review

# Cholesterol 7α-hydroxylase

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**Contents**



## I. THE 7α-HYDROXYL GROUP IN BILE ACIDS

A 7α-hydroxyl group is present in cholic acid and chenic (chenodeoxycholic) acid, the two primary bile acids synthesized from cholesterol in the livers of most mammals. A proportion of the primary bile acids reaching the lumen of the small intestine is modified by intestinal micro-organisms to form secondary bile acids lacking a 7α-hydroxyl group, with deoxycholate arising from cholic acid and lithocholate from chenic acid. The primary and secondary bile acids are partially reabsorbed, reaching the liver via the portal vein. In rats, the secondary bile acids are hydroxylated in the 7α position by hepatic enzymes and are then secreted into the intestine, together with re-absorbed and newly-synthesized primary bile acids. Since reabsorption of bile acids from the intestinal lumen is incomplete, bile acids must be continually synthesized in the liver to balance the daily loss via the feces. Furthermore, the rate of synthesis must be adjusted to meet the biological requirements of the animal. The enzyme system catalyzing the introduction of a 7α-hydroxyl group into the nucleus of cholesterol is present in liver microsomes and is known as cholesterol 7α-hydroxylase (EC 1.14). The enzymes responsible for the 7α-hydroxylation of secondary bile acids and of certain steroid hormones are almost certainly different from cholesterol 7α-hydroxylase and are not dealt with systematically in this review.

## II. HISTORICAL BACKGROUND

The biological derivation of bile acids from cholesterol was first suggested when their structural similarities became apparent in the 1920's. Proof that cholesterol is a precursor of bile acids was first obtained by Bloch, Berg, and Rittenberg in 1943 (1), who isolated highly labeled cholic acid from a dog given deuterium-labeled cholesterol. Subsequent work with [$^{14}$C]cholesterol (2, 3) showed that bile acids are the major end product of the metabolism of cholesterol and that in many species their excretion in the feces is the main route for the removal of exchangeable cholesterol from the body. (In man, only about half the cholesterol removed from the body is removed as bile acid.)

The steps involved in the formation of cholic and chenic acids from cholesterol include the oxidative cleavage of the side chain between C-24 and C-25, the formation of a 5β (A/B, *cis*) saturated ring system

Abbreviations: GLC, gas–liquid chromatography; MS, mass spectrometry; TLC, thin-layer chromatography.

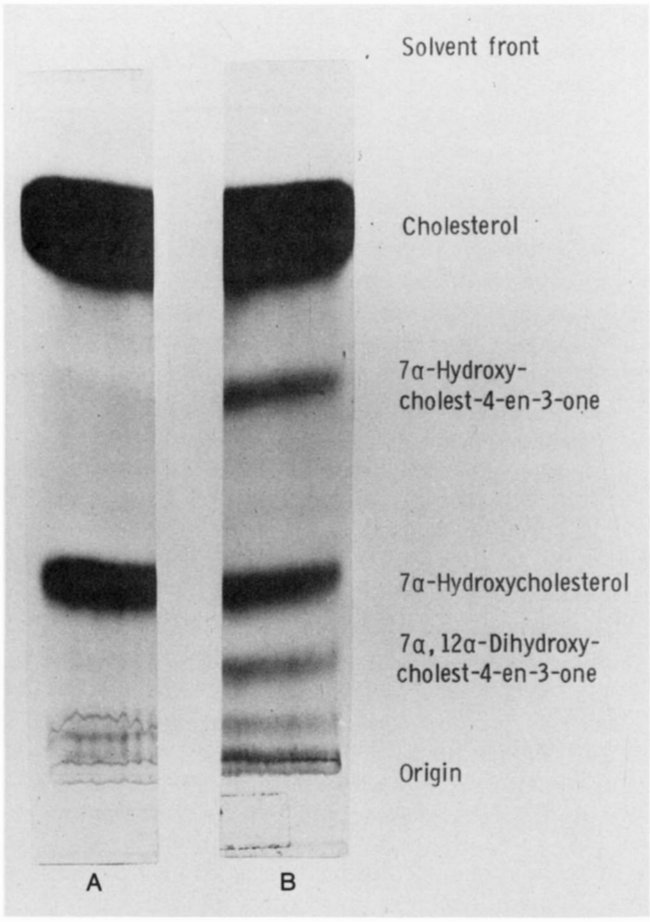


**Fig. 1.** Radioautographs of thin-layer chromatograms of the extracts from incubations of rat liver microsomes in the presence of [4-$^{14}$C]cholesterol. In both experiments the microsomes were prepared from livers homogenized in the presence of EDTA (1 mM). The standard incubation mixture contained, in a final volume of 5 ml, 3 ml of microsomal suspension (7 mg of protein), 0.1 M potassium phosphate buffer (pH 7.4), 30 mM nicotinamide, 5 mM $MgCl_2$, 1 mM NADP, 10 mM glucose 6-phosphate and 1 unit of glucose 6-phosphate dehydrogenase. In A, the standard incubation mixture was used; in B, the standard incubation mixture was supplemented with NAD (0.3 mM). In both, the gas phase was air and the incubation was carried out for 1 hr at 37°C. Note that in A the main radioactive product of the metabolism of cholesterol was 7α-hydroxycholesterol (containing 5% of the total radioactivity added as [$^{14}$C]cholesterol), whereas in B some of the 7α-hydroxycholesterol formed during the incubation was converted into 7α-hydroxycholest-4-en-3-one and 7α,12α-dihydroxycholest-4-en-3-one.

by saturation of the $\Delta^5$ double bond, inversion of the 3β hydroxyl group to the 3α-configuration and the introduction of hydroxyl groups into the 7α and 12α positions (cholic acid) or into the 7α position (chenic acid). During the 1950's and 1960's it was established beyond reasonable doubt that the first of these steps in the conversion of cholesterol into cholic acid in rat liver is the introduction of the 7α-hydroxyl group into the sterol ring system, leading to the formation of cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol). The evidence for this conclusion was derived mainly from experiments on the incorporation of labeled hypothetical intermediates into cholic acid in vivo and from the nature of the labeled intermediates that accumulate in cell-free preparations of liver incubated in the presence of labeled cholesterol. This evidence has been reviewed in detail elsewhere (4, 5) and need not be considered here.

Chenic acid is also formed from cholesterol in rat liver by a pathway in which the first intermediate is 7α-hydroxycholesterol, but there is evidence to suggest that some chenic acid is formed via another pathway in which hydroxylation and cleavage of the side chain of cholesterol precede modification of the ring system (6, 7). In this pathway, therefore, 7α-hydroxycholesterol is not an intermediate. The quantitative importance of the alternative pathway to chenic acid is difficult to assess. In any case, since cholic acid is the major bile acid formed by rats and human beings, the enzyme system responsible for the 7α-hydroxylation of cholesterol must play a major role in the overall synthesis of bile acids in rat and human liver.

Once it became clear that 7α-hydroxycholesterol is an intermediate in the biosynthesis of bile acids, attention was focused on the nature of cholesterol 7α-hydroxylase, the mechanism of the hydroxylation, the rate-limiting role of this step in the formation of bile acids from cholesterol and, finally, the physiological regulation of the enzyme. An experimental approach to these questions necessitated the development of methods for assaying cholesterol 7α-hydroxylase. Several methods for assaying the activity of this enzyme are in current use, though each one is open to theoretical or practical objections. Nevertheless, despite these shortcomings, much progress has been made during the past decade towards an understanding of cholesterol 7α-hydroxylase.

## III. ASSAY OF CHOLESTEROL 7α-HYDROXYLASE

### Incorporation of [$^{14}$C]cholesterol

If [4-$^{14}$C]cholesterol is incubated with a suspension of liver microsomes under suitable conditions, the radioactive cholesterol is converted into 7α-hydroxy[$^{14}$C]cholesterol at a rate related to the activity of the cholesterol 7α-hydroxylase in the microsomes. The radioactive 7α-hydroxycholesterol may then be isolated from the incubation medium and assayed for radioactivity. This is the principle underlying the methods first used for assaying cholesterol 7α-hydroxylase activity and of many of those still in current use (8–10). The most convenient method for isolating 7α-hydroxycholesterol from the incubation mixture is by thin-layer chromatography (TLC)

of the sterols extracted from the incubation medium. **Fig. 1** shows the radioautograph of a thin-layer chromatogram of the sterols obtained after incubation of an emulsion of [4-$^{14}$C]cholesterol in a suspension of liver microsomes containing NADPH and a system for continuous generation of NADPH from NADP.

In any assay system based on measurement of the incorporation of $^{14}$C from labeled cholesterol into 7$\alpha$-hydroxycholesterol in the presence of liver microsomes, certain conditions should be satisfied in addition to those required for enzymic hydroxylation (the presence of NADPH and molecular $O_2$).

