ASPECTS OF THE CATABOLISM OF CHOLESTEROL TO BILE ACIDS IN MAMMALS

by

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SUMMARY

(1) A cholesterol-7α-hydroxylase has been investigated in rat liver cell fractions; the enzyme is located in the endoplasmic reticulum (microsomes) and requires co-factors in the cytoplasm.

(2) The enzyme was assayed by following the metabolism of cholesterol-4-^{14}C; analysis was effected by thin layer chromatography followed by liquid scintillation counting.

(3) Cholesterol-7α-hydroxylase was found to be sensitive to prolonged homogenisation of the liver, long incubation periods, etc.; stimulation of the activity was observed only in the presence of NADPH.

(4) Preliminary studies showed that the enzyme was inhibited by carbon monoxide; it appeared that a carbon monoxide binding pigment may be involved in oxygen activation for the system. The enzyme is suggested to be a mixed function oxidase.

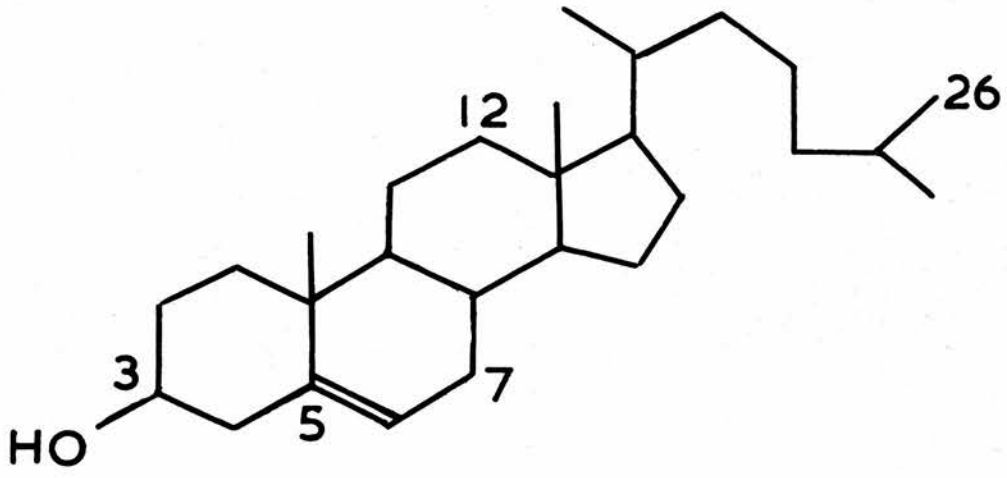
(5) The conversion of cholesterol to 7α-hydroxycholesterol can be increased several fold by preventing the reabsorption of bile salts from the gut; the significance of this enzyme as a rate-controlling enzyme in the overall catabolism to bile acids is discussed.

(6) Non-enzymic oxidation of cholesterol has been investigated in some detail in order to determine whether enzymic and non-enzymic cholesterol oxidation have any common characteristics. Evidence is presented to suggest that cholesterol can be oxidised in conditions which support peroxidation of unsaturated lipids.

CONTENTS

Abbreviations used in the text

$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced $NADP^+$
NAD^+	Nicotinamide adenine dinucleotide
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
5^1-AMP	Adenosine monophosphate
$3^1 5^1$-AMP	Cyclic adenosine monophosphate



Cholesterol (cholest-5-en-3β-ol)

Figure 1: Structure of cholesterol.

Section 1.

Introduction

Cholesterol is the most ubiquitous member of a group of crystalline alcohols of plant or animal origin termed sterols, and belongs to the class of biological materials known as lipids. In general, lipids are solubilised in tissues and body fluids as lipoproteins, composed of proteins in association with long chain fatty acid esters of cholesterol, non-esterified (free) cholesterol, triglycerides, etc.

Cholesterol can be synthesised by all tissues in the animal body, with the possible exception of adult brain and nervous tissue. It is therefore not an essential dietary component in mammals. This sterol is the precursor of the steroid hormones in endocrine tissue, but the most important quantitative metabolic route is the formation of the primary bile acids by the liver. In higher animals the major bile acid is cholic acid (figure 3), with smaller amounts of chenodeoxycholic, deoxycholic and lithocholic acids. In the rat only cholic and chenodeoxycholic acids are formed in quantity. Chenodeoxycholic acid is not a precursor of cholic acid, but can be metabolised to the two 6β-hydroxylated bile acids, α and β muricholic acids, which are found in varying but small quantities in

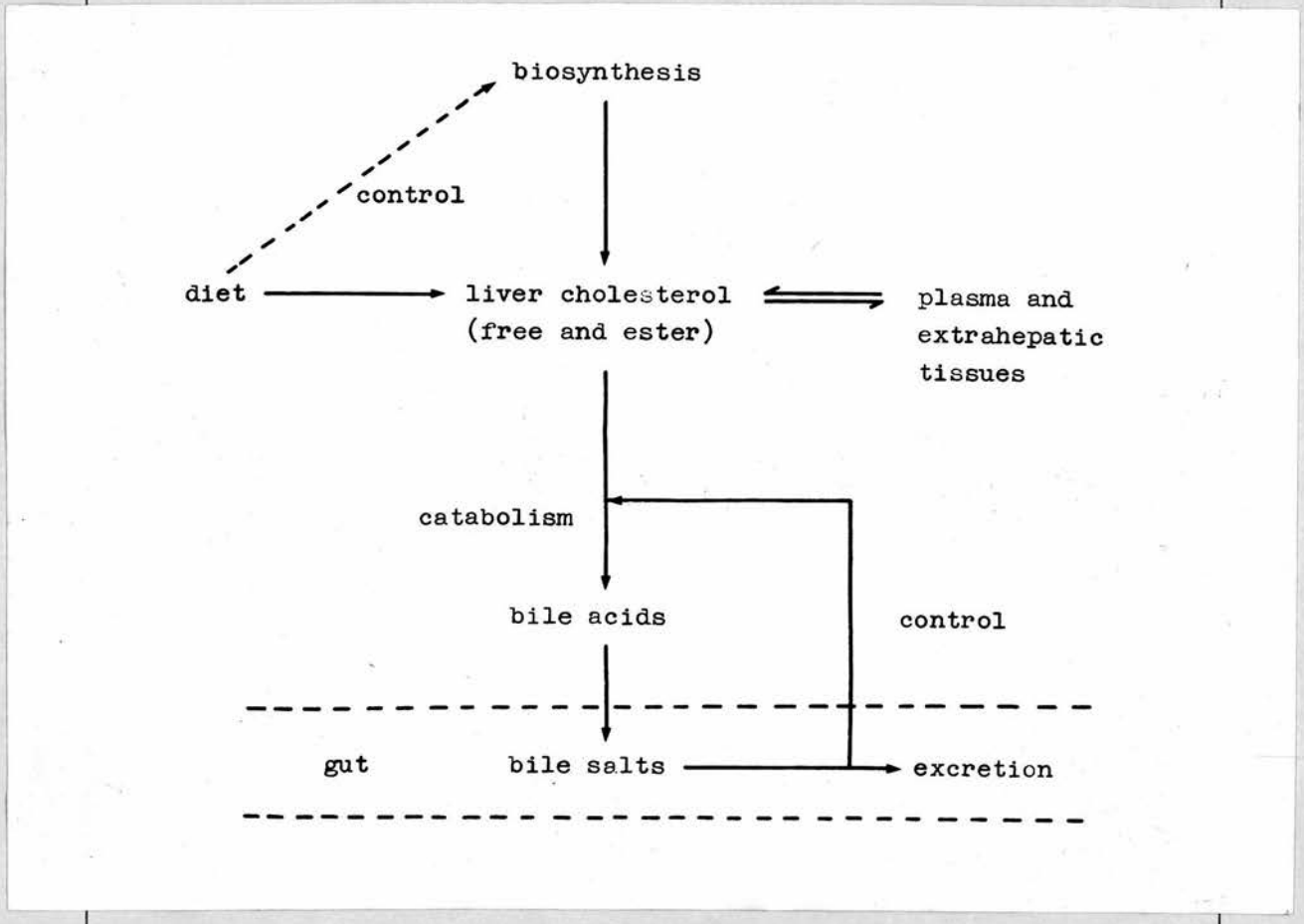


Figure 2: Schematic representation of the fate of liver cholesterol.

bile. Deoxycholic acid and lithocholic acid are formed from the primary bile acids by bacterial modification in the gut. There is evidence to suggest that lithocholic acid may be formed in the liver as a primary bile acid (figure 3).

Rat plasma contains about 50-70 mg. of cholesterol per 100 ml., while in liver the figure is about 180-200 mg. per 100 g. of tissue. Equilibration of plasma and liver cholesterol is rapid, and liver cholesterol is in a state of constant flux (figure 2). Consequently, the half-life of liver cholesterol in most animals is short, in the region of several hours, compared with an estimated half-life of total body cholesterol of 30 days in the rat, and 60-100 days in man[1,2]. Isotopic labelling has shown that the primary bile acids of the rat have a half-life of about 2-3 days.[3] Although most of the bile acids present in the gut as bile salts are reabsorbed through the enterohepatic circulation, a small quantity is excreted in the faeces so that new bile acids are constantly being synthesised to compensate for this loss. The circulating pool of cholic acid in the rat is estimated as about 10-20 mg.,[4] with a total daily production of cholic and chenodeoxycholic acids of the order of 5 mg. The rate of cholesterol breakdown to bile acids can be radically altered by the lipid content of the diet; the feeding of cholesterol in the

diet depresses cholesterol biosynthesis, especially in the liver and induces an increased rate of cholesterol catabolism. This catabolism is under homeostatic control, and interruption of the enterohepatic circulation by bile duct cannulation causes a marked increase in bile acid production,[5]; it has been concluded that the concentration of bile salts in the portal blood may regulate the catabolism of cholesterol. Total bile acids in the liver are present in a concentration of about 110 μg per gram of liver;[6] mostly in the cell fluid. The formation of bile acids in the liver of various species has been reviewed by Danielsson.[7]

Bile acid formation from cholesterol in the rat.

There have been numerous attempts to isolate intermediates in the catabolism of cholesterol to bile acids. Anfinsen and Horning[8] in 1953 described a fortified preparation of mouse liver which could oxidise cholesterol-26-^{14}C to $^{14}CO_2$; cell fluid (105,000 g supernatant) was essential for the activity of the system. Later studies[9] showed that the supernatant fraction could be replaced by a boiled preparation, from which most of the protein had been removed. This fraction became known as S.F. for Soluble Factor, and has since been found essential for maximal activity in many studies of cholesterol oxidat-

:ion; the factor or factors on which the activity of this preparation depends have not been identified. Whitehouse[10,11] confirmed the early findings with rat liver mitochondria in a fortified medium including S.F., NADPH and NAD. Kritchevsky[12] and Fredrickson[13,14] investigated the products of mitchondrial cholesterol oxidation and found a variety of neutral and acidic compounds. The acidic products were similar to deoxycholic and lithocholic acids but not identical; neutral metabolites were identified as 25- and 26-hydroxycholesterols. Danielsson[15,16] was not however able to confirm the enzymic formation of 25-hydroxycholesterol, although nine products more polar than cholesterol were isolated. He concluded that most of these products were formed non-enzymically and that the only enzymic product was 26-hydroxycholesterol. Recent work by Mitropoulos and Myant[17] has provided further evidence for the formation of 26-hydroxycholesterol as a likely precursor of chenodeoxycholic and lithocholic acid in fortified mitochondrial systems.

An experimental approach used to examine the degradative mechanisms of cholesterol is the study of the metabolism of hypothetical intermediates in animals subjected to biliary cannulation. Although the enterohepatic bile acid circulation is broken in such animals and they are therefore abnormal, valuable data can nevertheless be obtained. Possible

intermediates of cholesterol degradation can be administered by injection and their rates of conversion to bile acids calculated. With this 'in vivo' technique, 26-hydroxycholesterol can be metabolised to chenodeoxycholic acid but not to cholic acid, so it has been concluded that this sterol can only be involved in a route to one of the major bile acids (figure 3; route A).

Bergstrom demonstrated in 1955[18] that a route which did not involve 26-hydroxylation as the first step was a more likely pathway to both primary bile acids. A number of possible intermediates were investigated[19,20] and it appeared that nuclear modifications of the cholesterol molecule must necessarily precede side chain cleavage. Further investigation using bile duct cannulated animals and also in vitro studies showed that epimerisation of the 3β-hydroxyl group and double bond saturation occurred after insertion of the two hydroxyl groups at 7 and 12,[21,22] so that, of several mono-oxygenated sterols, only cholesterol could be catabolised to cholic acid.[23-28] In vitro studies by Danielsson and co-workers[29-33] and Mendelsohn and Staple [34-36] have provided considerable evidence for pathway B in figure 3. The demonstration of the postulated first step, the 7α-hydroxylation of cholesterol, has proved difficult; only small conversions to this

sterol have been reported, and this reaction has not been unequivocally established as the first catabolic step in the major route to the primary bile acids. 7α-hydroxycholesterol can be quantitatively metabolised by mitochondrial or microsomal preparations of liver,[37,38] implying that the formation of this intermediate is a rate-controlling process.

In contrast to the earlier work with mitochondrial preparations, enzymes of route B appear to be located in the microsomal fraction (endoplasmic reticulum) of the liver cell, where the bio-synthesis of cholesterol has recently been shown to occur.[39] Enzymes of the cell sap effect the reduction of the 3-keto intermediates (g,i) to hydroxyl groups, with the concomitant reduction of the 4-5 double bond to the coprostane series (5β). The latter stages of bile acid formation are thought to occur by formation of side chain hydroxylated sterols (tetrols and pentols) which undergo ω and β oxidation to the bile acids as Coenzyme A intermediates.[40-44] These reactions are probably mitochondrial events, with the microsomal catabolism proceeding only as far as trihydroxycoprostane (j) and dihydroxy-coprostane (l).

Cholic acid, the major bile acid, can only be formed from intermediates on the postulated microsomal route B. It has been established that the enzymes

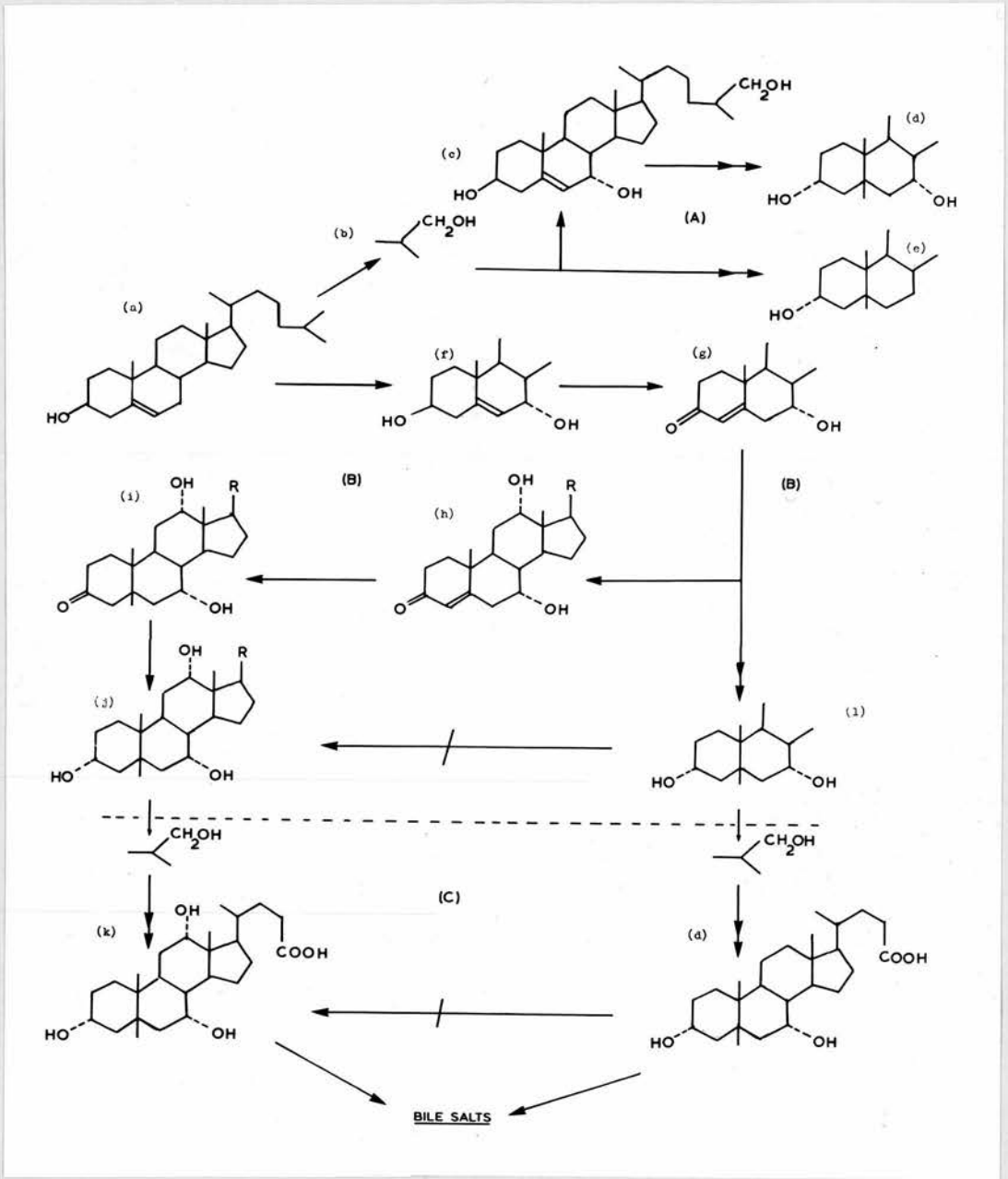


Figure 3: Possible routes for the catabolism of cholesterol by liver.
(A) Mitochondrial reactions
(B) Microsomal/cell fluid reactions
(C) Probable mitochondrial side chain cleavage reactions.

(a) cholesterol
(b) 26-hydroxycholesterol
(c) cholest-5-en-3β,7α,26-triol
(d) chenodeoxycholic acid
(e) lithocholic acid
(f) cholest-5-en-3β,7α-diol
(7α-hydroxycholesterol)
(g) cholest-4-en-3-on-7α-ol
(h) cholest-4-en-3-on-7α,12α-diol
(i) 5β-cholestan-3-on-7α,12α-diol
(j) 5β-cholestan-3α,7α,12α-triol
(trihydroxycoprostane)
(k) cholic acid
(l) 5β-cholestan-3α,7α-diol
(dihydroxycoprostane)

responsible for the formation of this bile acid possess an absolute specificity towards the structures of the substrates; no cholic acid can be formed if the modifications to the nuclear structure of the molecule are not completed in the sequence shown in figure 3. The route involving 26-hydroxylation as an early intermediate cannot therefore give rise to cholic acid.

7α-hydroxylation of cholesterol

Bergstrom has shown[45] by tritium labelling experiments that hydroxylation of cholesterol at C7 involves a direct replacement reaction rather than a hydration of an olefinic intermediate. The reaction appears to be similar in mechanism to the C11 hydroxylation in steroid hormone synthesis.[46] The highest reported yields of 7α-hydroxycholesterol in microsomal preparations are of the order of 0.5-1.9%.[32] Studies have been hampered by the ease with which cholesterol can be attacked non-enzymically by molecular oxygen to form autoxidation products, one of which is 7α-hydroxycholesterol; the formation of this sterol by such a mechanism probably accounts for a considerable portion of the "enzymic" 7α-hydroxylase activity reported by early workers. Autoxidation of cholesterol will be discussed in section 3.

Since this hydroxylation reaction has not been successfully investigated, the characteristics and

Oxygenases

1. Oxygen transferase

$$A + O_2 \longrightarrow AO_2$$

2. Mixed function oxidase (hydroxylase)

   a. internal

$$A\text{–}BH + O_2 \longrightarrow A^{2+}B(OH) + O^{2-}$$

   b. external

   (i) $A\text{–}B + O_2 + 2e \longrightarrow A(OH)\text{–}B(OH)$

   (ii) $AH + O_2 + 2e \longrightarrow A\text{-}OH + O^{2-}$

   2e supplied by NADPH
   NADH
   T.H. Folate
   Ascorbate

Figure 4: Oxygenases classified according to Mason.[50]

properties of the enzymes system must be inferred by consideration of other hydroxylations which have been investigated and purified. Many steroid hydroxylases have been studied with outstanding success, and some have been found to utilise molecular oxygen stoichiometrically.[47-49] Most require NADPH (some NADH) as a specific electron donor in the hydroxylation reaction, and are classified according to Mason[50] as mixed function oxidases, hydroxylases or mono-oxygenases. Such enzymes comprise a significant part of the large group of enzymes termed oxygenases (figure 4), which all show an absolute requirement for molecular oxygen. It has become apparent in the last decade that the fixation of oxygen by these enzymes forms an important part of the normal metabolism of cells; a wealth of data on the function and characteristics of oxygenases is now available.[51,52,53].

Concomitant with investigations of oxygenase-type enzymes, several heme-containing components have been detected and are likely to be involved in hydroxylation reactions.[54] These hemo-protein, cytochrome-like materials are implicated in the terminal stages of the electron transport systems from NADPH to oxygen, which must compete with the conventional electron pathways for oxygen and pyridine nucleotides. One of these hemo-proteins has emerged as a participant in a number of mixed function oxidations and has been termed

cytochrome P-450, since it can combine with carbon monoxide, to give a characteristic absorption band at 450mμ. This pigment represents a new type of oxidase which participates in the hydroxylation of drugs, carcinogens and steroids, and can apparently interact with oxygen and substrate during the hydroxylation reaction.[55-59]

In liver, the hemo-protein has been implicated as the oxygen activating enzyme for a number of NADPH-dependent microsomal hydroxylations.[60-64] Some of these hydroxylations appear to be non-specific and are part of the extensive capacity of liver microsomes for the metabolism of a large number of foreign compounds. Although most aerobic hydroxylations possess a specific requirement for NADPH, other physiological electron donors are utilised by many reactions. The electrons may be supplied by ascorbic acid, NADH, etc. Substituted pteridines such as tetrahydrofolic acid and biopterin are utilised by some hydroxylases,[79] which also require NADPH to maintain the pteridine moiety in the reduced state; the function of NADPH in these reactions is therefore not directly concerned with the actual hydroxylation step. The organisation of microsomal redox components has been extensively reviewed by Mason.[65] Soluble Factor (S.F.), used in studies of mitochondrial cholesterol oxidation, contains co-factors which can markedly enhance certain steroid

hydroxylations;[66] the comparable activity of S.F. and reduced pteridines has suggested that this fraction contains one or more pteridines having biological activity. Metal ions, also implicated in cholesterol oxidase activity, have been shown to be involved in reactions catalysed by oxygenase-type enzymes,[67] so that the mitochondrial side chain cleavage of cholesterol in liver may involve enzymes of the mixed function oxidase category.

Little is known of the co-factor requirements of the microsomal catabolism of cholesterol, especially the 7α- and 12α-hydroxylation steps. However, rat liver microsomes can efficiently 7α-hydroxylate various steroids such as deoxycholic acid[68], dehydro-epiandrosterone, pregnenolone[69] and 3β-hydroxy-Δ^5-cholenic acid[70], the latter compound having the same structure in rings A,B,C and D as cholesterol. These hydroxylations appear to be effected by mixed function oxidases, utilising molecular oxygen and NADPH.

On the basis of these findings, it is expected that cholesterol-7α-hydroxylase will also belong to this category of enzymes. The 7α-hydroxylation of cholesterol has been investigated in cell-free preparations of rat liver, in the light of current knowledge of hydroxylations reactions which may have similar mechanisms. The objects of the study were:

(1) the <u>in vitro</u> demonstration of cholesterol-

7α-hydroxylase.

(2) the investigation of factors influencing this reaction.

(3) an assessment of the 7α-hydroxylation of cholesterol as the first step in bile acid formation, and as a rate-controlling factor in cholesterol catabolism by the liver.

Section 2.

Methods

The rats used in this study were young adult males of the Wistar strain bred from the Departmental colony, and weighing from 180 to 240 g. Their diet consisted of rat cake (Oxoid 86; 75% whole meal flour, 20% fish and bone meal, 5% dried yeast), except in certain cases where a "soft diet" was used to facilitate mixing with various additions. The soft diet was composed of 70% whole meal flour, 25% skimmed milk powder and 5% yeast.

(i) Tissue preparation

The rats were killed by a sharp blow on the head and the livers (7-12 g.) rapidly excised. The liver was placed in a tared beaker containing 0.25 M. sucrose; this procedure allowed a rapid chilling of the organ and also served to remove extraneous blood. All subsequent operations were carried out at 0 to 5°.

The sucrose was removed and the liver finely minced with scissors. Homogenisation was effected in four times the tissue weight by volume of 0.25 M. sucrose, using a Teflon/glass homogeniser with a clearance of 0.15-0.20 mm. This process was kept to a minimum, and was continued only as long as pieces of

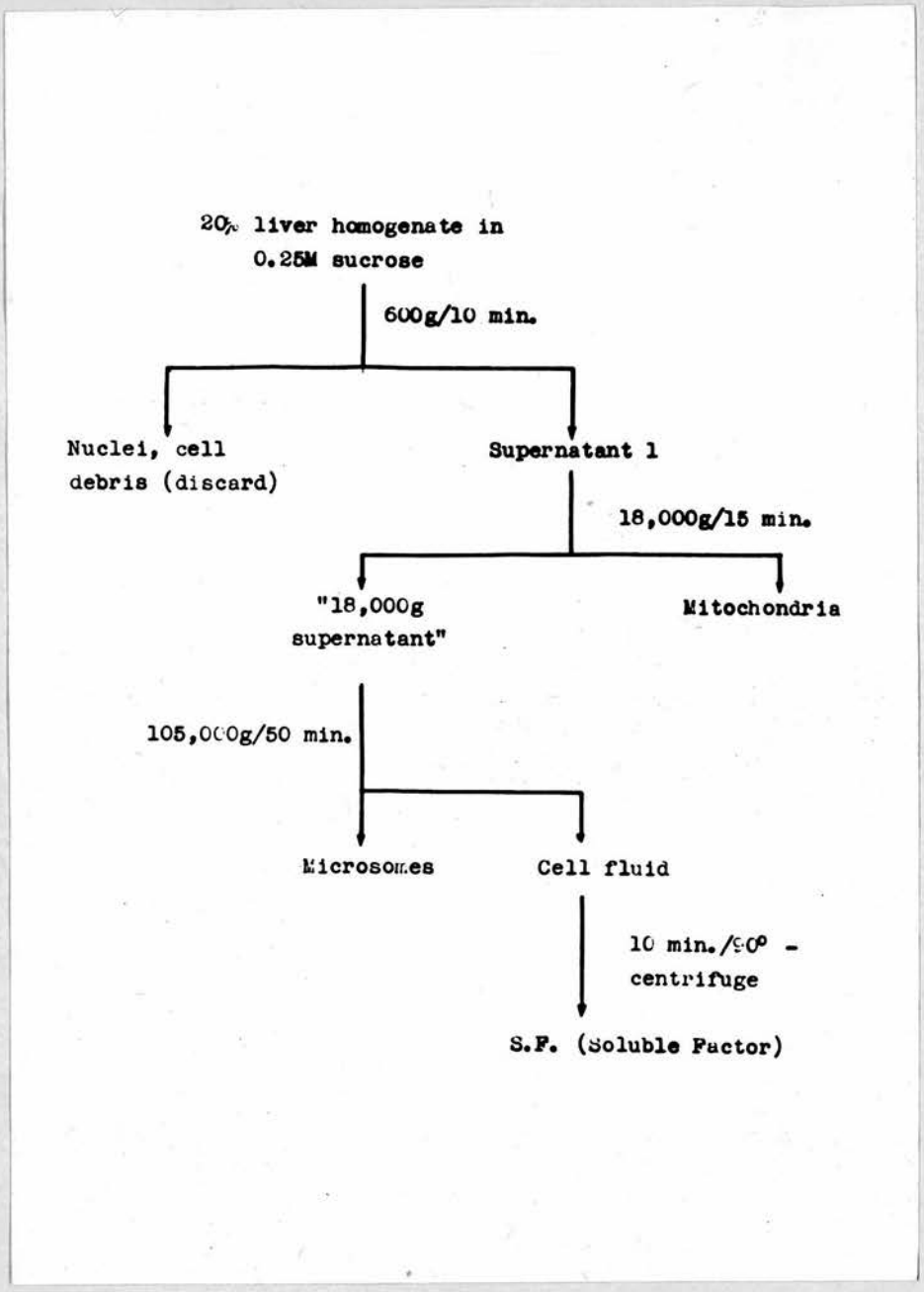


Figure 5: Flow diagram of cell fractionation.

whole liver were visible in the homogeniser tube. The homogenisation step was found to be a critical factor in the assay of enzymic activity (section 5).

The liver homogenate was subjected to differential centrifugation as shown in the flow diagram (figure 5).

Only a few experiments were performed with the mitochondrial fraction, so that normally the first low-speed centrifugation was omitted. After the second centrifugation, the 'supernatant' consisted of the endoplasmic reticulum in the cell sap, and this was the fraction most often used. This fraction is referred to as the 18,000 g supernatant. Further centrifugation afforded the microsomal and cell sap fractions, which have been used separately and in combination. In some cases the 105,000 g supernatant fraction was heated at 90° for 10 minutes, and centrifuged to remove denatured protein, leaving the Soluble Fraction (S.F.), described in the introduction. Washed preparations of microsomes were obtained by suspending the microsomal pellet in isotonic potassium chloride or 0.25 M. sucrose by gentle hand homogenising, and repeating the centrifugation. For addition to incubations, the microsomal fraction was taken up in one of these wash solutions so that the 4 ml. of the final suspension contained the microsomes from approximately 1.3 g. of liver. Each incubation sample contained 4 ml. of this

suspension or 4 ml. of the 18,000 g supernatant fraction, also equivalent to the 1.3 g. of whole liver. S.F. or 105,000 g supernatant from the same weight of liver was added in 4 ml. aliquots, made up to this volume with 0.25 M. sucrose. Mitochondria or microsomes were suspended in these fractions where required, in order to keep the final incubation volume constant. Every incubation therefore contained the appropriate cell fraction(s) from the same quantity of liver, so that the metabolism by the different preparations could be compared directly.

Acetone powders were prepared from the microsomal fraction. The microsomes, suspended in a small volume of 0.154 M. KCl., were poured with vigorous stirring into 40-50 volumes of acetone (-40°). The precipitate was rapidly filtered, washed twice with acetone and finally with cold ether. After removal of all solvent at room temperature under vacuum, the dry, friable powder was stored at -15°C. The microsomes from 1 g. of liver yielded about 60-70 mg. of powder. Lipid analysis of the powder showed that it contained only trace amounts of cholesterol, cholesterol esters and triglyceride. Each incubation contained 70 mg. of acetone powder (equivalent to 1.0 g. liver) suspended in 4 ml. 0.25 M. sucrose.

(ii) Incubations

Incubations with cholesterol-4-^{14}C were fortified with an NADPH generating system, except in those experiments designed to illustrate the requirements for this nucleotide. The NADPH generator was composed of:

0.5 ml. NADP solution (5 μ mole)

0.5 ml. glucose-6-phosphate solution (50 μ mole)

0.1 ml. glucose-6-phosphate dehydrogenase (1 unit, where 1 unit converts 1 μ mole of NADP into NADPH at 25° in the presence of glucose-6-phosphate). The system was pre-incubated at room temperature for 10 minutes, to ensure that most of the NADP was in the reduced form. These concentrations of the generator components are referred to as 'normal' in section 4.

Incubations were buffered with phosphate (Na_2HPO_4/NaH_2PO_4) at a molarity of 0.1 at pH 7.4. In a smaller number of experiments Tris/hydrochloride (Tris(hydroxy-methyl)-amino methane) was used at the same pH but with a molarity of 0.05. 2 ml. of the appropriate buffer was used in each sample. Unless otherwise stated, every incubation contained phosphate buffer and NADPH in these concentrations.

Cholesterol-4-^{14}C was purified before use by thin layer chromatography in the solvent system described below, in order to remove products of air oxidation.

This radioactive substrate was then stored in re-distilled acetone at -15° for less than four days before use. Each incubation sample received the substrate in 0.05 ml. of acetone (0.1-0.2 μC).

Additions of other co-factors were made as far as possible in distilled water, in a volume which did not exceed 0.3 ml., in order to minimise dilution effects. Where such materials were not water soluble, they were added in a small volume of acetone (0.05-0.1 ml.). Total incubation volume was 7.1-7.3 ml.

