STUDIES ON CHOLESTEROL 7α-HYDROXYLASE

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This thesis has been composed entirely by myself and all the experimental results described herein, except where otherwise acknowledged, are the product of my own work.

To G.S.B., M.E.L., and my other colleagues and friends in the laboratory.

ABBREVIATIONS

Cholesterol 7α-hydroxylase - cholesterol, reduced NADP : oxygen oxidoreductase, 7α-hydroxylating (EC 1.14.-.-.)

cholesterol	-	cholest-5-ene-3β-ol
7α-hydroxycholesterol	-	cholest-5-ene-3β, 7α-diol
nor-cholesterol	-	26 nor-cholest-5-ene-3β-ol
cholenol	-	chol-5-ene-3β-ol
bisnor-cholenol	-	23,24 bisnor-chol-5-ene-3β-ol
pregnenol	-	pregn-5-ene-3β-ol
sitosterol	-	24α-ethyl-cholest-5-ene-3β-ol
27 dimethyl norcholesterol	-	26 nor,27 dimethyl cholest-5-ene-3β-ol
desmosterol	-	cholest-5,24-diene-3β-ol

NADP	-	nicotinamide adenine dinucleotide phosphate
NADPH	-	reduced nicotinamide adenine dinucleotide phosphate
NADH	-	reduced nicotinamide adenine dinucleotide

ABSTRACT

Attempts were made to solubilize the enzyme cholesterol 7α-hydroxylase from native rat liver microsomes and from rat liver microsomal acetone and butanol powders. Mechanical techniques such as freezing and thawing, repeated homogenisation and sonication, and also the use of hydrolytic enzymes such as Phospholipase A and those contained in pancreatin and Naja naja venom, all failed to solubilize the enzyme. Solubilizing agents such as urea, n-butanol, sodium deoxycholate and cholate, cetyltrimethylammonium bromide, and all the non-ionic detergents tested, with the exception of Nonidet P40 and P42, failed to release into solution cholesterol 7α-hydroxylase activity or greatly inhibited this enzyme.

Nonidet P42 solubilized microsomes were applied to a column of DEAE-cellulose, and chromatography separated cytochrome P-450, cytochrome b_5 and NADPH-cytochrome c oxidoreductase from each other.

Fractions eluted from DEAE-cellulose contained very little or no cholesterol 7α-hydroxylase activity, but on recombination of the cytochrome P-450 fraction with a fraction containing NADPH-cytochrome c oxidoreductase, cholesterol 7α-hydroxylase activity was reconstituted. The interdependence of cytochrome P-450 and NADPH-cytochrome c oxidoreductase and the effect of cytochrome b_5 was investigated in the reconstituted cholesterol 7α-hydroxylase system.

Further attempts have been made to increase the purity of cytochrome P-450 and NADPH-cytochrome c oxidoreductase, and these partially purified fractions were recombined and tested for their ability to support the 7α-hydroxylation of cholesterol.

Some chemical and biochemical properties of Nonidet P42 solubilized rat liver microsomes and rat liver microsomal acetone and/

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butanol powders have also been investigated to characterise the system.

The effect of modifications to the cholesterol side chain on cholesterol 7α-hydroxylase activity has been observed. Studies on the substrate specificity of cholesterol 7α-hydroxylase have revealed that this enzyme is very sensitive to small changes in the side chain of the sterol.

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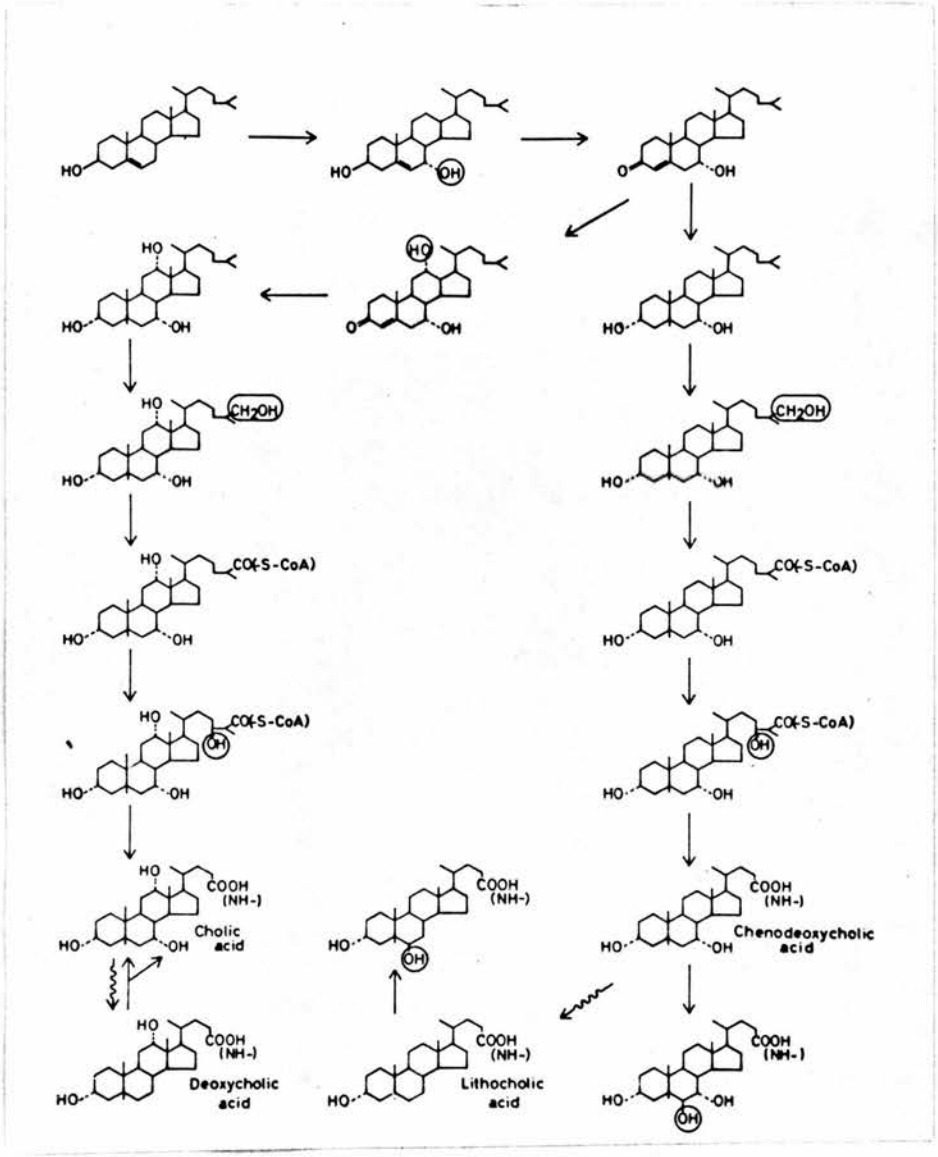


Figure 1,1.

Pathways for the biosynthesis and metabolism of bile acids in the rat.

⟶ Reactions catalyzed by liver enzymes

⟿ Reactions catalyzed by microbial enzymes

SECTION 1

INTRODUCTION

The tissue and body fluid cholesterol content appears to stay fairly constant over long periods. Maintenance of a constant level of cholesterol in the body requires that de novo synthesis and ingestion of dietary cholesterol should balance cholesterol metabolism and excretion. Quantitatively, the most important catabolic route of cholesterol is the transformation of this sterol to bile acids, approximately 50% of excreted cholesterol is in the form of these surface active agents, the other 50% consists of neutral faecal steroids such as cholestanol and coprostanol (1). Metabolism to steroid hormones and their catabolites is of minor quantitative importance. That bile acids are synthesized from cholesterol was first demonstrated by Bloch (2) who showed that deuterated cholesterol was transformed to deuterated bile acids.

Transformation of cholesterol to bile acids requires many chemical modifications involving two hydroxylations of the sterol nucleus at the 7α and 12α positions, oxidation of the 3β hydroxyl, a reduction of the Δ5 double bond to a 5β hydrogen and reduction of the 3 oxo group to a 3α hydroxyl. The side chain is reduced in length by a three carbon fragment by a series of hydroxylations and oxidations. A generally accepted pathway to the two primary bile acids is shown in Figure 1,1. These bile acids, conjugated with either taurine or glycine, are secreted from the bile duct into the duodenum in response to the gastric contents, in contact with the duodenal mucosa, exciting the release of cholecystokinin. The function of these bile salts is emulsification of fats prior to digestion by pancreatic lipase, the activation of this enzyme, and the efficient absorption of fat soluble vitamins.

Bile salts may also be further metabolised by bacteria in the intestinal tract. The processes catalysed are basically a dehydration followed by a reduction to form deoxycholate from cholate and lithocholate from chenodeoxycholate, hydrolysis of the peptide bond and oxidation of some secondary alcohols to ketones. The result of these bacterial transformations is the production of secondary bile acids in contrast to those synthesized by the liver which are termed primary bile acids.

It has been estimated that 90% of the bile acids are reabsorbed, particularly in the region of the lower ileum, and returned to the liver via the portal vein, and any secondary bile acids may be rehydroxylated to form the equivalent primary bile acid. Shefer et al (3) have demonstrated that the enterohepatic circulation of bile acids occurs about 10 times per diem.

Linstedt (4) deduced that perhaps the first step in the transformation of cholesterol to bile acids was the 7α-hydroxylation of cholesterol. By feeding rats the bile acid sequestering resin, cholestyramine, or by cannulation of the bile duct, the activity of cholesterol 7α-hydroxylase may be increased ten-fold. Not only is this activity increased but the output of both cholic and chenodeoxycholic acids from the liver are increased. As the pathways of these two primary bile acids diverge after the 7α-hydroxylation, and no other enzyme in the pathway is stimulated by cholestyramine treatment, nor is there any noticeable build up of intermediates in the pathway, cholesterol 7α-hydroxylase is considered the first and rate limiting step in the conversion of cholesterol to bile acids. The factors responsible for the regulation of activity of this enzyme are still far from being understood, and much of the problem resides in

the fact that cholesterol 7α-hydroxylase activity cannot be resolved from another regulatory enzyme involved in the transformation of acetate to bile acids, the β-hydroxy-β methyl glutaryl-Coenzyme A reductase (HMG-CoA reductase). The activity of HMG-CoA reductase and cholesterol 7α-hydroxylase often rise and fall in parallel. Both enzymes undergo a diurnal variation in activity (5,6,7) which is abolished by hypophysectomy (8.9). Furthermore, in the case of HMG-CoA reductase, the increase in activity has been shown to be the result of increased synthesis rather than a decrease in degradation rate (10). The half-lives of both enzymes are relatively short, being about 3 hours.

As a result of the parallel activities, it has been suggested that the product of HMG-CoA reductase, namely, the amount of microsomal cholesterol, regulates the apparent activity of the cholesterol 7α-hydroxylase. Thus cholestyramine treatment could (a) activate HMG-CoA reductase synthesis, increasing cholesterol concentration and therefore affecting cholesterol 7α-hydroxylase activity; Bjorkhem states that the enzyme is already saturated with cholesterol (11) and Brown et al (12) have shown that cholesterol concentration is unchanged by cholestyramine feeding, and under the conditions of cholesterol 7α-hydroxylase assay, no cholesterol synthesis takes place; (b) activate cholesterol 7α-hydroxylase reducing the cholesterol concentration and, as a result, increase HMG-CoA reductase, or (c) cholestyramine could co-ordinately induce both enzymes. Literature concerning the role of cholesterol in the regulation of cholesterol 7α-hydroxylase activity is confused. Siperstein and Wilson et al (13,14) have noted that feeding rats a diet rich in cholesterol increased the activity of cholesterol 7α-hydroxylase, but HMG-CoA reductase activity was reduced, and Myant and Eder (15) showed that the induction of

cholesterol 7α-hydroxylase by biliary drainage was preceded by an increase in the rate of cholesterol synthesis. Cayen (16) has observed that tomatine, a steroid glycoside which complexes with cholesterol and prevents its absorption from the gut, activates cholesterologenesis but the rate of bile acid synthesis was unaltered. However, as the decrease in bile acid concentration in the enterohepatic circulation precedes the rise in cholesterol 7α-hydroxylase activity, and as puromycin, actinomycin D and cycloheximide all inhibit the rise in activity (12,18), most workers are agreed that the increase in cholesterol 7α-hydroxylase activity is not due to relief of inhibition of this enzyme by reduction in concentration of portal bile salts (17,19,20,21,22).

The only organ in which the synthesis of bile acids from cholesterol has been demonstrated is the liver, and the rate determining enzyme in this transformation, cholesterol 7α-hydroxylase, occurs only in the microsomal fraction of liver. This fraction, capable of catalysing various reactions, contains many hydrolytic enzymes and is noticeably rich in haem. Two of the major haemoproteins are cytochrome b_5 and cytochrome P-450, which was first identified by Garfinkel (23) and Klingenberg (24) though its catalytic significance at that time was not known. It is now known that cytochrome P-450 is a component of most mixed function oxidases (25), capable of catalysing in the presence of NADPH and oxygen the hydroxylation of many endogenous and foreign compounds. This drug hydroxylating system is characterised by its inhibition by carbon monoxide, an inhibition optimally relieved by irradiation by light of 450 nm. Cholesterol 7α-hydroxylase is inhibited by carbon monoxide, and irradiation by light of 450 nm. optimally relieves the inhibition (26,27). Furthermore, antibody towards NADPH-cytochrome P-450

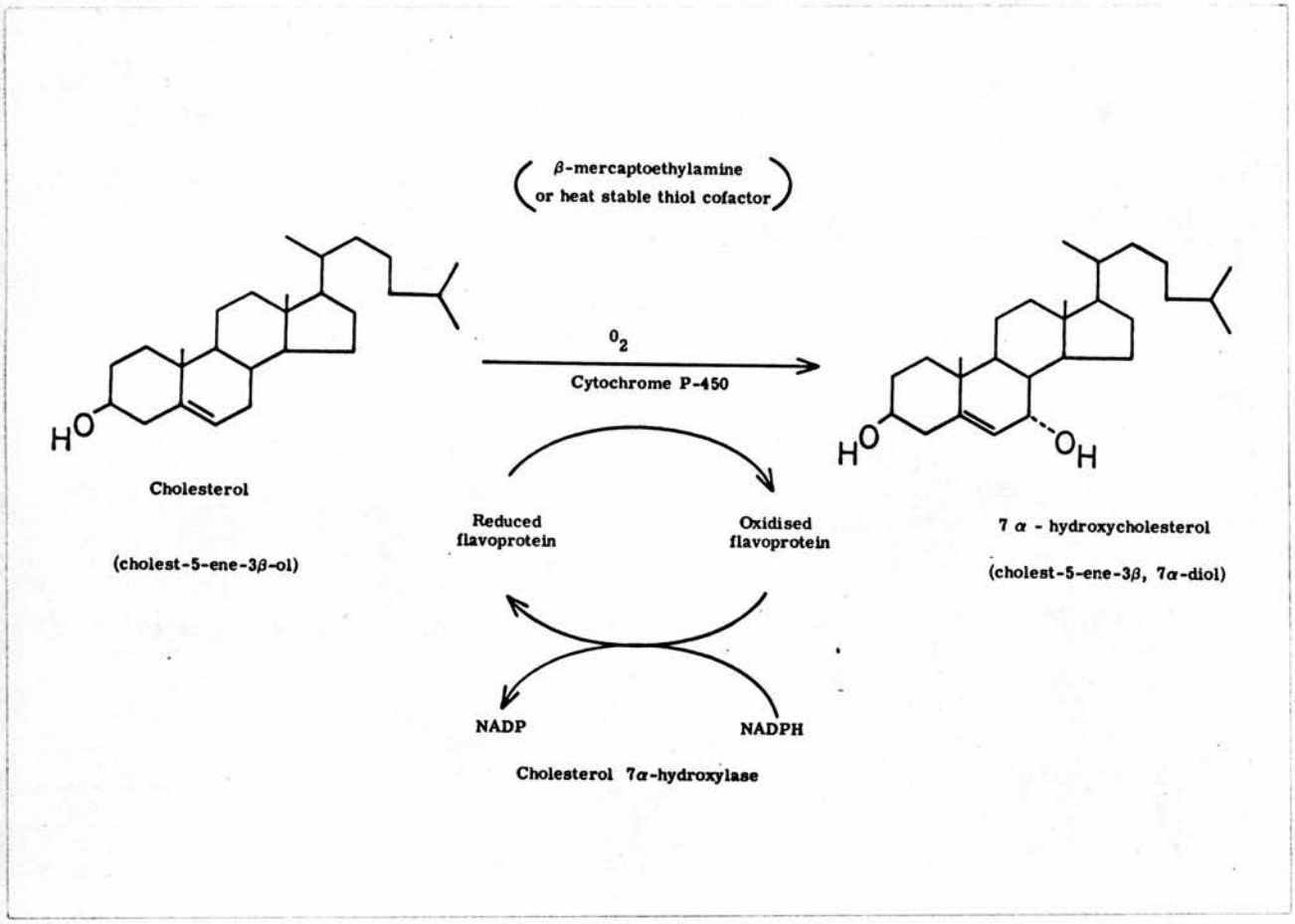


Figure 1,2.

The 7α-hydroxylation of cholesterol by the microsomal fraction of rat liver.

reductase inhibits the 7α-hydroxylation of cholesterol (28). It is therefore considered that the reaction shown in Figure 1,2 is catalysed by a mixed function oxidase, having cytochrome P-450 as the terminal oxidase. It has been found that if cholesterol is incubated with microsomes and NADPH, in the presence of oxygen, many products are formed, the major product being 7-oxo-cholesterol. It has been shown by Mitton et al (29) that the formation of these products may be greatly reduced by adding to the incubation glutathione and manganous ions, β-mercaptoethylamine or a heat stable thiol present in the microsomal supernatant.

The mixed function oxidase of rat liver microsomes can be induced by a variety of compounds such as phenobarbital, 3-methylcholanthrene, pregnenolone 16α-carbonitrile. All these agents increase the specific content of cytochrome P-450, but there also appears to be a preferential stimulation of, for example, polycyclic hydrocarbon hydroxylation by prior treatment with 3-methylcholanthrene, which also induces the formation of a cytochrome P-448 (30,31,32) and pregnenolone 16α-carbonitrile appears to induce ethylmorphine demethylase activity (33). Phenobarbital, in the strain of rats used in this laboratory, does not stimulate cholesterol 7α-hydroxylase activity although the cytochrome P-450 content is increased. Furthermore, cholestyramine feeding, which can induce cholesterol 7α-hydroxylase activity ten-fold, has no effect on the specific content of cytochrome P-450. It is, therefore, of interest to determine the essential components of the cholesterol 7α-hydroxylase multi-enzyme complex.

The aims of this study were four-fold:-

(i) To obtain a soluble active preparation of cholesterol 7α-hydroxylase to which purification techniques could be applied in an

attempt to move some way towards the isolation of the protein catalysing the rate determining step in the 7α-hydroxylation of cholesterol;

(ii) To investigate the chemistry and bio-chemistry of some of the characteristics of such a solubilized preparation;

(iii) To compare and contrast properties of cholesterol 7α-hydroxylase with those of the drug hydroxylating mixed function oxidase system;

(iv) To gain some knowledge concerning the active site of the enzyme with respect to its substrate specificity.

SECTION 2

MATERIALS AND METHODS

A Animals and their diets

Male rats of the Wistar strain were used and weighed approximately 200g. They were bred in the animal house of this department. Control diets consisted of 70% wholemeal flour, 25% skimmed milk powder and 5% dried yeast. Except where statements to the contrary are made, the rats were also fed the bile acid sequestering resin, cholestyramine or "Cuemid" at a level of 4% w/w in the soft diet. Phenobarbital was also administered to rats for certain experiments to increase the concentration of 'marker' cytochrome P-450; the phenobarbital was added to the drinking water for five days at a concentration of 1mg/ml.

B Perfusion of livers

In order to reduce the concentration of haemoglobin in the microsomal fraction, the rats were anaesthetized with ether, a needle was inserted into the portal vein and the liver perfused with 0.154M potassium chloride.

C Preparation of microsomes

The perfused haemoglobin-free liver was excised and chopped with scissors and homogenised by three passes of a 'Teflon' pestle to form a 25% w/v homogenate. This homogenate was centrifuged for 20 minutes at 18,000 x g to remove cell debris, nuclei and mitochondria. The supernatant from this centrifugation was centrifuged at 105,000 x g for 1 hour. The pellet resultant from this centrifugation contained the endoplasmic reticulum of rat liver. Microsomes were resuspended in distilled water, lyophilised, and stored at -20^{o}C.

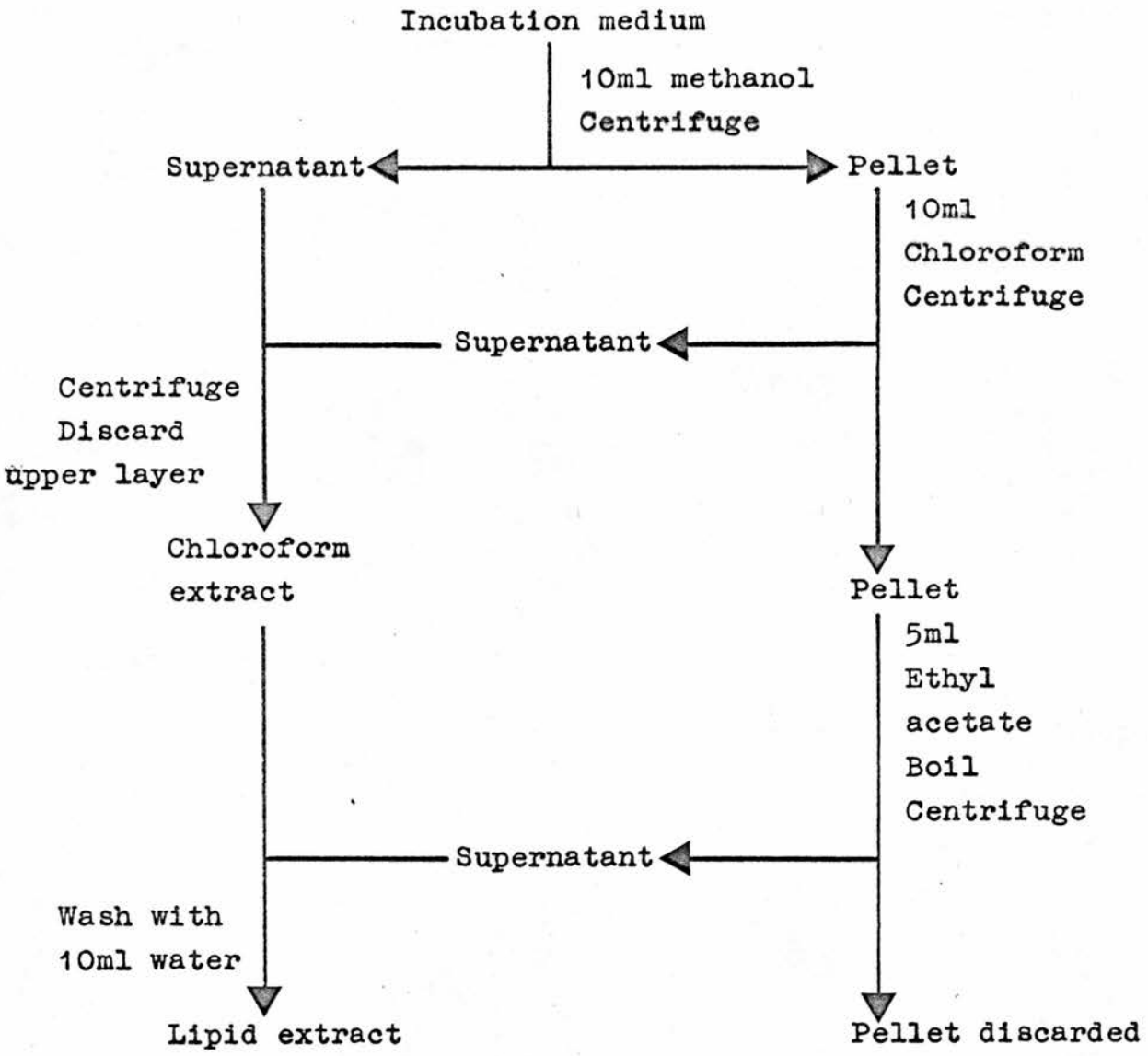


Figure 2,1

Scheme for the extraction of steroids from the cholesterol 7α-hydroxylase incubation medium.

D The cholesterol 7α-hydroxylase assay

[4 - ^{14}C] cholesterol was purified by t.l.c. using a solvent system of petroleum ether : diisopropyl ether, 30:70, prior to use. About 1μg (0.1μCi) radioactive cholesterol, dissolved in 50μl acetone, was added to the incubation medium of 7ml, containing 0.1M potassium phosphate buffer pH 7.4, 10mM β-mercaptoethylamine, 5μmoles NADP, 50μmoles glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase, and approximately 10mg microsomal protein which is equivalent to 1g wet weight of original liver sample.

It has been shown by Mitton et al (29) that these are optimal conditions for the enzyme assay, and Brown has demonstrated that the velocity of the reaction is proportional to the protein concentration, at least to 8mg protein per ml (34). The reaction rate is also linear up to 40 minutes incubation. Brown (34), Bjorkhem et al (11) and Van Cantfort (35) have also shown by g.l.c. and g.l.c. mass spectrometry that results expressed as percentage conversions of [4 - ^{14}C] cholesterol to [4 - ^{14}C] 7α-hydroxycholesterol are good approximations to those results obtained from the determination of the mass of 7α-hydroxycholesterol.

After incubation for 40 minutes at 37^{o}C with constant agitation, the reaction was terminated by addition of 10ml methanol. Neutral lipid was then extracted according to the scheme presented in Figure 2,1. The volume of the extract was reduced by evaporation and the residual volume applied to thin layer plates of silica gel. The products of reaction were separated from the substrate by developing in a solvent system of benzene and ethyl acetate, 7:13. Peaks of radioactivity were located on the chromatoplates by scanning with a Panax thin layer radioactive scanner. In the presence of 10mM β-mercapto-

	Microsomes	Acetone powder
Total protein,(mg)	187	185
Cytochrome P-450 (nmoles)	176	69
Cytochrome b_5 (nmoles)	58	56
NADPH-cytochrome c oxidoreductase (nmoles/min/mg protein)	82	66
Cholesterol µg	2860	140
Percentage cholesterol converted to 7α-hydroxy-cholesterol in 1hr.	10.2	4.4

4.5 ml of the microsomal suspension (42.5 mg protein/ml) was added to 500ml acetone to prepare an acetone powder (420mg),as described in the text.

Table 2,1.

Some properties of a rat liver microsomal acetone powder.

ethylamine, 7α-hydroxycholesterol is the only major product formed. Other products which may be formed in the absence of β-mercaptoethylamine, 7-oxo-cholesterol, 7α- and 7β-hydroxycholesterol and cholestan-3β,5α,6β-triol, have been characterized by Mitton et al (29). The silica gel was scraped from the plates into scintillation vials and 5ml of scintillant added. The scintillation fluid was prepared by adding 20g PPO and 150mg POPOP dissolved in 250ml methanol to 4,750ml of toluene. Radioactivity was then quantitatively determined in a Packard Tri-Carb liquid scintillation spectrophotometer. Addition of silica gel to the scintillant does not cause significant quenching, and the efficiency of counting ^{14}C disintegrations is about 80%.

When the percentage conversion of [4 - ^{14}C] cholesterol to [4 - ^{14}C] 7α-hydroxycholesterol was very low, 1cm sections of the plate were scraped into vials and these were counted to observe peaks of radioactivity. The activity of cholesterol 7α-hydroxylase was expressed as the percentage [4 - ^{14}C] cholesterol converted to [4 - ^{14}C] 7α-hydroxycholesterol.

E Preparation of rat liver microsomal acetone powder

Microsomes prepared in the usual way were resuspended in 0.154M KCl to give a protein concentration of about 30mg/ml. The suspension was added dropwise to 100 volumes of stirred acetone at -20°C. After filtering the solvent, the powder was washed with diethyl ether, then acetone, both at -20°C. The resultant powder was transferred to a vacuum desiccator and left in vacuo for about 1 hour at room temperature to remove traces of solvent. The material was then stored at -20°C in the deep freeze. Some of the properties of this powder are shown in Table 2,1.

	Microsomes	Butanol powder
Total protein,(mg)	260	243
Cytochrome P-450,(nmoles)	125	117
Cytochrome P-420,(nmoles)	16	14
Cholesterol,(μg/mg protein)	18	0.1
Phospholipid,(mg/mg protein)	0.6	0.3
Percentage conversion of cholesterol to 7α-hydroxy-cholesterol	9.4	10.0

Table 2,2.

Some properties of a rat liver microsomal butanol powder, prepared as described in the text.

F Preparation of a butanol powder from rat liver microsomes

Microsomes were resuspended in distilled water and freeze dried. About 3g of the lyophilised powder were then homogenised in 500ml of n-butanol, cooled to -30°C. Butanol at this temperature is rather viscous and filtration is very slow, therefore the homogenate was centrifuged in an MSE 1800, pre-cooling the rotor to -20°C, at 14,000 r.p.m. for approximately 5 minutes. The supernatant was discarded and the pellet resuspended in acetone at ca.-20°C, homogenised in about 300ml acetone, then filtered through a Buchner funnel. The powder was washed with acetone until all butanol was removed. After being kept in vacuo for 1 hour at room temperature the powder was kept at -20°C in the deep freeze. The powder was very stable and could be kept for two months at -20°C with no apparent loss of cholesterol 7α-hydroxylase activity. Some properties of the powder are shown in Table 2,2 and it is clear that loss of cytochrome P-450 to cytochrome P-420 in this preparation was reduced compared with the acetone powder preparation.

G Determination of cholesterol

Cholesterol was measured either by gas liquid chromatography or by a modification of the Liebermann-Burchard reaction (36). In both cases, [4 - ^{14}C] cholesterol was added to the microsomal suspension to serve as a recovery marker. Lipid was extracted by chloroform/ methanol, 1:1, and the extract applied to a thin layer plate which was developed in diisopropylether, petroleum ether, 70:30. The [4 - ^{14}C] cholesterol was located using a Panax thin layer radioactive scanner. The sterol was eluted from the silica gel and scraped from the plate eluting with either acetone or diethyl ether. A proportion of this extract was removed and the radioactivity counted in the liquid

scintillation spectrometer to determine the recovery of cholesterol.

The Liebermann-Burchard Test

The cholesterol sample was evaporated to dryness and redissolved in chloroform. 1ml of sample was added to 2ml of the reagent consisting of 95% acetic anhydride and 5% concentrated sulphuric acid. After leaving for exactly 0.5hr. at room temperature, the absorbance at 625nm was determined. In this colour reaction the chromophore obeys Beer's law up to 500 µg cholesterol in 3ml of the final reaction mixture.

Gas liquid chromatography

The cholesterol sample in acetone was applied to a column of 100-120 mesh Gas-Chrome Q on which was adsorbed 1% SE 30 in a Pye 104 gas chromatogram. The temperatures of the injection port, column oven and detector oven were 300°C, 235°C and 240°C respectively. The flow rates of both nitrogen carrier gas and hydrogen were 30ml/minute. Detection of the sample was by flame ionisation. As an internal standard, pregnenolone acetate was added to the sample and the ratio of peak heights of pregnenolone acetate to cholesterol was compared to a standard curve of actual ratio to observed ratio of pregnenolone acetate to cholesterol.

H Determination of phospholipid

Phospholipid was measured by a modification of the method of Zilversmit et al (37). Phospholipid was precipitated from the sample by 5% trichloroacetic acid, and after centrifugation the pellet was washed with 5% trichloroacetic acid. The pellet after centrifugation was suspended in 1ml water and 1ml 60% perchloric acid was added. The mixture was boiled until the brown colour which developed finally disappeared. Phosphate was then assayed by adding

water to 3.5ml followed by 0.5ml 8.3% ammonium molybdate, and 1ml of 1% amidol in 20% sodium metabisulphite. After exactly 15 minutes the absorbance at 720nm was determined. The mass of phosphorus so determined was multiplied by 25 to convert it into the mass of phospholipid. This colour reaction was useful in the range 1 to 25µg phosphorus.

I Glucose-6-phosphatase assay

Glucose-6-phosphatase was assayed by adding the test sample to 4ml 0.05M maleate buffer pH 6.5 containing 28mg glucose-6-phosphate, and incubating for 0.5hr. at 37°C. The reaction was stopped by addition of 0.5ml, 0.8% zinc sulphate and 0.5ml, 0.72% barium hydroxide. 1 drop of phenolphthalein solution was added and alkali added until the indicator turned red. The glucose liberated was assayed essentially as described by Gardner (38) by oxidation with glucose oxidase and reduction of the hydrogen peroxide formed with peroxidase with the concomitant oxidation of gum guaiacum. Absorbance of this chromagen at 625nm was measured. The determination of glucose was performed using the Technicon autoanalyser.

J Alkaline phosphatase

This enzyme was measured as described by Kachmar (39).

K Aryl esterase

This was determined according to the method of Rommerts (40).

L Aryl sulphatase was assayed by adding 0.2ml of the enzyme fraction to 1ml 0.1M acetate buffer pH 6.2 containing 0.4mg p-nitrophenyl sulphate.

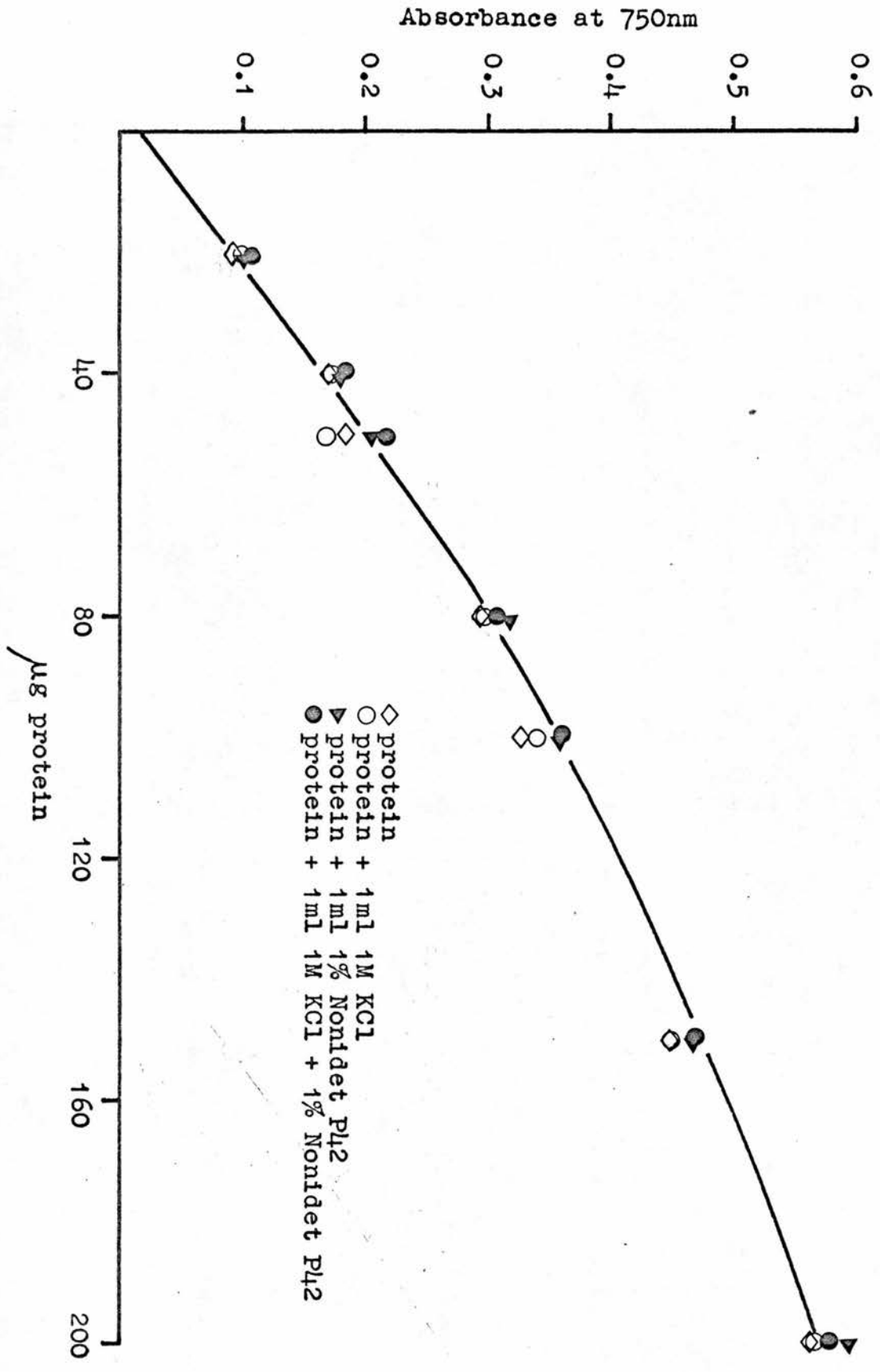


Figure 2,2.

The effect of Nonidet P42 and KCl on the measurement of protein.

After incubation for 25 minutes at 37°C, the reaction was stopped and the colour developed by addition of 2ml 0.5M NaOH. The absorbance at 405nm was determined.

M RNA and DNA were measured by the method of Schneider (41). No DNA was detectable in the microsomal fraction.

N Protein was assayed either according to the method of Lowry (42) or by the biuret method (43). When the protein sample contained either KCl or Nonidet P42 a precipitate formed in the presence of the Folin-Ciocalteu reagent, but Figure 2,2 shows that when the sample was centrifuged, no difference in the absorbance between the sample and the control was detected.

O Assay for benzphetamine demethylase activity

Benzphetamine is N-demethylated by liver microsomes in the presence of NADPH with the concomitant release of formaldehyde (32). To a final volume of 8ml of 0.1M potassium phosphate buffer pH 7.4 was added about 18mg microsomal protein or 7 nmoles cytochrome P-450, 2.74mg benzphetamine hydrochloride dissolved in 100µl ethanol to give a final concentration of 1.25mM, 5µmoles NADP, 50µmoles glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase. After 0, 5, 10 and 15 minutes, 1.5ml were removed and added to 1.5ml 15% trichloroacetic acid. After centrifugation, 2ml of the supernatant were removed and 2ml Nash reagent (2M ammonium acetate, 50mM acetic acid and 20mM acetyl acetone) were added (44). The colour was developed for 8 minutes at 60°C and the absorbance at 412nm measured using a Unicam SP 600. An absorbance of $8mM^{-1}cm^{-1}$ was used to determine the concentration of formaldehyde. It was shown that Nonidet P42 did not affect the development of the colour.

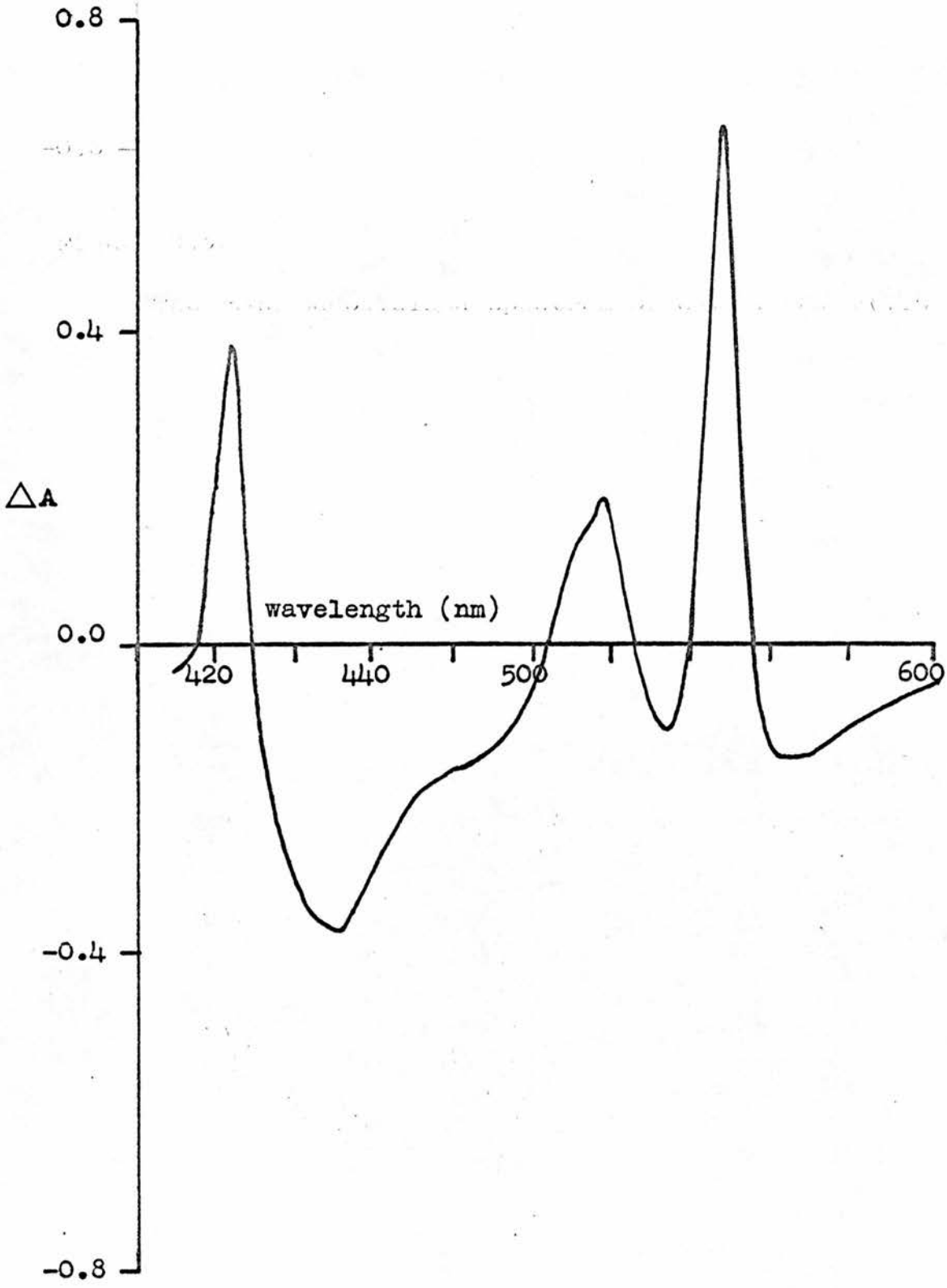


Figure 2,3.

The reduced-oxidized spectrum of cytochrome c(1.5mg/ml)

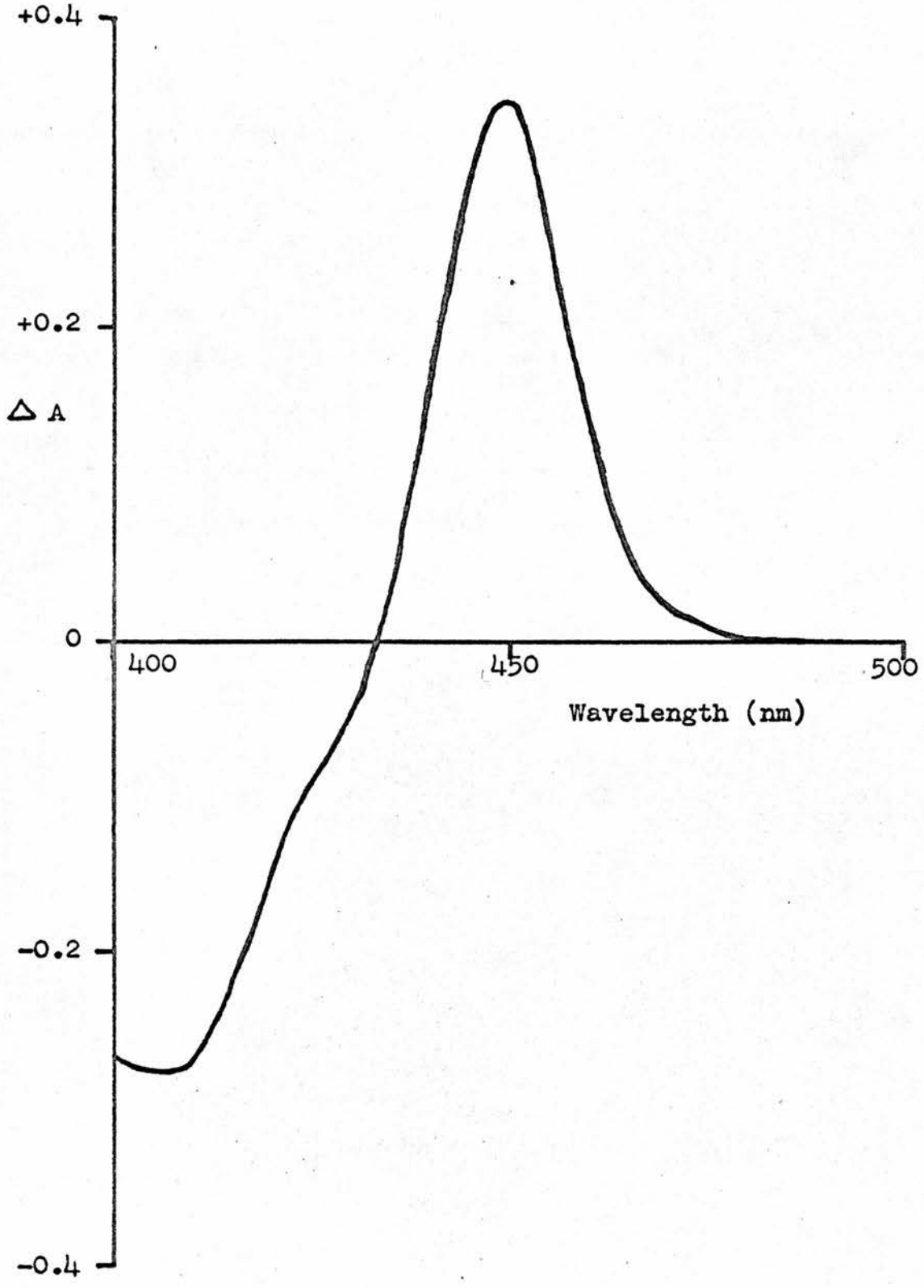


Figure 2,4.

The(reduced + CO) - (reduced) difference spectrum of rat liver microsomal cytochrome P-450.

P Assay of NADPH-cytochrome c oxidoreductase

To 2.8 ml of 0.1 M potassium phosphate buffer pH 7.7 were added 50 µl, i.e. 1 mg cytochrome c, 100 µl of NADPH generator (0.5 µmoles NADP, 5 µmoles glucose-6-phosphate and 0.5 I.U. of glucose-6-phosphate dehydrogenase). The reaction, performed at 25°C, was started by addition of the flavoprotein containing sample. The velocity of reaction was measured in an Aminco-Chance, dual wavelength/split beam recording spectrophotometer in the dual wavelength mode. The difference in absorbance between 551 nm and 540 nm was recorded as a function of time. An extinction coefficient of 19 $mM^{-1}cm^{-1}$ was used as the difference between reduced and oxidised cytochrome c. A reduced-oxidised difference spectrum of cytochrome c is shown in Figure 2,3. 1 unit is defined as being the amount of flavoprotein which will produce a change in $absorbance_{(551-540)}$ of 1 per minute.

Q Assay of cytochrome P-450

Cytochrome P-450 concentration was determined essentially as described by Omura and Sato (45) in an Aminco-Chance spectrophotometer in the split beam mode. Thus, to approximately 6 ml of sample were added a few grains of sodium dithionite. After recording a base line spectrum, carbon monoxide was gently bubbled through the sample cuvette for about 30 seconds. A recording was then made of the CO-reduced minus reduced difference spectrum and the concentration of cytochrome P-450 determined by measuring the absorbance at 450 nm relative to 490 nm, and using an extinction coefficient of 91 $mM^{-1}cm^{-1}$. A reduced CO-reduced spectrum of rat liver microsomes is shown in Figure 2,4.

R Measurement of cytochrome b_5

In the presence of cytochrome P-450 and NADH-cytochrome c

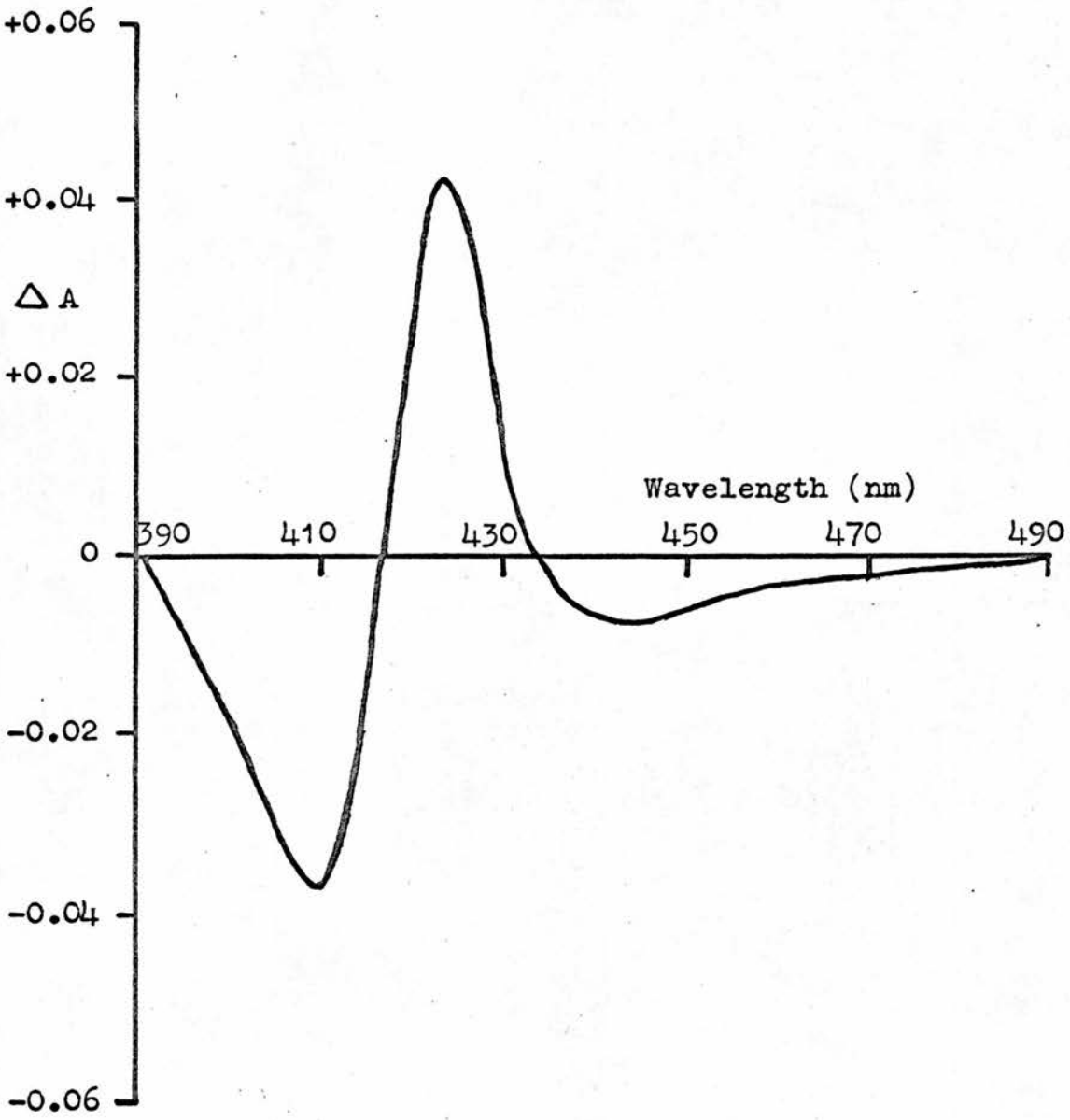


Figure 2,5.

A reduced-oxidized difference spectrum of cytochrome b_5.

reductase, cytochrome b_5 concentration was determined by observing the difference in absorbance between 424 and 409 nm. observed when a few grains of NADH are added to the sample cuvette during difference spectroscopy. In the absence of both NADH-cytochrome c reductase and cytochrome P-450, cytochrome b_5 was reduced by sodium dithionite. An extinction coefficient of 185 $mM^{-1}cm^{-1}$ was used for the difference in absorbance between 424 nm and 409 nm. A reduced-oxidised difference spectrum of cytochrome b_5 is shown in Figure 2,5.

S Measurement of difference spectra

Microsomal suspensions containing about 6 mg protein per ml were added to both sample and reference cuvettes in the Aminco-Chance spectrophotometer, set in the split beam mode. A scan of the spectrum was made before any addition of mixed function oxidase substrates, amines or ethanol. After addition of a solution of a compound to the sample cuvette and the same volume of solvent to the reference cuvette, a scan of the spectrum between 350 nm and 500 nm was made.

T Absolute spectra of partially purified cytochrome P-450 were recorded using a Pye-Unicam SP 1800.

U Electron paramagnetic resonance spectra were obtained using a Varian E4 machine, operating at -172°C.

V DEAE-cellulose was prepared as described by Peterson et al (46). K Cl concentrations were determined using an E.E.L. chloride meter, and phosphate concentrations during chromatography were determined conductimetrically. Linear gradients of KCl were generated as described by Peterson and Sober (46).

W Affinity chromatography

Cyanogen bromide activated Sepharose 4B was either purchased from Pharmacia, Uppsala, Sweden, or prepared by the method described by March et al (47).

Cytochrome c was covalently bound to the CNBr activated Sepharose 4B essentially as described by Golf et al (48). 30g CNBr activated Sepharose 4B (Pharmacia) was prepared by washing with 5 litres 1mM HCl pH 3. The beads were then equilibrated with 0.1M phosphate/borate buffer pH 9.0, and the suspension at 4°C was poured into a solution containing 2g cytochrome c to give a final volume of 200ml. The reaction was allowed to proceed for 20 hours at 4°C. At the end of this period, 1M ethanolamine was added and left for 3 hours at 25°C to allow reaction with unreacted CNBr. The beads were then washed with 0.1M sodium acetate buffer pH 4 and 1M sodium chloride, followed by 0.1M borate/phosphate buffer pH 9 and 1M sodium chloride. The process of washing was repeated twice.

Octylamine was bound to cyanogen bromide activated Sepharose 4B as described by Cuatrecasas (49).

X Phospholipase A was prepared from the venom of Crotalus adamanteus as described by Tzagaloff et al (50). The activity was tested by adding an aliquot of enzyme solution to a final volume of 3ml of 10mM glycylglycine buffer pH 8.95, containing 6.7mg lecithin/ml, 20 μl Tween 80/ml, 0.014mg cresol red/ml, 0.5mg $CaCl_2$/ml and 0.05mg $MgCl_2.6H_2O$/ml. 1 unit corresponds to a ΔA_{546}/min. of 0.110 at 25°C.

Y Materials

All common reagents were purchased from B.D.H. or Sigma

and were of Analar grade.

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

Crotalus adamanteus venom, Naja naja venom, pancreatin, sodium cholate, sodium deoxycholate, Lubrol WX and DEAE-cellulose (0.8meq/g) were obtained from Sigma, London.

Triton X-100, digitonin, saponin, Nonidet P40 and Nonidet P42, ammonium sulphate, polyethylene glycol 6000, calcium phosphate gel, egg lecithin and phenobarbital, were supplied by B.D.H. Chemicals Ltd., Poole, England.

Benzphetamine hydrochloride was obtained from Upjohn Co. Kalamazoo, Michigan.

DCC-Trypsin was supplied by Serva, London.

Tween 80 and cyanogen bromide, PPO and POPOP, were purchased from Koch-Light, England.

Bio-gel A 0.5M and hydroxylapatite were obtained from Bio-Rad, England.

Sephadex and Sepharose were supplied by Pharmacia, Uppsala, Sweden, and CM-cellulose and cellulose phosphate by Whatman, England.

Diamino-octane was purchased from Ralph N. Emanuel Ltd., London, and L-α-lecithin (β, γ dipalmitoyl) from Calbiochem, London.

Cholestyramine resin (Cuemid) and Kieselgel H were products of Merck, Sharp & Dohme.

$[4\text{-}{}^{14}C]$ cholesterol, $[22\text{-}23\text{-}{}^{3}H]$ sitosterol, $[26\text{-}{}^{14}C]$ desmosterol, were obtained from N.E.N. Chemicals GmbH. Other steroids radioactive and radioinactive were synthesized in this laboratory by Dr. K.E. Suckling.

Superoxide dismutase was a gift from Dr. R.C. Bray, Sussex.

SECTION 3

ATTEMPTS AT THE SOLUBILIZATION OF CHOLESTEROL 7α-HYDROXYLASE

Cholesterol 7α-hydroxylase activity resides in the microsomal fraction of rat liver, a fraction of which has been defined operationally as the high speed pellet resulting from the centrifugation of the post-mitochondrial supernatant (the 10,000 x g supernatant) at 100,000 to 250,000 x g for 1 to 2 hours (51). Microsomes are a heterogeneous mixture of the membranes of the endoplasmic reticulum and contain nucleic acids, and lipids which create a hydrophobic environment for the large number of enzymes contained in this cell fraction. It is still unclear how enzymes are organised in the microsomal membranes and how the phospholipid molecules are arranged around the microsomal protein. Schulze and Staudinger (52,53) have proposed a model in which the lipid is interspersed in bilayers as islands between the protein molecules. Archakov, on the other hand, suggests on different experimental evidence that there is sufficient lipid to form a continuous bilayer over which is bound, by hydrophobic interactions, a third layer of protein (54). The structure of membranes, however, does seem to be dependent on the lipid composition rather than the protein composition (55). Whatever the actual structure of endoplasmic reticulum or microsomes may be, the practical result of this protein-protein, protein-lipid interaction is that microsomal enzymes are insoluble. This insolubility could be the result of several factors. With cholesterol 7α-hydroxylase, insolubility could be the result of strong ionic interactions with other proteins, phospholipid or RNA. If this were the case, solubilization might be expected upon treatment of microsomes with salts at high ionic strength. Such an apparent solubilization has been reported by Scholan and Boyd (26) in which a desiccated preparation of microsomes was

suspended in 1M potassium phosphate buffer and subjected to centrifugation at 105,000 x g for 50 min. Treatment with 1M potassium phosphate buffer resulted neither in inhibition of cholesterol 7α-hydroxylase activity nor in degradation of cytochrome P-450 to cytochrome P-420.

The criterion of solubilization, that of the enzyme remaining in the supernatant fraction after centrifugation at 105,000 x g for 50 min., is questionable here since the density of 1M phosphate is relatively high and the "solubilization" may merely have been a buoyancy effect. This criterion in more general cases is also unsatisfactory as phospholipids and detergents may alter the partial specific volume of proteins (56). A more useful criterion is perhaps the ability to remove interfering enzymatic activities from the "solubilized" preparation by fractionation techniques such as chromatography or differential precipitation. The "solubilized" preparation described by Scholan (26) when applied to Sephadex G-200 partitioned into 2 gross fractions which when assayed alone for cholesterol 7α-hydroxylase activity resulted in a percentage conversion to 7α-hydroxycholesterol approximately one-third that when the two fractions were recombined. It was clear, therefore, that the enzyme complex was not completely resolved.

A more likely cause of the enzyme's insolubility is that of hydrophobic interactions between proteins or between protein and either neutral lipid or more particularly phospholipid, as microsomal membranes are composed of approximately 50% by weight of phospholipid. This idea of hydrophobic interaction is strengthened by the finding that cytochrome b_5 has two domains, a hydrophobic and a hydrophilic region, bridged by a part of the polypeptide chain rich in helical

structure (57). The hydrophobic domain may be separated from the hydrophilic domain by incubating microsomes with trypsin. Cytochrome b_5 may be split by this technique into a peptide which retains some of its original catalytic activity, and a peptide abnormally high in hydrophobic residues which is essential for binding to microsomes (58).

Another, perhaps less likely, possibility is that the enzyme complex is covalently linked to a macromolecular matrix of lipid, carbohydrate or other protein such as the hydrophobic protein found in mitochondria and described by Capaldi et al (59). With these possibilities in mind, methods were used to try to weaken or break the bonds associated with insolubility, or to make a quasi-aqueous environment, thermodynamically favourable to proteins which are essentially hydrophobic.

Physical Methods

Microsomes prepared as described in the Methods section were resuspended several times and rehomogenised in 0.154M KCl and centrifuged at 105,000 x g for 1 hour, but this repeated washing of native microsomes failed to solubilize any significant quantity of cytochrome P-450 or cholesterol 7α-hydroxylase activity.

Ultrasonication has been a technique used for the solubilization of a number of enzymes such as the NADP dependent 17β hydroxysteroid dehydrogenase from porcine testicular microsomes (60), though this method is only of value in releasing inherently water soluble proteins from an insoluble matrix. It has been reported that during ultrasonication localised temperatures of 10,000^{o}K may be momentarily generated, with pressure waves of 10^6 atmospheres (61). These shock waves are clearly disruptive and enzymic activities are often reduced. However, there is evidence to suggest that with molecules

the size of proteins, damage caused by chemical effects is greater than that due to mechanical disruption (62), and it is thought that these chemical effects are largely the result of generation of free radicals such as the hydroxyl or hydroperoxyl radical. Nevertheless, the use of this harsh technique failed to release into the supernatant cholesterol 7α-hydroxylase activity.

Freezing and thawing has recently been used by Heller and Gould (63) for the solubilization of β-hydroxyβ-methyl glutaryl-CoA reductase. This technique was applied to rat liver microsomes, lowering the temperature gradually to -70°C. After thawing the microsomes, they were resuspended in 0.1M potassium phosphate buffer and centrifuged for 1 hour at 105,000 x g. The resultant supernatant contained no cytochrome P-450 and little cholesterol 7α-hydroxylase activity.

Approximately 50% of the dry weight of rat liver microsomes is composed of lipid, most of which is phospholipid. Therefore, an acetone powder was made from a suspension of native microsomes as described in the methods section, and because of the reported insolubility of phospholipids in acetone (64), a lipid depleted preparation of microsomes was made by extraction of a lyophilized powder of microsomes with n-butanol. Some characteristics of these powders are shown in Tables 2,1 and 2,2. It is interesting that even after suspending and homogenizing desiccated microsomes in this organic solvent, only 50% of the original phospholipid could be removed. Either there is some phospholipid which is not readily soluble in butanol or the binding of residual phospholipid to protein is extremely tenacious.

When these powders were subjected to repeated washing or ultrasonic cavitation as before, no cholesterol 7α-hydroxylase was released into the 105,000 x g supernatant. Since an acetone powder

preparation of rat liver microsomes retains cholesterol 7α-hydroxylase activity, is partially lipid depleted, and provides a convenient stock of enzyme, subsequent investigation of methods to solubilize cholesterol 7α-hydroxylase were performed using such preparations.

