7α-Hydroxylation of cholestanol by rat liver microsomes

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ABSTRACT In a study of the mechanism whereby 5α-bile acids are formed from cholestanol, the 7α-hydroxylation of cholestanol was investigated in rat liver preparations in vitro. It was found that in the presence of NADPH and oxygen, rat liver microsomes catalyzed the 7α-hydroxylation of cholestanol to the same extent as that of cholesterol.

The rate of the hydroxylation was enhanced by prior treatment of the experimental rats with cholestyramine (a bile acid sequestrant) or by establishment of bile fistulas—i.e., by partial or complete removal of bile acids from the enterohepatic circulation. The 7-hydroxylation reaction was further stimulated by pretreatment of the animals with phenobarbital, a drug known to produce increased biosynthesis of hepatic endoplasmic membranes. The 7α-hydroxylase was inhibited by the reaction product, by sterols with 7-keto or 7β-hydroxyl groups, and also by mono- and dihydroxy bile acids of the 5β-series, although cholic acid or taurocholate produced no inhibition unless added in high concentrations.

The results of these studies are in accord with the concept that the presence of a Δ^5-double bond is not required for the enzymatic formation of the 7α-hydroxy derivative. The rate of this hydroxylation reaction in vitro appears to depend on the concentration of bile salts in the enterohepatic circulation of the experimental animals from whom the microsomes were obtained.

KEY WORDS 7α-hydroxylation · in vitro · rat liver microsomes · cholesterol · cholestanol · phenobarbital · cholestyramine · bile acids

THE BILE OF HIGHER VERTEBRATES contains small amounts of 5α(allo)-bile acids ranging from a fraction of a per cent in man (1) to more than 5% in the rabbit (2). In certain lower vertebrates allo bile acids may be the major or the only bile acid constituents (1). At present, three processes are known which could account for the presence of allo bile acids in bile. First, the stereospecific reduction of 7α,12α-dihydroxycholest-4-en-3-one by liver microsomes leads to the formation of 5α-cholestane-3α,7α,12α-triol which, presumably, can then be further oxidized to allocholic acid (3). Second, the transformation of deoxycholic acid to allodeoxycholic acid by intestinal microorganisms has been demonstrated in rats in vivo (4). Third, the formation of 5α-bile acids can be induced in the rat (5) and the rabbit (6) by administration of cholestanol, a 5α-stanol.

The metabolic pathway leading from cholestanol to 5α-bile acids is not known in detail. It seems safe to assume, however, that this process takes place with retention of the 5α-configuration throughout the biosynthetic sequence. In that case the initial step would be the 7α-hydroxylation of cholestanol. This paper presents evidence that rat liver preparations can catalyze this hydroxylation reaction in a manner entirely analogous to the 7α-hydroxylation of cholesterol.

EXPERIMENTAL PROCEDURES

Animals

Male rats of the Wistar strain were subjected to the following procedures designed to stimulate the 7α-hydroxylation reaction.

Cholestyramine Treatment. The rats were fed ground Purina rat pellets containing 2.5% cholestyramine ("Questran," Mead Johnson & Co., Evansville, Ind.). Cholestyramine is a quaternary ammonium anion-

Systematic names of the steroids referred to in the text by their trivial names are: cholestanol, 5α-cholestan-3β-ol; 7α-hydroxycholestanol, 5α-cholestane-3β,7α-diol; 7α-hydroxycholesterol, cholest-5-en-3β,7α-diol; 7-ketocholesterol, cholest-5-en-7-on-3β-ol; 7-ketocholestanol, 5α-cholestan-7-on-3β-ol; 7β-hydroxycholesterol, cholest-5-ene-3β,7β-diol; 7β-hydroxycholestanol, 5α-cholestane-3β,7β-diol.

exchange resin which binds bile salts at the pH of the small intestine.

Bile Duct Cannulation. The bile ducts of the experimental animals were cannulated under phenobarbital anesthesia and the animals were kept in restraining cages for 5 days. Care was taken to maintain adequate nutrition.