In a large number of incubations it was possible to mix the NADPH generator, buffer and tissue before division into a number of incubation flasks in order to eliminate errors in pipetting small volumes. The substrate was not included in this mixture and was added separately. Incubations were conducted at 37° in a shaking water bath, usually for one hour. The gas phase in each case was air, except in those experiments investigating the effects of carbon monoxide on the system. Glass tubes were filled with water from an aspirator; the water was then displaced with 700 ml. of oxygen-free nitrogen, and mixtures of carbon monoxide and oxygen in a total volume of 300 ml. By a manifold system, the gas could then be led through the incubation flasks, against a back pressure of about 3-4 cm. of water. The incubation flasks were thoroughly flushed

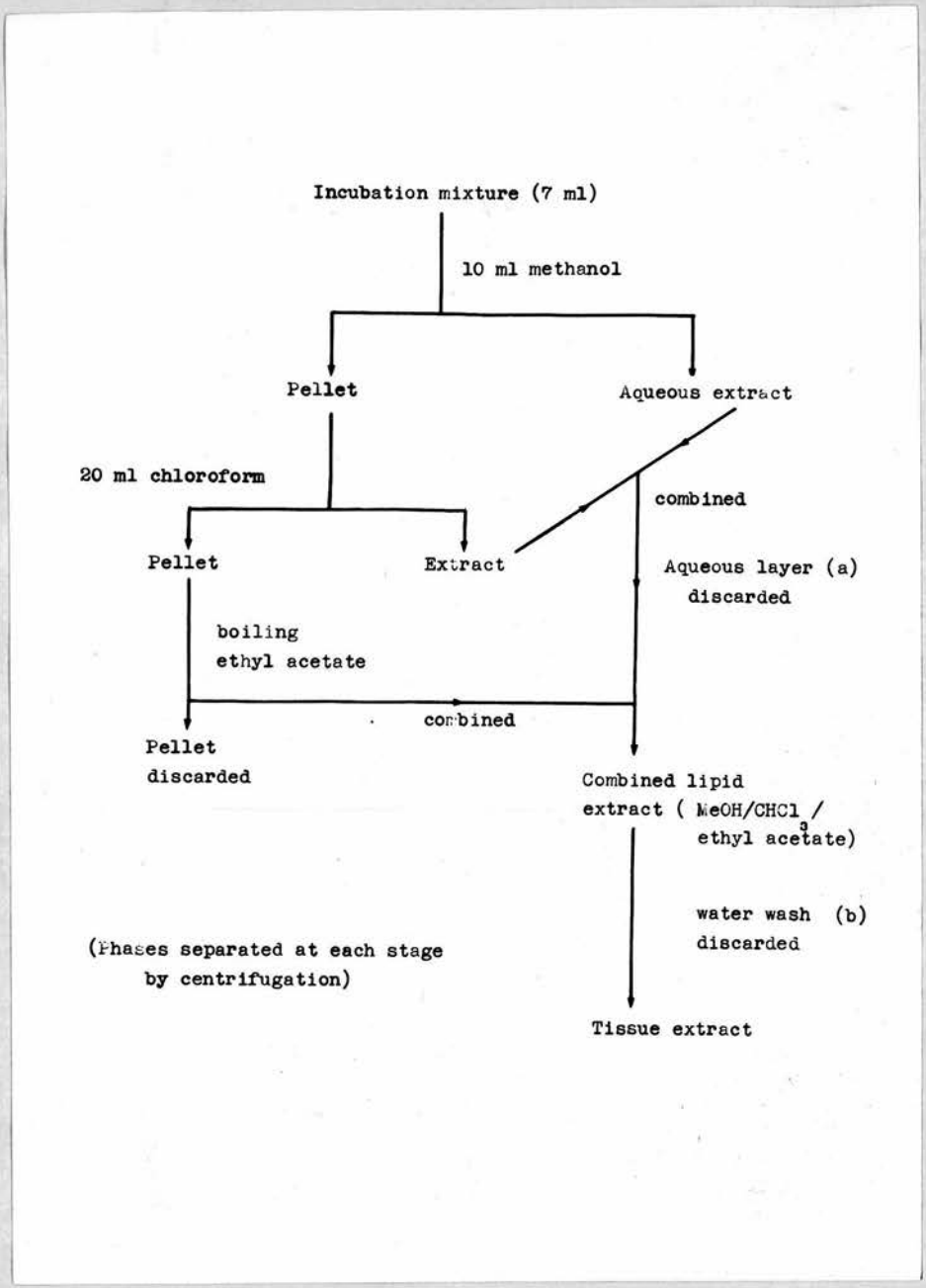


Figure 6: **Flow diagram of extraction procedure.**

for about 5 minutes before the addition of the substrate, then at frequent intervals over the period of the incubation.

(iii) Extraction

The reactions were stopped by the addition of 10 ml. of methanol, and extraction accomplished as shown in figure 6. No radioactivity was detected either in the aqueous layer (a), or in the final water wash (b). The extraction procedure shown was found to remove 98-100% of the added radioactivity; of this about 90% was removed by chloroform extraction alone and the rest by boiling ethyl acetate.

(iv) Chromatography

Although the extraction technique was efficient in removing radioactive lipid material from the samples, very little material was present in the final extract (c) which interfered with final analysis by thin layer chromatography. The dried extracts could therefore be transferred directly to thin layer plates (27 x 6 cm.). This was done by three washings of 0.2 ml. of chloroform using a fine capillary, which transferred 80-90% of the extract on to the plate.

Silica gel H (Merck) was used as adsorbent, and was slurried with water to give a viscous suspension. The glass plates were spread with a hand tool to give a

layer of 0.25 mm. when dry. Drying was effected by heating at 110-120° for 30 minutes. After spotting the samples and the standard mixture on the same plate, the plates were developed for 1.5-2 hours during which time the solvent ascended the gel for about 25 cm. The time taken for chromatography was rather long, but this was compensated by the excellent separation of the various sterols. The solvent system used in all of these experiments was benzene: ethyl acetate :: 7:13 and this mixture was found to give good separations both of metabolites of cholesterol and metabolites of 7α-hydroxycholesterol (section 9). Standard samples were visualised by spraying with dodeca-molybdo-phosphoric acid in ethanol; after heating the various materials appeared as orange or blue spots on a yellow background.

(v) Radioactive assay

The plates were assayed for radioactivity by a non-destructive method using a window-less gas-flow counter where the labelled products were required for further investigation. This method was not as efficient as liquid scintillation counting, which was used as a more absolute measure of radioactivity to obtain the results described in this thesis. The equipment used was a Packard Tri-carb Liquid Scintillation Spectrometer, using toluene with primary

and secondary scintillators and containing 4% methanol. Gel from the thin layer plates was scraped directly into the counting vials. Appendix 1 shows the reproducibility of counting with this equipment, and also that negligible quenching occurred in the presence of silica gel. The efficiency of the spectrometer was linear up to about 150,000 c/m; samples containing more radioactivity than this were divided into 2 or 3 smaller samples. Efficiency of counting for ^{14}C was about 65% and for ^{3}H about 40%.

(vi) Expression of results

The total radioactivity on the thin layer plates could be calculated from the results of liquid scintillation counting. The totals of each plate differed slightly since the lipid extracts were not transferred quantitatively to the thin layer plates. However, this was of no consequence, since the radioactivity corresponding to each standard was expressed as a percentage of the total radioactivity on the plate, which included the substrate cholesterol-4-^{14}C. This method was used to obtain the results shown in the figures and tables.

In all of the experiments described in this study where cholesterol-4-^{14}C was used as substrate, metabolism of this sterol was found to occur to 6 detectable

products, so that the radioactivity was confined to six areas of the plates. The amounts of these products were extremely variable, and the conditions under which they were formed are discussed later. Their Rf values correspond to the following standards:

cholestan-3β,5α,6β-triol

cholest-5-en-3β,7β-diol (7β-hydroxycholesterol)

cholest-5en-3β-ol-7-one (7-ketocholesterol)

cholest-5-en-3β,7α-diol (7α-hydroxycholesterol)

cholest-4-en-3-on-7α-ol

cholest-4-en-3on-7α,12α-diol

The first three of these products are formed by non-enzymic mechanisms from cholesterol as described in section 3. For most purposes in this work, the total metabolism of cholesterol-4-^{14}C attributable to these three compounds comprises the group of products termed 'autoxidation'.

The second group of compounds, in contrast, have all been shown to have physiological significance (section 1 - figure 3). The latter two, formed by oxidation and 12α-hydroxylation of 7α-hydroxycholesterol, were formed in small yield under the incubation conditions employed in these investigations, and in no case totalled more than 25% of the radioactivity found in the area attributed to 7α-hydroxycholesterol. In some experiments none of the 12α-hydroxylated derivatives

was detected. The three products have been grouped together and termed "enzymic products", although the purpose of this work has been to prove that this indeed is the case. The terminology of autoxidation and enzymic products is therefore largely one of convenience, the aim being to simplify the assessment of the overall metabolism of the substrate. The evidence justifying the distinction between "enzymic" and "autoxidation" products will be presented in section 3.

Section 3.

Interaction of cholesterol with molecular oxygen

The problems associated with the autoxidation of cholesterol were discussed briefly in section 1. Non-enzymic oxygen attack of cholesterol does not appear to occur *in vivo*; autoxidation products in tissue extracts identified by early workers were probably artifacts formed during extraction and analysis. This problem arises in *in vitro* studies with sub-cellular fractions, especially with preparations of liver. Little autoxidation appears to occur in cell fractions from other organs, e.g. bovine adrenal cortex mitochondria[71], and rat ovarian mitochondria[72], etc. The difference in susceptibility of cholesterol towards non-enzymic attack may be due to the amounts of free cholesterol in the preparation; lipoprotein bound or esterified cholesterol apparently is protected against autoxidation. The added cholesterol-4-^{14}C was in the form of the free sterol. Some tissues may afford a protection of the free sterol by high levels of 'anti-oxidants'; these may be free radical trapping agents, since autoxidation mechanisms may involve attack by free radicals (section 6).

Normally, an *in vitro* biochemical reaction is controlled by the inclusion of a boiled, enzymically

inactive sample, incubated and assayed under identical conditions; an estimate of non-enzymic activity can then be made. Unfortunately this technique cannot be used in this work since inactivation of the tissue by boiling actually enhances non-enzymic attack, giving a significant and random conversion of the substrate to a variety of products. Various sections in this thesis are concerned with the techniques which have been developed to minimise non-enzymic metabolism; in many studies, however, it has been necessary to allow, or correct, for autoxidative conversion of the substrate as a normal consequence of the in vitro assay system. Such products must be carefully considered so that they are not confused with enzymically formed products.

Since autoxidation is in reality a demonstration of the reactivity of the cholesterol molecule towards molecular oxygen, it is likely that the normal, enzymic reaction occurring in the liver cell also utilises this property of the cholesterol molecule. The reactive centre of cholesterol is located in the 5-6 double bond region of the molecule; positions 4 and 7 are allylic and are thus reactive (figure 1). 7α-hydroxycholesterol, postulated to be the first intermediate in cholesterol catabolism by the liver, has also been identified as one of the major products of autoxidation. Recently, more than thirty possible cholesterol autoxidation products have been found,[73] and the major ones shown to be

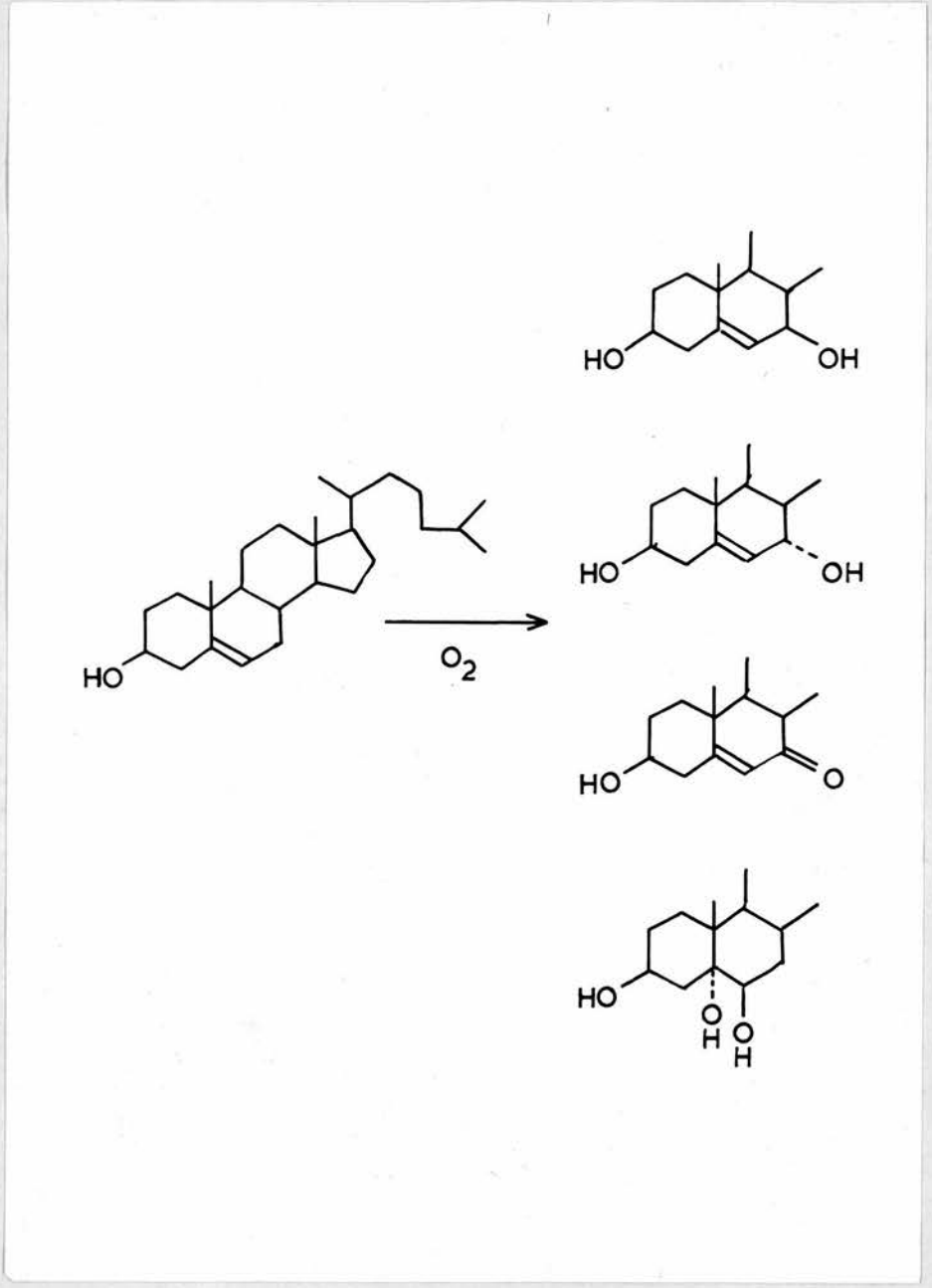


Figure 7: Major autoxidation products of cholesterol.

7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-keto-cholesterol and cholestan-3β,5α,6β-triol (figure 7). It is essential, therefore, that a distinction can be made between enzymic and non-enzymic activity. In order to do this it is necessary to consider as many of the known and postulated autoxidation products as possible, to determine the mechanisms by which they arise. It should then be possible to assess how they may interfere with the assay for enzymic activity.

One theory is that the attacking species is molecular oxygen itself, forming hydroperoxides of cholesterol. Hydroperoxides, which are generally unstable compounds, could then decompose in different ways to give the products which are eventually detected. Two hydroperoxides of cholesterol have been prepared chemically, the 5α and 7α-hydroperoxides (figure 8 and appendix 4). The 5α-hydroperoxide is the product of initial attack under the influence of light and can be isomerised to the 7α-position. There is also some evidence for a 6-hydroperoxide formed by attack at C_6. The 7α-hydroperoxide is interesting in that a simple reduction could form 7α-hydroxycholesterol, an autoxidation and a postulated enzymic product. This illustrates how enzymic and non-enzymic mechanisms might share a common oxygenated intermediate species. Product formation by the two routes would depend on the directional influences of the medium: where conditions

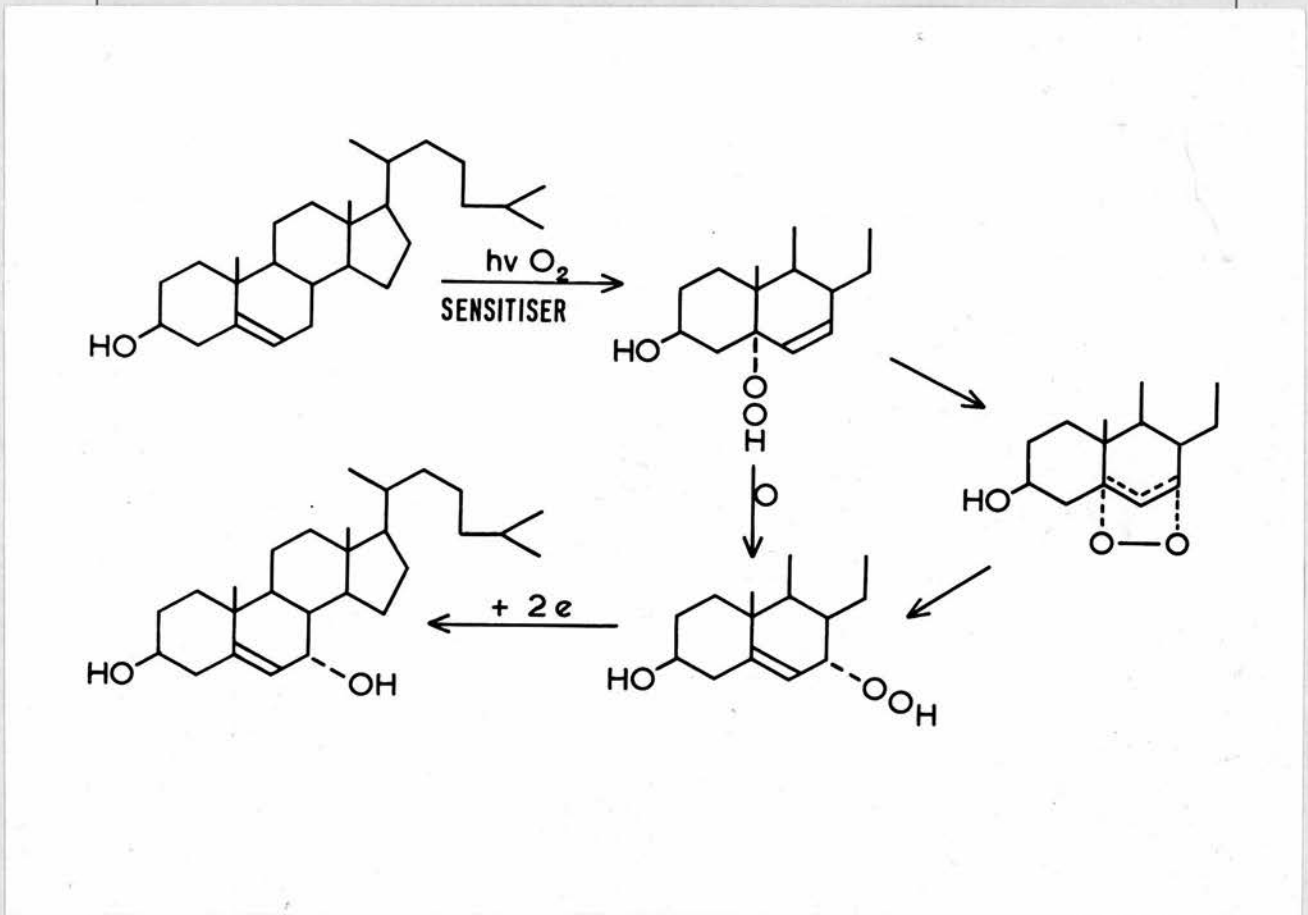
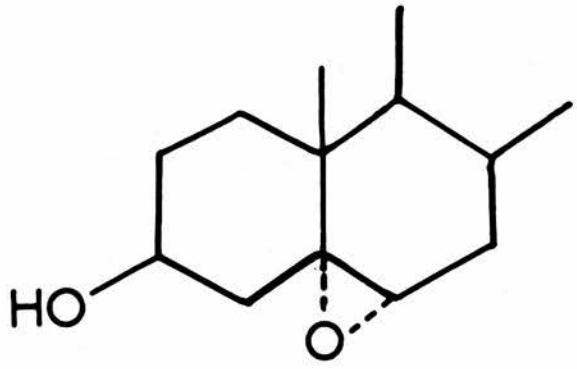
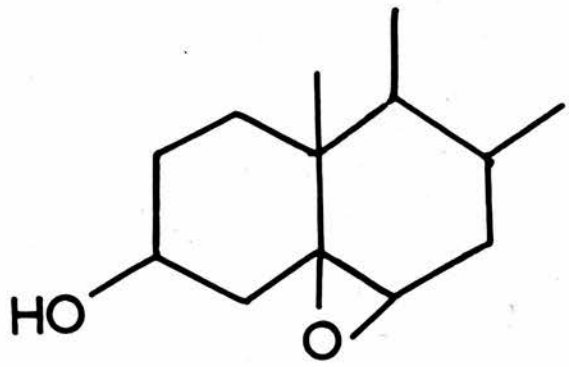


Figure 8: Formation of cholesterol-5α- and 7α-hydroperoxides.



Cholesterol-5α,6α-epoxide



Cholesterol-5β,6β-epoxide

Figure 9:

Table 1

Major products formed on incubation of cholesterol-5α- and 7α-hydroperoxides with S.F., microsomes, 18,000 g supernatant or microsomal acetone powder.

Incubation time 1 hour.

	5α-OOH	7α-OOH
7α-hydroxycholesterol	++	+++
7β-hydroxycholesterol	-	+
cholest-6-en-3β,5α-diol	++	-
7-keto-cholesterol	-	++

favoured enzymic activity, the predominant product would be formed enzymically. Mono-oxygenated derivatives of cholesterol, epoxides, can be prepared chemically. A steroid epoxide formed from a possible naturally occurring internal peroxide has also been reported.[78] The 5,6 α and 5,6 β epoxides of cholesterol are shown in figure 9.

The oxygenated compounds described above represent some of the possible intermediates which might be formed by oxygen attack, and which could be involved in both enzymic and non-enzymic mechanisms.

The experimental data presented in this section are the results of some investigations with cholesterol 5α- and 7α-hydroperoxides and with cholesterol-5α6α-epoxide to determine if any or all of these compounds are possible intermediates in autoxidative or enzymic mechanisms. The materials were prepared as described in the appendix and incubated as normal.

(i) <u>5α- and 7α-hydroperoxides</u>

Both hydroperoxies were unstable and were converted to other products on incubation with buffer at pH 7.4.

In the presence of any tissue preparation, boiled or native, the conversion of each compound to the products shown in table 1 was quantitative. The

7α-hydroperoxide formed 7α-hydroxycholesterol and 7-keto-cholesterol; major products from the 5α-hydroperoxide were cholest-6-en-3β,5α-diol, and 7α-hydroxycholesterol. The formation of 7α-hydroxy-cholesterol from the 5α-hydroperoxide suggested that isomerisation could occur on incubation. In both cases about six minor products were observed. These products had mobilities more and less than the parent compound; one of these derivatives appeared to be 7β-hydroxy-cholesterol. The conversion of cholesterol-5α- or-7α-hydroperoxide was not affected by NADPH.

(ii) Cholesterol-5α,6α-epoxide-^{3}H (Appendix 4)

This compound was also found to be unstable on incubation with buffer (table 2); NADPH again had little effect, but boiled tissue appeared to protect the substrate against decomposition. With unboiled 18,000 g supernatant, a 15% conversion was observed. The reaction products appeared to be cholestan-3β, 5α, 6β-triol (possibly formed by hydrolytic cleavage), cholestan-3β,5α-diol and a material with the mobility of 7β-hydroxycholesterol which was not identified further. In contrast to the hydroperoxides, which were decomposed equally well by boiled preparations, there may be an enzymic reaction which can metabolise this epoxide.

However, none of three compounds examined was

Table 2

Products formed by incubation for 1 hour of cholesterol-5α,6α-epoxide-^{3}H.

% Conversion	Buffer alone	NADPH	Boiled 18,000 g + NADPH	18,000 g + NADPH
cholestan-3β,5α,6β-triol	2.5	2.3	1.2	7.3
cholestan-3β,5α-diol	0.5	1.1	1.3	2.1
7β-hydroxycholesterol	3.0	1.7	1.0	5.8
% Total conversion	6.0	5.1	3.5	15.2

selectively converted into 7α-hydroxycholesterol by an enzymic mechanism, so that they are unlikely to be involved in enzymic 7α-hydroxylation. Nevertheless, the instability of such compounds has been demonstrated, and intermediate formation of materials of this type could well account for a number of autoxidation products. Although unstable intermediates are unlikely to survive in appreciable quantities after incubation, extraction, etc., some may be present in the final analysis of lipid extracts. Their detection at this stage would be difficult, since epoxide and hydroperoxide functions impart little additional polarity to the cholesterol molecule. The mobilities of the hydroperoxides and epoxides by thin layer chromatography were found to be very similar to 7-keto-cholesterol, from which they could not be separated (Appendix 2). The products formed from these derivatives, mainly diols, also tend to have similar polarities so that the dihydroxysterol area of the thin layer plate can contain:

7α- and 7β-hydroxycholesterol

cholestan-3β,5α-diol (from α- epoxide)

cholestan-3β,6β-diol (from β- epoxide)

cholest-6-en-3β,5α-diol (from 5α- OOH)

cholest-5-en-3β,4β-diol, which may also be an autoxidation product, has the same mobility as cholest-6-en-3β,5α-diol.

Fortunately, the products formed on incubation of cholesterol-4-^{14}C with liver cell fractions did not appear to be a complex mixture of these materials. Metabolism of this substrate under all conditions produced only six detectable and apparently homogeneous radioactive metabolites, formed in varying amounts dependent on the conditions of incubation. These compounds behaved on thin layer as the named standards, and were identified by the methods detailed in appendix 3.

(A) '<u>cholestan-3β,5α,6β-triol</u>'

Radioactive material isolated from incubation was co-chromatographed with cholestan-3β,5α,6β-triol, 5β-cholestan-3α,7α,12α-triol (trihydroxycoprostane), and cholest-5-en-3β,7α,26-triol. The physiological significance of the latter two triols has been discussed in section 1. The radioactivity ran with the autoxidation triol, which was more polar than the other two. A sample of the unknown material was oxidised with N-bromosuccinimide and again chromatographed. It has the Rf value of cholestan-3β,5α-diol-6-one, the oxidation product of cholestan-3β,5α,6β-triol. It was concluded that the metabolite was cholestan-3β,5α,6β-triol.

This trihydroxylated sterol was not metabolised further by liver cell fractions.

(B) '7β-hydroxycholesterol'

The radioactive metabolite was crystallised to constant specific activity with standard 7β-hydroxycholesterol. The radioactivity was retained by the crystals. It was concluded that the material was 7β-hydroxycholesterol; no further metabolism of this compound was observed.

(C) '7-keto-cholesterol'

Attempts to crystallise the 7-keto-cholesterol-like material were unsatisfactory; in some cases the radioactivity was retained, in others not. It was concluded that this fraction was of heterogeneous composition, and possibly contains 7-keto-cholesterol with one or more products in varying amounts. This metabolite will be further discussed in section 6.

A tritiated sample of 7-keto-cholesterol (prepared as in Appendix 4) was incubated with boiled and native 18,000 g supernatant fraction. With boiled tissue there was a small but detectable metabolism (table 3), presumably autoxidative, to several products which had the mobilities of cholestan-3β,5α,6β-triol, 7α- and 7β-hydroxycholesterol and less polar products. In native tissue, there was a significant, selective conversion to 7β-hydroxycholesterol, suggesting that a

Table 3

Metabolism of 7-keto-cholesterol-^{3}H in 1 hour at 37° by 18,000 g supernatant with NADPH.

% Conversion	Boiled	Native
cholestan-3β,5α,6β-triol	3.0	2.5
7α-OH chol.	1.4	1.2
7β-OH chol.	2.3	57.0
Non-polar metabolite	2.0	8.8
Total	8.7	69.5

7-keto reductase was present in the preparation.

7-keto-cholesterol may be the major source of 7β-hydroxycholesterol found in incubations; there is little evidence of significant 7α-hydroxycholesterol formation from this compound. The mechanisms of triol formation from 7-keto-cholesterol are not known.

The three metabolites identified above, together constitute the group of products referred to as 'non-enzymic' or 'autoxidation' throughout this work. It has been shown that active preparations may metabolise cholesterol-α-epoxide and 7-keto-cholesterol; there is little evidence of 7α-hydroxycholesterol being formed from these compounds. The term 'non-enzymic products' is therefore used to simplify assessment of several products, which do not include 7α-hydroxy-cholesterol; it does not imply that no enzymes are involved at any stage in the formation of these products.

The last three of the six metabolites together make up the group termed 'enzymic' and were identified as follows.

(A) '<u>7α-hydroxycholesterol</u>'

The radioactive material was crystallised to constant specific activity; the radioactivity was retained by

the crystals so that it was concluded that the radioactive metabolite was 7α-hydroxycholesterol.

(B) 'cholest-4-en-3on-7αol' and
'cholest-4-en-3-on-7α,12α-diol'

The two metabolites having the mobilities of these compounds (whose physiological significance was discussed in section 1) were not crystallised with standards since they were formed in small yields, and standard materials were in short supply. Evidence of their identities is circumstantial and is based on the following:

(1) Their mobilities were unlike any autoxidation products; they were not formed in boiled preparations.

(2) Their formation followed that of 7α-hydroxycholesterol and not the autoxidation products. 7α-hydroxycholesterol was the major product, with the two metabolites together accounting for less than 20% of the total activity.

(3) Subsequent study with 7α-hydroxycholesterol (section 9) showed that these compounds were the major metabolites of 7α-hydroxycholesterol.

The results described in the rest of this work are expressed as described in this section. On many occasions, it was evident that all of the 7α-hydroxycholesterol formed by a preparation could

not be attributed to an enzymic source, so that under these conditions an autoxidative mechanism for 7α-hydroxylation becomes significant. Where autoxidation in general was low, it was presumed that essentially all of the product was formed enzymically. Reference will be made to these effects when they are apparent.

Section 4.

(i) Metabolism of cholesterol-4-^{14}C by liver cell fractions.

Cell fractionation procedure is described in section 2. Each incubation sample contained whole homogenate, mitochondria or microsomes from about 1.3 g. of liver. Supernatant or S.F. fraction from the same weight of liver was added where indicated. The samples were buffered to a total volume of about 7 ml. with 0.1 M. phosphate pH 7.4; NADPH generator and substrate cholesterol-4-^{14}C were the only other components of the incubations, which were carried out for one hour at 37°. The results are shown in table 4, expressed as autoxidation and enzymic products as previously defined.

(a) Liver homogenate

Enzymic products formed in this fraction consisted of equal but small amounts of 7α-hydroxycholesterol and its oxidation product, cholest-4-en-3-on-7α-ol. Total non-enzymic products in liver homogenate accounted for about twice the radioactivity found in the enzymic products.

(b) Mitochondria

Conversion of cholesterol-4-^{14}C to

Table 4

Metabolism of cholesterol-4-^{14}C by liver cell fractions

M = mitochondria
Ms = microsomes
L.H. = liver homogenate
S = 105,000 g supernatant

(1)

Conversion %	L.H.	M	M.S.	M+S	M+S.F.	Ms+S	Ms+S.F.	18,000 g fraction	S	S.F.
Total	1.2	1.7	1.4	4.1	2.2	5.2	2.8	-	0	0
7α-OH chol.	0.2	0.2	0.3	0.9	0.4	2.4	0.9	-	-	-
cholest-4-en-3-on-7α-ol	0.2	0.3	0.2	0.2	0.2	0.5	0.2	-	-	-

(2)

Total	-	-	2.5	-	-	5.2	4.2	8.0	0	-
7α-OH chol.	-	-	1.3	-	-	3.4	2.2	7.5	-	-
cholest-4-en-3-on-7α-ol	-	-	0.1	-	-	0	0.3	0.5	-	-

7α-hydroxycholesterol by the mitochondrial fraction was approximately the same as that found with whole homogenate, but slightly more cholest-4-en-3-on-7α-ol was synthesised. More of the autoxidation products were formed.

(c) Microsomes

Formation of 7α-hydroxycholesterol by the microsomal fraction was greater than by mitochondria or by whole homogenate, but the total conversion to enzymic products was the same as that in mitochondria. Formation of non-enzymic products was lower.

(d) Supernatant fraction

No metabolism of cholesterol-4-^{14}C to either enzymic or non-enzymic products by the supernatant fraction was observed.

Comparison of the metabolism of cholesterol-4-^{14}C by whole homogenate, mitochondria or microsomes showed that none of these fractions was capable of effecting a significant conversion to 7α-hydroxycholesterol in the absence of autoxidation products. With the exception of the supernatant fraction (in which there was no oxidative metabolism) 7α-hydroxycholesterol together with its oxidation product accounted for only about one third of the total conversion by each fraction. None

of the 12α-hydroxylated derivative was detected in these samples. Autoxidation in these samples was high by comparison with the metabolism to 7α-hydroxycholesterol and cholest-4-en-3-on-7α-ol, so that it seems likely that a significant proportion of the 7α-hydroxycholesterol may have been formed by a non-enzymic route, in which case the true enzymic activity may be substantially lower than the above estimates.