Effect of hydrolytic enzymes

Considerable success has been achieved in the solubilization of lipid bound proteins using hydrolytic enzymes. Trypsin has been used for the purification of NADPH-cytochrome c oxidoreductase by Roerig et al (65), and cytochrome b_5 by Strittmatter (66,67,68); cathepsin D has been used by Ito (69) to solubilize NADH-cytochrome b_5 reductase, whilst leaving cytochrome b_5, NADPH-cytochrome c reductase and cytochrome P-450 still membrane bound. Lipase or steapsin has been useful in the solubilization of the NADPH-cytochrome c reductase first demonstrated in a whole liver acetone powder by Horecker (70) which was subject to further purification (71,72,73). It has been suggested, however, that the active principle in the lipase/steapsin preparation is a trypsin contaminant (74). As will be demonstrated in the section on reconstitution, the catalytic properties of such trypsin solubilized preparations can be fundamentally changed.

Action of Phospholipase A

It was possible that the binding of cholesterol 7α-hydroxylase to the microsomal membranes was dependent on the integrity of the phospholipid. Phospholipase A (EC 3.1.1.4) has been used with some degree of success in the solubilization of rat liver microsomal acetanilide-hydrolysing esterase. This lipase did not solubilize NADPH-cytochrome c reductase or cytochrome b_5, but did result in an inhibition of the esterase which was nevertheless solubilized (75).

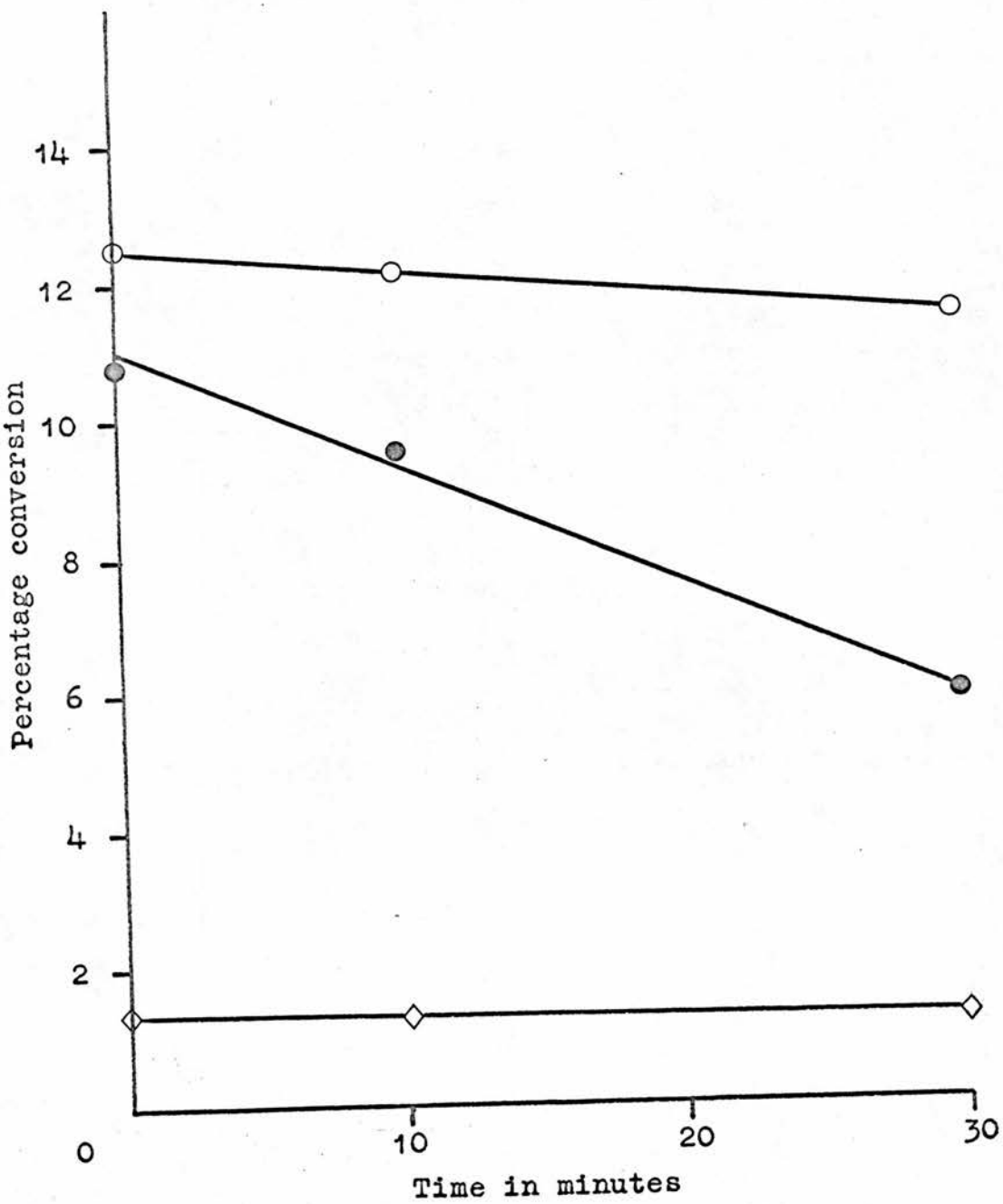


Figure 3,1.

The effect of incubating 120 μl phospholipase A with 100mg acetone powder at 37°C.

○ Control activity

◇ Supernatant activity

● Pellet activity

Phospholipase A was prepared from the venom of Crotalus adamanteus as described in the Methods section; this had an activity of 1 unit/ml. The results of incubating 120µl of phospholipase A with 100mg of an acetone powder are shown in Figure 3,1. The phospholipase A was incubated with acetone powder suspension for 0, 10 and 30 minutes at 37°C in 10mM Tris pH 7.7 containing 1mM $CaCl_2$ for the stimulation of phospholipase A activity. After cooling the reaction mixtures in ice, the suspension was centrifuged at 105,000 x g and the supernatant and resuspended pellet assayed for cholesterol 7α-hydroxylase in each case. No detectable cytochrome P-450 or protein was released into the supernatant, neither was there an increase in cholesterol 7α-hydroxylase activity. There was, however, a decline in activity after 30 minutes in the pellet when compared with control values. Since NADPH-cytochrome c reductase has been reported as not being solubilized, the inactivation of the enzyme on incubation with phospholipase A may be due to the detergency of lysolecithin, the product of reaction catalysed by this enzyme.

Effect of Naja naja venom

An acetone powder was incubated at 37°C for 0.5hr. with 5µg and 1mg venom/40mg acetone powder. Cytochrome P-450 was not released into the supernatant fraction after centrifugation at 105,000 x g neither was cholesterol 7α-hydroxylase activity solubilized. Little effect on either the resuspended pellet or the treated suspension was observed after incubation with N.naja venom.

Effect of pancreas acetone powder

Pancreatin is a complex mixture of enzymes such as α-amylase, lipase, zymogens, ribonuclease and deoxyribonuclease.

Incubation for 0.5hr. with 5mg pancreatin/60mg acetone powder suspension did not result in any solubilization of cholesterol 7α-hydroxylase activity.

Action of 3M urea

Treatment of an acetone powder with 3M urea at 0^{o}C led to total inactivation of cholesterol 7α-hydroxylase activity in the suspension, the supernatant and the resuspended pellet. Furthermore, disruption of cytochrome P-450 was so complete that neither a characteristic cytochrome P-450 nor a cytochrome P-420 spectrum could be observed.

Treatment of the acetone powder with n.butanol

The technique of disrupting protein-lipid interactions by n-butanol has been extensively reviewed by Morton (76) and many enzymes have been solubilized using n-butanol (77). The usefulness of this organic solvent over other solvents such as chloroform, ether and higher alcohols, appears to be due to its detergent-like properties, and it is thought that the bonds involved between phospholipid and protein are in competition with butanol and protein. In the attempt to solubilize cholesterol 7α-hydroxylase, both single phase and 2-phase systems were used. In the former, an 8% solution of butanol (max. solubility 10.5% at 0^{o}C) was used, and in the latter a 25% v/v mixture, in combination with sonication, was employed.

In the first experiment, treatment of an acetone powder suspension in 0.1M phosphate buffer with butanol added to produce an 8% v/v solution, followed by sonication for 2 minutes, resulted in cholesterol 7α-hydroxylase activity being released into the 105,000 x g supernatant. The percentage conversion of radioactive cholesterol to

radioactive 7α-hydroxycholesterol in this supernatant was 3.1% and in the pellet 1.8%. This compares with the control value of 7.1%. Because of this initial promising result, attempts were made to improve the technique, but these subsequent experiments did not result in solubilization of cholesterol 7α-hydroxylase, and the activity in the suspensions and pellets was inhibited or inactivated, this inhibition being more emphasised when the material was sonicated, or when a 2-phase system was used. Assay of the supernatant for cytochrome P-450 revealed that approximately 20% of cytochrome P-450 from the original acetone powder suspension was solubilized. One interesting observation which emerged from these studies was that on treatment with 8% butanol, formation of cytochrome P-420 was extensive. Upon dilution, however, the cytochrome P-450 content increased and the cytochrome P-420 content decreased. The formation of this catalytically inactive form of cytochrome P-450, therefore, seems to be partially reversible in this system on decreasing the concentration of n-butanol. This reconversion of cytochrome P-420 to cytochrome P-450 has also been demonstrated by Ichikawa and Yamano (78).

The effect of bile acids, sodium cholate and sodium deoxycholate on an acetone powder and cholesterol 7α-hydroxylase

Since 1968, the key to the solubilization of rat and rabbit liver microsomes has been the detergency of both sodium cholate and sodium deoxycholate. The original method of solubilization of cytochrome P-450 with retention of some enzymic activity was devised by Lu and Coon (79), and most other workers in this field of mixed function oxidase systems in liver microsomes have continued to use this general method (80,81,82). Sodium cholate has also been used in the solubilization of cytochrome P-450 from bovine adrenal mitochondria (83). An acetone powder was suspended in 0.1M phosphate buffer, pH 7.4 and

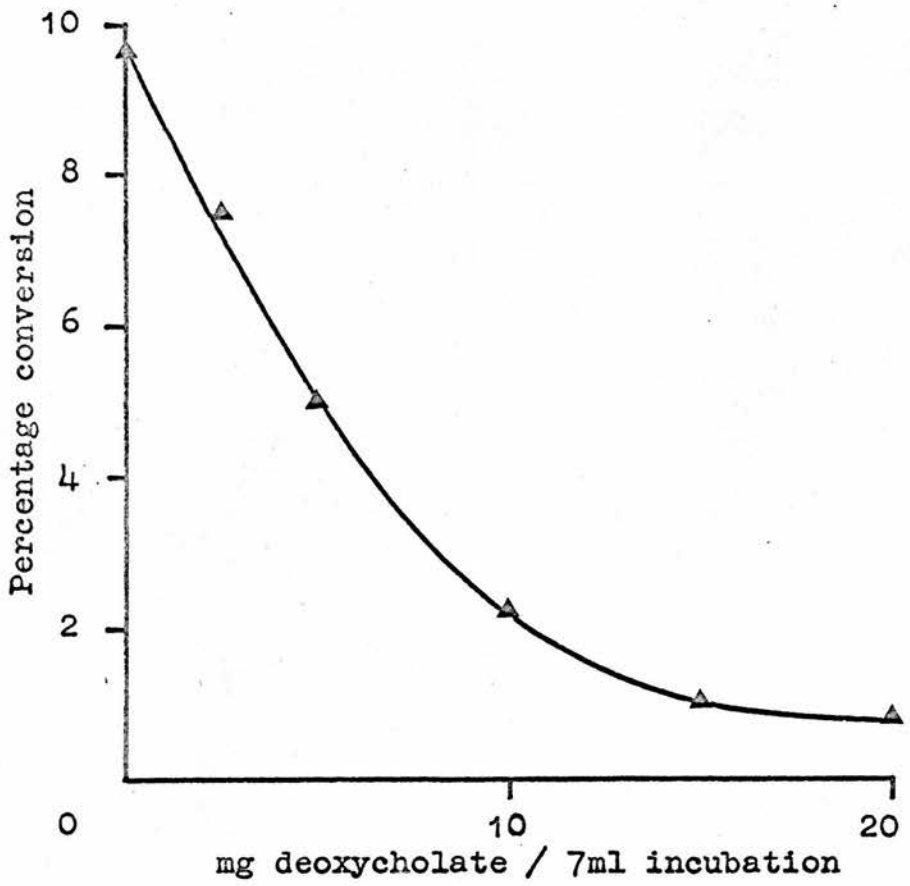


Figure 3,2.

The effect of sodium deoxycholate on cholesterol 7α-hydroxylase activity in 30mg (12mg protein) resuspended acetone powder.

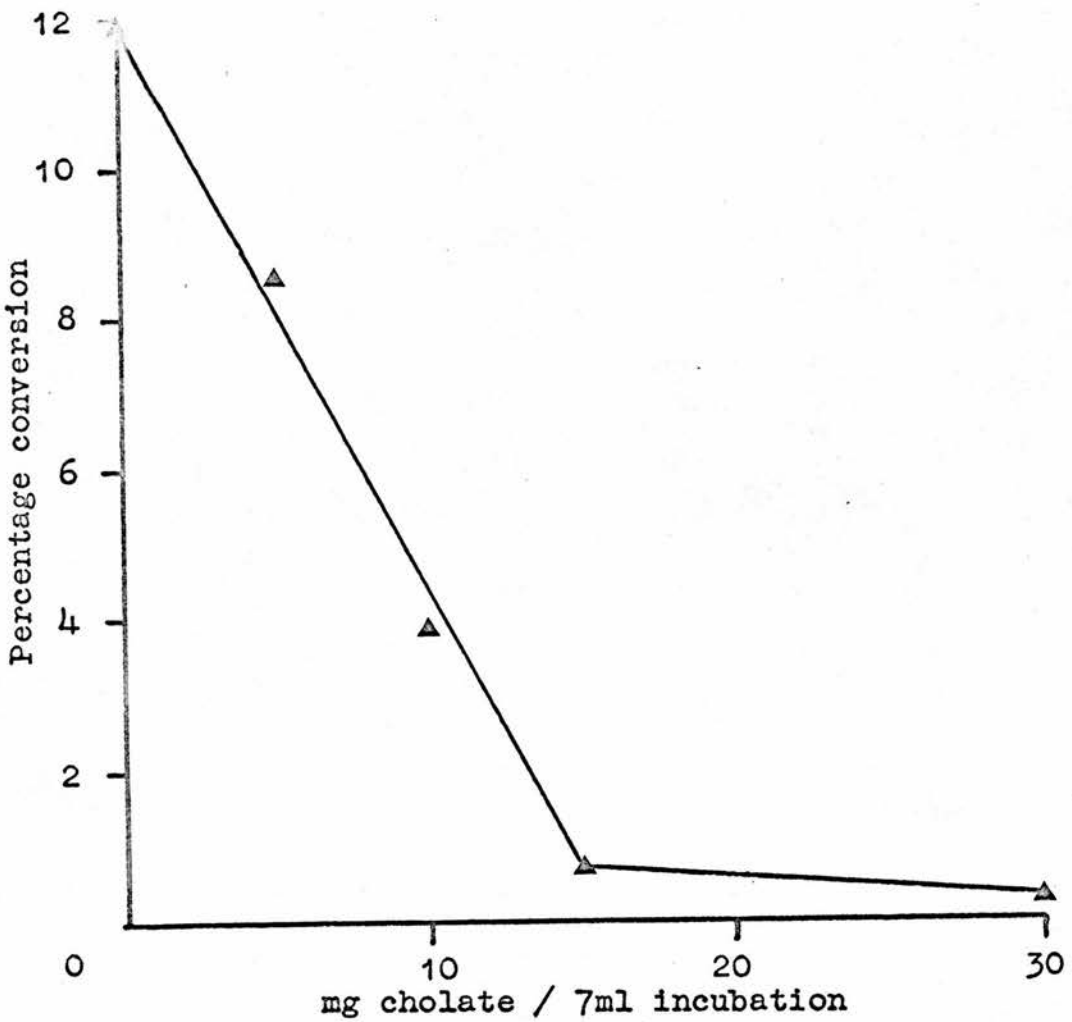


Figure 3,3.

The effect of sodium cholate on cholesterol 7α-hydroxylase activity in 30mg (12mg protein) resuspended acetone powder.

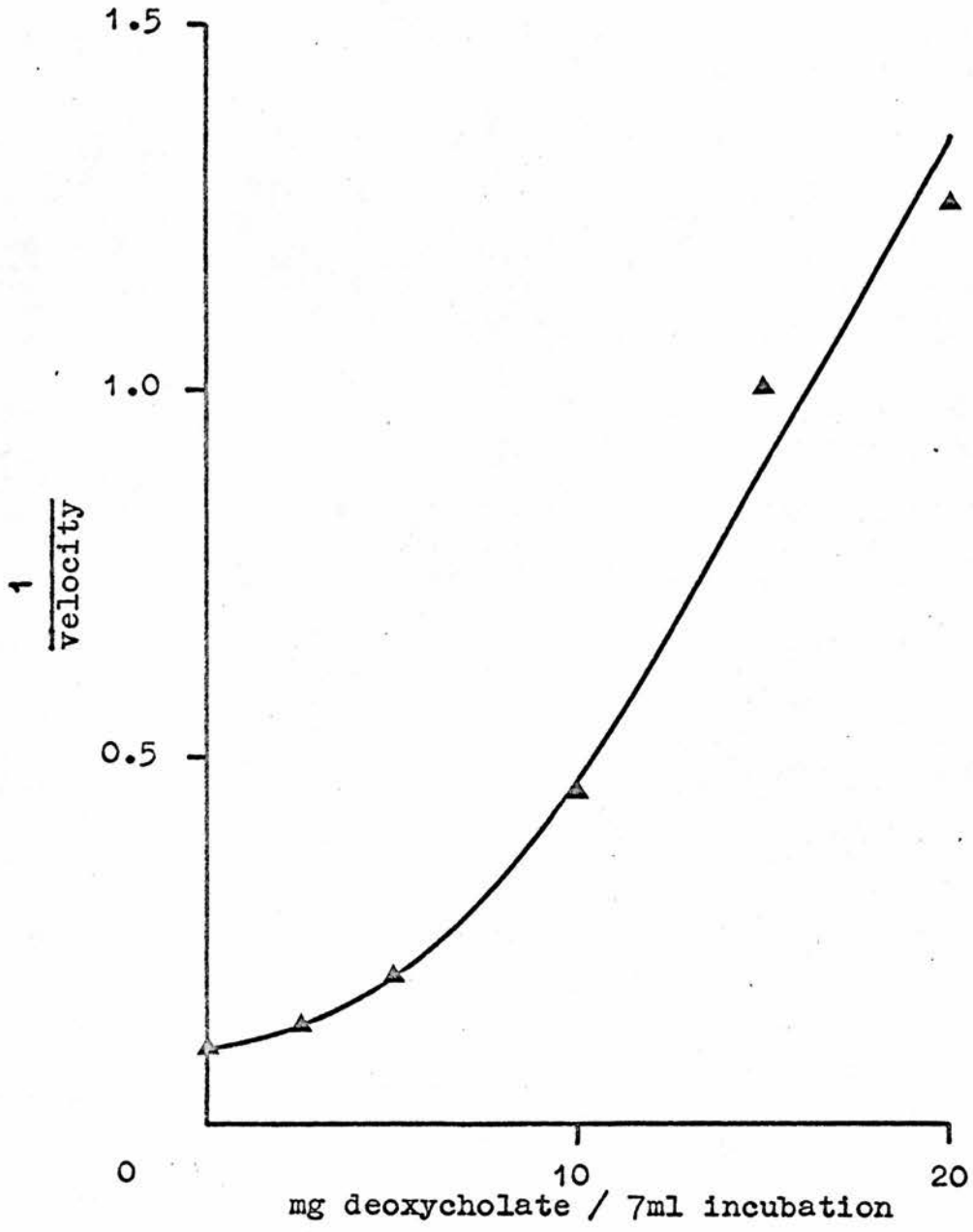


Figure 3,4.

A Dixon plot of results presented in figure 3,2.

(velocity is equivalent to percentage conversion)

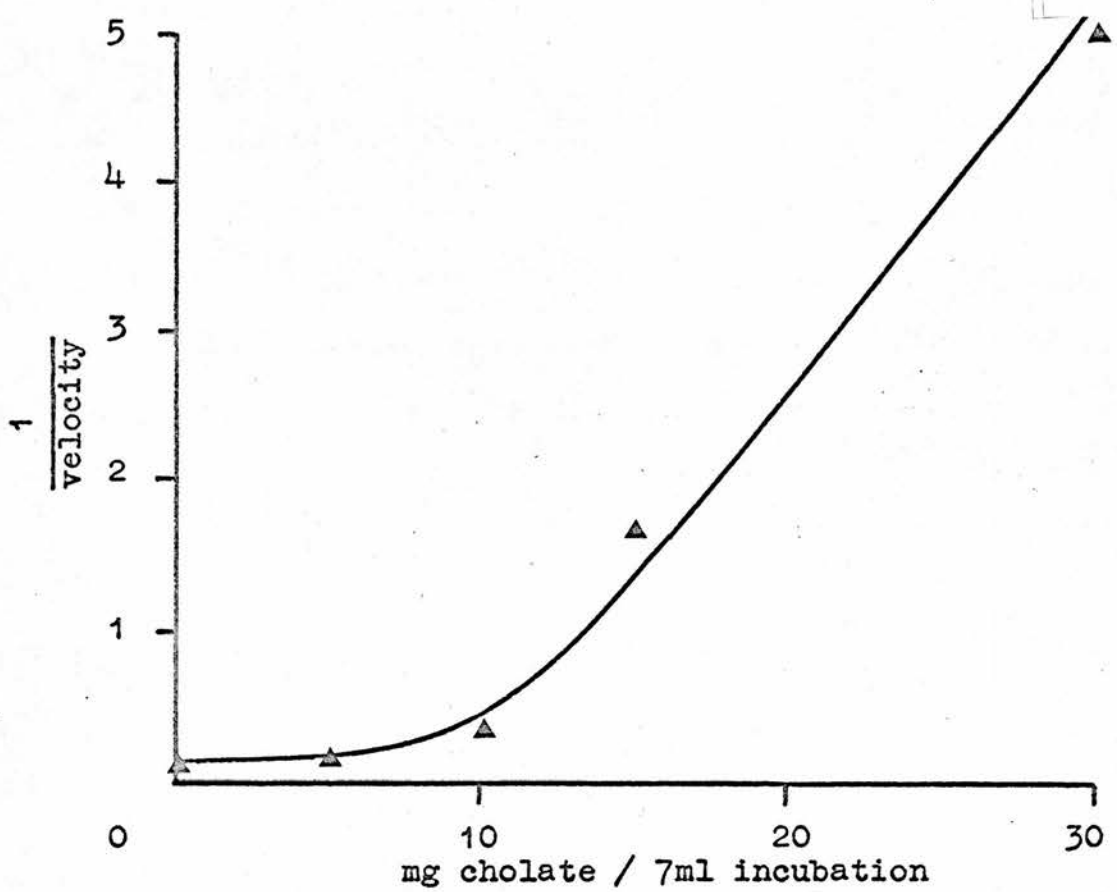


Figure 3,5.

A Dixon plot of results presented in figure 3,3.

(velocity is equivalent to percentage conversion)

increasing quantities of the bile salts, sodium cholate and sodium deoxycholate were added, and the results of these experiments are shown in Figures 3,2 and 3,3. Figures 3,4 and 3,5 are Dixon plots of $\frac{1}{v}$ vs $[I]$ (84). It is quite clear that both bile salts are very inhibitory, but the type of inhibition observed is complex. It is neither purely competitive nor purely non-competitive inhibition and might represent denaturation of the enzyme as well as inhibition.

The ratio of sodium cholate to microsomal protein generally used to solubilize mixed function oxidase systems is 1mg. cholate/mg. protein. This ratio was used by Bernhardsson et al (85) and Bjorkhem et al (86) in the solubilization ~~of~~ and reconstitution of the 12α-hydroxylase of 7α-hydroxycholest-4-ene-3-one, and the 7α-hydroxylase of cholesterol, but at this concentration the latter enzyme is inhibited by approximately 80%.

It has been reported by Dean et al (87) that deoxycholate used in the solubilization of hog liver microsomes suspended in 0.1M phosphate buffer could be removed by precipitation with $CaCl_2$. After solubilization of an acetone powder with deoxycholate and centrifugation at 105,000 x g, the resultant supernatant was treated with a mass of $CaCl_2$ equimolar to the deoxycholate and then re-centrifuged. Cholesterol 7α-hydroxylase activity in this treated supernatant could not, however, be demonstrated.

Having observed inhibition of cholesterol 7α-hydroxylase with anionic detergents, a cationic detergent, cetyl trimethylammonium bromide, was tested for its ability to solubilize a resuspended acetone powder. A 0.1% and 2% final concentration of this detergent was used, and even at the low concentration cholesterol 7α-hydroxylase was inactive, and it was also observed that the normally fine suspension of

acetone powder had formed a flocculent precipitate.

The effect of non-ionic detergents on the activity and solubilization of cholesterol 7α-hydroxylase

Although most of the reported successes in solubilization of mixed function oxidases from microsomes have resulted from the use of the two bile salts, cholate and deoxycholate, there nevertheless have been achievements using non-ionic detergents particularly from workers from the University of Osaka. One of the early reports showed the solubilization of cytochrome P-450, but no enzyme activity was demonstrated (88). Solubilization of a fatty acid ω-hydroxylase from porcine kidney microsomes (89) and porcine liver microsomes (90) using Triton X-100 has been reported, and for enzymes not sensitive to sodium cholate, the inclusion of this bile sale as well as the non-ionic detergent has been used (81,91,92).

There is now commercially available a very great variety of non-ionic detergents, and their advantages over ionic detergents are that they are relatively mild surface active agents. Furthermore, a solubilized preparation of microsomes can be applied to ion-exchange columns without the interference of the ionically charged detergents. Their major drawback, however, is that they are very difficult to dissociate from protein. Given such a large number of non-ionic detergents, it was clear that the necessary conditions for solubilization of cholesterol 7α-hydroxylase with all these compounds could not be examined. As a result, for each detergent tried, a low concentration, 0.1%, was used to determine whether this concentration was inhibitory, and a high concentration, 2%, was used to detect whether the detergent was capable of solubilization of the microsomal membranes.

Digitonin

When digitonin solution was added to a suspension of

acetone powder at 0°C, there was a very rapid clarification. Analysis of the 105,000 x g supernatant revealed that more than 95% of the cytochrome P-450 was solubilized and there was little or no degradation to cytochrome P-420. Difference spectroscopy of the supernatant showed that when aminopyrine was added to the sample cuvette, a type I difference spectrum was generated. This was further proof that digitonin solubilized cytochrome P-450 maintained some of its structural integrity. It was found that NADPH-cytochrome c reductase was solubilized with complete preservation of activity. When assayed for cholesterol 7α-hydroxylase activity in the digitonin treated suspension, the supernatant or pellet, no activity could be detected. Since digitonin forms insoluble aggregates with cholesterol, it was thought that this detergent might be sequestering the radioactive cholesterol tracer. For this reason a digitonin solubilized preparation was passed slowly down a column of small glass beads, on which were coated cholesterol. The object of using this technique was to remove excess digitonin from the solubilized preparation by binding to the cholesterol which was bound to the glass beads. The eluate from the column was turbid and required centrifugation at 105,000 x g for clarification. However, when the sample was tested, no cholesterol 7α-hydroxylase activity was observed.

Lubrol WX

This detergent was used by Gaylor et al to solubilize methyl sterol demethylase (93). Most of the cytochrome P-450 was solubilized by this detergent but no cholesterol 7α-hydroxylase activity was observed when the acetone powder suspension was treated with 0.1% Lubrol WX. A similar inactivation was observed by Gaylor (93) with the demethylase enzyme, but he found that when the concentration of Lubrol WX

was reduced by applying the 105,000 x g supernatant of solubilized microsomes to Sephadex LH 20, in the presence of 20% to 50% ethylene glycol, activity of the enzyme could be restored. Therefore, the supernatant of a Lubrol WX solubilized acetone powder was subjected to chromatography on Sephadex LH 20 and the eluate was tested for cholesterol 7α-hydroxylase activity, but even after this treatment no activity was detectable.

Lubrol W

The use of this non-ionic detergent led to the solubilization of cytochrome P-450 with no concomitant transformation to the inactive form cytochrome P-420, but the activity of cholesterol 7α-hydroxylase was inhibited by approximately 90% in the Lubrol W treated suspension.

Triton X-100

Again, solubilization of cytochrome P-450 with no degradation to cytochrome P-420 ensued when an acetone powder was treated with Triton X-100, but even at the low concentration of 0.1% detergent, cholesterol 7α-hydroxylase activity was abolished.

Saponin

Saponin, like digitonin, is a cardiac glycoside with detergent properties, and although 0.1% Saponin inactivated cholesterol 7α-hydroxylase, unlike the other non-ionic detergents tested, no solubilization of the acetone powder took place.

Tween 80

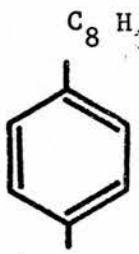
This detergent has been used as a vehicle in which the relatively insoluble cholesterol was delivered as a substrate to

native microsomes (94). 0.1% Tween 80 was capable of solubilizing cytochrome P-450, but inhibition of cholesterol 7∝-hydroxylase, although not as severe as other detergents tested, was nevertheless in the range of 50-75%.

Nonidet P40 and P42

When an acetone powder, resuspended in 0.1M potassium phosphate buffer pH 7.7, was subjected to a final concentration of 0.1% Nonidet P40, clarification of the suspension ensued, and the supernatant resultant from centrifugation at 105,000 x g contained 90% of the cytochrome P-450. In contrast to all other non-ionic detergents used, when the treated suspension or 105,000 x g supernatant was tested for cholesterol 7∝-hydroxylase activity, not only was the enzyme still active but the percentage conversion of [4 - ^{14}C] cholesterol to [4 - ^{14}C] 7∝-hydroxycholesterol was increased by 50-100% over cholesterol 7∝-hydroxylase activity in the control, untreated acetone powder.

Nonidet P42 is a 27% solution of Nonidet P40. This detergent is non-ionic and is an octylphenolethylene oxide condensate (average 9moles of ethylene oxide).



$C_8 H_{17}$

$O - (CH_2 - CH_2O)_n - H$

$n = 9$

SUMMARY

(1) Many procedures have been utilised in an attempt to solubilize rat liver microsomal cholesterol 7α-hydroxylase. These techniques have extended over the whole gamut of solubilization procedures.

(2) Mechanical disruption such as repeated washing and homogenisation, ultrasonication, freezing and thawing, were not effective in the solubilization of mixed function oxidase components.

(3) Treatment of acetone powders with traditional hydrophobic bond disrupting agents such as n-butanol or urea led to the inactivation of cholesterol 7α-hydroxylase.

(4) The use of hydrolytic enzymes was found to be of little value in the solubilization of cholesterol 7α-hydroxylase, or cytochrome P-450, although such treatment was not found to be particularly deleterious to the activity of the enzyme.

(5) The degree of inhibition caused by sodium cholate and sodium deoxycholate, which figure so prominently in the solubilization not only of rat liver microsomes and the associated mixed function oxidase activities, but also in the solubilization and preparation of mitochondrial cytochromes and sub-particles, was considered too great and therefore other detergents were utilised.

(6) With the exception of saponin, all the non-ionic detergents were efficient in the solubilization of cytochrome P-450, conversion to the inactive form, cytochrome P-420, being minimal, but in all of these, with the exception of Nonidet P40, solubilization was at the expense of cholesterol 7α-hydroxylase activity.

Conditions	Suspension	Pellet	Supernatant
Repeated washing and homogenization	Active	Active	Inactive
Ultrasonication	Active	Active	Inactive
Freezing and thawing	Active	Active	Inactive
Phospholipase A	-	Some loss of activity	Inactive
Naja naja venom	Active	Active	Inactive
Pancreas acetone powder	-	-	Inactive
3M urea	Inactive	Inactive	Inactive
8% n-butanol	Inactive	Inactive	Inactive
Sodium cholate 1.5mg/mg protein	70% inhibition	80% inhibition	70% inhibition
Sodium deoxycholate 1.5mg/mg protein	Inactive	inactive	Inactive

Table 3,1.

Conditions	Suspension	Pellet	Supernatant
Cetyl trimethyl-ammonium bromide	Inactive	-	-
Digitonin(1%)	Inactive	Inactive	Inactive
Lubrol WX(0.1% and 1%)	90% inhibition	Inactive	Inactive
Lubrol W (0.1%)	90% inhibition	Inactive	Inactive
Triton X-100 (0.1%)	Inactive	-	-
Saponin(0.1% and 2%)	Inactive	-	-
Tween 80(0.1%)	inhibition	inhibition	Inactive
Nonidet P40(0.1%)	Active	Active	loss of activity
Nonidet P42(0.1%)	Active	Active	loss of activity

Table 3,2.

Tables 3,1, and3,2, summarize the effects of solubilizing agents on cholesterol 7α-hydroxylase from rat liver.

(7) This enzyme can, therefore, be efficiently solubilized by Nonidet P42, with retention, and even increase, of its activity.

These results are summarised in Tables 3,1 and 3,2.

SECTION 4

DETERMINATION OF THE OPTIMAL CONDITIONS FOR THE SOLUBILIZATION OF CHOLESTEROL 7α-HYDROXYLASE

Membrane proteins have been described as being extrinsic or intrinsic by Capaldi and Green (95), this classification being an operational one based on the ease with which the proteins are released from the membrane. Cytochrome c is an example of a peripheral or extrinsic protein being easily removed from mitochondrial membranes. Because cytochrome b_5, NADPH-cytochrome c reductase and cytochrome P-450 can only be solubilized in a complete form by the use of detergents, these proteins are considered to be firmly embedded in the lipid matrix and therefore intrinsic. Because there is this difference between membrane proteins in their extent of binding to microsomal lipid, this section deals with solubilization of mixed function oxidase components and their correlation with cholesterol 7α-hydroxylase activity with increasing concentrations of Nonidet P42. A similar study has been reported by Bleecker et al using sodium deoxycholate to solubilize murine liver microsomes (80).

When detergent is added to membranes, it is primarily the monomeric unit which binds to these membranes (96,97,98). Therefore, if the affinity of the detergent for the membranous material is high, the concentration of free detergent is maintained at a level below the critical micellar concentration (CMC), and as a result, micelles of pure detergent are not formed. The degree of solubilization depends to a large extent on the amount of detergent which is, at any one time, bound to the microsomal membranes. However, the equilibrium which exists between free and bound detergent is apparently rather difficult to determine, but it has been found that there is a good correlation between the bound detergent to membrane ratio and the total

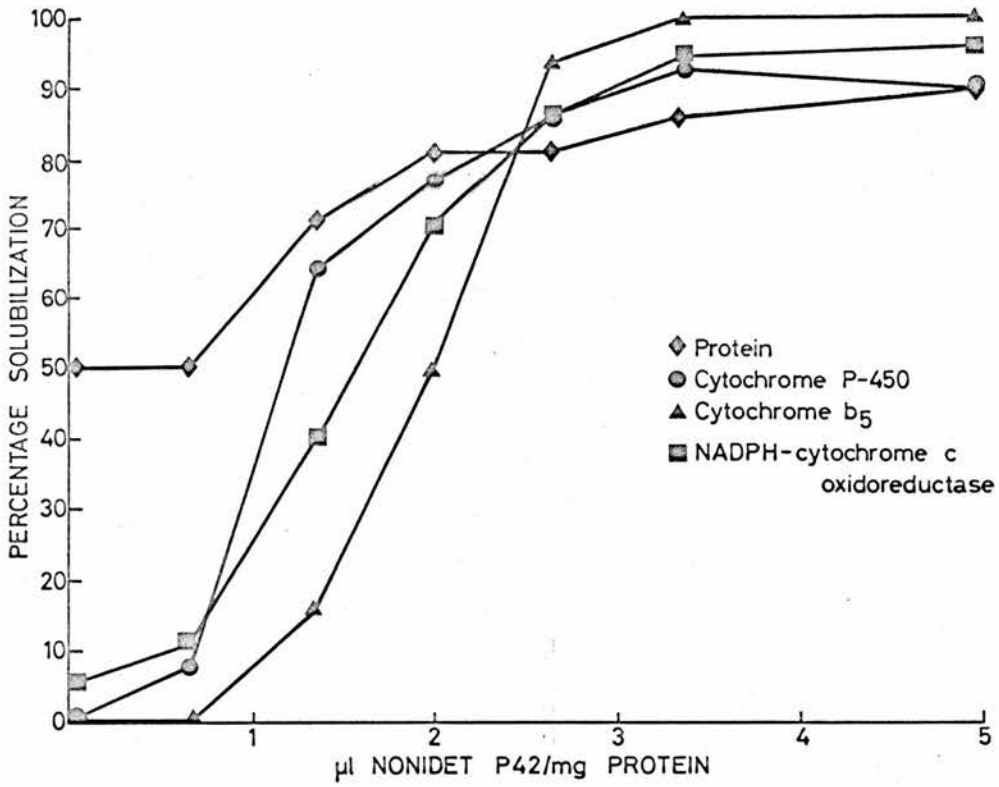


Figure 4,1.

The effect of adding to 30mg acetone powder (15mg protein) suspended in 10ml 0.1M potassium phosphate buffer,increasing quantities of Nonidet P42, on the solubilization of protein and mixed function oxidase components.

detergent to membrane ratio, at least when the concentration of membrane is relatively high. For these reasons, therefore, detergent concentration is expressed as the ratio µl Nonidet P42/mg microsomal protein.

The effect of increasing levels of Nonidet P42 on the extent of solubilization of mixed function oxidase components and cholesterol 7α-hydroxylase from an acetone powder

For the purpose of solubilization, it was found that before addition of Nonidet P42, the buffer containing the resuspended acetone powder must be cooled to 0-5°C. If Nonidet P42 were added to a suspension of acetone powder at 25°C, solubilization would not occur.

Figure 4,1 shows the result of increasing the ratio of Nonidet P42 to protein on the solubilization of protein and mixed function oxidase components. The extent of solubilization of the enzyme is expressed as the percentage solubilization or the ratio

$$\frac{\text{supernatant enzyme activity}}{\text{supernatant enzyme activity + pellet enzyme activity}} \times 100$$

after centrifugation at 105,000 x g for 1 hour.

This figure shows that as the detergent concentration was increased, solubilization of protein, cytochrome P-450, cytochrome b_5 and NADPH-cytochrome c reductase proceeded. Despite the difficulties (described below) involved in assaying these components, it does appear that cytochrome P-450 was more readily soluble in the presence of Nonidet P42 than NADPH-cytochrome c reductase, and that the least soluble component measured was cytochrome b_5. This difference in the ease with which the components were solubilized could represent either their affinity for Nonidet P42 or the strength of binding to the lipid-protein matrix.

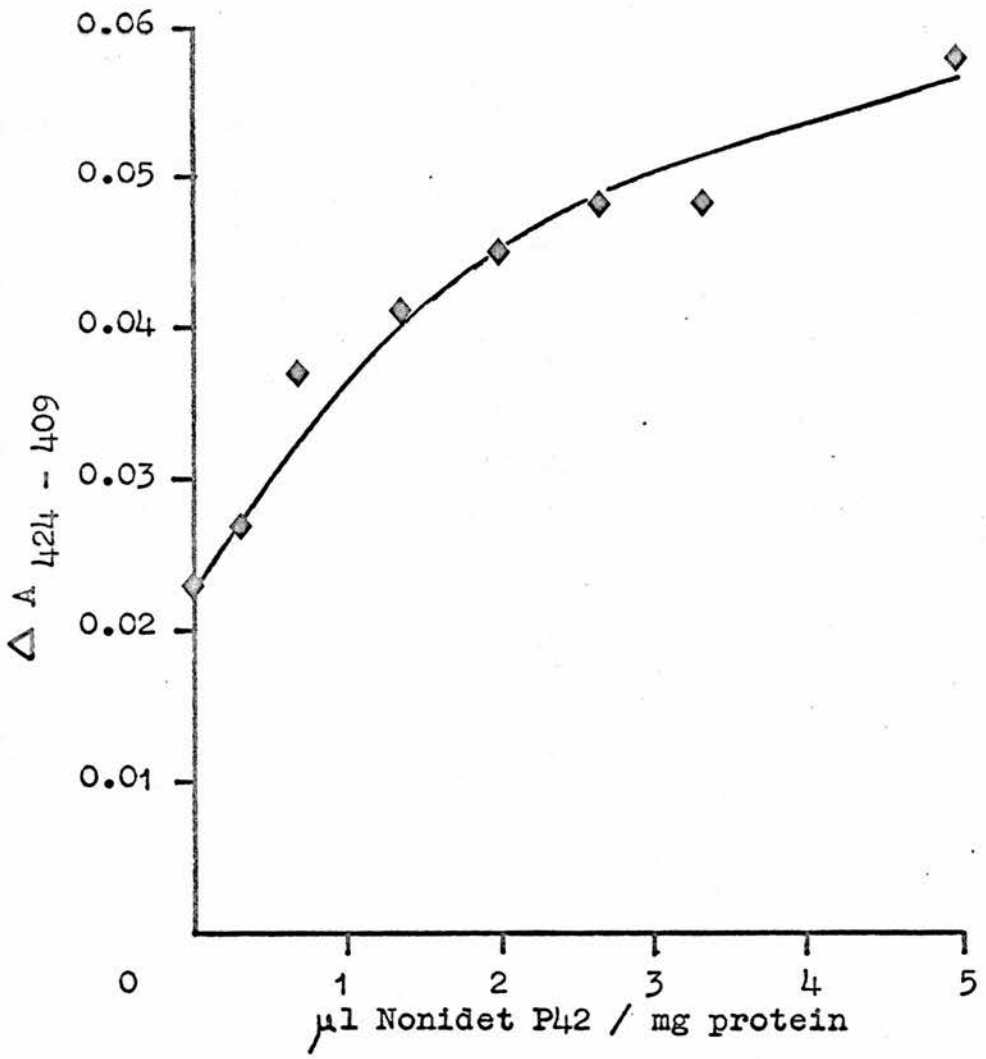


Figure 4,2.

The effect of Nonidet P42 on the magnitude of the reduced-oxidised difference spectrum of cytochrome b_5 from rat liver microsomal acetone powder.

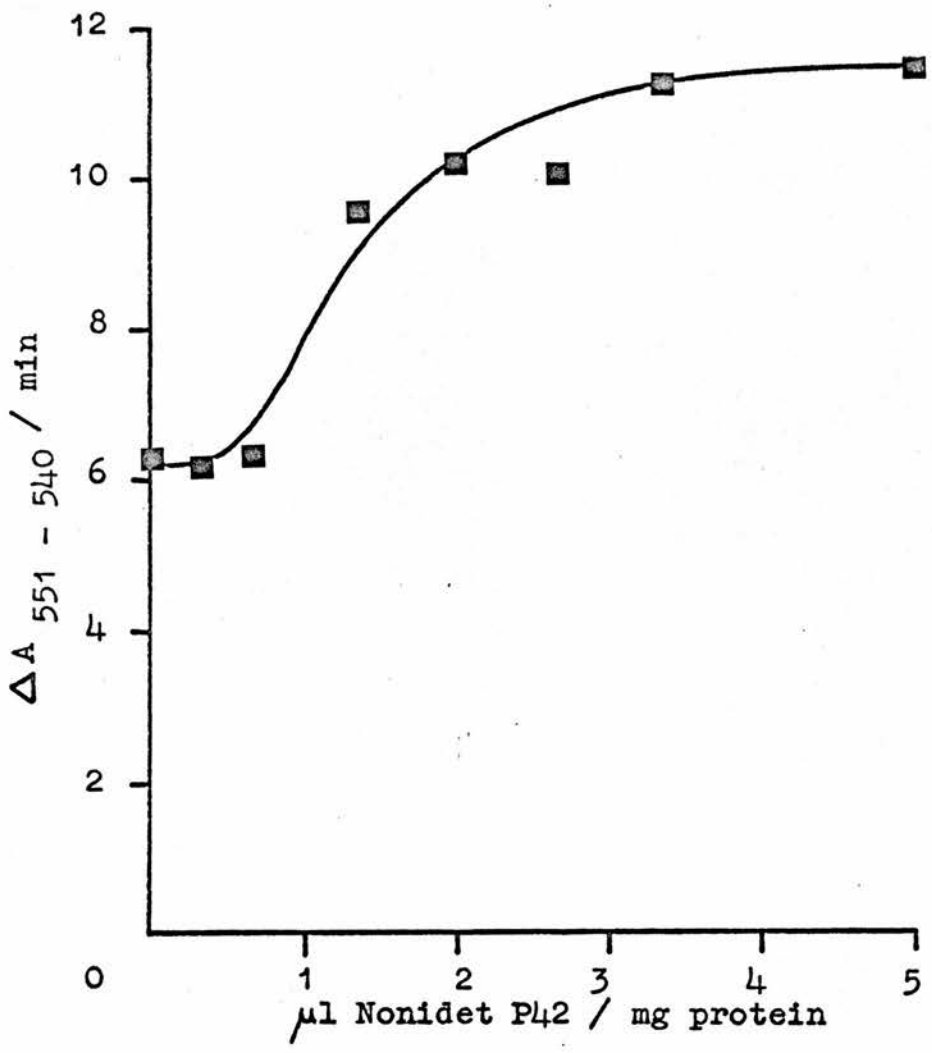


Figure 4,3.

The effect of Nonidet P42 on the activity of NADPH-cytochrome c oxidoreductase from rat liver microsomal acetone powder.

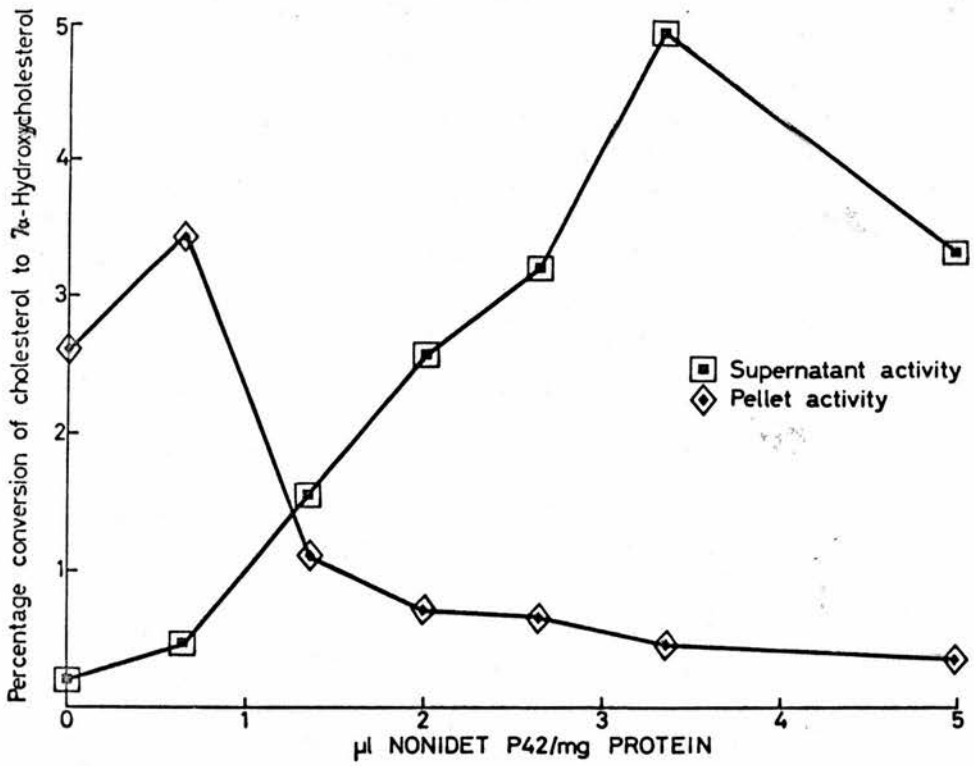


Figure 4,4.

The effect of Nonidet P42 on the solubilization of cholesterol 7α-hydroxylase from 30mg acetone powder.

The difficulty in measuring protein in the presence of detergent has been discussed in the Methods section. It was observed that as solubilization took place the extinction for the reduced-oxidised form of cytochrome b_5 in the summed pellet assay and supernatant assay gradually increased. This is shown in Figure 4,2. It is not known whether this increase in absorbance is due to more cytochrome b_5 being reduceable or that the same absolute amount is being reduced but with a change in the extinction coefficient. A similar effect was observed with NADPH-cytochrome c reductase. As the concentration of Nonidet P42 was increased, the total activity of this enzyme assayed in the supernatant and resuspended pellet also increased, and this increase in activity is shown in Figure 4,3. The reason for this increase in activity and apparent increase in cytochrome b_5 assay is not known. As a consequence of these observations, it was decided to express the results in the form shown in Figures 4,1 and 4,5.

The percentage conversion of cholesterol to 7α-hydroxy-cholesterol at low concentrations of Nonidet P42 was slightly increased, but as the ratio of detergent to protein increased, the cholesterol 7α-hydroxylase activity in the pellet rapidly decreased. Concomitant with this decrease was a rise in cholesterol 7α-hydroxylase activity determined in the 105,000 x g supernatant. These results are presented in Figure 4,4. Nonidet P42 solubilized an acetone powder suspension and the range of effectiveness for the solubilization of cholesterol 7α-hydroxylase spanned 2-6 μl Nonidet P42/mg protein. At a ratio of 3.5 μl Nonidet P42/mg protein, however, an apparent two-fold activation of the enzyme occurs. Such an activation may also be demonstrated in a lyophilised powder extracted with butanol when the concentration of Nonidet P42 is adjusted to 3.5 μl/mg protein. The reason for this activation remains unknown.

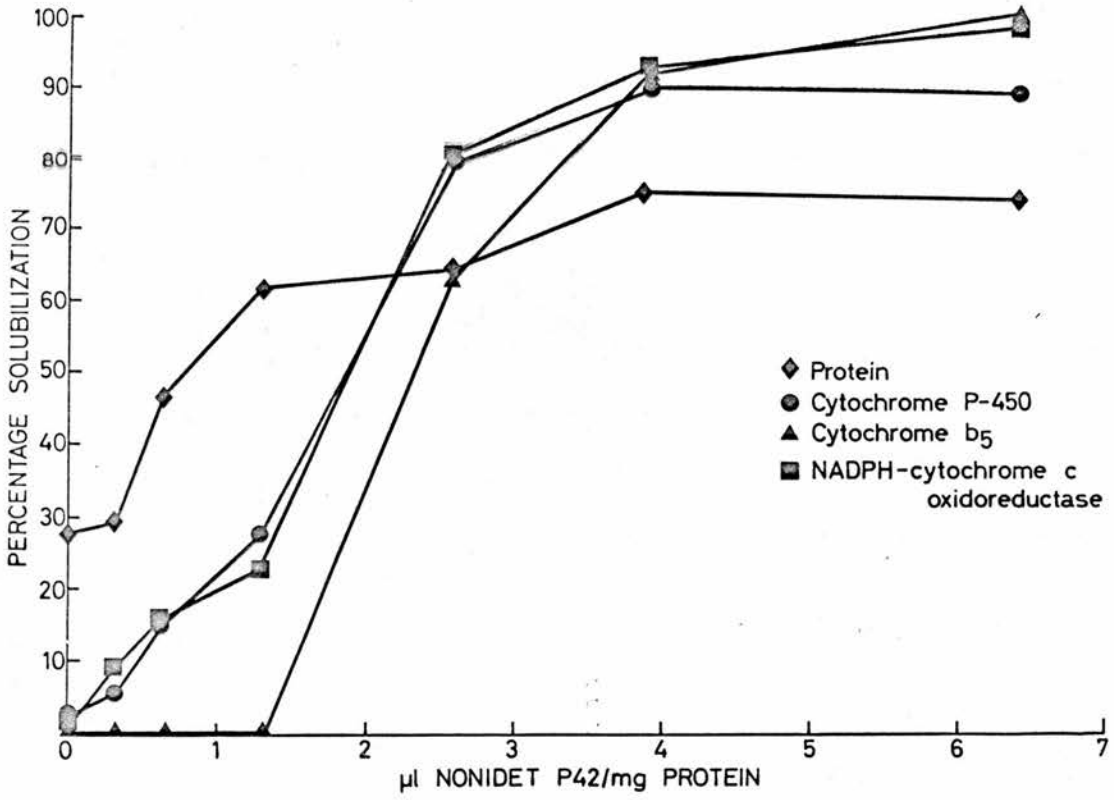


Figure 4,5.

The effect of Nonidet P42 on the solubilization of protein and mixed function oxidase components from native rat liver microsomes.

Effect of increasing levels of Nonidet P42 on the solubilization of rat liver microsomal mixed function oxidase components and cholesterol 7α-hydroxylase

Microsomes were resuspended in ice-cold 20mM potassium phosphate buffer pH 7.7 and subdivided into fractions which were then treated with increasing quantities of Nonidet P42. After leaving for 20 minutes, with occasional stirring, the treated suspensions were centrifuged at 105,000 x g for 1 hour. The pellets were resuspended in the same volume as the supernatants and analysed for cytochrome P-450, cytochrome b_5, NADPH-cytochrome c reductase and protein. In contrast to the cytochrome b_5, both cytochrome P-450 and NADPH-cytochrome c reductase were gradually solubilized to an extent of about 25% at 1.3 μl Nonidet P42/mg protein. Further increase of the detergent to protein ratio resulted in liberation of more of these two proteins into solution, and ultimately 90-95% of the total protein could be solubilized. Cytochrome b_5, on the other hand, resisted solubilization up to a level of 1.3 μl Nonidet P42/mg protein, and then was very rapidly released from the microsomal membrane. This observation perhaps reinforces the idea that cytochrome b_5 is more deeply buried in the lipid matrix than cytochrome P-450 or NADPH-cytochrome c reductase.

During the solubilization process, unlike that when an acetone powder was used, there was no increase in total cytochrome b_5 absorbance on solubilization from microsomes, nor was any increase in NADPH-cytochrome c reductase detected. The results of this experiment are shown in Figure 4,5.

In the assay of cholesterol 7α-hydroxylase activity in the supernatant after treatment of an acetone powder with Nonidet P42, no account was taken of the cholesterol content. This was because at the low level of cholesterol which occurs in an acetone powder, the velocity of cholesterol 7α-hydroxylase is directly proportional to the

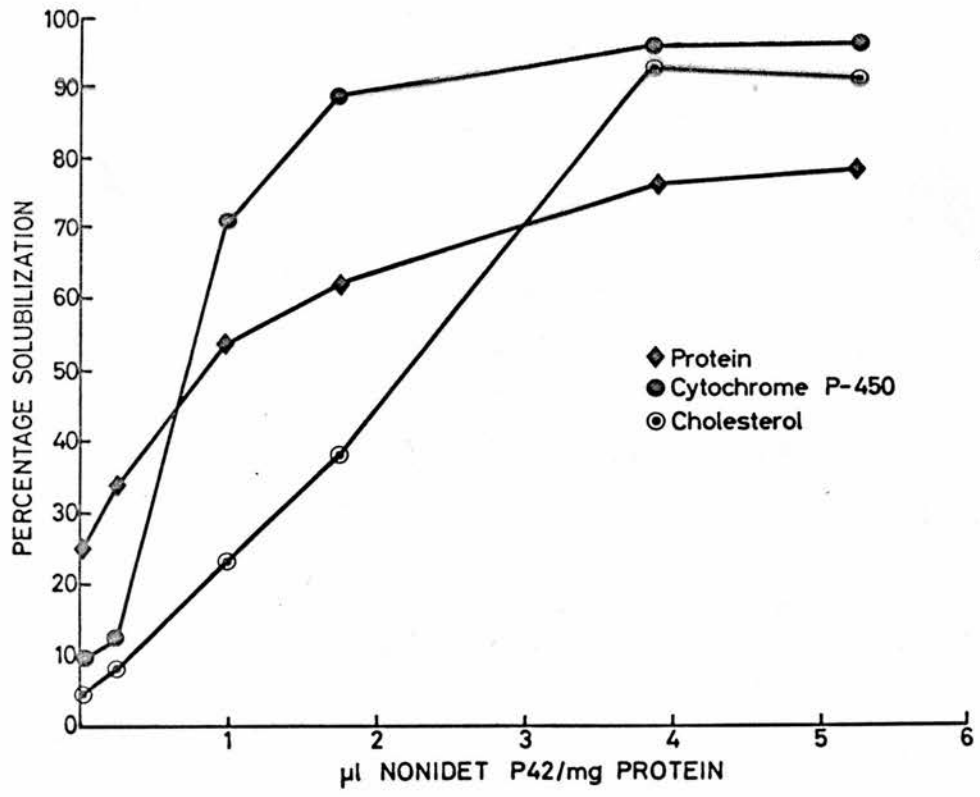


Figure 4,6.

The solubilization by Nonidet P42 of cholesterol, protein and cytochrome P-450 from native rat liver microsomes.

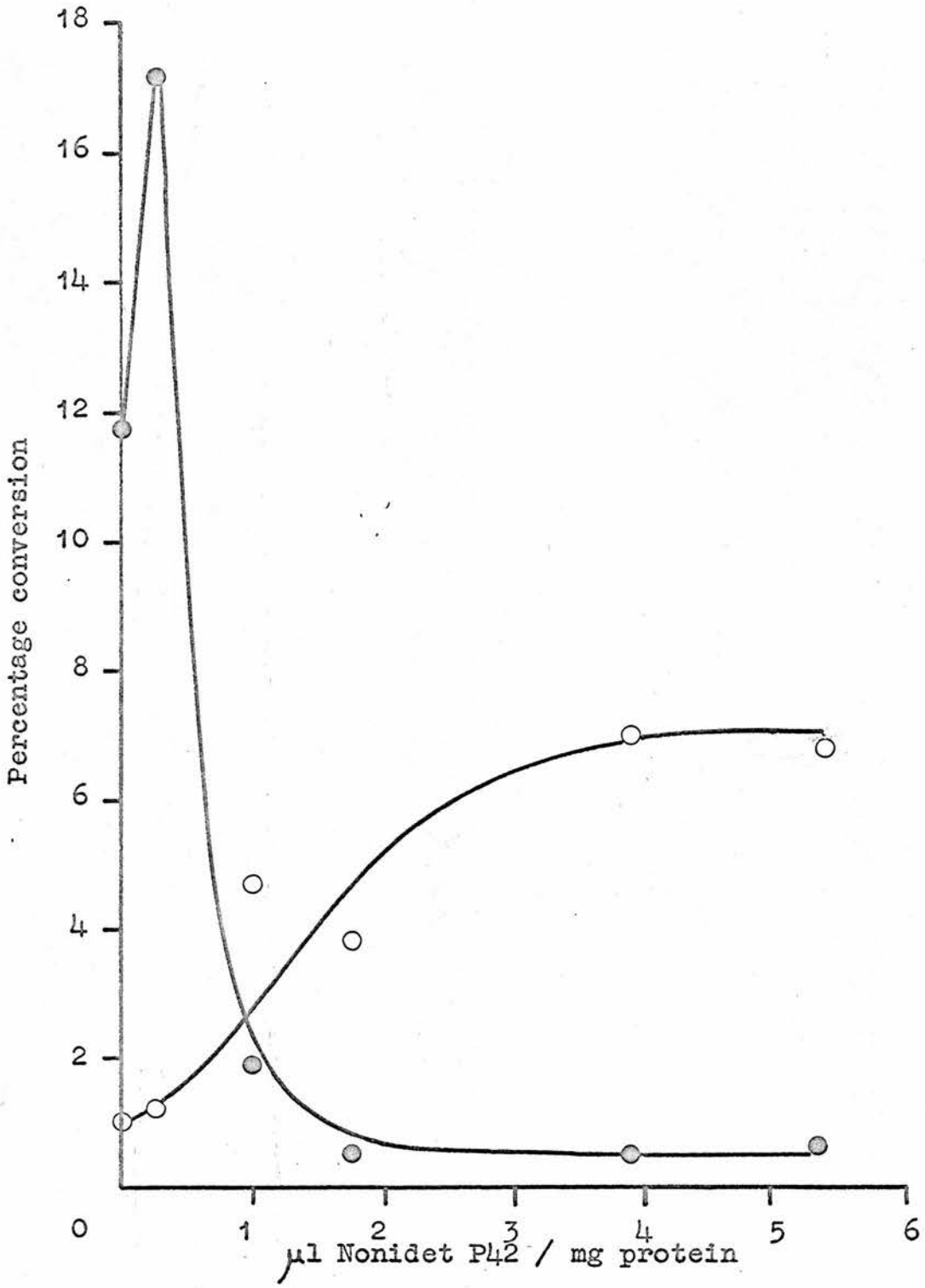


Figure 4,7.

The effect of increasing concentrations of Nonidet P42 on the solubilization of cholesterol 7α-hydroxylase activity.