(*a*) The formation of 7$\alpha$-hydroxycholesterol in the incubation mixture by nonenzymic autoxidation of cholesterol must be eliminated or minimized. This may be achieved by homogenizing the liver in the presence of EDTA (8) or by adding a sulfhydryl reagent such as $\beta$-mercaptoethylamine (10) to the incubation mixture. As an additional precaution, a correction for nonenzymic formation of 7$\alpha$-hydroxycholesterol may be made by running control incubations with boiled microsomes.

(*b*) The assay conditions must be such that the 7$\alpha$-hydroxycholesterol formed from cholesterol is not converted into intermediates further along the pathway to bile acids during the course of the incubation. In the intact liver cell, 7$\alpha$-hydroxycholesterol is converted enzymically into 7$\alpha$-hydroxycholest-4-en-3-one with NAD as cofactor. This compound is the major substrate for the enzyme catalyzing the introduction of a 12$\alpha$-hydroxyl group into the sterol ring system during the formation of cholic acid; the enzyme is present in liver microsomes and requires NADPH (11) and molecular $O_2$ (12). Hence, if NAD, in addition to NADPH and $O_2$, is present in the incubation mixture, some of the radioactive 7$\alpha$-hydroxycholesterol formed during the incubation will be removed by conversion into other intermediates and the net rate of accumulation of 7$\alpha$-hydroxy[$^{14}$C]-cholesterol in the incubation medium will be less than its rate of production. A one-step reaction, in which 7$\alpha$-hydroxycholesterol is the sole end product formed enzymically from cholesterol, may be achieved simply by omitting NAD from the reaction mixture, since the amount of endogenous NAD present in a suspension of liver microsomes is not sufficient to permit the formation of 7$\alpha$-hydroxycholest-4-en-3-one from 7$\alpha$-hydroxycholesterol. As may be seen in Fig. 1, in the absence of exogenous NAD, radioactive compounds other than 7$\alpha$-hydroxycholesterol are not formed in significant amounts during an assay carried out under the proper conditions.

(*c*) The form in which the [4-$^{14}$C]cholesterol is added to the incubation mixture must be standardized. The addition of a lipid substrate to an aqueous medium in a physical form in which it is fully accessible to an enzyme in the medium raises problems familiar to anyone who has worked with enzymes that use lipids as substrates. When the radioactive cholesterol used during an assay of cholesterol 7$\alpha$-hydroxylase is present in the incubation medium in the form of an emulsion, the rate at which the added cholesterol is 7$\alpha$-hydroxylated may be influenced by the particle size of the emulsion. Hence, apparent differences in enzyme activity under different experimental conditions may be due to differences in the physical state of the exogenous substrate rather than to differences in the capacity or degree of activation of cholesterol 7$\alpha$-hydroxylase itself. This, presumably, is why the measured activity of the enzyme in a given preparation of liver microsomes differs according to whether the radioactive cholesterol is added as an acetone solution or as an emulsion in a nonionic detergent (13, 14). A further problem arises from our lack of understanding as to how, and in what form, endogenous cholesterol gains access to the membrane-bound enzyme system in liver microsomes. Because of this uncertainty one cannot be sure that the behavior of the enzyme towards exogenous cholesterol added to an incubation medium is identical with its behavior towards the endogenous cholesterol that acts as its natural substrate.

One way of overcoming the problems arising from the use of exogenous substrate in the assay system would be to label the microsomal cholesterol in the liver by administration in vivo of a labeled precursor of cholesterol and then to measure the rate of production of labeled 7$\alpha$-hydroxycholesterol by the microsomes incubated in vitro. This approach has, in fact, been used (13) but is clearly not suitable for routine use and could not normally be used for the study of cholesterol 7$\alpha$-hydroxylase in human liver. Nor would it be applicable to kinetic studies of the relation between enzyme activity and substrate concentration. Despite these theoretical objections to assay methods based on the use of labeled exogenous cholesterol, extensive experience in many laboratories suggests that such methods are valid for comparative purposes, even if they may fail to give an absolute measurement of enzyme activity, provided that the method of adding labeled substrate to the assay system is rigidly standardized.

(*d*) If changes in the rate of product formation are to be interpreted unequivocally, it is usually essential to measure the activity of an enzyme under conditions in which substrate supply is not rate-limiting. Indeed, statements about changes in the activity of an enzyme are generally taken to imply that activity was measured in the presence of saturating concen-

trations of substrate. Unfortunately, it has not yet been possible to devise conditions under which cholesterol 7α-hydroxylase can be assayed in the presence of concentrations of cholesterol sufficient to saturate the enzyme (see Section V). Hence, changes in the measured activity of this enzyme (expressed as the rate of formation of 7α-hydroxycholesterol/mg of protein, irrespective of substrate concentration) may be brought about by changes in the amount or accessibility of microsomal substrate, as well as by changes in the capacity or state of activation of the enzyme.

### Tritiated cholesterol as substrate

The introduction of the 7α-hydroxyl group into the ring system of cholesterol by cholesterol 7α-hydroxylase is completely stereospecific, the hydroxyl group replacing the 7α hydrogen atom of cholesterol without change in the configuration of the 7β hydrogen atom (15). Hence, if [7α-$^3$H]cholesterol is used as substrate for cholesterol 7α-hydroxylase in an assay system, $^3$H will be released into the medium at a rate proportional to the rate of 7α-hydroxylation of the substrate. The activity of the enzyme may thus be assayed by measuring the amount of $^3H_2O$ that accumulates in the medium during a standard period of incubation. This, in principle, is the method introduced by Gielen, Van Cantfort, and Renson (16) for assaying cholesterol 7α-hydroxylase in liver microsomal preparations. The method gives results agreeing closely with those obtained with [4-$^{14}$C]-cholesterol as substrate and has been used extensively by Gielen and co-workers in their studies of the diurnal rhythm in cholesterol 7α-hydroxylase activity. However, the [$^3$H]cholesterol method has not yet been widely adopted, partly because it is difficult to synthesize tritiated cholesterol in which the tritium is present exclusively in the 7α position. As with assay methods based on the use of [4-$^{14}$C]cholesterol as substrate, it is essential, when using the method of Gielen et al. (16), to minimize nonenzymic autoxidation of the substrate and to standardize the form in which the substrate is added to the incubation mixture.

### Calculation of enzyme activity from isotope incorporation

In the early work on cholesterol 7α-hydroxylase, enzyme activity was expressed in terms of the percent of [$^{14}$C]cholesterol added to the incubation medium that was converted into 7α-hydroxycholesterol. Although this empirical approach yielded much valuable information about the influence of drugs and other factors on enzyme activity (reviewed in (5)), the catalytic properties of the enzyme cannot be investigated in any depth unless the results of an assay can be expressed in terms of the rate of formation of 7α-hydroxycholesterol. If there were no endogenous cholesterol in liver microsomes, the amount of 7α-hydroxycholesterol formed during an incubation could be calculated from the specific radioactivity of the exogenous cholesterol in the incubation medium and the radioactivity recovered in the product. However, liver microsomes contain cholesterol, and no method has yet been devised for removing this cholesterol without inactivating the microsomal cholesterol 7α-hydroxylase. Hence, estimates based on the amount of radioactivity recovered in the 7α-hydroxycholesterol formed during an incubation cannot be made without making assumptions as to how far the specific radioactivity of the exogenous cholesterol is diluted by equilibration with endogenous cholesterol in the microsomes. Some workers have assumed that equilibration is complete and have estimated the specific radioactivity of the substrate from the amount of radioactivity added as exogenous cholesterol and the total amount of cholesterol (exogenous plus endogenous) in the incubation mixture (9). Others have used high concentrations of labeled exogenous cholesterol and have assumed that, under these conditions, the endogenous cholesterol in the microsomes has no significant effect on the specific radioactivity of the exogenous cholesterol acting as substrate for cholesterol 7α-hydroxylase (8).