In view of the low enzymic activity of these cell fractions, combinations of mitochondria and microsomes with the 105,000 g supernatant and S.F. fractions were assayed for activity.

(e) 1. Mitochondria + supernatant

The presence of cell supernatant fraction with mitochondria increased the conversion of cholesterol-4-^{14}C to more than twice that found with mitochondria alone. However, this increase was due largely to an enhanced formation of the autoxidation products; enzymic activity was indeed larger but the two enzymic products accounted for little more than a quarter of the total metabolism. Nevertheless, this increase was accounted for by an increase only in 7α-hydroxycholesterol, and not by an increase in cholest-4-en-3-on-7α-ol. Metabolism to 7α-hydroxycholesterol by this preparation was more than four times that found in the mitochondrial fraction.

2. Mitochondria + S.F.

Metabolism of cholesterol-4-^{14}C by the mitochondrial fraction fortified by S.F. was slightly greater than that by mitochondria alone. Again autoxidation was enhanced as with native supernatant fraction, and there was also an increase in the amount of 7α-hydroxycholesterol formed. This increase gave a metabolism to this sterol which was twice that found with mitochondria alone.

(f) 1. Microsomes + supernatant

Of the total product formation by the combined microsomal and cell supernatant fractions, non-enzymic products accounted for less than half of the radioactivity, with 7α-hydroxycholesterol alone representing a conversion of 2.4% of cholesterol-4-^{14}C, making a total of 2.9% with its oxidised derivative, cholest-4-en-3-on-7α-ol.

2. Microsomes + S.F.

Fortified with S.F. fraction, microsomes effected a total metabolism slightly greater than that found in the corresponding mitochondrial preparation but the enzymic products were almost doubled, the increase appearing as a selective increase in 7α-hydroxycholesterol.

In the presence of native 105,000 g

supernatant fraction or S.F., metabolism of cholesterol-4-^{14}C by the mitochondrial and microsomal fractions was markedly enhanced. Cell supernatant was the more potent of the two preparations. Formation of non-enzymic products by fortified mitochondrial preparations was increased more than the enzymic products in the same samples; no increase in cholest-4-en-3-on-7α-ol, the oxidised metabolite of 7α-hydroxycholesterol, was observed, so that there appeared to be a significant stimulation of 7α-hydroxylase activity. The largest increases in the metabolism to 7α-hydroxycholesterol were found in the fortified microsomal preparations, in which the total conversions exceeded those in the corresponding mitochondrial preparations. With the 105,000 g supernatant microsomal conversion to 7α-hydroxycholesterol was much larger than any of the other combinations investigated. There appears to be little doubt that significant enzymic 7α-hydroxylase activity was present in this sample. Increased activity was also present in the microsomes + S.F. sample but was much lower than with untreated cell sap.

Although some 7α-hydroxylase activity was found in the fortified mitochondrial preparations (possibly due to microsomal contamination in the mitochondrial fraction) it was considered that microsomal preparations would be the obvious choice

for further investigation of the enzyme system which effects the 7α-hydroxylation of cholesterol-4-^{14}C.

(ii) Metabolism of cholesterol-4-^{14}C by microsomal preparations

The results shown in the lower part of table 4 were obtained under the same incubation conditions described above, but using preparations of liver microsomes only. The metabolism of cholesterol-4-^{14}C was studied in an isolated microsomal preparation, and in microsomes resuspended in the cell sap (supernatant) and in S.F. These preparations were contrasted with the metabolism by the 18,000 g supernatant fraction, i.e. a homogenate from which nuclei, cell debris and mitochondria have been removed, and which can be separated into the microsomal and 105,000 g supernatant fractions.

(a) Microsomes alone

Of the total metabolism of cholesterol-4-^{14}C by the microsomal fraction, more than half of the radioactivity was accounted for by the two enzymic products. Metabolism to 7α-hydroxycholesterol alone represented a conversion greater than to the three non-enzymic products.

(b) Microsomes + S.F.

This fortified microsomal preparation further enhanced the selective metabolism to 7α-hydroxycholesterol which accounted for 55% of the more polar products.

(c) Microsomes + separated cell sap

Almost twice as much radioactivity was found in the 7α-hydroxycholesterol area as was found in the three areas corresponding to the autoxidation products, which were formed to the same extent as in the microsomes + S.F. sample.

(d) Microsomes + supernatant (18,000 g supernatant).

A conversion of 8.0% to the two enzymic products was obtained by incubation of the unseparated microsomes + supernatant fraction. In the same sample, total metabolism to the three non-enzymic products accounted for only 0.5% of the added radioactivity. Under these conditions, it can be presumed that essentially all of the 7α-hydroxycholesterol has been formed by an enzymic mechanism.

The results from the two experiments shown in table 4 indicate that the overall metabolism of cholesterol-4-^{14}C can be enhanced by S.F., and by native

105,000 g supernatant fraction, the latter being the more effective. Conversion of the substrate to non-enzymic products was very similar in the duplicate samples of the two experiments. Enzymic activity, in contrast, was higher in the second experiment, specifically with respect to the 7α-hydroxylase activity, the effect being most pronounced in the microsomal and microsomes + S.F. samples. The difference in conversion to the enzymic products by the microsomes + cell sap fractions was not so marked. These results demonstrate the differences which are invariably found in the levels of enzymic activity present in identical preparations from different livers. The similarity in the samples fortified with 105,000 g supernatant therefore suggests that the enzyme system requires one or more co-factors, whose distribution between the cell fluid and microsomes is variable, accounting for lower and widely different enzymic activities in isolated microsomal fractions. Possibly a thermo-labile co-factor is involved, since metabolism by microsomes + S.F. is intermediate between microsomes and microsomes plus cell sap. However, none of these preparations of combined cell fractions showed the large, selective metabolism to 7α-hydroxycholesterol found to occur in the 18,000 g supernatant fraction, where the conversion was more than double that by the recombined microsomal and 105,000 g

supernatant fractions. This enhanced activity was observed in the presence of only about one third of the non-enzymic activity in the same sample. It was evident that a much more physiological system existed in this fraction than in any other previously examined, and this resulted in a selective metabolism to the enzymic product, 7α-hydroxycholesterol. The low level of the autoxidation products under these conditions has been found to be indicative of an active preparation; it is reasonable to postulate that an absence of non-enzymic activity suggests that the tissue preparation in the incubation medium provides an environment which more closely approximates to actual intra-cellular conditions. The substrate added to this enzyme solution is then less likely to be attacked by a mechanism which is not operative in the normal working of the cell; in this case the substrate is protected against non-enzymic attack, the autoxidation products (whose formation in vivo has never been substantiated) are kept to a minimum. Exactly why the sedimentation of the microsomal fraction should produce a less active but more autoxidation-sensitive system is not known.

Most of the results described in this thesis have been obtained using the unseparated 18,000 g supernatant fraction (apart from the present discussion, the

microsomes + supernatant fraction referred to in later work is synonymous with 18,000 g fraction). Not all of the experiments were found to have such a high level of enzymic activity as the preparation described in this section, nor do they all show the same selectivity in the conversion of cholesterol-4-^{14}C to 7α-hydroxycholesterol. However, the effects of S.F. and cell sap fractions have been well substantiated; the reasons for the differences in the metabolism by the different microsomal preparations will be later discussed after consideration of various factors which were found to influence the 7α-hydroxylase system.

(iii) Metabolism of cholesterol-4-^{14}C by 18,000 g supernatant (microsomes + supernatant)

(a) NADPH dependence

The experiments in the preceding two chapters of this section were performed in the presence of an NADPH generating system, whose components were added in the concentrations described in section 2. For the purposes of the following discussion, these concentrations are referred to as "normal". In table 5 are shown the results of an experiment to investigate the metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant fraction in the presence of different

Table 5

Metabolism of cholesterol-4-^{14}C by 18.000 g supernatant in 1 hour at 37° with different concentrations of NADPH.

N = 'normal' concentration defined in section 2.

% Conversion	$\frac{N}{10}$	N	N x 10
Total	2.0	3.4	4.3
Autoxidation	0.9	1.0	1.0
7α-OH chol.	0.6	2.2	3.0
cholest-4-en-3-on-7α-ol	0.5	0.2	0.3

concentrations of NADPH generator. The concentrations used corresponded to the "normal" concentration, and this concentration increased and decreased ten fold.

None of the three concentrations significantly affected the conversion of the substrate to non-enzymic products. Enzymic activity, in contrast, varied from 1.1% to 3.3% of the added radioactivity. Little additional metabolism to the enzymic products was obtained by increasing the NADPH concentration by a factor of ten, but there was a substantial decrease in these products with the lowest concentration. No evidence of the 12α-hydroxylated derivative of cholest-4-en-3-on-7α-ol was found in this preparation; however, a significant oxidation of 7α-hydroxycholesterol occurred in the presence of the low concentration of NADPH, suggesting that this oxidation reaction was enhanced by depletion of the reduced form of the co-factor.

On the basis of this evidence, it was considered that the concentration of NADPH defined as "normal" should be used as the standard NADPH concentration for further work. Although a higher level of enzymic activity was observed at the highest concentration, the extreme cost of material prohibited the use of this concentration in normal work; the level of reduced

co-factor in these circumstances is also very unphysiological. The selected concentration is probably much higher than that present in the intact cell but it was considered essential to maintain a high level of co-factor in the reduced form, in order to counteract the losses due to the high levels of glucose-6-phosphatase, and NADP'ase activities present in microsomal preparations. Oxidation of NADPH itself by the microsomal fraction is also very rapid.[74]

Summary:

1. Evidence presented in this section indicates that the enzyme system responsible for the 7α-hydroxylation of cholesterol is located in the endoplasmic reticulum of the liver cell, the most active enzyme fraction being the 18,000 g supernatant.

2. The 7α-hydroxylase system requires the addition of NADPH; in the presence of low concentrations of this nucleotide the enzymic activity was substantially reduced.

3. Non-enzymic activity, expressed as the total of the three autoxidation products, was in some cases large, and under these conditions it must be concluded that true enzymic 7α-hydroxylase activity may be much lower than the values found, due to the formation of 7α-hydroxycholesterol from a non-enzymic

source. In the 18,000 g supernatant fraction, the substrate appears to be protected against non-enzymic oxygen attack.

Section 5.

Factors affecting the activity of the cholesterol-7α-hydroxylase system in the 18,000 g supernatant fraction

In the previous section the metabolism of cholesterol-4-^{14}C was investigated in the 18,000 g supernatant fraction (microsomes + supernatant). The methods employed for cell fractionation and the subsequent incubations are described in section 2; these techniques are used by other workers in investigations of this type, and can be considered as fairly standard. However, the results obtained by incubation of the 18,000 g supernatant fraction indicated that large variations must be expected in the levels of 7α-hydroxylase activity. The present section deals with the further investigation of the techniques and conditions used in the assay system, in order to determine where a loss of 7α-hydroxylase activity might occur even before incubation of the tissue, and to ensure that, within narrow limits, conditions were optimal for the enzyme system.

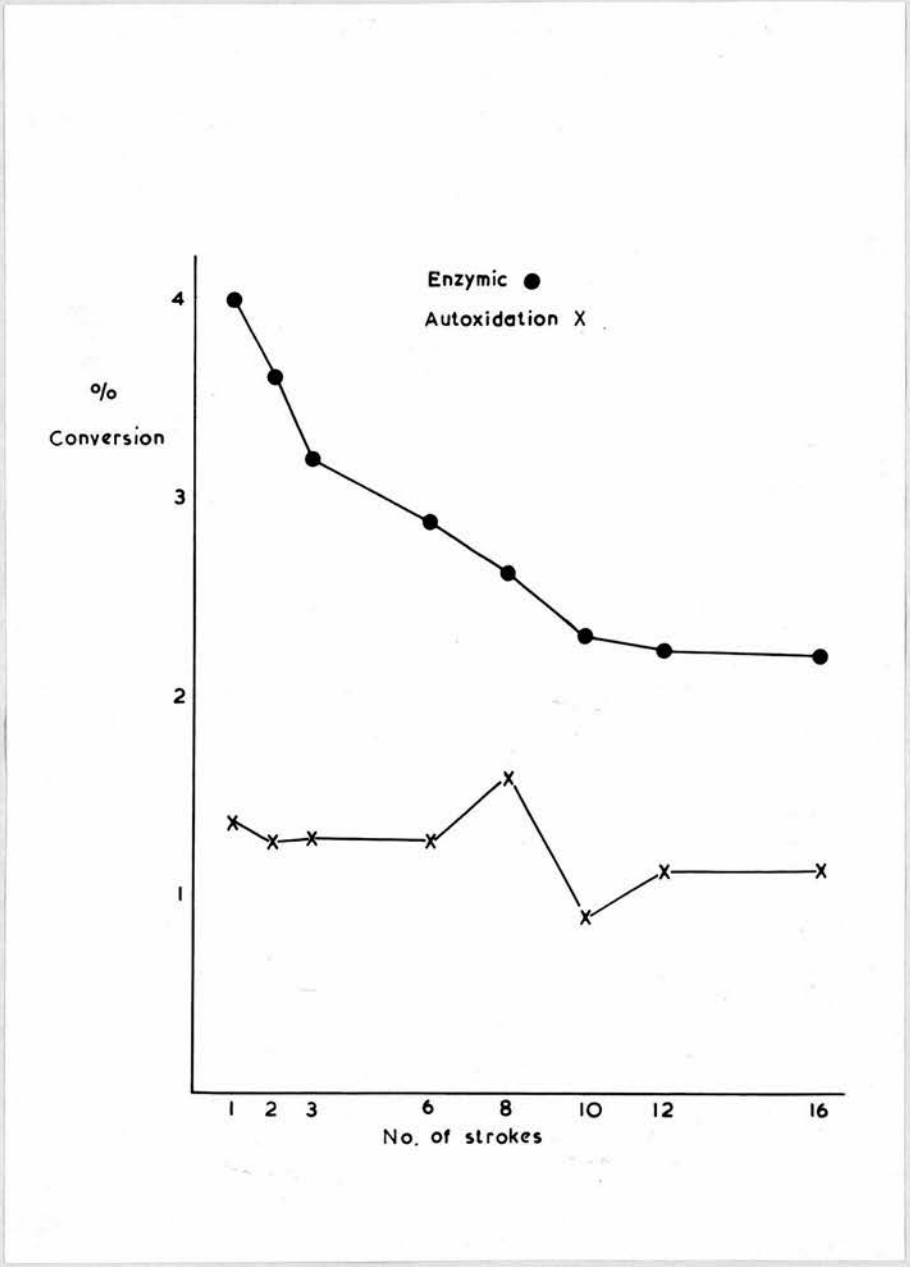


Figure 10: Metabolism of cholesterol-4-^{14}C by 18,000 g supernatant fraction after variable homogenisation of liver tissue. Incubation for 1 hour at 37^{0} with NADPH.

(i) Tissue homogenisation

After mincing, liver was homogenised as described in section 2. This process was, however, subject to extreme variation with the equipment employed. The power source, a small electric motor attached to the Teflon pestle, required constant adjustment during use to maintain a slow and smooth homogenisation, so that it was impossible to reproduce the same conditions exactly. Investigations of the "extent" of homogenisation were performed using a high torque motor, so that there was no significant change of speed when in use. The speed was fixed at 475 r.p.m. by a stepdown gear system. After the liver homogenate had been prepared by this method, it was centrifuged as usual at 18,000 g/15 minutes, and the preparation assayed for activity. The units of "homogenisation" are arbitrarily defined as the number of upward or downward strokes of the pestle.

In figure 10 are shown the results obtained by incubation of the various 18,000 g supernatants with cholesterol-4-^{14}C. Total enzymic activity, expressed as the percentage conversion of the substrate to 7α-hydroxycholesterol and its two metabolites, varied from 4.0 to 2.2%. Within these figures, 7α-hydroxycholesterol itself accounted for 80-90% of the enzymic products; the proportion of the diol in this experiment was rather lower and more variable than found in other circumstances.

The highest level of activity was observed where the tissue had been homogenised by one stroke of the pestle. Further homogenisation rapidly reduced the activity, although this decrease was not so marked after the initial few strokes, during which the activity was reduced by about 30%.

Non-enzymic products varied from 1.1 to 1.6% conversion of the substrate, and their formation tended to be rather erratic within these limits; no significant increase or decrease in the total of these three products was observed.

These results indicate that the organisation of the 7α-hydroxylase system is vulnerable to a homogenisation technique which cannot be considered as harsh. In subsequent investigations of the 18,000 g supernatant fraction, this method of homogenisation was used. Two strokes of the pestle were employed since it was found on some occasions that one stroke was not sufficient for complete homogeneity of the suspension.

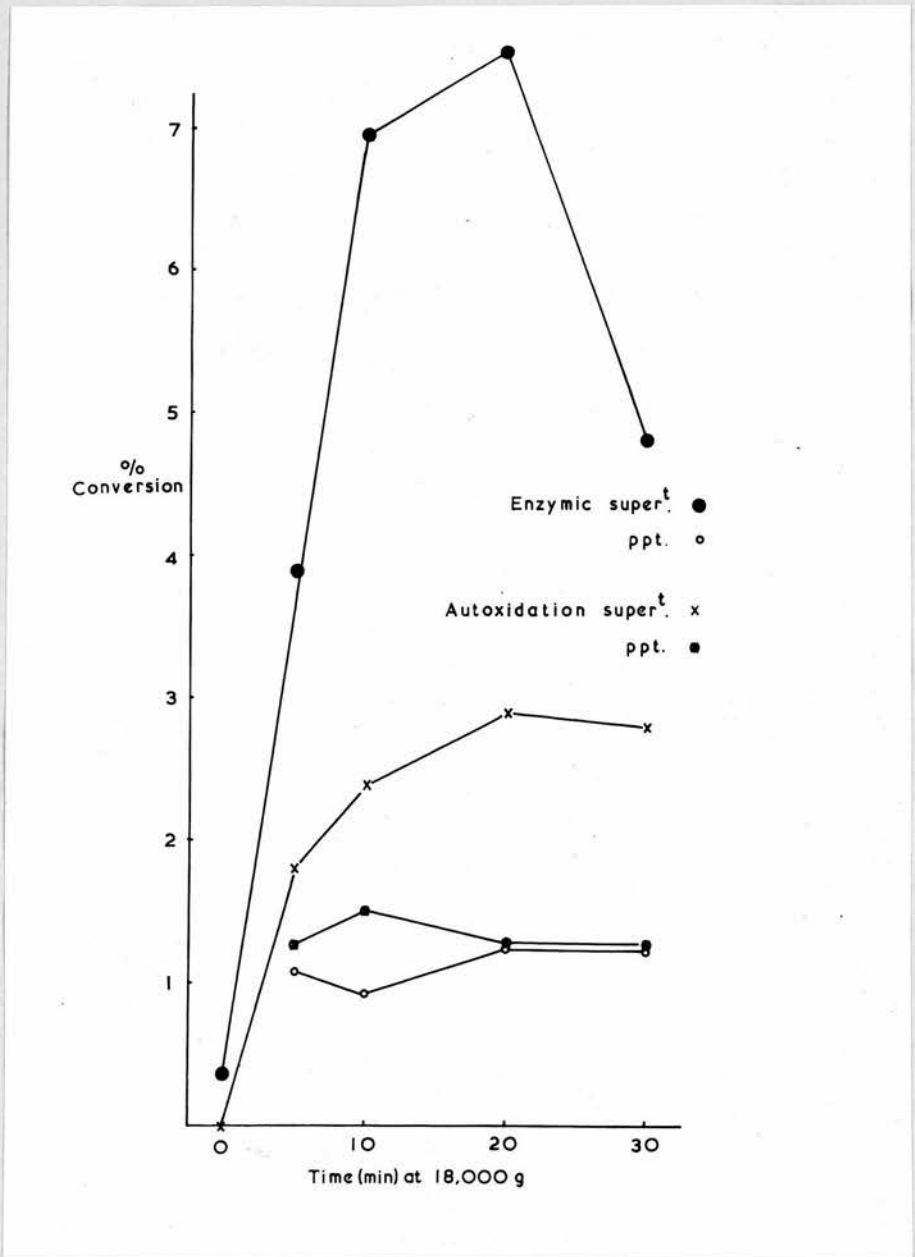


Figure 11: Metabolism of cholesterol-4-^{14}C by the supernatant and pellet fractions obtained by centrifugation for various times at 18,000 g.
Incubation for 1 hour at 37° with NADPH.

(ii) Speed of centrifugation.

In the preparation of the 18,000 g supernatant fraction, one centrifugation of the liver homogenate was employed to remove nuclei, cell debris and mitochondria. This centrifugation, equivalent to 4.5×10^3 g hours, is relatively high compared with values of 1×10^3 and 3×10^3 g hours used by other workers in the separation of the mitochondrial fraction. The results presented in this chapter were obtained by the incubation of the supernatant fraction and the pellet fraction after centrifugation of the liver homogenate for various times up to 30 minutes at 18,000 g. The two curves obtained for both enzymic and autoxidative activity the supernatant and pellet fractions are plotted in figure 11.

(a) Pellet

Enzymic and non-enzymic activity in the pellet from each centrifugation were approximately similar. After 10 minutes centrifugation, all of the mitochondria were probably sedimented, so that this low level of activity substantiates the finding above (section 4) that the mitochondrial fraction was not the site of the 7α-hydroxylase system.

(b) Supernatant

Whole liver homogenate which had not been centrifuged was almost devoid of activity (cf. section 4). Enzymic activity, expressed as the total of the three physiological products increased as the mitochondria were removed from the preparation. 7α-hydroxycholesterol accounted for 84-93% of the total of the three products, with maximal metabolism of the substrate occurring in the supernatant fraction isolated at between 10 and 20 minutes at 18,000 g. After 20 minutes the activity again fell. Metabolism to the three non-enzymic products increased to plateau level at about 20 minutes; no decrease after 20 minutes comparable to that found for the enzymic activity was observed.

It was concluded that the rapid increase in 7α-hydroxylase activity as the centrifugation time was increased was due to the removal of sub-cellular particles containing enzyme systems which compete with the hydroxylase system for the available co-factors, such as reduced NADP and other co-factors as yet unidentified. The drop in activity of the supernatant separated at 18,000 g for 30 minutes (9×10^3 g hours) cannot be explained on the same basis; the 7α-hydroxylase system has already been established to belong to the endoplasmic reticulum of the liver cell, and centrifugation for this time should not sediment

more than a small portion of the microsomes, which require about 100,000 g hours for complete sedimentation. However, it has been demonstrated that the enzyme system is sensitive to high speed centrifugation, whereby the endoplasmic reticulum is packed into pellet form. Possibly the reduction in activity observed in this experiment was due to an early stage in the packing process, implying that system is sensitive even to the preliminary accumulation of the endoplasmic reticulum prior to the formation of the microsomal fraction as a discrete pellet.

The results of this investigation show that the centrifugation time of 15 minutes at 18,000 g used for the isolation of the "microsomes + supernatant fraction" lay within the range determined for optimum 7α-hydroxylase activity.

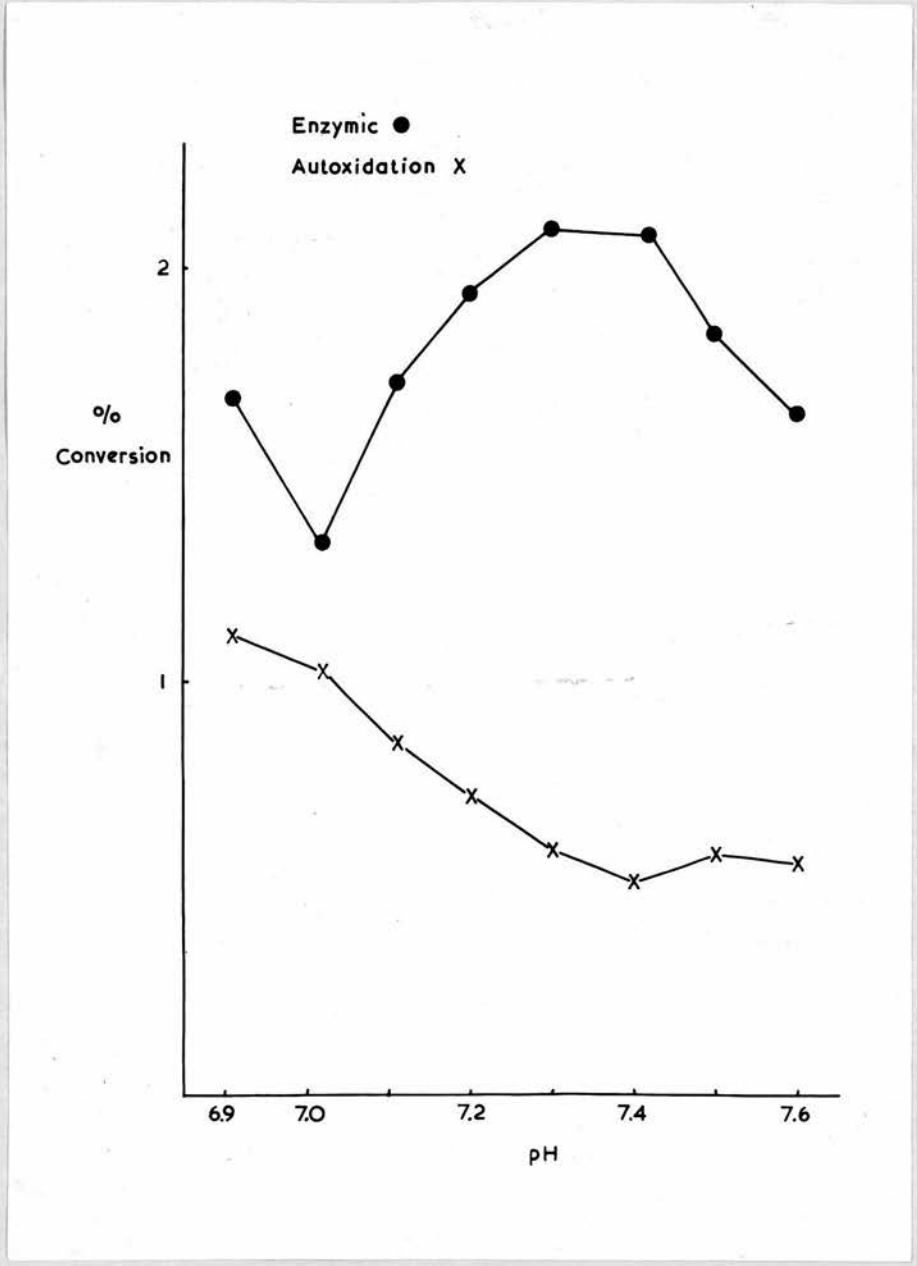


Figure 12: Metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant fraction at different pH values.
Incubation for 1 hour at 37° with NADPH.

(iii) Variation of the pH of the incubation medium.

The metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant was studied in incubation samples buffered with phosphate in the range 6.9 to 7.6 The results are shown in figure 12. 7α-hydroxycholesterol was the major enzymic product, accounting for 83-85% of the radioactivity in the three metabolites, except at pH 7.6 where this figure was 90%. The highest conversion to these products was found in the range pH 7.2 to 7.4, with the activity decreasing on either side of these limits. However, the enzymic products at pH 6.9 were found to be greater than the values found at pH 7.0; further investigation at lower pH values showed that this observation was indeed genuine. Autoxidation products, which remain fairly constant in the range of 7.2 to 7.6 also increased at pH values below 6.9. Rather erratic results were obtained at these lower proton concentrations, but it was evident that this was due to a significant stimulation of non-enzymic activity. Presumably the interruption of the smooth curve for the enzymic products in the range 7.0 to 7.6 was due to an enhanced formation of 7α-hydroxycholesterol from a non-enzymic source.

This investigation provides further indication that a substantial portion of the 7α-hydroxycholesterol isolated from incubations has arisen by an

autoxidative mechanism. However, the pH of the phosphate buffer normally used in incubations was 7.4 ± 0.02, which was within the range of pH values at which enzymic metabolism was optimum, but in the absence of a concomitant increase in non-enzymic activity.

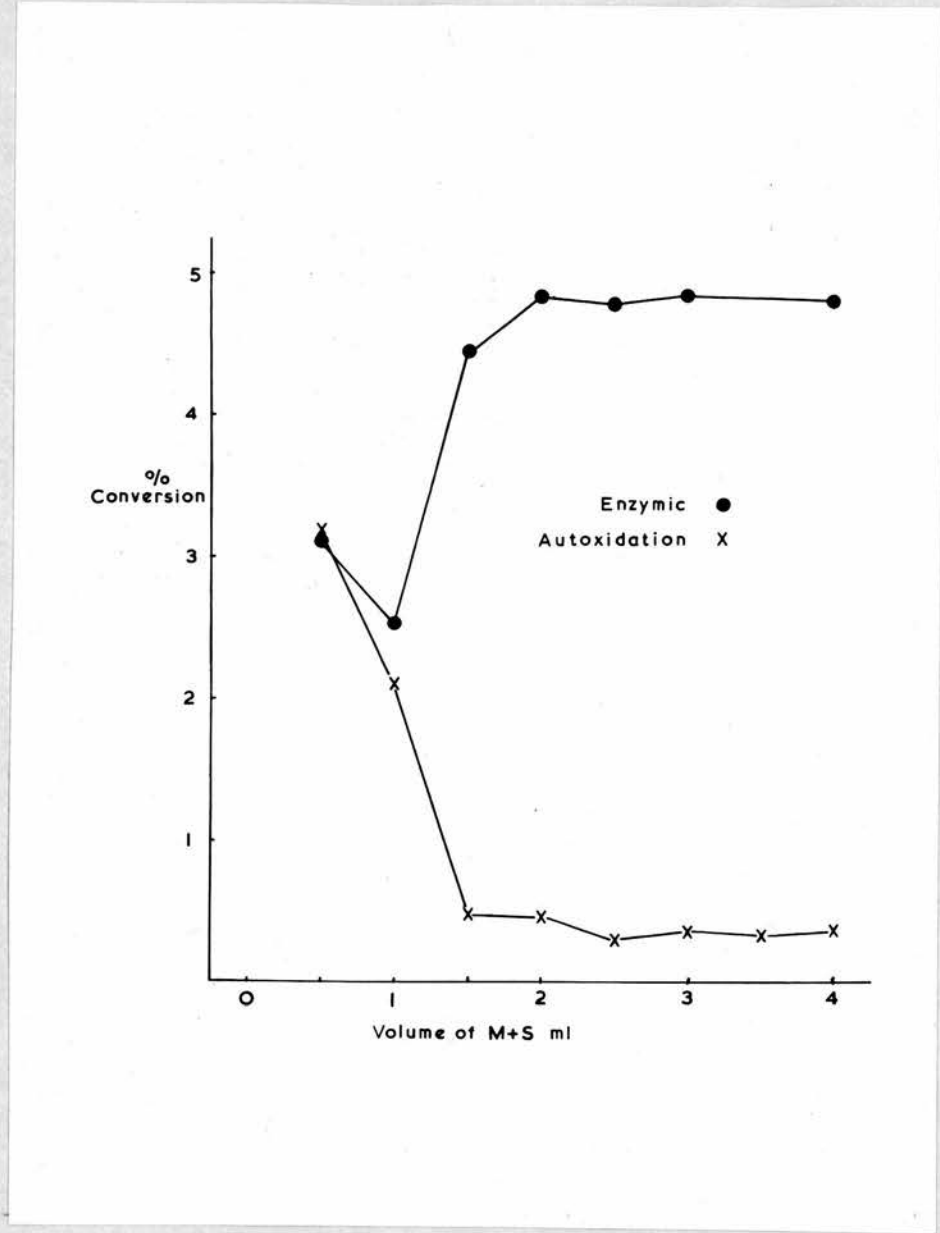


Figure 13: Metabolism of cholesterol-4-^{14}C by varying amounts of the enzyme preparation (ml. of 18,000 g supernatant fraction). Incubation for 1 hour at 37° with NADPH.

(iv) Metabolism of cholesterol-4-^{14}C by different concentrations of 18,000 g supernatant fraction

One of the large problems in the study of cholesterol catabolism by the liver is due to the large and variable amounts of free and ester cholesterol present in all of the sub-cellular fractions. In order to be able to study the metabolic processes with accuracy, it is necessary to use trace amounts of radioactively labelled cholesterol. This further complicates matters since there is no way of knowing exactly in what form and environment the endogenous cholesterol exists. It must be presumed that at least some of the labelled material equilibrates with the endogenous sterol, so that a radioactive assay of the metabolites can be used as a measure of the metabolism of the endogenous substrate. In this study, cholesterol-4-^{14}C was added as described in the form of the free sterol, in a small volume of acetone. If equilibration of this cholesterol with the endogenous pool destined for catabolism to the bile acids takes place, then it would be expected that, irrespective of the amount of enzyme present in the sample, the conversion of the labelled material to the various products would be invariable, depending only on the particular liver preparation under investigation.