○ Supernatant activity

● Pellet activity

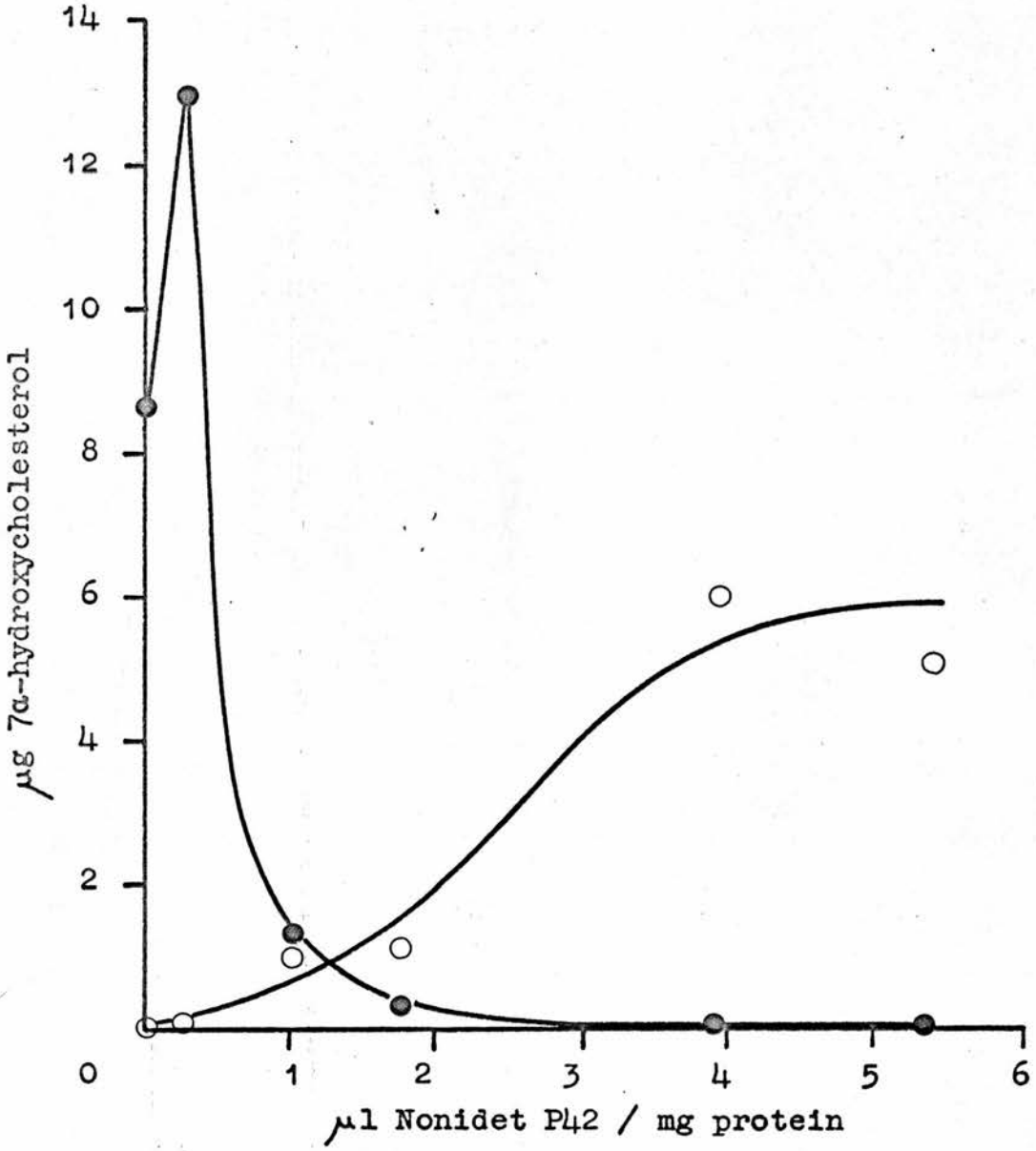


Figure 4,8.

The effect of increasing concentrations of Nonidet P42 on the solubilization of cholesterol 7α-hydroxylase activity.

○ **Supernatant activity**

● **Pellet activity**

cholesterol concentration (see Section 8). Therefore, cholesterol content in the supernatant will not affect the percentage conversion of [4 - ^{14}C] cholesterol to [4 - ^{14}C] 7α-hydroxycholesterol which is an indirect measure of the velocity of the enzyme. However, native microsomes contain 20-100 times as much cholesterol/mg protein as an acetone powder contains, i.e. approximately 20μg cholesterol/mg protein. Therefore, to obtain a more accurate picture of the solubilization of cholesterol 7α-hydroxylase activity from native microsomes, cholesterol in the supernatants and resuspended pellets was measured by g.l.c. In the same experiment, the results of which are shown in Figure 4,6, cytochrome P-450 and protein were measured. Contrary to expectation, cholesterol was solubilized in a manner quite different from that exhibited by cytochrome P-450. In previous experiments, cytochrome P-450, cytochrome b_5 and NADPH-cytochrome c reductase were solubilized in a sigmoidal fashion, but cholesterol was here solubilized in direct proportion to the quantity of Nonidet P42 added. This, therefore, is tentative evidence that cytochrome P-450 solubilized by Nonidet P42 is independent of cholesterol (lipid) interactions.

Cholesterol 7α-hydroxylase activity, measured on a percentage conversion basis, was stimulated by almost 50% at low ratios of Nonidet P42/mg protein in the pellet resulting from centrifugation at 105,000 x g. This increase in activity may also be demonstrated in a microsomal suspension which has not been centrifuged. As the ratio of detergent to protein is further increased, cholesterol 7α-hydroxylase activity in the resuspended pellet rapidly declines in a manner which mirrors very closely the release of cytochrome P-450 into solution. Similarly, cholesterol 7α-hydroxylase is released into solution and these results are expressed in Figure 4,7 on a percentage conversion basis, and in Figure 4,8 as μg 7α-hydroxycholesterol formed, assuming

equilibration of tracer [4 - ^{14}C] cholesterol with endogenous cholesterol. The activity of cholesterol 7α-hydroxylase solubilized was in this case about 70% of the control microsomes, but on numerous other occasions full activity of this enzyme was solubilized.

The criterion of solubility of cholesterol 7α-hydroxylase

Solubilization is an ill-defined term which is based arbitrarily on operational results. In the case of membranes, the actual process of solubilization may probably be nothing more than a reduction in size of membranous material. This distinguishes incomplete solubilization from complete solubilization which one could define as the liberation of single molecules into solution. The generally considered opinion that non-ionic detergents are mild leads to the suggestion by Razin (99) that these detergents may not give rise to complete solubilization. Centrifugation at 105,000 x g for 1 hour does not differentiate between complete and incomplete solubilization, and Kahane and Razin (100) have suggested supplementing this criterion with the following riders; that the solubilized material should not be excluded on a column of Sepharose 4B, i.e. the particle should have a molecular weight less than 3×10^6, and that no membranous structures should be visible when the preparation is examined by electron microscopy.

These two criteria were not applied to the Nonidet P42 solubilized acetone powder as they would not differentiate between complete and incomplete solubilization. Furthermore, more important than obtaining complete solubilization is the ability to remove proteins or lipids which may not have a role in the cholesterol 7α-hydroxylase reaction.

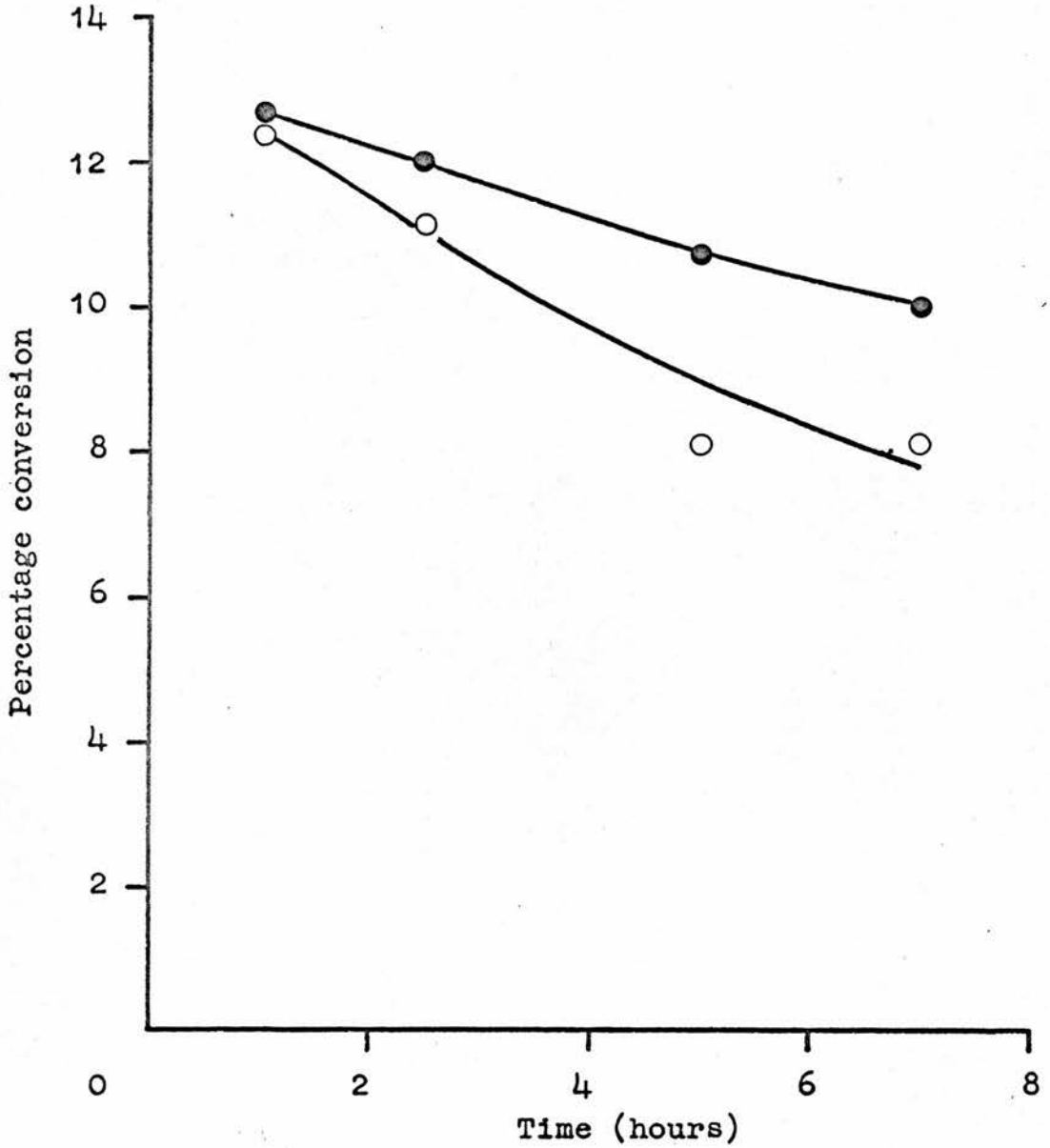


Figure 4,10.

The effect of centrifugation on cholesterol 7α-hydroxylase activity in the supernatant fraction compared with control experiments which were not centrifuged.

● Control activity

○ Activity in supernatant

The effect of centrifugation of a solubilized preparation of an acetone powder for 7 hours at 105,000 x g

Figures 4,9 and 4,10 show the results obtained during the centrifugation of an acetone powder (6.7mg/ml) suspended in 0.1M potassium phosphate buffer pH 7.7, and solubilized by Nonidet P42. In Figure 4,9 it may be seen that after an initial increase in sedimentable material, the percentage of the original cytochrome P-450 and protein remaining in the supernatant fraction plateaus, and after 44×10^6 g.min., approximately 70% of the original cytochrome P-450 and protein remains in the supernatant. Figure 4,10 demonstrates that even after 7 hours centrifugation at 105,000 x g, cholesterol 7α-hydroxylase activity still resides in the supernatant fraction and that this activity, expressed as a percentage conversion of $\left[4 - {}^{14}C\right]$ cholesterol to $\left[4 - {}^{14}C\right]$ 7α-hydroxycholesterol is 80% that of the control, Nonidet P42 treated acetone powder suspension.

SUMMARY

(1) Both rat liver microsomal acetone powders and native microsomes may be solubilized by Nonidet P42. Cholesterol 7α-hydroxylase and mixed function oxidase components were released into the supernatant fraction resultant from centrifugation for 1 hour at 105,000 x g.

(2) There appeared to be some sequential solubilization of the component enzymes involved in drug hydroxylation and in particular cytochrome b_5 seemed more resistant to solubilization than did cytochrome P-450 and NADPH-cytochrome c reductase. The solubilization profile of cholesterol was quite different from that of the proteins measured, and the amount of cholesterol solubilized was directly proportional to the amount of Nonidet P42 added to the microsomal suspension.

(3) When Nonidet P42 was added to an acetone powder suspension, cholesterol 7α-hydroxylase activity in the sedimentable fraction decreased whilst the activity in the supernatant rose. At a detergent to protein ratio of 3.5μl/mg protein virtually all the mixed function oxidase components were solubilized, and the activity of cholesterol 7α-hydroxylase was increased by as much as two-fold over the control value. Thus this value of 3.5μl Nonidet P42/mg protein is the optimal value for the solubilization of cholesterol 7α-hydroxylase in an acetone and butanol powder.

(4) Since with native microsomes no activation of cholesterol 7α-hydroxylase activity in the supernatant fraction was observed, a concentration of 4μl Nonidet P42/mg protein was considered optimal for its solubilization; activation of the enzyme in native microsomes was observed, however, at low detergent to protein ratio.

SECTION 5

DEAE-CELLULOSE CHROMATOGRAPHY OF THE NONIDET P42 SOLUBILIZED RAT LIVER MICROSOMES AND MICROSOMAL ACETONE AND BUTANOL POWDERS

Depending on which type of detergent is used for the solubilization of rat liver microsomes, subsequent behaviour on a DEAE-cellulose column of the solubilized preparation will be quite different. Thus, when microsomes are solubilized by an ionic detergent such as sodium cholate or sodium deoxycholate, cytochrome P-450 is bound to the column of diethylaminoethyl cellulose at pH 7.4 to 7.7. Lu and Coon (79) originally reported that cytochrome P-450 was quite strongly bound to this ion exchanger and required a molarity of KCl of approximately 0.2 for its elution. Similarly, Comai and Gaylor (101) demonstrated three forms of cytochrome P-450, separable on DEAE-cellulose, which showed spectral differences; two of these three forms were bound quite strongly to the column.

However, Miyake, Gaylor and Mason (88) also observed that when rabbit liver microsomes were solubilized with the non-ionic detergent Lubrol WX, and the resultant supernatant applied to a column of DEAE-Sephadex, cytochrome P-450 was eluted without adsorption leaving cytochrome b_5 adsorbed at the top of the column. The reason for the observed differences in behaviour on DEAE-cellulose depending on whether the microsomes were solubilized by deoxycholate or Lubrol WX is unclear but could be the result of incomplete solubilization in one case or by negative charges being conferred on cytochrome P-450 by adsorption of deoxycholate anions.

Behaviour of Nonidet P42 solubilized acetone powder on DEAE-cellulose

As in the case of Miyake's Lubrol WX solubilized preparation of liver microsomes, the bulk of cytochrome P-450 was not adsorbed to DEAE-cellulose. There was nevertheless a second peak of

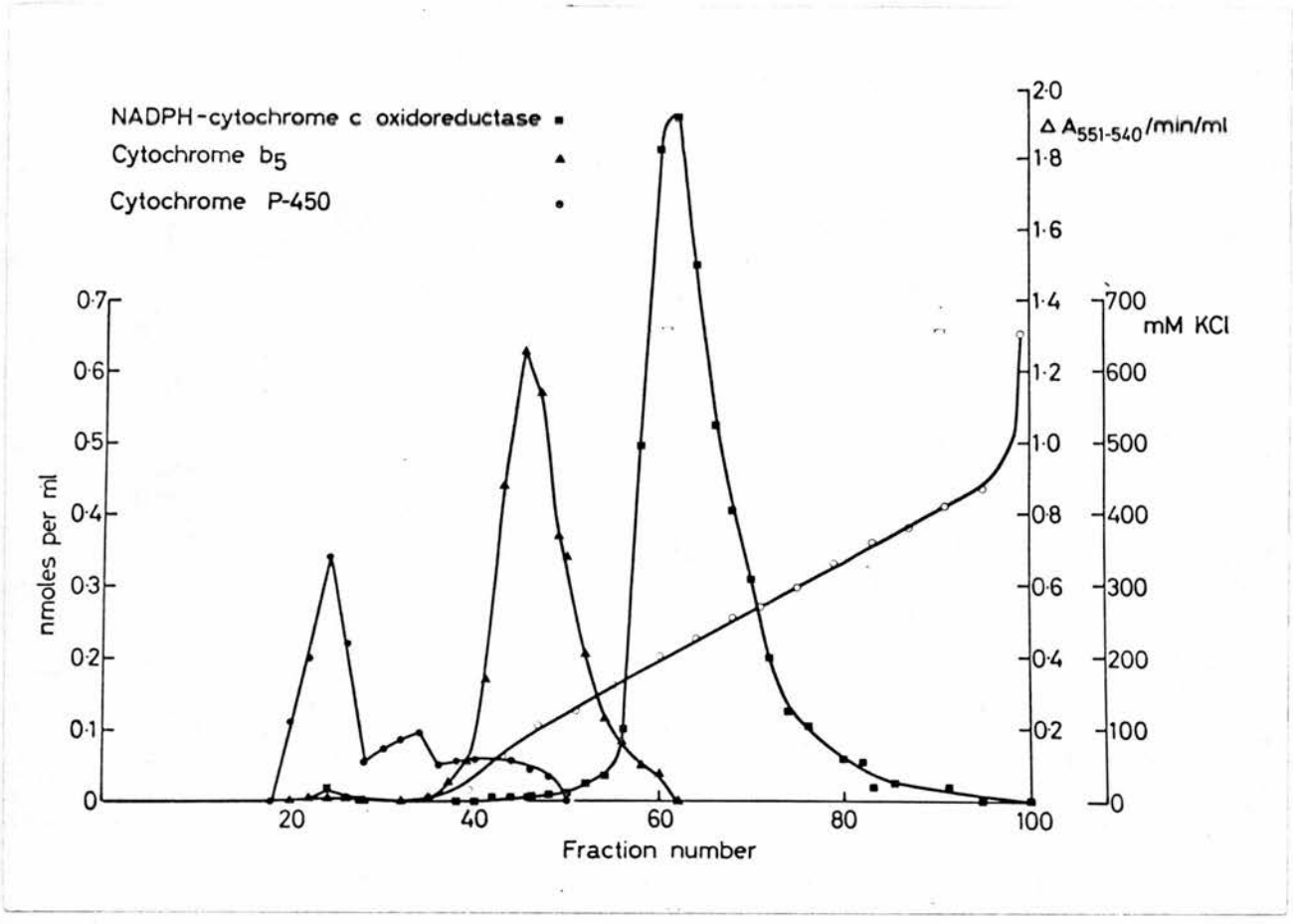


Figure 5,1.

The elution profile of cytochrome P-450, cytochrome b_5 and NADPH-cytochrome c oxidoreductase from 500mg acetone powder solubilized by Nonidet P42, eluted from a DEAE-cellulose column by a linear gradient of KCl.

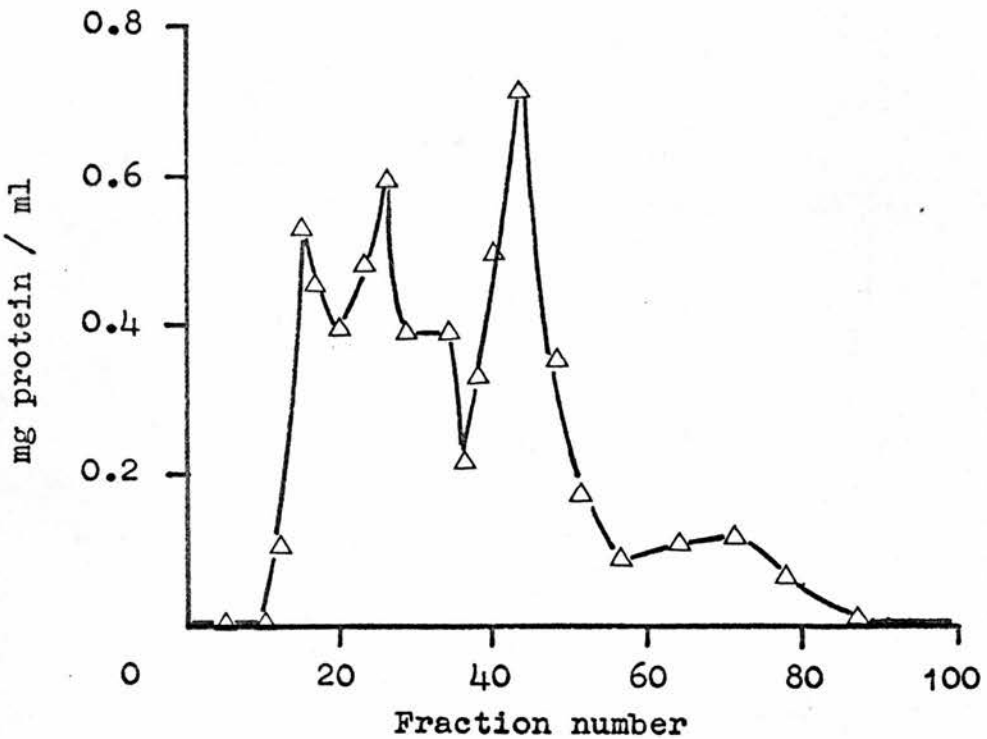


Figure 5,2.

500mg acetone powder (140mg protein) solubilized by Nonidet P42 were applied to a DEAE-cellulose column and eluted (as in figure 5,1.) with a linear KCl gradient.

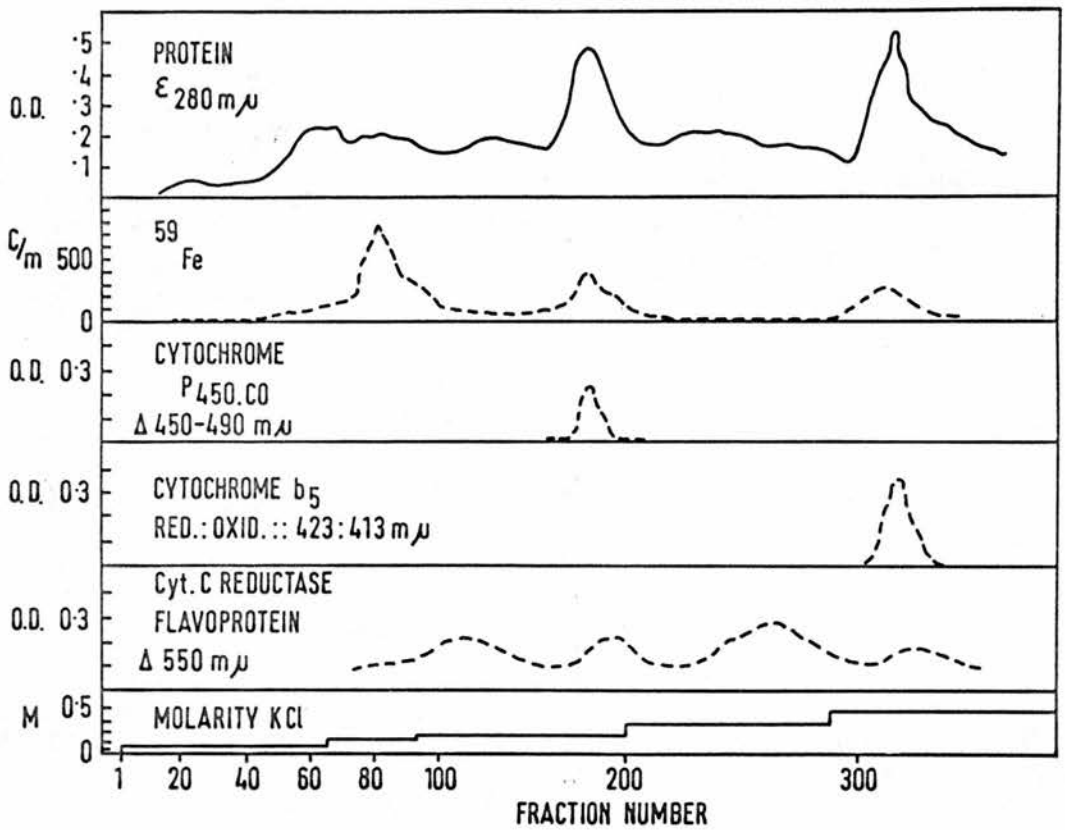


Figure 5,3.

The elution profile of rat liver microsomal mixed function oxidase components, solubilized by deoxycholate, from a DEAE-cellulose column.

cytochrome P-450 which eluted without any further increase in the ionic strength of the eluting buffer and a third peak which eluted at approximately ~~0.1M~~ 0.05M KCl. It is not known whether these three fractions of cytochrome P-450 are analogous to those found by Comai and Gaylor. The ionic strength of the eluting buffer was increased linearly as described in the methods section, and cytochrome b_5 was eluted as a single band at approximately ~~0.2M~~ 0.1M KCl. No detectable cytochrome b_5 was found in the first cytochrome P-450 fraction although the third cytochrome P-450 fraction was grossly contaminated with cytochrome b_5.

Increasing the ionic strength of the eluting buffer further, results in the elution at approximately ~~0.4M~~ 0.25M KCl of NADPH-cytochrome c oxidoreductase, again as a single peak, contamination of the two major microsomal cytochromes being minimal. The elution profile of solubilized acetone powder from DEAE-cellulose is shown in Figure 5,1. Figure 5,2 shows the pattern of elution of protein. Each eluted fraction was assayed by the Lowry method (42) for protein as monitoring the absorbance of the eluate at 280nm was not a useful measure of protein concentration since the aromatic ring of Nonidet P42 absorbs very strongly ultra-violet light in this region. Figure 5,3 shows data presented by Boyd et al (187) and demonstrates clearly that cytochrome P-450 binds strongly to DEAE-cellulose being eluted at 0.3M KCl and cytochrome b_5 even more strongly, 0.5M KCl being the required concentration for elution. But the interesting feature of this elution profile is that several peaks of NADPH-cytochrome c reductase were observed. This is in contrast to the Nonidet P42 solubilized system where a single peak is observed. Lu et al (102) have also made the statement that the limitation of the use of sodium cholate or sodium deoxycholate alone is that cytochrome P-450 and its reductase cannot easily be resolved.

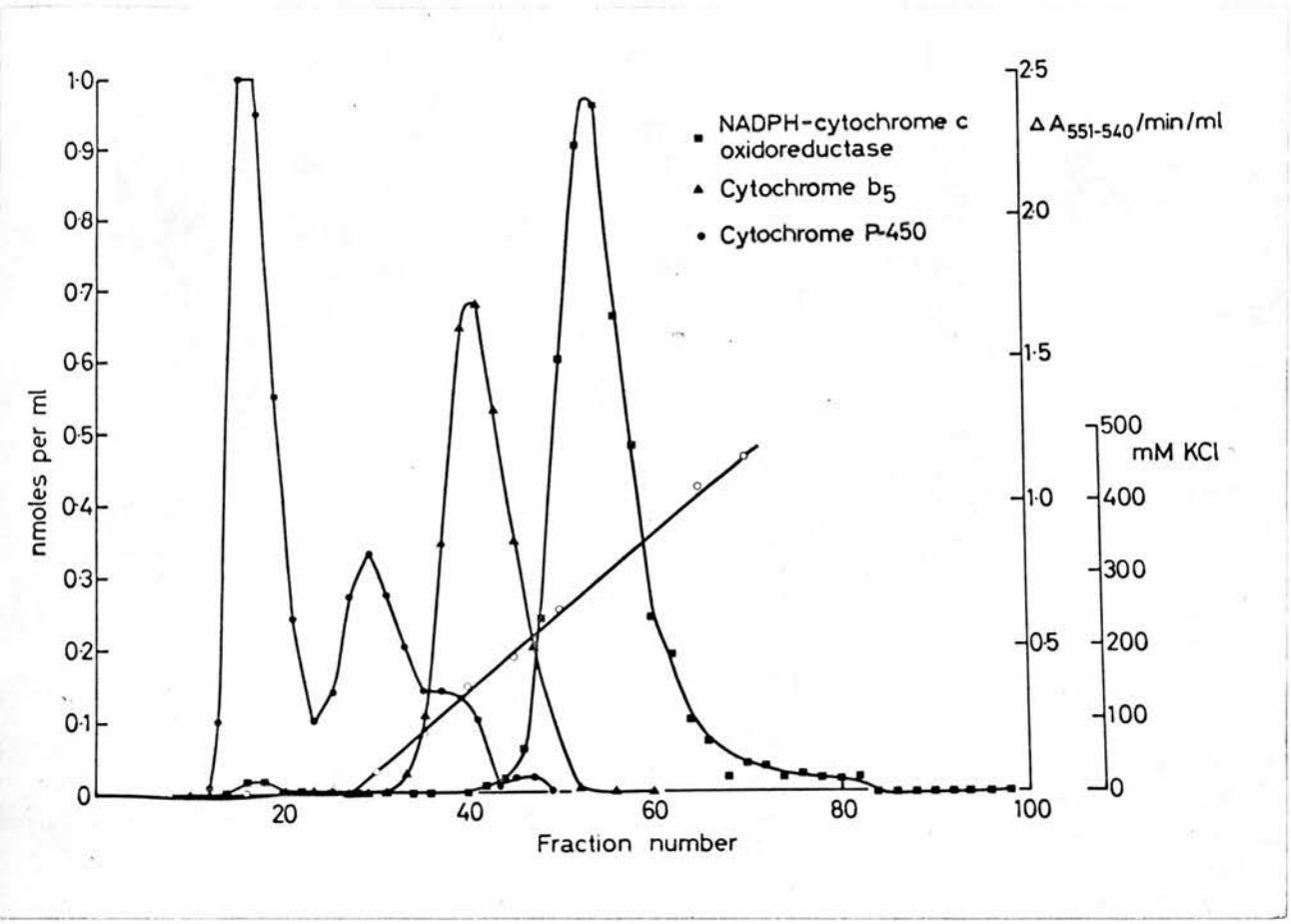


Figure 5,4.

The elution profile of mixed function oxidase components of rat liver microsomes solubilized by Nonidet P42, from a DEAE-cellulose column.

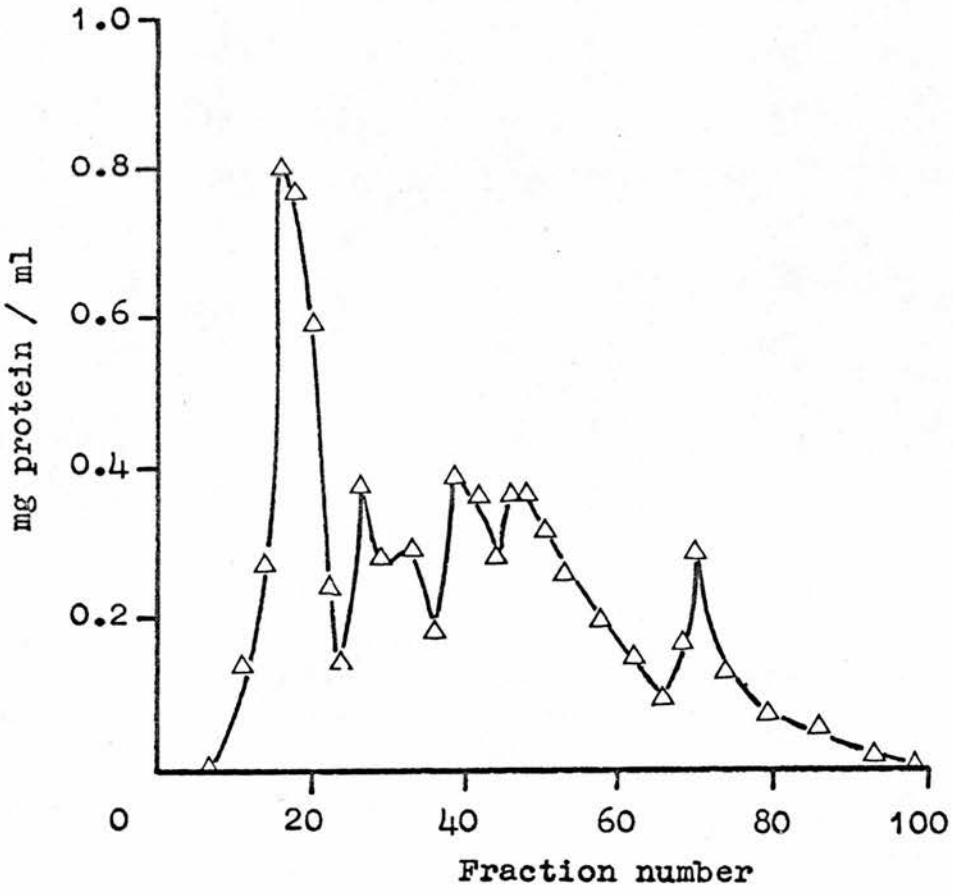


Figure 5,5.

230 mg microsomal protein solubilized by Nonidet P42 eluted from DEAE-cellulose using a linear gradient of KCl. (6ml fractions)

The conditions necessary for DEAE-cellulose chromatography

The use of Tris-HCl or potassium phosphate buffers, 20mM, pH 7.7 both resulted in similar elution profiles, but to prevent tailing, and for reproducibility in the elution pattern, it was found necessary to include in the buffer 0.4% Nonidet P42. These conditions were also applicable to chromatography of Nonidet P42 solubilized preparation of native microsomes. Chromatography was performed at 0-3°C.

DEAE-cellulose chromatography of Nonidet P42 solubilized native rat liver microsomes

Native microsomes were resuspended from the 105,000 x g pellet in ice-cold 20mM potassium phosphate buffer pH 7.7 and solubilized by addition of 4 µl Nonidet P42/mg protein. Native microsomes contain more lipid than an acetone powder and substantially more than a butanol powder whose characteristics when solubilized by Nonidet P42 upon DEAE-cellulose chromatography were very similar to those found in the solubilized acetone powder.

A solubilized preparation of native microsomes might, therefore, be expected to behave differently on a DEAE-cellulose column. Figure 5,4 shows the elution profile when solubilized native microsomes were applied to DEAE-cellulose; cytochrome P-450, cytochrome b_5 and NADPH-cytochrome c reductase were measured, and Figure 5,5 shows the elution profile of protein as determined by the Lowry method (42).

Cytochrome P-450 was again not bound by DEAE-cellulose, but there was nevertheless some retardation which gave rise to two other peaks of cytochrome P-450. Again there was no apparent contamination of the predominant peak of cytochrome P-450 with cytochrome b_5, which was eluted when the concentration of KCl in the eluting buffer

was raised to 0.15M. There was little contamination by cytochrome b_5 in this cytochrome P-450 up to a protein concentration in the supernatant of about 10mg/ml, whereupon some cytochrome b_5 failed to be adsorbed to the column and eluted with the bulk of cytochrome P-450. Increasing the molarity of KCl to approximately 0.3 brought about the elution of NADPH-cytochrome c reductase, again as one peak.

An interesting observation made was that upon solubilization of native microsomes there was from time to time some conversion of cytochrome P-450 to cytochrome P-420. This form of cytochrome could be partially removed from cytochrome P-450 as it appeared to bind more strongly to DEAE-cellulose. A similar separation of cytochrome P-450 from cytochrome P-420 has been observed by Ramseyer and Harding (103). The difference in cytochrome P-450 and cytochrome P-420 might here represent a difference in aggregation or an unfolding of the polypeptide chain to unmask further anionic residues.

Examination of the elution profiles of protein from chromatography of both Nonidet P42 solubilized native microsomes and acetone powder reveals that a great part of the microsomal protein did not bind to DEAE-cellulose and was eluted in the cytochrome P-450 fraction without adsorption. In order to characterise in more detail the behaviour of Nonidet P42 solubilized microsomes on DEAE-cellulose, a sample was applied to a column and eluted with a step-wise KCl gradient.

The step-wise elution of microsomal cytochromes and enzymes from DEAE-cellulose

3.3g of freeze-dried microsomes suspended in 20mM potassium phosphate were solubilized by addition of Nonidet P42. The 105,000 x g supernatant was applied to a DEAE-cellulose column 30cm x 3.5cm internal diameter and 300ml each of 0, 0.075M, 0.15M and 0.35M KCl in 20mM phosphate

containing 0.4% Nonidet P42 were used for elution. Cytochrome P-450, cytochrome b_5, NADPH-cytochrome c reductase, glucose-6-phosphatase, esterase, aryl sulphatase, alkaline phosphatase, protein, cholesterol and [4 - ^{14}C] cholesterol and RNA were measured.

The first peak which was eluted, unretarded, from the column contained all the various activities assayed with the exception of, fortuitously, cytochrome b_5 and NADPH-cytochrome c reductase. The elution profile of cholesterol measured by extraction of the steroid and measurement of the [4 - ^{14}C] cholesterol was very similar to that obtained when cholesterol was assayed quantitatively using cholesterol oxidase. No cholesterol was found other than in the first peak eluted from DEAE-cellulose. Although this first peak contained many enzymes, RNA and cholesterol, maximal activities did not occur in the same fraction within this peak. Thus, cytochrome P-450, cholesterol and alkaline phosphatase attained maximum activities in tube 14. This was followed in the next tube by the peak of activity of aryl sulphatase which was followed by the peak esterase activity. Glucose-6-phosphatase, although eluted in the fraction which was not adsorbed, was separated from the cytochrome P-450 peak by 6 tubes, or about 90ml. This peak of glucose-6-phosphatase activity coincided with a peak of RNA. There appears, therefore, to be some adsorption to DEAE-cellulose in the presence of the detergent of certain enzymes and RNA.

In another experiment, phospholipid and NADH-cytochrome c reductase were assayed in each fraction eluted from the column. As with cholesterol, phospholipid accompanied cytochrome P-450 and was eluted without adsorption. NADH-cytochrome c reductase also eluted with cytochrome P-450, but there was sufficient contamination of the cytochrome b_5 fraction to enable slow reduction of this cytochrome by NADH. Not all the original NADH-cytochrome c reductase in the

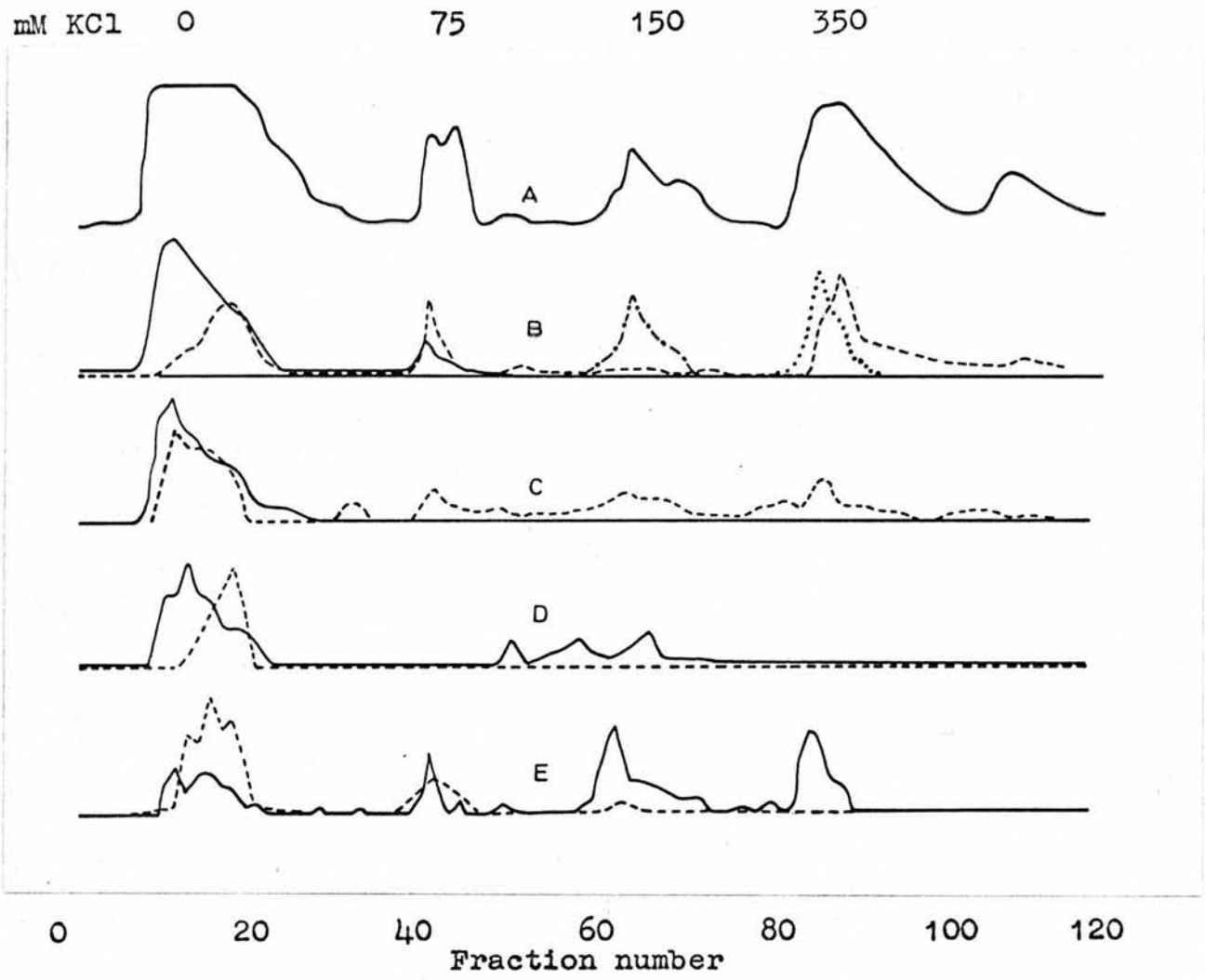


Figure 5,6.

DEAE-cellulose chromatography of Nonidet P42 solubilized microsomes

A Absorbance at 280nm

B ——— Cytochrome P-450
- - - - - Ribonucleic acid
-.-.-. Cytochrome b_5
..... NADPH-cytochrome c oxidoreductase

C ——— [4-^{14}C] cholesterol
- - - - - Protein

D ——— Aryl sulphatase
- - - - Glucose 6-phosphatase

E ——— Alkaline phosphatase
- - - - - Esterase

supernatant could be accounted for in the DEAE-cellulose eluate. This may have been due to inactivation of the enzyme on DEAE-cellulose or this could be a result of dilution (104).

The fraction eluting at 0.075M KCl in 20mM phosphate and 0.4% Nonidet P42 contained more RNA, alkaline phosphatase and esterase; further cytochrome P-450 was also eluted.

Application of 0.15M KCl to the DEAE-cellulose column brought about the elution of cytochrome b_5 and further alkaline phosphatase, but no RNA was associated with this fraction. Increasing the ionic strength of KCl to 0.35M resulted in the elution of NADPH-cytochrome c reductase and further alkaline phosphatase and RNA. These results are shown in Figure 5,6.

Cholesterol 7α-hydroxylase activity

None of these fractions eluted step-wise and none of the individual fractions resulting from the use of a linear KCl gradient had cholesterol 7α-hydroxylase activity, but evidence presented in section 7 showed that when the cytochrome P-450 fraction was recombined with NADPH-cytochrome c reductase then cholesterol 7α-hydroxylase activity was reconstituted.

SUMMARY

(1) Nonidet P42 solubilized preparations, whether of native microsomes, acetone or butanol powders, when applied to a column of DEAE-cellulose, equilibrated with 20mM potassium phosphate buffer, pH 7.7 and 0.4% Nonidet P42, produced the same pattern of elution.

(2) The bulk of the cytochrome P-450 was not adsorbed to DEAE-cellulose, though there was perhaps a part which was weakly bound and therefore retarded. The cytochrome P-450 fraction was not contaminated by cytochrome b_5 and there was little contamination by NADPH-cytochrome c reductase.

(3) Cytochrome b_5 was bound to the column but eluted at approximately 0.15M KCl. This cytochrome was contaminated by a small part of the cytochrome P-450.

(4) NADPH-cytochrome c reductase was the component of the mixed function oxidase system bound most strongly to DEAE-cellulose and this was eluted at 0.3M KCl.

(5) Examination of the fractions eluted by a step-wise gradient revealed that the first fraction not adsorbed to DEAE-cellulose contained a mixture of enzymes, phospholipids, cholesterol and RNA. There was, however, some retardation of glucose-6-phosphatase and esterases and also of RNA within this first unadsorbed fraction. The third fraction contained alkaline phosphatase and cytochrome b_5, eluted at 0.15M KCl, and the fourth fraction, eluted at 0.35M KCl, contained NADPH-cytochrome c reductase.

SECTION 6

PURIFICATION TECHNIQUES APPLIED TO CYTOCHROME P-450 AND NADPH-CYTOCHROME c OXIDOREDUCTASE

Evidence will be presented in Section 7 that for the reconstitution of cholesterol 7α-hydroxylase activity two fractions from DEAE-cellulose chromatography are required; one fraction contains cytochrome P-450 and the other NADPH-cytochrome c oxidoreductase. Further evidence in Section 7 suggests that the specificity of cholesterol 7α-hydroxylase resides in that fraction containing cytochrome P-450. For this reason, in further attempts at the purification of cholesterol 7α-hydroxylase, attention was devoted more to the cytochrome P-450 fraction than to the NADPH-cytochrome c reductase fraction.

Apart from the readily soluble cytochrome P-450 from Pseudomonas putida isolated by Yu and Gunsalus (105), three groups have reported the purification of rat liver microsomal cytochrome P-450 to near homogeneity (106,107,108), but without exception sodium cholate, which has been found inhibitory towards cholesterol 7α-hydroxylase, has been used. There has been as yet no report on the purification of rat liver microsomal cytochrome P-450 using non-ionic detergents. Furthermore, in those reports of homogeneous preparations of cytochrome P-450, the specific content of cytochrome P-450 in the microsomes was 3-7 nmoles/mg protein. This compares with a value of about 1 nmole cytochrome P-450/mg protein found in the phenobarbital treated rats used in these experiments.

Ammonium sulphate fractionation

Before application to a DEAE-cellulose column, Levin et al (81) partially purified the sodium cholate solubilized cytochrome P-450 from rat liver microsomes by ammonium sulphate fractionation. This

fractionation appears complex as increasing the cholate to protein ratio preferentially affects the salting out of cytochrome P-450, the saturation of ammonium sulphate required for precipitation being reduced from 42-50% to 37-45%. The other contaminating components of the mixed function oxidase system, cytochrome b_5, NADPH-cytochrome c oxidoreductase and phospholipid appear to be unaffected in their precipitation characteristics by the cholate to protein ratio (102).

When a Nonidet P42 solubilized acetone powder was subjected to ammonium sulphate fractionation, all the components necessary for the 7α-hydroxylation of cholesterol precipitated in the 0-30% saturation fraction, and there was no separation of cytochrome P-450 from NADPH-cytochrome c reductase. Similarly, when the cytochrome P-450 fraction pooled from DEAE-cellulose was treated with ammonium sulphate in 25% glycerol, 50% was recovered in the fraction precipitating between 0 and 15% saturation of ammonium sulphate. There was no concomitant purification of cytochrome P-450, although some cytochrome P-450 precipitating between 25 and 35% was slightly purer (1.0 nmoles/mg protein compared with 0.7 nmoles/mg) but the yield was only 15%. This low concentration of ammonium sulphate at which the bulk of the cytochrome P-450 is precipitated is probably due to the high concentration of Nonidet P42.

Polyethyleneglycol

Another of the disadvantages in the use of ammonium sulphate to fractionate Nonidet P42 solubilized cytochrome P-450 was that the protein which precipitated from solution by ammonium sulphate floated to the surface upon centrifugation to form an oily layer. This fraction proved very difficult to recover. Polyethyleneglycol has been observed to have an effect on proteins similar to that of ammonium

Nonidet P42 solubilized liver microsomal Cytochrome P-450 was eluted from DEAE-cellulose and pooled

	Cytochrome P-450(nmoles)	Protein (mg)	Specific Content (nmoles/mg protein)
Cyt P-450 from DEAE-cellulose	9.7	40	0.24
Precipitate from 10% PEG 6000	0.8	6	0.13
Precipitate from 13% PEG 6000	2.2	6.4	0.35
Precipitate from 15.5% PEG 6000	2.34	6.4	0.37
Precipitate from 17.5% PEG 6000	0.26	4.8	0.05
Supernatant from 17.5% PEG 6000	1.70	16.8	0.01

Table 6,1.

Purification of cytochrome P-450 using polyethyleneglycol 6000..

sulphate, and of the homologous series available, PEG 6000 appears to be the polymer most useful for fractionation (109). This material has been found useful in the purification of fatty acid synthetase from brewers' yeast (110) and has recently been used by van der Hoeven and Coon (82).

Table 6,1 shows the results of the fractionation of a Nonidet P42 solubilized preparation of cytochrome P-450 eluted from DEAE-celluiose in the absence of glycerol and at ca.3°C.

Turbidity did not develop until the PEG 6000 concentration was 10%. This was in contrast to van der Hoeven's sodium cholate solubilized rabbit liver microsomes where a precipitate was formed at 4% PEG 6000 (82). Increasing the concentration of polyethyleneglycol brought about the precipitation of further cytochrome P-450 and protein. Approximately 45% of the cytochrome P-450 precipitated between 10 and 15.5% PEG 6000, and the specific content of these fractions was increased 50% over the original material. Although with PEG 6000, precipitates formed pellets on centrifugation, at high concentrations, the suspension was viscous and required centrifugation at 16,000 r.p.m. for 0.5hr. (MSE 8 x 50 rotor) for separation of the supernatant from the pellet.

Gel exclusion chromatography

Sephadex G-100 and G-200

The estimated molecular weight of cytochrome P-450 by SDS gel electrophoresis is about 46 (111), 53 (82) and 45 (106) kilodaltons. Application of Nonidet P42 solubilized acetone powder to Sephadex G-100 and G-200 did not lead to any separation of the cholesterol 7α-hydroxylase components.

Sepharose 4B

Cytochrome P-450, purified two-fold by DEAE-cellulose

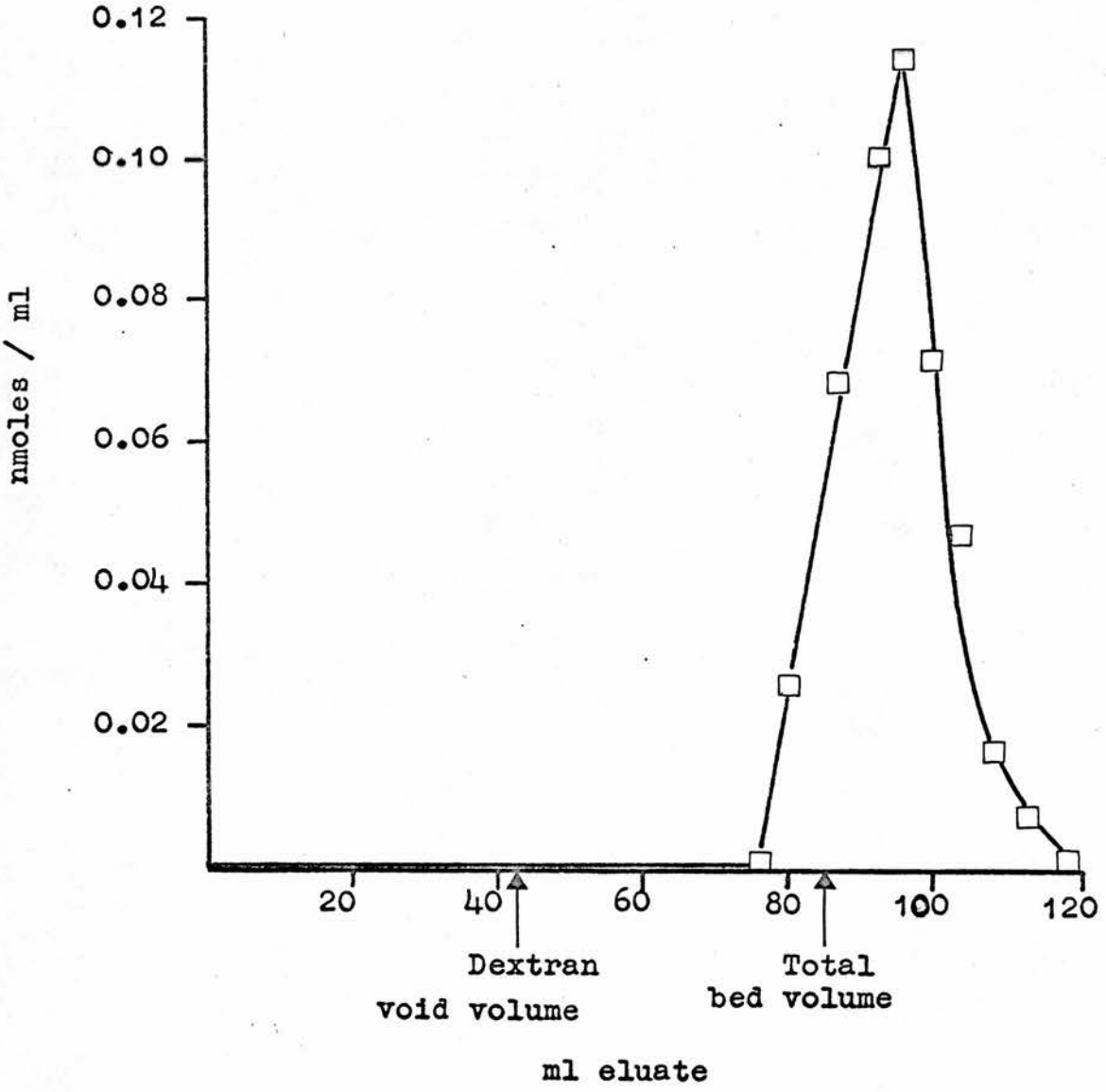


Figure 6,1.

Sepharose 4-B chromatography of partially purified cytochrome P-450.

	Cytochrome P-450(nmoles)	Protein (mg)	Specific Content (nmoles/mg protein)
Supernatant	852	1120	0.76
Cyt P450 fraction from DEAE-cellulose	470	378	1.25
Elution of Cyt P450 from hydroxylapatite with:-			
20 mM phosphate	0	27	0
50 mM phosphate	56	54	1.05
80 mM phosphate	99	60	1.65
150 mM phosphate	205	78	2.62
220 mM phosphate	69	50	1.39
400 mM phosphate	20	34	0.59

The phosphate buffer, pH 7.7 contained 20% glycerol and 1% Nonidet P42.

Table 6,2.

Purification of Cytochrome P-450 on hydroxylapatite.

chromatography, was concentrated by ultrafiltration over a PM 30 Diaflo membrane. In one case 3ml (8.4mg protein, 8.4 nmoles cytochrome P-450) were applied to a column of Sepharose 4B, total volume 85ml, blue dextran exclusion volume 42ml. Figure 6,1 shows that the only peak of cytochrome P-450 observed was spread over a volume of 36ml. This result and the fact that the elution volume at the peak is 96ml probably means that the preparation did bind to the Sepharose 4B. This may explain the poor yield, determined as protein or cytochrome P-450, of 25%. The increase in specific content in this peak as a result of Sepharose 4B chromatography was barely detectable.

Hydroxylapatite chromatography

Imai and Sato (106) have found that cytochrome P-450, solubilized by sodium cholate, could be purified two-fold by chromatography on hydroxylapatite in the presence of 20% glycerol and 0.2% Emalgen 913. Cytochrome P-450 from a DEAE-cellulose column was, therefore, diluted with glycerol to 20% and applied to a column of hydroxylapatite, equilibrated with 10mM phosphate buffer pH 7.7, 20% glycerol and 1% Nonidet P42. Increasing the phosphate concentration to 80mM eluted a fraction containing cytochrome P-450. This material was purified by 33% and a further peak of this cytochrome was eluted at 150mM which was purified 2.1 fold over the DEAE-cellulose eluate. As great difficulty was encountered in the flow rate, particularly when the hydroxylapatite had been re-cycled, a batch-wise procedure for the purification of cytochrome P-450 was attempted. The results of a typical purification are summarised in Table 6,2. It may be seen that virtually all of the cytochrome P-450 and 82% of the protein applied to hydroxylapatite was recovered and that approximately 46% of the initial haemoprotein was purified about two-fold.

100 nmoles Cytochrome P-450 eluted from DEAE-cellulose were applied to hydroxylapatite and eluted with either increasing concentrations of potassium phosphate buffer or increasing concentrations of potassium chloride in potassium phosphate buffer, both elution buffers containing 1% Nonidet P42 and 20% glycerol.

	Cytochrome P-450(nmoles)	Specific content (nmoles/mg protein)
20 mM phosphate	2	-
80 mM phosphate	23	1.0
150 mM phosphate	28	1.15
220 mM phosphate	9	0.5
300 mM phosphate	2	-
400 mM phosphate	-	-
20 mM phosphate	4	-
+ 0.1M KCl	7	0.4
+ 0.2M KCl	24	1.0
+ 0.3M KCl	12	0.8
+ 0.4M KCl	4	0.4
+ 0.5M KCl	-	-

Table 6,3.

Comparison of the elution of cytochrome P-450 from hydroxylapatite using either potassium chloride or phosphate.

140 nmoles cytochrome P-450 (124mg protein) were added to 3g calcium phosphate gel and eluted with increasing concentrations of potassium phosphate buffer, pH 7.7 containing 1% Nonidet P42 and 20% glycerol.

	Cytochrome P-450(nmoles)	Protein (mg)	Specific content (nmoles/mg protein)
20 mM phosphate	-	22	-
50 mM phosphate	6	14	0.44
100 mM phosphate	36	24	1.53
150 mM phosphate	34	21	1.66
200 mM phosphate	17	13	1.32
300 mM phosphate	5	8	0.63
500 mM phosphate	6	15	0.40

Table 6,4.

Purification of cytochrome P-450 using calcium phosphate gel.

As cytochrome P-450 did not bind to an ion exchange column, it was thought possible that this cytochrome had an overall positive charge at pH 7.7. It has been reported by Bernardi et al (112) that basic proteins may be eluted from hydroxylapatite by increasing concentrations of KCl. Therefore, the difference in elution profile of the same sample of cytochrome P-450 on hydroxylapatite using KCl or potassium phosphate as the eluting agent, was observed to see if there was any preferential elution of the cytochrome P-450. Table 6,3 shows the difference in purity of cytochrome P-450 eluted from hydroxylapatite using either KCl in 20mM phosphate or potassium phosphate alone. It is clear that although it was possible to elute cytochrome P-450 from hydroxylapatite with potassium chloride, elution with phosphate appeared to give a sharper profile concomitant with a slightly purer haemoprotein.

Calcium phosphate gel chromatography

Table 6,4 shows the scheme of purification of cytochrome P-450 pooled from the combined 80mM and 150mM eluates from hydroxylapatite on calcium phosphate gel. As the cytochrome P-450 fraction was eluted with greatest purity at 150mM potassium phosphate, being 62% more pure than the combined 80 and 150mM phosphate eluates from hydroxylapatite, it appears that calcium phosphate gel behaves similarly to hydroxylapatite gel.

Ammonium sulphate fractionation after removel of excess Nonidet P42 by binding cytochrome P-450 to hydroxylapatite

Since hydroxylapatite binds cytochrome P-450 strongly, advantage of this fact was taken to remove excess Nonidet P42, which interfered with ammonium sulphate fractionation. Cytochrome P-450 eluted batch-wise by 150mM phosphate buffer from hydroxylapatite was applied to a column of hydroxylapatite freshly suspended in 20mM

	Cytochrome P-450(nmoles)	Protein (mg)	Specific content (nmoles/mg protein)
Supernatant	500	620	0.81
Cyt P-450 from DEAE-cellulose	270	280	0.96
Cyt P-450 from hydroxylapatite (150 mM phosphate)	79	64	1.23
Removal of excess detergent	40	20.4	1.95
0-30% $AmSO_4$	-	0.25	-
30-40% $AmSO_4$	14.5	7.35	1.98
40-50% $AmSO_4$	15.3	5.1	3.0
50-70% $AmSO_4$	9	4.5	2.0
70% supernatant	0.8	1.4	0.6

Table 6,5.

Purification of cytochrome P-450 by ammonium sulphate fractionation.

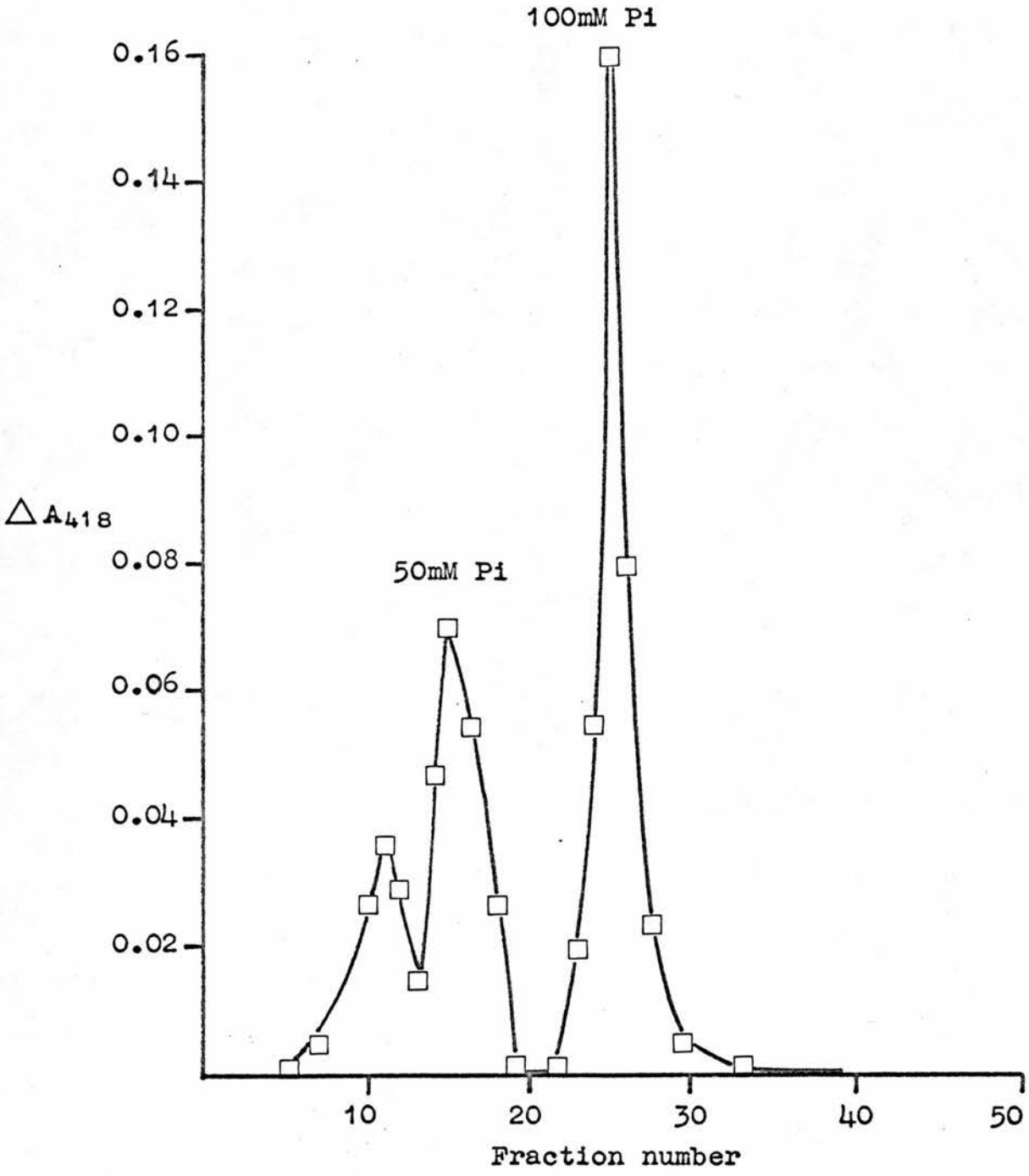


Figure 6,2.