### Measurement of the mass of 7α-hydroxycholesterol

The uncertainties in the assumptions that underlie determination of the activity of cholesterol 7α-hydroxylase by radioisotopic methods have led to the development of methods for measuring the mass of 7α-hydroxycholesterol formed during the assay procedure. In the first of these methods to be developed (17), a trace of [4-$^{14}$C]cholesterol was added to the incubation mixture as exogenous substrate. The radioactive 7α-hydroxycholesterol present at the end of the incubation was then isolated from the total sterol fraction by TLC and was acetylated with [$^3$H]acetic anhydride of known specific radioactivity. The [$^3$H,$^{14}$C]diacetate of 7α-hydroxycholesterol was purified and its $^3$H and $^{14}$C radioactivities were determined. The mass of 7α-hydroxycholesterol recovered as the diacetate could then be calculated from the known specific radioactivity of the [$^3$H]-acetyl group, measurement of the $^3$H/$^{14}$C ratio also making it possible to correct for losses occurring during TLC of the sterol fraction and the subsequent acetylation procedure. The mass of 7α-hydroxycholesterol formed during the incubation was estimated from the difference between the amounts pres-

ent at the beginning and end of the incubation. Since an essential part of this method was the measurement of the mass and $^{14}C$ radioactivity of 7$\alpha$-hydroxycholesterol, the specific radioactivity of the newly-formed 7$\alpha$-hydroxycholesterol could be compared with that of the exogenous cholesterol added to the microsomal suspension, thus opening the way to the study of the compartmentation of microsomal cholesterol discussed below. The acetylation method of Mitropoulos and Balasubramaniam (17) has been modified by Shefer, Nicolau, and Mosbach (18), with some simplication of the acetylation procedure and an improvement in the correction for incomplete recovery of 7$\alpha$-hydroxycholesterol.

Björkhem and Danielsson (14, 19) have developed an ingeniously simple method for assaying 7$\alpha$-hydroxycholesterol in suspensions of liver microsomes. A known amount of trideuterated 7$\alpha$-hydroxycholesterol is added to the suspension at the end of the incubation. The ratio of trideuterated to unlabeled molecules in the 7$\alpha$-hydroxycholesterol isolated from the incubation mixture is then determined by a combination of gas–liquid chromatography with mass spectrometry, after conversion of the 7$\alpha$-hydroxycholesterol into its trimethylsilyl ether. The mass of unlabeled 7$\alpha$-hydroxycholesterol present in the incubation mixture is calculated from the extent of dilution of labeled molecules with unlabeled molecules, no correction for incomplete recovery being required. This method is less time-consuming than the method of Mitropoulos and Balasubramaniam (17) and could be used to measure picogram amounts of 7$\alpha$-hydroxycholesterol. However, the instruments required for GLC–MS are very expensive.

Both the acetylation method and the GLC–MS method agree in showing that the amount of 7$\alpha$-hydroxycholesterol formed during an incubation of a suspension of liver microsomes may differ by a factor of two to three from the amount estimated from the incorporation of $^{14}C$ from [4-$^{14}C$]cholesterol into 7$\alpha$-hydroxycholesterol, if the calculation is based on the assumption that the exogenous cholesterol equilibrates completely with all the endogenous cholesterol in the microsomes (13, 14). The reasons for this discrepancy have been discussed by Balasubramaniam, Mitropoulos, and Myant (13) and by Björkhem and Danielsson (14). In brief, if the radioactive cholesterol is added in a form such that it equilibrates preferentially with the pool of endogenous cholesterol that is accessible to the enzyme (see Section V), enzyme activity will be overestimated by the radioisotopic method; if, on the other hand, some of the radioactive cholesterol remains inaccessible to the enzyme (e.g., by precipitation in the medium), enzyme activity estimated by the radioisotopic method will be erroneously low.

### Clinical applications

Assay methods based on measurement of the incorporation of radioactivity from [$^{14}C$]cholesterol into 7$\alpha$-hydroxycholesterol can be adapted for use with the milligram quantities of human liver obtainable by percutaneous biopsy (20). In view of the theoretical objections to radioisotopic methods discussed above, it would be preferable to use a method based on measurement of the mass of 7$\alpha$-hydroxycholesterol formed during an incubation. The GLC–MS method of Björkhem and Danielsson (19) would certainly be sensitive enough for this purpose. Alternatively, the feasibility of measuring 7$\alpha$-hydroxycholesterol by radioimmunoassay could be explored.

## IV. THE NATURE OF CHOLESTEROL 7$\alpha$-HYDROXYLASE

### Mixed-function oxidases

Cholesterol 7$\alpha$-hydroxylase belongs to a class of enzyme systems known as mixed-function oxidases. The term "mixed-function oxidation", introduced by Mason (21), refers to an enzyme-catalyzed hydroxylation in which one atom of a molecule of $O_2$ is introduced into the substrate, the other oxygen atom being reduced to water by a reduced coenzyme (usually NADPH). The stoichiometry of the reaction shows that two electrons are taken up for each molecule of $O_2$ consumed. Hence, the net reaction may be written

$$RH + NADPH \text{ (or } NADH) + H^+ + O_2 \rightarrow ROH + NADP^+ \text{ (or } NAD^+) + H_2O$$

where RH is the substrate.

Since the oxygen atom introduced into the substrate is derived from molecular $O_2$, the product will contain $^{18}O$ if the reaction is carried out in the presence of $^{18}O_2$. Moreover, if the H atom at the substrate position to be hydroxylated is labeled, the label will be displaced quantitatively from hydroxylated substrate molecules into the medium.

### Components and cofactor requirements of the enzyme system

Cholesterol 7$\alpha$-hydroxylase has certain features in common with several hepatic mixed-function oxidases, including those using drugs, steroids, fatty acids, and carcinogens as substrates. In all these enzyme systems, the activator of oxygen and the sub-

strate is cytochrome P450, a heme pigment discovered in liver microsomes in 1958 (22, 23) and so designated because the difference spectrum of the CO complex of the reduced ($Fe^{2+}$) form has a peak at 450 nm. All hepatic cytochrome P450-requiring hydroxylations use NADPH as the donor of electrons and a flavoprotein[1] (NADPH-cytochrome c reductase) that catalyzes the transfer of electrons from NADPH to cytochrome P450. Both cytochrome P450 and the reductase are present in liver microsomes. Hence, many hydroxylations of this class are catalyzed by suspensions of washed liver microsomes in the presence of NADPH, $O_2$, and a system for maintaining NADPH in the reduced state. Since CO combines reversibly with cytochrome P450 at its oxygen-combining site, hydroxylations requiring cytochrome P450 are inhibited if the reaction is carried out in the presence of CO as well as $O_2$, and the inhibition is reversed by monochromatic light of wavelength 450 nm.

Workers in several laboratories have shown that cholesterol 7$\alpha$-hydroxylase requires NADPH and $O_2$ for full activity (8, 10, 17) and that the microsomal 7$\alpha$-hydroxylation of cholesterol is inhibited by CO (10, 17, 24–26), with reversal of inhibition by light at 450 nm (27).

More direct evidence that cytochrome P450 is required for the 7$\alpha$-hydroxylation of cholesterol has recently been provided by the reconstitution experiments of Björkhem, Danielsson, and Wikvall (28), who used partially purified preparations of cytochrome P450 to restore the cholesterol 7$\alpha$-hydroxylase activity of inactive subfractions of liver microsomes. The evidence implicating NADPH-cytochrome c reductase in the 7$\alpha$-hydroxylation of cholesterol is largely inferential, although Wada et al. (29) have reported that an antibody to this flavoprotein inhibits the reaction. The 7$\alpha$-hydroxylation of cholesterol resembles other hepatic microsomal mixed-function oxidases in not requiring the iron–sulfur protein (adrenodoxin) that is essential for cytochrome P450-dependent hydroxylations occurring in adrenal mitochondria.

### Mechanism of the hydroxylation

Most of our ideas about the mechanism of cholesterol 7$\alpha$-hydroxylation are derived from work on the hydroxylation of drugs by cytochrome P450-requiring enzyme systems present in liver microsomes and certain bacteria. Little work has been done specifically on the mechanism of the 7$\alpha$-hydroxylation of cholesterol, largely because it is so difficult to achieve satisfactory experimental conditions. First, there is the problem, discussed above, of devising a valid assay method; second, there is the complication introduced by the unavoidable presence of endogenous substrate in the reaction mixture; third, it is difficult to isolate the components of the enzyme system without marked loss of activity. Investigation of the hydroxylation of drugs by liver microsomes, and by bacteria grown under conditions leading to induction of cytochrome P450, is largely free from these drawbacks. The cytochrome P450-dependent hydroxylation of a number of drugs by purified components obtained from these sources has been studied by following changes in the optical and EPR spectra of the cytochrome during the course of the reaction. As a result of such studies, summarized in reference (30), the initial sequence of reactions in these drug hydroxylations has been established. However, the later reactions, particularly those concerned in the transfer of the second electron and in the introduction of the activated oxygen atom into the substrate, are still the subject of speculation.

The first step in the reaction sequence is the reversible combination of the substrate with cytochrome P450 in the oxidized form ($Fe^{3+}$). This is followed by the reduction of the cytochrome P450–substrate complex to the $Fe^{2+}$ form by transfer of one electron from NADPH. Molecular $O_2$ then reacts reversibly with the reduced cytochrome–substrate complex to give a ternary complex of $Fe^{2+}$ cytochrome P450, substrate, and $O_2$. The subsequent steps involve the transfer of a second electron to the ternary complex, the introduction of one atom of oxygen into the substrate, expulsion of water from the complex, and the release of hydroxylated substrate and cytochrome P450 in the oxidized form.