Figure 13 shows the metabolism of a fixed amount of cholesterol-4-^{14}C by different amounts (volume) of the 18,000 g supernatant fraction. Samples containing less than 4 ml. of tissue preparation were made up to volume with 0.25 M. sucrose. The volumes of the microsomes + supernatant fraction were equivalent to 0-1.3 g. of whole liver.

No metabolism was observed in the absence of tissue. 7α-hydroxycholesterol, in the samples with 0.5 and 1.0 ml. of tissue accounted for 90 and 88% of the total enzymic products and from 1.5 to 4 ml. represented 96 to 92% of the total. Between 2.5 and 4 ml. of tissue, the metabolism to the enzymic products remained constant; the lowest value was found in the presence of 1.0 ml. of tissue, the sample preceding this being significantly higher. Non-enzymic product formation was greatest in the presence of the lowest concentrations of tissue, but this activity was quickly reduced as the amount of the enzyme increased. It appeared that susceptibility towards autoxidative attack increased as the samples were diluted, which also gave a less active enzymic system. The anomalous increase in the level of the "enzymic" products in the most dilute system was considered to be explicable in the same way as described above (iii), i.e. an enhancement of autoxidative activity results in an increase in 7α-hydroxycholesterol from a non-enzymic source.

These results show that within the range of 2.5 to 4 ml. of the 18,000 g supernatant in a total incubation volume of 7 ml. the measurement of the radioactivity in the products is a valid measure of the metabolism of endogenous substrate. Equilibration of the labelled sterol with endogenous cholesterol appeared to be excellent.

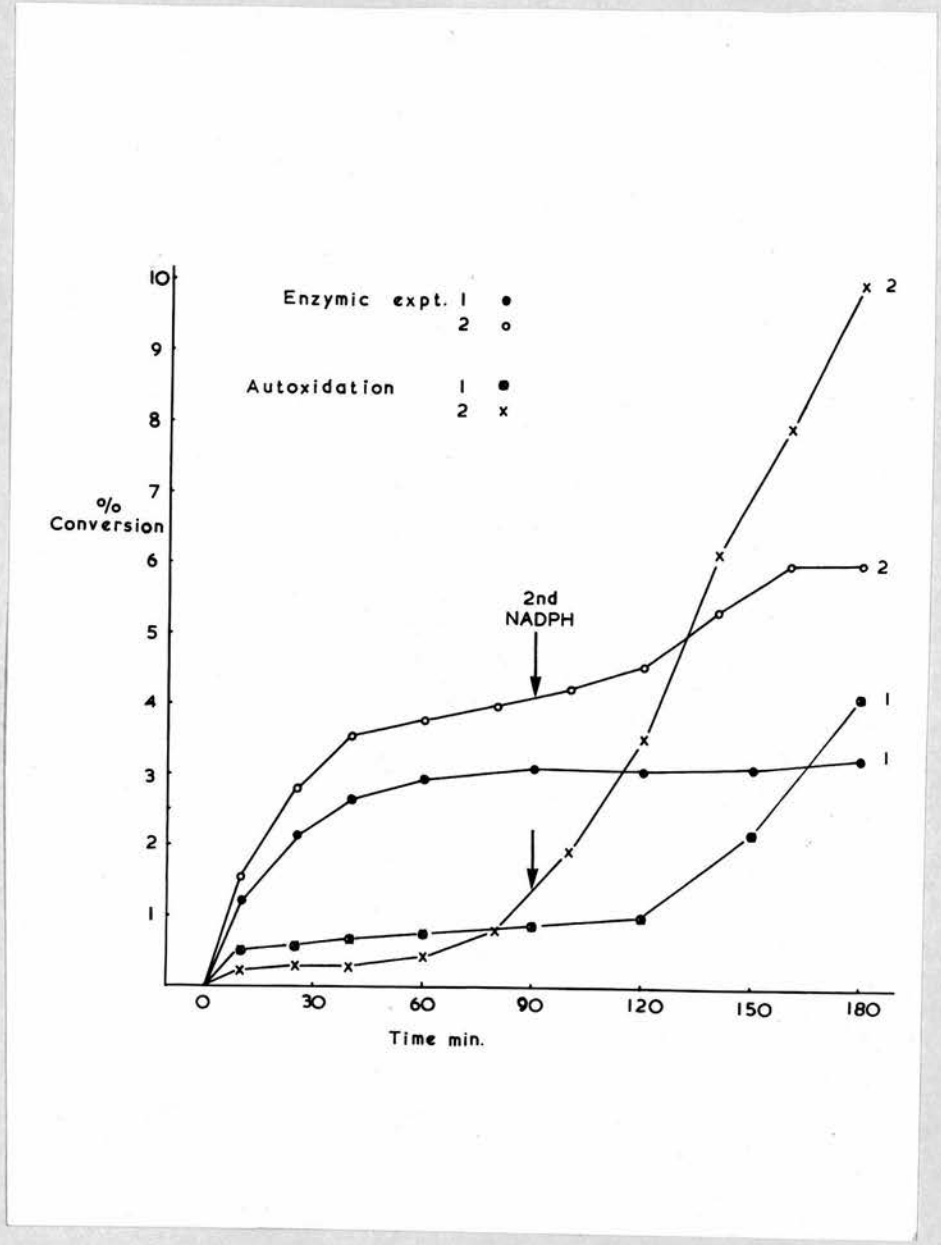


Figure 14: Metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant fraction. Incubation for 0-3 hours at 37° with NADPH.

(v) Time course of product formation by the 18,000 g supernatant

Figure 14 shows the formation of enzymic and autoxidation products by the 18,000 g fraction, in two experiments, over three hours.

Both graphs show that enzymic product formation was initially rapid, with a fall-off after 30-60 minutes. The product was almost entirely 7α-hydroxycholesterol during the initial rise. The oxidised product of 7α-hydroxycholesterol, cholest-4-en-3-on-7α-ol, slowly increased, especially in the plateau region after 30 minutes, suggesting that there may be a depletion of NADPH at this stage, allowing the oxidation reaction to proceed.

It will be noted that the level of activity in each experiment was markedly different, illustrating the variations which are found. Both curves have the same characteristics, and in both cases the level of autoxidation initially found was very low. At longer incubation times it was found that the autoxidation products rapidly increased until, in experiment 2, their total exceeded that of the enzymic products. The point at which this phenomenon occurred was different in the two experiments and this again must be explained by biological variation.

In experiment 2, a second addition of NADPH generator was made at 90 minutes, to determine if the lack of this co-factor was responsible for the effects. It appeared that this further addition did nothing to check the rise in the autoxidation products; it was also found that the initial rate of enzymic product formation could not be restored, although a small secondary stimulation was observed. However, in the presence of large amounts of autoxidation it is probable that a significant proportion of the 7α-hydroxycholesterol was formed non-enzymically, so that the actual stimulation of true enzymic activity was probably quite small.

It is considered that the failure to induce a secondary rate approximately the same as that found originally, was due to certain effects which became evident in the longer incubation times; these effects are also manifest in the large increase found in the autoxidation level occurring in this region. Possibly the tissue preparation, which was fortified only with NADPH, is unable to preserve its structural integrity for long periods; this results in a "decomposition" of the enzyme system and allows the cholesterol in the system to be exposed to an unphysiological environment in which autoxidation can rapidly occur.

In all of the experiments described in the later sections, incubations were conducted for no longer than

60 minutes, since longer incubation produced a rather confusing spectrum of products as shown in figure 14. However, in experiment 2, even at 60 minutes incubation the autoxidation products were beginning to increase, so that in those experiments where autoxidation was high, this effect may have become evident at an even earlier stage, and could account for the lower levels of enzymic activity which were found, relative to the formation of non-enzymic products.

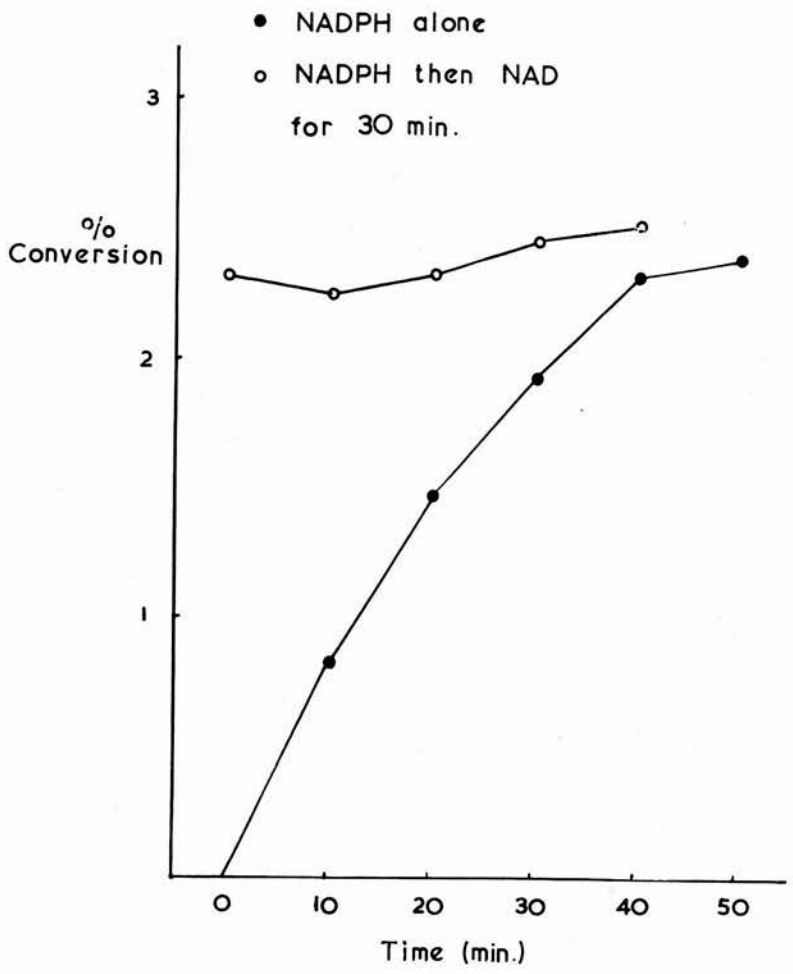


Figure 15: Metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant fraction with NADPH and NAD.
Incubation times shown in table 6.
NAD added to final concentration of 1.4 mM.

(vi) Product formation by the 18,000 g supernatant fraction with NADPH and NAD.

The metabolism of cholesterol-4-^{14}C was followed over 50 minutes under the conditions above in (v). Total enzymic products are plotted in figure 15, to give a curve of the form already described. 7α-hydroxycholesterol accounted for a high percentage of the enzymic products as shown in table 6.

At the incubation times shown, where a sample was removed for analysis, a duplicate sample was removed and incubated for a further 30 minutes after the addition of NAD. A series of samples was thus obtained, with total incubation times varying from 30 minutes to 70 minutes.

It was found that, irrespective of the length of "pre-incubation" period with NADPH, the same total metabolism of the substrate occurred to the three enzymic products, but the relative amounts of each of the products was radically altered by incubation with NAD which enhanced the further oxidation of 7α-hydroxycholesterol. NAD was therefore capable of increasing the rate of formation of the three products in the presence of NADPH, but could not affect the total conversion to these metabolites. It will be noted that the same conversion was attained by

Table 6

% 7α-hydroxycholesterol of total enzymic products in figure 15

7α-hydroxycholesterol
cholest-4-en-3-on-7α-ol
cholest-4-en-3-on-7α,12α-diol

Pre-incubation with NADPH (min.)	10	20	30	40	50	0	10	20	30	40
30 min. incubation with NAD	-	-	-	-	-	+	+	+	+	+
Total enzymic products	0.8	1.5	1.9	2.3	2.3	2.3	2.4	2.5	2.5	2.5
% 7α-OH chol.	61	75	73	74	71	29	37	36	36	34

incubating for 50 minutes with NADPH alone, which gave as the major product 7α-hydroxycholesterol.

This experiment shows that the availability of NADPH and NAD in the system can affect the amounts of the enzymic products relative to one another, but the total metabolism to these intermediates is regulated by some other factor in the preparation. This controlling influence does not appear to depend on the removal of 7α-hydroxycholesterol by further oxidation. NAD did not affect the autoxidation of cholesterol, the products of which totalled 1.5%.

The microsomal oxidation of 7α-hydroxycholesterol has been shown to be extremely efficient in the present of NAD[37], and NADP can also be utilised as the electron acceptor, although not as efficiently as NAD. Most of the incubations described in this work were found to effect a small and variable conversion of 7α-hydroxycholesterol to cholest-4-en-3-on-7α-ol and cholest-4-en-3-on-7α,12α-diol in the presence of an NADPH generating system; the results of the present experiment suggest that the oxidation of 7α-hydroxycholesterol depends on the relative concentrations of reduced and oxidised nucleotides in the preparation. The extent of the oxidation of this sterol may reflect the activities of the enzymes which catalyse the reduction, oxidation, hydrolysis, etc.

of pyridine nucleotides in different preparations. Variable concentrations of NADPH, NADP and NAD may therefore be provided for utilisation by the enzymes effecting the hydroxylation and oxidation of cholesterol and its metabolites.

Summary

1. The cholesterol-7α-hydroxylase system of the 18,000 g supernatant fraction was found to be sensitive to prolonged homogenisation.

2. The most active enzyme preparation was the supernatant fraction obtained by centrifuging liver homogenate at 18,000 g for 10-20 minutes.

3. The pH optimum of the hydroxylase system lay in the range pH 7.2-7.4

4. A trace amount of cholesterol-4-^{14}C was found to equilibrate with the endogenous cholesterol pool, allowing theradioactive assay to be used as a measure of the metabolism.

5. The 18,000 g supernatant rapidly metabolised cholesterol-4-^{14}C to 7α-hydroxycholesterol during the first 30 minutes of the incubation. At long incubation times, the situation was confused by large and rapid increases in the autoxidation products.

6. 7α-hydroxycholesterol generated in the incubation with the 18,000 g fraction could be oxidised by further incubation with NAD; this nucleotide cannot increase the overall enzymic conversion and affects only the rate of product formation and the distribution of the three products.

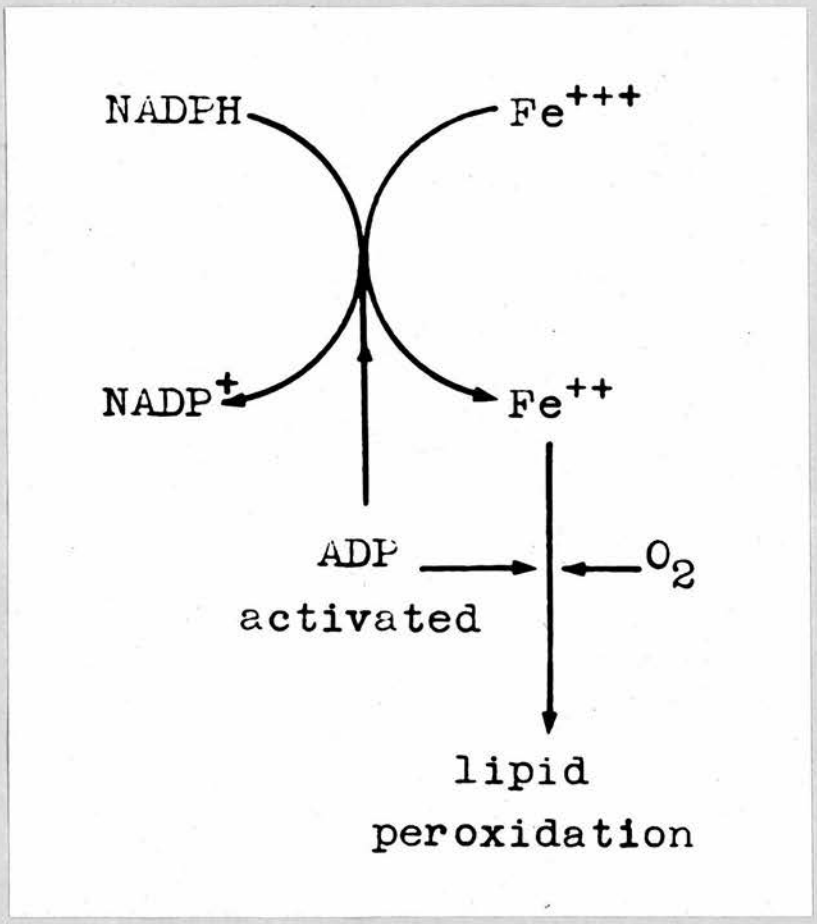


Figure 16: Schematic representation of microsomal lipid peroxidation catalysed by ADP, NADPH and Fe^{++}.

Section 6.

Metabolism of cholesterol-4-^{14}C under 'lipo-peroxidation conditions'.

In section 3 several oxygenated derivatives were investigated as possible intermediates in both enzymic and autoxidation mechanisms. It was concluded that the hydroperoxides of cholesterol could be involved in autoxidation, but there was no evidence for their involvement with enzymic 7α-hydroxylation.

The results presented in this section are concerned with the *in vitro* metabolism of cholesterol under conditions which favour peroxidation of unsaturated lipids. Evidence from various sources suggests that peroxidation of lipids catalysed by liver microsomes is linked to NADPH oxidation; various nucleotides such as ADP have been found to promote a large increase in oxygen uptake by microsomes with a concomitant increase in the aldehydic products of peroxidation.[75,76] A probable scheme is shown in figure 16. The potency of ADP is considered to be due to contamination with ferrous ions. In such investigations, no lipid peroxides can be isolated, and their existence is assumed from the nature of the products formed. This can be compared with the system postulated for initial oxygen attack on the cholesterol molecule, i.e. formation

Table 7

Comparison of normal assay system with 'lipo-peroxidation' system

	Normal	Lipoperoxidation
Tissue (1.3 g. liver)	4 ml. 18,000 g supernatant	4 ml. microsomes (0.154 M KCl)
Buffer	2 ml. 0.1 M phosphate pH 7.4	2 ml. 0.05 M tris pH 7.4
NADPH generator	1.1 ml.	1.1 ml.
ADP	-	0.7 mM
Ferrous Sulphate	-	2.5 mM

of an unstable oxygenated intermediate, a hydroperoxide, which decomposes to the products eventually detected.

The tissue fraction and incubation conditions used in this study were based on that of Ernster[75]. The system is composed as shown in table 7 of microsomes in tris/hydrochloride buffer, with NADPH in the normal concentration.

The terminology of enzymic and autoxidation products has been used in this section as in the rest of the thesis. In this section especially, it must be stressed that the use of these terms is basically to simplify the expression of results; it will become obvious under the conditions described here that the distinction between autoxidation and enzymically formed products cannot be made.

(i) Metabolism of cholesterol-4-^{14}C by fortified microsomal suspensions.

Table 8 shows the metabolism of cholesterol-4-^{14}C by microsomal preparations in tris/hydrochloride buffer under the influence of NADPH, ADP and ferrous ions and combinations of these co-factors. The concentrations used are those shown in table 7.

(1) The total products formed by the eight preparations tended to fall roughly into two groups - those having a high conversion of the substrate

Table 8

Metabolism of cholesterol-4-^{14}C by fortified microsomal suspensions

Addition	-	ADP	NADPH	ADP NADPH	Fe^{2+}	Fe^{2+} ADP	Fe^{2+} NADPH	Fe^{2+} ADP NADPH
Total conversion %	1.1	2.3	3.5	15.3	6.7	16.5	13.3	14.7
% of total products: 'Enzymic'	41	23	39	13	18	21	20	16
Autoxidation	59	77	61	87	82	79	80	84
7-keto-cholesterol	20	49	32	55	65	61	61	60

(samples d, f, g and h - averaging 15%) and those having a low conversion (a, b, c and e - averaging 3.4%). The most active systems in terms of overall metabolism of the substrate were the samples fortified with ferrous ions with one or both of the two nucleotides. ADP + NADPH with no added ferrous ions also produced an active system.

(2) Products defined in section 3 as 'enzymic'. Metabolism to 7α-hydroxycholesterol and cholest-4-en-3-on-7α-ol was small by comparison with the three 'autoxidation' products. Only in two preparations, (a) and (c) were the amounts of these two metabolites at all significant relative to the overall metabolism of the substrate; these preparations metabolised only a small amount of the substrate so that the enzymic product formation was small. 7α-hydroxycholesterol was the major of the two enzymic products in the samples with NADPH indicating that further oxidation to cholest-4-en-3on-7α-ol was suppressed in the strongly reducing systems. In none of these samples was there a selective conversion to 7α-hydroxycholesterol or its oxidation product; where there was significant metabolism to these products, there was a much larger conversion to the three autoxidation products. It was concluded in these circumstances that there was no evidence of enzymic 7α-hydroxylation of the substrate.

(3) Products defined as 'autoxidation'. A substantial conversion to the 'non-enzymic products' occurred in the samples described in (a') as having a high overall metabolism of the substrate. Of the three products, metabolism to one in particular was exceptionally high. This product, having the polarity of 7-keto-cholesterol and tentatively identified as this sterol, accounted for 55-65% of the total of all five products under the conditions shown.

(ii) Metabolism in fortified suspensions of acetone powders.

The metabolism of cholesterol-4-^{14}C was investigated under the influence of the same co-factors described above in (i) using suspensions of microsomal acetone powders. The results are shown in table 9, and indicate that extensive metabolism of the substrate occurred under the same conditions as with native microsomes. A low conversion was found in the sample with ferrous ions as the only addition. The reason for this is thought to be the lack of endogenous co-factors in the acetone powder (by virtue of its method of preparation section 2); these co-factors in native microsomes could produce a partially active system when stimulated by ferrous ions.

Table 9

Metabolism of cholesterol-4-^{14}C by fortified suspensions of microsomal acetone powder (70 mg/sample)

	a	b	c	d	e	f	g	h
Addition	-	ADP	NADPH		Fe^{2+}	Fe^{2+} ADP	Fe^{2+} NADPH	Fe^{2+} ADP NADPH
Total conversion %	0.7	1.6	3.0		1.4	12.1	14.8	14.6
% of total products: 'Enzymic'	43	25	30		29	16	15	14
Autoxidation	57	75	70		71	84	85	86
7-keto-cholesterol	14	31	30		36	62	62	62

Table 10

Comparison of the effects of ADP, ATP, 5^1-AMP and $3^1 5^1$-AMP on microsomal cholesterol oxidation

1 Hour incubation at 37° with NADPH, Fe^{2+}

Addition	ADP	ATP	5^1-AMP	$3^1 5^1$-AMP
% total products	9.9	6.4	1.6	1.6
*Enzymic products	1.8	1.2	0.4	0.4

(iii) Comparison of the effects of ADP, ATP, 5^1-AMP and $3^1 5^1$-AMP

The metabolism of cholesterol-4-^{14}C was investigated in microsomal suspensions, fortified with NADPH and ADP, ATP, 5^1-AMP or $3^1 5^1$-AMP. The results are shown in table 10. Overall conversion of the substrate was highest in the presence of ADP; ATP produced a less active system, but this was significantly more active than with 5^1-AMP or $3^1 5^1$-AMP.

Enzymic products, 7α-hydroxycholesterol with small amounts of cholest-4-en-3-on-7α-ol, were not formed selectively in the presence of any of these nucleotides. The 7-keto-cholesterol compound accounted for most of the more polar products in each case.

(iv) Comparison of phosphate and tris buffers.

Table 11 shows the product formation by microsomal suspensions in tris and phosphate buffers (0.05 M pH 7.4) and the effect of ADP on these preparations. Total metabolism in the phosphate systems was low, even in the presence of ADP. The tris buffered preparation required ADP before a significantly higher conversion was obtained. 7α-hydroxycholesterol in these samples again accounted for only a small portion of the total products.

Table 11

Comparison of the effects of tris and phosphate buffers on microsomal cholesterol oxidation

Incubation 1 hour at 37° with NADPH

	tris		phosphate	
Addition	-	ADP	-	ADP
% total products	1.5	6.9	1.0	2.5
'enzymic'	0.2	0.9	0.2	0.4

The results described in this section showed that metabolism of cholesterol-4-^{14}C by the microsomal fraction was markedly enhanced by NADPH, ADP and ferrous ions. ADP of several nucleotides tested gave the largest stimulation of cholesterol oxidation, and this has also shown to be the case with general peroxidation of unsaturated lipids by the microsomal fraction. The two systems therefore appeared to be stimulated under the same conditions. Cholesterol, itself a lipid, may be susceptible to a peroxidative attack; peroxidation of lipids probably occurs in the system described above, although this has not been measured, so that cholesterol oxidation may be one part of the extensive peroxidation system of the microsomes.

The major product formed from cholesterol under these conditions was tentatively identified as 7-keto-cholesterol from its mobility on thin layer chromatography. Identification of 7-keto-cholesterol as a major autoxidation product (section 3) was unsatisfactory, and the results suggested that, while 7-keto-cholesterol certainly appeared to be a major constituent of this fraction, at least one other product was also present. Reference was made in section 3 to various oxygenated sterols (hydroperoxides and epoxides of cholesterol) which have approximately the same mobility as 7-keto-cholesterol. 7α-hydroxycholesterol could be formed from the

hydroperoxides of cholesterol, but this could occur in boiled tissue samples; the spectra of products formed from these hypothetical intermediates indicated that they were not formed to a large extent in in vitro work. However, in the present section, a system has been described which could oxidise significant amounts of cholesterol-4-^{14}C in the presence of co-factors known to enhance the peroxidation of unsaturated lipids. Various investigations were performed on the apparently heterogeneous 7-keto-cholesterol fraction to find out if there was any evidence of hydroperoxide or epoxide formation in this system.

Investigation of the '7-keto-cholesterol' material.

(i) Re-incubation.

The labelled product was isolated after chromatography, and re-chromatographed to remove traces of other metabolites. It was then re-incubated under the conditions shown in table 12.

A significant but variable metabolism was observed in each case. The major products were cholestan-3β,5α,6β-triol, 7β-hydroxycholesterol and an unidentified non-polar metabolite. The amounts of 7α-hydroxycholesterol were small. The differences in the conversion of the substrate (31 to 83%) were not apparently due solely to the tissue preparation used; a sample incubated for 30 or 60 minutes with microsomal acetone powder gave almost the same metabolism of the substrate, suggesting that the 'substrate' remaining consisted of a material which could not be metabolised. The metabolism by identical tissue preparations of material isolated from different incubations, (samples with 18,000 g supernatant fraction) also appeared to differ markedly, suggesting that the unknown fraction had a variable composition.

The products of these incubations did not conform to a definite pattern, e.g. the amount of tri-hydroxylated sterol relative to 7β-hydroxycholesterol

Table 12

Re-incubation of 7-keto-cholesterol-like compound with NADPH.

System	Microsomes ADP, Fe^{2+}		Acetone powder ADP, Fe^{2+}		18,000 g supernatant
Time	30	60	60	60	60
% 'triol'	16	14	25	13	21	33
7α-OH chol.	1	3	0	2	4	0
7β-OH chol.	8	8	21	23	22	49
Non-polar material	6	10	26	27	10	1
Total conversion %	31	35	72	65	57	83

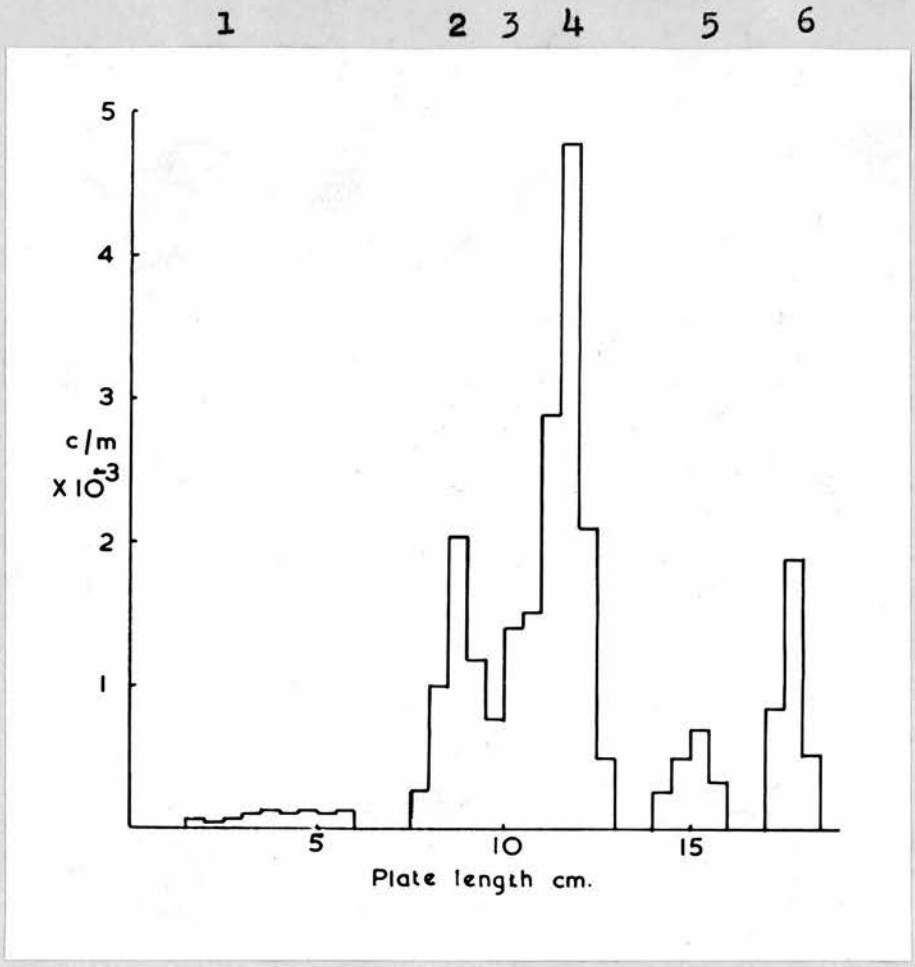


Figure 17: Reduction of the 7-keto-cholesterol-like material with lithium aluminium hydride.

1. cholestan-3β,5α,6β-triol
2. cholestan-3β,6β-diol
3. 7α-hydroxycholesterol
4. 7β-hydroxycholesterol
5. '7-keto-cholesterol'
6. non-polar product.

could be greater or less. The most obvious source of 7β-hydroxycholesterol is 7-keto-cholesterol (section 3). However, only small amounts of triol were shown to be formed from 7-keto-cholesterol, certainly in much smaller amounts than were found by incubation of the unknown material. Formation of 7α-hydroxycholesterol appeared to be about the same as that found by incubation of standard 7-keto-cholesterol, so that triol was the only product which could not be explained by the metabolism of 7-keto-cholesterol.

(ii) Reduction with lithium aluminium hydride.

A sample of the radioactive 7-keto-cholesterol fraction was reduced with lithium aluminium hydride (Appendix), to give the radioactive products shown in histogram form in figure 17. 7β-hydroxycholesterol was formed in large yield, with smaller amounts of 7α-hydroxycholesterol. Reduction of 7-keto-cholesterol gives predominantly the 7β-diol, with some 7α-epimer, so that these products would appear to have come from 7-keto-cholesterol. An unidentified non-polar compound was also formed, together with a material which was more polar than 7α-hydroxycholesterol. A sterol having such a mobility is cholestan-3β,6β-diol, which is the major product in the reduction of cholesterol-5β,6β-epoxide. It therefore appeared that this epoxide could be present in the

7-keto-cholesterol fraction, and this derivative could also account for the formation of the triol by hydrolytic cleavage in a similar manner to that described for the α-epoxide (section 3).

From the results presented in (i) and (ii) above it was concluded that at least two compounds were present in the 7-keto-cholesterol fraction from incubations. One of these compounds appeared to be 7-keto-cholesterol; the other may be a mono-oxygenated derivative of cholesterol formed by 'peroxidation' of cholesterol.

The investigations in the present section and in section 3 have failed to establish a possible mechanism of the non-enzymic formation of 7α-hydroxycholesterol. This sterol can be formed in small yield from various oxygenated sterols which could conceivably be produced by initial oxygen attack on the cholesterol molecule. No evidence was obtained for the existence of oxygenated intermediates like hydroperoxides, which have already been shown to be efficient precursors of 7α-hydroxycholesterol by a non-enzymic mechanism. Incubation and reduction studies of the 7-keto-cholesterol fraction indicated that only small amounts of 7α-hydroxycholesterol could be formed from this source.

Table 13

Effect of diphenyl-phenylenediamine on cholesterol oxidation by microsomes. 1 hour incubation at 37° with NADPH, Fe^{2+}, ADP.

DPPD Concentration μM	0	0.1	1.0	5.0
Total products %	11.5	1.3	1.2	1.0

It was evident however that 7α-hydroxycholesterol could be formed non-enzymically by tissue preparations, especially where enzymic conversion to this sterol was inhibited, so that a mechanism for this reaction does exist.