Chromatography on CM-Sephadex of partially purified cytochrome P-450, eluted with incresing concentrations of potassium phosphate buffer,pH 7.0.

phosphate buffer pH 7.7. The bound haemoprotein was then washed with 100ml of this buffer and then eluted with 400mM phosphate buffer. By comparison of the absorbance at 280nm before and after this treatment, and assuming an absorbance of 1 at this wavelength for a 1% protein solution, the concentration of detergent in the eluted cytochrome P-450 fraction was undetectable. The penalty for this reduction of Nonidet P42 concentration was a recovery of only 50% of the cytochrome P-450, although the specific content was increased by 60%. On certain occasions, removal of excess detergent led to the cytochrome P-450 being irretrievably bound to the hydroxylapatite column. However, as demonstrated in Table 6,5, ammonium sulphate fractionation led to a 2.5 fold purification over the 150mM phosphate hydroxylapatite eluate and the precipitate developed by this 40-50% saturation formed a pellet on centrifugation. Thus, although a four-fold purification over the solubilized microsomes had been achieved, the final yield was only 3%.

Cation exchange chromatography

After purification of cytochrome P-450 by DEAE-cellulose and hydroxylapatite chromatography, the cytochrome was applied to columns of both CM cellulose and cellulose phosphate, equilibrated at pH 6.2 in 5mM potassium acetate buffer. Although cytochrome P-450 bound to both columns (more strongly to cellulose phosphate), no purification of this haemoprotein was observed on elution with increasing concentrations of KCl. Figure 6,2 shows the elution profile of DEAE-cellulose and hydroxylapatite treated cytochrome P-450 when applied to CM-Sephadex C-25 equilibrated with 10mM phosphate, pH 7.0, 20% glycerol, 1% Nonidet P42. The eluted fractions were assayed for absorbance at 418 nm as this wavelength is an absorption maximum in preparations which contain no haem other than cytochrome P-450

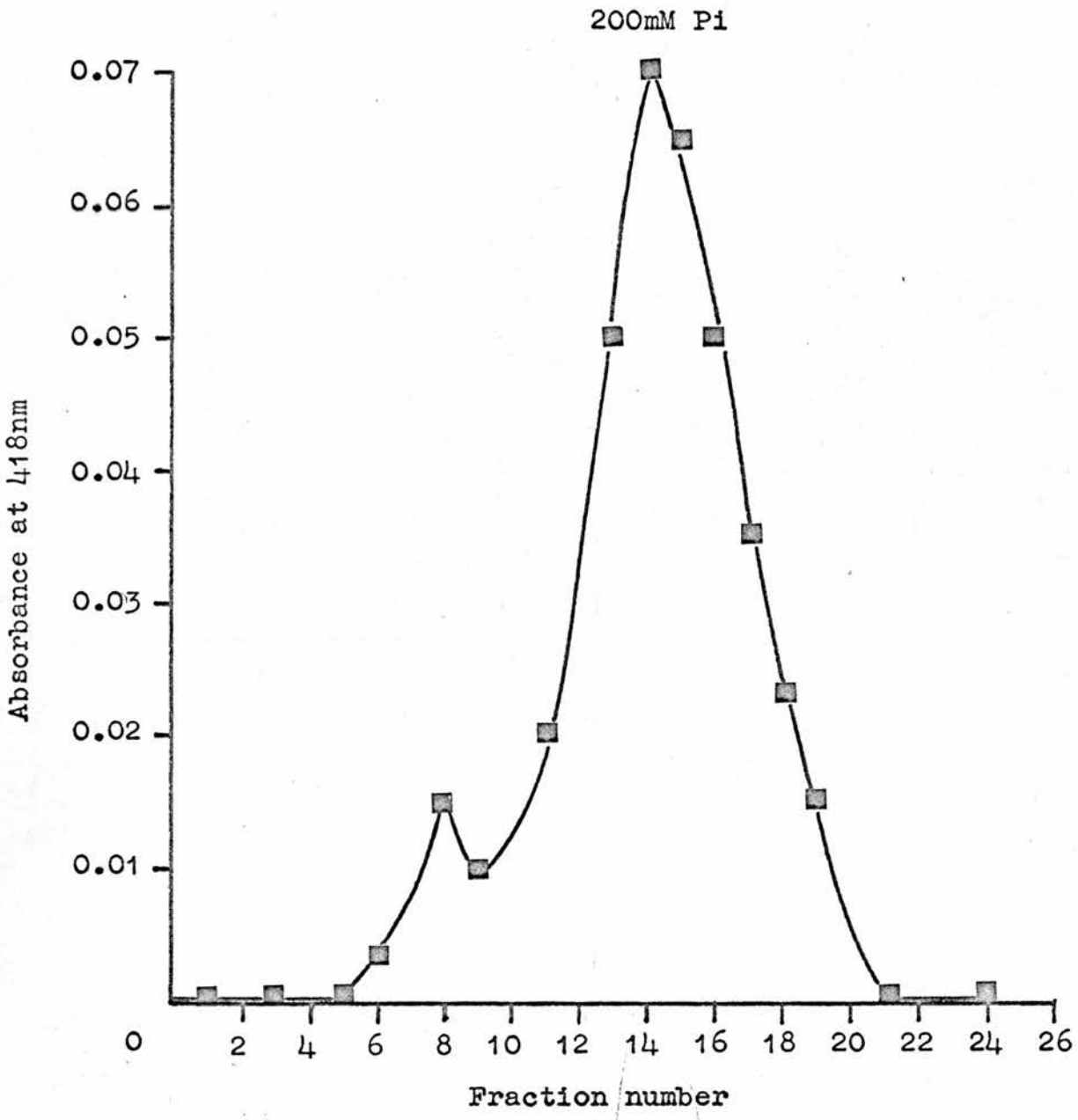


Figure 6,3.

Chromatography of partially purified cytochrome P-450 on cellulose phosphate, eluted by increasing concentrations of potassium phosphate buffer, pH 7.0.

3ml of partially purified cytochrome P-450 were applied to 1.5ml columns of Sepharose 4-B to which were covalently bound R-NH_2 where R = C_6, C_8, or C_{10}. The eluate was diluted to 6ml and cytochrome P-450 determined.

	Cytochrome P-450 in eluate (nmoles / ml)	Specific content (nmoles/mg protein)	Percentage cytochrome P450 bound
Eluate from C_6 column	0.275	0.35	16
Eluate from C_8 column	0.132	0.22	60
Eluate from C_{10} column	0.066	0.12	80

3ml cytochrome P-450 diluted to 6ml gave a final concentration of 0.33 nmoles / ml. (Specific content 0.40 nmoles / mg protein)

Table 6,6.

The binding of cytochrome P-450 to affinity / hydrophobic columns.

and cytochrome P-420. Two major peaks were obtained, one at 50mM phosphate and another at 100mM phosphate. It is, therefore, apparent that cytochrome P-450 at pH 7.0 binds quite strongly to a cation exchanger. However, when the two peaks were individually pooled, the specific content of both fractions was reduced when compared with the hydroxylapatite eluate.

Cytochrome P-450 eluted from hydroxylapatite by 0.5M KCl was applied to a column of cellulose phosphate equilibrated at pH 7.0 with 20mM phosphate, 20% glycerol, 1% Nonidet P42. The concentration of phosphate buffer was increased and at 200mM phosphate, cytochrome P-450 was eluted but the specific content of this haemoprotein of 1.1 nmole/mg protein, was exactly the same as the sample before application to the cellulose phosphate column. Cytochrome P-450 as shown in Figure 6,3 eluted as a sharp, symmetrical peak.

Affinity/Hydrophobic chromatography

The substrates of cytochrome P-450 are in general rather insoluble in aqueous media, and it is, therefore, possible that the catalytic centre of this enzyme is hydrophobic. It is also likely that a large part of this enzyme presents a predominantly hydrophobic surface. For this reason, a sample of cytochrome P-450 (0.41 nmoles/mg protein) was applied to three columns of Sepharose 4B to which were covalently attached a C_6, C_8 and C_{10} side chain. The results are presented in Table 6,6 and show that as the chain length increased, more cytochrome P-450 was bound. The observation that the specific content of cytochrome P-450 in the material not bound decreased suggests that there had been preferential binding of cytochrome P-450. However, the bound haemoprotein which appeared as a red band at the top of the columns could not be eluted by molar KCl or aminopyrine (a substrate for cytochrome P-450) at 240mM in 25% glycerol or 1% Nonidet P42.

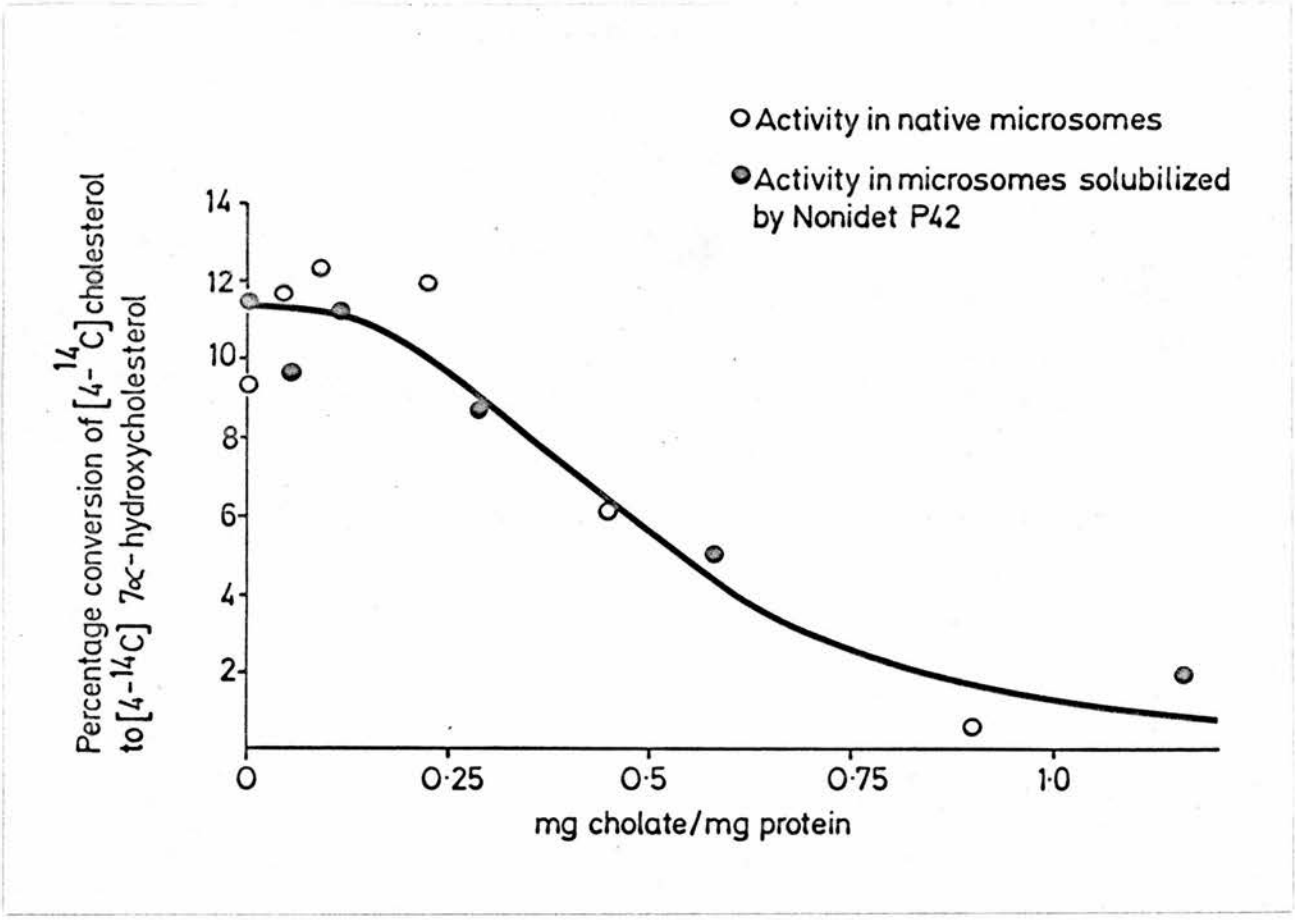


Figure 6,4.

The effect of sodium cholate on cholesterol 7α-hydroxylase activity in native and Nonidet P42 solubilized rat liver microsomes.

Iso-electric focussing

Cytochrome P-450 partially purified by DEAE-cellulose and hydroxylapatite chromatography was applied to a glycerol gradient containing Ampholines. Electrophoresis was continued for 20 hours at 3°C to focus the proteins at their iso-electric points. However, it was observed that after electrophoresis cytochrome P-450 precipitated.

Effect of sodium cholate on the activity of Nonidet P42 solubilized cholesterol 7α-hydroxylase

To the present time no purification of rat liver microsomal cytochrome P-450 to homogeneity has proceeded without the use of the surface active agent, sodium cholate. It has been reported by Yonetani (113) that the non-ionic detergent Emasol 4130 protected cytochrome a from the inhibitory effects of sodium cholate, and for these two reasons the effect of sodium cholate on cholesterol 7α-hydroxylase in native microsomes and the same sample solubilized by Nonidet P42 was observed. The results are shown in Figure 6,4 and show that Nonidet P42 did not influence the inhibitory properties of sodium cholate.

Further purification of NADPH-cytochrome c reductase

Gel filtration

Flavoprotein eluted from DEAE-cellulose was concentrated by ultrafiltration and applied to columns of Sephadex G-200, Sepharose 4B and Bio-gel A 0.5M. The flavoprotein was retarded by these columns but the molecular weight of this species was approximately 400,000, estimated by determination of K_{av}. The eluted peaks were also symmetrical but SDS-gel electrophoresis revealed that the protein was not homogeneous. When a similar preparation was subjected to electrophoresis on a 5% polyacrylamide gel using [4,4' - biphenyldi (2,5-diphenyltetrazolium chloride);NT] as a substrate, and NADPH as a source of reducing

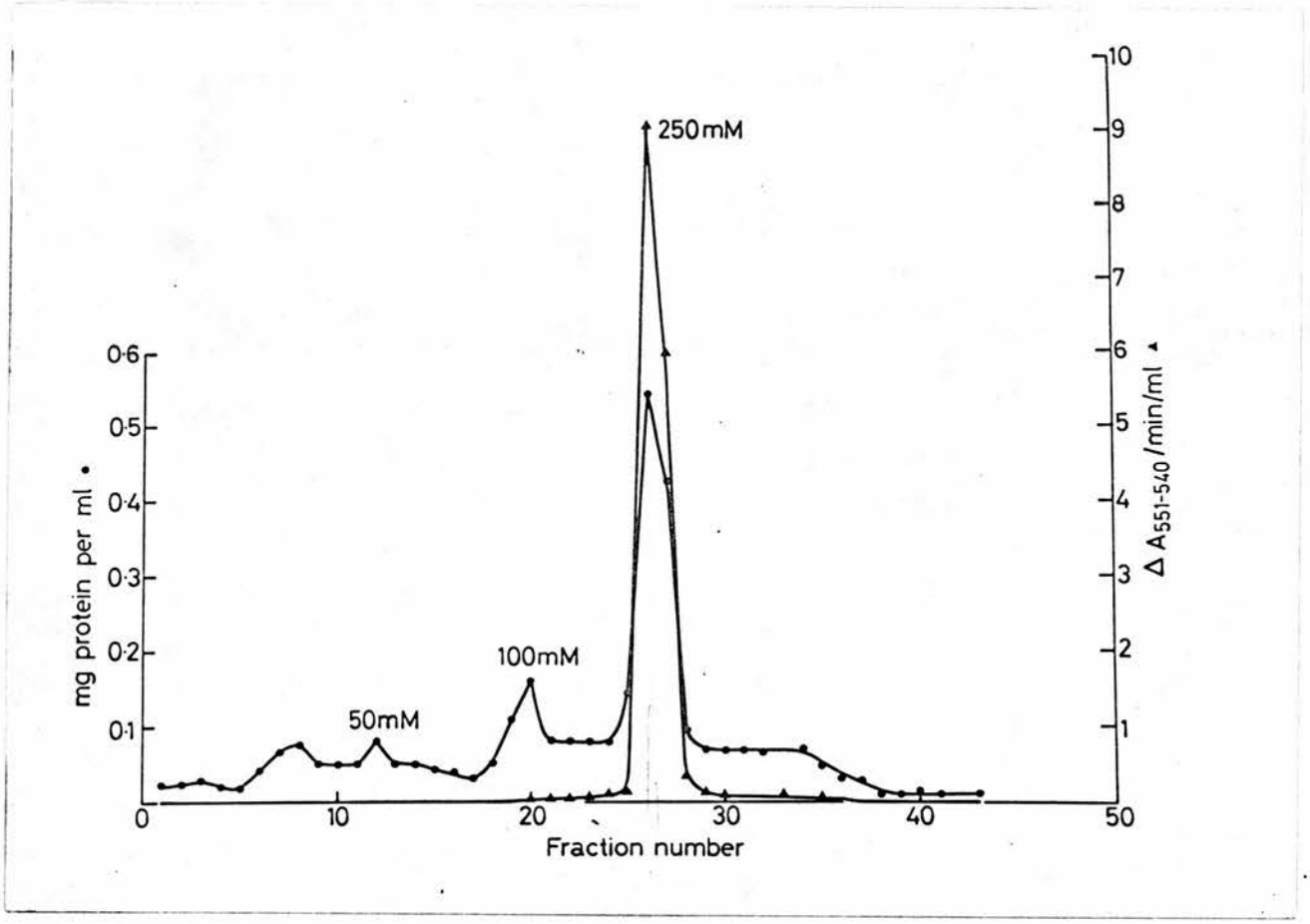


Figure 6,5.

The elution of NADPH-cytochrome c oxidoreductase from a cytochrome c affinity column, by increasing concentrations of KCl.

equivalents, two bands of activity were observed. On one occasion, which has not been possible to reproduce, application of flavoprotein to Sepharose 4B revealed two peaks of NADPH-cytochrome c reductase activity, one of high molecular weight and one which when applied to a Sephadex G-200 column, eluted with a K_{av} equivalent to a molecular weight of 70,000. When this low molecular weight material was subjected to gel electrophoresis, one of the bands of activity was lost, and a single NADPH-neotetrazolium chloride reductase band was observed. It is, therefore, likely that the preparation contains only one NADPH-diaphorase activity.

Cytochrome c affinity column

Cytochrome P-450 reductase has a wide substrate specificity and may transfer electrons from NADPH to cytochrome P-450, neotetrazolium salts, dichlorophenolindophenol, potassium ferricyanide and cytochrome c. Golf et al (48) have utilised this observation by preparing a column of Sepharose 4B on which cytochrome c was immobilised by covalent linkage.

Figure 6,5 shows the elution profile of protein and NADPH-cytochrome c oxidoreductase following step-wise increase of potassium chloride in 5mM potassium phosphate buffer pH 7.7 containing 1% Nonidet P42. Cytochrome b_5, which sometimes is a contaminant of NADPH-cytochrome c oxidoreductase eluted from a DEAE-cellulose column, was eluted at 100mM KCl in the above buffer, and was therefore quite strongly adsorbed. It is not clear whether the adsorption is due to the basicity of cytochrome c or whether cytochrome c is acting as a substrate for cytochrome b_5. The flavoprotein was eluted at 250mM KCl and the specific activity of NADPH-cytochrome c oxidoreductase was increased three-fold over that found in the DEAE-cellulose eluate.

SUMMARY

(1) The use of cation exchange and gel exclusion chromatography did not lead to any increase in the specific content of cytochrome P-450, nor of NADPH-cytochrome c oxidoreductase specific activity, even though the haemoprotein was retarded on the cation exchange resins and both proteins retarded by Sepharose 4B.

(2) Polyethyleneglycol 6000 fractionation of cytochrome P-450 led to a small increase in specific content, but ammonium sulphate fractionation, after removal of Nonidet P42, led to a 2.5 fold purification.

(3) Both hydroxylapatite and calcium phosphate gel chromatography resulted in approximately two-fold purification of cytochrome P-450.

(4) Hydrophobic chromatography and iso-electric focussing were not found useful in the attempt to further purify cytochrome P-450.

(5) Nonidet P42 was found not to affect the degree of inhibition of cholesterol 7α-hydroxylase by sodium cholate and, therefore, this bile acid detergent could not be used as an aid to purification.

SECTION 7

THE RECONSTITUTION OF RAT LIVER MICROSOMAL CHOLESTEROL 7α-HYDROXYLASE

Although solubilization is generally assumed to have occurred when the enzyme in question remains in the supernatant fraction after centrifugation for 1 hour at 105,000 x g, a more useful criterion in the case of a multi-enzyme complex is separation of the components by standard biochemical techniques of purification, and reconstitution of enzymic activity upon recombination of partially purified components. Demonstration of absolute requirements necessitates the purification of a protein to homogeneity and recombining with a fraction which by itself has no activity. As important and informationally useful is the elimination of contaminating enzymes which might interfere with, or be components of, a multi-enzyme complex. In the case of cholesterol 7α-hydroxylase, these could be such enzymes as cytochrome b_5 and NADH-cytochrome c reductase. By such elimination it should be possible to show whether the microsomal components are absolute requirements or play just a facilitating role.

As a result of these reconstitution experiments it has been possible to demonstrate that in the recombined fractions solubilized by the method of Lu and Coon (79), phosphatidylcholine was required for maximal drug hydroxylation activity (114). Bjorkhem et al (86) have reported that NADPH-cytochrome c reductase from phenobarbital fed rats was twice as efficient in supporting the 7α-hydroxylation of cholesterol in a reconstituted system solubilized by sodium cholate as reductase from control or cholestyramine fed rats. Reconstitution experiments have also shown that the four types of

	Percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] 7α-hydroxy-cholesterol
1.3 nmoles cytochrome P-450	-
3.0 nmoles cytochrome b_5	-
2.0 units NADPH-cytochrome c reductase	-
1.3 nmoles cytochrome P-450 + 3.0 nmoles cytochrome b_5	-
3.0 nmoles cytochrome b_5 +2.0 units NADPH-cytochrome c reductase	-
1.3 nmoles cytochrome P-450 +2.0 units NADPH-cytochrome c reductase	6.1
1.3 nmoles cytochrome P-450 +3.0 nmoles cytochrome b_5 +2.0 units NADPH-cytochrome c reductase	3.0

Table 7,1.

A rat liver microsomal acetone powder solubilized by Nonidet P42 was subjected to DEAE-cellulose chromatography and fractions containing cytochrome P-450, cytochrome b_5 and NADPH-cytochrome c reductase were pooled. These fractions were tested alone and recombined with each other for their ability to support the 7α-hydroxylation of cholesterol.

cytochrome P-450 recently partially purified may have different specificities towards the hydroxylation of drugs and steroids (107).

This section, therefore, deals with the requirements for activity of the reconstituted cholesterol 7∝-hydroxylase complex and the interdependence of these components.

The determination of the components of rat liver microsomal mixed function oxidase necessary for reconstitution of cholesterol 7∝-hydroxylase

400mg of an acetone powder having been solubilized with 0.6ml Nonidet P42 was applied to a DEAE-cellulose column, and cytochrome P-450, cytochrome b_5 and NADPH-cytochrome c reductase separated from each other. When each tube was assayed individually low to negligible cholesterol 7∝-hydroxylase activity could be observed. Previous evidence has suggested that cytochrome P-450 and NADPH-cytochrome c reductase were involved in the 7∝-hydroxylation of cholesterol and therefore these two fractions, and also a fraction containing cytochrome b_5, eluted from DEAE-cellulose, were separately pooled. Table 7,1 shows the results when these three fractions were assayed for cholesterol 7∝-hydroxylase activity either alone or recombined. The fraction containing cytochrome P-450 contained no cytochrome b_5 or NADPH-cytochrome c reductase and had no cholesterol 7∝-hydroxylase activity. The cytochrome b_5 fraction which was pooled contained no detectable cytochrome P-450 or its reductase and was inactive in the hydroxylation of cholesterol. The reductase fraction contained no detectable cytochromes P-450 or b_5, and was also inactive in the 7∝-hydroxylation of cholesterol. When cytochrome P-450 was recombined with cytochrome b_5 , or when cytochrome b_5 was recombined with NADPH-cytochrome c reductase in the presence of NADPH and radioactive $\left[4 - {}^{14}C\right]$ cholesterol, no cholesterol 7∝-hydroxylase

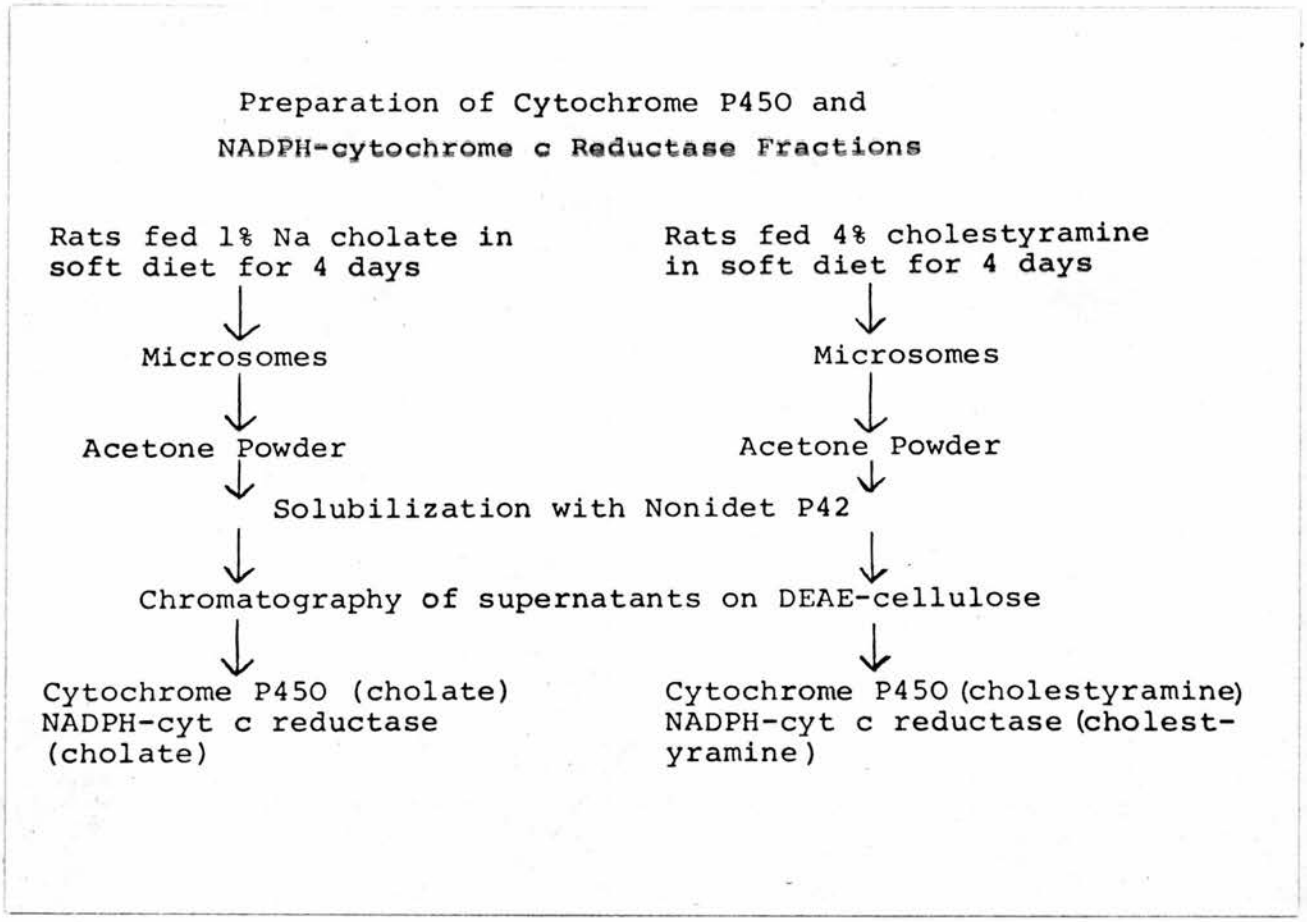


Table 7,2.

The preparation of cytochrome P-450 and NADPH-cytochrome c reductase from rats fed either sodium cholate or cholestyramine in the diet.

activity could be detected. However, when a fraction containing cytochrome P-450 was recombined with the NADPH-cytochrome c reductase fraction and a NADPH generating system, 6.1% of the radioactive cholesterol was converted to [4 - ^{14}C] 7α-hydroxycholesterol after incubation at 37^{o}C for 1 hour. This same recombination when incubated with the fraction containing cytochrome b_5 converted only 3% of the radioactive cholesterol to [4 - ^{14}C] 7α-hydroxycholesterol. Thus, at first sight, only the cytochrome P-450 and NADPH-cytochrome c reductase fractions appear to be necessary for the enzymic 7α-hydroxylation of cholesterol, and the cytochrome b_5 fraction appears inhibitory.

Attempts to establish in which fraction cholesterol 7α-hydroxylase specificity resides

Two groups of rats were fed in one case a diet containing 4% w/w cholestyramine resin, which increases the hepatic cholesterol 7α-hydroxylase activity, and in another, the same diet containing 0.75% sodium cholate which lowers the hepatic cholesterol 7α-hydroxylase activity (115). Liver microsomes were made in the usual way and acetone powders were prepared from both groups of rats. In both cases 300mg of the powders were solubilized with 0.45ml Nonidet P42, and after centrifugation at 105,000 x g for 1 hour, the supernatants were applied to two DEAE-cellulose columns similarly constructed. A linear gradient of KCl was applied to each column and fractions containing cytochrome P-450, cytochrome b_5 and NADPH-cytochrome c reductase eluted from the column were collected and pooled. This is summarised in Table 7,2. The specific content of cytochrome P-450 in both the fractions pooled from the two columns were fortuitously identical. The pooled NADPH-cytochrome c reductase fraction from the column to which solubilized liver microsomal acetone powder from the

	Percentage Conversion of cholesterol to 7α hydroxycholesterol
2.6 nmoles cyt P450 (cholate)	0.1
2.6 nmoles cyt P450 (cholestyramine)	n.d.
7.2 units NADPH-cyt c reductase (cholate)	n.d.
7.2 units NADPH-cyt c reductase (cholestyramine)	n.d.
2.6 nmoles cyt P450 (cholestyramine) + 7.2 units NADPH-cyt c reductase (cholestyramine)	4.7
2.6 nmoles cyt P450 (cholestyramine) + 7.2 units NADPH-cyt c reductase (cholate)	4.9
2.6 nmoles cyt P450 (cholate) + 7.2 units NADPH-cyt c reductase (cholestyramine)	1.0
2.6 nmoles cyt P450 (cholate) + 7.2 units NADPH-cyt c reductase (cholate)	1.0
Supernatant activity (cholestyramine)	4.6
Supernatant activity (cholate)	0.7

n.d. - not detectable

Table 7,3.

The reconstitution of cholesterol 7α-hydroxylase activity using the fractions prepared as in Table 7,2.

cholate fed rats was applied, had 33% more activity per ml (expressed as nmoles cytochrome c reductase reduced per minute) than the other pooled fraction. Thus, in the reconstitutions, 3ml of the flavoprotein from the cholate fed rats, were used. In Table 7,3 the results of recombining the same quantity of liver microsomal cytochrome P-450 from cholate or cholestyramine fed rats with NADPH-cytochrome c reductase from either cholate or cholestyramine fed rats, are shown. When 2.56 nmoles of liver microsomal cytochrome P-450 from cholestyramine fed rats were incubated with 7.2 units of reductase from both preparations, the percentage conversion of $\left[4 - {}^{14}C\right]$ cholesterol to $\left[4 - {}^{14}C\right]$ 7α-hydroxycholesterol were 4.7% and 4.9%. Thus, there is no substantial difference in the ability of the two preparations of reductase to support the 7α-hydroxylation of cholesterol. When 2.56 nmoles of cytochrome P-450 from the cholate fed rats was recombined with 7.2 units of both preparations of reductase, only 1.0% conversion of radioactive cholesterol to $\left[4 - {}^{14}C\right]$ 7α-hydroxycholesterol occurred in both cases. Again there was no difference in the efficiency of the reductases in supporting the 7α-hydroxylation of cholesterol, although the activity of the reconstituted system was only 20% that of the reconstituted system in which the cytochrome P-450 was obtained from rats fed the bile acid sequestering resin, cholestyramine. When an aliquot of the supernatants from the solubilized liver microsomal acetone powders of both groups of rats were assayed for cholesterol 7α-hydroxylase activity, the percentage conversion of radioactive cholesterol to $\left[4 - {}^{14}C\right]$ 7α-hydroxy-cholesterol in the supernatant from the cholestyramine fed rats was 4.6, and in the case of the cholate fed rats was 0.7. Although the results of this experiment do not constitute absolute proof that the specificity of cholesterol 7α-hydroxylase resides in the cytochrome

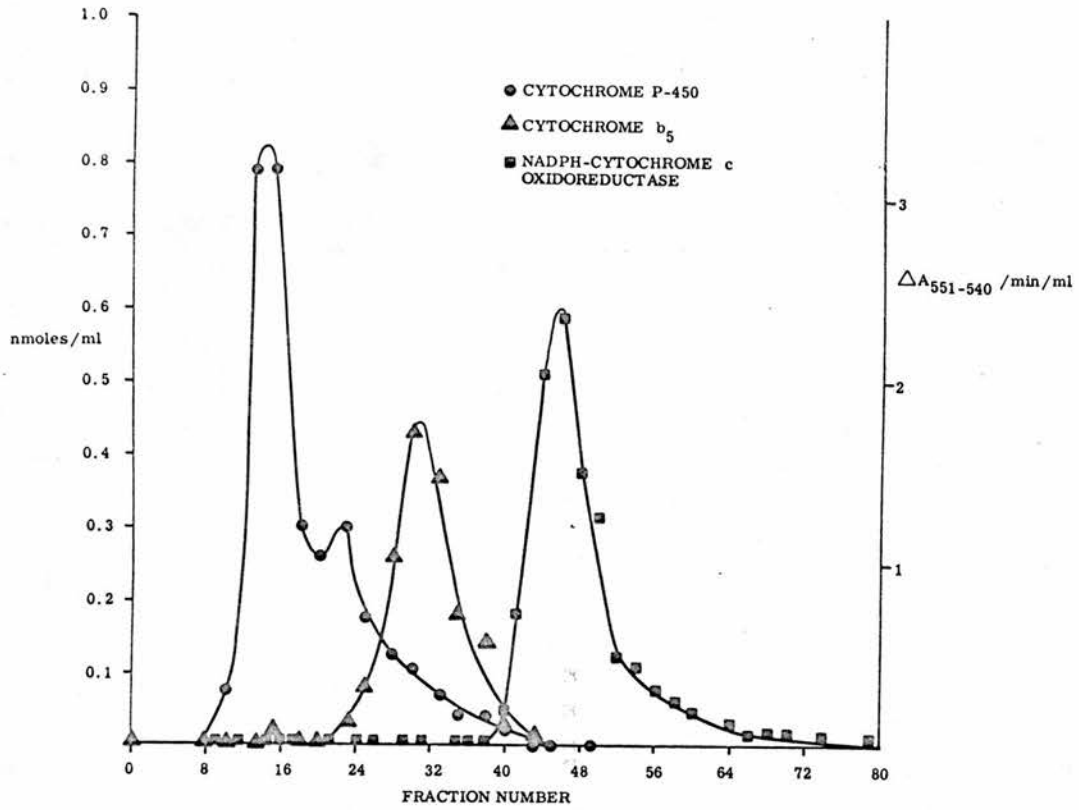


Figure 7,1.

The elution profile of Nonidet P42 solubilized microsomal cytochrome P-450, cytochrome b5, and NADPH-cytochrome c oxidoreductase from a DEAE-cellulose column, eluted with a linear gradient of KCl.

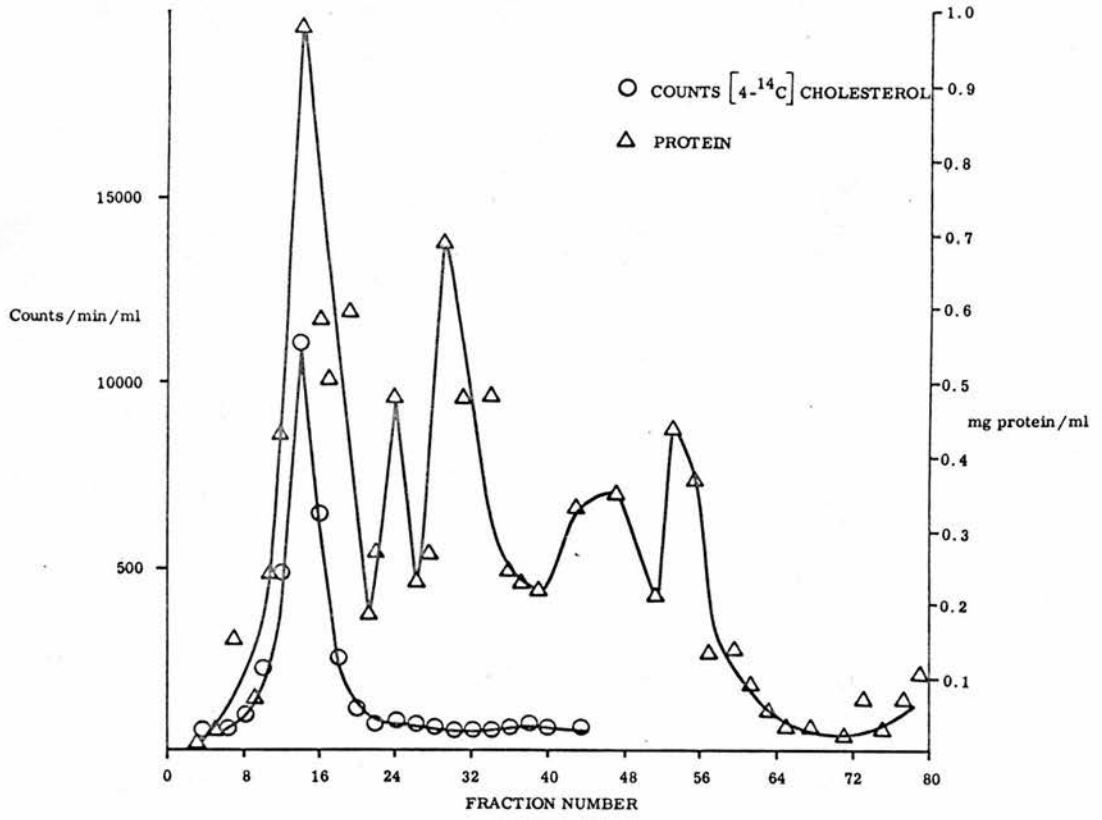


Figure 7,2.

[4-^{14}C]-cholesterol was added to the supernatant of Nonidet P42 solubilized microsomes, and applied to a DEAE-cellulose column. Cholesterol and protein were eluted using a linear gradient of KCl.

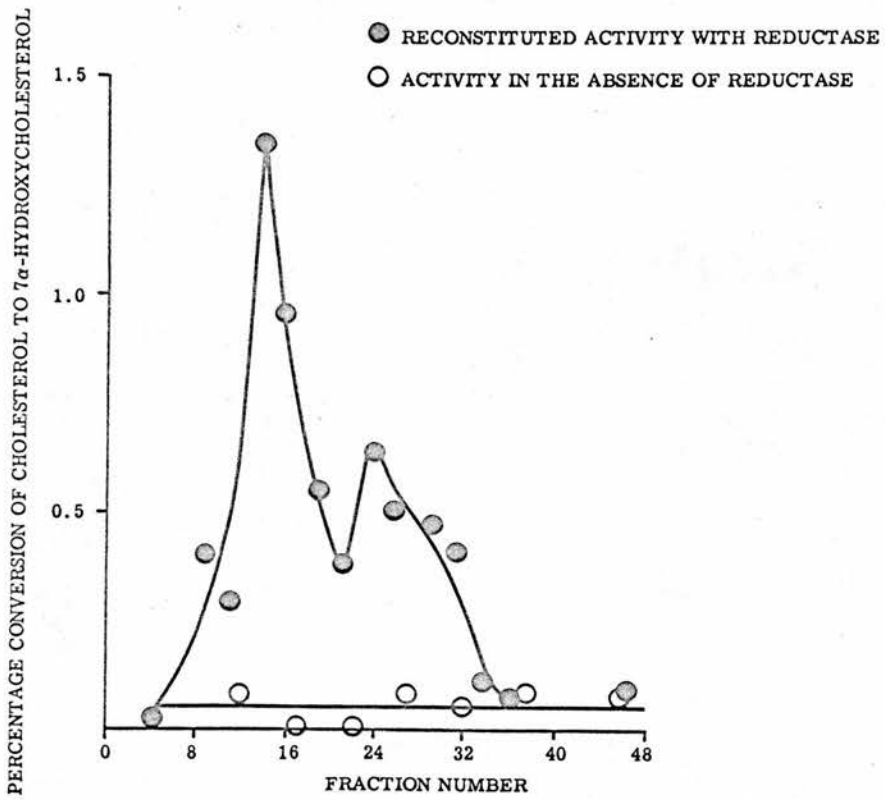


Figure 7,3.

After pooling the NADPH-cytochrome c oxidoreductase, this fraction (which itself contained no cholesterol 7α-hydroxylase activity) was recombined with other fractions eluted from the DEAE-cellulose column, and tested for their ability to catalyse the 7α-hydroxylation of cholesterol. Control incubations contained no NADPH-cytochrome c oxidoreductase.

P-450 fraction, there is reasonable indication that this is the case.

Cholesterol 7α-hydroxylase activity in DEAE-cellulose eluate

It had been previously observed that when rat liver microsomes were solubilized by Nonidet P42 and applied to a column of DEAE-cellulose, on elution at least two, and probably three, peaks of cytochrome P-450 were detected. Therefore, to determine whether any minor peak was solely responsible for the catalysis of the 7α-hydroxylation of cholesterol, Nonidet P42 solubilized microsomes were applied to a DEAE-cellulose column and eluted with a linear gradient of KCl. Elution of cytochrome P-450, cytochrome b_5 and NADPH-cytochrome c oxidoreductase is shown in Figure 7,1. Elution of [4 - ^{14}C] cholesterol added to the supernatant fraction before application to the column, and protein, is shown in Figure 7,2. The NADPH-cytochrome c oxidoreductase fractions were pooled and recombined with fractions 10 to 38 eluted from the column. The reconstituted activities, together with those fractions incubated in the absence of reductase, were measured as percentage conversion of [4 - ^{14}C] cholesterol to radioactive 7α-hydroxycholesterol. The results are shown in Figure 7,3. It is apparent that cholesterol 7α-hydroxylase activity is located wherever cytochrome P-450 is found and is not restricted to any one of the three peaks of cytochrome P-450 eluted.

The interaction of partially purified cytochrome P-450 with DCC-trypsin solubilized NADPH-cytochrome c oxidoreductase

A number of extrinsic microsomal proteins may be solubilized by hydrolytic enzymes. Velick, Strittmatter and Omura et al (66,67,68,117) have published extensively on cytochrome b_5 and its reductase which was initially solubilized by cobra venom (117).

	Percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] 7α-hydroxy cholesterol.
Supernatant activity	16.0
2.0 nmoles cytochrome P-450	0.12
2.0 nmoles cytochrome P-450 + 5.0 units trypsin solubilized NADPH-cytochrome c reductase	0.44
2.0 nmoles cytochrome P-450 + 10 units trypsin solubilized NADPH-cytochrome c reductase	0.26
2.0 nmoles cytochrome P-450 + 15 units trypsin solubilized NADPH-cytochrome c reductase	0.32

Table 7,4.

The effect of adding to a partially purified preparation of cytochrome P-450,NADPH-cytochrome c oxidoreductase solubilized by treatment of rat liver microsomes with trypsin, on its ability to 7α-hydroxylate cholesterol.

NADPH-cytochrome c reductase has also been solubilized by a number of agents, notably by pancreatin or steapsin (70), but also by bromelain (118) and subtilisin (119).

Only recently has the purification of NADPH-cytochrome c oxidoreductase been reported using detergents (120) although this flavoprotein had been solubilized by detergent and its molecular weight determined some years before (118). In an effort to obtain a large stock of pure NADPH-cytochrome c oxidoreductase with which to reconstitute cholesterol 7α-hydroxylase activity, microsomes were prepared from 50 rat livers. The 1.5g of microsomal protein obtained was suspended in 130ml of 0.1M potassium phosphate buffer pH 7.7 containing 1mM EDTA. 6mg DCC-trypsin (a chymotrypsin/trypsin mixture in which the chymotrypsin had been inactivated with diphenylcarbamoyl chloride) was added to the above suspension at 0^{o}C and left for approximately 14 hours at 0-3^{o}C with stirring and under a positive nitrogen pressure. After centrifugation at 105,000 x g for 1 hour, the supernatant was subjected to ammonium sulphate treatment. The 45-75% cut was applied to Sephadex G-100 and the reductase fractions applied to DEAE-cellulose. A linear gradient of KCl was used to elute the flavoprotein and the reductase fractions pooled, desalted on Sephadex G-25, and lyophilised.

234 units of NADPH-cytochrome c oxidoreductase activity were present in the microsomal fraction, and after solubilization 380 units were observed. The reason for this activation is not known. The final lyophilised preparation had a total activity of 230 units and the $\Delta A_{551-540}$/min/mg was 38 compared with the microsomal specific activity of 0.17. Thus, the enzyme had been purified approximately 220-fold. Table 7,4 shows the effect of increasing the amount of NADPH-cytochrome c oxidoreductase activity to 2 nmoles cytochrome P-450,

	Percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] 7α-hydroxy-cholesterol.
Supernatant activity	7.28
2.5 nmoles cytochrome P-450	0.04
6 units Nonidet P42 solubilized NADPH-cytochrome c reductase	-
7.4 units trypsin solubilized NADPH-cytochrome c reductase	-
2.5 nmoles cytochrome P-450 + 6 units Nonidet P42 solubilized NADPH-cytochrome c reductase	7.72
2.5 nmoles cytochrome P-450 + 7.4 units trypsin solubilized NADPH- cytochrome c reductase	0.53
2.5 nmoles cytochrome P-450 + 7.4 units trypsin solubilized NADPH-cytochrome c reductase + 1mg lecithin	0.56

Table 7,5.

The ability of trypsin and Nonidet P42 solubilized NADPH-cytochrome c oxidoreductase to support the 7α-hydroxylation of cholesterol when recombined with partially purified cytochrome P-450.

purified from cytochrome b_5 and NADPH-cytochrome c oxidoreductase. The control activity showed a 16% conversion of [4 - ^{14}C] cholesterol to [4 - ^{14}C] 7α-hydroxycholesterol, though very little, if any, reconstitutional activity could be demonstrated with the trypsin solubilized reductase. In a subsequent experiment an acetone powder was solubilized and fractionated in the usual way, and 2.5 nmoles of cytochrome P-450 were recombined with 6 units of Nonidet P42 solubilized reductase or 7 units of trypsin solubilized reductase. These studies were made with and without 1mg egg lecithin. The results are presented in Table 7,5. Cytochrome P-450 and both preparations of reductase, when assayed for cholesterol 7α-hydroxylase activity in the standard incubation medium, showed no activity. However, when the haemoprotein was recombined with Nonidet P42 partially purified NADPH-cytochrome c oxidoreductase, the reconstituted enzyme was active, 7.7% of the radioactive cholesterol being converted to 7α-hydroxycholesterol. The trypsin solubilized reductase supported only 0.5% conversion of tracer cholesterol to 7α-hydroxycholesterol, and this activity was not increased when 1mg egg lecithin was added. It is, therefore, clear that (a) during the purification of the trypsin solubilized reductase some other essential factor is lost, or (b) because part of the reductase is lost upon trypsin solubilization (118) it is unable to interact with cytochrome P-450. This inability of trypsin solubilized reductase to support the 7α-hydroxylation of cholesterol correlates with the recent comment in the review by Lu and Levin (102) that reductase prepared by trypsin digestion fails to transfer electrons from NADPH to cytochrome P-450, even in the presence of phospholipid. It therefore appears from these results and those of Okuda et al (121) that the 'hydrophobic' part of membrane bound proteins is important, if not essential, for the proper functioning of their catalytic activities.

	Percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] 7α-hydroxy-cholesterol.
0.7 nmoles cytochrome P-450 eluted from DEAE-cellulose + 10 units NADPH-cytochrome c reductase	3.3
0.6 nmoles cytochrome P-450 eluted by 80mM phosphate buffer from hydroxylapatite + 10 units NADPH-cytochrome c reductase	4.8
0.6 nmoles cytochrome P-450 eluted by 150mM phosphate buffer from hydroxylapatite + 10 units NADPH-cytochrome c reductase	3.7

Neither the cytochrome P-450 fractions alone nor the NADPH-cytochrome c oxidoreductase alone contained cholesterol 7α-hydroxylase activity.

Table 7,6.

Cytochrome P-450 was eluted from hydroxylapatite by increasing concentrations of potassium phosphate buffer, pH 7.7 containing 1% Nonidet P42 and 20% glycerol, and recombined with partially purified NADPH-cytochrome c oxidoreductase.

The reconstitution of cholesterol 7α -hydroxylase after DEAE-cellulose and hydroxylapatite chromatography of liver microsomal cytochrome P-450

Cytochrome P-450 eluted from DEAE-cellulose was pooled and subjected to hydroxylapatite chromatography in the presence of 20% glycerol and 1% Nonidet P42. Two fractions of cytochrome P-450, further purified by approximately two-fold, were eluted at 80mM and 150mM phosphate and tested for their ability to support the 7α -hydroxylation of cholesterol in the presence of NADPH-cytochrome c oxidoreductase. It may be seen from Table 7,6 that with 0.6 nmoles of cytochrome P-450 the percentage conversion of [4 - ^{14}C] cholesterol to [4 - ^{14}C] 7α -hydroxycholesterol was 4.8% in the fraction eluted at 80mM phosphate and 3.7% in the 150mM phosphate fraction. This compares with the 3.3% conversion observed in the pooled cytochrome P-450 from DEAE-cellulose.

Reconstitution of cholesterol 7α -hydroxylase activity after calcium phosphate gel chromatography

The 80mM and 150mM phosphate eluates from hydroxylapatite were pooled, dialysed overnight against 10mM phosphate and 20% glycerol. Calcium phosphate gel was added and eluted with increasing concentrations of phosphate buffer. Three fractions were eluted which yielded cytochrome P-450 with a higher specific content than the hydroxylapatite eluate. Table 7,7 shows the results when these fractions were incubated with reductase and [4 - ^{14}C] cholesterol, and demonstrate that cholesterol 7α -hydroxylase activity is not lost when cytochrome P-450 is further purified by calcium phosphate gel.

Reconstitution of cholesterol 7α -hydroxylase activity after removal of excess detergent

A lyophilised powder of rat liver microsomes was solubilized and applied to a DEAE-cellulose column. Cytochrome P-450

	Percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] 7α-hydroxy-cholesterol.
Supernatant activity	6.0
2.0nmoles cytochrome P-450 eluted from DEAE-cellulose + 11 units NADPH-cytochrome c reductase	4.5
1.0 nmole cytochrome P-450 from the pooled 150mM and 80mM phosphate eluates + 11 units NADPH-cytochrome c reductase	6.6
1.0 nmole cytochrome P-450 from which excess Nonidet P42 had been removed + 11 units NADPH-cytochrome c reductase	12.1

Table 7,8.

The effect of removing excess Nonidet P42 from the cytochrome P-450 fraction on cholesterol 7α-hydroxylase activity in a reconstituted system.

	Percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] 7α-hydroxy-cholesterol.
0-30% $AmSO_4$, 0.0 nmoles cytochrome P-450	0.0
30-40% $AmSO_4$, 2.4 nmoles cytochrome P-450	10.3
40-50% $AmSO_4$, 2.6 nmoles cytochrome P-450	10.3
50-70% $AmSO_4$, 1.6 nmoles cytochrome P-450	4.6
70% supernatant, 0.05 nmoles cytochrome P-450	0.0

All cytochrome P-450 fractions were recombined with 8.4 units NADPH-cytochrome c reductase.

Table 7,9.

Cholesterol 7α-hydroxylase activity in cytochrome P-450 fractionated by ammonium sulphate precipitation, when recombined with NADPH-cytochrome c oxidoreductase.

fractions , and also NADPH-cytochrome c oxidoreductase, were separately pooled. On hydroxylapatite, again it was observed that those fractions eluted at 80mM and 150mM contained cytochrome P-450 of the highest specific content and also the greatest cholesterol 7α-hydroxylase activity. When these two fractions were pooled, applied to a column of hydroxylapatite and the excess Nonidet P42 removed, on elution with 300mM phosphate a cytochrome P-450 fraction was obtained with a specific content increased by 50% and which, on recombination with reductase, was able to convert 12.1% of the [4 - ^{14}C] cholesterol to [4 - ^{14}C] 7α-hydroxycholesterol. These results are summarised in Table 7,8.

Reconstitution of cholesterol 7α-hydroxylase activity after ammonium sulphate fractionation

Cytochrome P-450 from a lyophilised powder whose cholesterol 7α-hydroxylase activity was represented by a percentage conversion of radioactive cholesterol to [4 - ^{14}C] 7α-hydroxy-cholesterol of approximately 10%, was purified 2.5 fold by DEAE-cellulose and batch-wise hydroxylapatite chromatography followed by chromatography on a column of hydroxylapatite to remove excess Nonidet P42. This final preparation was treated with ammonium sulphate to give five fractions, the properties of which are given in Table 6,5. These fractions were dialysed and 1ml of each recombined with 8.4 units of reductase. The reconstituted cholesterol 7α-hydroxylase activities are shown in Table 7,9 and demonstrate that the activity remains with the cytochrome P-450 containing fractions.

The interdependence of cytochrome P-450, cytochrome b_5 and NADPH-cytochrome c oxidoreductase in the cholesterol 7α-hydroxylase complex

Because of the poor yields obtained during attempted

purification of the mixed function oxidase components, the following experiments were performed on the components which were, as far as could be detected, free from each other.

The cytochrome P-450 fraction was prepared by DEAE-cellulose chromatography followed by chromatography on hydroxylapatite. This resulted in a 3.5 fold purification, the final specific content being 2.62 nmoles/mg protein. It had been observed previously that the peak of NADH-cytochrome c reductase activity resided in the cytochrome P-450 fraction. It is, therefore, apparent that some cytochrome b_5 must have been contained in this fraction since the reduction of cytochrome c by NADH is dependent on cytochrome b_5 (121,122). However, the total NADH-cytochrome c reductase activity eluted from DEAE-cellulose represented less than 2% of the original activity. This was presumably because the bulk of the cytochrome b_5 activity was separated from cytochrome P-450, or that this reductase was inactivated by DEAE-cellulose. There is also the possibility that NADH-cytochrome c reductase was inactivated due to dilution (104) but this is unlikely as the activity in the DEAE-cellulose eluate was compared with the supernatant fraction diluted 12-fold.

Cytochrome b_5 was assayed in the cytochrome P-450 fraction by the addition of NADH. This haemoprotein could not be detected even with the addition of NADH-cytochrome c reductase. Endogenous cytochrome b_5 reductase was known to be present as 5μl of the cytochrome P-450 fraction supported the rapid reduction of cytochrome b_5 by NADH. It was also known from previous experiments that in similar preparations, if 2% of the original cytochrome b_5 contaminated the cytochrome P-450 fraction, this could be very clearly demonstrated. Cytochrome b_5 cannot, of course, be assayed by the

Specific content of cytochrome P-450 (nmoles / mg protein)	2.7
Specific content of cytochrome b_5 (nmoles / mg protein)	2.3
Specific activity of NADPH- cytochrome c oxidoreductase ($A_{551-540}$ / min / mg protein)	13.4

Table 7,10.

The purity of rat liver microsomal mixed function oxidase components used in the experiments described in figures 7,4, to 7,9.

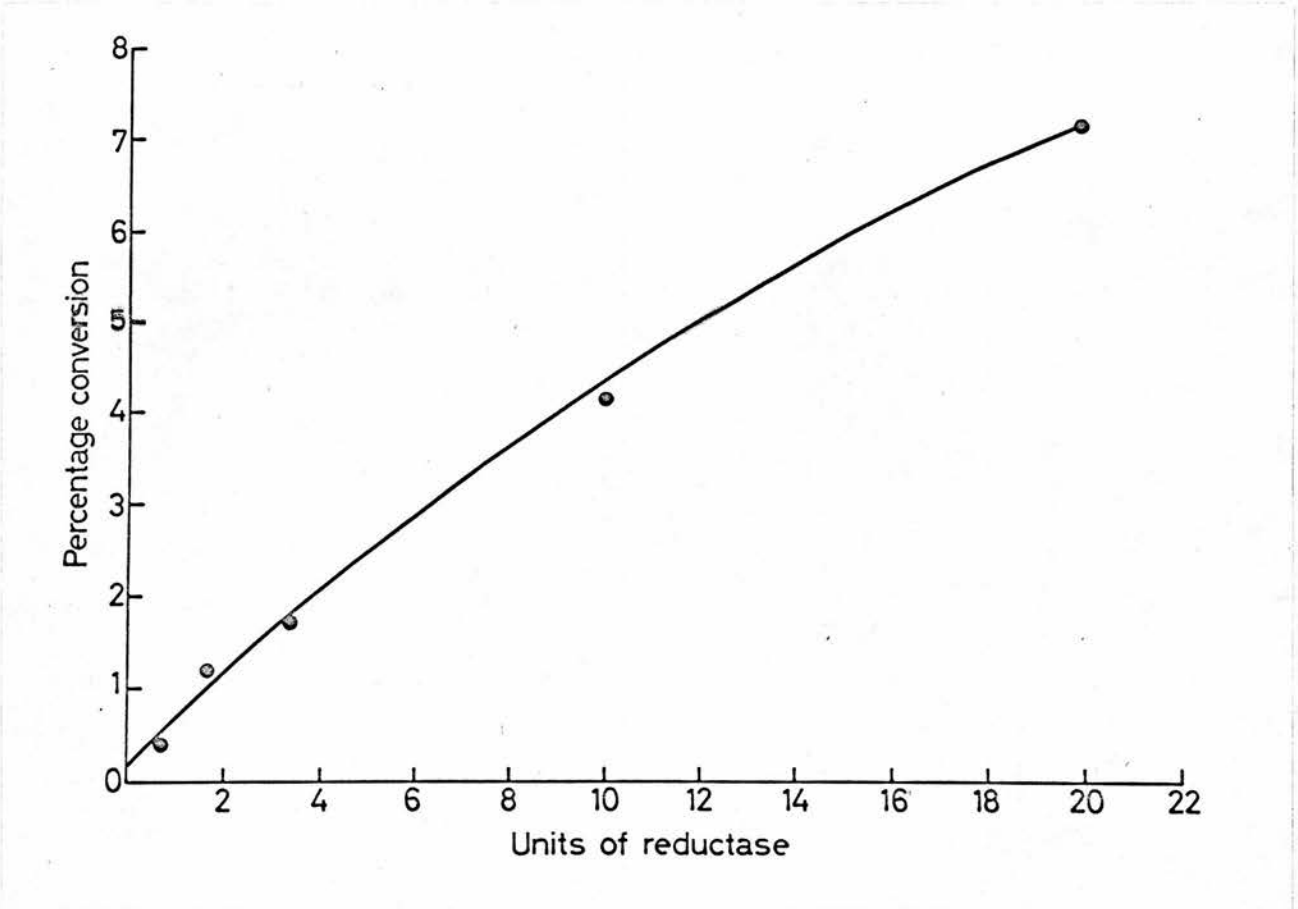


Figure 7,4.

The effect of increasing concentrations of NADPH-cytochrome c oxidoreductase on the velocity of cholesterol 7α-hydroxylase, when added to 2.7nmoles of cytochrome P-450.

addition of sodium dithionite in the presence of cytochrome P-450 because of the negative absorption of cytochrome P-450 at approximately 420nm in the reduced-oxidised difference spectrum. After hydroxylapatite chromatography, cytochrome b_5, NADH-cytochrome c reductase and NADPH-cytochrome c reductase could not be detected.

The removal of cytochrome P-450 from cytochrome b_5 was more difficult than the removal of cytochrome b_5 from cytochrome P-450, but after repeated chromatography on DEAE-cellulose, a preparation of cytochrome b_5 was obtained which did not contain cytochrome P-450 or P-420. No NADPH-cytochrome c oxidoreductase activity was observed but NADH could reduce cytochrome b_5 very slowly; in effect, the complete reduction would have taken approximately 45 minutes, but addition of 2 μl of supernatant to the 3ml cuvette enabled rapid reduction by NADH to completion as assayed by the addition of sodium dithionite.

NADPH-cytochrome c reductase was purified 10-fold by DEAE-cellulose chromatography, cytochrome c affinity chromatography and further chromatography on DEAE-cellulose. Cytochrome P-450 could not be detected, neither could cytochrome b_5 by the addition of sodium dithionite or by NADH in the presence of NADH-cytochrome c reductase. Table 7,10 shows the final activities of the prepared components of the mixed function oxidase.

Dependence of cholesterol 7α-hydroxylase activity on NADPH-cytochrome c oxidoreductase

Figure 7,4 shows the results of incubating 2.7 nmoles of cytochrome P-450 with increasing quantities of NADPH-cytochrome c oxidoreductase, which itself contained negligible amounts of cholesterol. It is quite clear that increasing the concentration of

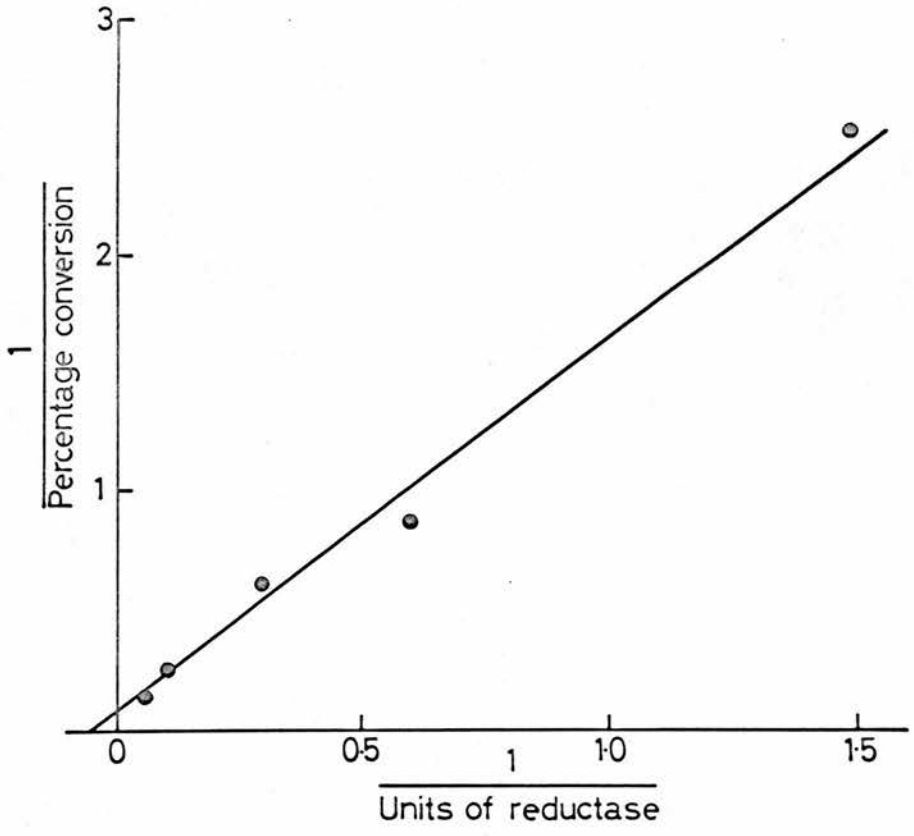


Figure 7,5.