Phenobarbital Treatment. The animals were injected intraperitoneally daily for 4–5 days with 100 mg of phenobarbital per kg body weight. This drug produces proliferation of the smooth endoplasmic reticulum of the liver, thereby increasing the activity of drug-metabolizing (hydroxylating) enzymes in the microsomal fraction of rat liver homogenates (7).

Administration of Sterols. Two groups of rats were treated with cholestyramine and either 1% cholesterol or 1% cholestanol for 5 days. The sterols were incorporated into the diets in ether solution (8).

Preparation of Enzyme

Male albino rats (200 g) of the Wistar strain were killed by cervical dislocation and their livers were removed immediately and chilled on ice. All subsequent operations were carried out at 0–5°C. Portions of liver (4 g) were homogenized in a Potter-Elvehjem homogenizer with 6 ml of a solution containing sucrose, 0.25 M; nicotinamide, 0.075 M; and neutralized EDTA, 2.5 mM. Washed microsomes were prepared as described previously (9). When stored at −15°C they lost 50% of their activity during a storage period of 1 month. A typical preparation had a protein concentration of 10 mg/ml (10).

Assay

The complete system contained in a volume of 2.4 ml: potassium phosphate buffer pH 7.4, 0.167 mmole; $MgCl_2$, 11 μmoles; $NADP^+$, 3.0 μmoles[1]; glucose-6-phosphate, 6.0 μmoles; glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 5 IU; cholestanol-4-^{14}C or cholesterol-4-^{14}C, 0.5 μmole, solubilized with 0.75 mg of Cutscum[2]; and washed microsomes containing approximately 5 mg of protein. The specific activity of the substrate was 5.6×10^5 cpm/μmole. The incubation mixture was shaken in air or oxygen at 37°C for 30 min, care being taken to exclude light (11) (the results obtained with these two gas phases were identical, within the precision of measurement). The reaction was stopped by the addition of 15 volumes of methylene dichloride–ethanol 5:1 to an aliquot (0.4 ml) of the incubation mixture. 1 ml of water was added, the tube was shaken vigorously in a Vortex mixer for 1 min and centrifuged, and the methylene dichloride layer was pipetted into a glass-stoppered centrifuge tube. The organic solvent was removed at 60°C under a current of nitrogen. Under these conditions 85–95% of the steroids were recovered from the incubation mixture.

The dried steroid fraction was dissolved in 40 μl of $CHCl_3$, and half of this solution was applied as a spot to a 250-μ thick layer of Silica Gel G. Unlabeled 7-ketocholesterol and 7α- and 7β-hydroxysterols (30 μg of each) were applied at the same spot, the nature of the reference compounds depending upon the substrate used. The plate was developed with ether in a tank without previous equilibration at 10°C, dried, and sprayed with a 0.2% (w/v) solution of 2′,7′-dichlorofluorescein in methanol. The position of the spots was marked under UV illumination (3660 A); the pertinent areas were removed from the plate by suction, transferred to scintillation vials containing 0.6% (w/v) 2,5-diphenyloxazole in toluene, and counted in a Nuclear-Chicago "Unilux" scintillation counter. Suitable corrections were made for background and quenching. The average R_f values observed were: cholesterol and cholestanol, 0.88; 7α-hydroxycholesterol, 0.40; 7β-hydroxycholesterol, 0.54; 7-ketocholesterol, 0.63; 7α-hydroxycholestanol, 0.55; 7β-hydroxycholestanol, 0.47; 7-ketocholestanol, 0.66. Since the specific activity of a given substrate was known, the radioactivity data could be expressed in terms of mμmoles of substrate converted to a given product.

Labeled Compounds

Cholestanol-4-^{14}C (New England Nuclear Corp. Boston, Mass.) was purified by chromatography on a $AgNO_3$–silicic acid column (12). The purified product contained less than 0.04% cholesterol, 0.04% 7α-hydroxycholestanol, and 0.01% 7β-hydroxycholestanol.