These studies gave some indication of the complexities of non-enzymic cholesterol oxidation, with the peroxidative effect appearing as a markedly enhanced autoxidative effect. Staudinger[77] has shown that simple systems with metal ions, an electron donor and oxygen can hydroxylate organic substrates, and has postulated the formation of free radicals such as •OH and •OOH as the hydroxylating species. Preliminary investigations of cholesterol oxidation in the presence of diphenyl-phenylenediamine, a free radical trapping agent, showed that low concentrations of this compound could effect a substantial reduction of the oxidation (table 13). This suggests that non-enzymic cholesterol oxidation may involve free radical attack; lipid peroxidation is affected in the same way by free radical trapping agents, indicating a further similarity between the two systems.

Summary

(1) Cholesterol-4-^{14}C can be oxidised in large yield by the microsomal fraction fortified with co-factors known to enhance general peroxidation of unsaturated lipids. "Peroxidation" of cholesterol appeared to be an enhancement of autoxidation.

(2) The heterogeneous nature of the 7-keto-cholesterol was investigated; 7-keto-cholesterol appeared to be the major component of this fraction which alone accounted for more than 50% of the oxidation products of cholesterol.

(3) Conclusive evidence of a non-enzymic route to 7α-hydroxycholesterol was not obtained.

(4) Preliminary investigation with a free radical trapping agent suggested that autoxidation or peroxidation of cholesterol occurred by a free radical mechanism.

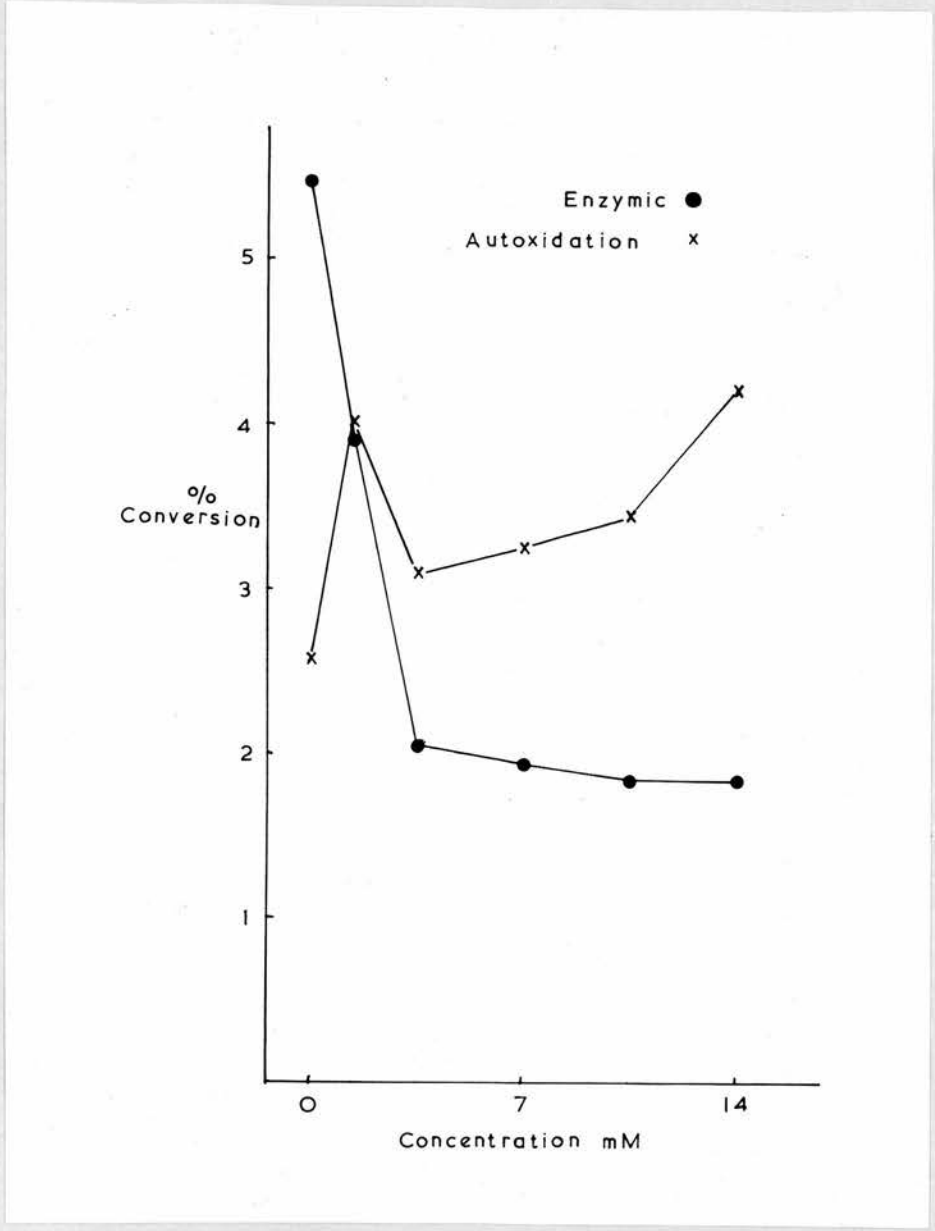


Figure 18: Metabolism of cholesterol-4-^{14}C by the 18,000 supernatant fraction with NADPH in the presence of magnesium ions. 1 hour at 37°.

Section 7.

(1) Metabolism of cholesterol-4-^{14}C in the presence of metal ions

(a) Magnesium

(i) 18,000g supernatant fraction

The metabolism of cholesterol-4-^{14}C was studied in the presence of various concentrations of magnesium sulphate (0-14 mm.). The results are plotted in figure 18.

As the magnesium ion concentration increased, formation of the enzymic products, 7α-hydroxycholesterol, cholest-4-en-3-on-7α-ol, and cholest-4-en-3-on-7α,12α-diol, from cholesterol decreased from 5.5% to 1.9%. Of these products, about 0.5% was accounted for by the two metabolites of 7α-hydroxycholesterol, which were found in approximately equal amounts. The formation of these compounds appeared to be unaffected by the presence of magnesium ions. Hence, 7α-hydroxycholesterol was the only product which was affected by the magnesium ion concentration. Inhibition was observed at 1.4 mM, increasing to about 65% inhibition at 3.5 mM magnesium ions. Formation of autoxidation products was found to increase from 2.6% to 4.2% conversion. The reason

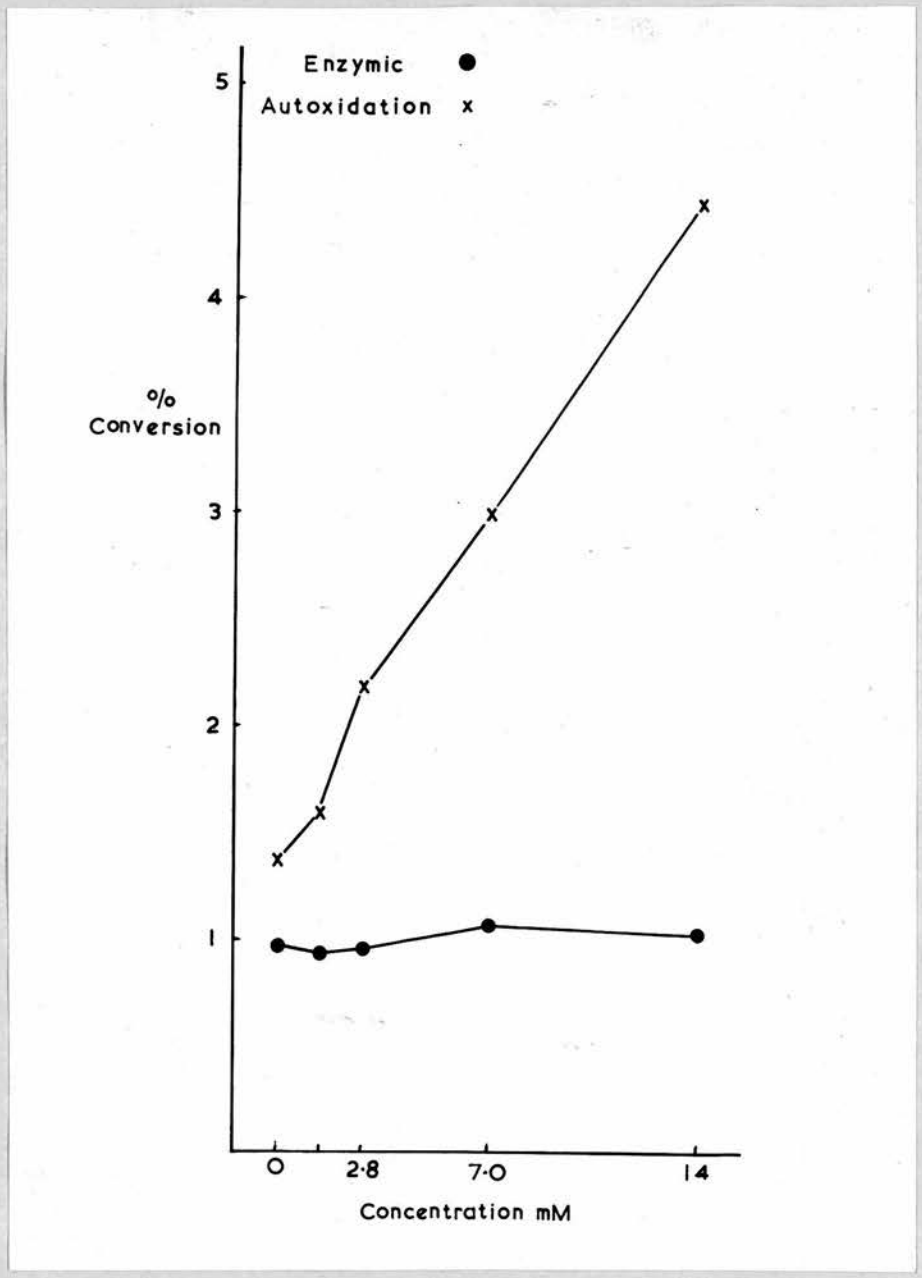


Figure 19: Metabolism of cholesterol-4-^{14}C by the microsomal fraction with NADPH in the presence of magnesium ions. 1 hour at 37°.

for the apparently anomalous kinetics of the increase in autoxidation with respect to metal ion concentration is not known. The overall effect of magnesium ions appeared to be stimulation of the formation of non-enzymic products.

(ii) Microsomal fraction

In figure 19 are shown the results of an experiment performed under the same conditions as described in (i), using the microsomal fraction in place of the 18,000 g supernatant.

Autoxidation was again markedly stimulat-:ed, and showed an increase from 1.4% to 4.4% total conversion. No anomalies were observed in the curve. In contrast to the 18,000 g supernatant fraction, no inhibition of enzymic activity was evident in the microsomal fraction. The enzymic product formation was low (1.0-1.1% total conversion) and about 0.6% was accounted for as 7α-hydroxycholesterol, the remainder being cholest-4-en-3-on-7α-ol. No 12α-hydroxylated derivative was formed in this experiment. In both the microsomal fraction and the 18,000 g supernatant fraction, the effect of added magnesium ions on the formation of autoxidation products was similar. A stimulation of non-enzymic products was observed up to a final concentration of magnesium ions of 14 mM.

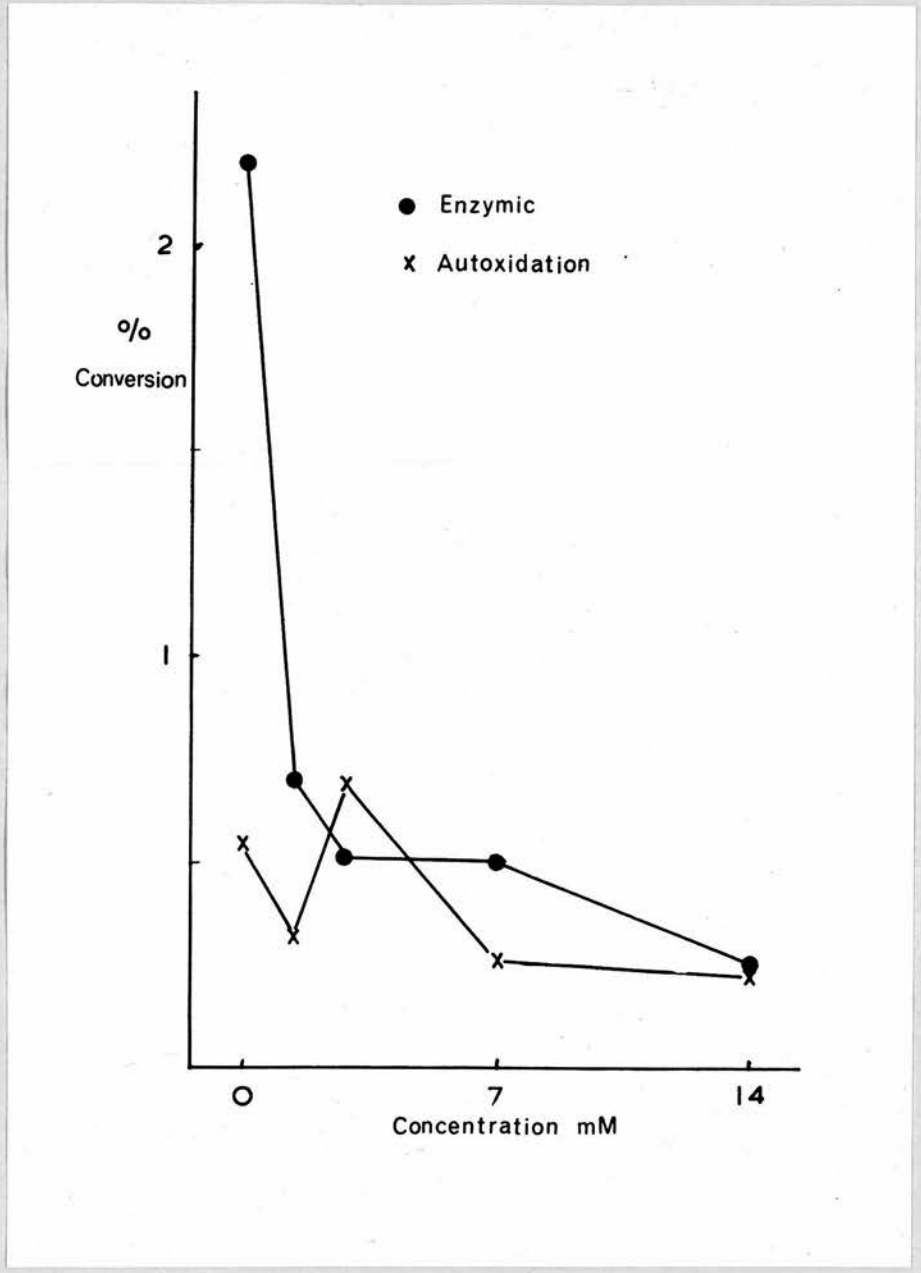


Figure 20: Metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant fraction with NADPH in the presence of manganese ions. 1 hour at 37°.

In view of the great increase in the amounts of autoxidation products, a significant proportion of the 7α-hydroxycholesterol may have been formed by a non-enzymic route. There appeared to be almost total inhibition of enzymic activity at concentrations of about 3.5 mM with the 18,000 g supernatant. Thus the apparent lack of inhibitory effect on the microsomal enzymic activity may be due to the fact that a significant portion of this sterol has been formed non-enzymically. Enzymic activity in the separated microsomal fraction has already been shown to be low (section 4).

(b) Manganese ions

Incubations were conducted with the addition of manganese chloride in increasing concentrations from 0-14 mM. Only the 18,000 g supernatant fraction was used in this investigation. The results are plotted in figure 20.

Total enzymic activity decreased from 2.2% in the absence of added manganese ions to 0.2% at the highest concentration. 0.4% of the first total was accounted for by the two oxidised derivatives of 7α-hydroxycholesterol, and thereafter these products collectively accounted for 0.2% of the enzymic total. The formation of neither of these products appeared to

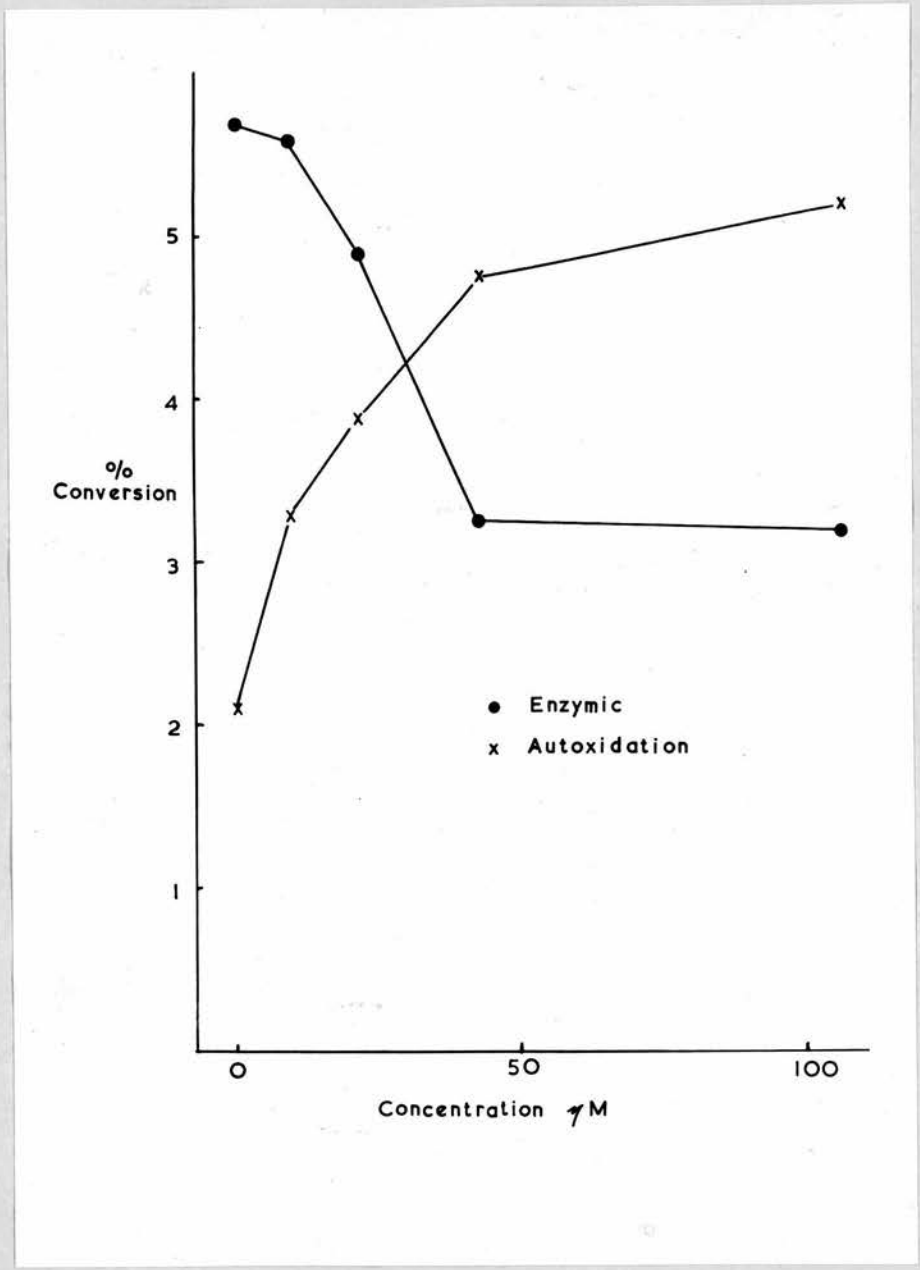


Figure 21: Metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant fraction with NADPH in the presence of cupric ions. 1 hour at 37°.

be completely inhibited by manganese ions. 7α-hydroxylase activity however was significantly depressed in the presence of 1.4 mM final concentration of this ion. With increasing concentrations there was a gradual decrease to about 60% of the original enzymic activity of the control sample.

In contrast to the effect of magnesium ions, manganese ions did not produce a marked rise in the level of autoxidation products, except in the sample with 0.28 mM manganese ions (cf. magnesium).

(c) Copper

Figure 21 shows the results of an experiment of the type described above with the 18,000 g supernatant fraction in the presence of increasing concentrations of cupric ions.

Conversion to the enzymic products, 7α-hydroxycholesterol with cholest-4-en-3-on-7α-ol, varied from 5.7% in the control sample to 3.2% in the presence of 0.1 mM cupric ions. No cholest-4-en-3-on-7α,12α-diol was detected in this experiment. Formation of cholest-4-en-3-on-7α-ol was apparently unaffected by variations in the cupric ion concentration. Over the range studied, an inhibition of about 50% in the 7α-hydroxylase activity was observed.

In contrast to the inhibitory effect of cupric ions on the enzymic activity, the autoxidation products were increased from about 2 to 5%.

The ability of the three ions to stimulate autoxidative conversion was different. Cupric and magnesium ions both caused significant increases in the autoxidation products, while manganese ions inhibited. The effect of the latter ion was small by comparison with the other two, but nevertheless gave a significant reduction of autoxidation over the range tested. Whatever the site of action of these ions, the effects cannot be attributed to an effect induced by di-valent ions on the autoxidation mechanisms - two of the ions stimulated such mechanisms, while the third was inhibitory. Possibly these effects must be considered separately from the effects on the enzymic mechanisms, since it is presumed that formation of the two groups of enzymic and autoxidative products are different. It will be noted that the inhibition by cupric ions occurred at a much lower concentration than the other two. In general, it appeared that metal ions increased the "unphysiological" nature of the tissue preparation (cf. ferrous ions in section 6) and in doing so promoted the non-enzymic attack of cholesterol with a concomitant reduction in enzymic activity. The

further oxidation of 7α-hydroxycholesterol was not markedly inhibited by these ions.

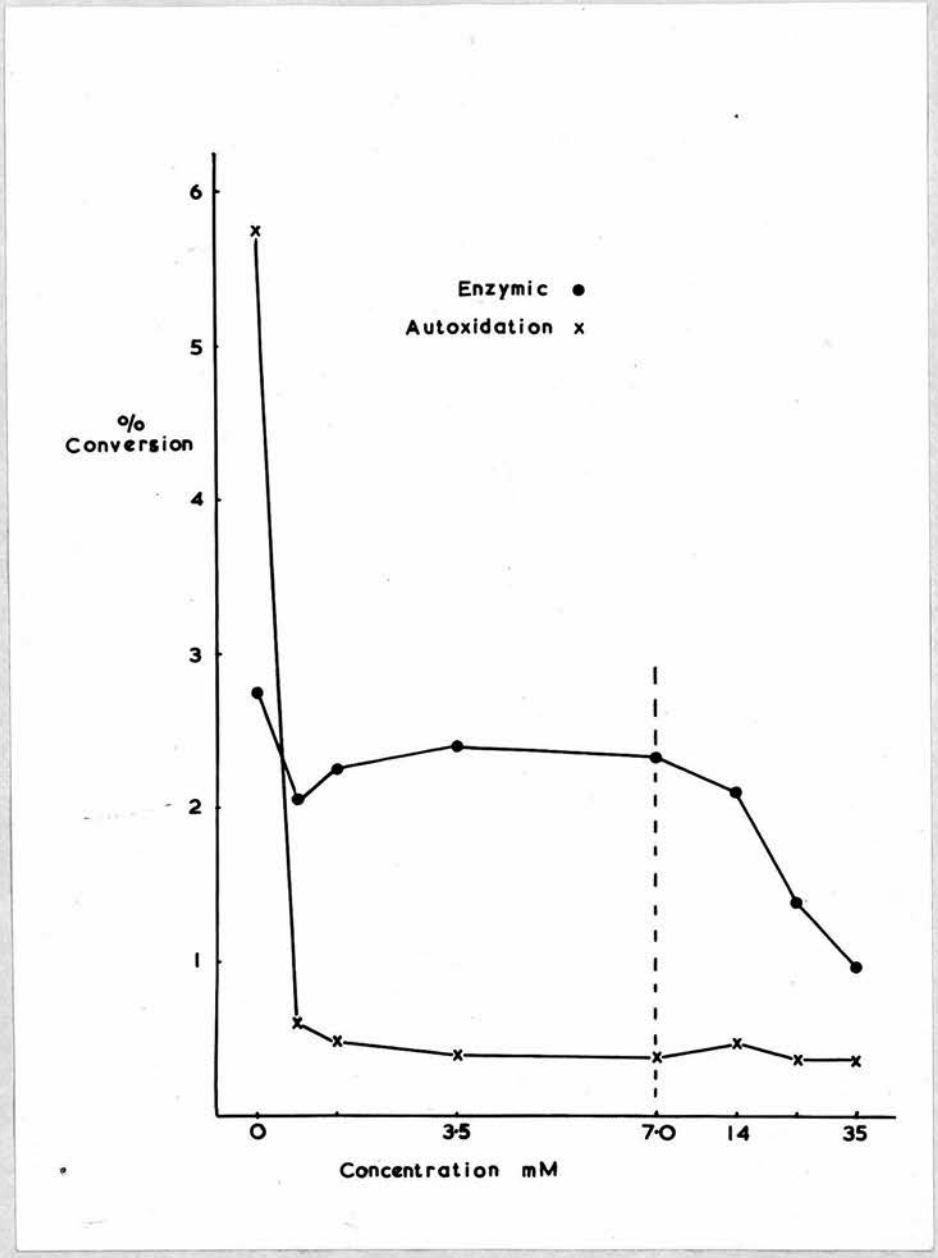


Figure 22: Metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant fraction with NADPH in the presence of EDTA.
1 hour at 37°.

(2) Metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant fraction in the presence of metal ion sequestering agents.

(i) EDTA (ethylenediamine tetra-acetic acid).

In the first part of this section, the inhibitory effects of various metal ions were discussed. The endogenous concentrations of metal ions in subcellular preparations may vary considerably, and it is conceivable that a certain amount of inhibition may be present in the preparations, or that the 7α-hydroxylase system requires certain metal ion(s) for activity.

Figure 22 shows the conversion of the substrate cholesterol by the 18,000 g supernatant fraction in the presence of concentrations of EDTA varying from 0 to 35 mM. Enzymic products consisted of 7α-hydroxycholesterol, cholest-4-en-3-on-7α-ol and the 12α-hydroxylated derivative. A slight stimulation of the enzymic products was found in the concentration range from 1.4-7 mM. At the lowest concentration of EDTA, the enzymic total was lower than the control value with no EDTA. Throughout the range of concentrations used, the ratio of 7α-hydroxycholesterol to the total of the enzymic products remained constant, indicating that there was no stimulation of the further metabolism of 7α-hydroxycholesterol under these

conditions.

Non-enzymic product formation, in the control sample representing 5.8% conversion of the added cholesterol, was almost entirely eliminated at the lowest EDTA concentration used. The total of these autoxidation products remained at 0.6-0.4% over the rest of the range.

It was observed that the dramatic fall in the non-enzymic activity in the presence of 0.7 mM EDTA occurred at the same time as the fall in the enzymic products. This finding supports the view that where autoxidation effects were large, a significant proportion of the metabolism to 7α-hydroxycholesterol was by a non-enzymic pathway; in the present investigation, the difference in the values for the enzymic activity in the control sample and at 0.7 mM represents that portion of the total 7α-hydroxy-cholesterol which has arisen non-enzymically, and which has been almost completely inhibited in the presence of EDTA. In this event, the enzymic activity of the control sample probably more closely approximates to the activity found at 0.7 mM EDTA; the difference between this sample and the activity in the plateau region would therefore be a stimulation of about 13%.

The results indicate that EDTA complexes with

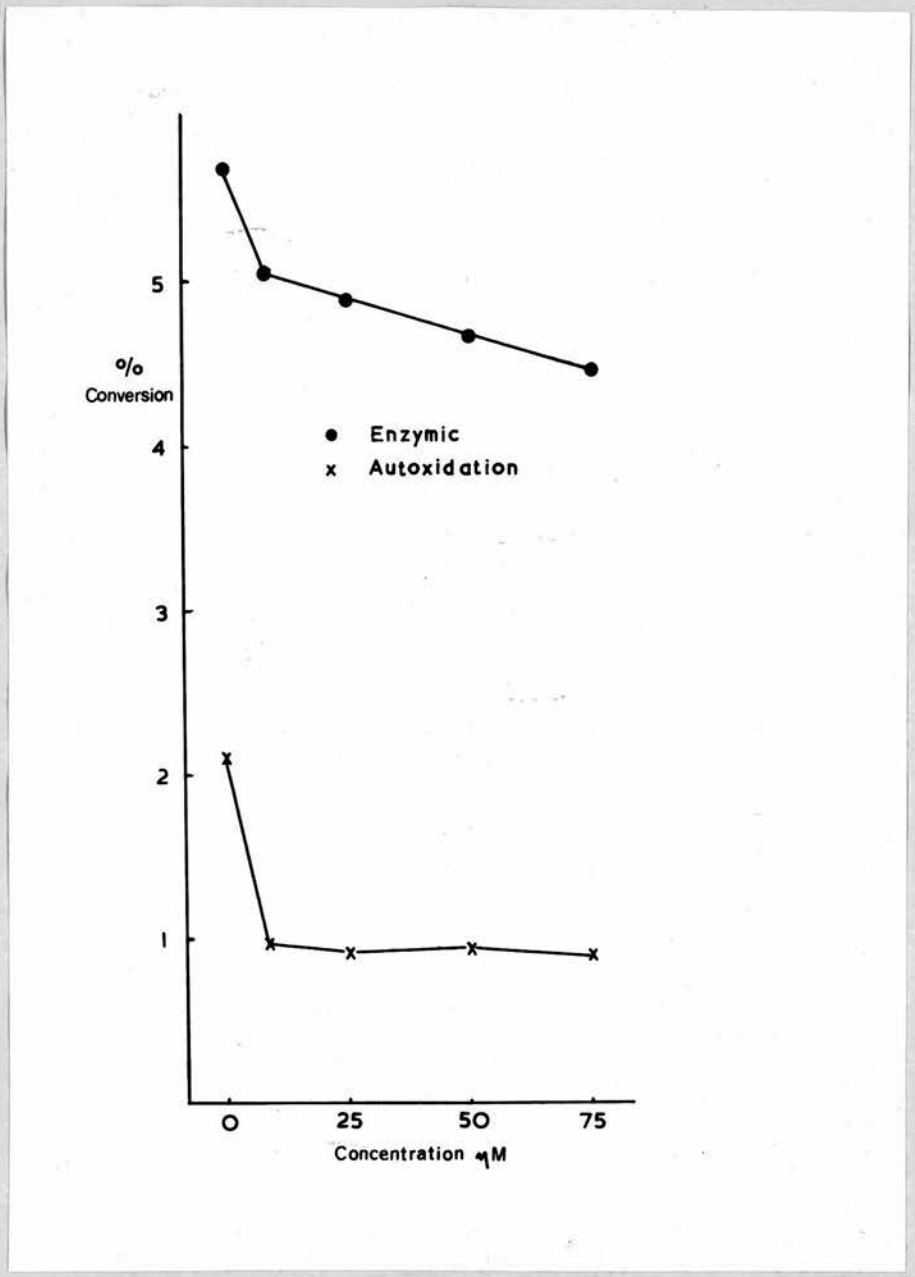


Figure 23: Metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant fraction with NADPH in the presence of sodium diethyldithiocarbamate.
1 hour at 37°.

ions in the system which are concerned in the autoxidative mechanisms. Inhibition of the enzymic system in the presence of EDTA concentrations from 7-35 mM is considered to be due to the gross effects induced by the high concentrations of the sequestering agent in the incubation medium.

(ii) Sodium diethyl dithiocarbamate (NaDDC).

NaDDC is a complexing agent which combines specifically with cupric ions. Figure 23 shows the metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant in the presence of increasing concentrations of NaDDC. As described above, cupric ions in the system are inhibitory towards the 7α-hydroxylase system, so that the presence of these ions in appreciable concentration in the incubations might cause a substantial reduction in the amounts of the enzymically formed products.

