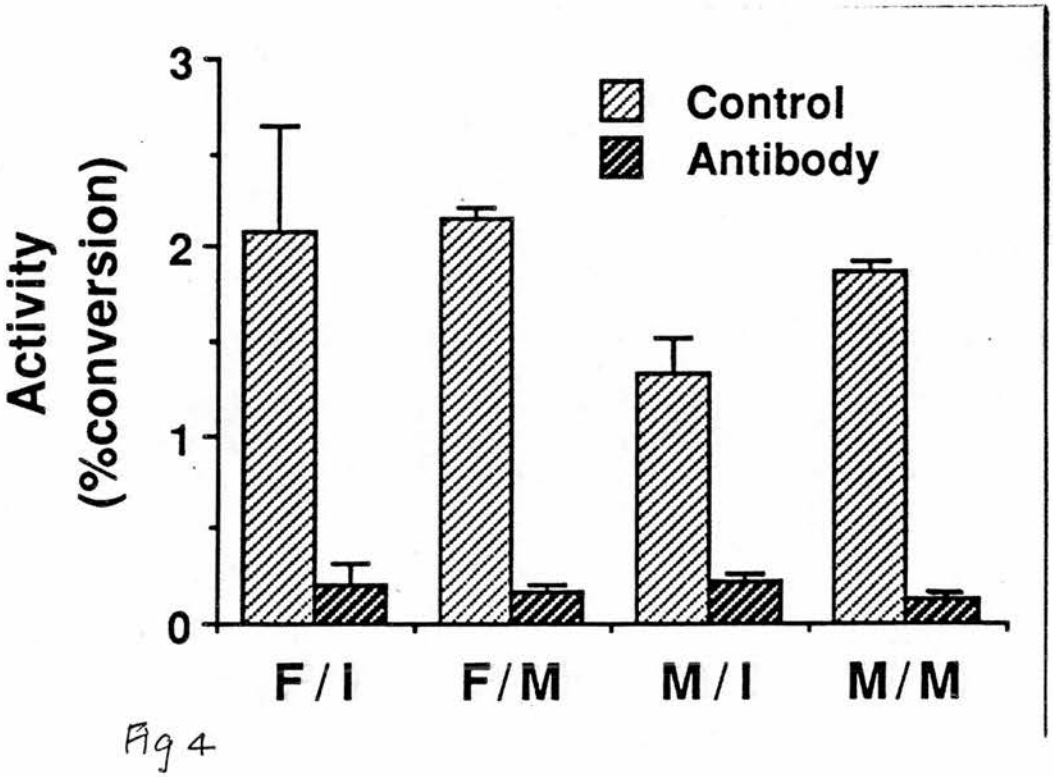
Fig 3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F/I
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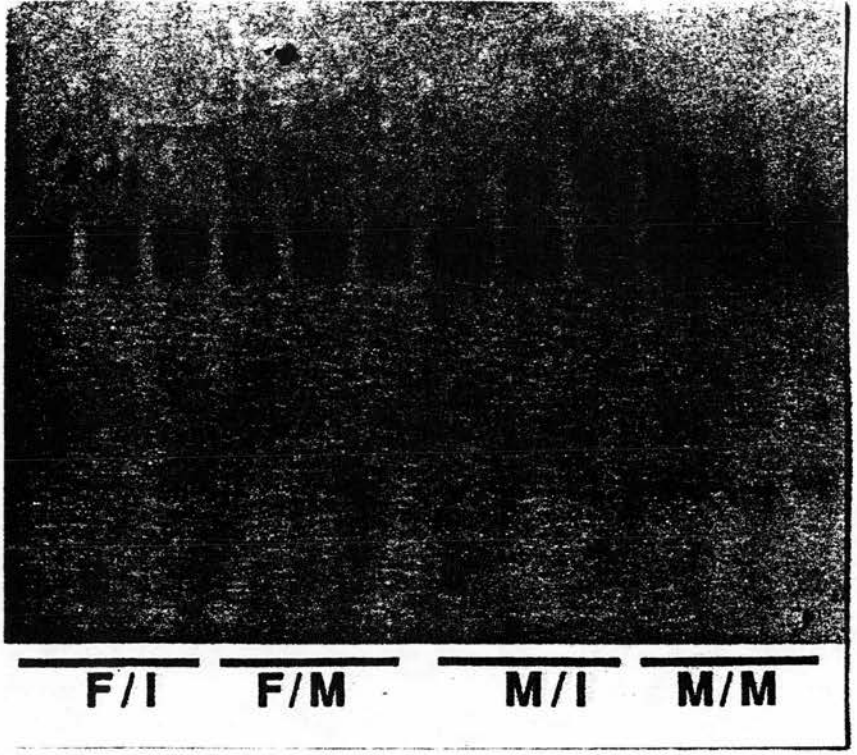
Fig 4