Work with solubilized preparations of cytochrome P450 from liver microsomes has shown that lecithin is required for full drug-hydroxylating activity of reconstituted systems, the lecithin possibly playing a role in the transfer of the first electron from NADPH (31). There is some direct evidence for the formation of oxygenated substrate–cytochrome intermediates (32) during the hydroxylation of drugs by cytochrome P450. However, little is known about the acceptor for the second electron or about the intramolecular events leading to the insertion of O into the substrate. It has been suggested that cytochrome $b_5$ participates in the transfer of the second electron during the cytochrome P450-dependent hydroxylation of certain drugs by liver microsomes (33). On the other hand, cytochrome $b_5$ is not a component

[1] Some authors refer to this enzyme as NADPH-cytochrome P450 reductase. Since it is assayed in terms of its ability to catalyze the reduction of cytochrome c by NADPH, we prefer to call it NADPH-cytochrome c reductase.

of the solubilized microsomal hydroxylating system described by Coon et al. (34).

The experiments on which much of this evidence is based were made possible by the development of methods for obtaining soluble preparations of cytochrome P450 that were free from the other components of the oxidase system and that retained their catalytic activity (35). Solubilization of the microsomal cytochrome P450 required for the 7$\alpha$-hydroxylation of cholesterol has proved to be very difficult, largely owing to loss of activity of this enzyme system during contact with the ionic detergents used during the purification procedures. Recent progress in the solubilization of membrane-bound cholesterol 7$\alpha$-hydroxylase from rat liver microsomes (36, 37) suggests that this problem may eventually be overcome and that it will be possible to study the reaction mechanisms involved in 7$\alpha$-hydroxylation of cholesterol. In the meantime, there is no reason to doubt that the reactions in this hydroxylation are essentially the same as those occurring in other cytochrome P450-requiring hydroxylations.

There is no direct evidence for the formation of a cholesterol–cytochrome P450 complex during the 7$\alpha$-hydroxylation of cholesterol by liver microsomes. However, the adrenal mitochondrial cytochrome P450 that catalyzes the cleavage of the cholesterol side chain between C-20 and C-22 (cytochrome $P450_{scc}$) has been obtained in a state of purity sufficient for examination of the interaction between cytochrome and substrate (38–40). This work has shown that cholesterol combines reversibly with oxidized cytochrome $P450_{scc}$, leading to a change in the optical and EPR spectrum consistent with a change in the spin state of the $Fe^{3+}$ of the cytochrome from low spin to high spin when complexed with cholesterol. The formation of an oxygenated ferrous cytochrome P450 complex with cholesterol has not yet been demonstrated with cytochrome P450 from liver microsomes or adrenal mitochondria. The most recent work with solubilized cytochrome P450 and NADPH-cytochrome c reductase from rat liver[2] (36) has failed to provide any evidence that either cytochrome $b_5$ or a phospholipid play an essential role in cholesterol 7$\alpha$-hydroxylation.

## V. THE SUBSTRATE FOR CHOLESTEROL 7$\alpha$-HYDROXYLASE

The sterol substrate for the enzyme system needs to be considered from two points of view. First, there is the question of substrate specificity; second, there is the question of the origin of the intracellular cholesterol that acts as preferential substrate for the enzyme in the intact liver cell.

### Substrate specificity

Information about the influence of structural modifications to the cholesterol molecule upon the rate of hydroxylation by cholesterol 7$\alpha$-hydroxylase would be bound to throw light on the mechanism of the interaction between the enzyme and its natural substrate. However, experiments on the ability of liver microsomes to catalyze the introduction of a 7$\alpha$-hydroxyl group into analogues of cholesterol may be difficult to interpret. Since the enzyme system in the microsomes is embedded in a lipoprotein membrane, it may not be directly accessible to potential substrates added to an incubation medium. Hence, differences in the rate of 7$\alpha$-hydroxylation of various sterol analogues could be due to differences in the rate at which the substrate reaches the active site of the enzyme, rather than to differences in the intrinsic ability of the enzyme to interact catalytically with the substrate.

Another difficulty in interpretation arises from the probable presence of more than one cytochrome P450-dependent hydroxylating system in liver microsomes (see Section VII). There is a good deal of evidence to suggest that cholesterol 7$\alpha$-hydroxylase is different from other hepatic mixed-function oxidases, including those responsible for the hydroxylation of various drugs and for the 7$\alpha$-hydroxylation of taurodeoxycholate. Hence, the demonstration that a liver microsomal preparation can hydroxylate a steroid other than cholesterol in the 7$\alpha$ position does not necessarily have any bearing on the substrate specificity of cholesterol 7$\alpha$-hydroxylase. A possible way of distinguishing between a 7$\alpha$-hydroxylation due to cholesterol 7$\alpha$-hydroxylase, and one due to some other microsomal mixed-function oxidase present in the microsomal preparation, would be to test the effect of inducing agents on the hydroxylation in question. If it is catalyzed by cholesterol 7$\alpha$-hydroxylase, it should be enhanced by treatment of the animal with cholestyramine, but not, in most strains of rat, by treatment with phenobarbitone. However, as with so many of the problems relating to the mechanism of action of this enzyme, definitive experiments will not be possible until the membrane-bound components of the system have been purified. In the meantime, certain experimental observations on the microsomal 7$\alpha$-hydroxylation of sterols other than cholesterol are worth mentioning.

Shefer, Hauser, and Mosbach (8) have shown that

[2] Hattersley, N. G., and G. S. Boyd. Personal communication.

suspensions of rat liver microsomes catalyze the 7α-hydroxylation of 5α-cholestan-3β-ol (cholestanol) in the presence of $O_2$ and NADPH at a rate comparable with the 7α-hydroxylation of cholestanol, and that the 7α-hydroxylation of cholestanol is stimulated by treating the animal with cholestyramine. This suggests that cholestanol and cholesterol are equally efficient as substrates for cholesterol 7α-hydroxylase. This would not be surprising since the two sterols differ only by one nuclear double bond and, moreover, the conformation of the ring system of cholestanol is closely similar to that of cholesterol. Boyd et al. (41) have measured the rate of 7α-hydroxylation, by rat liver microsomal preparations, of several analogues of cholesterol in which the side chain has been modified. They find that slight changes in the side chain of cholesterol lead to marked loss of ability to act as substrate for 7α-hydroxylation. Thus, loss of a terminal methyl group from the side chain (26-nor-cholesterol) reduces the rate of 7α-hydroxylation to about half that for cholesterol, and the addition of an ethyl group at C-24 (β-sitosterol) leads to almost complete loss of 7α-hydroxylating activity. Boyd et al. (41) conclude that the side chain plays an important part in the binding of cholesterol to the enzyme.

### Microsomal pool of substrate for the enzyme

When [$^{14}$C]cholesterol is incubated with liver microsomes under conditions in which 7α-hydroxylation takes place, the specific activity of the 7α-hydroxycholesterol formed during the incubation is lower than that of the added cholesterol (13, 17). This shows that some of the endogenous cholesterol in the microsomes equilibrates with the radioactive cholesterol and is accessible to the enzyme. Balasubramaniam, Mitropoulos, and Myant (13) propose the term "substrate pool" for that fraction of the microsomal cholesterol that acts as substrate for cholesterol 7α-hydroxylase and they have attempted to estimate the size of this pool on the assumption that it equilibrates instantaneously and completely with all the added radioactive cholesterol. If this assumption is valid, the size of the substrate pool would be equal to the ratio R/S, where R is the amount of radioactive cholesterol added to the incubation mixture and S is the specific activity of the 7α-hydroxycholesterol formed enzymically (which must, of course, be the same as that of the pool of cholesterol from which it arises). It has not been possible to test the assumption that all the added radioactive cholesterol mixes with the substrate pool. Indeed, experiments to test the effect of adding the radioactive cholesterol in different forms have shown that exogenous cholesterol is more accessible to the enzyme system if it is added in Tween 80 than if it is added in acetone (13, 14). For these reasons, Björkhem and Danielsson (14) have expressed doubt as to the usefulness of the term "substrate pool". Nevertheless, even though it does not seem possible to measure the absolute size of the pool, it is reasonable to assume that valid comparisons of pool size under different physiological conditions may be made, provided that the method of adding the exogenous cholesterol to the incubation mixture is standardized. As discussed in Section VI, such comparisons may provide useful information about the way in which cholesterol 7α-hydroxylase activity is regulated.