Enzymic products were found to be significantly reduced in the sample containing NaDDC at a concentration of 10 μM. Autoxidation was reduced more than the enzymic metabolites; in this respect the effect of the copper sequestering agent differs from the effect of EDTA described above in that the effective removal of the ions from the system does not induce a stimulation of the enzymic activity. It was noted that the

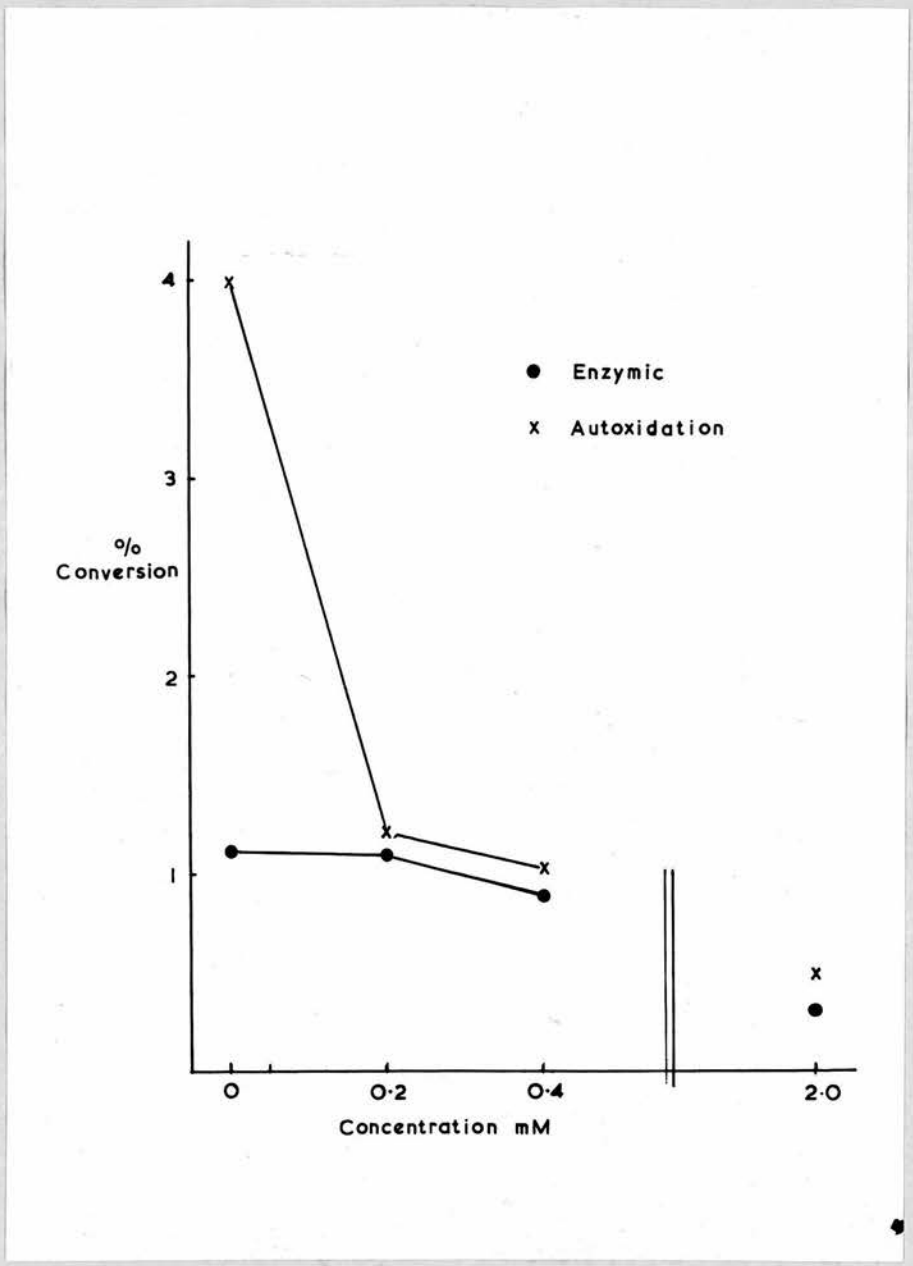


Figure 24: Metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant fraction with NADPH in the presence of 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine.

inhibition by NaDDC also occurred at much lower concentrations.

From these investigations it was concluded that the effect of free cupric ions in the incubations could for all practical purposes be neglected. It was considered that these ions were not present in sufficient concentration to affect the 7α-hydroxylase system to a significant extent nor were these ions required in the enzymic 7α-hydroxylation reaction.

(iii) 2-Amino-4-hydroxy-6,7 dimethyl-tetrahydro-pteridine (PtH_4).

In section 1, the role of pteridine in certain hydroxylation reactions was discussed. Various investigations were conducted using the above pteridine in order to determine if this compound could stimulate 7α-hydroxylation of cholesterol. The data are presented in this section since the effect of this agent appeared to be similar to that of the metal sequestering agents EDTA and NaDDC. Preparation of the reduced pteridine is described in Appendix 4.

(a) Concentration

The metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant fraction was investigated in the presence of reduced pteridine from 0 to 2.0 mM (figure 24). A significant inhibition of both enzymic

Table 14

Metabolism of cholesterol-4-^{14}C by 18,000 *g* supernatant fraction.

1 hour at 37°. PtH_4 0.2 mM.

Addition	NADPH	PtH_4	NADPH + PtH_4
Products %			
Enzymic	0.5	0.1	0.5
Autoxidation	1.3	0.5	0.5

Table 15

Addition	NADPH	Mg^{++} NADPH	Mg^{++}, PtH_4 NADPH
Products %			
Enzymic	2.3	1.0	2.0
Autoxidation	3.0	3.8	3.1

and non-enzymic activity was observed with increasing concentrations. The reduction of autoxidation appeared, however, to be more selective than the reduction of the hydroxylase activity.

(b) NADPH

In hydroxylation reactions utilising a reduced pteridine, an electron donor is required to maintain the co-factor in the reduced (active) form. Table 14 shows the metabolism of cholesterol-4-^{14}C in the presence of added, reduced pteridine with and without NADPH. In the absence of NADPH, enzymic activity was very low. It has already been shown that the 7α-hydroxylase system requires this co-factor, so that it appears that the reduced pteridine could not substitute for NADPH. Metabolism to the autoxidation products did not depend on the presence of NADPH; addition of reduced pteridine markedly inhibited the formation of these products in the presence or absence of NADPH.

(c) Magnesium ions

Table 15 shows the results of an experiment to study the effects of reduced pteridine in the presence of magnesium ions. The sample with magnesium ions as the only addition, gave a low enzymic activity. The established inhibitory effect of these ions was

evident by comparison with the control sample. Where both reduced pteridine and magnesium ions were present, the inhibition of the metal ions alone was partially compensated, with a concomitant large reduction in non-enzymic activity. It appeared that the action of reduced pteridine was similar to that of EDTA, i.e. a slight stimulation of enzymic metabolism at the expense of autoxidative activity.

The effects of the reduced pteridine described above have been well substantiated. However, the magnitude of the effects varied a lot from one preparation to the next, so that a concentration of this co-factor could not be selected as one at which a preferential stimulation of enzymic hydroxylation occurred.

(d) Investigations with the 6-methyl tetrahydropteridine, shown to be generally more active than the 6,7-dimethyl compound in hydroxylation reactions, indicated that there was no difference in effect between the two pteridines. It was concluded that reduced 2-amino-4-hydroxy-pteridines did not enhance 7α-hydroxylase activity by substituting for, or by stimulating, any of the natural co-factors of the reaction. Their inhibitory influence on autoxidation may be due to complexing with endogenous metal ions.

Summary

(1) Magnesium, manganese and cupric ions inhibited the 7α-hydroxylase system. The influence of these ions on autoxidative mechanisms appeared to be different.

(2) EDTA, a heavy metal chelator, appeared to cause a slight stimulation of enzymic activity, and reduced autoxidation to insignificant levels. Sodium diethyl dithiocarbamate (NaDDC) inhibited enzymic activity.

(3) Reduced 2-Amino-4-hydroxypteridines appeared to function as metal ion sequestering agents by reducing autoxidation. Their action seemed to depend on the basic molecular structure, and was independent of oxidation state of the molecule or substitution with methyl groups.

Section 8.

Characteristics of cholesterol-7α-hydroxylase and its role in bile acid formation

In previous sections, some of the factors and conditions influencing the *in vitro* 7α-hydroxylation of cholesterol were described. The present section describes investigations of a preliminary nature to study the effects of various factors which might control this reaction, and which might be important in assessing the significance of this enzyme in the overall catabolism to bile acids.

(1) Cholesterol-7α-hydroxylase as a mixed function oxidase.

It has been shown (section 4) that the enzymic 7α-hydroxylation of cholesterol required NADPH, suggesting that this hydroxylation may be a mixed function oxidation. Bergstrom has shown that the reaction proceeds as a direct replacement reaction at C_7, inferring that molecular oxygen is employed, rather than the hydration of an olefinic intermediate. The incorporation of $^{18}O_2$ into the molecule has not, however, been demonstrated.

Initial studies carried out under anaerobic conditions (N_2) using the gassing apparatus described in section 2, showed that a rigorous exclusion of oxygen (by gassing and boiling where possible) was necessary to reduce the 7α-hydroxylase activity of the 18,000 g supernatant fraction by a detectable amount. In some cases, the activity was independent of the N_2/O_2 gas phase from 0-100%.

It has been suggested that liver microsomes contain two terminal oxidases,[77] one having a high affinity for oxygen ($K_m = 2 \times 10^{-6}$M), the other having a low affinity ($K_m = 1.5 \times 10^{-4}$M). If a pigment having a low Km for oxygen uptake were involved in the cholesterol-7α-hydroxylase system, the uptake of oxygen might not be a limiting factor, especially in view of the small amount of substrate which is hydroxylated. Also a large number of reactions occur in liver microsomes using molecular oxygen, and it is unlikely that any methods for the determination of oxygen uptake would be sufficiently accurate to permit the demonstration of the utilisation of molecular oxygen by the 7α-hydroxylase system in its present crude form. The problem of measuring oxygen uptake has been encountered in many investigations where only small amounts of reaction product are formed. A number of aerobic hydroxylations have been successfully studied in recent years, and it has become obvious that

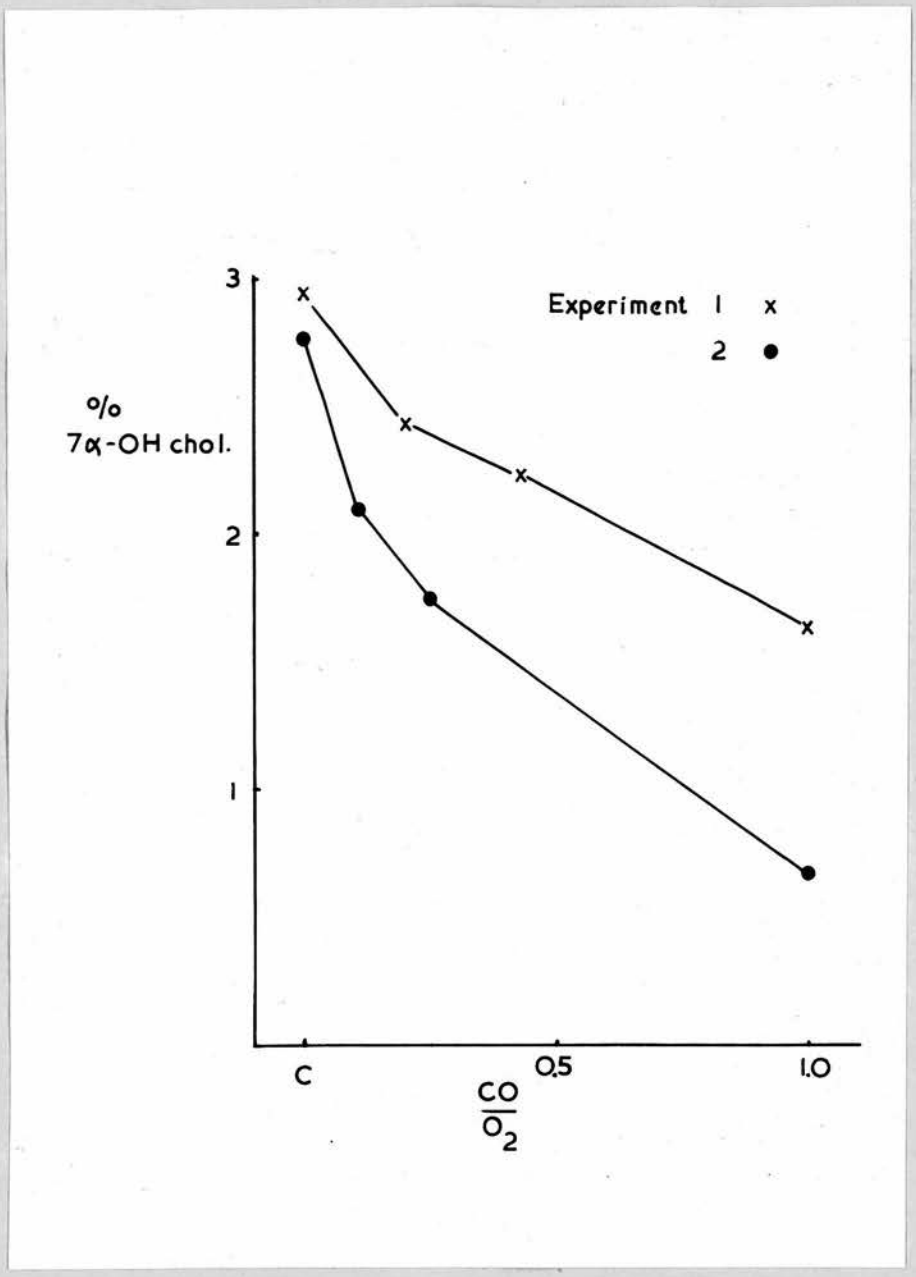


Figure 25: Metabolism of cholesterol-4-^{14}C by 18,000 g supernatant fraction in the presence of increasing $\frac{CO}{O_2}$ ratios.

Incubation 1 hour with NADPH.

pigments such as cytochrome P-450 are intimately involved as terminal electron acceptors in the reactions. This pigment, which recent evidence suggests may be a family of pigments, can combine with carbon monoxide, and in doing so inhibits the uptake of oxygen by a large number of steroid and non-steroid substrates, implying that this hemo-protein is involved in oxygen activation.

The metabolism of cholesterol-4-^{14}C was studied in the presence of CO/O_2 ratios from 0.1 to 2.0. The results are shown in figure 25 as the amount of 7α-hydroxycholesterol formed, plotted against the CO/O_2 ratios. If it is presumed that carbon monoxide combines at the same site as oxygen we obtain (Warburg[80]):

$$E.O_2 + CO \rightleftharpoons E.CO + O_2$$

$E.O_2$ and E.CO represent the concentrations of bound O_2 and CO.

$$K(\text{equilibrium constant}) = \frac{E.O_2 \cdot CO}{E.CO \cdot O_2}$$

$$\frac{E.O_2}{E.CO} = \frac{E.O_2}{E - E.O_2} \quad \text{(where E is the total enzyme concentration)}$$

$$= \frac{\frac{E.O_2}{E}}{1 - \frac{E.O_2}{E}}$$

Since r_{CO} (rate in presence of CO) $\propto E.O_2$

and r_0 (rate in absence of CO) $\propto E$

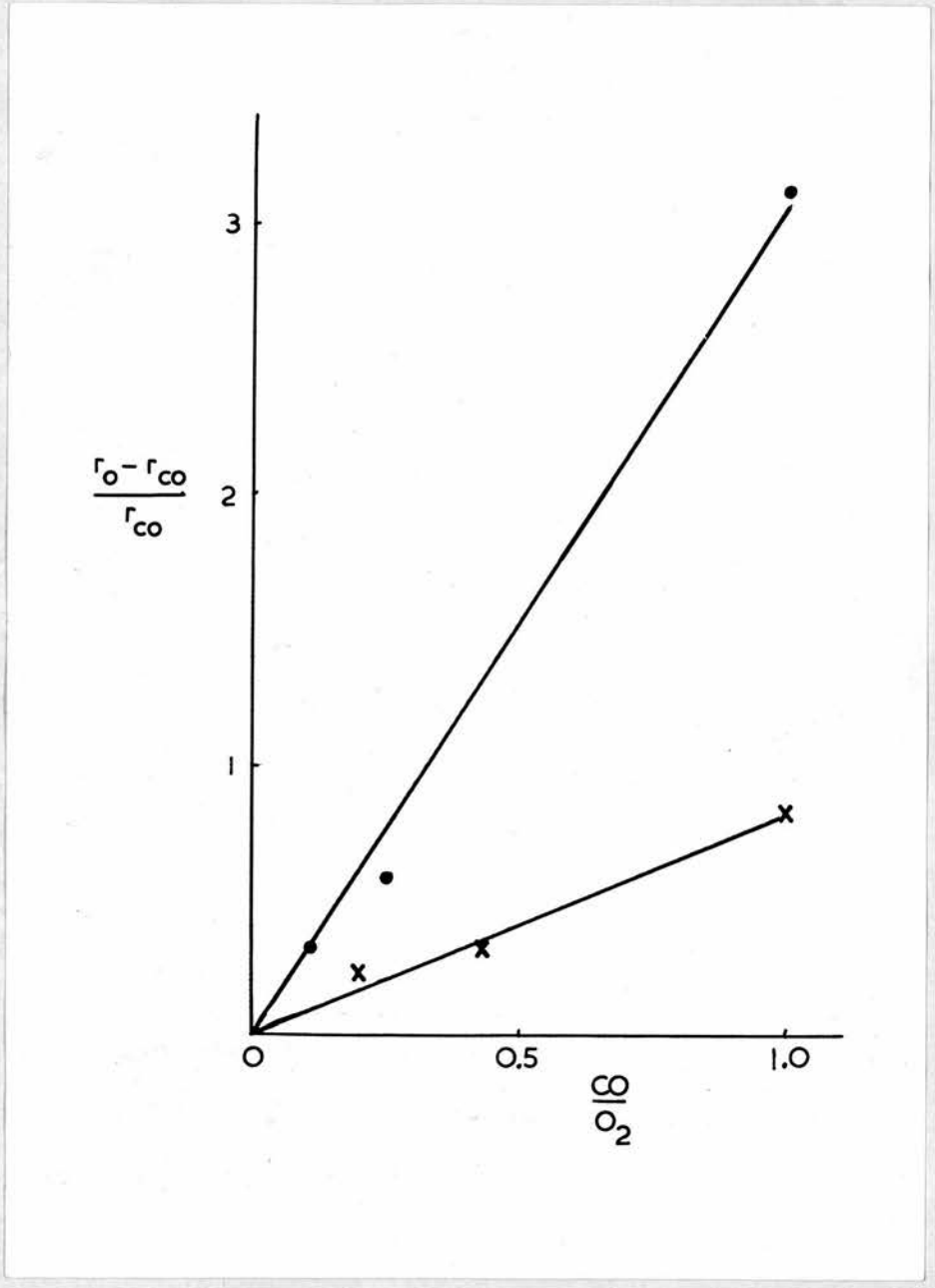


Figure 26: Best straight lines plotted by the method of least squares from data in figure 25, expressed as described in the text.

$$\frac{\frac{E.O_2}{E}}{1 - \frac{E.O_2}{E}} \quad \text{becomes} \quad \frac{\frac{r_{CO}}{r_O}}{1 - \frac{r_{CO}}{r_O}}$$

$$\therefore K = \frac{1 - \frac{r_{CO}}{r_O}}{\frac{r_{CO}}{r_O}} \cdot \frac{O_2}{CO}$$

$$= \frac{r_O - r_{CO}}{r_{CO}} \cdot \frac{O_2}{CO}$$

A plot of $\frac{r_O - r_{CO}}{r_{CO}} / \frac{CO}{O_2}$ is a straight line of gradient $\frac{1}{K}$ passing through the origin.

The partition constant (K) between carbon monoxide and oxygen for cytochrome P-450 has been calculated to lie in the range 0.5-2.0, compared with 5×10^{-3} for hemoglobin and 10-20 for cytochrome oxidase.[62]

The best straight lines from the data in figure 25 were calculated. The gradients of the two plots were 3.08 ± 0.29 and 0.82 ± 0.10 (figure 26), giving values for K of 0.32 and 1.22 respectively. Both values are of the correct order for cytochrome P-450, although the first is rather low. Further investigation is required to produce a more significant value for K, since there appeared to be considerable variation in different experiments. The results, however, suggest that the 7α-hydroxylation

of cholesterol utilises molecular oxygen, activated by a pigment which appears to be similar to cytochrome P-450. On this evidence, it seems likely that cholesterol-7α-hydroxylase belongs to the mixed function oxidase category of enzymes. Enzymes utilising molecular oxygen have also been implicated in the biosynthesis of cholesterol from acetate and mevalonate[81]; the 12α-hydroxylation step in the formation of cholic acid, which has not been investigated, is probably a mixed function oxidase like cholesterol-7α-hydroxylase, so that several enzymes of the oxygenase classification are involved in the de novo synthesis of bile acids by the liver.

It is difficult to correlate the significance of individual enzymes of this type with the overall picture of cell metabolism. During incubation in an aerobic environment, the enzymes are exposed to much higher oxygen tensions than are present in the liver cell, so that results obtained in this way must be regarded with a certain amount of caution. Since the recognition about ten years ago that molecular oxygen was utilised in a large number of systems in normal cellular processes, data on oxygen fixation has rapidly accumulated, but it is not possible at present to explain how such reactions affect the energetics of the cells, or indeed to describe unequivocal mechanisms for oxygen activation.

If pigments like cytochrome P-450 are involved in the assimilation of oxygen for utilisation by aerobic enzymes, it may be difficult to show that the oxygen activation step is rate-limiting, since the affinity of such hemo-proteins for oxygen is very high. (see above). Liver microsomes especially contain appreciable amounts of these pigments, which are involved in the detoxification mechanisms of drugs and foreign compounds. The synthesis of cytochrome P-450 can be induced by the administration of appreciable quantities of drugs such as phenobarbitone[63], but normally these hemo-proteins may be present in the microsomes in concentrations which are more than adequate to support the normal aerobic reactions of the cell. Furthermore, if cytochrome P-450 is really a generic term for several pigments, small changes in one of these hemo-proteins may not be detectable.

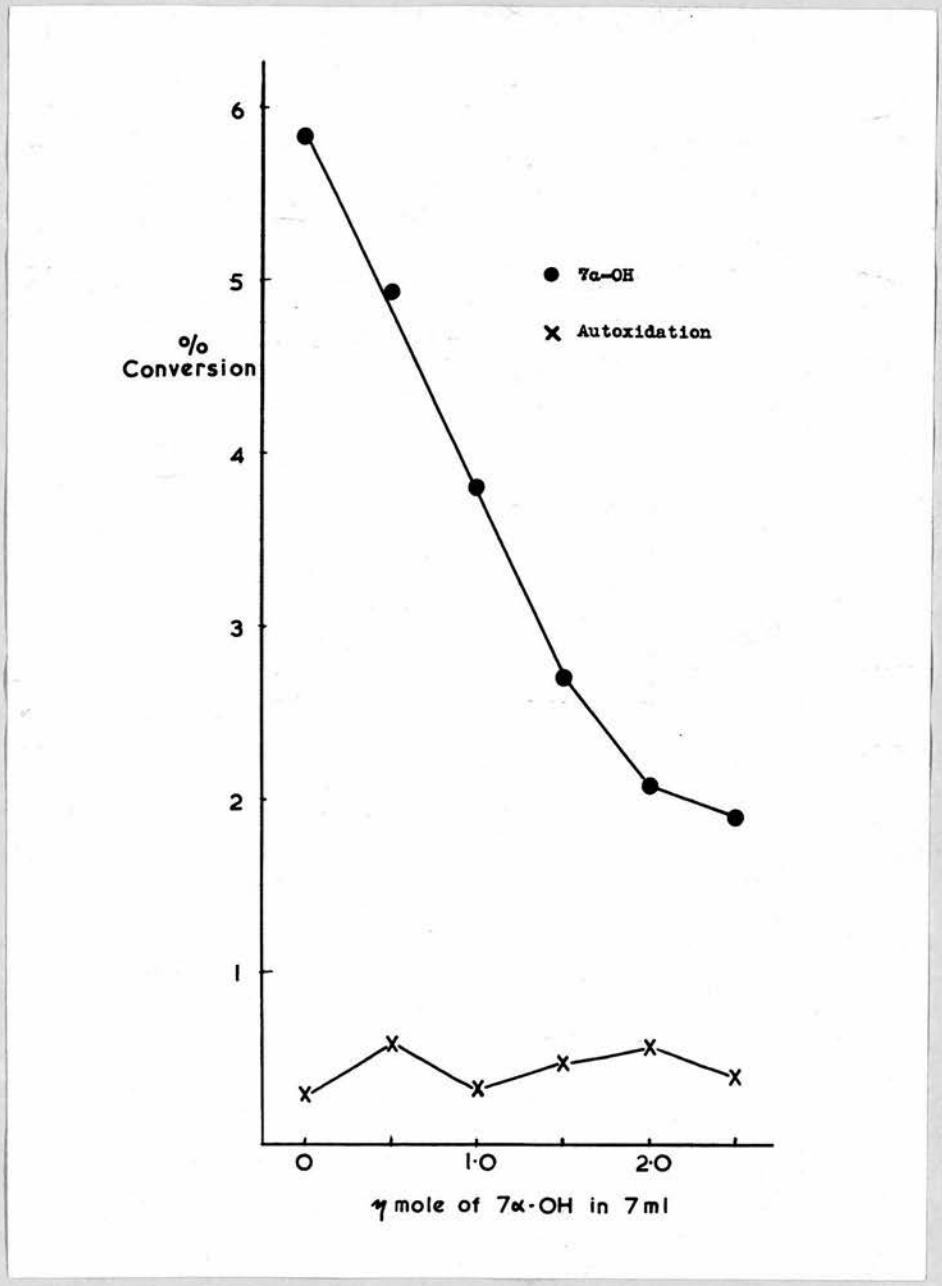


Figure 27: Metabolism of cholesterol-4-^{14}C to 7α-hydroxycholesterol by the 18,000 g supernatant fraction in the presence of increasing amounts of 7α-hydroxycholesterol. Incubation for 1 hour with NADPH.

(ii) Inhibition by 7α-hydroxycholesterol of cholesterol-7α-hydroxylase

The possible control of the catabolism of cholesterol to bile acids was discussed in section 1. Various workers have been unable to isolate intermediates in this catabolism, suggesting that the first step may be rate-limiting. Evidence has been presented in this thesis to show that the first reaction is the 7α-hydroxylation of cholesterol; as a mixed function oxidation, this reaction would be virtually irreversible, and since 7α-hydroxycholesterol has no known fate in the liver other than its conversion to the primary bile acids, the hydroxylation may be a rate-controlling process in the catabolism.

Figure 27 shows the conversion of cholesterol-4-^{14}C to 7α-hydroxycholesterol-4-^{14}C by the 18,000 g supernatant fraction in the presence of increasing concentrations of added 7α-hydroxycholesterol. Inhibition of the cholesterol-7α-hydroxylase system was observed under these conditions. Evidence discussed in section 5 (vi) above indicated that the accumulation of the product of the hydroxylation, 7α-hydroxylation was not inhibitory, since the removal of this intermediate by further oxidationdid not stimulate the conversion of cholesterol to this sterol. In the present experiment, however, the concentrations of

7α-hydroxycholesterol in the incubation were much larger and could account for the difference in the effect.

The inhibition produced by the addition of 7α-hydroxycholesterol was difficult to assess for several reasons. In order to be able to calculate a rate constant for the reaction from data of this sort, a knowledge of the substrate concentration is required. Unfortunately this could not be found, due to the large and variable pools of cholesterol in the liver. Furthermore, the addition of even small amounts of 7α-hydroxycholesterol to the aqueous incubation medium must induce precipitation of the lipo-soluble sterol, so that the concentration of 7α-hydroxycholesterol in contact with the enzyme is variable.

From these tentative investigations, it can only be concluded that 7α-hydroxycholesterol as the product of the reaction can inhibit the cholesterol-7α-hydroxylase system of the 18,000 g supernatant fraction when added in appreciable concentrations. The dynamic aspects of this inhibition must await further purification of the enzyme system.

Table 16

Metabolism of cholesterol-4-^{14}C by 18.000 g supernatant from cholestyramine-fed rats.
(5-14 days / 6% diet)
Incubation for 1 hour with NADPH.

%	Test	Controls
Total enzymic products	10.6 ± 1.8	1.9 ± 0.7
7α-hydroxycholesterol	9.7 ± 1.8	1.5 ± 0.6
Autoxidation products	1.1 ± 0.6	0.9 ± 0.5

Conversions ± standard deviations from
7 test animals and 6 controls.

(iii) Entero-hepatic circulation of bile salts.

Cannulation of the common bile duct in the rat has been shown to cause a large increase in the production of bile acids.[5] This increase can be as high as ten times the normal, but it has not been shown to be due to an increase in the rate of a specific catabolic step. The 7α-hydroxylation of cholesterol, described above as a possible rate-limiting step, may be the reaction which is affected by the removal of the circulating bile salts. The method of biliary cannulation is not altogether satisfactory, since the animal is considerably stressed by surgery, and becomes abnormal through the loss of salts in the bile. Cholestyramine, an anionic resin ("Cuemid" - Merck, Sharp and Dohme, West Point, Pa.), can combine with bile salts and acids in the gut, and in so doing prevents their re-absorption by the portal system; this compound fed to the rats in a soft diet (section 2) was used to accomplish the same change in bile acid production as would be produced by bile duct cannulation. The animals were fed on the supplemented diet (6%) for 5-14 days before killing; cholesterol-7α-hydroxylase in the 18,000 supernatant fraction was then assayed in the usual way.

In table 16 are shown the results from several treated animals compared with controls fed on the same diet without cholestyramine. The results are expressed

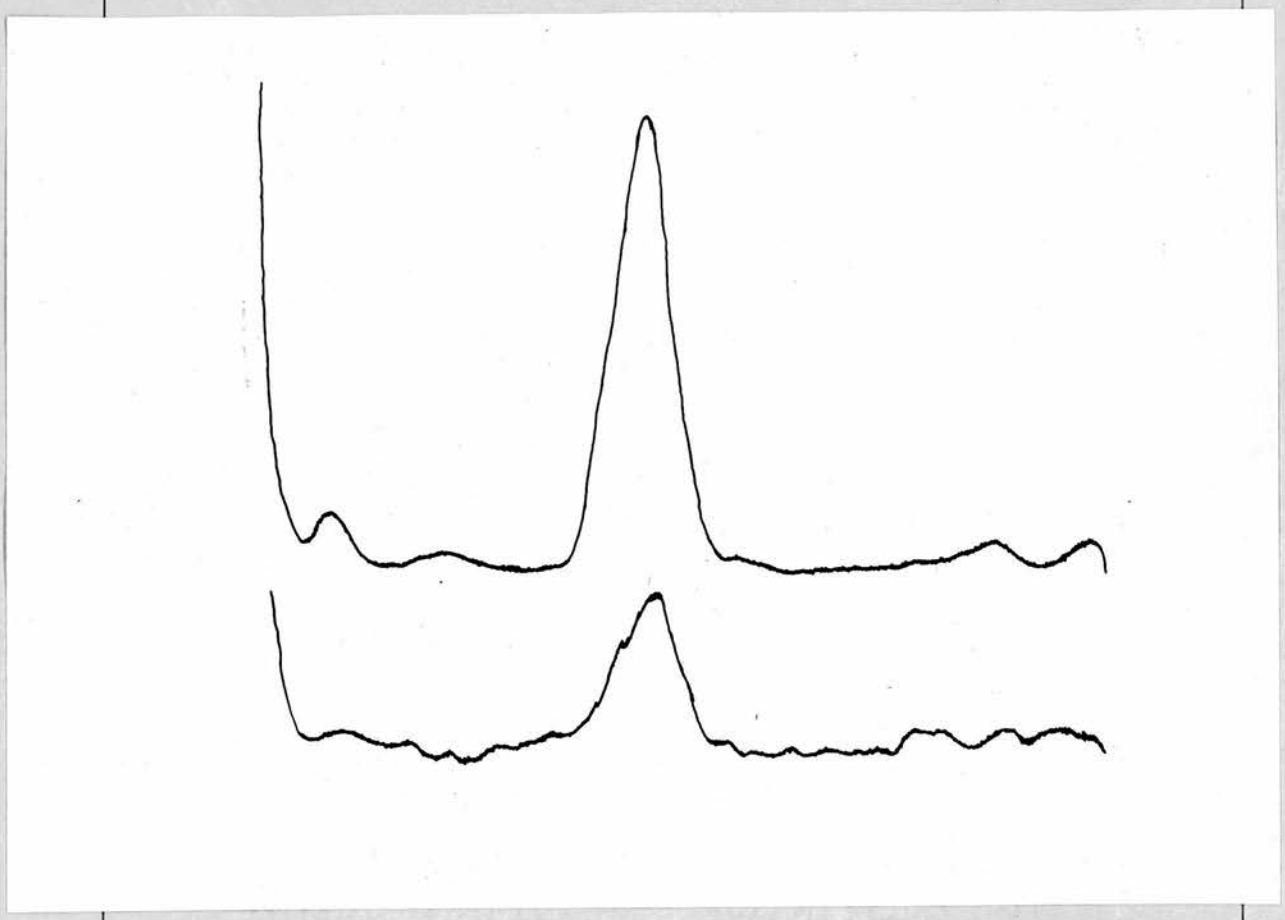


Figure 28: Comparison of metabolism of cholesterol-4-^{14}C to 7α-hydroxycholesterol by 18,000 g supernatant of cholestyramine-fed and normal animals.