Cholesterol-4-^{14}C (New England Nuclear Corp.) was purified by chromatography on a silicic acid column in the presence of unlabeled 7α- and 7β-hydroxycholesterol (13). The purified product contained less than 0.06% 7α-hydroxycholesterol.

Reference Compounds. 7-ketocholesterol and 7α- and 7β-hydroxycholesterol were synthesized as described by Fieser, Herz, Klohs, Romero, and Utne (14). 7-ketocholestanol and 7α- and 7β-hydroxycholestanol were synthesized as described by Wintersteiner and Moore (15).

Conjugated and free bile acids were purchased from Calbiochem, Los Angeles, Calif. The allo bile acids were a gift from Dr. A. F. Hofmann.

RESULTS

Identification of Reaction Products

7α-Hydroxycholestanol. The contents of 10 incubation tubes were extracted and combined. Unlabeled carriers

[1] This NADPH-generating system gave twice the activity of 3 μmoles of NADPH and ten times the activity of NADH.

[2] Cutscum is a detergent sold by Fisher Scientific Company. It is an isooctyl phenoxypolyoxyethylene ethanol.

(cholestanol, 7-ketocholestanol, 7α-hydroxycholestanol, and 7β-hydroxycholestanol) were added and the steroid mixture was chromatographed on a column of Bio-Rad silicic acid deactivated by the addition of 20 ml of water per 100 g of silicic acid. Only the peaks corresponding to cholestanol and 7α-hydroxycholestanol had measurable radioactivity. The effluent containing 7α-hydroxycholestanol was evaporated and the 7.26 mg of 7α-hydroxycholestanol obtained was further diluted with 10 mg of unlabeled carrier. This sample (2500 cpm/μmole) was divided into two portions. The first was crystallized from acetone–water and then from methanol–water. The specific radioactivities remained constant during these crystallizations (2390 and 2470 cpm/μmole). The second portion was oxidized with CrO_3 (16) to the 3,7-diketone and this derivative was chromatographed on a silicic acid column. The specific radioactivity of this compound, 2420 cpm/μmole, remained unchanged during crystallizations from acetone–water and methanol–water (2370 and 2440 cpm/μmole).

7α-Hydroxycholesterol. The formation of 7α-hydroxycholesterol from cholesterol in rat liver preparations has been reported previously (17, 18), and no further identification studies were carried out.

Intracellular Localization of 7α-Hydroxylase. A 40% homogenate of the liver of a cholestyramine-treated rat was separated centrifugally into mitochondria, microsomes, and final supernatant solution. It was found that the hydroxylation was catalyzed predominantly by the microsomal fraction (Table 1), and that cholesterol and cholestanol reacted at similar rates.

Table 2 illustrates the effect of various types of treatment on the activity of the microsomal 7α-hydroxylase. It is evident that the rate of hydroxylation was enhanced in microsomal preparations of rats with bile fistula and those treated with cholestyramine or phenobarbital. The effects of cholestyramine and phenobarbital appeared to be additive. Feeding sterols in addition to cholestyramine did not produce increased hydroxylation.

Optimal reaction rates were obtained when the final pH of the microsomal system was between 6 and 7. Under the assay conditions described above, the rate of the 7α-hydroxylation reaction was approximately linear with respect to time for the first 10 min, and linear with respect to protein concentration up to 10 mg of protein per tube. The effects of changes in substrate concentration on the reaction rate are illustrated in Fig. 1. At low substrate concentration (below 100 mμmoles of cholestanol per tube), more than 10% of the starting material was converted to 7α-hydroxycholestanol. The results obtained with cholesterol were quite similar to those observed with cholestanol.