A double reciprocal plot of results presented in figure 7,4.

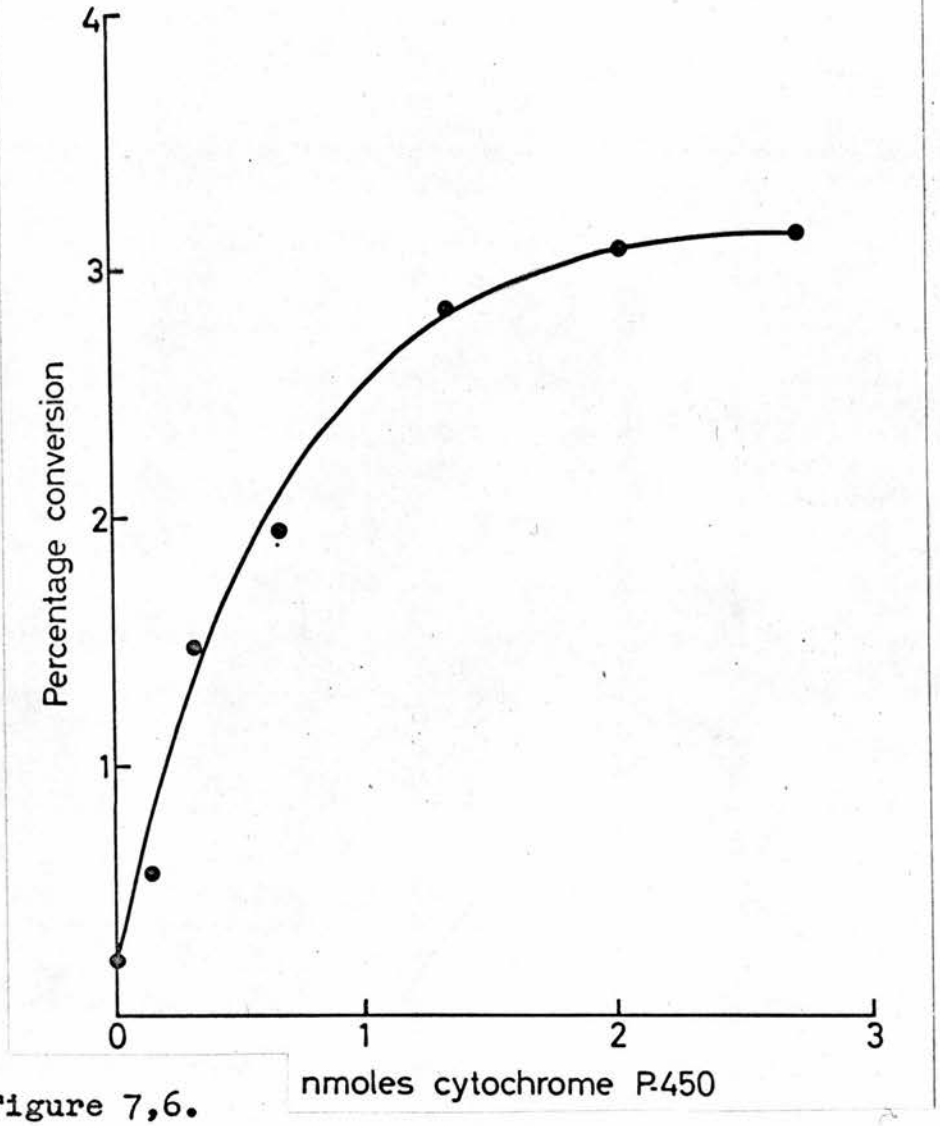


Figure 7,6.

The effect of adding to incubations containing a constant concentration of NADPH-cytochrome c oxidoreductase and cholesterol, increasing masses of cytochrome P-450.

reductase increases the velocity of cholesterol 7α-hydroxylase and a double reciprocal plot of $^{1}/v$ vs $^{1}/[S]$ in Figure 7,5 shows that the flavoprotein behaves as though it were saturating the cytochrome P-450 fraction, and at infinite concentration of reductase the maximum velocity is represented as a percentage conversion of [4 - ^{14}C] cholesterol to [4 - ^{14}C] 7α-hydroxycholesterol of approximately 12%. This is equivalent to the formation of 4.5 nmoles/min. of 7α-hydroxycholesterol, assuming complete equilibration of tracer cholesterol with endogenous cholesterol, but of course does not represent the real V_{max} of the reconstituted enzyme as the velocity was determined at only one, low, concentration of cholesterol. [Using a K_m for cholesterol of 50μM (determined on a soluble preparation of an acetone powder) the V_{max} at infinite reductase and cholesterol concentrations would be approximately 1125 nmoles/min.] This rate is equivalent to the formation of 0.42 nmoles 7α-hydroxy-cholesterol/nmole cytochrome P-450/min. This is in good agreement with that rate found in native microsomes and is two orders of magnitude less than the rate of benzphetamine demethylase.

<u>The effect of increasing the concentration of cytochrome P-450 on the activity of cholesterol 7α-hydroxylase</u>

Using an amount of NADPH-cytochrome c oxidoreductase which from the last experiment was known not to saturate the cholesterol 7α-hydroxylase system, incubations were carried out with increasing masses of cytochrome P-450. The results are presented in Figure 7,6. Evidence such as difference spectra and related electron paramagnetic resonance spectroscopy indicate that substrates of the liver microsomal mixed function oxidase system bind and interact directly with cytochrome P-450. Thus, although the dependence of cholesterol 7α-hydroxylase activity on NADPH-cytochrome c oxidoreductase may show

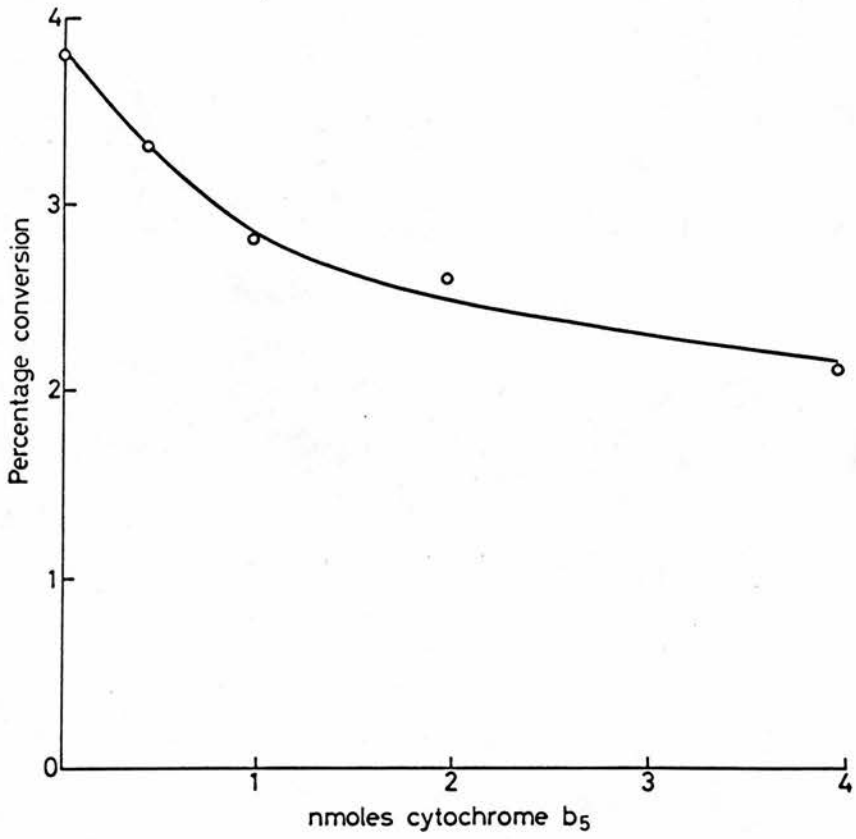


Figure 7,7.

The effect of adding cytochrome b_5 to incubations containing partially purified cytochrome P-450 and NADPH-cytochrome c oxidoreductase,on cholesterol 7α-hydroxylase activity.

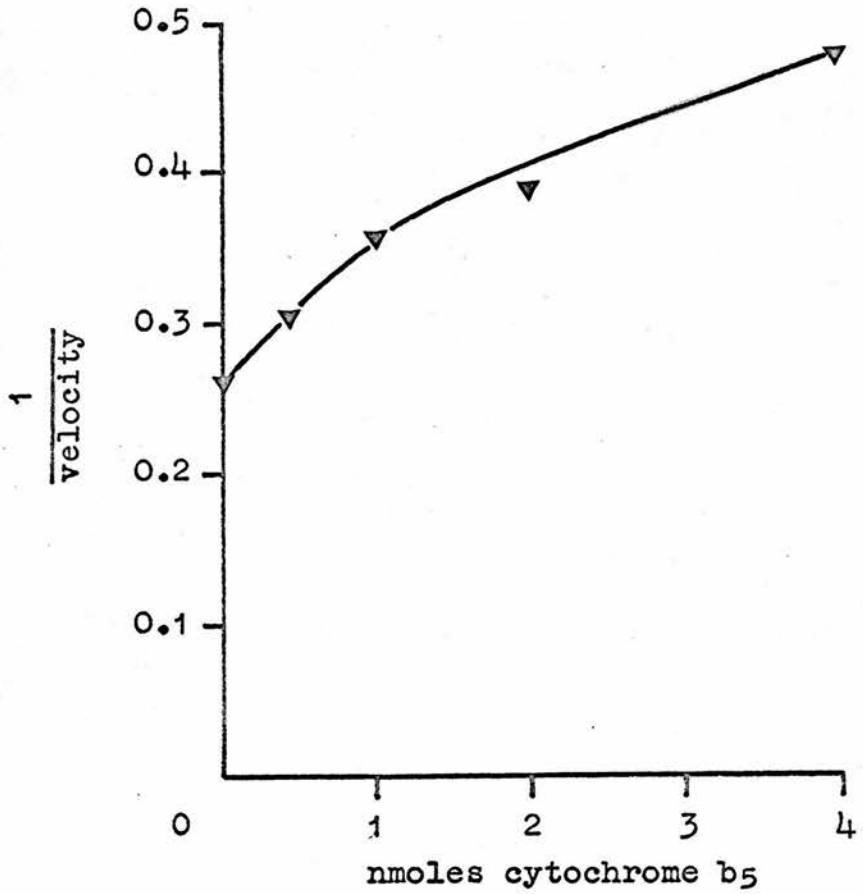


Figure 7,8.

A Dixon plot of results presented in figure 7,7. (velocity is equivalent to percentage conversion)

saturation kinetics, that is, it behaves as a substrate, it would be expected that increasing cytochrome P-450 with saturating NADPH-cytochrome c oxidoreductase would lead to an initial directly proportional dependence of cholesterol 7α-hydroxylase activity on cytochrome P-450 concentration. The results indicate that this was the case but that as the concentration of cytochrome P-450 increased, NADPH-cytochrome c oxidoreductase became rate-limiting. Since the preparation of cytochrome P-450 contained significant amounts of cholesterol, this substrate was added to ensure the same concentration in each incubation.

The effect of adding a preparation containing cytochrome b_5 to a reconstituted cholesterol 7α-hydroxylase preparation

It has been suggested by Estabrook, Cohen and Hildebrandt (123,124,125,126) on the basis that NADH increases the velocity of drug hydroxylation when the NADPH concentration is suboptimal, and from spectral studies, that the first of the two electrons necessary for hydroxylation is donated by NADPH and the second from NADH or NADPH, cytochrome b_5 acting as the carrier. Correia and Mannering (127,128) also concluded that cytochrome b_5 was an essential cofactor in the cytochrome P-450 dependent hydroxylation of the substrates tested. Figure 7,7 shows the effect of adding to a reconstituted cholesterol 7α-hydroxylase system, a fraction containing cytochrome b_5 but no detectable cytochrome P-450 or NADPH-cytochrome c oxido-reductase. It may be seen that addition of this fraction caused a marked decrease in cholesterol 7α-hydroxylase activity and at a ratio of cytochrome b_5 to cytochrome P-450 observed in the original supernatant fraction, the inhibition was approximtely 27%. A Dixon plot of these results, Figure 7,8, perhaps indicates that the

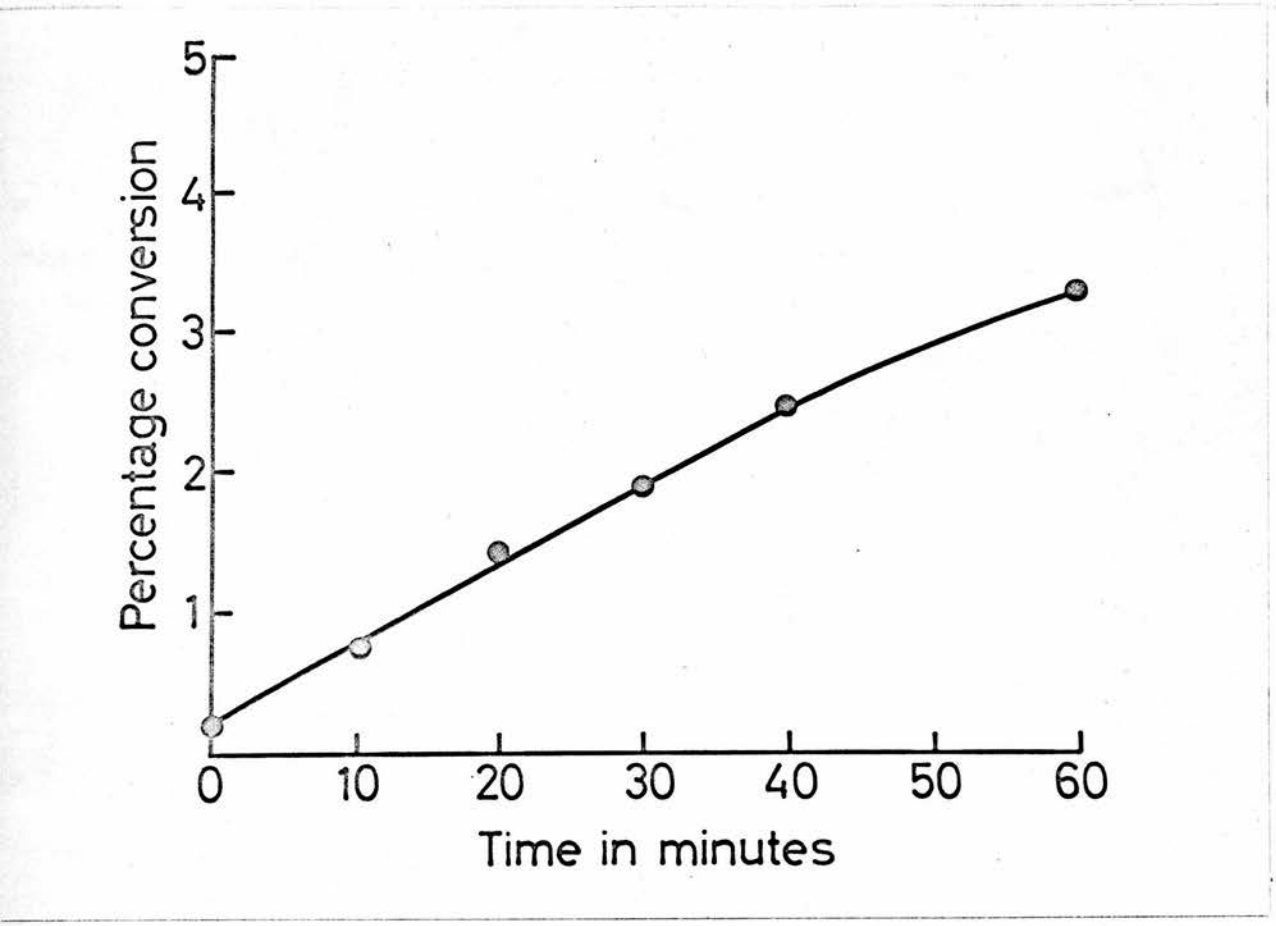


Figure 7,9.

The percentage conversion of cholesterol to 7α-hydroxycholesterol with time, in a reconstituted system.

inhibition was not simply a single competitive phenomenon as sequestration of electrons by cytochrome b_5 would suggest, but appears to be biphasic. It would, therefore, seem that the results of this experiment and the previous reconstitution experiment indicate not only that cytochrome b_5 is not an obligatory component of cholesterol 7α-hydroxylase but perhaps is also inhibitory. However, this does not exclude the possibility that cytochrome b_5 is involved in the synergistic action of NADH during hydroxylation under sub-optimal NADPH concentrations.

Figure 7,9 shows that the reaction rate in a reconstituted system is linear for 40 min.

SUMMARY

(1) It has been shown in this section that two fractions are essential for the reconstitution of cholesterol 7α-hydroxylase activity, one containing cytochrome P-450 and the other containing NADPH-cytochrome c oxidoreductase. It also appears that all three fractions of cytochrome P-450 which are eluted from DEAE-cellulose contain cholesterol 7α-hydroxylase.

(2) The specificity of this enzyme was also shown to reside in the cytochrome P-450 fraction as reductase from rat liver microsomes both low and high in cholesterol 7α-hydroxylase activity were equally capable of supporting this enzymic activity when recombined with the cytochrome P-450 from both preparations.

(3) Trypsin solubilized NADPH-cytochrome c oxidoreductase was incapable of supporting cholesterol 7α-hydroxylase activity.

(4) When the cytochrome P-450 was subjected to various techniques to increase the specific content, cholesterol 7α-hydroxylase was still present.

(5) The reconstitution of cholesterol 7α-hydroxylase activity using a fixed mass of cytochrome P-450 has been shown to be dependent on the concentration of reductase and this dependency was manifested as saturation kinetics. Using a fixed mass of NADPH-cytochrome c oxidoreductase, cholesterol 7α-hydroxylase activity was shown to be dependent on cytochrome P-450 concentration to the point where the reductase became rate-limiting. Cholesterol 7α-hydroxylase appeared not only to be independent of cytochrome b_5 but also to be inhibited by this haemoprotein.

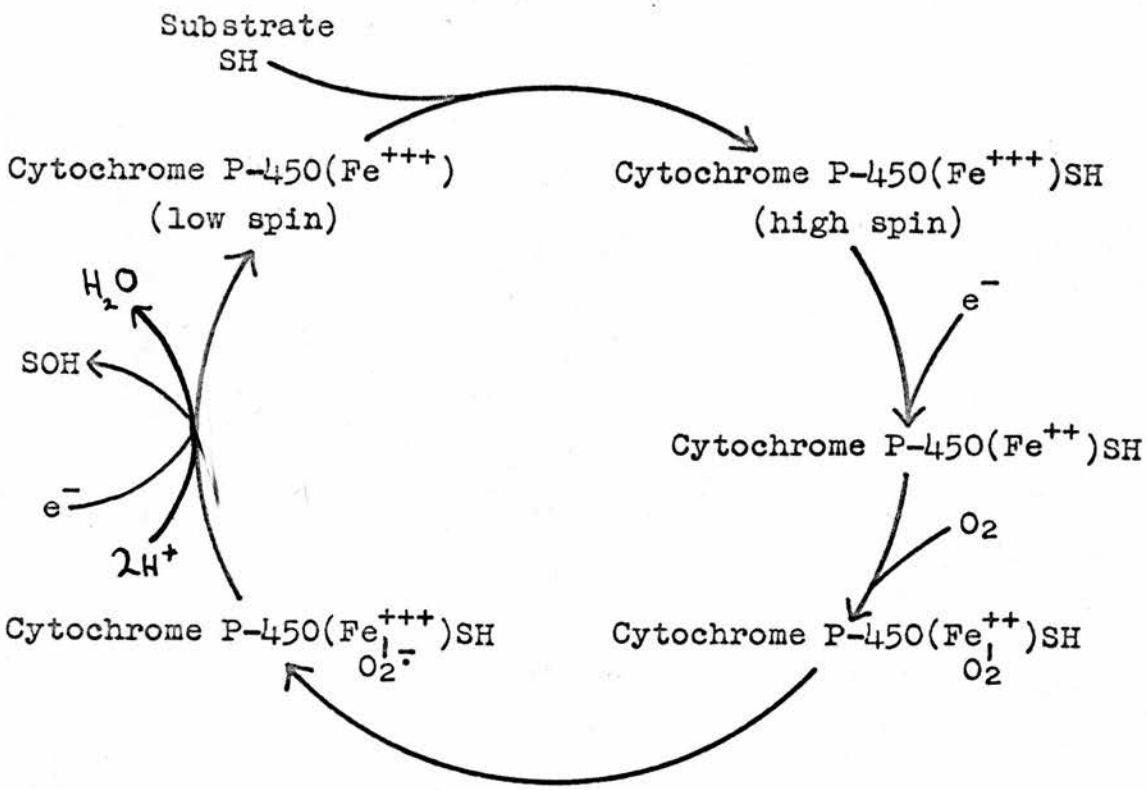


Figure 8,1.

The cyclical transformation of the spin state of cytochrome P-450 during the oxidation of substrate.

SECTION 8

PROPERTIES OF NONIDET P42 SOLUBILIZED MICROSOMES AND CHOLESTEROL 7α-HYDROXYLASE

Some spectral properties of cytochrome P-450 solubilized with Nonidet P42

It has been deduced by Estabrook et al (129) that cytochrome P-450 undergoes cyclical transformations during the hydroxylation of foreign compounds and steroids, as depicted in Figure 8,1. Cytochrome P-450 prepared by the usual techniques exists as the oxidised form, and the ferric ion may be in the high spin (5 unpaired electrons) or low spin (1 unpaired electron) state. Transformation from the low spin state to the high spin state is at least partially dependent on the strength of the ligand field. When foreign compounds are added to liver microsomes, changes in the spectral properties occur which are readily detectable using difference spectroscopy. These changes in absorbance have been shown to reflect the direct interaction of these compounds with cytochrome P-450 (130,131). Furthermore, these changes have also been observed in purified cytochrome P-450 from Pseudomonas putida. Correlation of the optical spectra with electron paramagnetic resonance spectra reveals that two of the three fundamental types of spectral change are associated with the alteration of the cytochrome P-450 high spin/low spin ratio. These three types of spectral change have been described by Schenkman (130). A type I spectral change is characterised by the appearance of a peak at about 390nm and a trough at about 420nm, and represents a change of the ferric ion from a low spin to a high spin state. The magnitude of the spectral change is dependent on the concentration of the xenobiotic, ligand or steroid, and the spectral dissociation constant, K_s, has been found in many cases to be very similar to the Michaelis constant, K_m, for the enzyme-substrate complex (130). It has also been the general

observation that compounds exhibiting the type I binding spectrum are also substrates for the microsomal mixed function oxidase. The converse, however, is not true. The mirror image of this optical spectrum may also be formed by the addition of ethanol, agroclavine, testosterone or even tryptophan, and is characterised by a trough at 390nm and a peak at 420nm. These spectra have been termed modified type II (130), inverse type I (133) or a reverse type I (132). Addition of primary aliphatic or aromatic amines results in a peak at about 430nm and a trough around 390-410nm. (Type II spectrum) Because this spectral change is competitively inhibited by carbon monoxide and therefore probably by oxygen, it is considered that binding of, say, octylamine or aniline, which has been shown to involve direct interaction of the basic nitrogen with haem (130), is at a site different from the substrate binding site, otherwise each substrate in the hydroxylation reaction would compete for the same binding site. However, aniline may also bind at the substrate binding site to give a type I binding spectrum at low concentrations, which is swamped on increasing the aniline concentration by the appearance of the type II spectrum. It is, therefore, apparent that these spectra must be interpreted with caution, as the magnitude of the spectral change bears no simple relationship between the K_m or the maximal velocity of the hydroxylation, and for many compounds the spectral changes are probably composites of all three types, thus a single compound could generate type I, inverse type I and also type II spectra.

Amine binding spectra

As previously referred to in the Introduction, both octylamine and aniline bind to cytochrome P-450 and cause spectral changes characteristic of type II binding. Jefcoate et al (134,135) observed

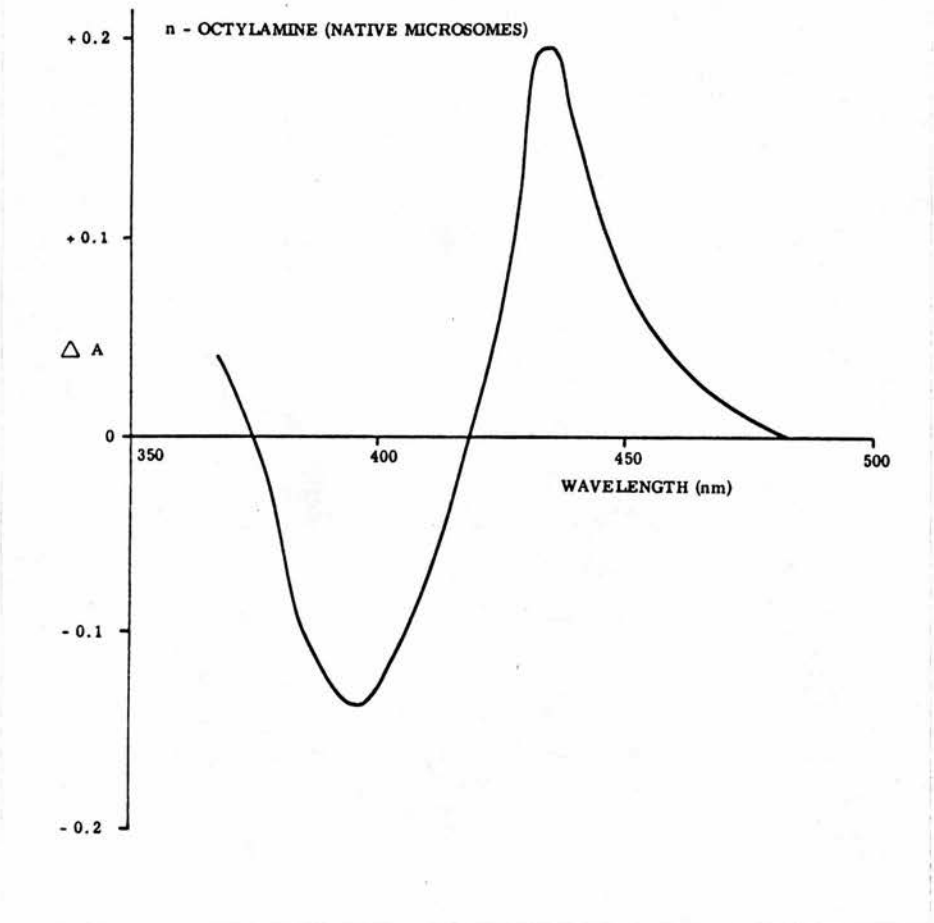


Figure 8,2.

The difference spectrum induced by the addition of octylamine to rat liver microsomes.

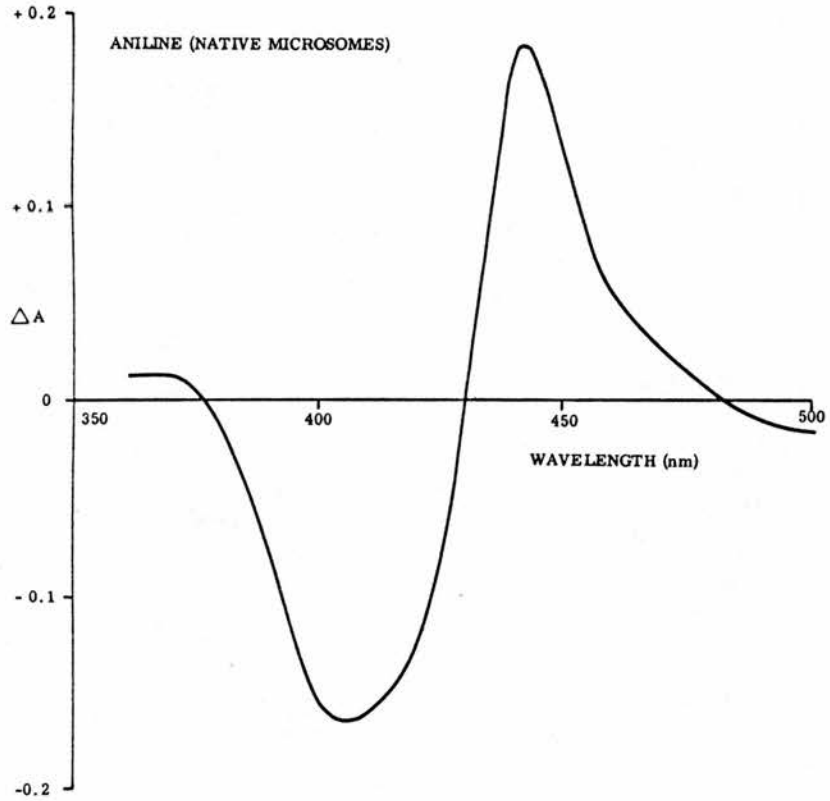


Figure 8,3.

The difference spectrum induced by the addition of aniline to rat liver microsomes.

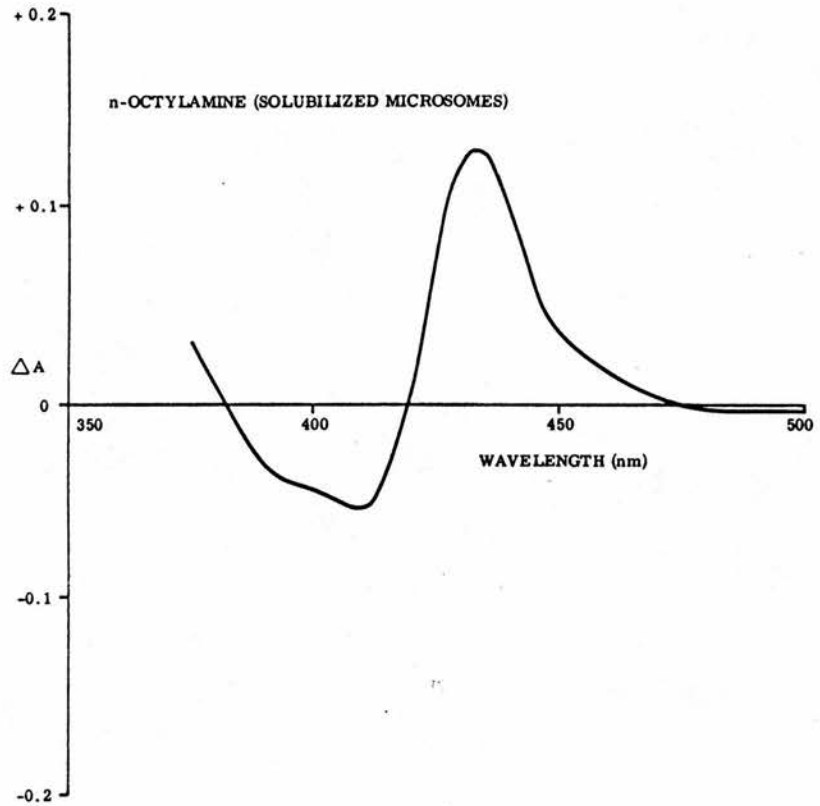


Figure 8,4.

The difference spectrum induced by the addition of octylamine to Nonidet P42 solubilized rat liver microsomes.

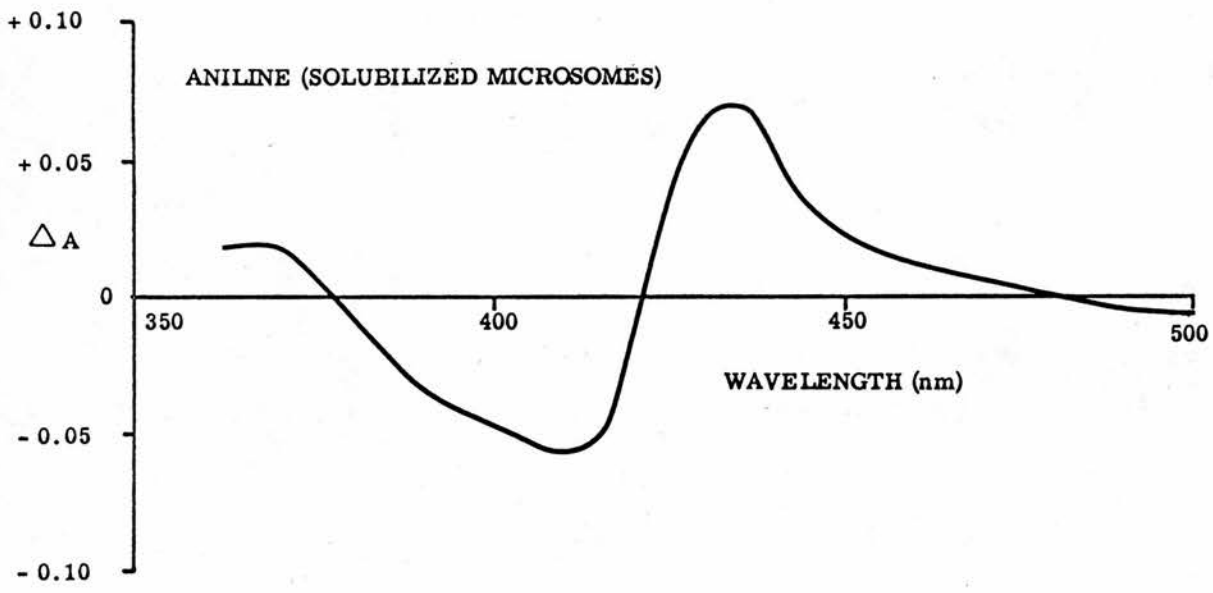


Figure 8,5.

The difference spectrum induced by the addition of aniline to Nonidet P42 solubilized rat liver microsomes.

that this spectral change was a composite of two spectra, and that the relative changes in absorbance of the two species, type a and type b, could be markedly changed by pre-treatment of the rabbits with phenobarbital and 3-methylcholanthrene. Type a, λ_{max} 427nm, λ_{min} 392nm, and type b λ_{max} 432nm, λ_{min} 410nm, were also shown to correspond to the high and low spin states respectively of cytochrome P-450 and thus pre-treatment of rabbits with 3-methylcholanthrene favours the synthesis of a high spin cytochrome P-450 or cytochrome P-448 (32).

In the mouse, the difference in substrate specificity between liver microsomal cytochrome P-448 and P-450 appears to be independent of the spin state of these haemoproteins, and induction of cytochrome P-448 does not result in an increase of the high spin species (136). Phenobarbital pre-treatment, however, increased both forms but favoured type b (134).

Figures 8,2 and 8,3 show the difference spectra obtained when native microsomes were treated with octylamine (2mM) and aniline (3mM). The spectra are qualitatively very similar, both having λ_{min} at 396nm and λ_{max} at about 433nm. The $\Delta A_{433-396}$ in both cases was 0.335. Using the empirical equation of Jefcoate et al (134) and using the data from the octylamine difference spectrum, 33% of the total P-450 was determined to be high spin (type a). Figures 8,4 and 8,5 show the difference spectra obtained when the same compounds are added to Nonidet P42 solubilized rat liver microsomes. The concentration of cytochrome P-450 in this preparation was about 50% that of the native rat liver microsomes, i.e. $\Delta A_{450-490}$ was 0.32. The preparation also contained cytochrome P-420. However, it is known that n-octylamine does not combine with cytochrome P-420 (134,135). It is clear from the difference spectra that a change in spin state of cytochrome P-450 has occurred on solubilization of microsomes by Nonidet P42 as the greatest negative

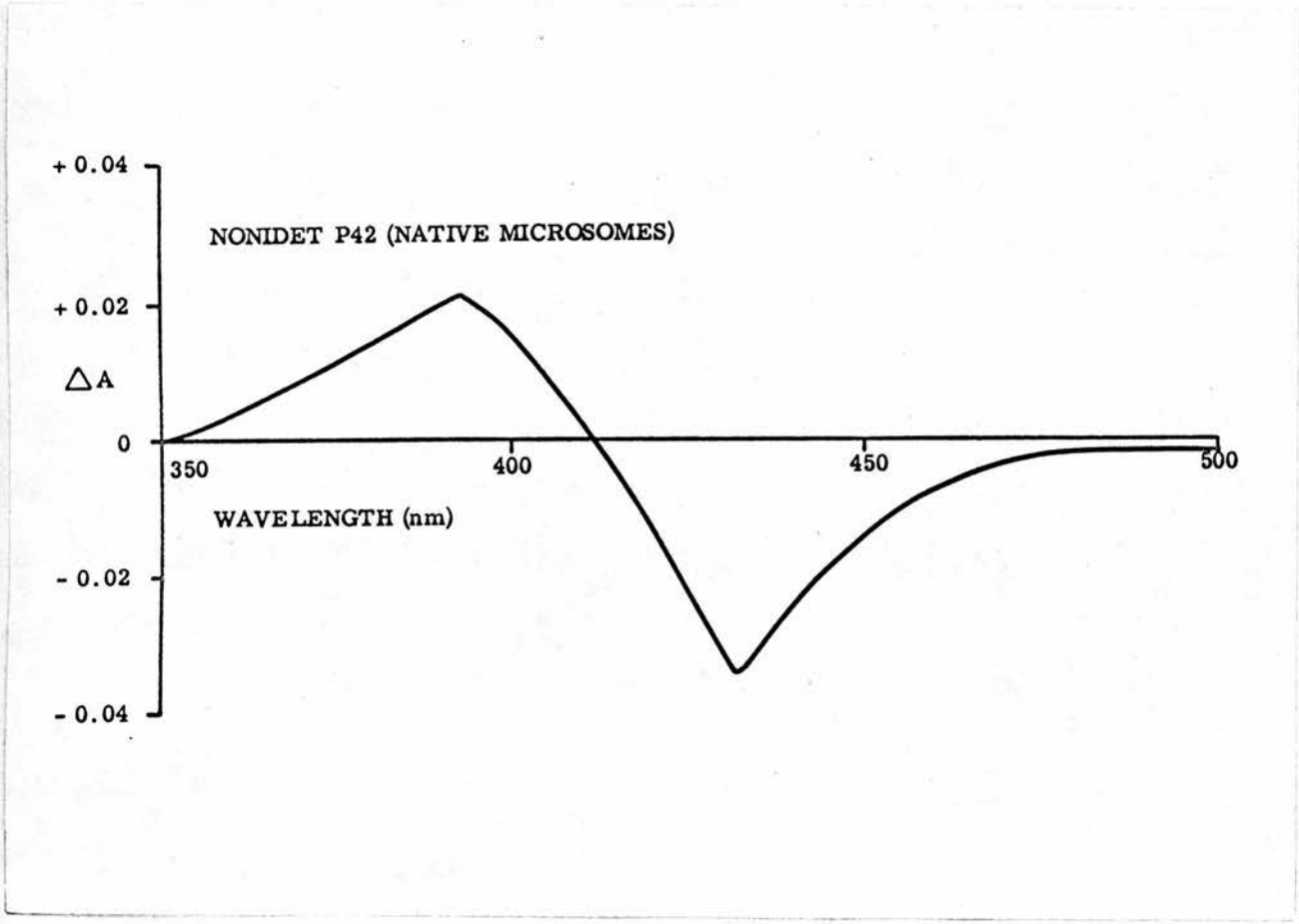


Figure 8,6.

The difference spectrum induced by the addition of Nonidet P42 to rat liver microsomes.

absorbance peak is at approximately 410nm in both the n-octylamine and aniline induced type II spectrum change. The absorption maximum was unchanged, being again at 433nm. Using the empirical formula of Jefcoate et al (134), the percentage high spin cytochrome P-450 in the solubilized microsomes was reduced from 33% to 8%. As 85-90% of the cytochrome P-450 was solubilized, even if the remainder of the cytochrome P-450 in the pellet were 100% high spin, which is unlikely as the solubilization process seems non-specific, this would still not account for the loss of high spin cytochrome P-450. It would, therefore, appear that in common with the results of Jefcoate (134) where microsomes were treated with Lubrol WX, high spin to low spin ratios of cytochrome P-450 were reduced on treatment of microsomes with Nonidet P42. However, there is also the possibility that the cytochrome P-420 present in the preparation to a large extent (ca. 28% of the initial cytochrome P-450) was specifically generated from the original high spin species of cytochrome P-450, this would mean that high spin cytochrome P-450 was more susceptible than the low spin species to degradation to cytochrome P-420.

Effect of Nonidet P42 at low concentrations on the spin state of cytochrome P-450

The effect of Nonidet P42 on the difference spectrum of native liver microsomes was observed only at low concentration as, clearly, at high concentrations one cuvette would be optically clear and the other turbid. Under such conditions the new base line would be grossly different from the original. Figure 8,6 shows the difference spectrum induced by adding the equivalent of 1 μl Nonidet P42 to the sample cuvette containing 18mg protein. Thus, the ratio Nonidet P42/mg protein (0.05 μl/mg protein) is well below that required for solubilization. The absorption maximum occurred at 392nm and the minimum at 432nm, which

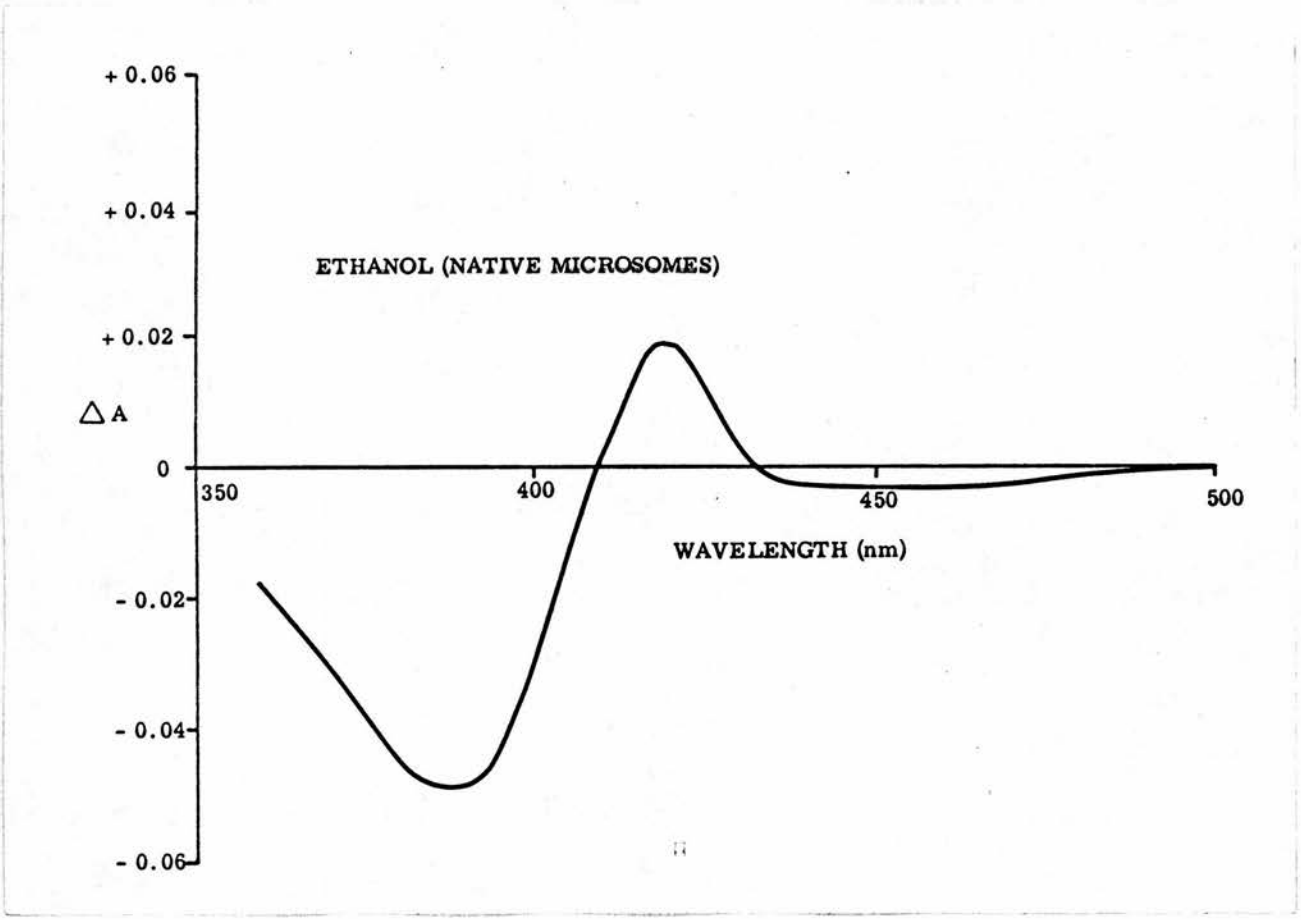


Figure 8,7.

The difference spectrum induced by the addition of ethanol to rat liver microsomes.

is indicative of a transformation of part of the cytochrome P-450 to the high spin form. It is, therefore, likely that Nonidet P42 interacts with and is susceptible to oxidative attack by the microsomal mixed function oxidase. It is interesting to note, however, that the cytochrome appears mainly in the low spin state upon solubilization. Perhaps Nonidet P42, like phenacetin and agroclavine (137) at low concentrations binds to cytochrome P-450 to induce a type I spectral change, but that as the concentration is increased, substrate already bound to cytochrome P-450 (high spin) is displaced by the Nonidet P42, causing the high spin species to revert to low spin, this process manifesting itself as an inverse type I spectral change.

The effect of ethanol on the difference spectrum of rat liver microsomes

Figure 8,7 shows the difference spectrum obtained when 100 µl ethanol were added to the sample cuvette. With a minimum absorbance at 388nm and a maximum at 420nm, this spectrum is a mirror image of the type I binding spectrum. The explanation offered for the induction of such a spectrum is that substrates are displaced from the binding sites in such a way that the strength of the ligand field is decreased and low spin cytochrome P-450 is formed. When ethanol was added to the solubilized preparation, no difference spectrum was detectable. However, most of the cytochrome P-450 was already in the low spin state, and as mentioned earlier, this may be the result of Nonidet P42 displacement of substrate. This explanation, however, does not seem entirely satisfactory as Nonidet P42 itself gives a type I difference spectrum and might be expected to displace endogenous substrate with maintenance of the high spin state of cytochrome P-450.

The effect on the difference spectra of binding mixed function oxidase substrates

Benzphetamine hydrochloride added to the sample cuvette

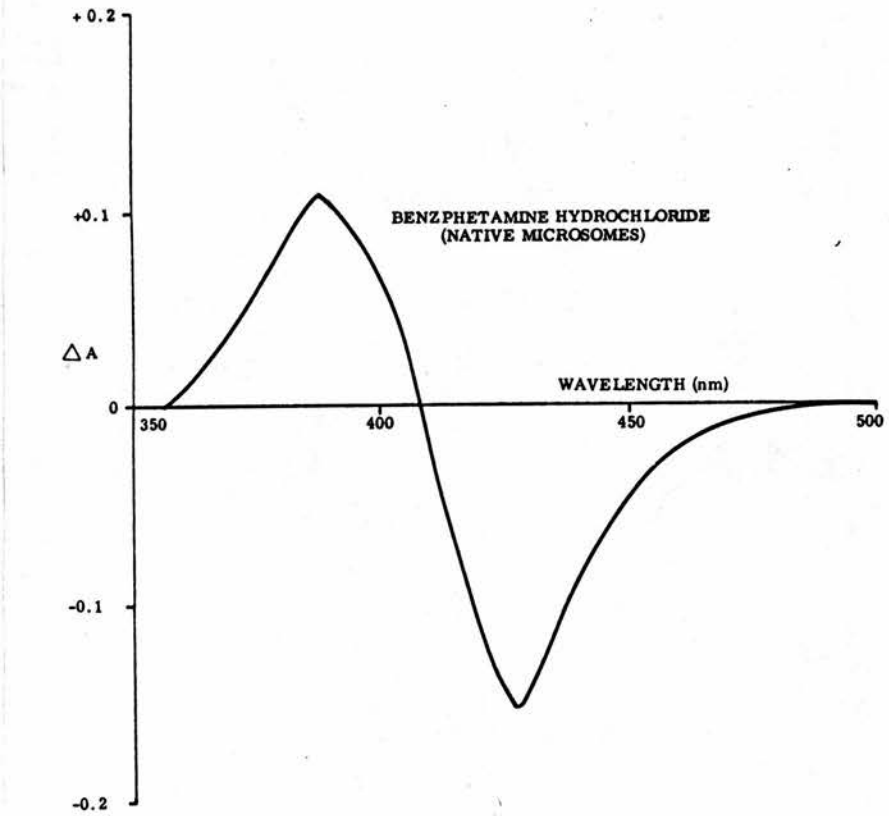


Figure 8,8.

The difference spectrum induced by the addition of benzphetamine hydrochloride to rat liver microsomes.

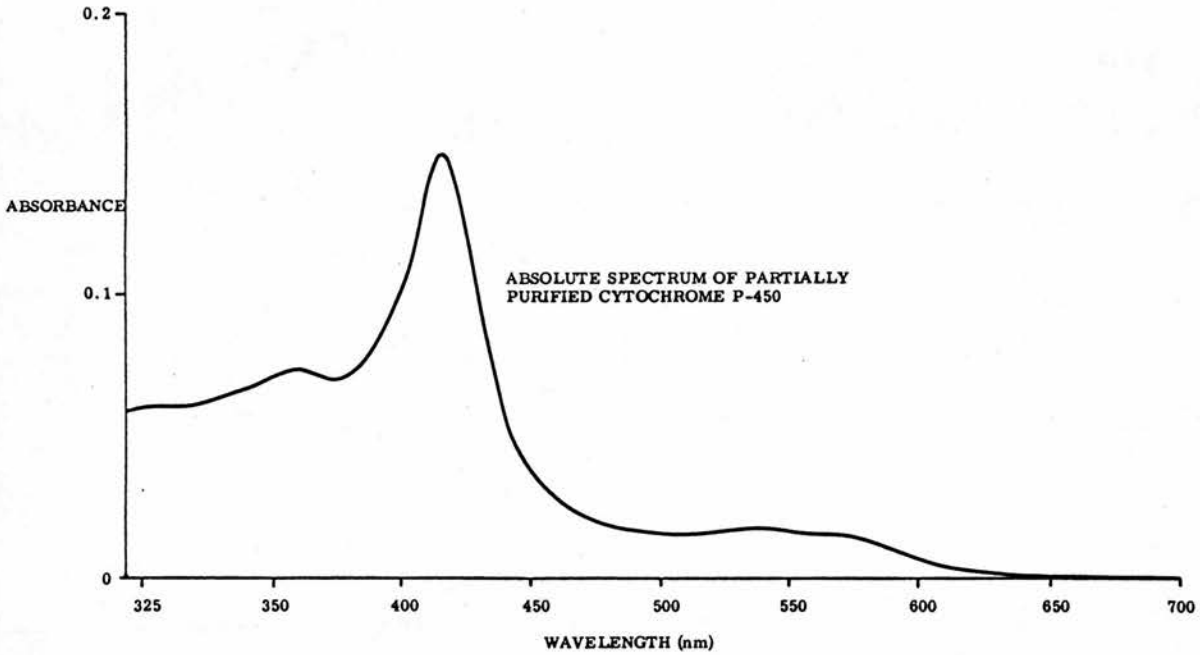


Figure 8,9.

The absolute spectrum of partially purified cytochrome P-450.

containing native liver microsomes to a final concentration of approximately 0.5mM, gave a type I difference spectrum as shown in Figure 8,8. The $\Delta A_{387-426}$ was 0.262. The $\Delta A_{450-490}$ was 0.64 and 33% of this cytochrome P-450 was already high spin. However, when this drug was added to solubilized liver microsomes, the $\Delta A_{390-424}$ was only 0.04. The $\Delta A_{450-490}$ was 0.32, but only 8% of this was already in the high spin state. It therefore appears that Nonidet P42 inhibits the binding of benzphetamine hydrochloride.

Iso-octane and cyclohexane were also added to native liver microsomes and both gave rise to type I spectra with $\Delta A_{388-424}$ of 0.174 and 0.275 respectively. When these hydrocarbons were added to Nonidet P42 solubilized liver microsomes, although type I spectra were discernible, the $A\lambda_{max} - \lambda_{min}$ was again greatly reduced to 0.016 and 0.058 respectively. It would, therefore, seem probable that on solubilization substrates binding to cytochrome P-450 to give type I difference spectra have a reduced affinity for this haemoprotein.

The absolute spectrum of partially purified cytochrome P-450

Cytochrome P-450 was prepared essentially free of cytochrome b_5 and containing little cytochrome P-420 by DEAE-cellulose chromatography of Nonidet P42 solubilized liver microsomes. Figure 8,9 shows the absolute spectrum of this preparation which contained 1.6 nmoles/ml cytochrome P-450. The Soret maximum is exhibited at 417nm which is indicative of a low spin cytochrome P-450, but there are small peaks at approximately 530 and 570nm, which is also suggestive of the cytochrome P-450 being in a low spin state. There is also a peak at 360nm which has been observed by Miyake et al (88) in his Lubrol WX solubilized preparation.

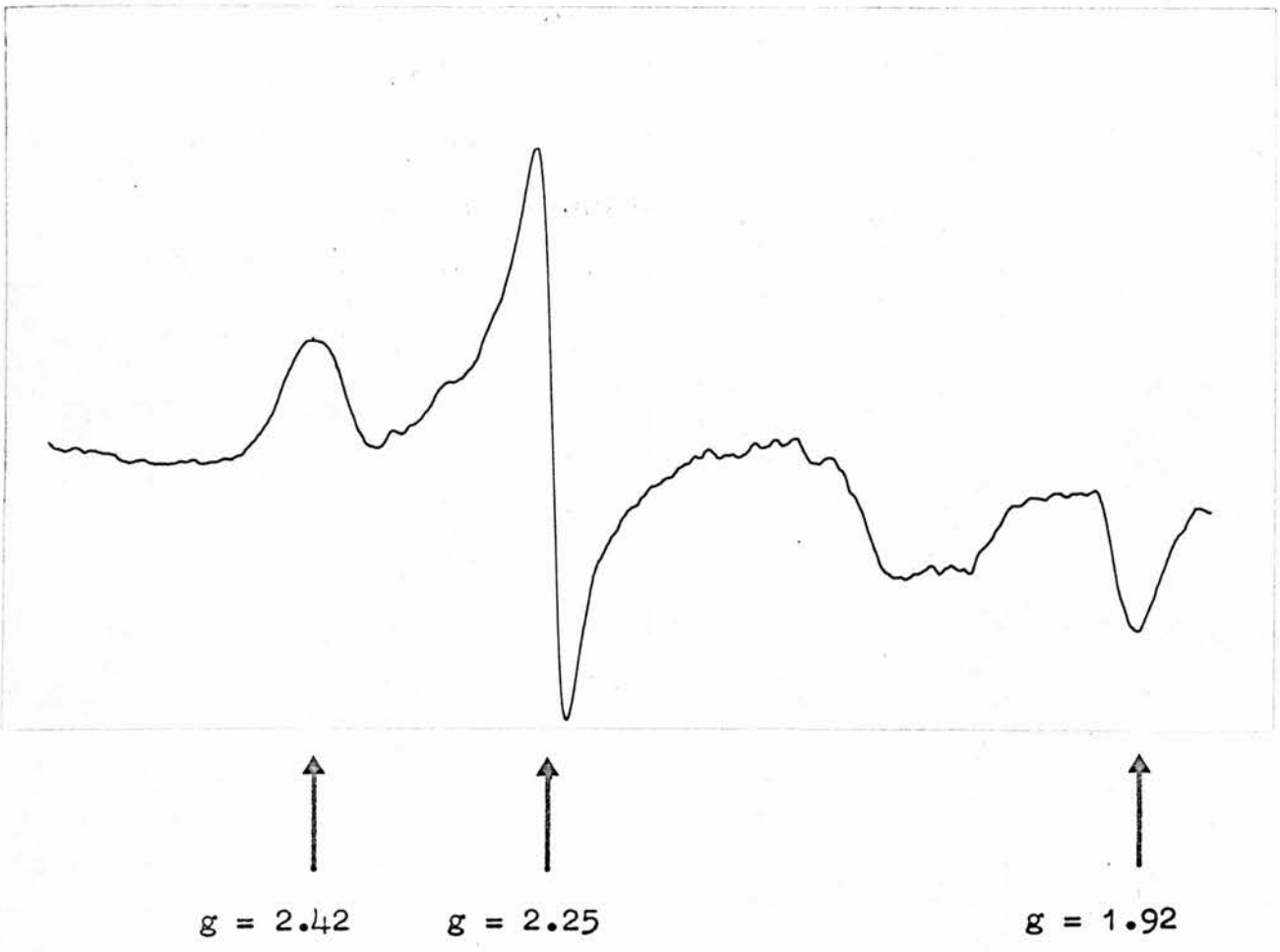


Figure 8,10.

The e.p.r. spectrum of native rat liver microsomes.

Scan range 1×10^3 Gauss

Field set 2965 Gauss

Modulation amplitude 1.25×10^1 Gauss

Modulation frequency 100 KHz

Receiver gain 1.25×10^3

Temperature $-172^{\circ}C$

Microwave power 50mW

Microwave frequency 9.145 GHz

Time constant 1.0 second

Scan time 4min

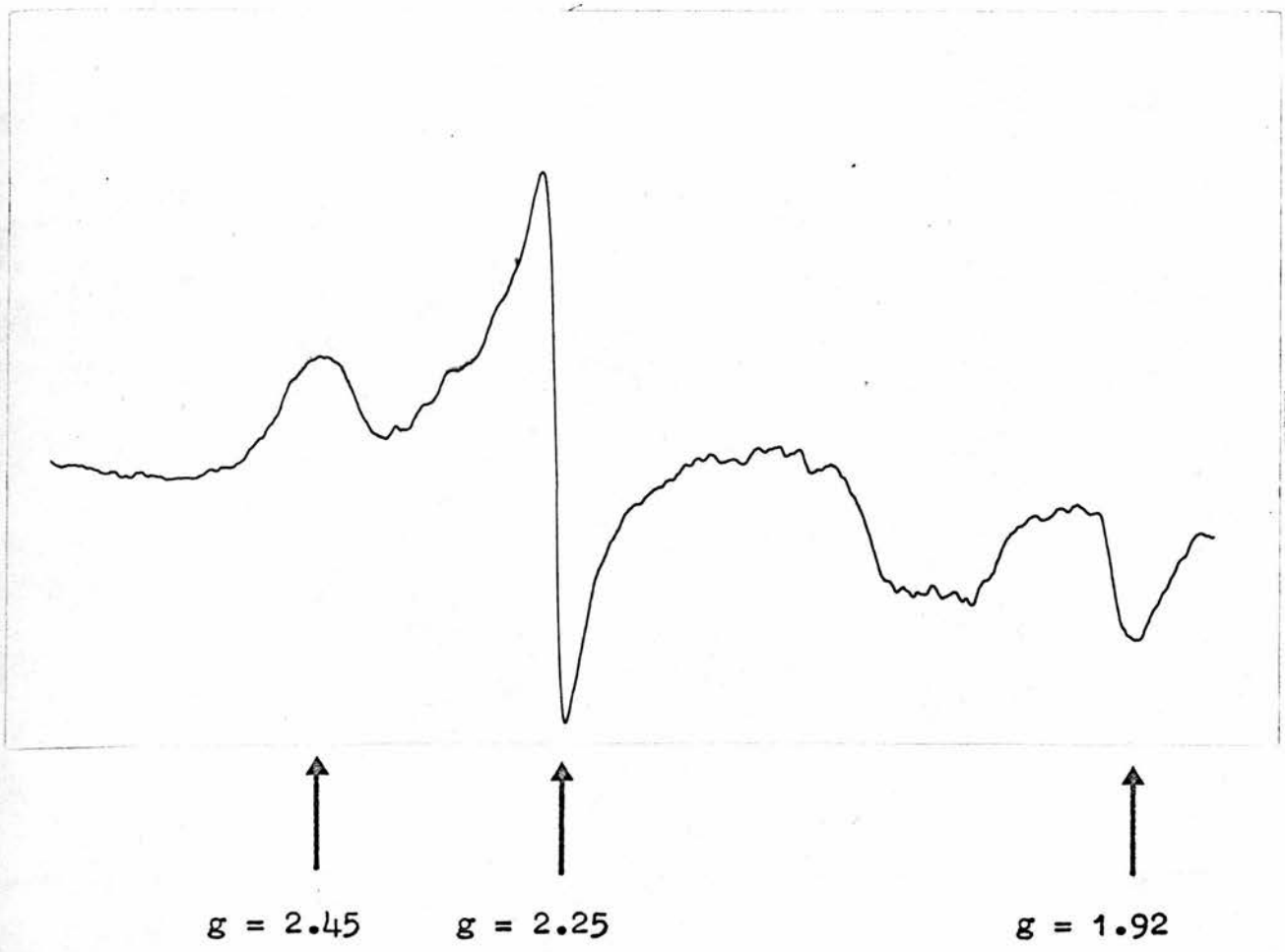


Figure 8,11.

The e.p.r. spectrum of Nonidet P42 solubilized rat liver microsomes, recorded under the same conditions as in figure 8,10, except that the receiver gain was 1.60×10^3.

	Values of g		
	g_z	g_y	g_x
Cytochrome P-450 from native microsomes	2.42	2.25	1.92
Cytochrome P-450 from Nonidet P42 solubilized microsomes	2.42	2.25	1.92

Table 8,1.

The g values of cytochrome P-450 in native microsomes and in Nonidet P42 solubilized microsomes observed during electron paramagnetic resonance spectroscopy at 100°K.