1 cholesterol
2 cholest-4-en-3-on-7α-ol
3 7-keto-cholesterol
4 7β-hydroxycholesterol
5 7α-hydroxycholesterol
6 cholest-4-en-3-on-7α,12α-diol
7 cholestan-3β,5α,6β-triol
8 Origin

as the mean values with standard deviations. Metabolism of cholesterol-4-^{14}C to 7α-hydroxycholesterol was more than six times that found in the control samples, with no significant change in the level of autoxidation products between treated and control rats.

Figure 28 is a photograph of traces from a radiochromatograph assay of the products of incubation of cholesterol-4-^{14}C with 18,000 g supernatant from fed and control rats, and illustrates pictorially the large increase in 7α-hydroxylase activity found by feeding cholestyramine.

The results of these investigations are important for the following reasons:

(1) The specific increase in cholesterol-7α-hydroxylase activity showed that this enzyme catalyses a rate-limiting step in cholesterol catabolism by the liver.

(2) There was no evidence of an increase in any other metabolites of cholesterol except those formed from 7α-hydroxycholesterol, implying that 7α-hydroxycholesterol alone is the first product of the only route for cholesterol catabolism by the 18,000 g supernatant fraction.

A limited number of experiments have been performed using cholestyramine fed animals; it is not

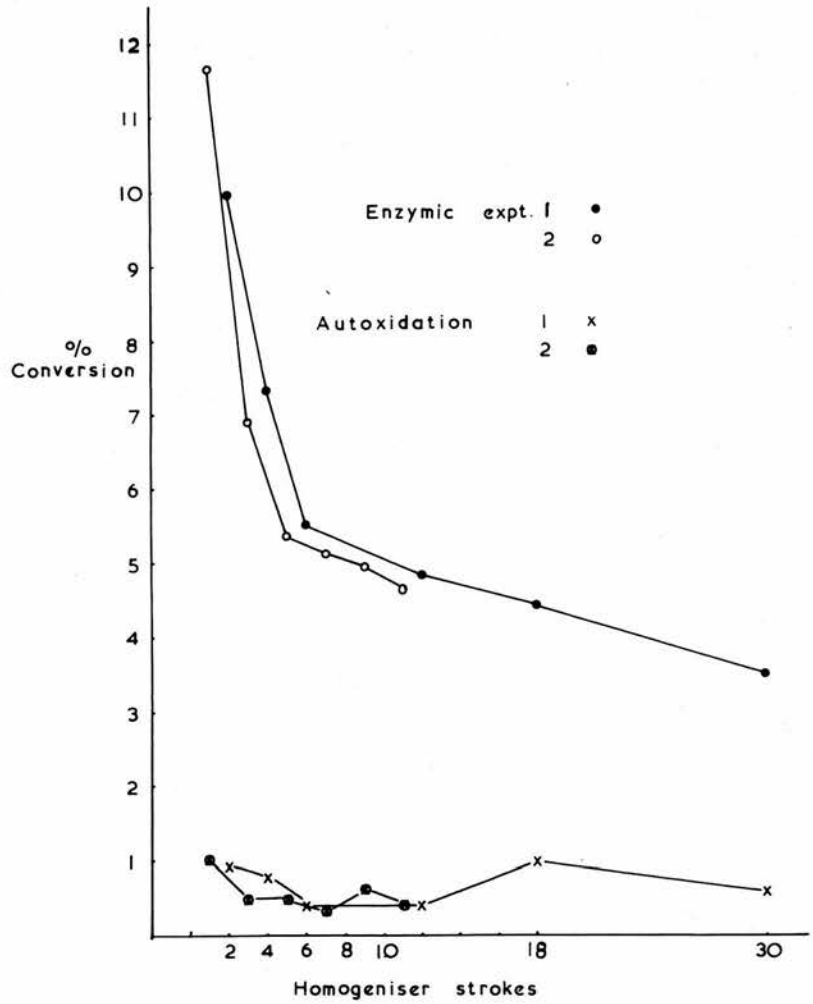


Figure 29: Metabolism of cholesterol-4-^{14}C by the 18,000 g supernatant fraction from cholestyramine-fed rat after variable homogenisation.
Incubation for 1 hour with NADPH.

yet known if the cholesterol-7α-hydroxylase system in the cholestyramine fed animal is subject to the same influences as the system in the normal animal. However, preliminary investigations indicated that the highly active system in the 18,000 g fraction of the liver of the treated rat was affected under the same conditions. Figure 29 shows the activity of the 18,000 g supernatant 7α-hydroxylase system after homogenising the liver as described in section 5 (i). As with normal liver, a substantial loss of activity occurred by prolonged homogenisation in both of the experiments shown in the figure. Rather more activity was lost by this process in tissue from a cholestyramine-fed animal, suggesting that the cholesterol-7α-hydroxylase system may be even more sensitive to harsh treatment.

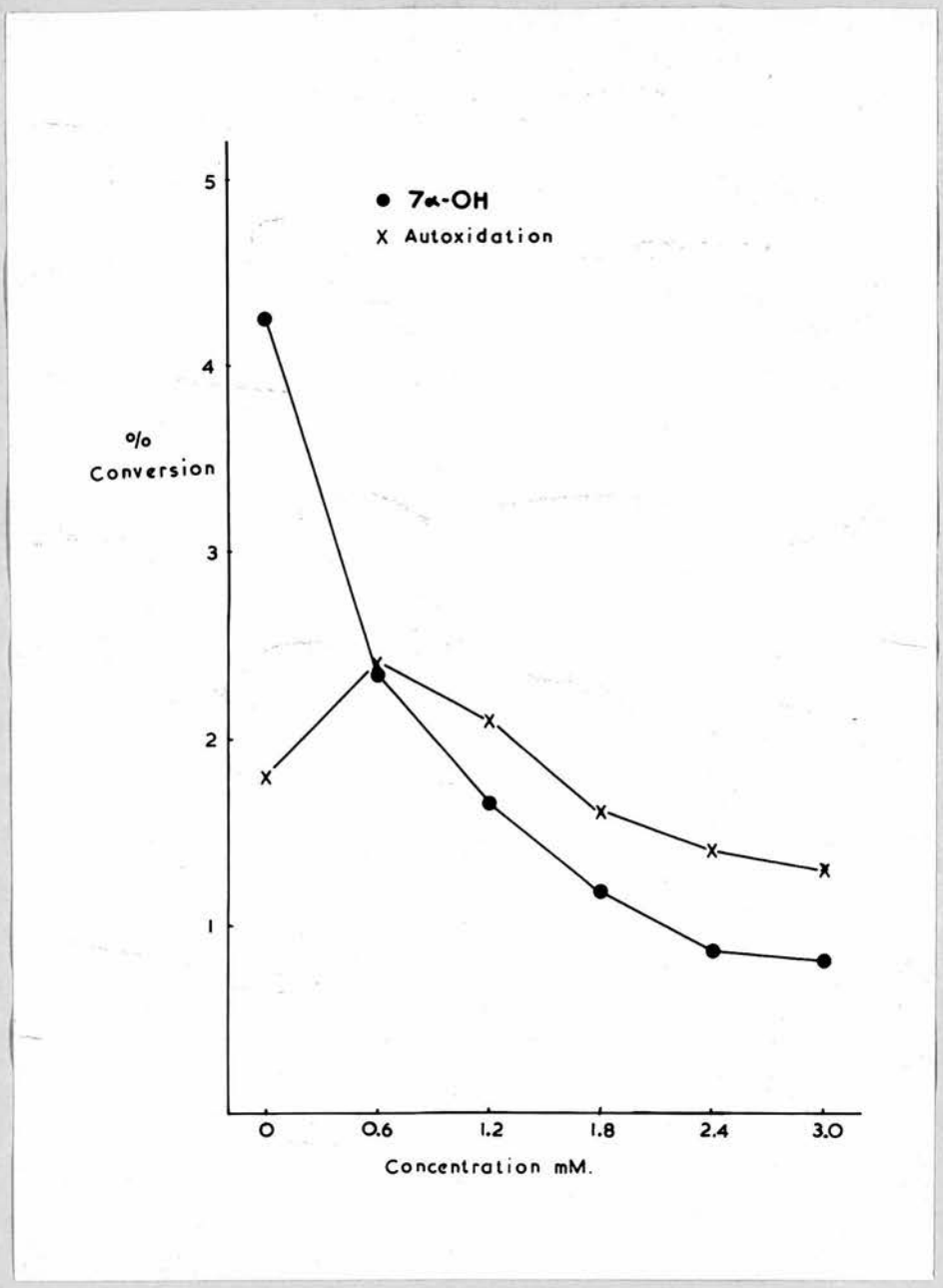


Figure 30: Metabolism of cholesterol-4-^{14}C to 7α-hydroxycholesterol by the 18,000 g supernatant fraction in the presence of increasing concentrations of sodium taurodeoxycholate.
Incubation 1 hour with NADPH.

(iv) Inhibition by taurodeoxycholic acid.

Taurodeoxycholic acid, formed from cholic acid by bacterial action in the gut, is found in appreciable concentrations in portal blood and in the liver cell. This salt can be efficiently re-hydroxylated to cholic acid by the microsomal fraction of the liver, and it is conceivable that this hydroxylation has similar characteristics to the hydroxylation of cholesterol by the same fraction. The hydroxylation of taurdeoxycholic acid could exert a moderating influence on the cholesterol-7α-hydroxylase system, by utilising the same co-factors such as NADPH, O_2, etc., as an important aspect of the homeostatic regulation of cholesterol catabolism by the bile salts.

Figures 30 and 31 show the conversion of cholesterol-4-^{14}C to 7α-hydroxycholesterol by the 18,000 g supernatant fraction from a normal and a cholestyramine-fed rat, in the presence of increasing concentrations of sodium taurodeoxycholate. Inhibition of cholesterol-7α-hydroxylase was observed in both experiments, but only at high concentrations of the added bile salt. It is estimated[6] that normal liver contains about 15 μg. of this salt per g. of liver, so that the inhibition occurred at concentrations very much higher than this.

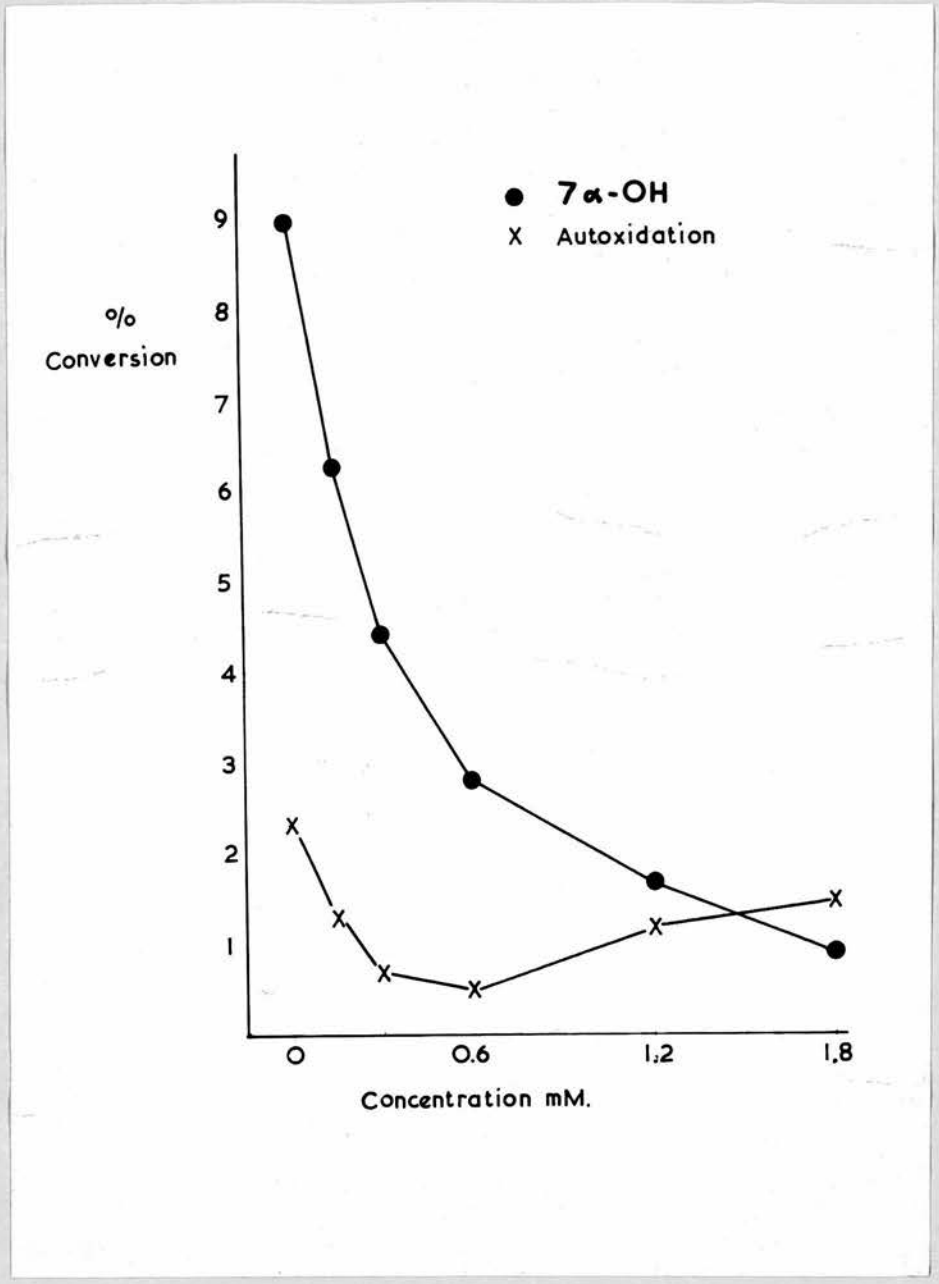


Figure 31: Metabolism of cholesterol-4-^{14}C to 7α-hydroxycholesterol by the 18,000 g supernatant fraction from a cholestyramine-fed rat in the presence of increasing concentrations of sodium taurodeoxycholate. Incubation 1 hour with NADPH.

A major problem created by the use of taurodeoxycholate in incubations is that this salt is a powerful detergent, and may inhibit cholesterol-7α-hydroxylase by solubilising the membranes of the endoplasmic reticulum; it has already been established that this enzyme system is vulnerable even in relatively mild conditions.

These investigations are of a preliminary nature; further study of the cholesterol-7α-hydroxylase system in the presence of taurodeoxycholate with normal and cholestyramine-fed rats may give valuable information concerning the control of this reaction, and it may be possible to show that the 7α-hydroxylation of cholesterol is dependent on the 7α-hydroxylation of taurodeoxycholate. It would be of great interest to determine the factors which affect the 7α-hydroxylation of cholesterol as a rate-limiting process to find out if the increase in enzymic activity is due to an increased synthesis of this enzyme or to the removal of some inhibitor.

Summary.

(1) Carbon monoxide inhibited the cholesterol-7α-hydroxylase system of the 18,000 g supernatant fraction. Calculation of K, the partition constant between carbon monoxide and oxygen, from the available data suggested that cytochrome P-450 or a related pigment was involved in the reaction, inferring that cholesterol-7α-hydroxylase was a mixed function oxidase.

(2) The conversion of cholesterol-4-^{14}C to 7α-hydroxycholesterol was increased several-fold by feeding cholestyramine to the rats. The effect was thought to be due to the combination of this resin with bile salts in the gut, which prevented their re-absorption, and allowed the hydroxylation reaction to be increased unchecked by the regulatory influence of the salts.

(3) The presence of 7α-hydroxycholesterol or sodium taurodeoxycholate in the incubation inhibited the cholesterol-7α-hydroxylase system.

The investigations described in this section were of a preliminary nature. The short discussions in relation to each topic are based only on the experimental evidence available. Positive proof

of the results will be obtained after more intensive research, and in some cases, only after extensive purification of the enzyme preparation.

Table 17

Metabolism of 7α-hydroxycholesterol-4-^{14}C by 18,000 g supernatant fraction in one hour at 37°

Addition	NADPH	NAD	NAD followed by NADPH
% conversion			
cholest-4-en-3-on-7α-ol	2.1	38.4	30.7
cholest-4-en-3-on-7α,12α-diol	1.0	20.5	14.2
trihydroxy-coprostane	0.3	1.0	4.0
5β-cholestan-3-on-7α,12α-diol	0.3	2.1	6.3
Total %	3.7	62.0	55.5

Section 9.

Further metabolism of 7α-hydroxycholesterol.

Although the main object of these investigations was the study of the 7α-hydroxylation of cholesterol, the two oxidised derivatives of this sterol, cholest-4-en-3-on-7α-ol and cholest-4-en-3-on-7α,12α-diol were formed under most conditions in small yield. These products were identified as described in section 3, and are thought to be involved in the major catabolic route as shown in figure 3. Their formation occurred even in the presence of NADPH, although other workers have shown that the oxidation to cholest-4-en-3-on-7α-ol requires NAD or NADP. Possibly the 12α-hydroxylation step, which has been little studied, is also a mixed function oxidation so that it would be expected to be stimulated in the presence of an electron donor.

Table 17 shows the products formed by incubation of 7α-hydroxycholesterol-4-^{14}C (appendix 4) with the 18,000 g supernatant fraction in the presence of NADPH or NAD. The products formed in these investigations are implicated in the catabolic route as shown in figure 3. The preparation with NADPH metabolised only a small amount of the substrate to the products shown; in contrast, NAD catalysed a quantitative conversion

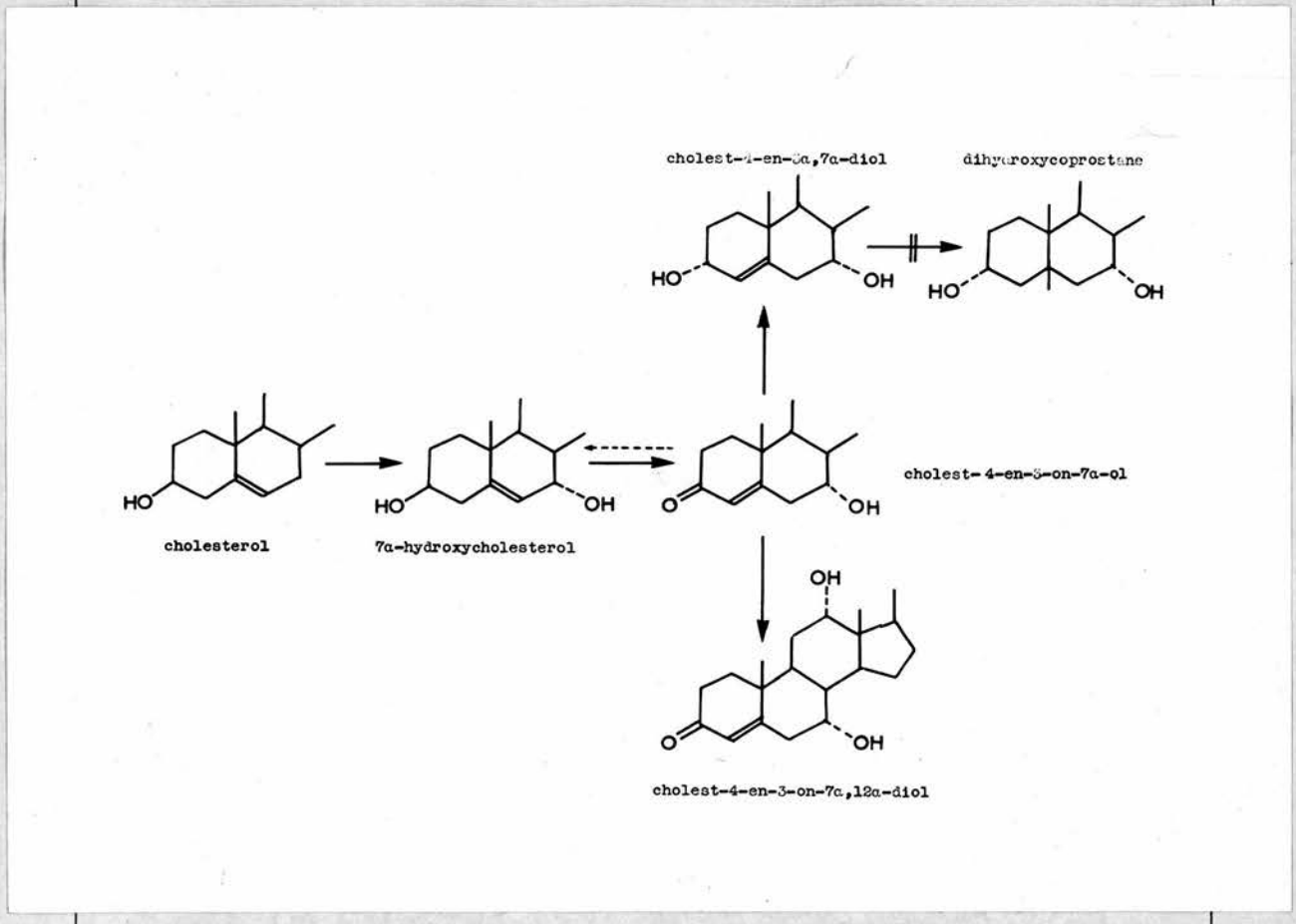


Figure 32: Major products obtained by incubation of cholesterol-4-^{14}C and 7α-hydroxy-cholesterol-4-^{14}C with the 18,000 g supernatant fraction. (cf. figure 3)

to two products in particular, identified as cholest-4-en-3-on-7α-ol and cholest-4-en-3-on-7α,12α-diol. Subsequent incubation with NADPH of the sample fortified with NAD, showed that the amounts of the reduced intermediates, trihydroxycoprostane and 5β-cholestan-3-on-7α,12α-diol could be increased as these reductive steps were stimulated. The formation of cholest-4-en-3-on-7α,12α-diol was not enhanced by the prolonged incubation with NADPH, implying that a co-factor other than NADPH is required in the 12α-hydroxylation of cholest-4-en-3-on-7α-ol. It was also observed that the total conversion of 7α-hydroxycholesterol appeared to be decreased by incubation with NADPH; it is probable, however, that this discrepancy is due to the formation of cholest-4-en-3α,7α-diol (figure 32), an intermediate found by other workers to be formed from cholest-4-en-3-on-7α-ol in certain circumstances. This sterol has not been shown to have physiological significance, since it can not be converted into dihydroxycoprostane, a postulated intermediate in the formation of chenodeoxycholic acid. Figure 32 shows the main products formed from cholesterol and 7α-hydroxycholesterol by the 18,000 g supernatant. The co-factors required for each step have been omitted, since it appeared that the requirements for some of these steps were apparently anomalous. It seems likely that some of these

reactions must be considered as a concerted series of steps and not as isolated individual reactions; the enzyme catalysing each step may supply the enzyme effecting the next step with both substrate and co-factor in the appropriate oxidation state. This could result in the apparent stimulation of one reaction by a particular co-factor, when in fact the stimulus should be assigned to an earlier step. By the same argument, the observed metabolism of an intermediate added in acetone may give misleading results; the formation of cholest-4-en-3α,7α-diol may be an example of this, where a reductase of the cell sap had reduced the 3-keto group in preference to the double bond. Had the double bond reductase operated before the 3-keto reductase, a physiological product, dihydroxycoprostane would have been formed. This suggests that the substrate was not available to the enzymes in the correct sequence. It will be noted that, although the 18,000 g supernatant fraction contains cell sap, no evidence of significant formation of dihydroxycoprostane was observed; the supernatant reductases are known to be very active, and in separated cell sap can convert cholest-4-en-3-on-7α-ol quantitatively into dihydroxycoprostane. It was evident that a regulatory factor present in the 18,000 g fraction controlled these catabolic steps, and under the conditions quoted prevented these reactions from taking place. In this preparation of liver, therefore, there was evidence only of intermediates on the

postulated route to cholic acid (tri-hydroxy); no intermediates on a parallel route to chenodeoxycholic acid through dihydroxycoprostane were detected (cf. figure 3).

Summary:

(1) 7α-hydroxycholesterol can be efficiently converted into two products by the 18,000 g supernatant fraction, tentatively identified as cholest-4-en-3-on-7α-ol and cholest-4-en-3-on-7α,12α-diol.

(2) The 12α-hydroxylation step was not apparently stimulated by NADPH and occurred in the presence of added NAD.

(3) Some of the results suggested that the catabolic sequence should be regarded as a concerted reaction sequence, and it was concluded that product formation was influenced by a factor in the 18,000 g supernatant fraction.

(4) The intermediates detected are implicated on a probable route to cholic acid but not to chenodeoxycholic acid.

The results discussed in this section are obviously incomplete; considerably more investigation

is required to elucidate the factors which control the metabolic sequence after the formation of 7α-hydroxycholesterol.

Section 10.

Discussion

(i) Location of cholesterol-7α-hydroxylase.

Investigation of sub-cellular fractions of rat liver has shown that only microsomal preparations possess significant cholesterol-7α-hydroxylase activity. Metabolism of cholesterol-4-^{14}C to 7α-hydroxycholesterol-4-^{14}C varied from about 1 to 8% conversion, averaging 4%. The enzyme preparation used in these studies was the supernatant fraction obtained by centrifuging liver homogenate at 18,000 g for 15 minutes; this fraction contains endoplasmic reticulum in the cell sap.

The preparation was not simplified further, since a significant portion of the hydroxylase activity was lost during microsomal sedimentation. Cytoplasm (105,000 supernatant) and S.F. (boiled cell fluid) were devoid of 7α-hydroxylase activity but could enhance the microsomal conversion of the substrate to 7α-hydroxy-cholesterol. Native cell fluid was the more potent of the two soluble fractions, and it was concluded that probably more than one factor in the cytoplasm is essential to the cholesterol-7α-hydroxylase system. The co-factor(s) in S.F. must be thermo-stable, by virtue of its method of preparation.

Recent work in the same laboratory where this study was performed suggests that a considerable simplification of the enzyme preparation may be possible by "protecting" the microsomal fraction during sedimentation by thiol compounds such as β-mercapto-ethylamine which appears to preserve most of the activity in a microsomal preparation in S.F. Such a simplification would eliminate possible ancillary reactions in the cell sap; it may eventually be possible to replace the soluble co-factors of the 7α-hydroxylation reaction found in cell sap and S.F. with synthetic co-factors.

Cholesterol-7α-hydroxylase is located in the same part of the liver cell as the enzymes responsible for the biosynthesis of cholesterol in the liver.[39] The enzymes effecting the catabolism of cholesterol to tri-hydroxycoprostane (a proposed intermediate immediately before side chain cleavage - figure 3, section 1) also appear to be located in the endoplasmic reticulum, with the participation of enzymes and co-factors in the cytoplasm. Mitochondria appear to be involved only in the final stages of side chain cleavage.

(ii) Conditions for optimum assay of the enzyme

Only NADPH of a number of possible co-factors caused a significant stimulation in the activity of

cholesterol-7α-hydroxylase. An NADPH generating system was used in preference to NADPH itself. Liver microsomes possess high levels of glucose-6-phosphatase activity and NADP hydrolase activity, so it was necessary to add these NADPH generator components in excess. NADPH, in contrast to $NADP^+$, does not appear to be cleaved hydrolytically by microsomal enzymes, but it can, however, be oxidised. Bogdanski[74] has investigated the oxidation of NADPH by liver microsomes, and has shown that the oxidation is markedly stimulated by metal ions such as magnesium. This author has found that NADPH added to a suspension of microsomes without generator components can be completely oxidised after about 15 minutes, a rate of 10 μg of NADPH/min./mg. of microsomal protein in the presence of 0.3 mM magnesium ions. Magnesium and other metal ions were found to inhibit cholesterol-7α-hydroxylase; the enhanced oxidation of NADPH, an essential co-factor in the hydroxylation reaction, may be partly responsible for the lower activity. Bogdanski has deduced from optical measurements that metal ions alter the structure of the microsomes, which must apparently be retained for optimum cholesterol-7α-hydroxylase activity.

The loss of enzymic activity in the 18,000 g supernatant fraction could not be attributed to a lack of NADPH, however, as shown by prolonged incubation of

the enzyme system. It appeared that the system was inactivated after about one hour's incubation, and it was presumed that this was due to a radical change in the structural integrity of the microsomes in a relatively simple system, which contained no agents for the "protection" of the enzyme. In general, the cholesterol-7α-hydroxylase system was susceptible to inactivation even in mild conditions; considerable activity was lost by removing the mitochondria at 18,000 g for more than 20 minutes, suggesting that a preliminary packing process in the formation of the microsomal pellet was sufficient to 'damage' the system.

Homogenisation of liver was shown to be a critical factor in maintaining a reasonable level of 7α-hydroxylase activity, assayed as the conversion of the labelled substrate. Variation in the"extent" of the homogenisation may not affect all of the tissue to that same extent. Certain structures may be relatively unaffected by mild treatment, and could remain as large particles; these structures would therefore be removed on low speed centrifugation. Inhibitors of the cholesterol-7α-hydroxylase system may be removed in this "heavy" fraction, which would normally be present in the microsomal fraction. Further study on the effects of "mild homogenisation" may allow a considerable purification of the enzyme preparation

with a concomitant increase in the 7α-hydroxylase activity.

Work with metallic ion sequestering agents showed that there was little inhibition of enzymic activity from heavy metals in the assay system, and that free metal ions did not appear to be involved in the hydroxylation reaction. With EDTA there was a slight increase in enzymic 7α-hydroxylation of cholesterol, and this was attributed to the removal of ferrous ions from the fraction.

Reduced substituted pteridines could not replace or enhance the activity of NADPH; these compounds, including folic acid, have been shown to exert a specific stimulation in some hydroxylation reactions; Kaufman has shown that biopterin is the natural co-factor for phenyl-alanine hydroxylase in rat liver,[79] and this co-factor must be present in the 18,000 g fraction used in these studies.

In assessing the findings of this work, it must be borne in mind that the enzyme fraction is extremely crude. It is possible to postulate that the complexity of the preparation may prevent the determination of the true effects of materials added as possible co-factors of the reaction. Conclusions drawn from various aspects of the work must therefore

be based on the obvious gross effects on the system. Other workers have tended to use heavily fortified assay systems in the study of cholesterol catabolism, e.g. ATP, AMP, NAD, etc., so that the results were even more difficult to interpret than using an 18,000 g fraction fortified only with NADPH. Danielsson, who has used a range of possible co-factors for the cholesterol-7α-hydroxylase system, has failed to show a dependence on NADPH, but has reported only small yields of 7α-hydroxycholesterol.

The pH optimum for cholesterol-7α-hydroxylase is in the range 7.3-7.4, a range also determined to be optimum for the microsomal oxidation of 7α-hydroxy-cholesterol.[37] There was no evidence for enzymic activity at values significantly beyond these limits. pH values as high as 8.5 to 9.0[11,17] have been quoted as optimum for the mitochondrial oxidation of cholesterol.

(iii) Difficulties of assay

Non-enzymic oxygen attack on the cholesterol molecule has been discussed elsewhere in this thesis. A thorough investigation of these products was essential in order to be able to differentiate enzymically and non-enzymically formed products in the assay system, especially since 7α-hydroxycholesterol is considered to be a major autoxidation product.

It was apparent that cholesterol could be converted into a number of autoxidation products, depending on the conditions of the incubation. Attempts were made using various oxygenated derivatives of cholesterol to demonstrate the involvement of such compounds as unstable intermediates in enzymic and/or autoxidation mechanisms. The results, however, did not implicate these derivatives in the enzymic 7α-hydroxylation of cholesterol, or in the non-enzymic mechanisms which were prevalent under the assay conditions. Furthermore, a non-enzymic mechanism for the formation of 7α-hydroxycholesterol could not be demonstrated although it was apparent that this sterol could be formed autoxidatively under conditions which favoured the formation of other recognisable autoxidation products. It must be concluded, therefore, that such a mechanism does exist. In general, it was possible to assay 7α-hydroxycholesterol in the relative absence of autoxidation products, when it was presumed that little or no 7α-hydroxycholesterol had been formed by a non-enzymic mechanism.

The autoxidation products formed in the incubations were identified as cholestan-3β,5α,6β-triol, 7β-hydroxycholesterol and 7-keto-cholesterol. The formation of these sterols was markedly enhanced in the presence of metal ions; various workers have suggested that autoxidation is enhanced by metal ions and this

appears to be borne out by these investigations. The microsomal fraction, fortified with ferrous ions, ADP and NADPH could convert cholesterol in high yield to these products; peroxidation of microsomal lipids is known to occur under these conditions, and an attempt was made to isolate an unstable intermediate, perhaps a hydroperoxide, from the incubation. This was unsuccessful, but it was found that there was present in the 7-keto-cholesterol fraction at least one other compound, whose reduction product was similar in polarity to that of cholesterol-5β,6β-epoxide. These studies of the oxidation of cholesterol under "lipoperoxidation" conditions were extremely interest-:ing, since they gave a considerable amount of information on cholesterol oxidation; unfortunately, there appeared to be little in common between the autoxidation of cholesterol and the enzymic 7α-hydroxylation reaction.