Results of studies dealing with the inhibition of the 7α-hydroxylase by the reaction products and other sterols

TABLE 1 INTRACELLULAR LOCALIZATION OF 7α-HYDROXYLASE

Fraction	5α-Cholestane-3β,7α-diol Formed from Cholestanol	Cholest-5-ene-3β,7α-diol Formed from Cholesterol
	mμmoles/mg protein	
Whole homogenate	0.08	0.1
Mitochondria	0.4	0.4
Microsomes	4.9	4.6
Final supernatant solution	0.0	0.0
Mitochondria + final supernatant solution	0.2	N.D.*
Microsomes + final supernatant solution	2.2	N.D.
Microsomes + mitochondria	1.9	N.D.
Microsomes + mitochondria + final supernatant solution	1.55	N.D.

Tissue fractions prepared from livers of male Wistar rats pretreated with 2.5% dietary cholestyramine and incubated as described in Experimental Procedures.

* N.D. = not determined.

TABLE 2 EFFECT OF TREATMENT OF RATS ON 7α-HYDROXYLASE ACTIVITY OF LIVER MICROSOMES

Treatment	7α-Hydroxy Compound Formed from: Cholestanol	Cholesterol
	mμmoles	
None	2.6	4.0
Bile fistula, 5 days	15.3	16.9
Phenobarbital, 100 mg/kg, i.p., 5 days	9.8	18.7
Cholestyramine, 2.5% of diet	8.1	13.1
Cholestyramine + phenobarbital	15.5	29.2
Cholestyramine + 1% cholestanol, 10 days	9.2	13.4
Cholestyramine + 1% cholesterol, 10 days	7.7	13.1

Liver microsomes prepared and assayed as described in Experimental Procedures.

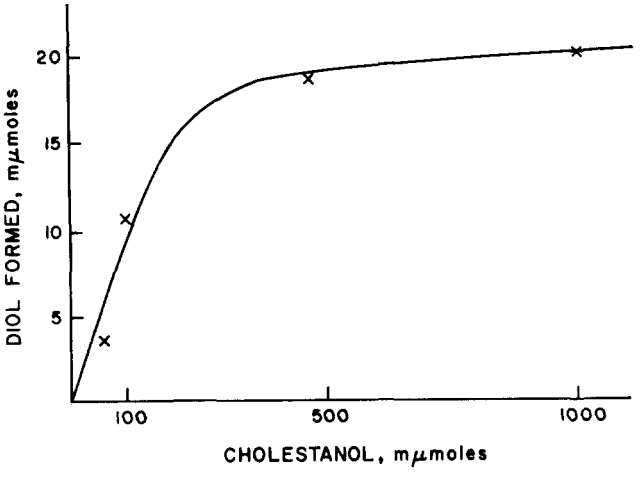

FIG. 1. Effect of substrate concentration on the amount of hydroxylation of cholestanol. Standard assay conditions (30 min) as described in Experimental Procedures. Diol, 5α-cholestane-3β,7α-diol.

are given in Table 3. Under the conditions employed, the unsaturated 7α-hydroxy compounds inhibited the hydroxylation of cholesterol and cholestanol to a greater extent than the saturated diols. In general, the 7β-hydroxy and the 7-keto derivatives of cholesterol and cholestanol were far more inhibitory than the 7α-hydroxy compounds. Cholesterol inhibited the 7α-hydroxylation of cholestanol by 68%; cholestanol inhibited the hydroxylation of cholesterol to the same degree.

Table 4 illustrates the inhibition of the 7α-hydroxylase by Na salts of free and conjugated bile acids. The degree of inhibition produced by the 5β-compounds was inversely related to the number of hydroxyl groups in the molecule. In fact, under the conditions employed, sodium taurocholate and cholate stimulated the hydroxylation to a considerable extent. A stimulation was also observed with allodeoxycholate and allolithocholate. Further experiments (Fig. 2) showed that higher concentrations of taurocholate were inhibitory.