When increasing amounts of exogenous cholesterol are added to a suspension of liver microsomes, the estimated size of the substrate pool (13) and the rate of formation of 7α-hydroxycholesterol (13, 14) increase progressively. This shows that the size of the substrate pool in normal liver microsomes is not sufficient to saturate their cholesterol 7α-hydroxylase. Nor does it seem to be possible to saturate the enzyme by adding exogenous cholesterol to the medium at the highest concentrations at which it remains in solution (13, 14).

### Origin of the substrate pool

An important question concerns the origin of the microsomal cholesterol used preferentially by the hydroxylase. Balasubramaniam et al. (13) isolated liver microsomes from rats shortly after the animals had been given intravenous [$^{14}$C]cholesterol and noted that the specific activity of the 7α-hydroxycholesterol formed by the microsomes in vitro was lower than that of the total cholesterol in the microsomes. They concluded that the pool of cholesterol from which 7α-hydroxycholesterol is synthesized does not equilibrate rapidly with the plasma cholesterol and they therefore suggested that this pool is derived preferentially from cholesterol newly synthesized in the liver. Independent evidence for the view that the preferred substrate for cholesterol 7α-hydroxylase is newly synthesized cholesterol was subsequently obtained from experiments in vivo. When bile-fistula rats are given intravenous infusions of [2-$^{14}$C]mevalonic acid at a constant rate, the specific activity of the bile acids secreted in the bile falls during the night, when the rate of synthesis of cholesterol is maximal (42). This relationship between the specific activity of bile acids and the rate of synthesis of cholesterol would be expected if bile acids are formed preferentially from newly synthesized cholesterol, since an in-

crease in the rate of formation of cholesterol from acetyl-CoA in the liver would lead to a fall in the specific activity of the cholesterol formed from [2-$^{14}$C]mevalonate. More recently, Björkhem and Danielsson (14) have shown that the specific activity of the 7$\alpha$-hydroxycholesterol formed from [5-$^{3}$H]-mevalonic acid during incubations of rat liver microsomes is higher than that of the total cholesterol of the microsomes. This observation provides further evidence that bile acids are synthesized preferentially from newly synthesized cholesterol.

It should be noted that the experiments described above show only that cholesterol newly synthesized in hepatic microsomes is the preferred substrate for cholesterol 7$\alpha$-hydroxylase and not that it is the sole substrate. All the exchangeable cholesterol in the body is potentially accessible to the enzyme. This is obvious from the well-known observation that the bile acids become labeled if ring-labeled cholesterol is injected intravenously. The situation revealed by observations on the relative specific activities of microsomal cholesterol and 7$\alpha$-hydroxycholesterol under different experimental conditions may perhaps be expressed most simply by saying that the pool of cholesterol that provides substrate for cholesterol 7$\alpha$-hydroxylase equilibrates more rapidly with cholesterol molecules that have just been synthesized in hepatic microsomes than with molecules reaching the microsomes from other sources.

On a historical note, it may be mentioned that in 1958 Kendall and co-workers (43) showed that, when rats are given single injections of radioactive acetate, the specific activity of the biliary bile acids is initially higher than that of the biliary cholesterol. In discussing the significance of this observation, Kendall suggested that some bile acid might be synthesized directly from acetate, without the intermediacy of cholesterol. In retrospect, it now seems more likely that Kendall's finding was due to preferential utilization of newly synthesized cholesterol for bile acid synthesis.

### Cholesteryl esters as precursors of bile acids

The identification of fatty acid esters of 7$\alpha$-hydroxycholesterol in the liver (44) has led to the suggestion that the substrate for cholesterol 7$\alpha$-hydroxylase is esterified cholesterol rather than free cholesterol. In keeping with this possibility, the liver is known to contain enzymes capable of esterifying free cholesterol and of hydrolyzing 7$\alpha$-hydroxycholesteryl esters to 7$\alpha$-hydroxycholesterol, which could then undergo further metabolism to bile acids. The question whether free or esterified cholesterol is the natural substrate for 7$\alpha$-hydroxylation has been surprisingly difficult to resolve. Liver homogenates do not 7$\alpha$-hydroxylate cholesteryl esters (45); but this could be due to failure of nonpolar exogenous substrates to reach the 7$\alpha$-hydroxylating enzyme system. Experiments in vivo, in which the specific activity of the biliary bile acids is compared with that of hepatic free and esterified cholesterol after intravenous injection of [$^{14}$C]cholesterol (46, 47), are also inconclusive since they do not exclude the existence of multiple pools of microsomal cholesteryl ester, one of which acquires a specific activity higher than that of the free cholesterol. More recent work (48), in which the rates of esterification and 7$\alpha$-hydroxylation of cholesterol by liver microsomes were varied independently, suggests that free cholesterol is the substrate for cholesterol 7$\alpha$-hydroxylase and that 7$\alpha$-hydroxycholesteryl esters arise by esterification of 7$\alpha$-hydroxycholesterol rather than by 7$\alpha$-hydroxylation of cholesteryl esters.

## VI. REGULATION OF THE 7$\alpha$-HYDROXYLATION OF CHOLESTEROL

### 7$\alpha$-Hydroxylation of cholesterol as a rate-limiting step

Some years ago, Bergström (49) suggested that the 7$\alpha$-hydroxylation of cholesterol might be the rate-determining step in the synthesis of bile acids. Although it remains possible that the rate of hepatic synthesis of cholesterol from acetyl-CoA determines the rate of formation of bile acids under some conditions, it is now generally agreed that the 7$\alpha$-hydroxylation of cholesterol is the step that determines the rate of conversion of cholesterol into bile acids in most physiological conditions. This view has generated a great deal of experimental work on the influence of various factors upon the activity of cholesterol 7$\alpha$-hydroxylase. Indeed, much of the evidence for the rate-limiting role of the step catalyzed by this enzyme is derived from measurements of cholesterol 7$\alpha$-hydroxylase activity under conditions known to alter the rate of bile acid formation.

It has not been possible to measure the capacities of the enzymes catalyzing all the steps in the pathway from cholesterol to bile acids, or to measure steady-state concentrations of the intermediates in this pathway. Nevertheless, most of the available evidence is consistent with the assumption that once a cholesterol molecule in a liver cell has been hydroxylated in the 7$\alpha$ position, the subsequent steps in the path-

way leading to bile acids are not normally rate-limiting.

**Enterohepatic circulation of bile acids**

The synthesis of bile acids is regulated in part by a homeostatic mechanism in which bile salts returning to the liver from the intestine inhibit their own synthesis. The existence of this mechanism was first suggested by the observations of Thompson and Vars (50) and of Eriksson (51), who showed that the rate of synthesis of bile acids in rats increases about tenfold when a bile fistula is made. The subsequent demonstration that bile acid synthesis can be restored to the normal rate in bile-fistula rats by duodenal infusions of taurochenate (52) or taurocholate (53) indicated that bile salts are responsible for the normal feedback inhibition of bile acid synthesis in intact rats.

That the major site of this regulation is the 7$\alpha$-hydroxylation of cholesterol has been demonstrated by Mosbach and his co-workers, using two different experimental approaches. These workers have shown (*a*) that the incorporation into bile acids of [$^{14}$C]-acetate, [$^{14}$C]mevalonate, and [$^{14}$C]cholesterol, but not of 7$\alpha$-hydroxy[$^{14}$C]cholesterol, is stimulated in perfused livers of cholestyramine-treated rabbits (54), and (*b*) that the incorporation of radioactive acetate, mevalonate, and cholesterol, but not of 7$\alpha$-hydroxy-[$^{14}$C]cholesterol, is inhibited in bile-fistula rats by duodenal infusions of taurocholate (55). These experiments show that there are essentially no rate-limiting steps beyond 7$\alpha$-hydroxycholesterol in the pathway from acetate to bile acids in rats; they do not exclude the possibility that there may be some conditions under which the rate of conversion of hepatic hydroxymethylglutaryl-CoA (HMG-CoA) into mevalonic acid becomes rate-limiting for bile acid synthesis.

If the 7$\alpha$-hydroxylation of cholesterol is a rate-limiting reaction in the formation of bile acids, the activity of cholesterol 7$\alpha$-hydroxylase should increase selectively in the livers of animals given a bile fistula. Danielsson, Einarsson, and Johansson (56) have shown that, when bile acid synthesis in rats is stimulated by making a bile fistula, cholesterol 7$\alpha$-hydroxylase activity (assayed by a radioisotopic method) increases in parallel with the increase in bile acid synthesis, but that the activities of the enzymes catalyzing two of the subsequent steps in the formation of cholic acid are not consistently influenced by the operation. An increase in cholesterol 7$\alpha$-hydroxylase activity in the livers of bile-fistula or cholestyramine-treated rats has subsequently been demonstrated by others, using radioisotopic methods of assay (8, 10) or by direct measurement of the rate of production of 7$\alpha$-hydroxycholesterol by liver microsomes in vitro (14, 57).