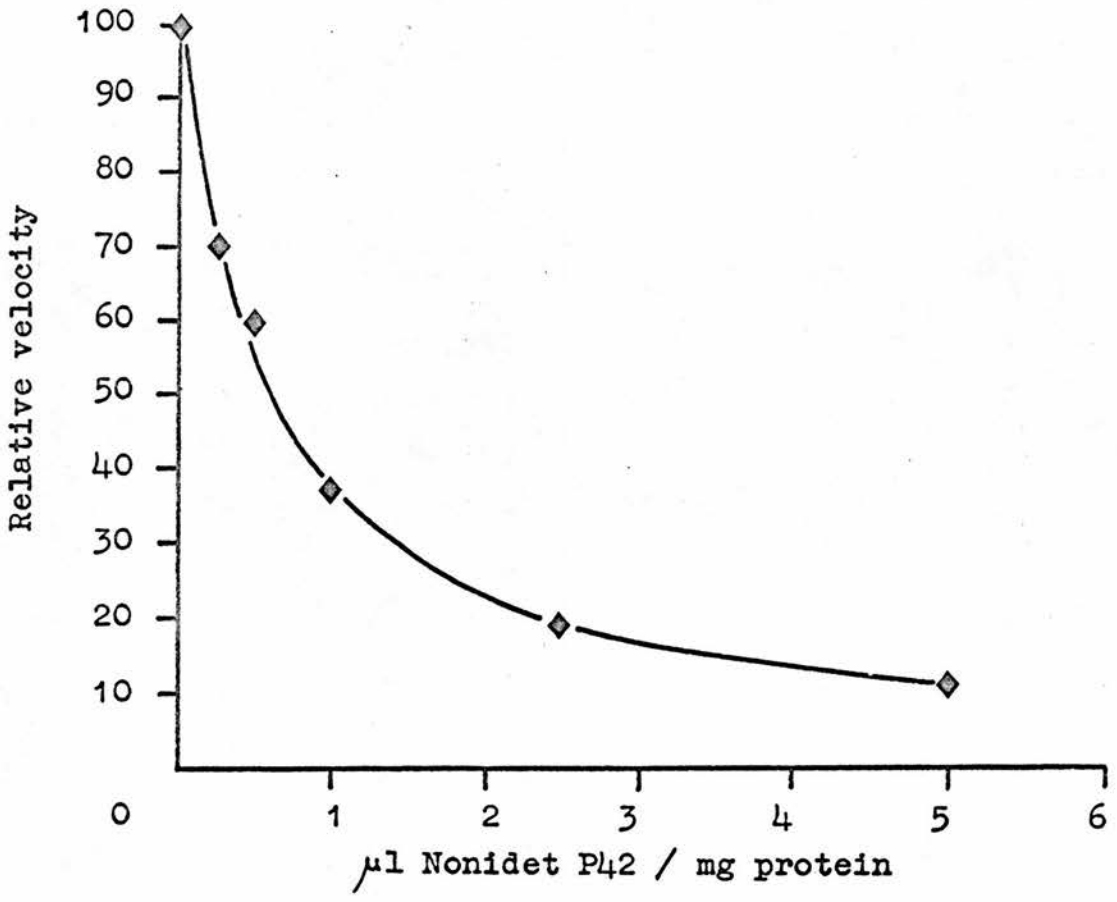


Figure 8, 12.

The effect of Nonidet P42 on benzphetamine demethylase activity.

Electron paramagnetic resonance spectroscopy of Nonidet P42 solubilized cytochrome P-450

Figures 8,10 and 8,11 show the e.p.r. spectra of native and Nonidet P42 solubilized liver microsomes containing 42 and 21 nmoles cytochrome P-450/ml respectively. At the temperature that these spectra were recorded, -172°C, only the low spin form of cytochrome P-450 is detectable. The g values for the three main absorbing species in both native and Nonidet P42 solubilized liver microsomes are given in Table 8,1. These values are very close to those recognised by Hashimoto et al (138) as being a low spin haemoprotein (termed at that time Fe_x) which was later equated with cytochrome P-450 by Mason, North and Vanneste (139). Since e.p.r. spectroscopy is a sensitive test of perturbation of the immediate environment of the free radical, in this case the ferric ion, it appears that upon solubilization of rat liver microsomes with Nonidet P42, the conformation of cytochrome P-450 (low spin) is not grossly altered.

The effect of Nonidet P42 on benzphetamine demethylase

Because rat liver microsomes may be solubilized by Nonidet P42 without loss of activity of cholesterol 7α-hydroxylase, it was of interest to observe whether the drug hydroxylating system was also solubilized. Benzphetamine is very readily hydroxylated by rat liver microsomes in the presence of cofactors, and therefore this substrate, final concentration 1mM, was incubated with 20mg microsomal protein in 8ml with increasing concentrations of Nonidet P42. The velocity of the reaction was determined by plotting the formation of formaldehyde after 0, 4, 8 and 12 minutes of incubation. In the absence of Nonidet P42, the velocity is expressed as 100%, and Figure 8,12 shows the effect on the relative velocity of increasing Nonidet P42 concentration.

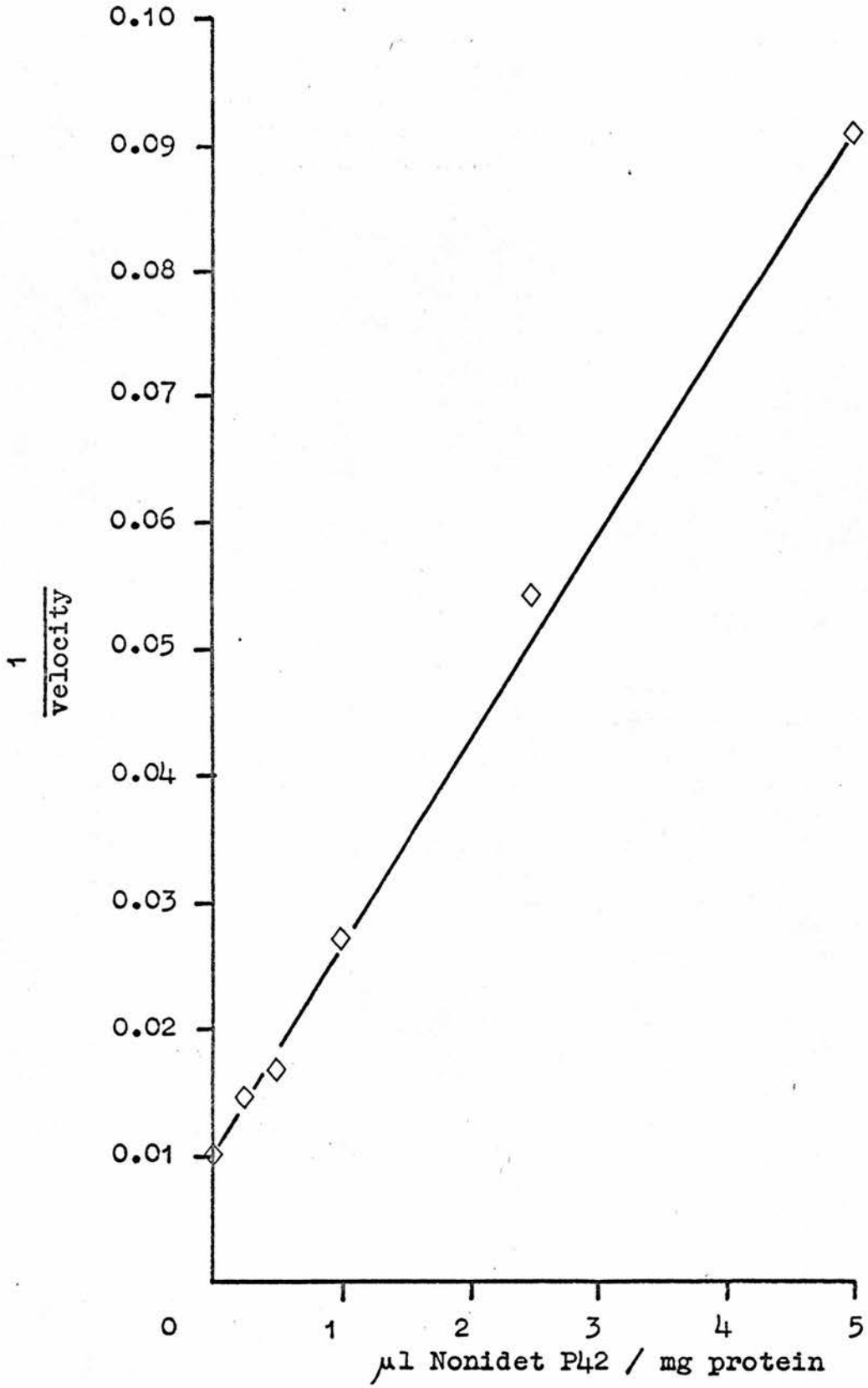


Figure 8,13.

A Dixon plot of results presented in figure 8,12.

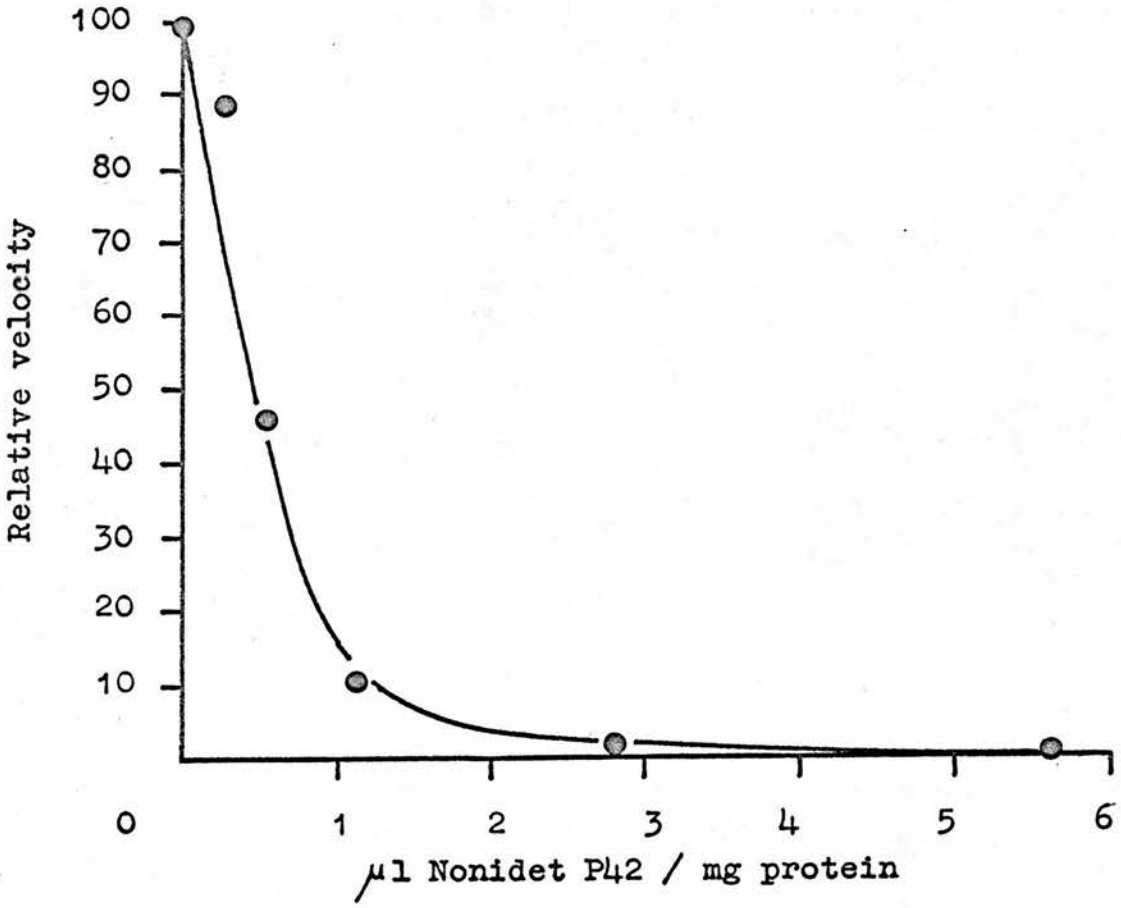


Figure 8,14.

The effect of Nonidet P42 on the rate of reduction of cytochrome P-450

It is clear that treatment with this detergent results in very substantial inhibition, the concentration required for 50% inhibition is only 0.6μl/mg protein and therefore the inhibition was not related to solubilization of the microsomal membranes. A Dixon plot is presented in Figure 8,13 and shows a simple relationship between the velocity and Nonidet P42 concentration. Nonidet P42 affects the binding of benzphetamine to cytochrome P-450 and could, therefore, inhibit the demethylation by reducing the affinity of the enzyme for benzphetamine. However, the reaction mechanism is complex and the inhibition could also be due to reduced affinity for O_2; this is unlikely as CO and octylamine binding appear unaffected by Nonidet P42. Miyake et al (88) have observed that their Lubrol WX solubilized preparation of cytochrome P-450 could not be reduced by NADPH in the presence of NADPH-cytochrome c reductase, therefore the rate of reduction of this haemoprotein was observed in the presence of increasing concentrations of Nonidet P42.

The effect of Nonidet P42 on reduction of cytochrome P-450 by NADPH

Using the same preparation of rat liver microsomes as was used in the experiment in which the effect of Nonidet P42 on benzphetamine demethylase was studied, the effect of this surface active agent on the reduction of cytochrome P-450 by NADPH was observed. Using the Aminco-Chance spectrophotometer in the dual wavelength mode, the rate of reduction was measured by adding NADPH generated by the oxidation of glucose-6-phosphate by NADP and glucose-6-phosphate dehydrogenase to the microsomal suspension, saturated with CO, and recording the difference in absorbance between 450nm and 490nm with time. Because the velocity of reduction was so great at 25°C, the sample was cooled to 0°C and measured in the spectrophotometer at 0°C. Figure 8,14 shows the effect of Nonidet P42 on the velocity of reduction. The effect was similar to

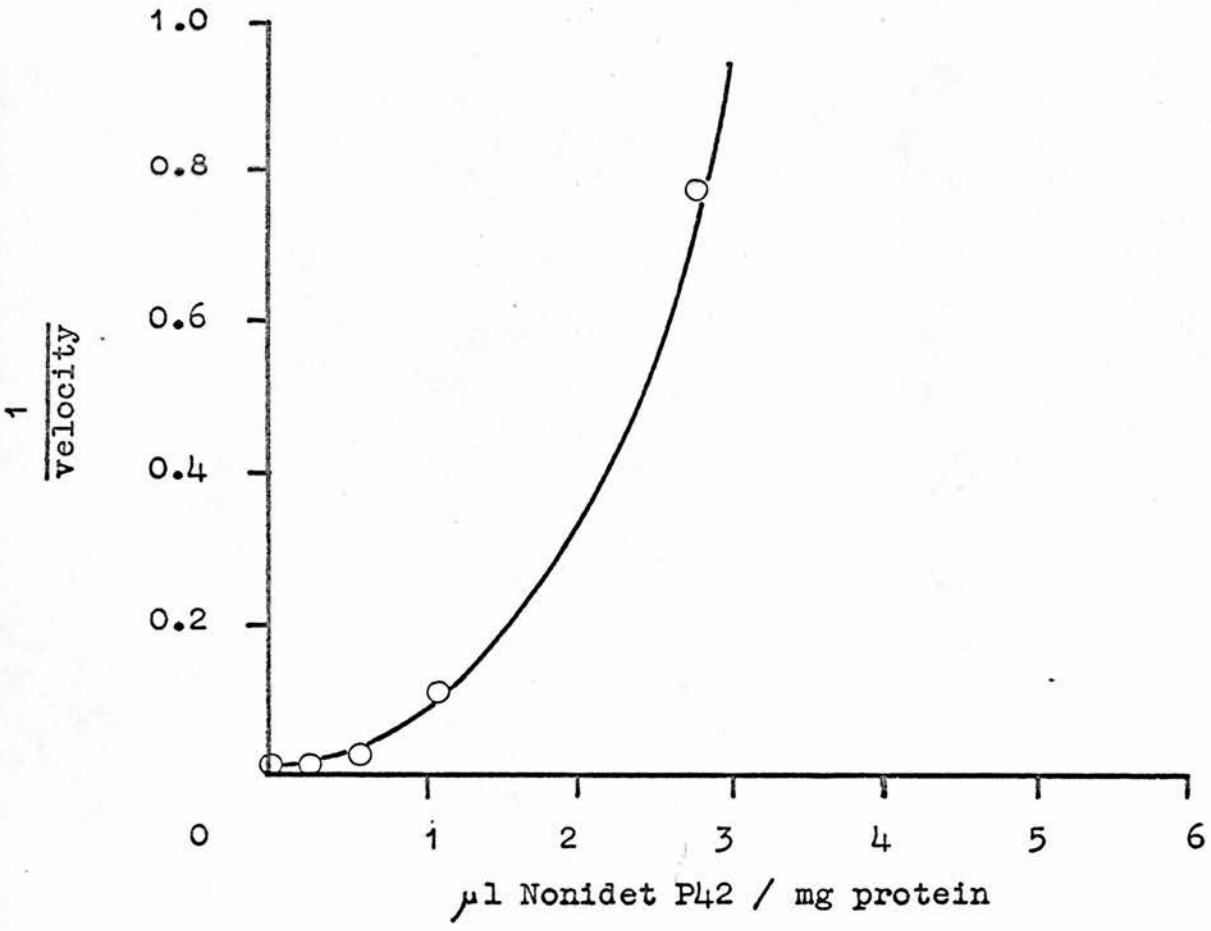


Figure 8,15.

A Dixon plot of results presented in figure 8,14.

that observed in the benzphetamine demethylase reaction, Nonidet P42 strongly inhibiting the reduction of cytochrome P-450. However, the degree of inhibition was much more marked at higher concentrations of Nonidet P42, and a Dixon plot, Figure 8,15, shows that the inhibition is more complex than that observed in the benzphetamine demethylase reaction.

Reduction of cytochrome P-450 by NADPH in a solubilized acetone powder could not be observed, either, even after addition of cholesterol or lauric acid. A preparation of partially purified cytochrome P-450 in which excess detergent was removed by binding the haemoprotein to hydroxylapatite was also tested, but again no reduction could be detected on addition of NADPH and NADPH-cytochrome c oxidoreductase. In both solubilized liver microsomes and in an acetone powder, NADPH-cytochrome c oxidoreductase was fully active or even activated.

This result is surprising in view of the fact that cholesterol 7α-hydroxylase may be solubilized by Nonidet P42 without loss of activity. Explanation for these apparently contradictory results would be that (a) reduction of cytochrome P-450 is not a rate-limiting step in the hydroxylation of cholesterol, (b) that cholesterol 7α-hydroxylase cytochrome P-450 represents only a small part of the bulk of this cytochrome in microsomes, and thus its reduction would not be easily observed, or (c) that cytochrome P-450 is not involved in the hydroxylation of cholesterol. However, cholesterol 7α-hydroxylase is inhibited by CO and the inhibition is relieved optimally by light of wavelength 450nm. Reconstitution experiments also show that two fractions are necessary for activity, one containing cytochrome P-450 and the other its reductase. The reaction is also inhibited by those reagents leading to conversion to cytochrome P-420 and by antibody towards

NADPH-cytochrome c oxidoreductase. From these observations it appears that there is good evidence for the participation of cytochrome P-450 and its reductase in the 7α-hydroxylation of cholesterol.

The Michaelis constant of cholesterol 7α-hydroxylase for cholesterol and the effect of Nonidet P42

The determination of K_m when the substrate is relatively insoluble presents many problems in interpretation as the value of K_m assumes that the substrate exists in solution as single molecular species. In the following experiments, acetone powder preparations of rat liver microsomes were used as the endogenous cholesterol concentration was reduced from 20μg/mg protein to 1μg/mg protein in the acetone powder. It is clear that when cholesterol is added to microsomes and acetone powder suspensions, the substrate is adsorbed on to the membranes, as when radioactive cholesterol is added to a microsomal suspension, all the radioactivity is found in the pellet after centrifugation at 105,000 x g for 1 hour (140). It is also apparent that when the concentration of a relatively insoluble substrate increases above the critical micellar concentration, micelles or emulsions are formed. This difference in physical state of the substrate again raises difficulties as the enzyme may use only molecular species of the substrate. In this case, V_{max} would be reached at the critical micellar concentration. If, however, the enzyme operated on micelles, the velocity would depend to a large extent on the surface area of the micelles. Mitropoulos (141) and Bjorkhem (11) have observed that the specific activity of cholesterol in microsomes to which radioactive cholesterol had been added was different from the specific radioactivity of 7α-hydroxycholesterol formed by these microsomes. This is suggestive of compartmentation and makes even less meaningful the value of K_m. It is hoped that in a soluble system such compartmentation will

Exogenous cholesterol µg/7ml	Percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] 7α-hydroxy-cholesterol.	µg 7α-hydroxy-cholesterol formed in 40 min
75	1.65	1.24
150	1.77	2.66
200	1.26	2.52
250	1.45	3.63
300	1.74	5.22
350	1.91	6.69
400	1.81	7.24

Table 8,2.

The effect of adding exogenous cholesterol to a resuspended acetone powder (9mg protein) on the percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] 7α-hydroxy-cholesterol.

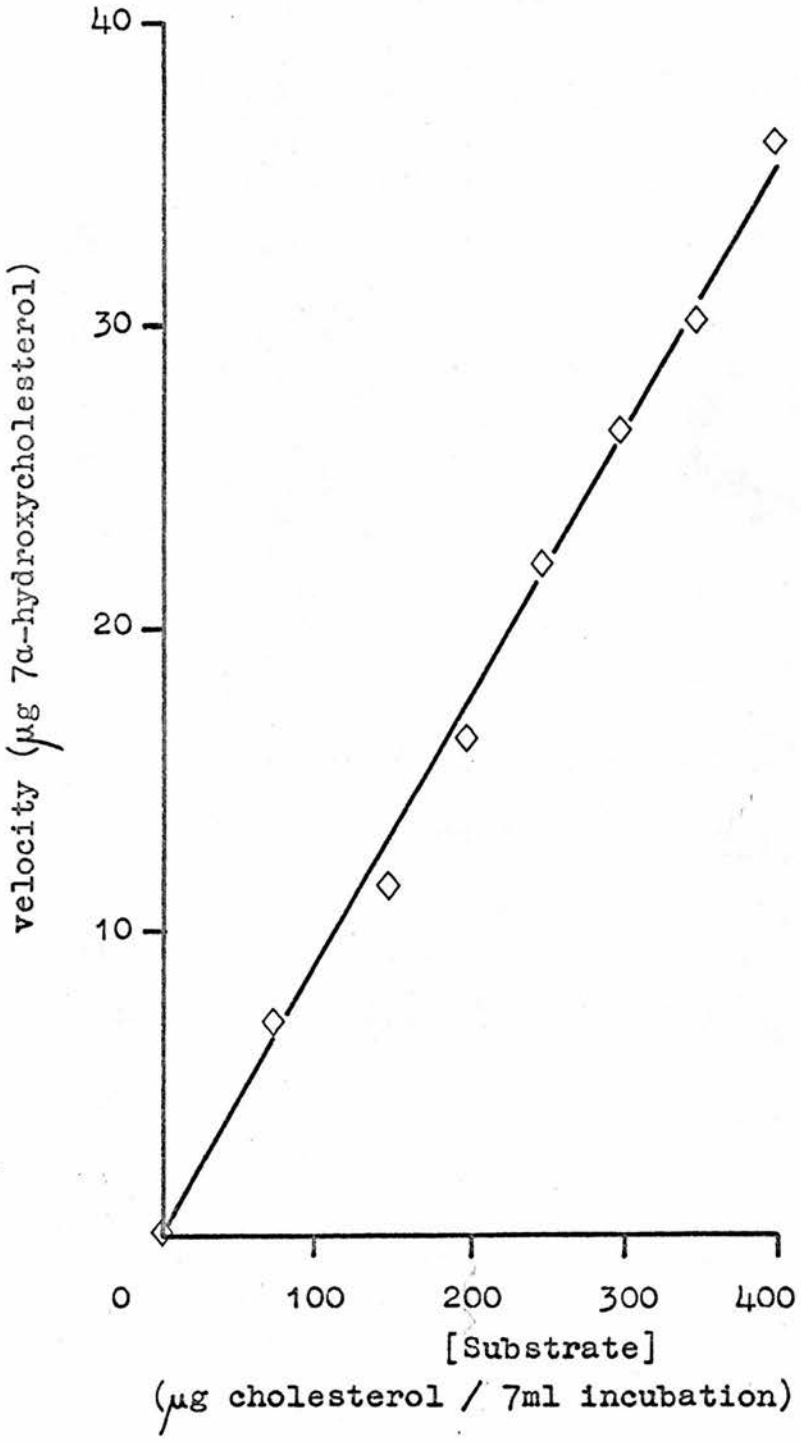


Figure 8,16.

The effect of adding to a Nonidet P42 treated acetone powder (1.2µl / mg protein) increasing concentrations of cholesterol on cholesterol 7α-hydroxylase activity.

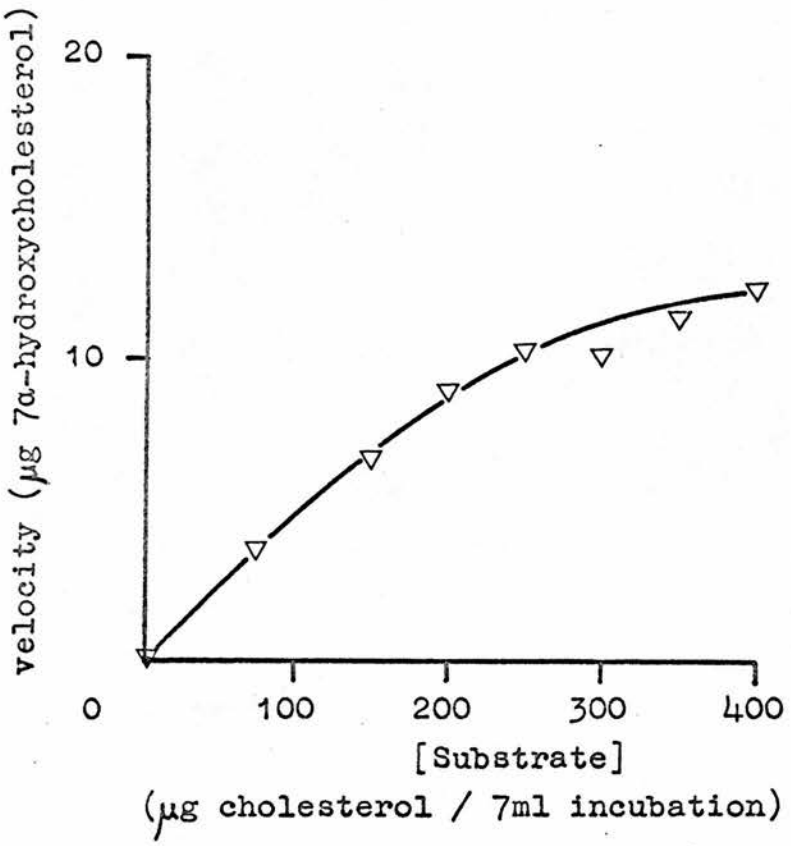


Figure 8,17.

The effect of adding to a Nonidet P42 treated acetone powder (1.8µl / mg protein) increasing concentrations of cholesterol on cholesterol 7α-hydroxylase activity.

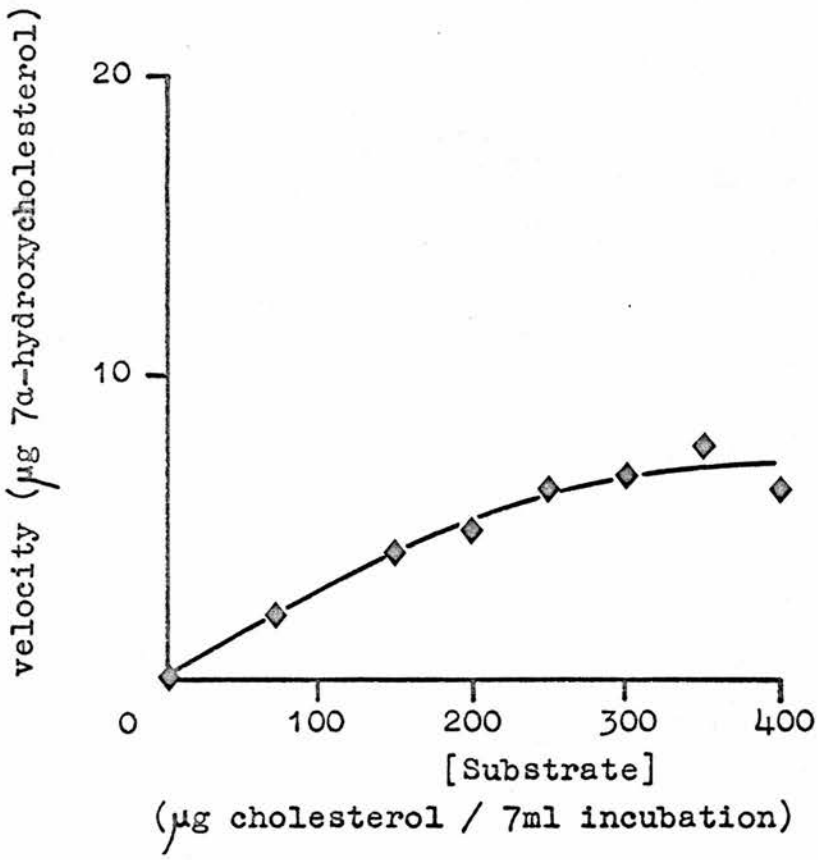


Figure 8,18.

The effect of adding to a Nonidet P42 treated acetone powder (2.4µl / mg protein) increasing concentrations of cholesterol on cholesterol 7α-hydroxylase activity.

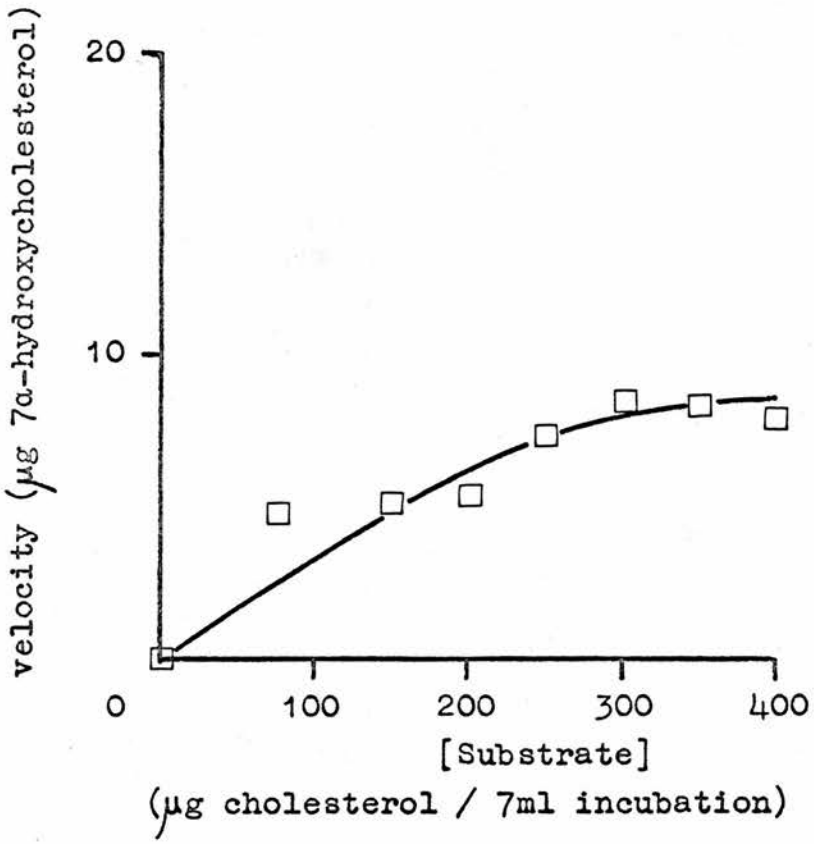


Figure 8,19.

The effect of adding to a Nonidet P42 treated acetone powder (3.0µl / mg protein) increasing concentrations of cholesterol on cholesterol 7α-hydroxylase activity.

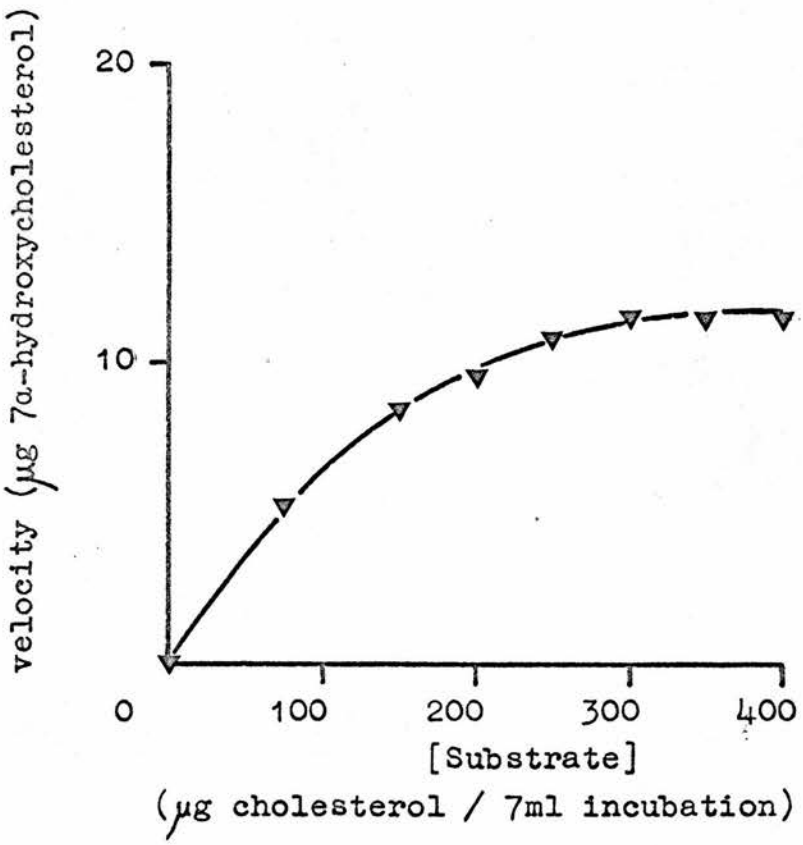


Figure 8,20.

The effect of adding to a Nonidet P42 treated acetone powder (3.6µl / mg protein) increasing concentrations of cholesterol on cholesterol 7α-hydroxylase activity.

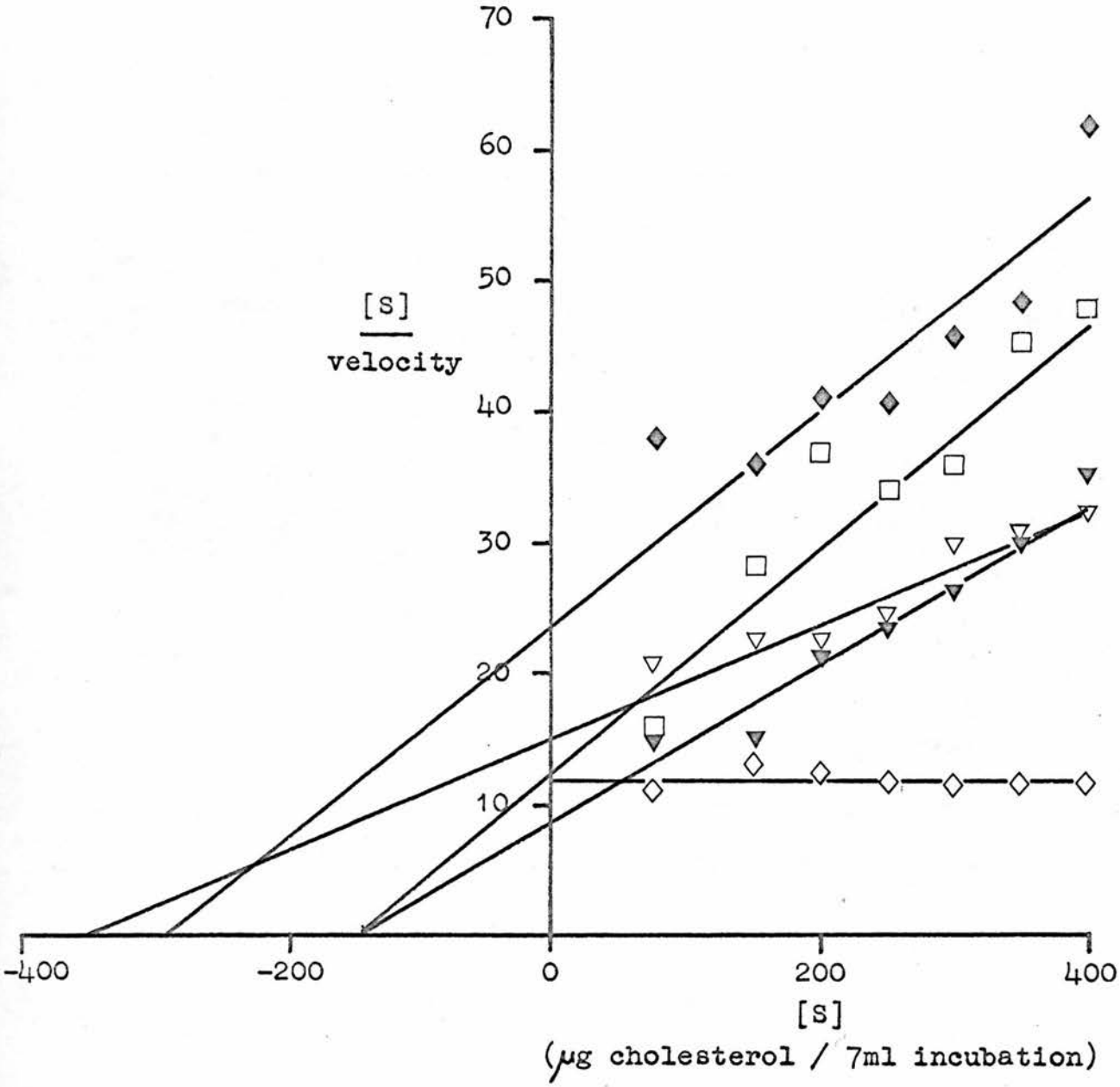


Figure 8,21.

$\frac{[S]}{v}$ vs [S] plots of data presented in figures 8,16 to 8,20.

	µl Nonidet P42 / mg protein				
	1.2	1.8	2.4	3.0	3.6
K_m (µM)	1053	128	107	54	52
V_{max} (µg 7α-hydroxy-cholesterol formed in 40min)	264	23	13	11	16

Table 8,3.

The effect of Nonidet P42 on the apparent K_m and V_{max} for cholesterol and cholesterol 7α-hydroxylase in a rat liver microsomal acetone powder.

be destroyed, and also that the Michaelis-Menten constant more accurately represents the concentration of cholesterol necessary for half the maximal velocity.

K_m for cholesterol in an acetone powder

Table 8,2 shows the effect of increasing the mass of cholesterol to incubations containing approximately 9mg protein on percentage conversion to radioactive 7α-hydroxycholesterol, and on μg product formed, assuming equilibration of $\left[4 - {}^{14}C\right]$ cholesterol. A plot of cholesterol 7α-hydroxylase activity against substrate concentration is linear, and an $[S]/v$ vs $[S]$ plot has a gradient of zero. Therefore, in an acetone powder it appears that the enzyme cannot be saturated with cholesterol. The most favourable value of K_m determined was 3500 μM, but as the maximum concentration of cholesterol added was approximately 150 μM, such a figure is of little value.

The effect of Nonidet P42 on the K_m for cholesterol

Thirty further incubations containing about 8mg protein were prepared consisting of five groups of six. Each group contained a fixed ratio of Nonidet P42/mg protein, and within the group the concentration of cholesterol was increased, but the concentration of acetone (the vehicle of added cholesterol) was constant. The amount of endogenous cholesterol, approximately 8 μg, was negligible in relation to exogenous substrate. After incubation for 40 minutes, the percentage conversion of $\left[4 - {}^{14}C\right]$ cholesterol to $\left[4 - {}^{14}C\right]$ 7α-hydroxy-cholesterol was determined. Figures 8,16 to 8,20 show v vs $[S]$ plots and Figure 8,21 shows all these results linearised by plotting $[S]/v$ vs $[S]$. Table 8,3 presents the values of K_m and V_{max} obtained for each ratio of

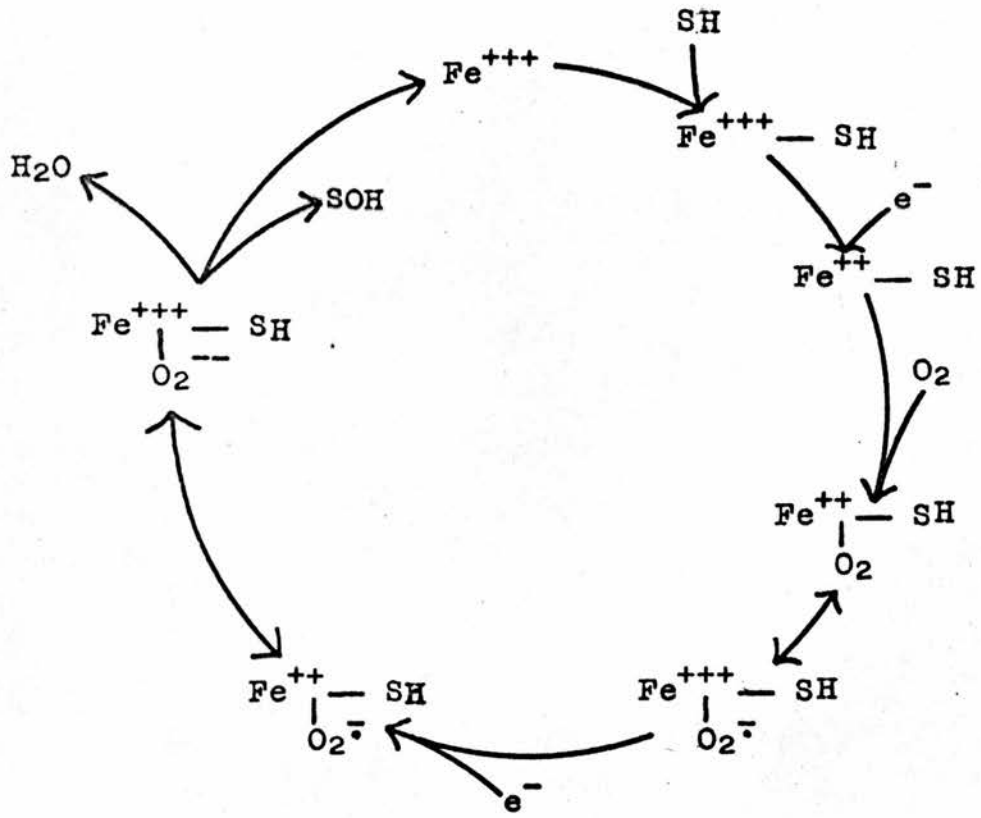


Figure 8,22.

A scheme showing how the superoxide anion could be formed during the hydroxylation of substrate by cytochrome P-450.

Nonidet P42/mg protein. These values were calculated by computer, programmed for the Wilkinson method for determination of Michaelis-Menten parameters (142). Again at low ratios, K_m could not be realistically measured, and the best value obtainable, 1050 μM, was six-fold greater than the highest concentration of cholesterol used. On increasing the ratio of Nonidet P42/mg protein and therefore an increased solubilization, the K_m decreased, and at the highest concentrations of detergent K_m seemed to reach a limiting value of approximately 50 μM. This value is of the same order of magnitude as that determined recently by Van Cantfort and Gielen (94). It is also interesting to note that the value of the apparent V_{max} fell in much the same way as the value of K_m. This relationship is also mentioned in a different context in Section 9.

The effect of superoxide dismutase on cholesterol 7α-hydroxylase solubilized by Nonidet P42

During the process of hydroxylation, it is likely that the substrate or molecular oxygen, or both, become activated. The substrate may be activated by formation of a free radical $SH^{\bullet}$ from SH_2, but if the abstraction of hydrogen from the substrate were a rate-limiting step, then if the hydrogen were exchanged for deuterium or tritium, the rate of reaction would be slower. Such an isotope effect is not observed in the case of cholesterol 7α-hydroxylase and thus the breaking of the C-H bond is not likely to be the rate determining step (143). An active species of oxygen is superoxide. This univalently reduced form of oxygen could theoretically be formed during the hydroxylation process as depicted in Figure 8,22. The superoxide anion has already been detected during the oxidation of xanthine by xanthine oxidase (144). Other possible intermediates have been discussed by Ullrich and Staudinger (145). Fridovich and Handler (146) demonstrated that the

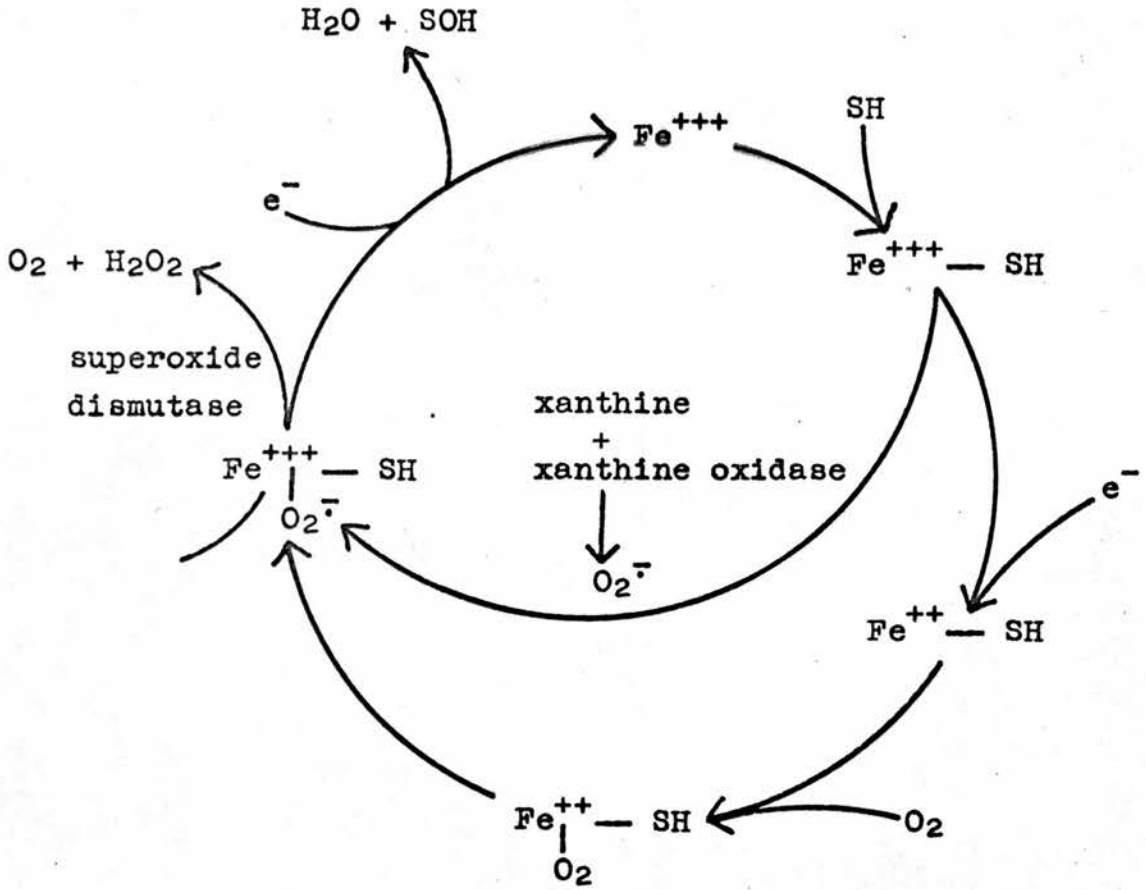


Figure 8,23.

A possible mechanism by which superoxide anion generated by the oxidation of xanthine by xanthine oxidase could support the demethylation of benzphetamine hydrochloride.

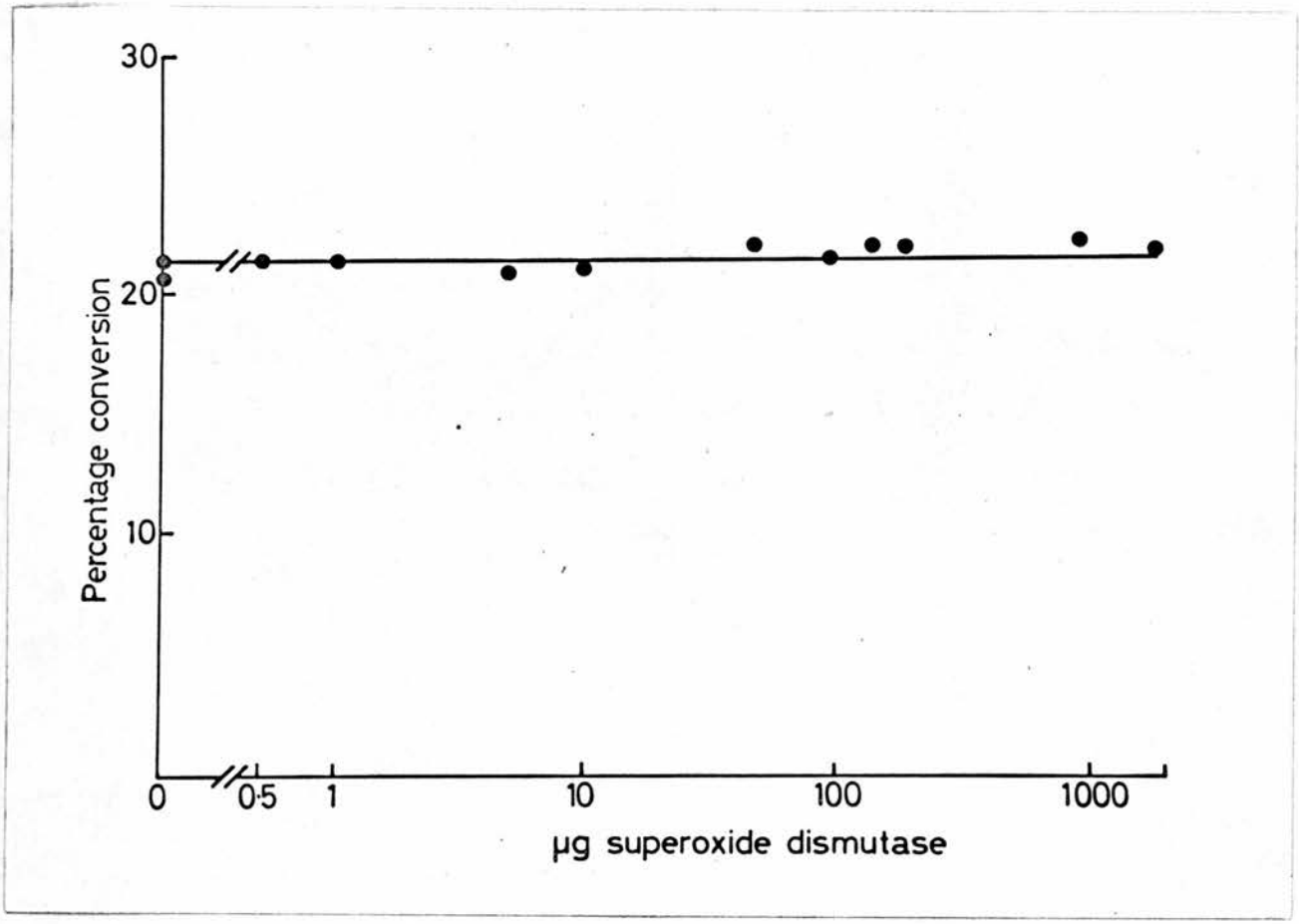


Figure 8,24.

The effect of adding increasing masses of superoxide dismutase to a 7ml incubation containing Nonidet P42 solubilized cholesterol 7α-hydroxylase.

reduction of cytochrome c by xanthine and xanthine oxidase could be stimulated by oxygen. The reaction involved the formation of free radicals and they proposed that the active species was the superoxide anion $O_2^{\bullet -}$. Knowles (144) suggested that superoxide existed in equilibrium with the dimer O_4^{--} and that this species underwent a disproportionation reaction, i.e. $2O_2^{\bullet -} \rightleftharpoons O_4^{--} + 2H^+ \rightarrow H_2O_2 + O_2$. McCord and Fridovich (147) later purified a protein capable of catalysing the disproportionation and of inhibiting the reduction of cytochrome c by xanthine and xanthine oxidase. This copper containing protein, superoxide dismutase, was shown to be identical to erythrocuprein. Coon et al (148) demonstrated that superoxide dismutase inhibited the demethylation of benzphetamine at low concentrations (ca. 100μg) and could be completely inhibited by further addition of 0.6M NaCl. Furthermore, it was demonstrated that a system generating superoxide could support the hydroxylation of benzphetamine in the absence of NADPH. A scheme proposing how the mechanism may occur is given in Figure 8,23.

An acetone powder preparation of rat liver microsomes was solubilized in the usual way and incubated for 30 minutes with increasing masses of purified superoxide dismutase. Cholesterol 7α-hydroxylase activity was measured and the results are shown in Figure 8,24. It may be seen that the preparation was very active, 23% of [4 - ^{14}C] cholesterol being converted [4 - ^{14}C] 7α-hydroxycholesterol in 30 minutes. No effect on this activity could be demonstrated even when 2mg purified superoxide dismutase were added to the 7ml incubation. Two interpretations of these results can be made (i) that superoxide anion was not formed during the transformation of cholesterol to 7α-hydroxycholesterol, or (ii) that superoxide radicals were produced but that superoxide dismutase was unable to disproportionate this species at a rate which inhibited cholesterol 7α-hydroxylase.

The effect of phospholipid on cholesterol 7α-hydroxylase activity

Since the initial solubilization and partial purification of rat liver microsomal mixed function oxidase (79), it has been apparent that an essential component of reconstituted drug hydroxylating systems was a heat stable lipid fraction, the active component of which was later identified as being phospholipid, in particular phosphatidylcholine (114). However, the question of cytochrome P-450 dependent reactions having an absolute requirement for phospholipid is controversial and much of the experimental evidence is contradictory. Thus, treatment of microsomes with phospholipase C removed about 70% of the phospholipid and decreased the metabolism, as well as abolishing the binding, of type I compounds (149). Coon, however, suggests that phosphatidylcholine does not serve as a substrate or induce a spectral change when added to cytochrome P-450, nor does it affect the dissociation constant of haemoprotein and laurate or benzphetamine complex (150). Cater et al (151) demonstrated that sodium deoxycholate inhibited the metabolism of compounds inducing a type I difference spectrum and found that this inhibition could be relieved by addition of phospholipid. He also showed that the loss of aminopyrine demethylase activity resulting from incubation of microsomes with phospholipase C could not be reversed by addition of lecithin. Recently, Vore et al have prepared microsomes in which up to 70% of the phospholipid had been removed by extraction of lyophilized microsomes with n-butanol (152). In this preparation 3,4- benzpyrene hydroxylase activity was reduced to 40-60% of the original activity, but when phospholipid was added, full activity could be restored. Phosphatidylcholine does not cause aggregation of cytochrome P-450 or NADPH-cytochrome P-450 reductase, or of these two components with each other, as evidenced by the ultracentrifugal studies of Autor et al (153), but Strobel has demonstrated

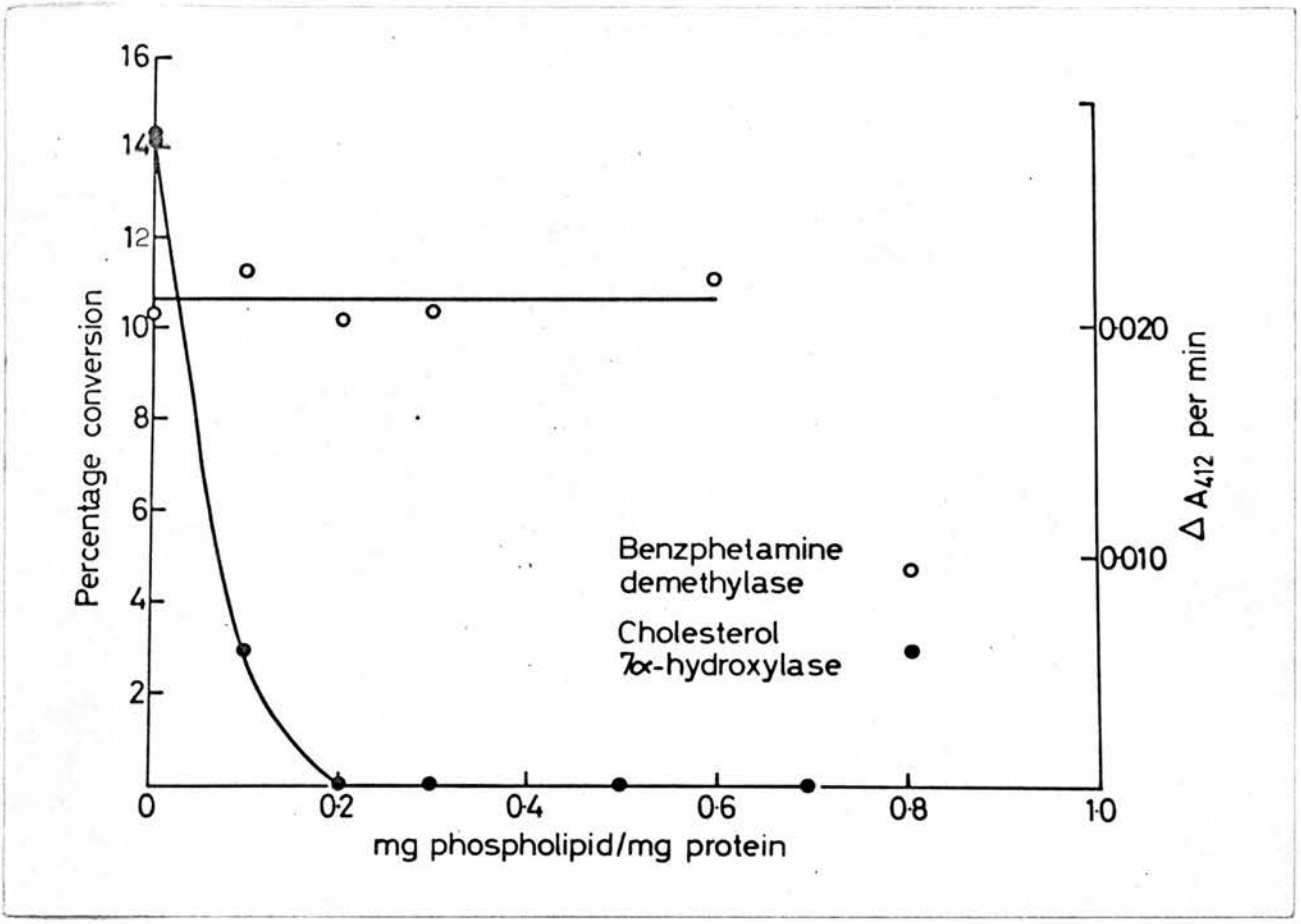


Figure 8,25.

The effect of adding phosphatidyl choline to a rat liver microsomal butanol powder on cholesterol 7α-hydroxylase and benzphetamine demethylase activities.

that the rate of reduction of cytochrome P-450 by NADPH is greatly increased in the presence of phosphatidylcholine (114) though it is difficult to envisage this compound as an electron carrier. Imai and Sato (106) and Bjorkhem et al (86) in the reconstitution of aniline hydroxylase and cholesterol 7α-hydroxylase respectively, have shown that in the absence of added phosphatidylcholine the activities were reduced by only 20% from the activities when phospholipid was added.

Rat liver microsomes containing 0.6mg phospholipid/mg protein were desiccated and then extracted with butanol and acetone. 50% of the phospholipid was removed by this treatment. Figure 8,25 shows the effect of adding phospholipid (dipalmitoyl glyceryl-3-phosphorylcholine) to the resuspended butanol powder. The addition was as follows:- To 3ml of the suspension was added 2ml of the phospholipid, suspended in 0.1M phosphate buffer by sonication. The mixture was sonicated for 10 secs. at 0°C, then incubated at 37°C for 25 minutes. Cofactors were then added and finally [4 - ^{14}C]cholesterol in acetone. After incubation for 40 minutes at 37°C, the cholesterol 7α-hydroxylase activity was measured. Taking into consideration the fact that the butanol powder already contained 0.3mg phospholipid/mg protein, the addition of a further 0.3mg/mg protein would only bring the ratio up to that observed in the original native microsomes. It is clearly seen, however, that at the ratio of 0.6mg phospholipid/mg protein, the apparent cholesterol 7α-hydroxylase activity was abolished. Using the same preparation and procedure, the effect of adding back phospholipid on the demethylation of benzphetamine was also observed. Using a final concentration of 1.25mM benzphetamine hydrochloride, the velocity was determined at each phospholipid/protein ratio by observing the formation of formaldehyde after 0, 5, 10 and 15 minutes of incubation at 37°C. These results are also shown in Figure 8,25 and

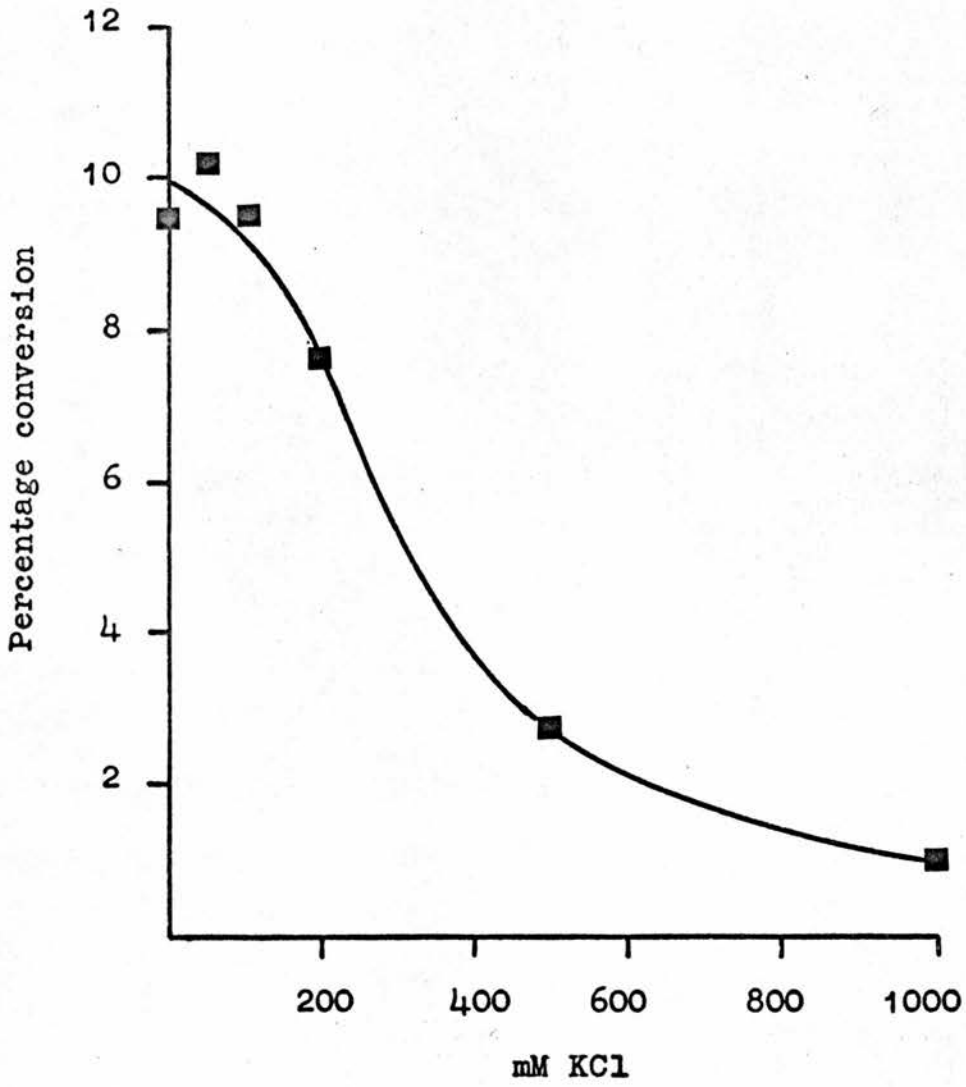


Figure 8,26.