TABLE 3 INHIBITION OF 7α-HYDROXYLASE BY STEROLS

Inhibitor	5α-Cholestane-3β,7α-diol Formed from Cholestanol	Inhibition	Cholest-5-ene-3β,7α-diol Formed from Cholesterol	Inhibition
	mμmoles	%	*mμmoles*	%
None	7.3	—	7.0	—
5α-Cholestane-3β,7α-diol	4.8	34	4.8	32
5α-Cholestane-3β,7β-diol	1.7	77	2.8	60
Cholest-5-ene-3β,7α-diol	2.8	62	3.2	54
Cholest-5-ene-3β,7β-diol	1.4	81	2.0	72
Cholesterol	2.3	68	—	—
Cholestanol	—	—	2.2	68
5α-Cholestan-3β-ol-7-one	1.5	98	0.4	94
Cholest-5-en-3β-ol-7-one	0.7	99	0.6	91

Concentration of inhibitor, 1 μmole per tube. Inhibitor and substrate were solubilized with Cutscum and incubated with liver microsomes of cholestyramine-treated rats as described in Experimental Procedures.

TABLE 4 INHIBITION OF 7α-HYDROXYLASE BY BILE ACIDS

Additions (Sodium Salts)	5α-Cholestane-3β,7α-diol Formed	Inhibition	Cholest-5-ene-3β,7α-diol Formed	Inhibition
	mμmoles	%	*mμmoles*	%
None	7.4	—	7.0	—
Taurocholate	7.7	−4	11.6	−66
Taurodeoxycholate	1.55	79	3.5	50
Taurochenodeoxycholate	1.65	78	3.1	56
Taurolithocholate	0.66	91	1.1	84
Cholate	11.5	−55	11.5	−64
Deoxycholate	6.4	14	5.0	28
Chenodeoxycholate	3.6	51	5.2	26
Lithocholate	4.4	41	3.6	49
Allodeoxycholate	11.2	−51	8.4	−20
Allolithocholate	12.5	−69	10.9	−56

Concentration of inhibitors, 1 μmole per tube. Conjugated and free bile acids were added in aqueous solution.

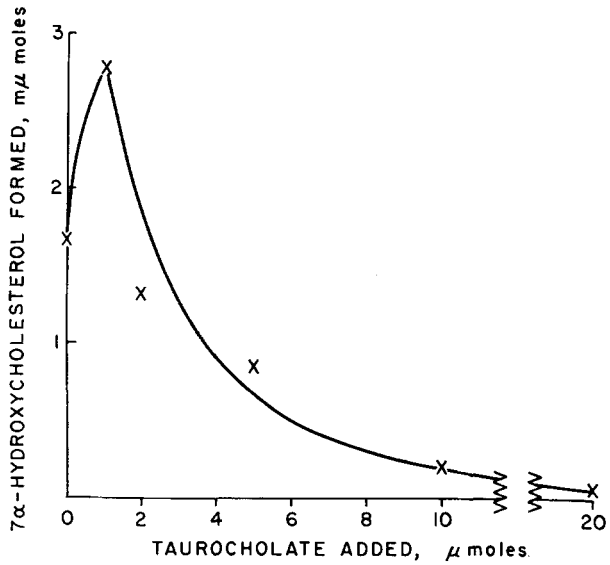

FIG. 2. Effect of increasing amounts of sodium taurocholate on the 7α-hydroxylation of cholesterol. Standard assay conditions.

Preliminary experiments on the effect of carbon monoxide indicated that the hydroxylation was inhibited approximately 50% in carbon monoxide–oxygen atmospheres containing 50 or 80% CO. Complete inactivation of the microsomal oxygenase was never observed under these conditions.