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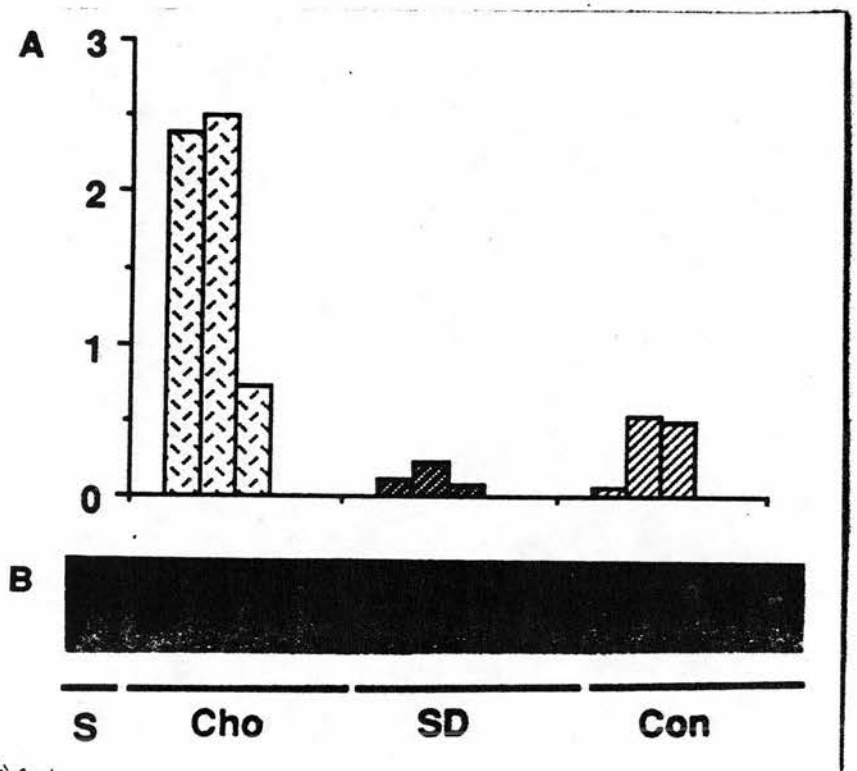
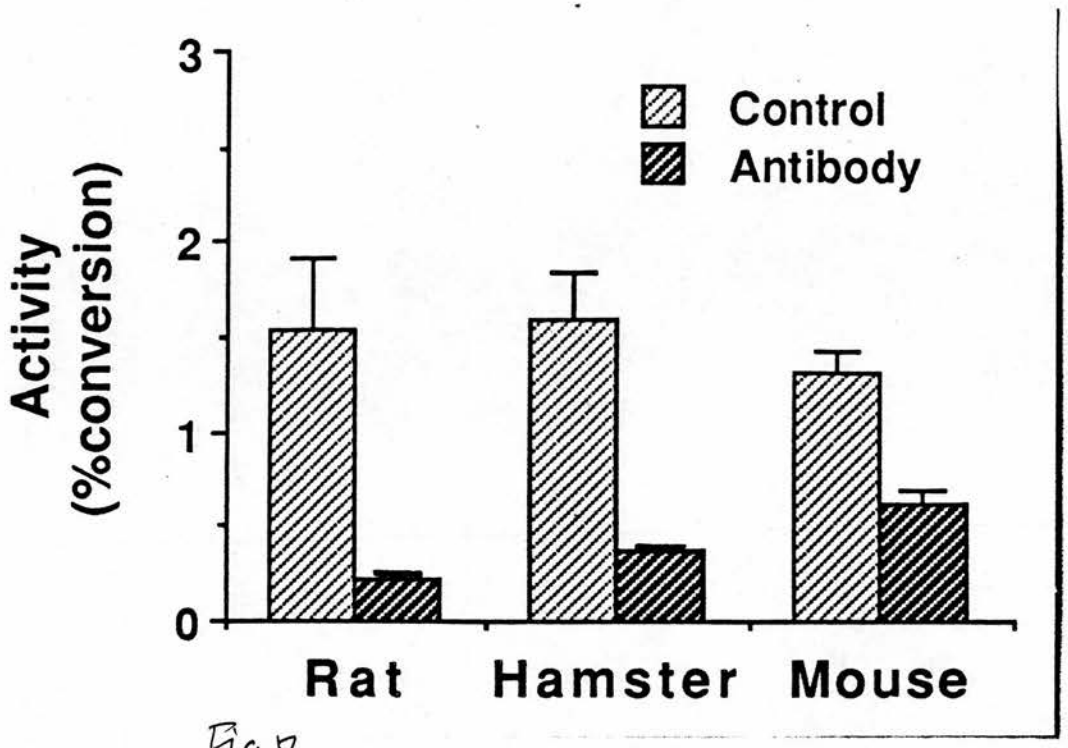


Fig 6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Rat
Hamster
Mouse

Fig 7