The effect of increasing ionic strength on cholesterol 7α-hydroxylase activity.

demonstrate that added phospholipid did not affect the rate of hydroxylation of benzphetamine. Phosphatidylcholine readily forms mixed micelles with cholesterol, and it is possible that the loss of cholesterol 7∝-hydroxylase activity was a reflection of the sequestration of tracer substrate by lecithin, making the cholesterol unavailable to the enzyme. However, the results of the experiment mean that demonstration of an absolute requirement for phospholipid will be difficult.

The effect of ionic strength on the activity of Nonidet P42 solubilized cholesterol 7∝-hydroxylase

It has been previously observed by Bjorkhem and Danielsson (154) that cholesterol 7∝-hydroxylase is more sensitive to increasing concentrations of KCl than other cytochrome P-450 dependent reactions. A butanol powder was solubilized in 20mM phosphate buffer pH 7.4 and incubated for 40 minutes with increasing concentrations of KCl. The results are shown in Figure 8,26 and demonstrate that up to 0.1M KCl the activity of the enzyme was not greatly affected, but at higher concentrations the enzyme was inhibited and at 1M KCl the activity was only 10% that of the control. The ionic strength of the 20mM potassium phosphate buffer, pH 7.4, was 0.04M.

The effect of pH on Nonidet P42 solubilized cholesterol 7∝-hydroxylase

The pH optimum of cholesterol 7∝-hydroxylase in native rat liver microsomes has been determined by Boyd et al and was found to have a value of approximately 7.4. This value is close to that found in human liver (155) and pigeon liver (156).

A butanol powder was solubilized with Nonidet P42 and 8mg protein was incubated in 7ml containing the necessary cofactors and

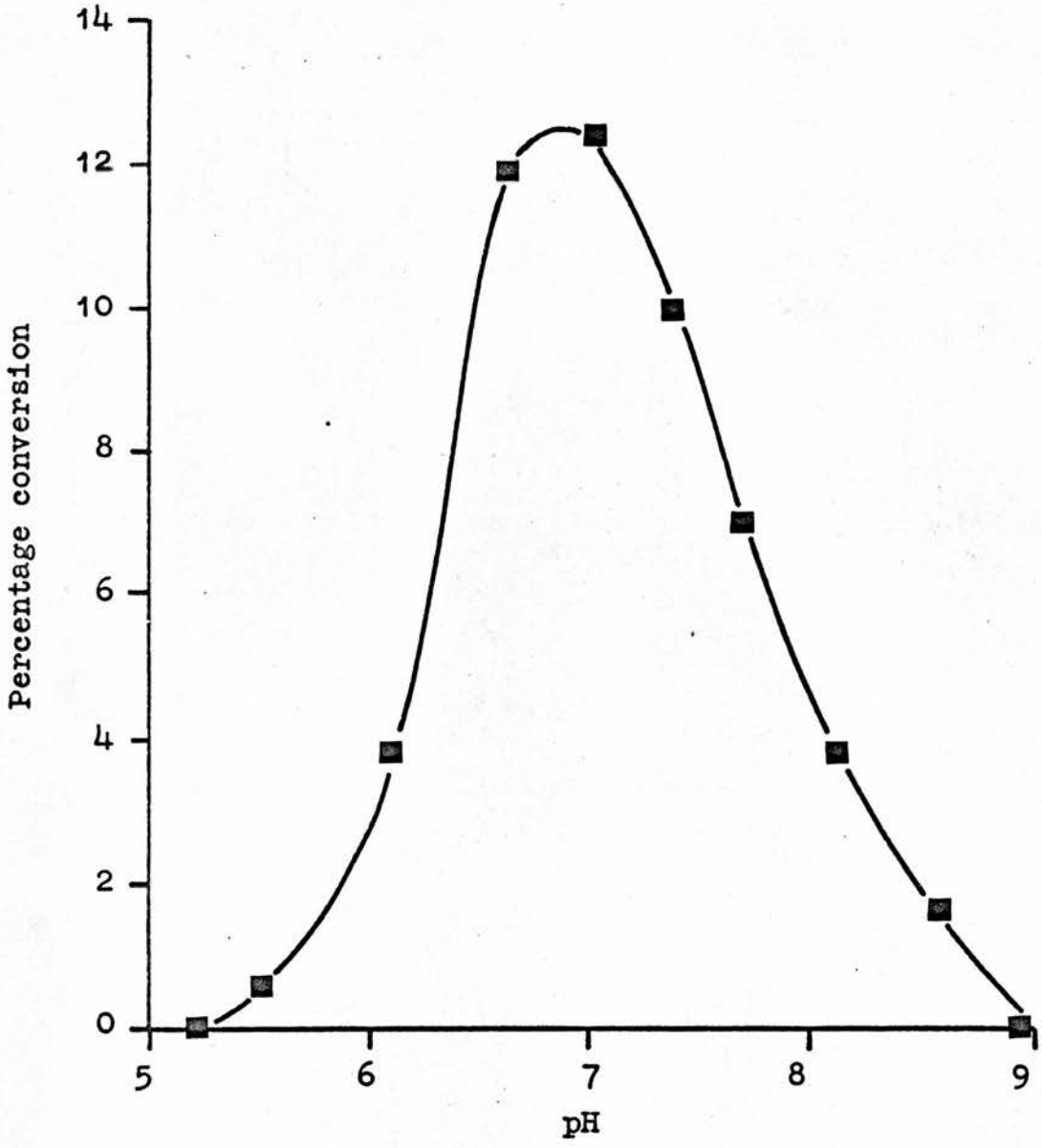


Figure 8,27.

The effect of pH on cholesterol 7α-hydroxylase activity.

50mM Tris-phosphate buffer. The pH of the incubations were measured at the beginning, in the middle and at the end of the 40 minute incubation, but no change was observable. Figure 8,27 shows the velocity of cholesterol 7α-hydroxylase, measured on a percentage conversion basis of [4 - ^{14}C] cholesterol to [4 - ^{14}C] 7α-hydroxy-cholesterol related to pH.

In contrast with other observed pH optima, the pH optimum here was approximately 7.0, and the fall off in activity either side of neutrality was steep. It is possible that the enzyme solubilized from a butanol powder by Nonidet P42 has a conformation different from that in native microsomes, and as a result a slightly lower pH is required to maximise cholesterol 7α-hydroxylase activity.

SUMMARY

(1) Both native and Nonidet P42 solubilized microsomes exhibit amine binding spectra, and using the empirical formula of Jefcoate (134) some high spin cytochrome P-450 appears to be transformed to low spin cytochrome P-450 upon solubilization with Nonidet P42.

(2) Type I binding spectra were generated on addition to native microsomes of various substrates. The magnitude of the absorbance change was decreased upon solubilization.

(3) Electron paramagnetic resonance spectroscopy of the low spin species of cytochrome P-450 in native microsomes and Nonidet P42 solubilized showed the g values to be the same. This, therefore, is evidence that the environment of the ferric ion of cytochrome P-450 is not greatly altered on solubilization.

(4) The absolute spectrum of cytochrome P-450 solubilized by Nonidet P42 and partially purified, further demonstrated that the haemoprotein contained low spin ferric ion.

(5) Nonidet P42 was shown to inhibit both the demethylation of benzphetamine and also the reduction of cytochrome P-450 by NADPH.

(6) The K_m for cholesterol of cholesterol 7α-hydroxylase depended on the concentration of Nonidet P42, and the values of both K_m and V_{max} fell to a limiting value at high concentrations of the detergent.

(7) The role of superoxide in the 7α-hydroxylation of cholesterol could not be determined as the addition of superoxide dismutase to a Nonidet P42 solubilized rat liver microsomal acetone powder did not lead to any inhibition.

(8) Addition of phospholipid to a preparation partially depleted of phospholipid led to apparent inactivation of cholesterol 7α-hydroxylase, but the rate of demethylation of benzphetamine was unaffected.

(9) The solubilized preparation of cholesterol 7α-hydroxylase was shown to be sensitive to the concentration of potassium chloride and was inhibited in a manner similar to that demonstrated in native microsomes by Bjorkhem et al (154).

(10) The pH optimum of cholesterol 7α-hydroxylase from a Nonidet P42 solubilized butanol powder was found to be approximately 7.0. This is in contrast to that determined in native microsomes where a value of 7.4 was observed.

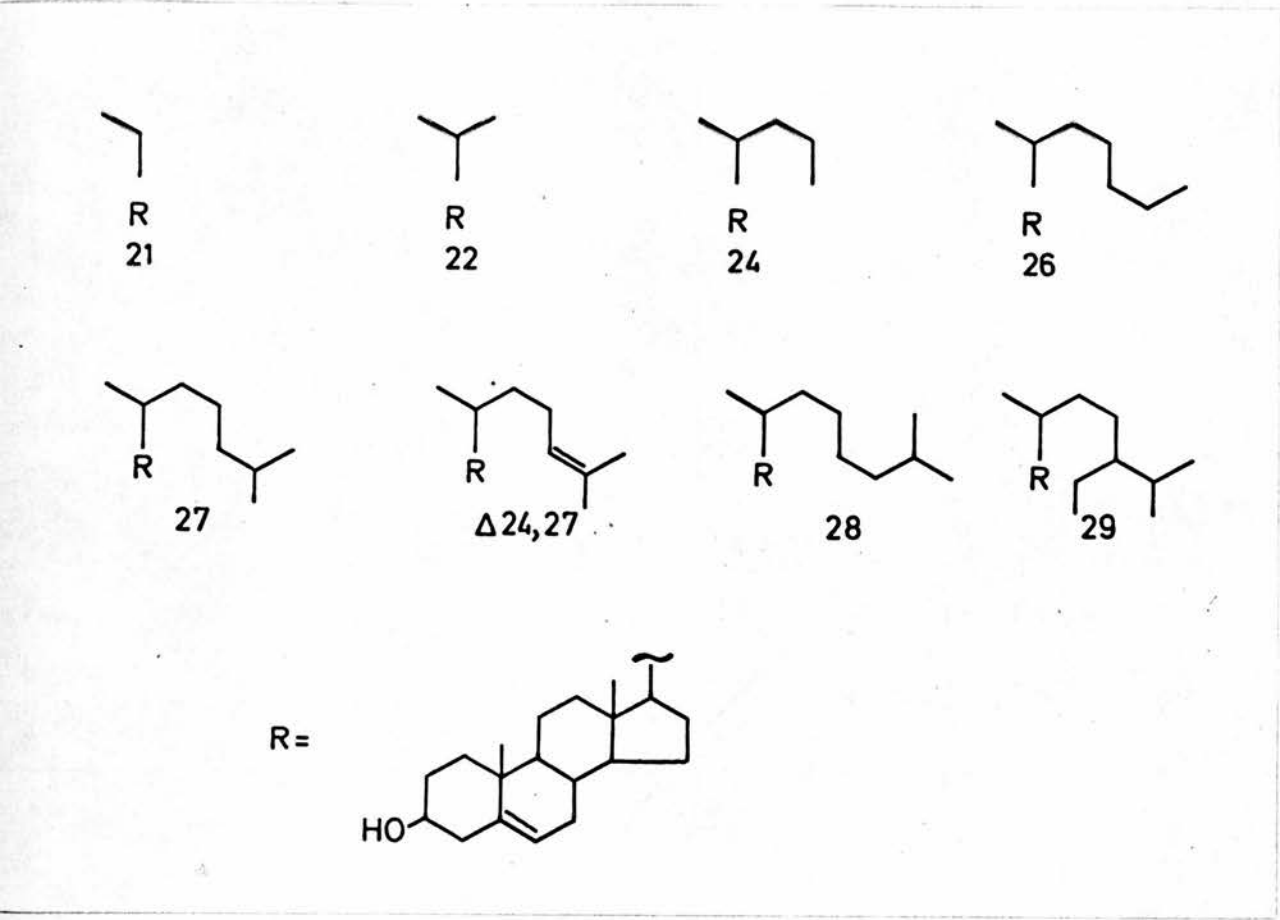


Figure 9,1.

The structure of sterols used in the study of the specificity of cholesterol 7α-hydroxylase.

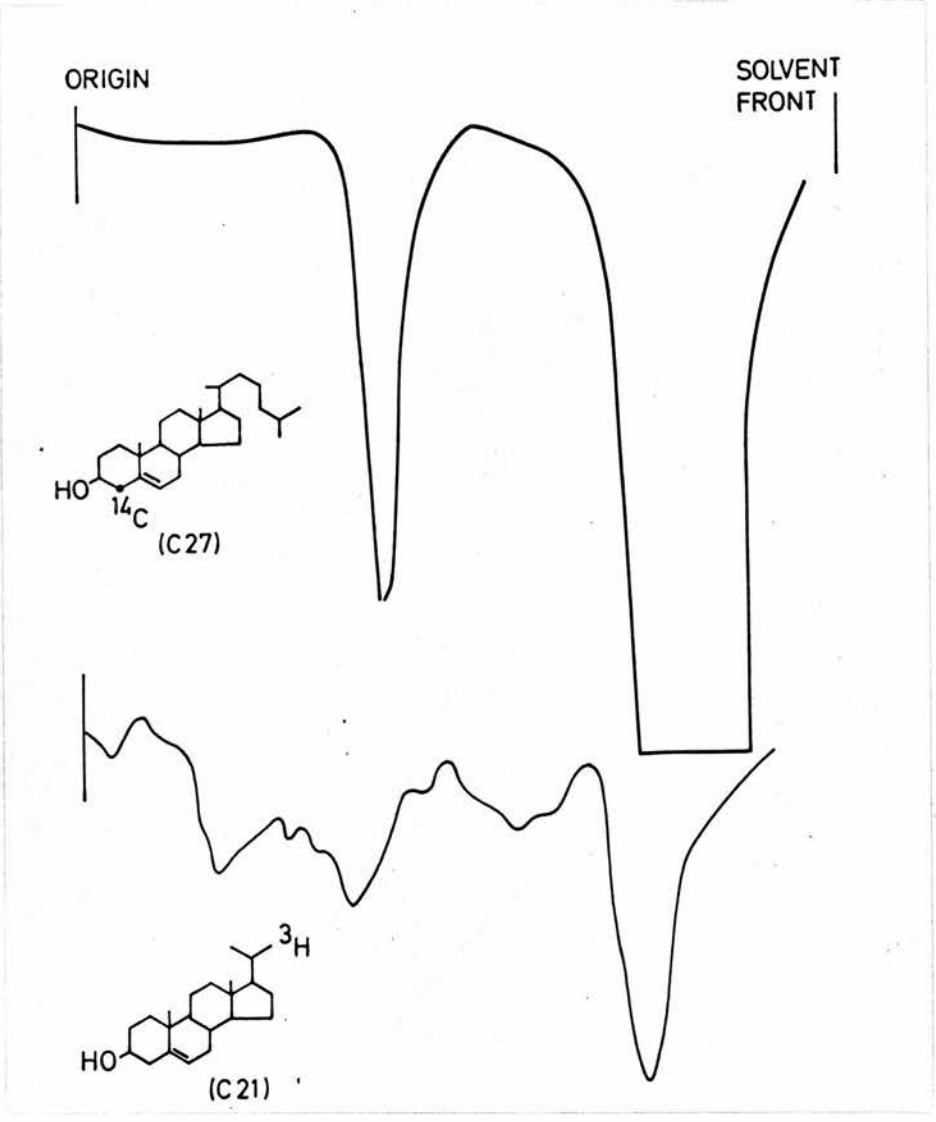


Figure 9,2.

Thin layer chromatography of the oxidation products formed on incubation of cholesterol and pregn-5-ene-3β-ol with rat liver microsomes.

SECTION 9

THE SUBSTRATE SPECIFICITY OF CHOLESTEROL 7α-HYDROXYLASE

The drug hydroxylation system of rat liver microsomes has a remarkable range of substrate specificity. Thus compounds as diverse as ethylmorphine, aminopyrine, cyclohexane, aniline, paraffins, may be hydroxylated. The specific activity of many of these hydroxylases may be increased by phenobarbital treatment of the rats, but the hydroxylation of cholesterol is unaffected by this treatment in the strain used in this laboratory (12). It was, therefore, of interest to determine whether the range of specificity displayed by cholesterol 7α-hydroxylase was as great as that shown by the drug hydroxylating system. Studies on this aspect have previously been made by Johansson (157) and Brown et al (12), but the structural changes in the side chain in these experiments were extensive. In order to probe the structural requirements for the side chain for cholesterol 7α-hydroxylase activity more closely, β-sitosterol (24, ethyl-cholesterol), cholesterol and desmosterol (Δ_{24} cholesterol) were purchased, and pregn-5-ene-3β-ol, bisnorcholenol, cholenol, 26 norcholesterol and 26 dimethyl norcholesterol were synthesized in radioactive and non-radioactive form. The cholesterol 7α-hydroxylase preparation used was a butanol powder solubilized by Nonidet P42. This removed the endogenous, perhaps competing, sterols. Protein (11mg) was added to each incubation and 4.3 nmoles of each steroid (approximately 2μg) in 50μl acetone, in a final volume of 7ml. After 40 minutes incubation at 37°C in the presence of 10mM β-mercaptoethylamine the sterols were extracted in the usual way and oxidised products separated from the substrate by t.l.c. Figures 9,1 and 9,2 show the structures of the sterols and two scans for radioactivity after t.l.c. showing the difference in the pattern of oxidation between

	Percentage conversion	% oxidation of steroid / % oxidation of cholesterol
Pregnenol	14.4 (many products)	1.13
Bis-norcholenol	5.1 (many products)	0.40
Cholenol	2.6	0.20
Norcholesterol	8.6	0.68
Desmosterol	11.9	0.94
Cholesterol	12.7	1.00
Dimethylnorcholesterol	1.2	0.09
β-sitosterol	-	-

Table 9,1.

The percentage conversion of radioactive sterols to oxidized products on incubation with Nonidet P42 solubilized butanol powder (11mg protein) for 40 minutes.

long and short side chain sterols. Table 9,1 shows the percentage conversion of these sterols into oxidised products.

Of the sterols which are hydroxylated to give a single product, cholesterol is the preferred substrate, but desmosterol, in which the two terminal methyl groups are in a configuration quite different from that of cholesterol, was demonstrated to be hydroxylated to an extent of 95% that of cholesterol; although this product has not been identified as being 7α-hydroxydesmosterol, since desmosterol may be efficiently converted into bile acids (159) when the conversion of desmosterol to cholesterol is inhibited by triparanol, it is probable that cholesterol 7α-hydroxylase was responsible for the oxidation.

26 norcholesterol, a sterol which differs from cholesterol only by the loss of a single methyl group at C_{25}, was hydroxylated to 7α-hydroxynorcholesterol only at 65% the extent of the cholesterol hydroxylation. The product has been positively identified as 7α-hydroxynorcholesterol by repeated crystallization to constant specific activity with authentic radioinactive 7α-hydroxynorcholesterol.

When the sterol side chain was lengthened by one carbon atom to form 26 dimethylnorcholesterol, and this substrate incubated with the solubilized butanol powder, a single product was formed. This is consistent with a 7α-hydroxylation but the product was not identified. The extent of conversion to this product was only 1.2%, that is, only 10% that of the cholesterol oxidation.

β-sitosterol was not oxidised to any detectable products. This result is in agreement with that of Aringer and Eneroth (158).

Approximately 2.6% of the C_{24} sterol, cholenol, was

	Percentage conversion	% oxidation of sterol / % oxidation of cholesterol
Pregnenol	50 (many products)	54
Bis-norcholenol	21.2 (many products)	1.48
Cholenol	0.85	0.07
Norcholesterol	0.96	0.56
Cholesterol	-	1.0
Dimethylnorcholesterol	0.50	0.04

Table 9,2.

The percentage conversion of radioactive sterols to oxidised products on incubation with native rat liver microsomes for 40 minutes.

oxidised to a single product, which is again consistent with a 7α-hydroxylation, but the product was not identified. This degree of oxidation was about 20% that of cholesterol.

Shortening the side chain further appears to introduce a completely different mechanism of oxidation. Both the C_{22} (bisnorcholenol) and C_{21} (pregn-5-ene-3β-ol) compounds were oxidised to many products, as is shown in Figure 9,2 of the t.l.c. scan. The formation of a range of products is not consistent with a specific 7α-hydroxylation, and it is postulated that other enzyme systems were responsible for this oxidation.

The oxidation of cholesterol analogues by native rat liver microsomes

Table 9,2 shows the extent of oxidation of the sterols expressed as a percentage of the control - cholesterol. As in the case of the butanol powder, C_{24}, C_{26}, C_{27}, C_{28} compounds all gave one product on incubation with rat liver microsomes (10mg protein), which suggests that the oxidation was a 7α-hydroxylation. 26 dimethyl-norcholesterol and cholenol were hydroxylated only 5% as efficiently as cholesterol and, as was observed in the solubilized liver microsomal butanol powder, 26 norcholesterol 7α-hydroxylase activity was greatly reduced in comparison with cholesterol 7α-hydroxylase activity. With the two shortest side chains tested, the C_{22} compound bisnorcholenol was extensively oxidised to give many products, and in the case of pregnenol, over 50% of the substrate was multiply oxidised. All these incubations were performed in the presence of 10mM β-mercapto-ethylamine.

These results indicate that the structure of the side chain is important in determining the efficiency of 7α-hydroxylation of the

sterol analogue. From these experiments it appears that lengthening the side chain has a greater effect than shortening, as β-sitosterol was not hydroxylated at all, and the addition of 1 carbon atom to the side chain to form 26 dimethylnorcholesterol reduced the activity of the enzyme towards this substrate to 5-10% of the control value. Although removal of 1 carbon atom led to the loss of 7α-hydroxylase activity by approximately 40%, even after removal of three carbon atoms 10-20% of an apparent 7α-hydroxylase activity remained. Such a degree of specificity is unusual as it is applied to part of the substrate which is remote from the C_7 position and even further away from the hydroxyl group at position 3, known to be a requirement for cholesterol 7α-hydroxylase activity. How this degree of specificity is accomplished is difficult to envisage. With polar substrates, the molecule under attack may be oriented by hydrogen bonds and electrostatic bonds. In the side chain of cholesterol, no such bonds could be formed and the hydrophobic bonding which may occur can probably not fulfil the requirements for the determination of specificity.

For this reason it would seem more likely that the lack of reactivity of both sitosterol and 26 dimethylnorcholesterol was due to steric hindrance by the 24 ethyl group of sitosterol or by the extra carbon in the C_{28} compound. It is interesting to conjecture at this point what are the factors determining the apparent specificity of hydroxylation of many of the foreign compounds which are substrates for the liver microsomal mixed function oxidase. In the case of xenobiotics, where there is an observed specificity of hydroxylation, one either has to postulate a specific cytochrome P-450 for each substrate, or a specifier protein, which donates or carries the substrate to the haemoprotein, or thirdly, it is possible that the

	Pregnenolone formed (nmoles/min/mg protein)	Pregnenolone from sterol / Pregnenolone from cholesterol
Bis-norcholenol	0.21	0.96
Cholenol	0.16	0.73
Norcholesterol	0.29	1.32
Cholesterol	0.22	1.00

Table 9,3.

The conversion of cholesterol analogues to pregnenolone by the side-chain cleavage enzyme system of a bovine adrenal mitochondrial acetone powder.

substrates bind to a single species of cytochrome P-450 whose active site could accommodate all the substrates, but the specificity is not determined by steric forces but by the thermodynamically most favourable configuration within a large hydrophobic cleft.

Because the effect of side chain structure on cholesterol 7α-hydroxylase proved so interesting, these same analogues were tested for their ability to be cleaved to pregnenolone by a bovine adrenal mitochondrial acetone powder. The unpublished results of these studies by J. Arthur, G.S. Boyd, J.I. Mason and K.E. Suckling are given in Table 9,3. The product pregnenolone was measured by a radioimmunoassay method (160).

In comparison with cholesterol, norcholesterol was more rapidly cleaved to pregnenolone, and bisnorcholenol was as effectively converted to pregnenolone as was cholesterol. Cholenol itself was metabolized by the side chain cleavage system at about 75% the rate of cholesterol. It is interesting that an enzyme which metabolizes the cholesterol side chain shows much less specificity towards the side chain structure than cholesterol 7α-hydroxylase.

The binding sites of the adrenal side chain cleavage system and the hepatic cholesterol 7α-hydroxylase enzyme are different inasmuch as in the side chain cleavage enzyme the steroid nucleus and only three carbon atoms in the side chain are necessary for activity, and lengthening of the side chain does not affect this activity. However, in the case of cholesterol 7α-hydroxylase, small changes in the length of the side chain greatly affect the activity.

The K_m and V_{max} of cholesterol and norcholesterol in a Nonidet P42 solubilized rat liver microsomal acetone powder

Because norcholesterol added to native microsomes or an

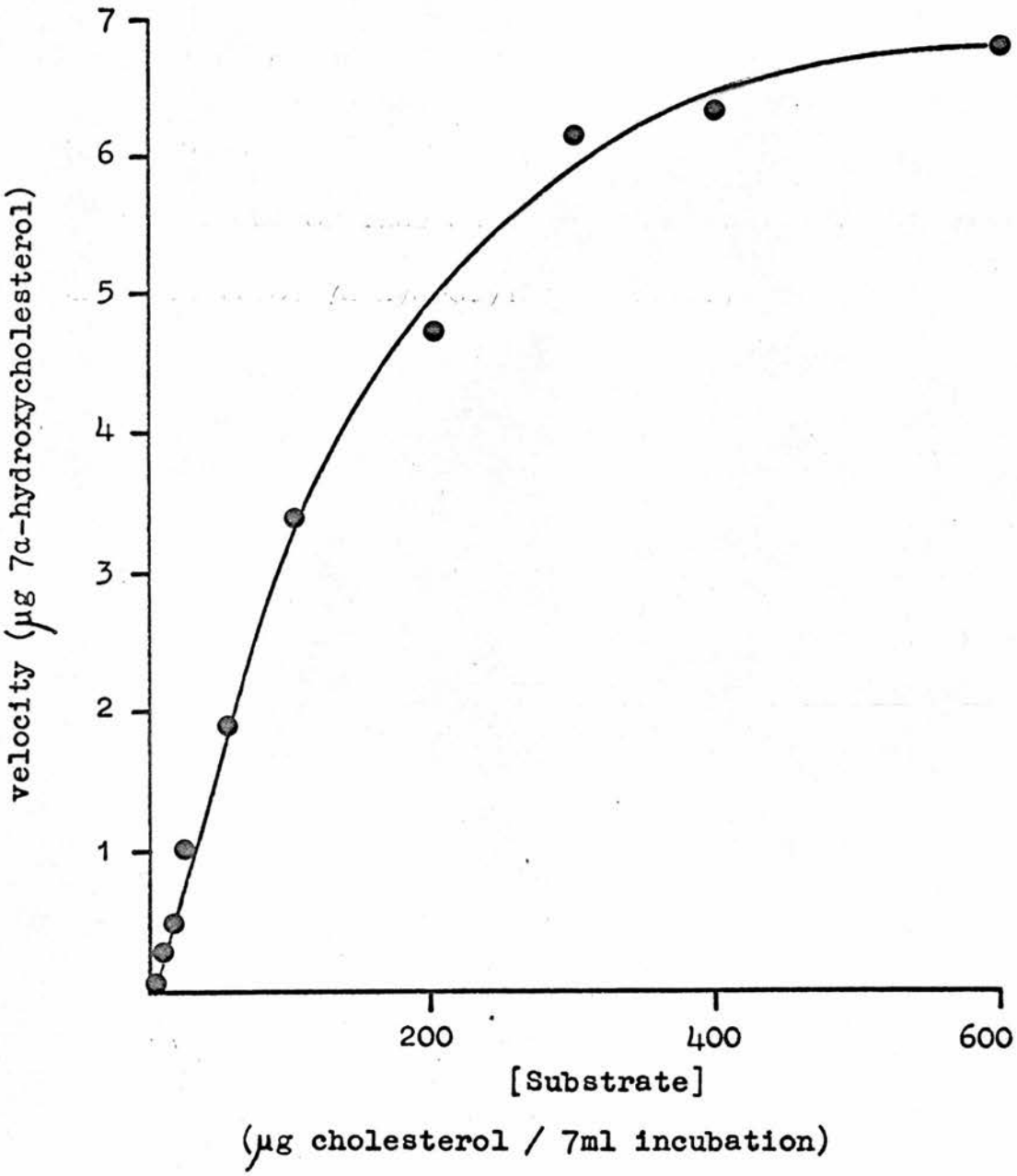


Figure 9,3.

The effect of increasing concentrations of cholesterol on cholesterol 7α-hydroxylase activity.

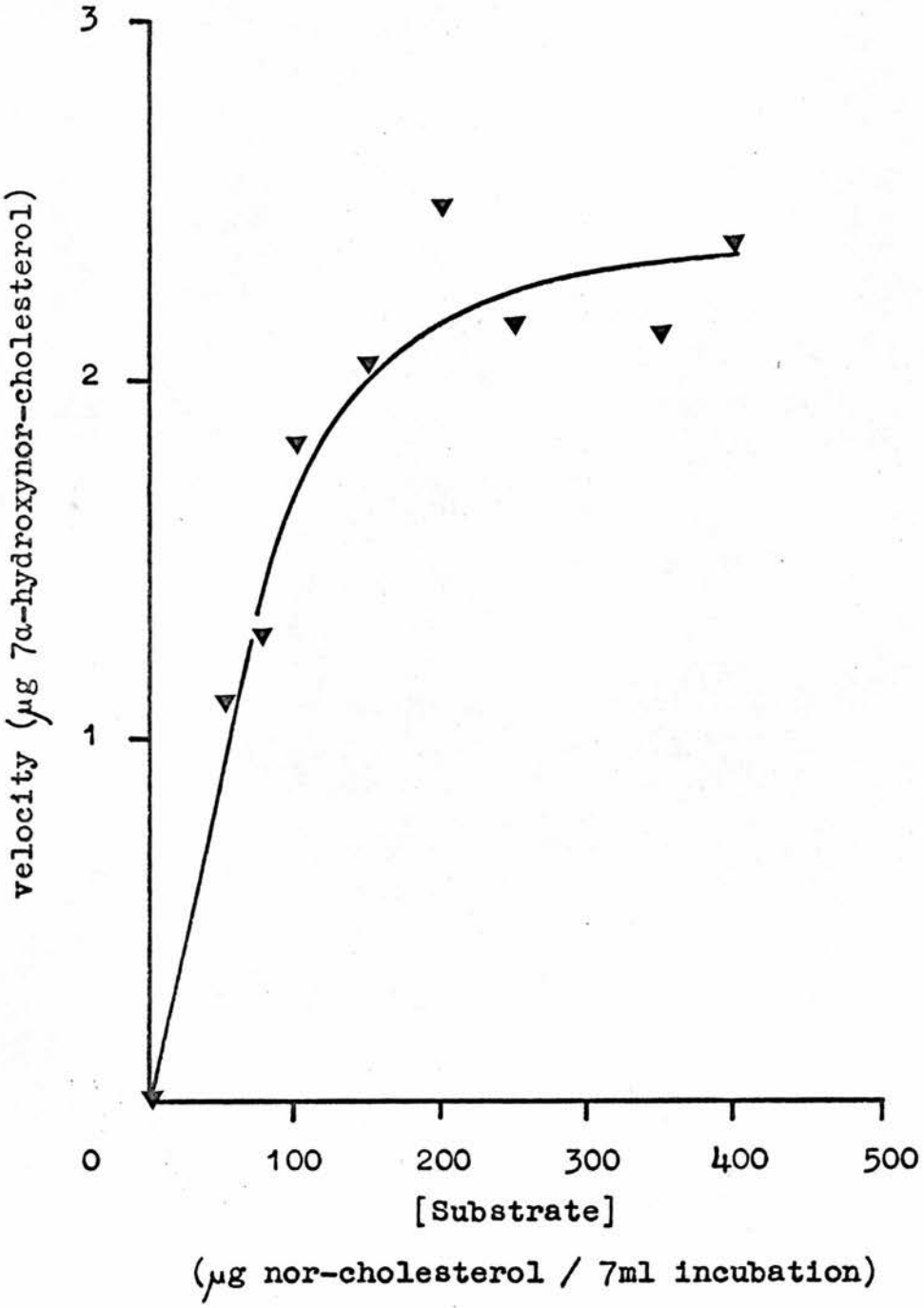


Figure 9,4.

The effect of increasing concentrations of nor-cholesterol on nor-cholesterol 7α-hydroxylase activity.

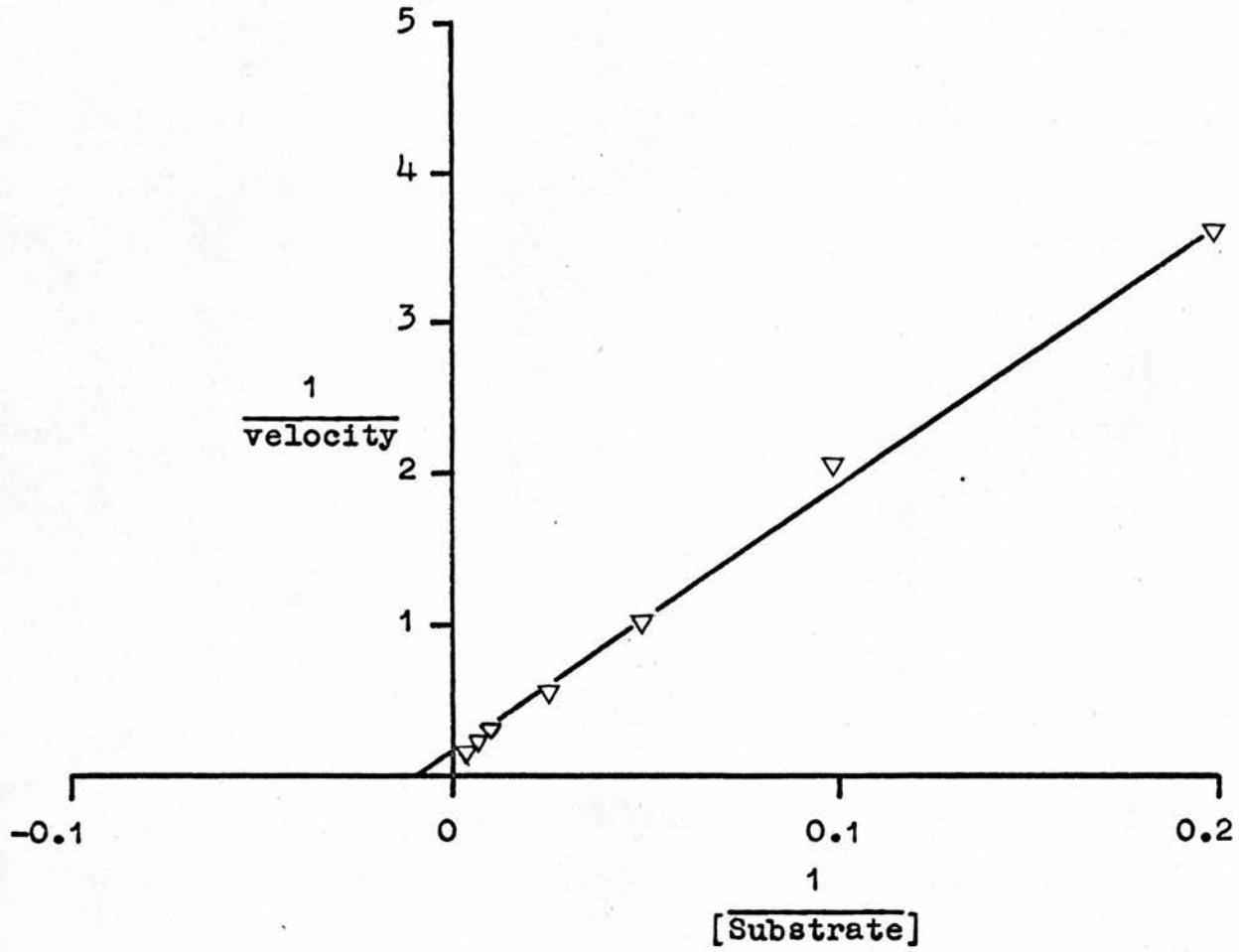


Figure 9,5.

A double reciprocal plot of results presented in figure 9,3.

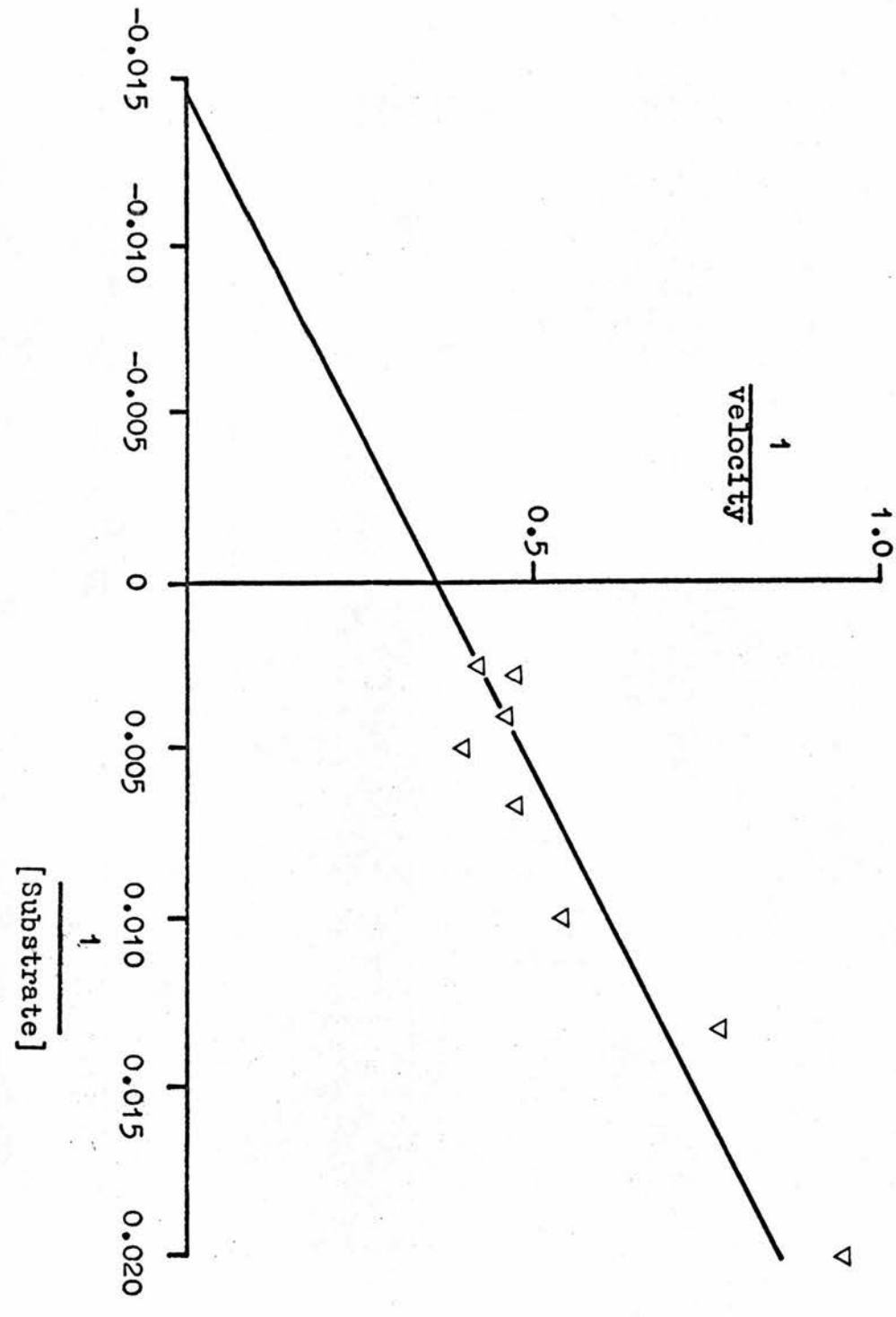


Figure 9,6.

A double reciprocal plot of results presented in figure 9,4.

	K_m	V_{max}
Cholesterol	85 M ± 11 M	10.5 g/40min ± 0.8
Norcholesterol	26 M ± 7 M	2.8 g/40min ± 0.2

Table 9,4.

The Michaelis-Menten parameters of cholesterol 7α-hydroxylase in a rat liver microsomal acetone powder, solubilized by Nonidet P42. Each incubation contained 12mg microsomal protein.

acetone powder was not as actively hydroxylated in the 7α position as was cholesterol, an attempt was made to determine whether the maximal velocity of the hydroxylation was altered, or whether the affinity of the enzyme for the new substrate was reduced.

Figures 9,3 and 9,4 are v vs $[S]$ plots of cholesterol and norcholesterol 7α-hydroxylase activity in the Nonidet P42 solubilized acetone powder. In each incubation of 7ml there was approximately 8mg protein (20mg acetone powder). The concentration of cholesterol or norcholesterol was increased, but the volume of added acetone was constant. $[4 - {}^{14}C]$ cholesterol, (2μg), and $[25 - {}^{3}H]$ norcholesterol, (50μg), was added to the respective incubations, and the mass of product formed was measured by assuming equilibration of the radioactive tracer with the substrate. Figures 9,5 and 9,6 are double reciprocal plots of the velocity vs substrate concentration. The results, computed by the Wilkinson method (142), gave values for K_m and V_{max}, given in Table 9,4. V_{max} for norcholesterol can be seen to be only 27% of the V_{max} of cholesterol. However, the K_m for norcholesterol was also reduced to 30% of the K_m for cholesterol, that is $\frac{K_m \text{ norcholesterol}}{K_m \text{ cholesterol}} = 0.30$

and $\frac{V_{max} \text{ norcholesterol}}{V_{max} \text{ cholesterol}} = 0.27$

A reduction in the value of K_m could mean that the enzyme had a greater affinity for norcholesterol. However, as observed previously, Nonidet P42 affected the apparent K_m and there was a parallel change in V_{max}. In the case of the observed values of K_m and V_{max} for norcholesterol, both were reduced by approximately the same extent from the cholesterol values.

It is possible that since the K_m and V_{max} values appear

to change in parallel, then K_m, which in the generalised scheme, $E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$ is equal to $\frac{k_2 + k_3}{k_1}$ might approximate to $\frac{k_3}{k_1}$ if k_2 is small in relation to k_3, that is, if the rate constant of dissociation of the enzyme-substrate complex is small in relation to the catalytic rate constant. In such a case, if the rate constant of binding of substrate to the enzyme did not vary, then K_m would parallel V_{max}.

The induction of type I binding spectra by cholesterol analogues

Pregn-5-ene-3β-ol, bisnorcholenol, cholenol, norcholesterol, cholesterol, desmosterol and sitosterol were added to native microsomes and Nonidet P42 solubilized microsomes containing about 6mg protein/ml. Difference spectra generated by the addition of 0.2mg sterol were measured as described in the Materials and Methods section.

Sitosterol, cholesterol, desmosterol, norcholesterol and cholenol did not induce any changes in absorption in either native or solubilized microsomes. That the cytochrome P-450 was already saturated with cholesterol is unlikely to be the sole reason for the lack of formation of difference spectra, as a butanol powder containing only approximately 0.1μg cholesterol/mg protein was also incapable of generating a type I difference spectrum on addition of cholesterol. A more plausible explanation would be that the amount of cytochrome P-450 involved in the 7α-hydroxylation of cholesterol is small in contrast to that associated with drug hydroxylation and that this quantity of cytochrome P-450 cannot be spectroscopically observed. Using the Aminco-Chance spectrophotometer, the limit of detection of substrate binding would be approximately that absorbance which would be 2% of the $\Delta_{450-490}$ absorbance of the total cytochrome P-450.

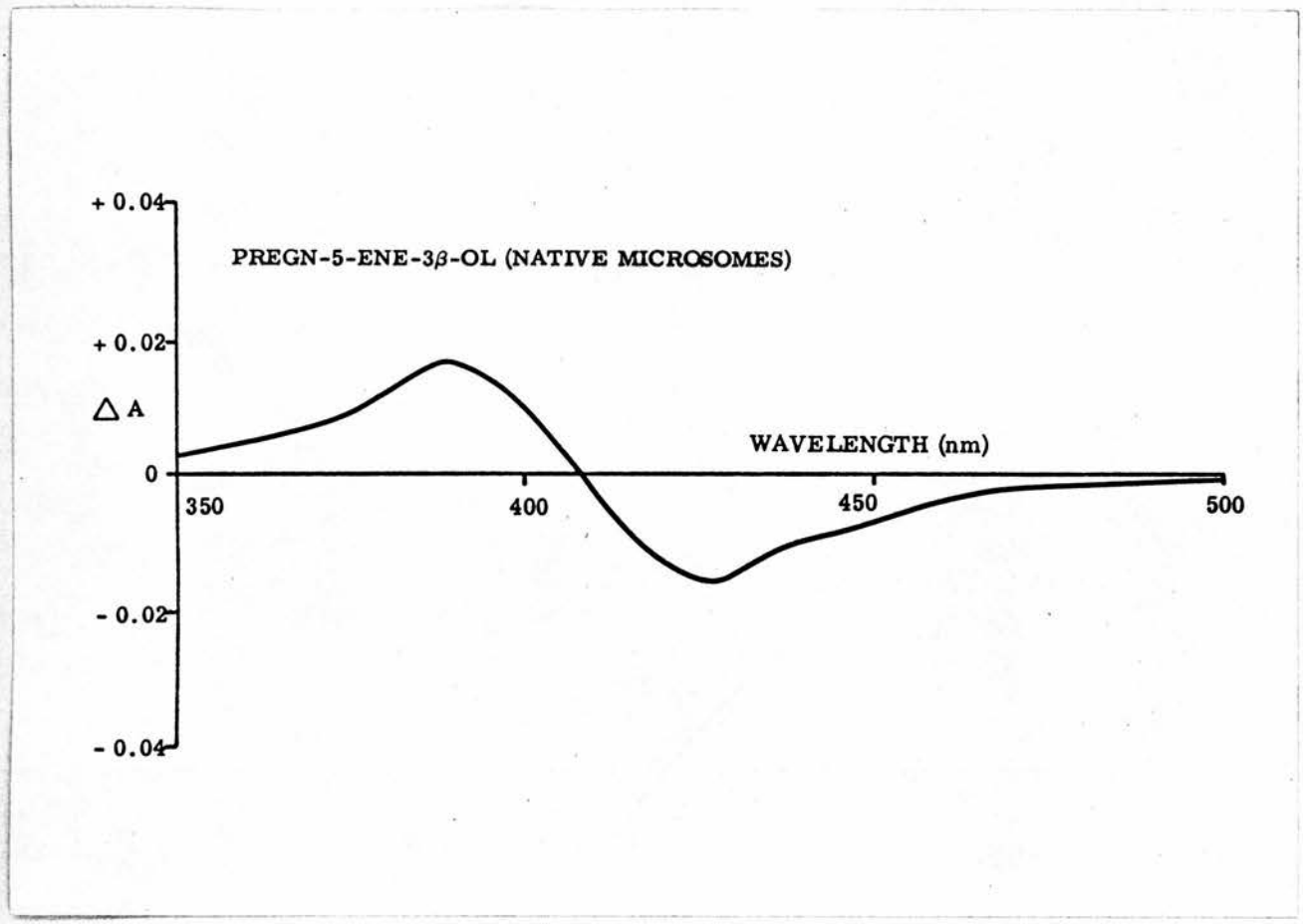


Figure 9,7.

The difference spectrum induced by the addition of pregn-5-ene-3β-ol to rat liver microsomes.

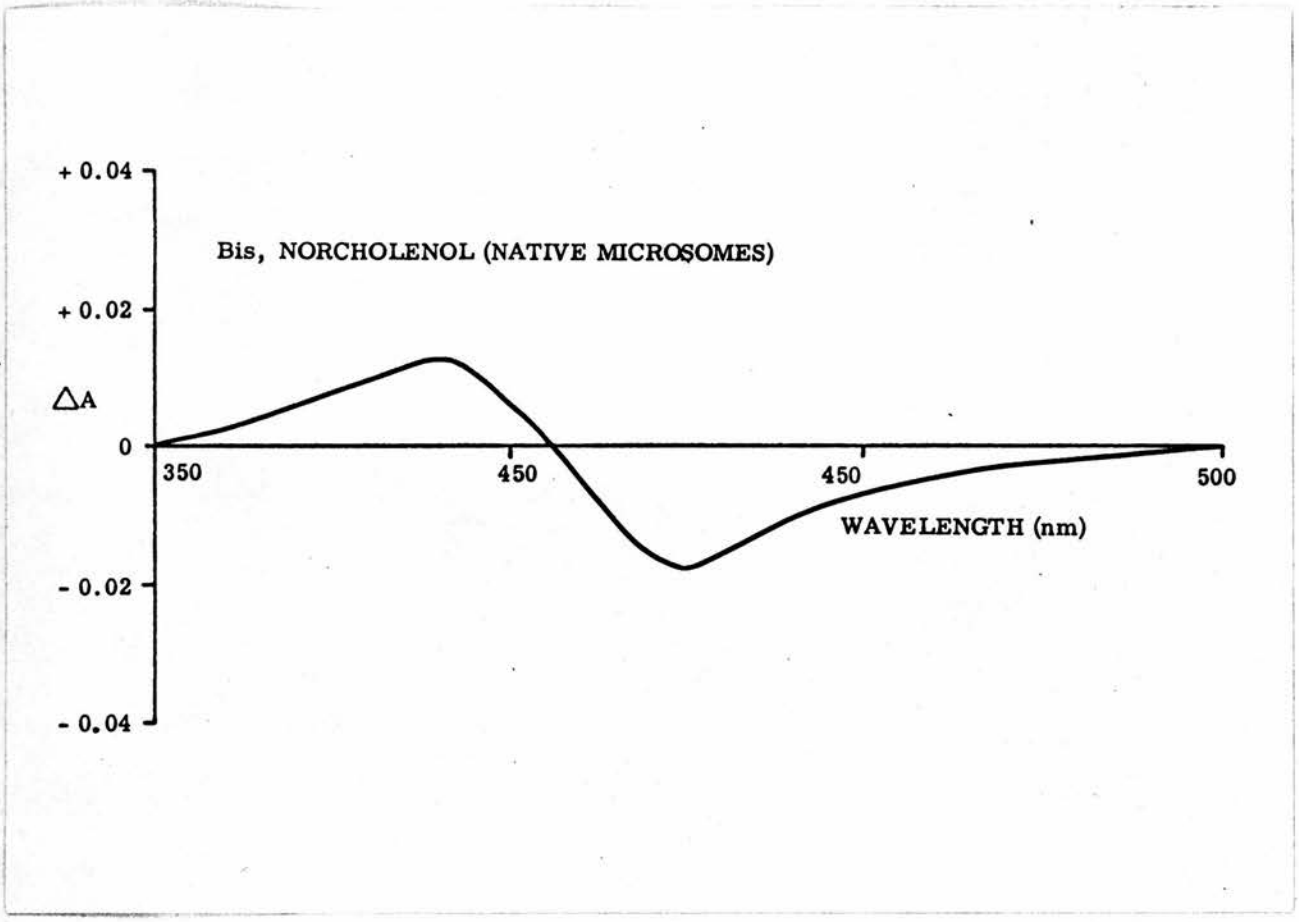


Figure 9,8.

The difference spectrum induced by the addition of bis-norcholenol to rat liver microsomes.

The sterols with the shortest side chain, pregnenol, bisnorcholenol, both produced type I binding spectra, λ max being at 389nm and λmin at 426nm. Spectra for these compounds are shown in Figures 9,7 and 9,8. No such spectra could be observed in the Nonidet P42 solubilized microsomes.

The most interesting feature of the results of this experiment is that difference spectra were only observed when the side chain was shorter than cholenol. It has previously been demonstrated that these compounds when incubated with native microsomes produce several oxidation products in contrast with those compounds which do not induce type I difference spectra, which are either not hydroxylated or are hydroxylated to form only one product.

This is perhaps further evidence that sterols with side chains shorter than cholenol bind to a general pool of cytochrome P-450 which is capable of hydroxylation at many sites. In this respect, such a lack of specificity in the hydroxylation process is similar to that shown by drug hydroxylating cytochrome P-450.

SUMMARY

(1) Cholesterol 7α-hydroxylase has been shown to exhibit a high degree of specificity towards the side chain of cholesterol. This is in contrast with the side chain cleavage enzyme of bovine adrenal cortical mitochondria.

(2) The kinetics of hydroxylation of 26 norcholesterol and cholesterol have been compared, and it has been shown that both the K_m and V_{max} for the hydroxylation of norcholesterol were similarly reduced.

(3) Sterols which were hydroxylated at several sites produced difference spectra on addition to native microsomes in contrast to those sterols which were either not metabolised or hydroxylated to a single product; these did not produce any difference spectra.

SECTION 10

DISCUSSION

The regulation of bile acid synthesis was shown by Bergstrom et al (161) to be dependent at least in part on the concentration of bile acids returning to the liver in the portal blood. The exact mechanism by which bile acids regulate their own synthesis is still obscure since the conditions which affect the rate of bile acid synthesis also affect the rate of synthesis of cholesterol, from which the bile acids are derived. Thus an increase in bile acid synthesis is often associated with an increase in cholesterol synthesis, and it is still not clear whether the increase in cholesterol synthesis precedes, is concomitant with, or follows, the increase in the rate of bile acid synthesis. This relationship is discussed more fully in the Introduction. It is now clear, however, that the rate of synthesis of both cholic and chenodeoxycholic acids is dependent primarily on the activity of the enzyme catalysing the first step in the transformation of cholesterol to bile acids, namely, the cholesterol 7α-hydroxylase. Purification of this enzyme will provide some answers relating to the hydroxylation of cholesterol to 7α-hydroxycholesterol, but would not reveal the mechanism of regulation of bile acid synthesis or cholesterol 7α-hydroxylase activity in the endoplasmic reticulum. However, purification will show the essential components of the cholesterol 7α-hydroxylase complex and whether the concentration of any of these components is increased upon induction of the hydroxylase by breaking the enterohepatic circulation through cannulation of the bile duct or by feeding cholestyramine resin. Also possible would be observation of the effects of modifiers on cholesterol 7α-hydroxylase in the

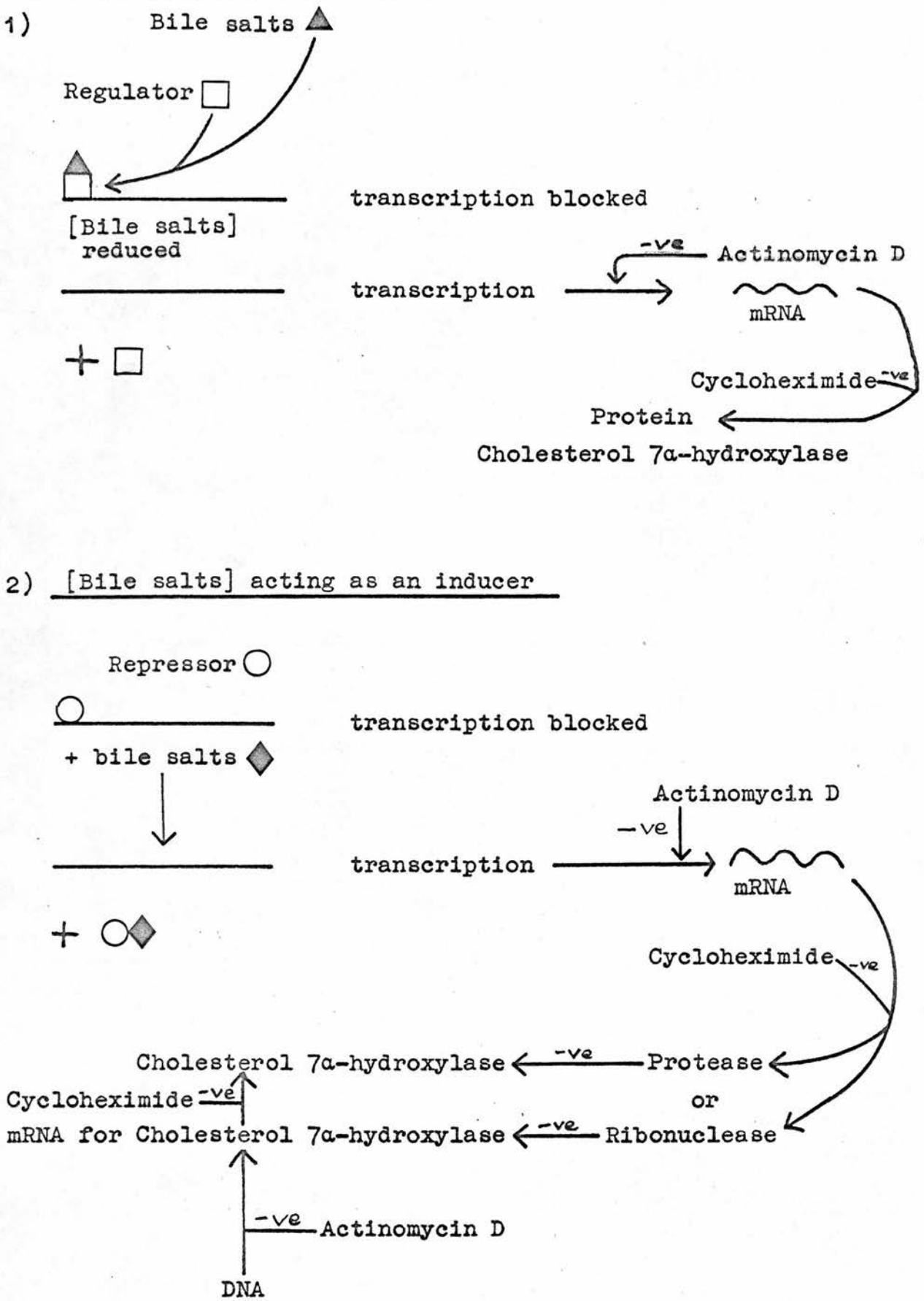


Figure 10,1.

Schemes showing how the concentration of bile salts could regulate the activity of cholesterol 7α-hydroxylase.

absence of complications due to impurities. The mechanism of catalysis and the stoichiometry of the 7α-hydroxylase reaction could be studied unhindered by the multiplicity of reactions in which NADPH and oxygen are involved in native microsomes. Purification of the protein synthesized in response to cholestyramine feeding, and which is responsible for the increase in activity of cholesterol 7α-hydroxylase, would allow the study of the mechanism of induction of this enzyme. This would be of interest not only to students of cholesterol 7α-hydroxylase but also to those interested in hepatic protein synthesis, as cholestyramine feeding is known to induce few proteins other than cholesterol 7α-hydroxylase. It is interesting at this point to speculate how bile acids could regulate the activity of cholesterol 7α-hydroxylase since a direct effect, as discussed in the Introduction, can be ruled out. At least three possibilities exist and these are presented in Figure 10,1.

The first mechanism involves bile acids entering the hepatocyte and combining with a regulator protein in the nucleus to form a complex which binds to the operator of a gene preventing transcription. Reducing the concentration of bile salts would cause dissociation of the regulator from the gene allowing transcription of a messenger RNA specific for a protein necessary for the 7α-hydroxylation of cholesterol.

The second and third possibilities are similar inasmuch as a messenger RNA would be synthesized in response to bile salts combining with a regulator protein; this message would code for either a specific ribonuclease or a protease which would decrease the half-life of either the cholesterol 7α-hydroxylase message or its product. It has not yet been established whether the increase in cholesterol 7α-hydroxylase activity is due to an increased rate of

its synthesis or a decrease in the rate of its degradation, although Gielen et al (18) have concluded that since the half-life of cholesterol 7α-hydroxylase measured from the rate of decay during the diurnal rhythm is less than that in the absence of protein synthesis, (i.e. cycloheximide treated), that an active degradation mechanism, requiring the synthesis of protein, is involved in the regulation of cholesterol 7α-hydroxylase activity.

There is substantial evidence that both RNA synthesis and protein synthesis are necessary for the rise in cholesterol 7α-hydroxylase activity (12,17,18). Proof of any mechanism requires the isolation of the protein necessary for the 7α-hydroxylation of cholesterol.

One of the major problems associated with the study of cholesterol 7α-hydroxylase in native microsomes is the large amount of endogenous substrate. In control rats the amount of cytochrome P-450/mg protein is about 0.3 nmoles, and cholesterol 7α-hydroxylase represents perhaps 1% of this, i.e. 0.003 nmoles/mg protein. The mass of cholesterol/mg protein is approximately 20μg which is about 50 nmoles/mg protein. Thus in control native microsomes, endogenous cholesterol could be present in a 17,000 fold molar excess over cytochrome P-450 involved in its hydroxylation if all the sterol could act as substrate. The method used in this laboratory for the assay of cholesterol 7α-hydroxylase is to add $\left[4 - {}^{14}C\right]$ cholesterol to the microsomes. Balasubramaniam et al (162) have demonstrated that there is compartmentation of cholesterol and that only 70% of the total is available to the enzyme during the course of the assay.