Since there is a delay of at least 24 h in the rise in cholesterol 7$\alpha$-hydroxylase activity that occurs after interruption of the enterohepatic circulation of bile salts (56), and since the rise is prevented by treating the animal with actinomycin D (58), it is likely that the increase in enzyme activity is due to induction of some component of the enzyme system, or of some factor required for normal access of the enzyme to its substrate, rather than to activation of pre-existing enzyme. Inhibition of the enzyme system by bile salts or bile acids added to microsomal suspensions (8) is probably due to a nonspecific detergent effect. In passing, it may be noted that the rate of fall of enzyme activity after administering actinomycin D to a bile-fistula (58) or intact (59) rat suggests that the half-life of cholesterol 7$\alpha$-hydroxylase is 2–3 hr.

The mechanism by which bile salts repress the enzyme system is not understood. Interruption of the enterohepatic circulation of rats does not increase the activity of NADPH-cytochrome c reductase (41), or the concentration of total cytochrome P450 (10, 29, 60) or the estimated size of the substrate pool of cholesterol (57) in liver microsomes. The finding that the rise in enzyme activity is not accompanied by a rise in the size of the substrate pool led Mitropoulos, Balasubramaniam, and Myant (57) to conclude that bile salts do not regulate the activity of the enzyme primarily by controlling the supply of cholesterol to the substrate pool, despite the fact that hepatic synthesis of cholesterol rises in parallel with the rise in bile acid synthesis when a bile fistula is made (56, 61). Mitropoulos et al. (57) interpreted the concomitant increase in the two biosynthetic processes in terms of a primary increase in cholesterol 7$\alpha$-hydroxylase activity, coupled with an increase in the supply of newly-synthesized cholesterol as substrate for an enzyme that is not normally saturated. Thus, we are left in the uncomfortable position of being unable to explain the regulation of cholesterol 7$\alpha$-hydroxylase activity by bile salts in terms of an effect on any of the known components of the enzyme system. The possibility that there is a small pool of a cytochrome P450 specific for the microsomal 7$\alpha$-hydroxylation of cholesterol is considered below.

Another puzzling feature of the bile acid regulatory mechanism is Mosbach's finding (62), not fully confirmed by Danielsson (63), that the cholesterol 7$\alpha$-hydroxylase of intact rats is suppressed to a greater extent by feeding taurocholate than by feeding taurochenate. This is surprising in view of the comparable effectiveness of taurochenate and taurocholate in suppressing bile acid synthesis in rats (52, 64).

## Diurnal rhythm in cholesterol 7α-hydroxylase activity

Gielen et al. (65) have shown that if rats are kept under conditions of controlled lighting and feeding, the activity of cholesterol 7α-hydroxylase in the liver (assayed by a radioisotopic method) varies diurnally, reaching a maximum during the night and falling to a minimum during the day. This observation has been confirmed in several laboratories (66–68) and has been shown to be valid when enzyme activity is assayed by measurement of the absolute rate of product formation (69). We now know a good deal about the characteristics of the diurnal variation in cholesterol 7α-hydroxylase activity in rats, but the underlying mechanism responsible for maintaining this rhythm remains something of a mystery.

In rats allowed food only during the dark period of the diurnal cycle, the nocturnal rise in cholesterol 7α-hydroxylase activity is not accompanied by an increase in the activity of NADPH-cytochrome c reductase, the concentration of total cytochrome P450, the microsomal content of cholesterol (expressed per mg of microsomal protein or per g of fresh liver), or the size of the substrate pool of cholesterol in liver microsomes (66). However, the rise in enzyme activity is prevented by treating the rats with actinomycin D or cycloheximide (66).

Taken together, these observations suggest that the diurnal rise in cholesterol 7α-hydroxylase activity is due, as in the bile-fistula rat, to induction of a rate-limiting component of the enzyme system, though they leave open the question as to which component is induced. Again, as in animals in which the enterohepatic circulation is interrupted, the diurnal rise in cholesterol 7α-hydroxylase activity is accompanied by an increase in the rate of synthesis of bile acids (57, 70). This similarity raises the possibility that the rise in the synthesis of bile acids and in the activity of cholesterol 7α-hydroxylase are initiated by increased withdrawal of bile salts from the liver, perhaps as a mechanical consequence of feeding. However, this is unlikely to be the sole cause of the diurnal rhythm in enzyme activity, since the rhythm persists during fasting (66, 67) and after total diversion of the bile through a bile fistula (57, 67). On the contrary, it seems likely that there is an inherent periodicity in cholesterol 7α-hydroxylase activity that is independent of the immediate presence of external stimuli such as the taking of food or a diurnal alternation of darkness and light, though the amplitude of the variations in enzyme activity may be influenced by the presence of these stimuli. In the longer term, external stimuli may determine the timing of the cyclical variations in enzyme activity, since the diurnal rhythm can be reversed if either the time during which food is allowed or the time of illumination is reversed for long enough for the colony of rats to become adapted synchronously to the changed environment (68, 71).

A dual mechanism, in which external stimuli modify an intrinsic periodicity, would explain why in bile-fistula animals, in which there can be no diurnal rhythm in the return of bile salts to the liver, a diurnal oscillation in cholesterol 7α-hydroxylase activity is maintained, but at a higher than normal level (57, 67). The existence of an intrinsic periodicity, independent of any diurnally variable external stimuli, is also suggested by Van Cantfort's report that a diurnal rhythm in cholesterol 7α-hydroxylase activity is maintained for at least 8 days in rats kept in continuous darkness (71), and that genetically blind mice appear to display some rhythmicity in the activity of this enzyme (72). It must be admitted, however, that it is difficult, if not impossible, to carry out any experiment on the earth's surface in which all diurnal influences due to the rotation of the earth are excluded.

Recent work on the manner in which the diurnal rhythm in cholesterol 7α-hydroxylase is controlled has been focused increasingly on the role of hormones. This is most appropriately considered in the next section.

## Effects of hormones

(*a*) Possible effects of *thyroid hormones* on the activity of cholesterol 7α-hydroxylase are of interest in view of the influence of the thyroid on bile acid metabolism. In rats, triiodothyronine and thyroxine in noncalorigenic doses markedly increase the ratio of chenic to cholic acid in the bile (73, 74), and triiodothyronine increases the rate of synthesis of total bile acids (74). Daily injections of thyroxine lead to a two- to threefold increase in the activity of cholesterol 7α-hydroxylase per mg of microsomal protein (29, 75). Thyroidectomy, on the other hand, decreases the activity of the enzyme (75, 76). In both thyroxine-treated and thyroidectomized rats the diurnal rhythm in cholesterol 7α-hydroxylase activity is maintained, though the mean level of activity is higher than normal in thyroxine-treated animals and is lower than normal after thyroidectomy (75, 76). Since Story, Tepper, and Kritchevsky (77) have estimated that the total activity of cholesterol 7α-hydroxylase per liver is not significantly altered in hyperthyroid rats, the increase in enzyme activity per mg of protein can hardly explain the effects of thyroid hormones on bile acid metabolism. It seems more likely that the

relative increase in chenic acid synthesis in thyroxine-treated rats is due to decreased activity of the enzyme catalyzing the introduction of the 12$\alpha$-hydroxyl group of cholic acid (78) and an increase in the rate of the 26-hydroxylation that precedes cleavage of the sterol side chain (79). The concentration of total cytochrome P450 in liver microsomes is decreased by thyroxine treatment and is increased after thyroidectomy (78).

(*b*) The hormones of the *adrenal cortex* are of particular interest in relation to cholesterol 7$\alpha$-hydroxylase because of their possible role in the maintenance of a diurnal rhythm in the activity of this enzyme. Plasma corticosterone concentration varies diurnally in rats adapted to regulated lighting conditions, rising to a maximum at the end of the light period (80, 81). Hence, it has been suggested that the diurnal fluctuations known to occur in the activities of several inducible hepatic enzymes, including HMG-CoA reductase, cholesterol 7$\alpha$-hydroxylase and tyrosine transaminase, are responses to changes in the rate of secretion of adrenal cortical hormones.

The administration of single or repeated doses of corticosteroids to intact rats leads to an increase in the activity of cholesterol 7$\alpha$-hydroxylase after a lag of 3 or more hr (75, 76, 82) and the increase is prevented by pretreatment of the animal with actinomycin D (82). An increase in the activity of this enzyme has also been shown to occur in rats after a brief exposure to ether sufficient to cause a rapid and transient rise in plasma corticosterone concentration (71). Gielen et al. (65) and Van Cantfort (82) have reported complete loss of diurnal rhythm in cholesterol 7$\alpha$-hydroxylase activity in rats after bilateral adrenalectomy or after feeding betamethasone in doses sufficient to suppress the secretion of adrenocorticotrophin, but not sufficient to stimulate the activity of the enzyme. Balasubramaniam, Mitropoulos, and Myant (75) have also observed a greatly reduced amplitude of the diurnal variation in cholesterol 7$\alpha$-hydroxylase activity in adrenalectomized rats, with no change in microsomal cytochrome P450 concentration.