Of the autoxidation products which were identified, only 7-keto-cholesterol was metabolised further by the 18,000 g supernatant fraction. An enzyme was found in this fraction which converted 7-keto-cholesterol quantitatively into 7β-hydroxycholesterol, so that this sterol could be accounted for by this mechanism. Various authors have suggested the presence of a 7β-hydroxylase(s) in liver microsomes which can hydroxylate 3β-hydroxycholenic acid[70], androstene-

:dione[82], and cholesterol[83]. The evidence presented in this work suggests that cholesterol-7α-hydroxylase is not in fact a hydroxylase but is a reductase, acting on a substrate which is formed non-enzymically.

Preliminary investigation of non-enzymic cholesterol oxidation by a free radical mechanism showed that free radical trapping agents, such as diphenyl-phenylenediamine, could almost eliminate autoxidation. Various workers[77] have shown that free radicals such as •OH and •OOH can be generated in simple systems with metal ions, oxygen, etc.; a similar mechanism for non-enzymic cholesterol oxidation may be postulated on this basis.

(iv) Cholesterol-7α-hydroxylase as a mixed function oxidase

Cholesterol-7α-hydroxylase has been shown to require reduced NADP, suggesting that this enzyme belongs to the class of enzymes which utilise molecular oxygen and an electron donor. All steroid hydroxylases studied appear to conform to the general equation:

$$RH + O_2 + NADPH \longrightarrow ROH + H_2O + NADP^+$$

Some aerobic hydroxylations have been shown to utilise stoichiometric amounts of NADPH and O_2. Attempts were made to show that the exclusion of oxygen from the cholesterol-7α-hydroxylase assay system inhibited

the system. These investigations were not successful, probably because a terminal oxidase with a high affinity for oxygen is involved in the reaction; only small amounts of the substrate are hydroxylated and would require extremely small volumes of oxygen. It was considered that the complexity and crudeness of the enzyme preparation precluded the study of both oxygen uptake by the 7α-hydroxylase or the demonstration of a stoichiometric utilisation of NADPH. Investigations in this field will probably be fruitless until a purified enzyme source can be obtained. The lability of cholesterol-7α-hydroxylase certainly suggests that it is similar in nature to other mixed function oxidases, whose characterisation has been hindered by the loss of enzymic activity during purification. Consequently, other, more indirect, methods were employed to establish the enzyme as a mixed function oxidase.

Cholesterol-7α-hydroxylase is inhibited by carbon monoxide, suggesting that a carbon monoxide-binding pigment is involved in the reaction. Cytochrome P-450 is present in liver microsomes where it forms part of the extensive detoxification mechanisms of drugs and xenobiotic compounds. This hemo-protein, recently suggested to be actually several pigments of related structure, has been shown to have a partition constant between carbon monoxide and oxygen of about 1.0, and has been implicated as the terminal oxidase of many hydroxylation systems. Calculation from the

available data of partition constants for the 7α-hydroxylase system gave values of 0.32 and 1.22, indicating that cytochrome P-450 may be involved in the activation of molecular oxygen in this reaction.

(v) 7α-hydroxylation of cholesterol as a rate-limiting reaction in the catabolism to bile acids.

Current evidence suggests that the rate of cholesterol catabolism by the liver is controlled by a homeostatic regulation operated by the bile salts (section 1). The intermediates of cholesterol catabolism are not found to accumulate, so that the first reaction may be rate-limiting. The first reaction from the evidence discussed above appears to be the 7α-hydroxylation of cholesterol, which may also be a mixed function oxidation. As such, it would be virtually irreversible, and since 7α-hydroxycholesterol has no known fate in the liver other than its conversion to bile acids, this reaction may be an important control point.

Investigations with cholestyramine, an anionic resin which combines with bile salts in the gut and prevents their re-absorption, showed that cholesterol-7α-hydroxylase activity increased several-fold, indicating that this reaction was rate-limiting. Cholesterol-7α-hydroxylase is therefore an important

enzyme in the overall catabolism to bile acids. These results show that a system is now available which can convert cholesterol-4-^{14}C to 7α-hydroxycholesterol in quite large yield (10-15%). Initial studies indicate that the enzyme in these circumstances is subject to the same factors as were found to influence the 7α-hydroxylase system of the normal liver. The effect of cholestyramine feeding may be similar to that obtained by bile duct cannulation, where bile acid production rises to a maximum value about 36-48 hours after surgery.[5] It is thought that during this time, increased enzyme synthesis may occur, so that in a cholestyramine fed animal the increase in activity may be due to larger concentrations of cholesterol-7α-hydroxylase.

A few tentative studies were performed to investigate the possible inhibitory effects of the product of cholesterol-7α-hydroxylase; the enzyme certainly was inhibited at high concentrations of this sterol, but not apparently by the amounts of 7α-hydroxycholesterol which would normally be found in the tissue. Inhibition by sodium taurodeoxycholate was also studied in normal and cholestyramine-fed animals. The re-hydroxylation of this salt by liver microsomes, a very efficient process, might exert a regulatory influence on the 7α-hydroxylation of cholesterol. Again the addition of this bile salt

inhibited the reaction, but at much higher concentrations than are found in the liver cell. The interpretation of these inhibitory effects must await further work in this field; it may ultimately be possible to show that the two 7α-hydroxylation reactions exert a moderating influence on one another. Investigation of the hydroxylation of taurodeoxycholate may show that this reaction is markedly decreased in microsomes from the liver of a cholestyramine-fed rat.

The investigation of the cholesterol-7α-hydroxylase of the 18,000 supernatant fraction was the main object of these investigations; it was obvious however that the metabolism of cholesterol proceeded further than 7α-hydroxycholesterol in the enzyme fraction used. Two products, having the mobilities of cholest-4-en-3-on-7α-ol and cholest-4-en-3-on-7α,12α-diol were detected. These products were not conclusively identified by crystallisation, etc.; their identities were presumed from evidence discussed in section 3, and their formation from 7α-hydroxycholesterol (section 9), with reference to their significance in the overall catabolism of cholesterol. In most of the studies of the cholesterol-7α-hydroxylase system, these products were formed in variable but usually small yields. Their formation was interesting, in that it occurred in the presence of NADPH generator, although in much smaller yields than

were obtained by incubating 7α-hydroxycholesterol (added exogenously or generated from cholesterol) with NAD. Most of the factors which seriously affected the metabolism of cholesterol to 7α-hydroxycholesterol did not markedly affect the further oxidation of this sterol. Evidence was presented to suggest that the oxidation of 7α-hydroxycholesterol in apparently reducing conditions occurred because the enzymes catalysing each step formed a concerted reaction sequence. Significant differences in product formation could be created by incubation of exogenous substrate or with substrate which had been formed from its natural precursor in the enzyme preparation. If a linked series of reactions effects the catabolic steps in this region of cholesterol catabolism, it is reasonable to speculate that perhaps most of the catabolic steps and possibly some of the bio-synthetic steps occur as a concerted process. The accumulation of cholesterol in this sequence may be controlled by a mechanisms as yet unidentified.

Scope for further work

The work described in this thesis was performed with the ultimate aim of identifying, purifying and characterising a cholesterol-7α-hydroxylase in rat liver. These objects were achieved with varying degrees of success. A microsomal preparation of liver, as yet in a very crude form, can metabolise cholesterol-4-^{14}C to 7α-hydroxycholesterol in yields of 10-15%. Recent evidence indicates that a considerable simplification of this system may be obtained by protecting the labile microsomal enzyme with thiol compounds, allowing the sedimentation of this fraction with little loss of enzymic activity. Synthetic co-factors may then be substituted for the natural co-factors of the reaction in the cytoplasm.

! cf. pg 109

Investigations of a rather incomplete nature have shown that cholesterol-7α-hydroxylase is probably an important rate-controlling enzyme in the formation of bile acids by the liver. Further study on this aspect of the catabolism may elucidate the mechanisms of regulation; it is essential to determine whether the observed increase in the activity of the enzyme is due to an increase in activity of the normal system or to an increased synthesis of the enzyme.

With a purified enzyme source it may be possible to assess the role of cholesterol-7α-hydroxylase in terms

of the dynamic aspects of the intact liver cell. The study of enzymes utilising molecular oxygen is a rapidly developing aspect of biochemistry; one can only speculate on the integration of these enzymes with electron transport systems, especially the newly-discovered pigments which are concerned with the activation of molecular oxygen. The investigation of cholesterol-7α-hydroxylase in the light of recent developments in this field may prove invaluable in determining the significance of this enzyme in the formation of the primary bile acids in mammalian liver.

Appendix 1

Efficiency of counting ^{14}C by Packard Liquid Scintillation Spectrometer in the presence of silica gel H.

	1	2	3
counts	14,300 ± 230	14,050 ± 230	13,600 ± 320
gel added	$1cm^2$	$2cm^2$	$4cm^2$
counts	14,120 ± 260	13,940 ± 240	13,780 ± 290

Standard deviations from 8 counts.

The gel added is expressed as an area of gel (approximately 0.25mm thick) removed from the thin layer plate, and inserted in the counting vial.

Appendix 2

Relative R_f values of compounds mentioned in the text.

Solvent system benzene:ethyl acetate :: 7:13; silica gel H; running time 2 hours.

Trivial names are used where applicable.

Proposed intermediates in bile acid formation (section 1)	R_f
cholesterol	0.78
7α-hydroxycholesterol	0.30
cholest-4-en-3-on-7α-ol	0.70
cholest-4-en-3-on-7α,12α-diol	0.26
5 -cholestan-3-on-7α,12α-diol	0.50
trihydroxycoprostane	0.14
dihydroxycoprostane	0.49
26-hydroxycholesterol	0.58
cholest-5-en-3β,7α,26-triol	0.14
cholest-4-en-3α,7α-diol (section 9)	0.31
Autoxidation products, oxygenated sterols and reduction products.	
7-keto-cholesterol	0.52
7β-hydroxycholesterol	0.39
cholestan-3β,5α,6β-triol	0.10
cholesterol-5α-hydroperoxide	0.55
-7α-hydroperoxide	0.55
-5α,6α-epoxide	0.53
-5β,6β-epoxide	0.53

cholest-6-en-3β,5α-diol	0.43
cholestan-3β,5α-diol	0.35
cholestan-3β,6β-diol	0.25

Appendix 3

1. Crystallisation to constant specific activity

A sample of the unknown metabolite was purified by thin layer chromatography. 20 mg. of standard were added, and the sample crystallised three times from hot aqueous acetone. Samples were removed in duplicate at each stage for radioactive and colorimetric assay by the Lifschütz reaction.[84]

The method was tested by crystallising a sample containing a small amount of contaminating radioactivity. Three crystallisations removed 98% of the radioactivity from the crystals.

7α-hydroxycholesterol

Crystallisation	Specific activity (c/m/mg)
-	2550
1	2500
2	2450
3	2600

7β-hydroxycholesterol

-	370
1	380
2	330
3	350

Attempts to crystallise '7-keto-cholesterol' to constant specific activity (radioactive assay / $O.D._{238}$) were not satisfactory (section 3).

2. Cholestan-3β,5α,6β-triol.

This autoxidation product was not characterised by crystallisation to constant specific activity, since a convenient colorimetric assay was not available. Radioactive material was mixed with a few mg. of standard cholestan-3β,5α,6β-triol (preparation ref. 85), dissolved in 10% aqueous dioxan and about 10 mg. of N-Bromosuccinimide added. The solution was allowed to stand at room temperature for 30 minutes, then extracted with ether. Oxidation of the trihydroxylated sterol occurs in these conditions to cholestan-3β,5α-diol-6-one[86]; thin layer chromatography of the product showed that the radioactivity was confined to the area of the oxidation product (R_f 0.28).

3. Reduction with lithium aluminium hydride

7-keto-cholesterol, cholesterol-5α-and 7α-hydro-peroxides, cholesterol-5α,6α- and 5β,6β-epoxides were reduced with lithium aluminium hydride in dry tetrahydro-furan by refluxing for 1 hour. Excess of the reagent was destroyed with ethyl acetate, and the mixture treated with a saturated solution of Rochelle salt (sodium potassium tartrate). The reduction

products were extracted with ether.

The major reduction products of the various compounds are shown below (according to Fieser[87]).

	Major product
7-keto-cholesterol	7β-hydroxycholesterol
cholesterol-5α-hydroperoxide	cholest-6-en-3β,5α-diol
-7α-hydroperoxide	7α-hydroxycholesterol
-5α,6α-epoxide	cholestan-3β,5α-diol
-5β,6β-epoxide	cholestan-3β,6β-diol

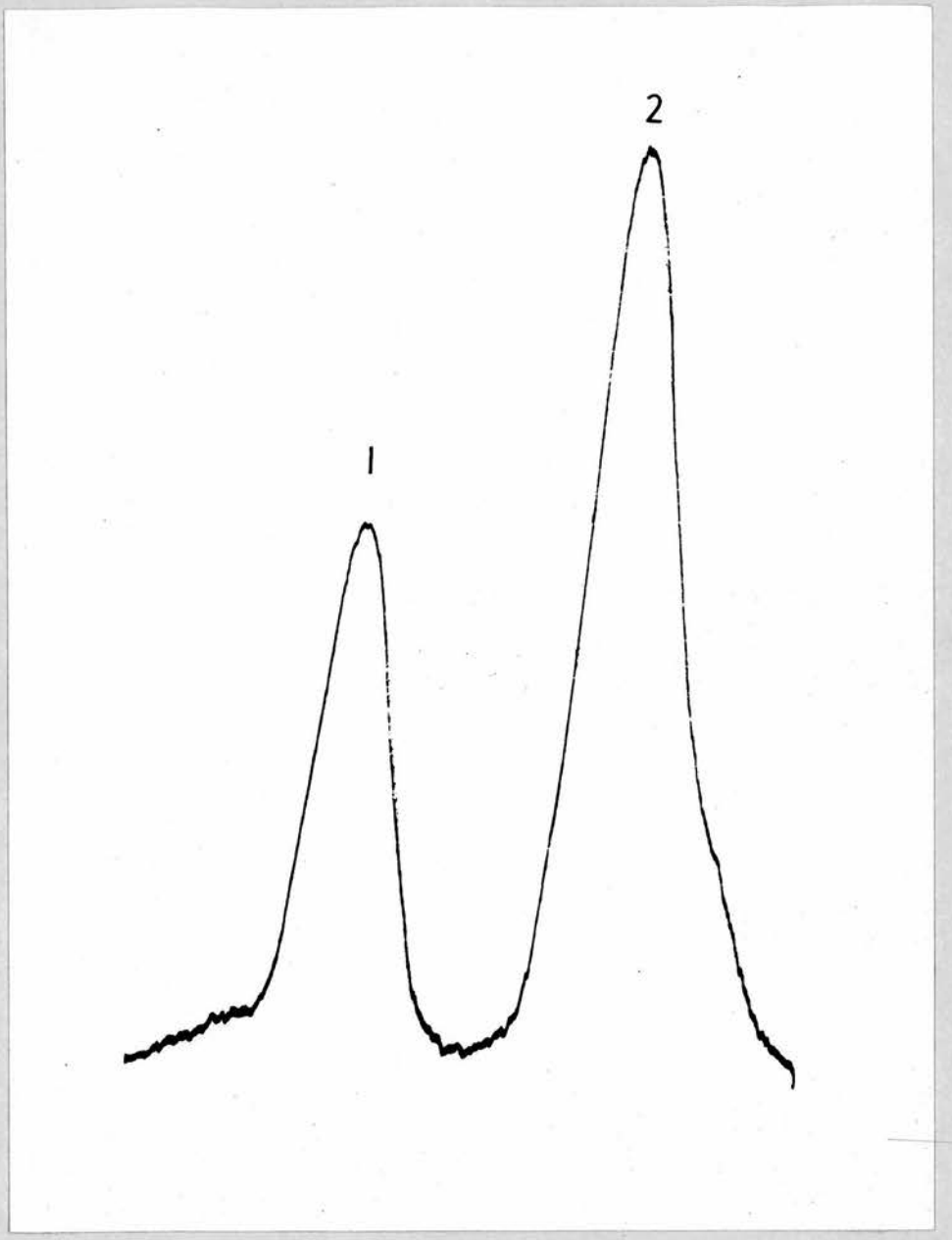


Figure A: Trace by gas-flow counter of the products formed after 10 hours photo-oxygenation of cholesterol-4-^{14}C.

1. cholesterol
2. cholesterol-5α-hydroperoxide.

Appendix 4

Chemical Synthesis

Most of the materials used in the work were available commercially or had been synthesised by previous workers.

1. Preparation of 7α-hydroxycholesterol-4-^{14}C by photo-oxygenation of cholesterol

The photo-oxygenation of cholesterol has been thoroughly investigated by Naqvi[88] and Ali[89]. The reaction was carried out in pyridine with hematoporphyrin as catalyst for 10 hours. A diluted radioactive sample of cholesterol was used (20 mg. = 18 μC).

Figure A shows a radioactive assay by gas-flow counter of the sample after 10 hours irradiation. Cholesterol was converted in about 60% yield to a product with the mobility of cholesterol-5α-hydroperoxide.

After removal of the solvent (at 30° under reduced pressure), the sample was dissolved in dry chloroform and allowed to stand for 18 hours. Isomerisation of the 5α-hydroperoxide function takes place in chloroform by a mechanism which has not yet been elucidated (cf. figure 8, section 3).

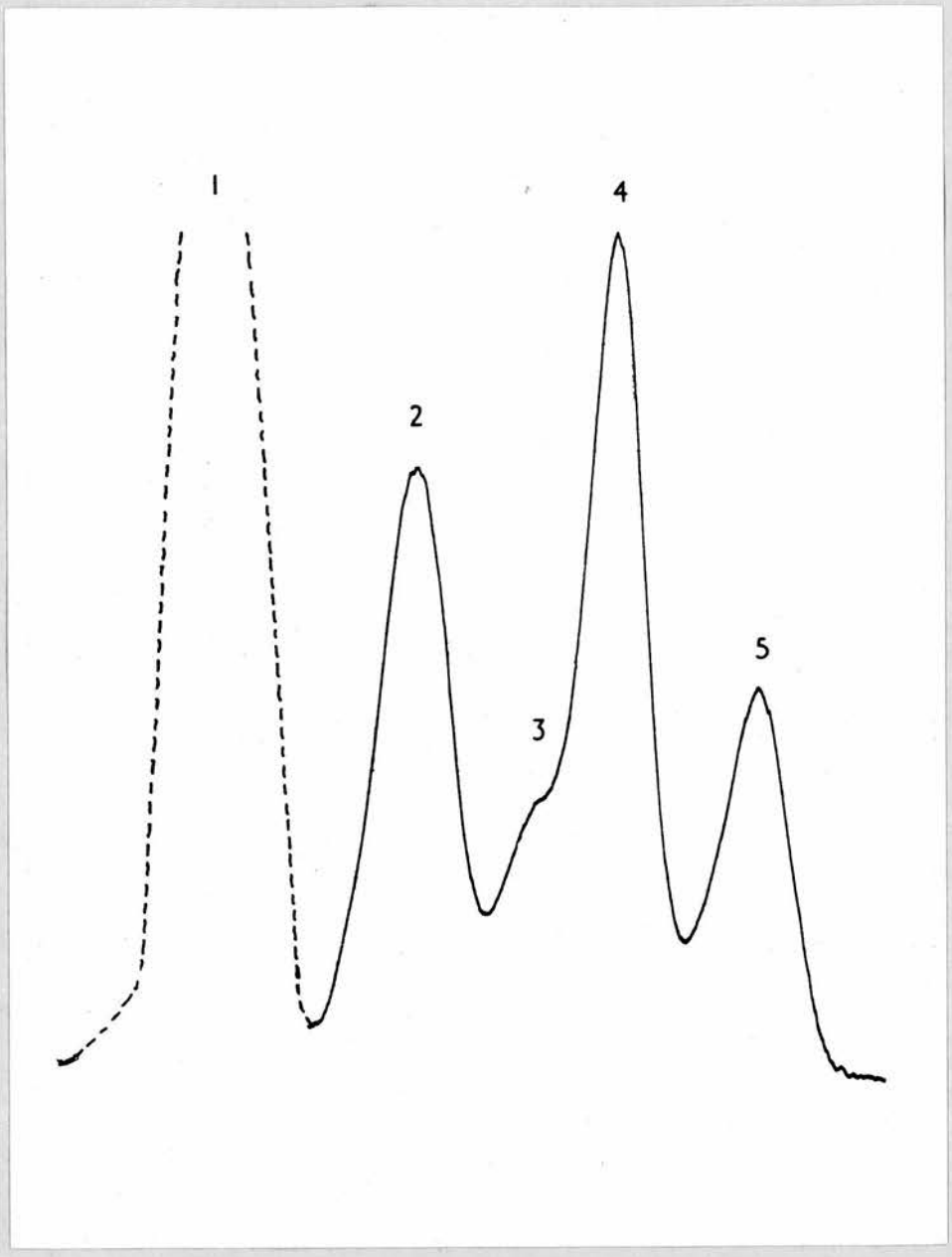


Figure B: Trace by gas-flow counter of the reduction products of cholesterol-hydroperoxide after isomerisation in chloroform.

1. cholesterol-hydroperoxide
2. cholest-6-en-3β,5α-diol
3. 7β-hydroxycholesterol (probably)
4. 7κ-hydroxycholesterol
5. cholesten-3β,6β-diol (estimated)

The sample was reduced with lithium aluminium hydride to give the spectrum of products shown in figure B. The major products were as shown; it was evident that isomerisation had been incomplete, since cholest-6-en-3β,5α-diol was formed in large yield. A product with the mobility of cholestan-3β,6β-diol was also formed; it is conceivable that the mobility of this sterol is similar to cholesten-3β,6β-diol which could be formed by reduction of a 6 -hydroperoxide, suggesting that this hydroperoxide may also be formed by photo-oxygenation of cholesterol.

The radioactive 7α-hydroxycholesterol was purified by thin layer chromatography; subsequent assay showed the sample had a specific activity of 1,750 c/m/μg.

2. Preparation of cholesterol-^{3}H

Randomly tritiated cholesterol was prepared by exposing a sample to tritium gas at low pressure for 7 days.[88] After equilibration, the sample was purified by thin layer chromatography and found to have a specific activity of about 16,000 c/m/μg. (Liebermann-Burchard/radioactive assay).

3. Cholesterol-5α,6α-epoxide-^{3}H

This epoxide was prepared by the method of Spring and Swain[90] from tritiated cholesterol.

4. 7-keto-cholesterol-^{3}H

Tritiated cholesterol acetate was prepared by refluxing cholesterol-^{3}H with acetic anhydride and pyridine. Oxidation to 7-keto-cholesterol was effected with chromic oxide in anaerobic condition.[91] The product was hydrolysed by refluxing for 30 minutes with 5% sodium hydroxide in methanol. After purification by thin layer chromatography 7-keto-cholesterol-^{3}H was obtained with a specific activity (counting/$O.D._{238}$) of 9,000 c/m/μ g. The yield of 7-keto-cholesterol was about 10%.

5. Reduction of 2-amino-4-hydroxy-6-methyl-and 6,7-dimethyl-pteridines

The properties of substituted pteridines have been thoroughly investigated by Viscontini and co-workers.[92]

The pteridines were reduced to the tetrahydro-derivatives in 5N HCl with 2 mg. of palladium on charcoal in the presence of gaseous hydrogen. The mixture was shaken for three hours in the dark.

The catalyst was removed by centrifugation and the solvent evaporated at 40° under reduced pressure. The white crystalline product in the form of the hydrochloride was crystallised from water : ethanol : acetone, 1 : 1 : 3 and stored at -15° in the

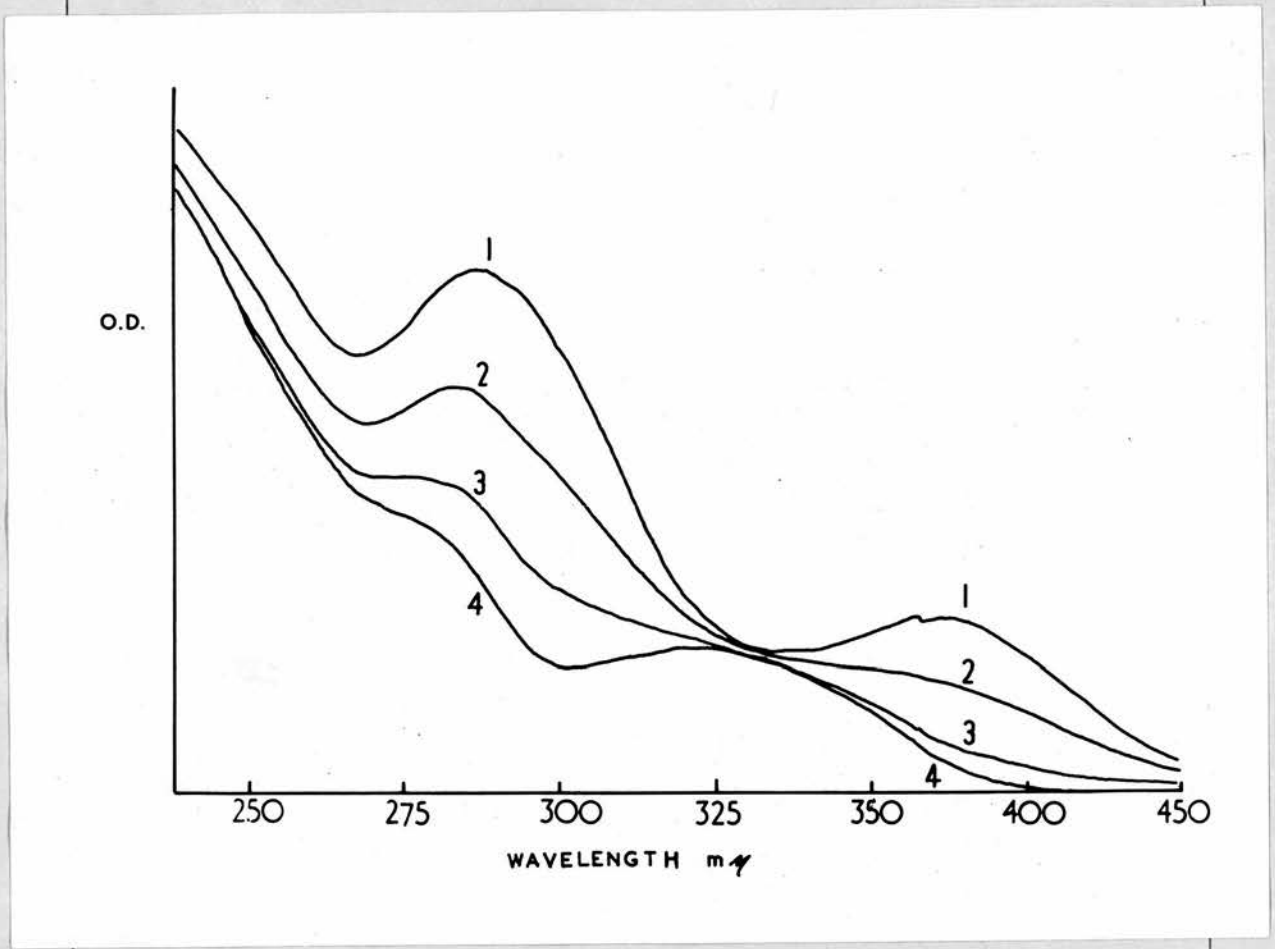


Figure C: Decomposition spectrum of 2-amino-4-hydroxy-6-methyl-tetrahydropteridine in 0.1 M NaOH.

1 - time zero
4 - after 10 minutes.

dark in 0.005 N hydrochloric acid.

Reduced pteridines tend to be unstable to light and oxygen; samples were assayed immediately before use by an optical method. Figure C shows the rapid decomposition of one of the reduced pteridines in 0.1 N sodium hydroxide. The characteristic peak of the reduced pteridine at about 280 mμ disappears rapidly under these conditions.

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The Hydroxylation of Cholesterol by Liver Microsomes

By J. R. MITTON and G. S. BOYD. (*Department of Biochemistry, University of Edinburgh*)

Various studies have indicated that one of the early stages in the formation of bile acids from cholesterol is the introduction of a hydroxyl group at C-7, (Lindstedt, 1957) suggesting that cholest-5-ene-3β,7α-diol may be an intermediate in bile acid biosynthesis. The stereochemistry of this hydroxylation at C-7 has been examined by Bergstrom, Lindstedt, Samuelsson, Corey & Gregoriou (1958) and shown to be achieved with retention of the configuration at that position. Certain hydroxylation reactions have been shown to require oxygen together with NADPH and are located in the liver microsomal fraction (Brodie *et al.* 1955). Danielsson & Einarsson (1964) reported the presence of a 7α-hydroxylating system which was not influenced by the addition of NADPH.

Studies on the phenylalanine hydroxylase system by Kaufman (1963) have demonstrated the participation of dihydrobiopterin in this system. We have investigated the mechanism of microsomal cholesterol hydroxylation and studied the effects of NADPH, ADP and certain pteridines on the reaction.

The livers of normal young rats were homogenized in 0·25M-sucrose and the microsomal fraction isolated in the usual way. 4-[^{14}C]Cholesterol was purified by chromatography immediately before use, and added to the microsomal preparations in acetone solution together with cofactors such as NADPH, 6,7-dimethyltetrahydropteridine, ADP, etc., in different combinations and concentrations. The mixtures were incubated in oxygen at 37° with shaking for periods from 10 to 60min. and then extracted with methanol, chloroform and ethyl acetate. The lipid extracts were subjected to a preliminary separation on alumina columns followed by final resolution by thin-layer chromatography.

It has been found that the microsomal fraction of rat liver in the presence of oxygen and NADPH is capable of metabolizing added cholesterol to a variety of products. In the presence of a tetrahydropteridine the principal metabolic product was cholest-5-ene-3β,7α-diol, suggesting that a reduced pteridine exerts an influence in the microsomal 7α-hydroxylation of cholesterol. Investigations using different buffer systems and ADP showed the formation of material with an R_F similar to cholest-5-en-3β-ol-7-one. Re-incubation of this substance produced a substantial conversion to cholest-5-ene-3β,7β-diol and another product with the R_F suggestive of a trihydroxylated sterol. The significance of these studies in relation to the overall conversion of cholesterol to bile acids in rat liver will be discussed.

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The Enzymic Hydroxylation of Cholesterol by Microsomal Preparations of Rat Liver

By J. R. MITTON and G. S. BOYD. (*Department of Biochemistry, University of Edinburgh*)

There is evidence that 7α-hydroxycholesterol is an intermediate in the conversion of cholesterol to the primary bile acids by mammalian liver (Lindstedt, 1957). Recent evidence (Danielsson & Einarsson, 1966) suggests that the 7α-hydroxylation of cholesterol and metabolism to 3α,7α,12α-trihydroxycoprostane is effected by the liver endoplasmic reticulum (microsomes) together with the cell sap. Factors influencing the 7α-hydroxylation of cholesterol and the metabolism of this intermediate in microsomal preparations of rat liver have been studied.

Livers from normal male rats were homogenized in 0·25M-sucrose and centrifuged at 20000*g* for 20min. to remove nuclei, cell debris and mitochondria, leaving only the microsomes and cell supernatant. The microsomal fraction was sedimented at 105000*g* for 50min. [4-^{14}C]Cholesterol was purified before use by thin-layer chromatography and added to incubations in a small volume of acetone. Further additions were made in water to give a final volume of about 7ml. with phosphate buffer, pH 7·4. Incubations were conducted at 37° with shaking in air. Lipids were extracted with methanol, chloroform and ethyl acetate and the lipid extract was finally washed with water. Final analysis was effected by thin-layer chromatography with subsequent elution and liquid-scintillation counting.

The most active 7α-hydroxylation system was found to be the 20000*g*/20min. supernatant (microsomes plus cell sap) fortified with NADPH. Re-combination of the separated microsomal and supernatant fractions gave a reduced activity, as did the microsomal fraction alone, indicating that some inactivation occurs during the fractionation procedure and suggesting the presence of a 'co-factor' in the supernatant. The only addition which produced a marked stimulation of the 7α-hydroxylase activity was NADPH in contrast to the finding of Danielsson & Einarsson (1964).

Autoxidation of cholesterol was minimal in this system but was increased in the presence of magnesium ions, which also produced a decrease in the enzymic 7α-hydroxylase activity. In a previous communication (Mitton & Boyd, 1965), some of the complexities of the autoxidation problem were described; it appears that cholesterol forms such products by a 'lipoperoxidation' mechanism in the presence of ferrous ions, nucleotides, phosphates etc.

The intermediate, 7α-hydroxycholesterol, is efficiently converted by the microsomal fraction in the presence of NAD^+ into 7α-hydroxycholest-4-en-3-one. In the presence of the microsomes plus supernatant fraction, 12α-hydroxylated products have been detected (cf. Danielsson & Einarsson, 1966). The factors and conditions influencing these two hydroxylations will be discussed.

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