In another paper, Mitropoulos suggested that newly synthesized cholesterol was the preferred substrate for the enzyme (163). This idea of different cholesterol pools is shown in

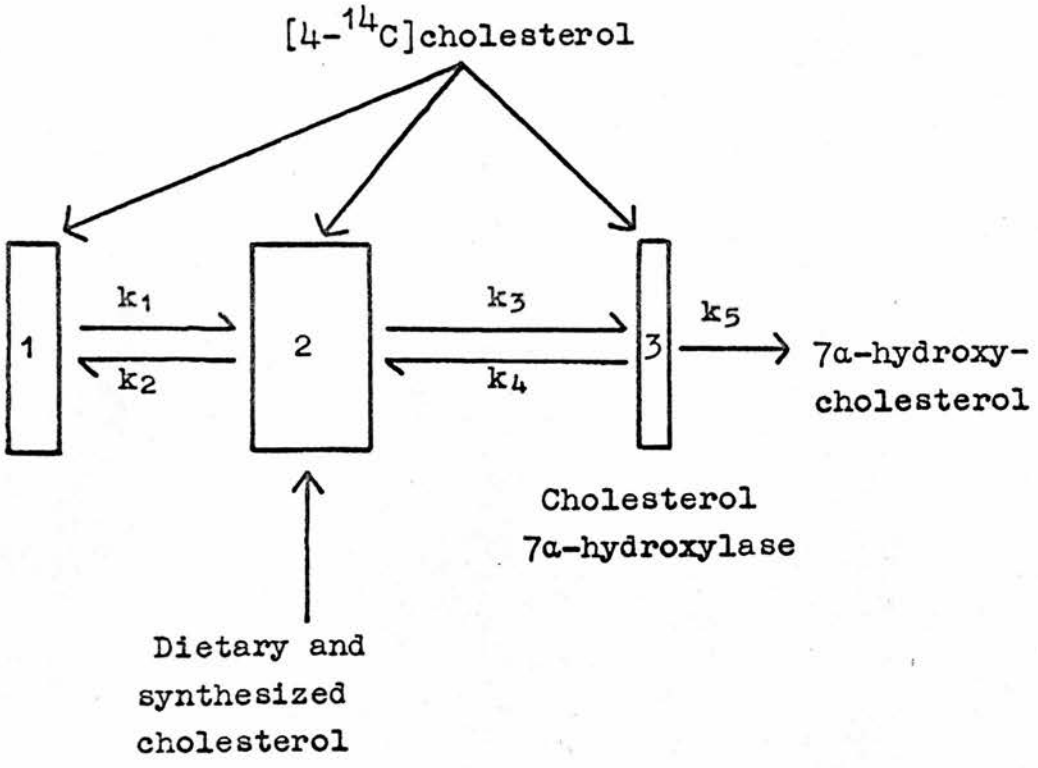


Figure 10,2.

A model to show the compartmentation of cholesterol in rat liver microsomes.

Figure 10,2. It has been stressed by Bjorkhem et al (11) that this compartmentation is of minor consequence when considering the results obtained by measuring the percentage conversion of [4 - ^{14}C] cholesterol to [4 - ^{14}C] 7α-hydroxycholesterol and those obtained by the laborious (and not entirely quantitative) technique of Mitropoulos (141).

During the purification of an enzyme it is desirable, though not always possible, to have available a quantitative assay for the determination of specific activity which may be carried out in minutes. In the case of cholesterol 7α-hydroxylase, the time required for assay is at least 4 hours and this, as Gaylor (93) has pointed out, is a serious handicap. It is clear that a purified system would allow measurement of NADPH oxidation and also oxygen consumption.

Cholesterol 7α-hydroxylase resisted all attempts to release it from the microsomal membrane by mechanical methods, and also by enzymatic techniques, and therefore the technique devised by Lu et al (79) was used. As described earlier, many hydroxylating systems involving cytochrome P-450 have been solubilized by sodium cholate and deoxycholate, and the singular sensitivity of cholesterol 7α-hydroxylase to these bile acids is further evidence that this mixed function oxidase is different from the drug hydroxylating mixed function oxidase. A reconstituted cholesterol 7α-hydroxylase system, solubilized by sodium cholate, has been reported by Bjorkhem et al (86) but this preparation has an activity which is stimulated only four-fold by addition of reductase to the cytochrome P-450 fraction, and the percentage conversion of cholesterol to 7α-hydroxycholesterol was maximally 0.4%. No mention was made of the activity in microsomes

before solubilization. The sensitivity of this enzyme to bile salts, particularly deoxycholate, might only be a property of the enzyme found in rat liver. In man, deoxycholate represents a substantial proportion of the circulating bile acids. This bile acid also appears in the bile duct and gall bladder. This is in contrast to the rat where it has been demonstrated that any deoxycholate returning to the liver is very rapidly converted to cholate by a specific tauro-deoxycholate 7α-hydroxylase. This has led to the suggestion that the rat is particularly sensitive to this powerful detergent (164). In the same way, chenodeoxycholate, which is a strong detergent, is rapidly converted by a 6β- hydroxylation to β muricholic acid, a weak detergent (165,166). Of the other detergents used, only Nonidet P40 solubilized cholesterol 7α-hydroxylase with retention of activity. The trial and error nature of finding a detergent which solubilizes without inhibition is demonstrated by the fact that Triton X-100, p-t-octylphenol polyethyleneglycol condensate (9-10 moles PEG) and to a lesser degree Tween 80, polyethyleneglycol sorbitol mono-oleate (20 moles PEG), both having very similar empirical formulae to Nonidet P40 (polyethyleneglycol, p-t-octylphenol, 9 moles PEG) inhibit strongly the activity of cholesterol 7α-hydroxylase. The use of Nonidet P40 in the solubilization of proteins has not been extensive but has been used in the preparation of polyribosomes (167) and of H2 alloantigens (168) and also in the isolation of protein bodies from developing wheat endosperm (169,171).

In relation to the observed compartmentation of cholesterol in native microsomes by Balasubramaniam et al (162), it is possible that one of the results of solubilization of microsomes by Nonidet P42 is that the pools of sterol which obtain in native microsomes are destroyed, so that radioactive cholesterol equilibrates with a

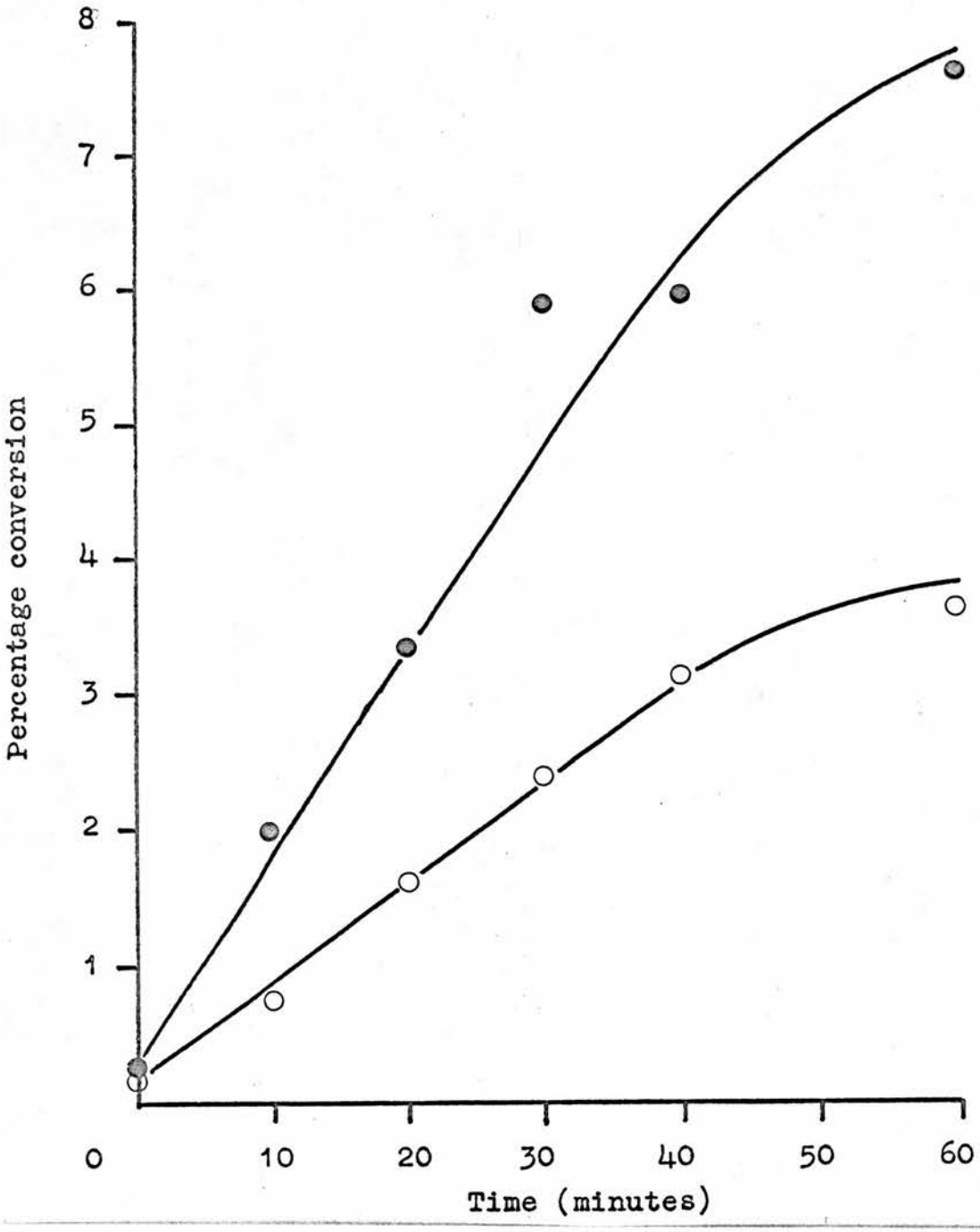


Figure 10,3.

The effect of adding Nonidet P42 (3.5µl/mg protein) to a butanol powder, on cholesterol 7α-hydroxylase activity.

○ Control activity

● Activity with Nonidet P42

homogeneous endogenous cholesterol pool, all of which may be equally accessible to cholesterol 7∝-hydroxylase.

It was observed that when very low concentrations of detergent were added to native microsomes, activation of cholesterol 7∝-hydroxylase occurred, and therefore the rate determining step in the hydroxylation was increased. But at this concentration cytochrome P-450 reductase was inhibited, and it therefore seems unlikely that reduction of cytochrome P-450 is rate limiting in the 7∝-hydroxylation of cholesterol. Nonidet P42 does, however, give a type I binding spectrum at this concentration, and the activation may be associated with this observation. It has been shown that aminopyrine which also binds to cytochrome P-450 to generate a type I difference spectrum activates cholesterol 7∝-hydroxylase by an unknown mechanism (34). Another possibility is that the detergent makes the membrane more fluid, allowing more rapid access of the enzyme to subtrate, and allowing the constituent proteins of the enzyme complex to move more rapidly.

Both acetone and butanol powders were activated when solubilized by the addition of 3.5µl Nonidet P42/mg protein. Figure 10,3 demonstrates that the increase in activity is a result of an increased initial velocity, linear to 40 min, but because the shape of the percentage conversion vs time plot for both insoluble and soluble butanol powders are the same, there is a relationship between the velocity of cholesterol 7∝-hydroxylase and the extent of conversion after 1 hour.

It is possible that during the preparation of an acetone or butanol powder, phospholipid is removed and Nonidet P42 mimics the lipid environment. Schulze et al (170) have observed that if microsomes are treated with phospholipase C or acetone:water, 9:1, loss of phospholipid

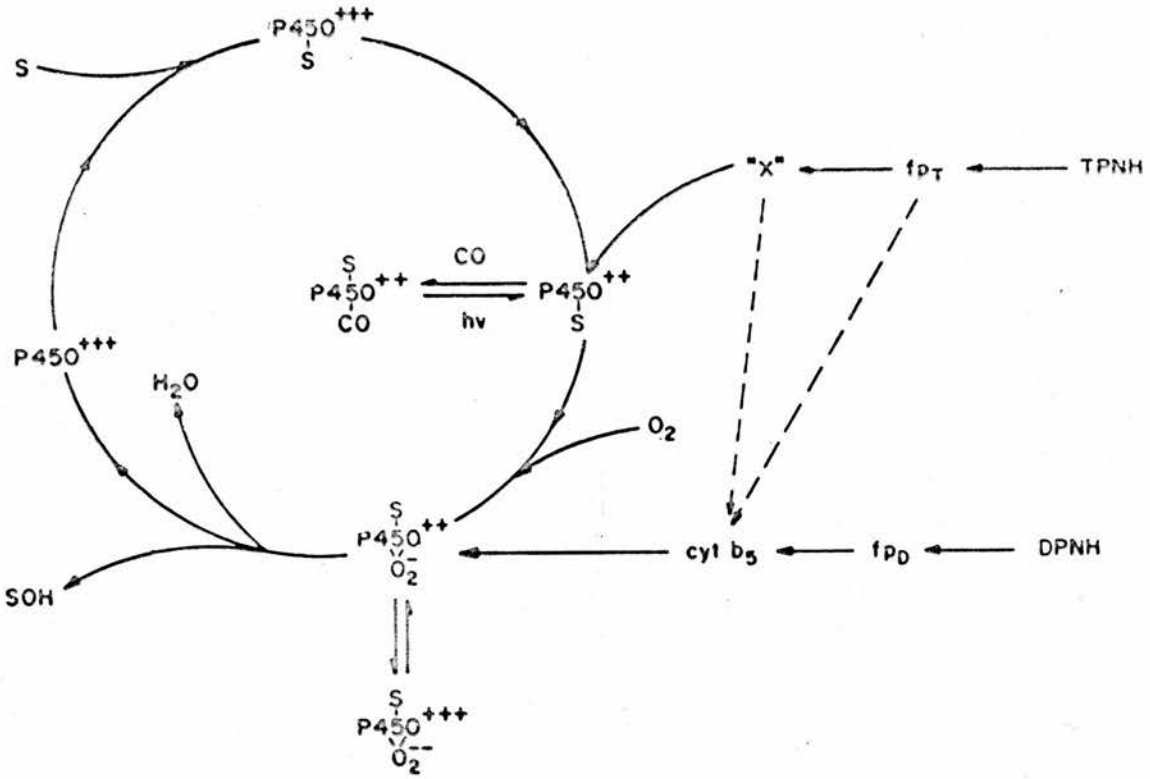
parallels loss of NADH-semidehydroascorbic acid oxidoreductase. This loss of activity could not be reversed by addition of phosphatidyl choline but could be reversed by a variety of detergents, the most active being Triton X-114, an octyl phenol polyethylene oxide (av. 7.5 moles ethylene oxide). It is interesting that knowledge concerning the effects of detergents on biological systems is scant, and no predictions can be made as to how a detergent may react on different enzymes. The activation of cholesterol 7α-hydroxylase by Nonidet P42 while diminishing other associated reactions is not without precedent as it has been observed that Tween 80 stimulates aniline 4-hydroxylation but inhibits biphenyl hydroxylation (172).

A preparation of microsomes treated with Nonidet P42 is soluble as judged by the criterion of prolonged ultracentrifugation. It has been suggested that this criterion does not differentiate between complete (molecular species) and incomplete solubilization. Nevertheless, centrifugation is an extremely useful test of solubilization, and particle size resultant from solubilization is certainly no criterion of the ability to purify a constituent protein to homogeneity. It has been shown by Holtzman (173) that the initial solubilization of microsomes by deoxycholate, a bile salt which has been found by Lu et al (79) to be very useful in the purification of cytochrome P-450, leads to particles, measured by inelastic laser beam scattering, of sizes ranging from 76 nm to 265 nm in diameter, suggesting that the mixed function oxidase components are present as aggregates. Much more important than the size of the particle is the observed behaviour of Nonidet P42 solubilized microsomes on DEAE-cellulose. It is clear that three of the known components of the microsomal mixed function oxidase system are separable from each other, and that none of these fractions has cholesterol 7α-hydroxylase activity.

However, on recombination of the cytochrome P-450 fraction with the NADPH-cytochrome c oxidoreductase fraction, cholesterol 7α-hydroxylase activity was restored. This reinforces the idea that cholesterol 7α-hydroxylase is a mixed function oxidase having as the terminal oxidase cytochrome P-450. Of these two fractions necessary for reconstitution of activity, it was demonstrated that the factor governing specificity resided in the cytochrome P-450 fraction. For this reason, several techniques were employed in an effort to obtain homogeneous cytochrome P-450 so that its capacity to transform cholesterol to 7α-hydroxycholesterol could be tested. The cytochrome P-450 containing fraction, however, proved difficult to purify, but when cytochrome P-450 was purified further, on recombination with NADPH-cytochrome c oxidoreductase, cholesterol 7α-hydroxylase activity could be reconstituted.

Another interesting point arising from this experiment of recombining cytochrome P-450 from liver microsomes of cholate fed and cholestyramine fed rats with flavoprotein was that under the conditions of assay, because the rate of reaction was proportional to cholesterol concentration, the percentage conversions would not be affected by relatively large changes in sterol concentration. That there was a large difference in percentage conversion is further evidence that in cholestyramine fed rats the increase in 7α-hydroxylation rate is dependent on enzyme concentration rather than, as has been suggested, by cholesterol concentration.

Cholesterol 7α-hydroxylase was active under conditions where cytochrome b_5 was undetectable. Furthermore, addition of this haemoprotein inhibited the activity. The role of cytochrome b_5 in hydroxylation reactions is still unresolved, and although it may be involved in the synergistic effect of adding NADH to incubations



b) NADH → Putidaredoxin reductase (flavoprotein) → Putidaredoxin (non-haem iron) → Cytochrome $P\text{-}450_{cam}$

c) NADPH → Adrenoredoxin reductase (flavoprotein) → Adrenoredoxin (non-haem iron) → Cytochrome P-450

Figure 10,4.

Electron transport systems involving cytochrome P-450, in a) rat liver microsomes

b) Pseudomonas putida

c) rat adrenal mitochondria

containing sub-optimal concentrations of NADPH, it appears not to be an obligatory component of cytochrome P-450 dependent reactions. Cytochrome b_5 has been found to be a necessary component of a reconstituted desaturase system (174) for which there is also immunochemical evidence (177), and is thought to play a part in the NADH and NADPH dependent hydroxylation of lauric acid (175). An interesting observation by West et al (176) was that in the NADPH dependent hydroxylation of chlorobenzene by cytochrome P-450, cytochrome b_5 was required for maximal activity; such an involvement was not necessary for maximal activity when cytochrome P-448 was the terminal oxidase. However, it is still not clear whether cytochrome P-450 is a different haemoprotein from cytochrome P-448, the different spectral and catalytic properties may be a reflection of different environments of the haemoprotein. Thus cytochrome b_5 may alter the environment of cytochrome P-450 in the above case without being involved in electron transfer. In the case of cholesterol 7α-hydroxylase, activity could be reconstituted when cytochrome P-450 and NADPH-cytochrome c oxidoreductase, neither of which contained detectable cytochrome b_5, were recombined. Cytochrome b_5, therefore, does not appear to play an obligatory role in this reaction.

One of the unusual features of the liver microsomal mixed function oxidase is the apparent absence of non-haem iron. In the bacterial system of Pseudomonas putida, NADPH transfers electrons to a flavoprotein putidaredoxin reductase. This reductase in turn reduces a non-haem iron putidaredoxin which donates electrons to cytochrome P-450. Mitochondria of the adrenal cortex contain a similar electron transport chain containing the non-haem iron adrenodoxin. These systems are presented in Figure 10,4.

Analysis of total iron in microsomes revealed that if there

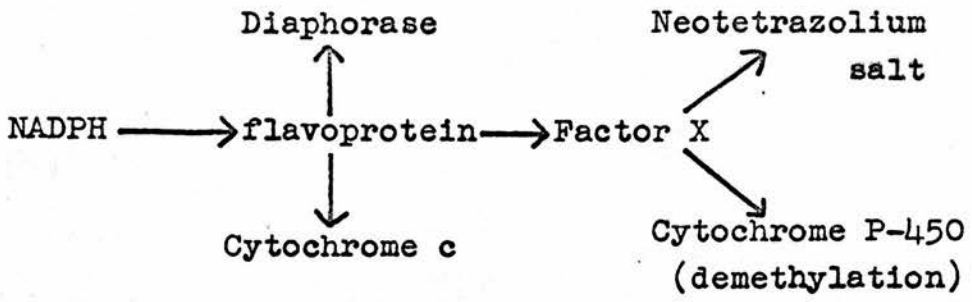
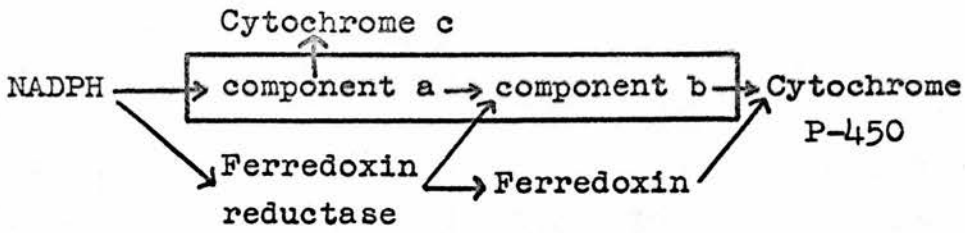


Figure 10,5.

The pathway of electrons to Neotetrazolium chloride and Cytochrome P-450,(after Siekevitz).

was non-haem iron present it would contribute less than 10% of the total iron (178). Non-haem iron is not necessarily a sine qua non, and an intermediate carrier is postulated becase of the large difference in redox potential of about 500 mvolts between flavoprotein and cytochrome P-450. Very recently, Salerno et al (179) have reported the presence of an e.p.r. signal at g = 1.93 and g = 1.88, which they suggest could be manifested by an iron sulphur protein. However, this species has a mid-point potential of -0.27 volts and would not, therefore, seem to satisfy the requirements of an intermediate carrier, Factor X. Factor X was postulated by Siekevitz (180) as he observed in the livers of rats from 18-day foetus to 8 days of age, that NADPH-cytochrome c oxidoreductase and cytochrome P-450 rose to 100% and 80% respectively of the adult level after +7 days, but that neotetrazolium-NADPH oxidoreductase and demethylation rose only to about 30% of the adult level. Thus Factor X would be a rate limiting factor in the electron transport chain to neotetrazolium and cytochrome P-450, as depicted in Figure 10,5. NADPH-cytochrome c oxidoreductase solubilized by trypsin was found not to support the hydroxylation of cholesterol, and Lu et al have observed that this preparation will not reduce cytochrome P-450 (181). In this respect it is interesting that trypsin solubilized NADPH-cytochrome c oxidoreductase will not reduce neotetrazolium salts except in the presence of lecithin (65). Ichihara et al (90) have reported that NADPH-cytochrome c oxidoreductase solubilized by Triton X-100 could support the ω-hydroxylation of lauric acid when recombined with cytochrome P-450. Treatment of this fraction by Sephadex G-100 or with trypsin yielded preparations which were indistinguishable from steapsin or trypsin solubilized NADPH-cytochrome c oxidoreductase. This preparation would not support fatty acid ω-hydroxylation except in the presence of NADPH-ferredoxin reductase.



Component a + component b = NADPH-cytochrome c oxidoreductase solubilized by Triton X-100

Figure 10,6.

The possible transport of electrons from NADPH to cytochrome P-450,(after Ichihara).

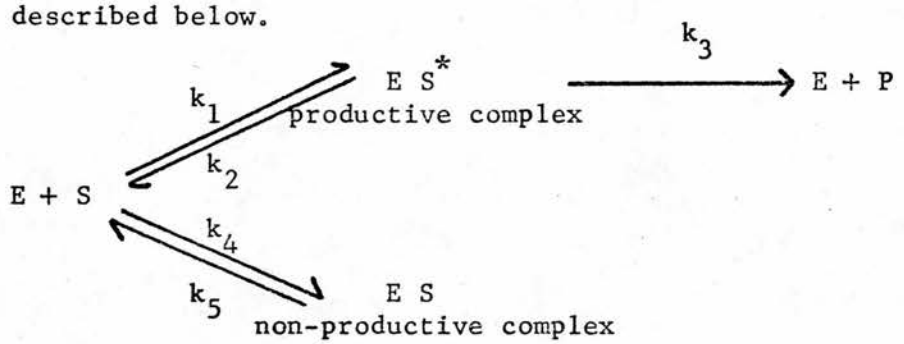
It is possible that the detergent solubilized flavoprotein contains two electron carrier components necessary for the transfer of electrons to cytochrome P-450. Such a scheme is demonstrated in Figure 10,6.

The effect of Nonidet P42 on NADPH-cytochrome P-450 reductase is interesting as it could mean that the rate of reduction of the cytochrome is not the rate determining step in the hydroxylation of cholesterol. The other possibilities are that the reduction of cholesterol specific cytochrome P-450 is so small as to be unobservable, or that another, as yet unidentified, terminal oxidase is involved in the hydroxylation. If the reduction of this haemoprotein is not the rate limiting step, it is interesting to speculate on which step is, in fact, rate determining. Evidence presented by Brown et al (12) was used to support the idea that the formation of the enzyme-cholesterol complex is the slow step in the overall reaction of the 7α-hydroxylation of cholesterol. However, it was observed that the Michaelis-Menten parameters, K_m and V_{max}, for cholesterol and cholesterol 7α-hydroxylase varied in parallel on addition of Nonidet P42 to an acetone powder. The K_m and V_{max} for norcholesterol were also reduced by the same factor when compared with the K_m and V_{max} for cholesterol. This could be interpreted as the Michaelis constant approximating to $\frac{k_3}{k_1}$ in the scheme:-

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\,S \xrightarrow{k_3} E + P$$

Since V_{max} in such a scheme only involves k_3, such a suggestion would mean that k_1 was unaltered even though K_m changed. When a butanol powder was incubated with equimolar masses of cholesterol

and norcholesterol, the latter was hydroxylated to an extent 60% that of the cholesterol hydroxylation. If the mechanism postulated were applicable, such a decrease in velocity would not be accounted for by a decrease in the rate of formation of the enzyme-substrate complex, that is, by a change in k_1. Another consequence of such a mechanism would be that the rate determining step in the hydroxylation of cholesterol and norcholesterol would occur after the formation of the enzyme-substrate complex; such a change in k_3 would also have to account for the remarkable specificity of cholesterol 7α-hydroxylase. Another mechanism which would also lead to parallel changes of K_m and V_{max} is described below.



Assuming

1) $\frac{d\left[E\,S^*\right]}{d\,t} = 0 = k_1\left[E\right]\left[S\right] - (k_2 + k_3)\left[E\,S^*\right]$

2) velocity, $v = k_3\left[E\,S^*\right]$

3) max velocity, $V_{max} = k_3\left[E\right]_{total}$

4) $\left[E\right]_{total} = \left[E\right] + \left[E\,S\right] + \left[E\,S^*\right]$

5) $\frac{d\left[E\,S\right]}{d\,t} = 0 = k_4\left[E\right]\left[S\right] - k_5\left[E\,S\right]$

$$\left[E\,S\right] = \frac{k_4}{k_5}\left[E\right]\left[S\right] = \frac{1}{K_{np}}\left[E\right]\left[S\right]$$

then

$$v = \frac{V_{max}\left[S\right]}{K_m\left(1 + \frac{[S]}{K_{np}}\right) + [S]} = \frac{\frac{V_{max}}{1 + \frac{K_m}{K_{np}}} \cdot [S]}{\frac{K_m}{1 + \frac{K_m}{K_{np}}} + [S]}$$

Thus as $K_{np} \rightarrow \infty$, $v = \dfrac{V_{max}[S]}{K_m + [S]}$

but as the degree of non-productive binding increases, i.e. as K_{np} decreases, both V_{max} and K_m are decreased by the factor $\left(\dfrac{1}{1 + \frac{K_m}{K_{np}}}\right)$.

The suggestion here is that a reduction in V_{max} represents an increase in binding of substrate to the "enzyme" in a position which is not favourable for hydroxylation. Such a decrease would result in a decrease in K_m.

In relation to the rate determining step in hydroxylation reactions, it has been observed by Strobel et al (114) that phospholipid (lecithin) was required for maximal reconstitution activity. It was not found necessary to supplement the reconstituted cholesterol 7α-hydroxylase, solubilized by either cholate (143) or Nonidet P42, and this could be taken as further evidence that the mechanism of the 7α-hydroxylation of cholesterol is different from drug hydroxylation. To obtain more information regarding the absolute requirement of phospholipid, a butanol powder was prepared from rat liver microsomes in which the phospholipid content was reduced to ca.50%. In contrast to the results of Vore (152) no activation of benzphetamine demethylase occurred on addition of phospholipid, neither was there inhibition. Cholesterol 7α-hydroxylase, on the other hand, was apparently inactivated by the addition of phospholipid. Phospholipid has been suggested as being the Factor X between reductase and cytochrome P-450, but this seems unlikely as no readily oxidised or reduced moiety is present in lecithin. It is more likely that phospholipid creates a membrane-like environment for cytochrome P-450 which alters the conformation of this haemoprotein, as Narasimhulu (182) found that on butanol extraction of bovine adrenal microsomes 80% of the

phospholipid was removed, and 90% of the steroid 21 hydroxylation was lost. Addition of phospholipid only partially restored activity, but Triton X-114 fully restored the activity.

Studies on the specificity of cholesterol 7α-hydroxylase demonstrated that this enzyme was very sensitive to changes in the side chain of sterols. The fact that nor-cholesterol, which differs from cholesterol by only a loss of a single methyl group at C25, is 7α-hydroxylated very much less efficiently than cholesterol, and 26 dimethyl norcholesterol even less efficiently suggests that the binding site governing specificity must span a distance of over 2 nm. It is not understood why the liver microsomal cholesterol 7α-hydroxylase should have such stringent requirements for the structure of the side chain, particularly when contrasted with the rat adrenal cortical mitochondrial enzyme which is associated with the hydroxylation of the side chain, the cholesterol side chain cleavage enzyme. Apart from differences in the rate of 7α-hydroxylation, or the rate of formation of a single product, consistent with a 7α-hydroxylation, of the analogues tested it was also observed that when the side chain was short, i.e. bis-norcholenol and smaller, the sterol was multiply oxidised. This increase in the number of products formed could be correlated with the appearance of a type I binding spectrum of these sterols with cytochrome P-450. This perhaps suggests that (a) the mechanism of hydroxylation of the short chain sterol, e.g. pregn-5-ene-3β-ol is different from the hydroxylation of longer chain sterols, e.g. cholesterol, and (b) that a different cytochrome P-450 species is responsible for the 7α-hydroxylation of cholesterol from that species required for the hydroxylation of the short chain sterols.

In the case of pregn-5-ene-3β-ol, it has been shown by Brown (34) that β-mercaptoethylamine inhibits the formation of all

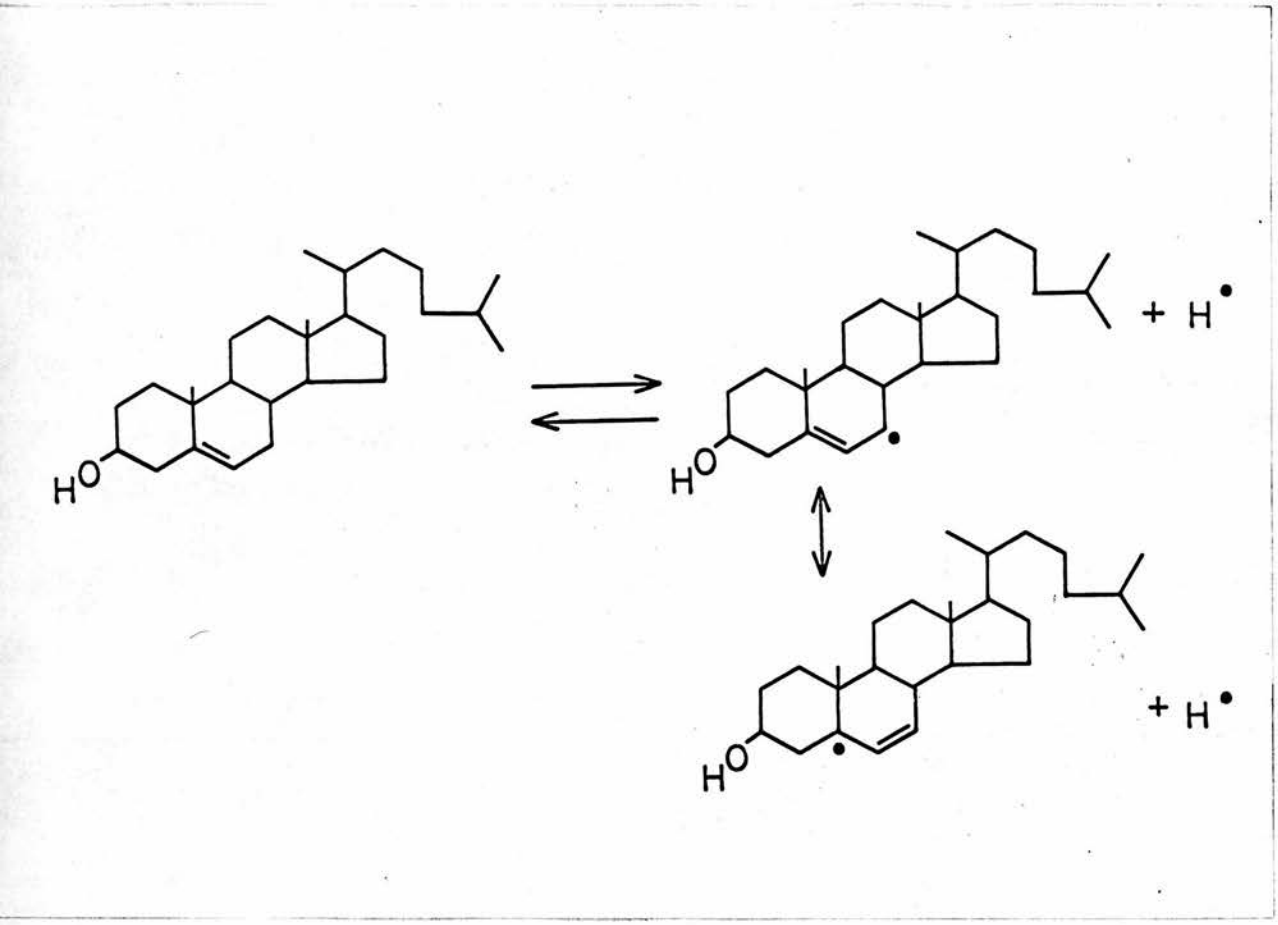


Figure 10,7.

The probable stabilization of a free radical at Carbon atom 7 by the 5 double bond.

products resultant from incubation of this sterol with microsomes. In connection with this observation, it has recently been found that β-mercaptoethylamine abolishes ethylmorphine demethylase activity (183). This amine has been used for several years in this laboratory as it prevents the formation of autoxidation products of cholesterol, as also does addition of the post-microsomal supernatant (184). It has been suggested by Mitton (185) that because the 7 position in cholesterol is allylic, free radical formation at the 7 position could be stabilised as in Figure 10,7. Certainly such autoxidation problems are not observed when cholestanol is used as a substrate for 7α-hydroxylation. It is, therefore, possible that oxidation of the short chain sterols proceeds by a free radical mechanism perhaps different from the 7α-hydroxylation of cholesterol. This might also explain why superoxide dismutase, which inhibits the demethylation of benzphetamine, has no effect on the 7α-hydroxylation of cholesterol. It would be interesting to observe whether superoxide dismutase inhibited the formation of products when pregn-5-ene-3β-ol was incubated with solubilized microsomes.

The distinction between two possible mechanisms by which the specificity of cholesterol 7α-hydroxylase is governed is not yet clear from the combined results of partial purification and substrate specificity. That the enzyme is different from the drug hydroxylation system seems clear, but specificity could be the result of a cholesterol specific cytochrome P-450 or the result of some other specifier protein conferring on a general pool of drug hydroxylating cytochrome P450, characteristics of cholesterol 7α-hydroxylase. It seems likely that the substrate is held in a hydrophobic pocket of the cytochrome P-450, probably by interaction of the 26 and 27 methyl groups of cholesterol to form a productive complex. Such a complex would, in

all probability, require that the 7 position of cholesterol was juxtaposed to an oxygen molecule, activated in some as yet unspecified way by binding to the iron ion, held in the tetrapyrole ring system. The observation that 26 dimethylnorcholesterol and β-sitosterol are very poor substrates for cholesterol 7α-hydroxylase, probably means that the side chains of these sterols are too large to bind to the enzyme to form a productive complex. In the case of norcholesterol and cholenol, it is possible that these sterols are not constrained in a complex which is productive and may be tumbling in the hydrophobic cleft but nevertheless can bind to form non-productive complexes. Desmosterol, on the other hand, by virtue of its two terminal methyl groups, 26 and 27, can be held in a configuration where 7α-hydroxylation can take place and is hydroxylated as readily as cholesterol.

Although no evidence can be produced for the existence of a cholesterol specific cytochrome P-450, a recent paper by Haugen et al (107) demonstrated the existence of four cytochromes P-450 which were induced by phenobarbital or β-naphthoflavone, and which showed different reactivities to substrates such as benzphetamine, p-nitroanisole, testosterone and biphenyl. These proteins have different electrophoretic mobilities and slightly different absorption maxima of the reduced CO - reduced complex. Apart from the possibility that there may be a specific cytochrome P-450 for the 7α-hydroxylation of cholesterol, Alvares and Siekevitz (186) have suggested that cytochrome P-450 exists as a tetramer, only some of these sub-units containing haem, the others binding substrates differentially. It was suggested that upon induction with phenobarbital or methylcholanthrene, the different catalytic activities were a reflection of a different composition of the tetramer by the different sub-units.

The number of compounds which may act as substrates for the rat liver microsomal mixed function oxidase system is vast. Apart from the large number of foreign compounds which may be hydroxylated, naturally occurring compounds such as sterols are extenisvely oxidised. Probably only the quaternary carbon atoms C 10 and C 13 of the sterol nucleus may not be hydroxylated by cytochrome P-450. To postulate a specific cytochrome P-450 for each substrate seems unattractive in terms of both the energy required for their synthesis and also the number of cistrons required for their coding. For this reason it is more attractive to envisage a general pool of a few species of cytochromes P-450 which have the capacity to support the hydroxylation of the great number of substrates which differ markedly in their structure. In this sense, the enzyme is envisaged as being non-specific. Any apparent specificity observed would not be due to the enzyme binding preferentially a particular substrate with the exclusion of other substrates of different structure, but due to the substrate in a large hydrophobic cleft assuming a conformation which is most thermodynamically favourable for hydroxylation. Thus aniline would perhaps be hydroxylated in the para position because the amine activates this position by donating electrons to the aromatic ring.

There seems little doubt that the 7α-hydroxylation of cholesterol is catalysed by a cytochrome P-450 since the reaction is inhibited by carbon monoxide, an inhibition optimally relieved by light of wavelength 450 nm, but many observations lead to the conclusion that this cytochrome P-450 is different from the bulk of this haemoprotein. Cholesterol 7α-hydroxylase, in contrast to the drug hydroxylases, is not induced by phenobarbital or 3-methylcholanthrene, but is stimulated by biliary drainage or treatment with cholestyramine resin. The enzyme is also more sensitive to detergents

and ionic strength than other drug hydroxylating enzymes.

The half-life of cholesterol 7α-hydroxylase of about 3 hours is probably necessary as the enzyme is immersed in a membrane containing its substrate cholesterol, and possibly 70% of this sterol is available to the enzyme as substrate. It is likely, therefore, that a rapidly turning over enzyme is required to control quickly the formation of bile acids and maintain the integrity of the microsomal membrane.

The rate of formation of 7α-hydroxycholesterol from cholesterol is about two orders of magnitude lower than the rate observed for the demethylation of aminopyrine (12), but the energy of activation for both hydroxylations is comparable. This, perhaps, is evidence that there is present in microsomes a cytochrome P-450, specific for cholesterol whose concentration is only 1% that of the bulk cytochrome P-450. The other possibility is that another protein is necessary to donate cholesterol to a general pool of cytochrome P-450 for specific 7α-hydroxylation. Whether such a specifier protein or a specific cytochrome P-450 is postulated, it is now known that the structural requirements for the smooth 7α-hydroxylation of substrate are stringent, in contrast to the drug hydroxylations. The idea of a specifier protein is not as appealing as a specific cytochrome P-450, as one might expect cholesterol in the absence of a specifier protein to be extensively oxidised. That this is not the case might suggest that the binding site of the drug hydroxylating cytochrome P-450 is not large enough to accommodate cholesterol, which has a bulky side chain, an important feature in the determination of substrate specificity. Certainly as the length of the side chain is reduced, hydroxylation more typical of the drug hydroxylation system takes place. However, these two possibilities by which specificity can be governed can only be distinguished by purification of cytochrome P-450 to homogeneity.

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SOLUBILIZATION OF RAT LIVER MICROSOMAL CHOLESTEROL 7 α-HYDROXYLASE

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1. Introduction

The 7 α-hydroxylation of cholesterol is considered to be the first and rate limiting step in the transformation of cholesterol to bile acids [1,2]. Cholesterol 7 α-hydroxylase (EC 1.14) is located in the microsomal fraction of rat liver and requires NADPH, molecular oxygen, and an electron transport system involving cytochrome *P*-450 and NADPH-cytochrome *c* oxido-reductase [3–7]. When the concentration of bile acids in the enterohepatic circulation is lowered, either by cannulation of the bile duct or by feeding rats a bile acid sequestrant, the specific content of cytochrome *P*-450 remains constant, but the specific activity of cholesterol 7α-hydroxylase is increased 3-fold [1,3]. Cholesterol 7α-hydroxylase appears to be more sensitive to detergents and salts than other cytochrome *P*-450 dependent reactions [8]. It is therefore likely that the bulk of the liver microsomal cytochrome *P*-450 is not associated with cholesterol 7α-hydroxylase activity. To study this enzyme in greater detail, it is first necessary to release it from the microsomal membrane. The mixed function oxidase system from rat liver microsomes has previously been solubilized and fractionated [9], but these methods rely on the detergency of sodium cholate or sodium deoxycholate. Since these detergents are both strong inhibitors of cholesterol 7α-hydroxylase it was necessary to explore other methods for the solubilization of this mixed function oxidase.

2. Materials and methods

Liver microsomes from rats fed a diet containing 4% w/w cholestyramine (a bile acid sequestering agent) were prepared in the usual way [10]. Microsomes from four livers (total 32 g wet weight) were resuspended in 0.154 M KCl to give a final volume of 7 ml. An aliguot of this suspension was added dropwise to 100 vol of stirred acetone cooled to approx. −30°C. The acetone was immediately filtered through a Buchner funnel, and the powder washed with diethyl ether then acetone, both cooled to −30°C. The powder was kept in vacuo at room temperature for one hour, then stored at −20°C. Cholesterol 7α-hydroxylase activity was retained for several weeks.

Cholesterol 7α-hydroxylase activity was assayed as described previously [10].

Cytochrome *P*-450 and cytochrome b_5 were measured according to the method of Omura and Sato [11].

NADPH-cytochrome *c* oxidoreductase activity was assayed by adding an aliquot of the sample to 2.8 ml 0.1 M potassium phosphate buffer pH 7.55, 1 mg cytochrome *c*, 100 μl NADPH generator (0.5 μmol $NADP^+$, 5 μmol glucose 6-phosphate and 0.5 IU glucose 6-phosphate dehydrogenase). The difference in absorbance between 551 nm and 540 nm was measured.

Protein was determined by the biuret method [12]. Cholesterol was measured by gas-liquid chromatography on a 1% SE30 column using pregnenolone acetate as the internal standard.

Nonidet P40 was obtained from BDH Chemicals Ltd., Poole, England. This surface active agent is an octyl-phenol-ethylene-oxide condensate, (average nine mol ethylene oxide). Other reagents used were

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the highest grade commerically avaible.

For solubilization, the acetone powder or microsomal suspension in 0.1 M phosphate buffer pH 7.6 was cooled to 0–4°C. After addition of Nonidet P42 detergent, the suspension was kept on ice for 20 min, stirring occasionally.

3. Results

An acetone powder was used to test the ability of enzymes and surface active agents to solubilize cholesterol 7α-hydroxylase. The results of these tests are summarized in table 1. Although most detergents

Table 1
Effect of solubilizing agents on the activity of cholesterol 7α-hydroxylase in rat liver microsomal acetone powder

Conditions	Suspension	Supernatant	Pellet
Sodium cholate 1.5 mg/mg protein	70% inhibition	70% inhibition	80% inhibition
Sodium deoxycholate 1.5 mg/mg protein	inactive	inactive	inactive
Phospholipase A (from *Crotalus adamanteus* venom, 4 μU/mg acetone powder)	–	inactive	some loss of activity
3 M Urea	inactive	inactive	inactive
8% Butanol	inactive	inactive	inactive
Pancreatin 5 mg/60 mg acetone powder	–	inactive	–
Naja naja venom; 5 μg and 1 mg/40 mg acetone powder	active	inactive	active
0.1% and 1% Lubrol WX	90% inhibition	inactive	inactive
0.1% Lubrol W	90% inhibition	inactive	inactive
0.1% Triton X-100	inactive	–	–
1% Digitonin	inactive	inactive	inactive
0.1% and 2% cetyl trimethylammonium bromide	inactive	–	–
0.1% and 2% Saponin	inactive	–	–
0.1% Tween 80	inhibition	inhibition	inactive
0.1% Nonidet P40	active	active	loss of activity
0.1% Nonidet P42	active	active	loss of activity

Acetone powder (approx. 50 mg.) was suspended in 12 ml 0.1 M potassium phoshate buffer pH 7.7 and treated with the solubilizing agents shown in the table. Cholesterol 7α-hydroxylase activity was measured as described previouslv [10].

solubilized cytochrome *P*-450, all but Nonidet P42 led to substantial inhibition of cholesterol 7α-hydroxylase. The surfactant Nonidet P42 (a 27% solution of Nonidet P40 in water) solubilized the components of the microsomal mixed function oxidase, and the resultant supernatant after centrifugation at 100 000 *g* for 1 hr still retained cholesterol 7α-hydroxylase activity.

3.1. *Effect of concentration of Nonidet P42 on the solubilization of acetone powder*

To determine the optimal conditions for solubilization of cholesterol 7α-hydroxylase, an acetone powder was suspended in 0.1 M phosphate buffer pH 7.6 at 0°C and increasing amounts of Nonidet P42 added. The percentage solubilization of protein, cytochrome *P*-450, cytochrome b_5, NADPH-cytochrome *c* reductase, and the activity of cholesterol 7α-hydroxylase is plotted against Nonidet P42 concentration in figs.1 and 2. From these graphs it

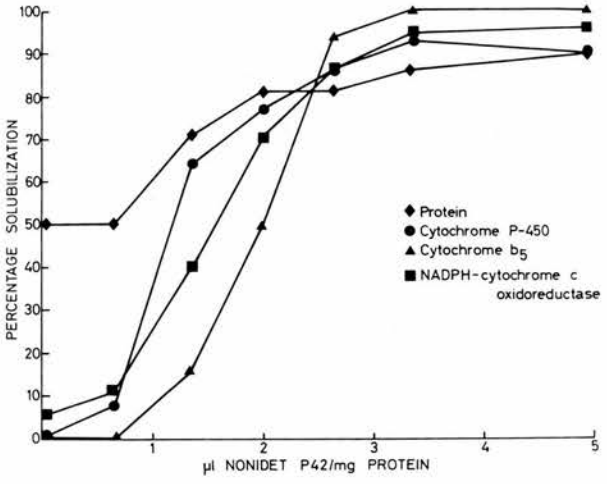

Fig.1. 30 mg liver microsomal acetone powder (15 mg protein) were suspended in 10 ml of 0.1 M phosphate buffer, 1 mM EDTA pH 7.55, and solubilized, as described in the text, with increasing quantities of Nonidet P42. After centrifugation at 105 000 *g* for 1 hr, the pellets were resuspended in 10 ml of buffer and the suspensions and supernatants assayed for protein, cytochrome *P*-450, cytochrome b_5, and NADPH cytochrome *c* oxidoreductase. Percentage solubilization is the ratio

$$\frac{\text{supernatant assay}}{\text{supernatant assay + pellet assay}} \times 100$$

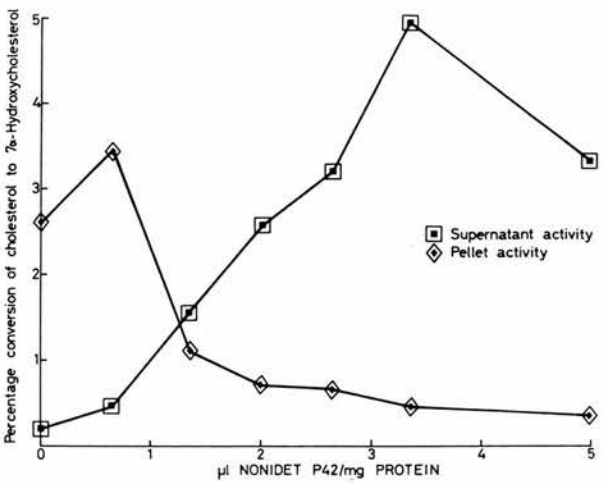

Fig.2. Experimental details were as in fig.1. Cholesterol 7α-hydroxylase activity was determined in the supernatants and resuspended pellets and expressed as the percentage conversion of [4-^{14}C] cholesterol to [4-^{14}C] 7α-hydroxycholesterol.

may be seen that the gradual release of cholesterol 7α-hydroxylase into solution follows the progress curve of solubilization of the mixed function oxidase components. Nonidet P42 solubilizes effectively over the concentration range 2–6 µl Nonidet P42/mg protein, but there is a narrow concentration range where substantial activation of cholesterol 7α-hydroxylase occurs.

3.2. *Criterion of solubility*

A solubilized rat liver microsomal acetone powder was centrifuged at 100 000 *g* for 7 hr, and after this period, results showed that approximately 75% of the enzyme activity still remained in the supernatant fraction. Since the enzyme may have lipoprotein characteristics, the specific gravity of the supernatant was determined and found to be 1.014.

3.3. *Effect of Nonidet P42 on native microsomes*

The applicability of the method used on a microsomal acetone powder to solubilization of native microsomes was studied using rat liver microsomes. A gradual release of mono-oxygenase components into solution on increasing the concentration of Nonidet P42 was again observed, and at a concentration of 4 µl Nonidet P42 × (mg protein)$^{-1}$ over 90% of

these components were solubilized. Cholesterol 7α-hydroxylase was measured as a function of Nonidet P42 concentration and results showed an activation of the enzyme at low concentration 0.3 μl Nonidet P42 × (mg protein)$^{-1}$.

Increasing detergent concentration caused a loss of activity in the pellet and a concomitant increase in cholesterol 7α-hydroxylase activity in the supernatant after centrifugation. Maximum cholesterol 7α-hydroxylase activity was observed in the supernatant fraction when 4 μl Nonidet P42 × (mg protein)$^{-1}$ was used for solubilization.

4. Discussion

Microsomes are a heterogeneous and complex mixture and study of the components necessitates solubilization. Cytochrome *P*-450 has proved to be difficult to resolve and where enzymic activity is to be studied, gentle solubilization procedures with minimal loss of activity are usually necessary prerequisites. Sodium deoxycholate holds the most prominent position in solubilization of microsomes as evidenced by the successful solubilization and reconstitution of several cytochrome *P*-450 dependent reactions, based on the method originally devised by Lu et al. [13]. Non-ionic detergents have been used by Miyake, [14] and more recently by Sato [15], Imai and Sato [16] and Ichihara et al. [17]. Because of the considerable inhibition of cholesterol 7α-hydroxylase by sodium deoxycholate, trial and error use of other surface active agents was employed, and it was found that although many detergents were capable of solubilizing microsomes, most inhibited or inactivated cholesterol 7α-hydroxylase even when the cytochrome *P*-450 content was not altered. Nonidet P42 solubilized both microsomal acetone powder and native microsomes with retention of cholesterol 7α-hydroxylase activity. The apparent activity of cholesterol 7α-hydroxylase solubilized from a liver microsomal acetone powder is at least as great as the control microsomal acetone powder with no added detergent and in most cases, at a concentration of approx. 3.5 μl Nonidet P42/mg protein, there is a very substantial activation. The reason for this activation is not understood.

With this preparation, resolution of the soluble cholesterol 7α-hydroxylase is now in progress in this laboratory.

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STUDIES ON THE SPECIFICITY OF THE RAT LIVER MICROSOMAL CHOLESTEROL 7α-HYDROXYLASE

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SUMMARY

The hydroxylation of several cholesterol analogues by rat liver microsomal cholesterol 7α-hydroxylase has been studied. Δ5-Pregnen-3β-ol is hydroxylated to several major products none of which is consistent with 7α-hydroxylation. β-Sitosterol (24α-ethyl-Δ5-cholesten-3β-ol) is not hydroxylated to any significant degree. 26-Norcholesterol (26-nor-Δ5-cholesten-3β-ol) is hydroxylated specifically in the 7α-position but at a lower rate than cholesterol. These results suggest that the sterol 7α-hydroxylation enzyme has a specific apolar binding site for the side-chain of cholesterol.

INTRODUCTION

Recent work in this [1] and other laboratories [2, 3] has suggested on the basis of several criteria that the cholesterol 7α-hydroxylase system of rat liver is distinct from the drug hydroxylation system which is also located in the microsomal fraction of rat liver. These studies have shown that the cholesterol 7α-hydroxylase has a much more specific substrate requirement compared to the drug hydroxylation system which will hydroxylate a wide range of substrates of very different chemical structure. Here we report further studies which suggest that the substrate requirement of cholesterol 7α-hydroxylase is very much more precise than had previously been expected.

MATERIALS AND METHODS

[4-^{14}C]Cholesterol (Δ5-cholesten-3β-ol) was obtained from the Radiochemical Centre (Amersham) and was diluted to the required specific activity. β-[22,23-$^{3}H_2$]-Sitosterol (24α-ethyl-Δ5-cholesten-3β-ol) was a product of NEN Chemicals GmbH (Frankfurt/Main). 26-[25-^{3}H]Norcholesterol (26-nor-Δ5-cholesten-3β-ol) and Δ5-

Abbreviations: Cholesterol, Δ5-cholesten-3β-ol. Norcholesterol, 26-nor-Δ5-cholesten-3β-ol. epiandrosterol, 24α-ethyl-Δ5-cholesten-3β-ol. Pregnenolone, 20-oxo-Δ5-pregnen-3β-ol. Dehydro-β-Sito-stenone, 19-oxo-Δ5-androsten-3β-ol.

[20-^{3}H]pregnen-3β-ol were synthesised from the corresponding 25- and 20-oxo compounds, respectively, by methods which will be described elsewhere. The appropriate oxo-steroid was reduced with NaB^3H_4 and the resulting alcohol converted to the corresponding tosylate which was reduced with $LiAlH_4$ to afford the required steroid. Cholestyramine (Cuemid) was a product of Merck, Sharpe and Dohme. NADP, glucose 6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim GmbH (Mannheim). β-Mercaptoethylamine was obtained from British Drug Houses Ltd (Poole). 7α-Hydroxycholesterol and 7α-hydroxy-26-norcholesterol were synthesised from cholesterol and norcholesterol, respectively, by a photo-oxygenation procedure [4]. Steroids obtained in sufficient quantity were characterised by infrared and NMR spectroscopy, by mass spectrometry and by preparation of their benzoate derivatives. The purity of all compounds was monitored by thin-layer chromatography, and radiochemical purity was established by thin-layer chromatography coupled with a thin-layer chromatographic scanner and crystallisation to constant specific activity.

Rats were treated as described previously [1] and the liver microsomal preparation obtained as previously described [5].

Incubations contained the substrate under examination added in solution in 50 μl acetone [5] and β-mercaptoethylamine (10 mM) was included in all incubations.

In the dilution experiment (Table II) 15 mg each of cholesterol and 7α-hydroxycholesterol, norcholesterol and 7α-hydroxy-norcholesterol were added to the appropriate incubation mixtures after the ending of the incubation in solution in methanol. The steroids were extracted as before [5] and were separated by thin-layer chromatography on plates 1-mm thick (Kieselgel H, Merck, developing with benzene–ethyl acetate, 7:13, v/v). After elution of the compounds from the appropriate bands with ethyl acetate and evaporation of the solvent the steroids were recrystallised to constant specific activity from methanol. The benzoates of the steroids were also prepared (benzoyl chloride–pyridine) and the derivatives recrystallised to constant specific activity from methanol–ether.

RESULTS

Previous studies [1–3] have investigated the action of rat liver microsomes on 25-hydroxycholesterol, 26-hydroxycholesterol, pregnenolone (20-oxo-Δ5-pregnen-3β-ol) and Δ5-pregnen-3β-ol. We have further examined the oxidation of Δ5-pregnen-3β-ol and in addition that of β-sitosterol and 26-norcholesterol. The results are given in Table I.

The table shows a clear difference in the treatment of the test substrates. Experiment 1 shows that, as observed before [1], Δ5-pregnen-3β-ol is substantially oxidised to give several products, none of which has characteristics which suggest that it might be the product of 7α-hydroxylation. In Experiment 2 very little oxidation took place and it was not possible to detect any significant product by thin-layer chromatography. The product of Experiment 3 was unambiguously identified as 7α-hydroxy-26-norcholesterol by addition of the appropriate steroids to the incubation mixtures after incubation followed by isolation and recrystallisation to constant specific activity of both the steroids and of their benzoate derivatives. The specific activities obtained are given in Table II.

TABLE I

Incubations contained a microsomal preparation from 1 g of liver, 0.1 M potassium phosphate, pH 7.4, NADP (5 μmoles), glucose 6-phosphate (50 μmoles), glucose-6-phosphate dehydrogenase (1 unit), and β-mercaptoethylamine (10 mM) in a total volume of 7 ml. The substrate was added in solution in acetone (50 μl). Incubations were carried out in air for 1 h at 37 °C in a shaking water bath.

Expt	Steroid	Activity (dpm/mg)	Weight	Percentage hydroxyl-* ation with respect to control	Products
1	Δ5-Pregnen-3-βol	$5 \cdot 10^7$	50 μg	54	Several products
2	β-Sitosterol	$1.7 \cdot 10^8$	6 ng	<0.005	Not detectable
3	26-Norcholesterol**	$3.8 \cdot 10^7$	50 μg	0.56	7α-Hydroxy-26-nor-cholesterol

* (Percentage total products of oxidation/percentage 7α-hydroxycholesterol in control) × 100. In Expts 1 and 3 the specific activity of the [4-^{14}C]cholesterol in the control incubation was adjusted to the same level as that of the test substrates, and each incubation contained 50 μg of substrate.

** m.p. 127–129 °C. Mass spectrum *m/e* 372 (molecular ion).

TABLE II

SPECIFIC ACTIVITIES OF STEROIDS AND THEIR BENZOATE DERIVATIVES

	Spec. act. (dpm/mmole × 10^6)	
	Steroid	Derivative
Cholesterol	15.9 ± 0.4	16.3 ± 0.4
7α-Hydroxycholesterol	0.28 ± 0.01	0.30 ± 0.02
Norcholesterol	64.0 ± 3.6	62.5 ± 1.9
7α-Hydroxynorcholesterol	0.62 ± 0.05	0.67 ± 0.02

DISCUSSION

The present work together with the work cited above [1–3] surveys the mode of oxidation by rat liver microsomes on compounds which retain the Δ5, 3β-hydroxy steroid nucleus and in which the side-chain is varied in length and polarity. It is clear from the previous work that steroids with polar side chains are not good substrates for cholesterol 7α-hydroxylase. Thus pregnenolone, dehydroepiandrostenone (19-oxo-Δ5-androsten-3β-ol), 25-hydroxycholesterol and 26-hydroxycholesterol were not hydroxylated by rat liver microsomes to give uniquely a 7α-hydroxylated product. This is consistent with the cholesterol 7α-hydroxylase system having a side-chain binding site which has apolar character and which cannot interact strongly with a polar side-chain.

The results in Table I suggest that this apparent specificity is extended to apolar side-chains. Steroids with short side-chains (Expt 1, [1, 2]), Δ5-pregnen-3β-ol and Δ5-androsten-3β-ol, are not hydroxylated to products which are consistent with attack by cholesterol 7α-hydroxylase.

With steroids which have bulky side-chains the rate of oxidation is too slow to be detectable under our standard assay conditions (Expt 2). Our findings with β-sitosterol in agreement with those of Aringer and Eneroth [6] who have carried out similar experiments, and are consistent with those of Subbiah and Kuksis [7, 8] who showed that the rate of metabolism of β-sitosterol in whole animals is very much lower than that of cholesterol. It may be that the slow rate of metabolism of β-sitosterol in

intact rats is due to the great stability that β-sitosterol shows to oxidation by rat liver microsomal cholesterol 7α-hydroxylase. β-Sitosterol can be photo-oxygenated as readily as cholesterol showing that chemically the bulky side-chain has no effect, steric or otherwise, on the reactivity at C_7.

From Experiments 1 and 2 we may predict that for optimum binding to this enzyme a side-chain which is approximately of the same length as that of cholesterol and not containing any bulky branching groups is required. The removal of one carbon from the C_8 side-chain of cholesterol might be expected to be not too great an alteration and we might expect 26-norcholesterol to be smoothly hydroxylated in the 7α-position by the microsomal preparation.

Table II shows that this is indeed the case, but, as can be seen from Table I, 26-norcholesterol is hydroxylated with only about half the efficiency of cholesterol. A substrate with a side-chain shorter than the natural substrate by only one carbon is hydroxylated with reduced efficiency and this was the only substrate tested which was oxidised to give solely the 7α-hydroxy product.

These results suggest that the cholesterol 7α-hydroxylase of rat liver microsomes has a specific apolar binding site for the side-chain of cholesterol. This binding may be required for the orientation of the substrate on the enzyme to allow the hydroxylation to occur stereospecifically at the 7α-position [9]. It is interesting to note that the mitochondrial cholesterol side-chain cleavage system is not similarly sensitive to the side-chain of cholesterol [10, 11] (Arthur, J., Boyd, G. S. and Suckling, K. E., unpublished).

We are further investigating the cholesterol 7α-hydroxylase system and related enzymes in order to establish in more detail the nature and significance of the substrate binding sites.

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