These results suggest that the normal diurnal rhythm in the activity of this enzyme is due to periodic induction by corticosterone, leading to a peak in enzyme activity several hours after each peak in plasma corticosterone concentration, and that the diurnal rhythm in plasma corticosterone concentration is due indirectly to a diurnal rhythm in adrenocorticotrophin secretion. In keeping with this interpretation, the diurnal rhythm in cholesterol 7$\alpha$-hydroxylase activity is abolished by hypophysectomy (65) (see below). Moreover, observations on the activity of cholesterol 7$\alpha$-hydroxylase in newborn rats (71) have shown that a diurnal rhythm in enzyme activity is not established until about the 20th day after birth; this is the stage in postnatal development at which a diurnal rhythm in adrenocorticotrophin secretion and in plasma corticosterone concentration is first detectable in rats (83). However, Mitropoulos and Balasubramaniam (84) have shown that a diurnal variation in cholesterol 7$\alpha$-hydroxylase activity with low amplitude is detectable in rats 14 days after adrenalectomy, and that although a single injection of hydrocortisone into adrenalectomized rats at the beginning of the dark period restores the peak activity to the level observed in intact rats, injection of the glucocorticoid 3 hr before the expected minimum does not cause any change in enzyme activity 3 hr later. These observations suggest that glucocorticoids are not the only factor responsible for the diurnal rhythm in cholesterol 7$\alpha$-hydroxylase activity.

(*c*) Removal of the *pituitary* has profound effects on the activity of cholesterol 7$\alpha$-hydroxylase. In rats kept in conditions of regulated feeding and lighting, the diurnal rhythm in enzyme activity is abolished (65), due mainly to absence of the rise in activity that normally occurs during the dark period of the diurnal cycle (67). Mayer (67) has also reported that the increase in cholesterol 7$\alpha$-hydroxylase that occurs in response to cholestyramine feeding and the decrease in response to feeding cholic acid are abolished by hypophysectomy.

No attempt to reverse these effects of hypophysectomy by administering specific pituitary hormones has been reported. However, in view of the known relation between the adrenal cortex and cholesterol 7$\alpha$-hydroxylase activity, it is reasonable to suppose that at least some of the effect of hypophysectomy on the diurnal rhythm of enzyme activity is due to absence of the normal oscillation in the secretion of adrenocorticotrophin. It is not easy to visualize a mechanism that would require an intact pituitary both for repression of cholesterol 7$\alpha$-hydroxylase activity in response to cholic acid feeding and for induction of the enzyme in response to cholestyramine.

(*d*) In rats, single or repeated injections of *insulin* have little or no effect on cholesterol 7$\alpha$-hydroxylase activity. Nor is the diurnal rhythm in enzyme activity significantly affected by diabetes induced by streptozotocin (76).

### Diet, including cholesterol

The feeding of cholesterol-rich diets to animals of several species, including dogs (85), rats (86), squirrel monkeys (87), and rhesus monkeys (88) has been

shown to lead to increased synthesis of total bile acids. Surprisingly, cholesterol feeding appears to have little or no influence on bile acid synthesis in man (89, 90), despite the fact that his natural diet contains moderate amounts of cholesterol, whereas that of most subhuman primates must contain very little.

Attempts to explain the effect of cholesterol feeding on bile acid synthesis in animals in terms of an effect on cholesterol 7$\alpha$-hydroxylase have led to contradictory results. Some workers have observed an increase in enzyme activity (10, 57, 91, 92) after cholesterol feeding, whereas others (14, 62) have failed to observe any significant change. These discrepancies cannot be explained by differences in the amounts of cholesterol fed to the experimental animals since, in all cases, the cholesterol-rich diets contained 0.5–2% of cholesterol. Measurement of the rate of synthesis of bile acids in the same cholesterol-fed rats that are used for assay of cholesterol 7$\alpha$-hydroxylase activity might help to resolve these contradictions, since it has not yet been proved that a rise in bile acid synthesis in response to cholesterol feeding can occur in the absence of an increase in the activity of cholesterol 7$\alpha$-hydroxylase.

When rats are starved, there is a marked fall in the activity of cholesterol 7$\alpha$-hydroxylase, the amplitude of the diurnal rhythm in activity being much reduced but not completely abolished (66, 67). Little experimental work has been done on the effects of other dietary modifications upon cholesterol 7$\alpha$-hydroxylase activity in animals. A fall in enzyme activity has been reported in rats fed a fat-rich diet containing a high proportion of saturated fat (93), and an increase in enzyme activity has been noted in rats fed glucose after a period of fasting (94). A marked fall has also been noted in scorbutic guinea-pigs (95). In view of the probable influence of certain dietary constituents on the plasma cholesterol concentration in man, studies of the influence of dietary fat and fiber upon cholesterol 7$\alpha$-hydroxylase activity in human liver might well provide useful information.

### Drugs and other factors

As discussed in Section IV, certain drugs share with cholesterol an absolute requirement for cytochrome P450 in their oxidative metabolism by liver microsomes. The effect of some of these drugs, particularly phenobarbitone, on the 7$\alpha$-hydroxylation of cholesterol has been investigated extensively in the hope that this will throw light on the mechanism of the reaction. Phenobarbitone, given in doses large enough to induce enzymes of the hepatic endoplasmic reticulum, increases bile acid output in rhesus monkeys (96) and human subjects (97). However, the effect of phenobarbitone treatment on cholesterol 7$\alpha$-hydroxylase activity in rats has varied from one laboratory to another. Some workers have found that phenobarbitone in doses sufficient to cause an increase in the concentration of total cytochrome P450 in liver microsomes does not change (10, 98), or slightly diminishes (60), the activity of cholesterol 7$\alpha$-hydroxylase. Others (24, 25) have found a stimulatory effect in some (24) but not all (99) strains of rat. These discrepancies cannot be explained by differences in the strain of rats used, since rats of the Sprague-Dawley strain have given different responses to phenobarbitone treatment in two different laboratories (25, 98). What does seem clear is that the concentration of total cytochrome P450 in rat liver microsomes *can* rise in response to phenobarbitone induction without any increase in cholesterol 7$\alpha$-hydroxylase activity. The significance of this fact is considered in Section VII.

Mayer (67) has reported the presence in rat bile of a substance that inhibits the 7$\alpha$-hydroxylation of cholesterol by liver microsomes in vitro. This substance is not removed by dialysis, suggesting that it is a peptide.

Legitimate opportunities for studying cholesterol 7$\alpha$-hydroxylase in human liver do not often occur. Hence, there have been few reports of the effects of drugs or diseases on the activity of this enzyme in man. Observations reported in preliminary form (100) suggest that cholesterol 7$\alpha$-hydroxylase activity is subnormal in patients with gallstones and that large doses of chenic acid depress the activity of the enzyme in these patients. Setoguchi et al. (101) have shown that cholesterol 7$\alpha$-hydroxylase activity is increased in the livers of patients with cerebrotendinous xanthomatosis, a rare inborn error of metabolism in which bile acid synthesis is diminished owing to deficiency of an enzyme catalyzing a step in the oxidative cleavage of the sterol side chain. This is the only known condition in which the rate of synthesis of bile acids is limited by a step later in the biosynthetic sequence than the 7$\alpha$-hydroxylation of cholesterol.

## VII. WHAT DETERMINES CHOLESTEROL 7$\alpha$-HYDROXYLASE ACTIVITY?

The rate of hydroxylation of many drugs by liver microsomes seems to be determined, under most conditions, by the concentration of cytochrome P450. For example, the rate of oxidative demethylation of

TABLE 1. Effect of various modifying factors on the components of the cholesterol 7α-hydroxylase system and on ethylmorphine demethylase in rat liver microsomes

| Modification | Cholesterol 7α-Hydroxylase (activity) | Cytochrome P450 (total concentration) | NADPH-Cytochrome c Reductase (activity) | Ethylmorphine Demethylase (activity) | References |
|---|---|---|---|---|---|
| Bile fistula or cholestyramine | ↑ | → | → | → | 41, 60 |
| Portacaval anastomosis | ↑ | ↓ | ↓ | ↓ | 107, 108 |
| Diurnal rhythm (dark period) | ↑ | → | → | → | 65, 69 |
| Thyroid hormone | ↑ | ↓ | ↑ | ↓ | 60, 75, 76, 78 |
| Thyroidectomy | ↓ | ↑ | ↓ | → | 60, 75, 76, 78 |
| Phenobarbitone | → or ↓ or ↑ | ↑ | ? | ↑ | 60, 98, 99, 102 |
| Corticosteroid | ↑ | ? | ? | ? | 75, 76, 82 |
| Adrenalectomy | ↓ | → | ? | → | 60, 75, 76, 82 |

↑ = increase; ↓ = decrease; → = no significant change.
Enzyme activities and cytochrome concentration are per mg of microsomal protein.

aminopyrine by rat liver microsomes rises concomitantly with the induction of cytochrome P450 (102) and the rate of oxidative demethylation of ethylmorphine rises or falls in parallel with changes in the concentration of microsomal cytochrome P450 brought about by a variety of experimental modifications (60). The supply of reductive hydrogen may become rate-limiting for some microsomal hydroxylations; for example, when the microsomal concentration of NADPH is diminished and that of cytochrome P450 is increased by a combination of starvation and treatment of the animal with phenobarbitone (103). But conditions in which this occurs are exceptional. The activity of cholesterol 7α-hydroxylase, on the other hand, appears to bear no consistent relation either to the concentration of total cytochrome P450 or to the activity of NADPH-cytochrome c reductase in liver microsomes.

Several examples of a dissociation between enzyme activity and total cytochrome P450 concentration have already been referred to and are summarized in **Table 1.** Some examples of the changes in NADPH-cytochrome c reductase activity that accompany changes in cholesterol 7α-hydroxylase activity are also included in this table. These observations show that the activity of cholesterol 7α-hydroxylase is not normally limited by the concentration of total cytochrome P450 or by the activity of NADPH-cytochrome c reductase. This is seen most clearly in the change in hydroxylase activity that occurs in response to interruption of the enterohepatic circulation of bile salts and during the dark period of the diurnal cycle. In both cases there is a severalfold increase in hydroxylase activity without detectable change in either of the other two known components of the hydroxylase system. This raises the question as to what it is that is induced when, under a variety of different conditions, cholesterol 7α-hydroxylase activity increases. Two possible answers are worth considering.

It is becoming increasingly clear that there is more than one form of cytochrome P450 in mammalian cells. Adrenal cortex mitochondria have been shown to contain at least two distinct forms, one interacting specifically with deoxycorticosterone and the other with 20α-hydroxycholesterol (104). Haugen, Coon, and Nebert (105) have also shown that rat and rabbit liver microsomes contain several different forms of cytochrome P450, all of which are concerned in the mixed-function oxidation of drugs. These forms differ in substrate specificity, subunit molecular weight, electrophoretic behavior, and inducibility by different drugs. The existence of multiple forms of cytochrome P450 raises the possibility that liver microsomes contain a specific form of the cytochrome that catalyzes the 7α-hydroxylation of cholesterol and whose activity is rate-limiting for this reaction. If this specific form comprised only a small fraction of the total cytochrome P450 in the liver, there might be no detectable change in the microsomal concentration of total cytochrome P450 when the specific fraction was induced or repressed. Thus, in terms of this hypothesis it would be possible to explain all the observations listed in Table 1 without invoking any additional components in the hydroxylase. It should be noted, however, that the inducible component of the enzyme system must have a half-life of only 2–3 hr, since this is the rate at which the activity of the hydroxylase declines after reaching its peak during the dark phase of the diurnal cycle. There is nothing inherently improbable in this, since some other components of the endoplasmic

TABLE 2. Effect of various modifying factors on the activities of HMG-CoA reductase and cholesterol 7α-hydroxylase in rat liver microsomes

| Modification | HMG-CoA Reductase | Cholesterol 7α-Hydroxylase | References |
|---|---|---|---|
| Bile fistula or cholestyramine | ↑ | ↑ | 8, 10, 56[a], 61[a] |
| Portacaval anastomosis | ↑ | ↑ | 107, 108 |
| Diurnal rhythm (dark period) | ↑ | ↑ | 65, 68, 69, 109, 110 |
| Thyroid hormone | ↑ | ↑ | 60, 75, 76, 111 |
| Adrenalectomy | ↓ | ↓ | 75, 76, 82, 112[b] |
| Glucose after starvation | ↑ | ↑ | 94[a] |
| Fasting | ↓ | ↓ | 67, 71, 113 |
| Cholesterol feeding | ↓ | ↑ or → | 10, 14, 57, 62, 91, 114, 115 |
| Tomatine | ↑ | → | 41 |
| β Sitosterol | ↑ | → or ↗ | 41, 62 |

↑ = increase; ↗ = slight but significant increase; ↓ = decrease; → = no change.

Activities are per mg of microsomal protein.

[a] Cholesterol synthesis measured by incorporation of [$^{14}$C]acetate.

[b] Values obtained 3 days to 3 months after adrenalectomy.

reticulum are known to have equally short half-lives, though the half-life of the total cytochrome P450 of liver microsomes is about 50 hr (102).

An alternative explanation of the findings shown in Table 1 is that cholesterol 7α-hydroxylase contains an additional, as yet unidentified, component that determines the activity of the enzyme and whose induction and repression are responsible for the modifications of enzyme activity shown in the table. There are various roles that an additional component could play: for example, that of an inducible protein carrier that facilitates access of substrate cholesterol to the catalytic site of the enzyme system. The presence of a saturable carrier, with a role such as this, is not inconsistent with the possibility that the rate of formation of 7α-hydroxycholesterol is influenced by the size of the substrate pool of cholesterol.

It is difficult to see how either of these working hypotheses can be tested experimentally until much more progress has been made in the purification and recombination of all the components required for the 7α-hydroxylation of cholesterol. Although the physiological regulation of cholesterol 7α-hydroxylase is largely independent of that of most cytochrome P450-dependent hydroxylations of drugs (compare, for example, ethylmorphine demethylase with cholesterol 7α-hydroxylase (Table 1)), there appears to be some interaction between the 7α-hydroxylation of cholesterol and the demethylation of ethylmorphine (60). This suggests that the specificity of the rate-determining component of cholesterol 7α-hydroxylase is not absolute.

## VIII. CORRELATION BETWEEN HMG-CoA REDUCTASE AND CHOLESTEROL 7α-HYDROXYLASE ACTIVITY

In the course of this review we have referred to several conditions in which an increase in cholesterol 7α-hydroxylase activity is accompanied by a rise in the activity of hepatic HMG-CoA reductase. These, together with other examples of parallel changes in the activities of the two enzyme systems, are listed in **Table 2.** The fact that, for example, both enzymes are induced during the dark period and are repressed during the light period of the diurnal cycle in rats, suggests that there is an obligatory link between the regulation of hepatic HMG-CoA reductase activity and that of cholesterol 7α-hydroxylase activity. Coordination of the two enzyme systems would make sense from the point of view of the functional requirements of the hepatocyte if, as we have argued, newly-synthesized cholesterol is the preferred substrate for cholesterol 7α-hydroxylase. The link could be brought about by interaction between the two enzyme systems. For example, increased activity of HMG-CoA reductase might lead automatically to increased activity of cholesterol 7α-hydroxylase in conditions in which the latter enzyme was not saturated with substrate; such a situation is suggested by the observations of Takeuchi, Ito, and Yamamura (94), who found that glucose feeding after a fast led to increased synthesis of cholesterol in the liver and that this was followed later by an increase in cholesterol 7α-hydroxylase activity. In other conditions, the

primary event might be an increase in the activity of cholesterol 7$\alpha$-hydroxylase, leading to induction of HMG-CoA reductase as a consequence of increased removal of cholesterol. A possible example of this is the effect of thyroxine treatment, which leads to increased cholesterol 7$\alpha$-hydroxylase activity followed by increased HMG-CoA reductase activity after a lag period of several hours (75). In cases where the activities of the two enzymes change more or less simultaneously, as in the rhythmic rise and fall during the diurnal cycle in rats (68), it seems more likely that the two enzymes are responding to a common signal, e.g., a diurnal variation in plasma corticosteroid concentration. It is implicit in this discussion that cholesterol 7$\alpha$-hydroxylase activity is not necessarily regulated by the same mechanism under all conditions. In this respect, the regulation of cholesterol 7$\alpha$-hydroxylase resembles that of other enzyme systems in higher animals (see (106) for discussion of this point). It is unlikely that the similarities in the regulation of hepatic HMG-CoA reductase and cholesterol 7$\alpha$-hydroxylase are due to coordinate induction, in the sense in which this term is used by microbial geneticists, since at least two examples are known in which induction of the two enzymes does not occur in parallel (cholesterol feeding and tomatine feeding (see Table 2)).

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