DISCUSSION

The bile salts of certain lower vertebrates such as the iguana and the carp contain 5α-steroids as the sole or predominating constituents (3, 19, 20). In these species, as in all animals studied so far, the major sterol of liver is cholesterol, cholestanol being present only in trace amounts (21). Since these trace amounts of cholestanol presumably do not have a rapid turnover, it is assumed at present that the major pathway in the formation of the naturally occurring 5α-bile acids and alcohols involves cholesterol as the main or sole precursor. According to this concept, cholesterol is converted to 7α,12α-dihydroxycholest-4-en-3-one, which is then transformed stereospecifically to 5α-bile salts via 5α-cholestane-3α,7α,12α-triol. This assumption is based on in vivo experiments with carp (19, 20) (in which 7α-hydroxycholesterol proved to be an efficient precursor of 5α-cyprinol) and on in vitro experiments with iguana liver microsomes (3) (in which 7α,12α-dihydroxycholest-4-en-3-one was found to be converted rapidly to 5α-cholestane-3α,7α,12α-triol).

In the present study it was shown that rat liver microsomes catalyze the 7α-hydroxylation of cholestanol under the same conditions and at the same rate as the hydroxy-

lation of cholesterol. It seems likely, therefore, that the 7α-hydroxylation of cholestanol is the initial step in the formation of 5α-bile acids in animals receiving large amounts of this stanol in their diet. The possibility that a Δ^4-3-keto intermediate is involved can be ruled out, since the administration of labeled cholestanol to rabbits does not produce appreciable quantities of labeled 5β-bile salts (E. H. Mosbach, unpublished observations).

At present it cannot be decided whether in certain species such as the rabbit, that have small proportions of cholestanol in the liver sterol fraction and excrete similar proportions of 5α-bile acids in bile, cholestanol or cholesterol serves as the precursor of these 5α-bile acids.

Danielsson, Einarsson, and Johansson recently showed (22) that the 20,000 *g* supernatant solution prepared from the livers of rats with bile fistula exhibits a greatly increased activity (compared to normal rats) in the biosynthesis of cholesterol from acetate and in the 7α-hydroxylation of cholesterol. [The stimulation of cholesterol biosynthesis in liver slices of rats with bile fistula had previously been demonstrated by Myant and Eder (23)]. The present studies confirm the stimulation of the 7α-hydroxylase in liver microsomes of rats with bile fistula and, in addition, demonstrate that treatment of the experimental animals with cholestyramine or phenobarbital enhances the hydroxylation to a similar extent. These two drugs probably have a different mechanism of action since their combined efforts were roughly additive. Phenobarbital presumably stimulated the formation of endoplasmic reticulum (7) so that more microsomal enzyme was available per g of liver, while the bile acid-sequestering resin acted like biliary drainage, i.e. by removal of bile acids from the enterohepatic circulation (24). The addition of sterols to the diet of cholestyramine-treated rats produced no additional increase in the rate of hydroxylation. This finding does not necessarily imply that excess exogenous sterol will not produce increased hepatic hydroxylation. Since, in the cholestyramine-treated animals, the intestinal bile salt concentration was low, little dietary sterol was absorbed and was therefore not available for conversion to bile acids by the liver.

Our results differ from those of Danielsson et al. (22) in that the addition of 100,000 *g* supernatant solution to the microsomes was not required for optimal hydroxylation of the sterols. This may merely reflect differences in the techniques used for solubilizing the substrate.[3]

The inhibition of the hydroxylase by bile salts in vitro observed in the present experiments (Table 4) was expected in view of similar inhibitory effects observed in in vivo studies (25). Nevertheless the inhibitory effects demonstrated here may be nonspecific and can perhaps be ascribed to a disruptive effect of the bile salts on the microsomes. This difficulty of demonstrating inhibition by bile salts upon various enzymes of cholesterol metabolism in vitro has been discussed by Lee and Whitehouse (26) and by Miller and Gaylor (27). In view of recent studies demonstrating that bile salts inhibit the formation of cholesterol from acetate in intestinal mucosa, and, less strongly, in the liver (24), the possibility that bile salts control bile acid biosynthesis mainly by their effect on cholesterol biosynthesis cannot be ruled out. These inhibitory effects of bile salts may be related to changes in the concentration of one or more enzymes of cholesterol biosynthesis rather than a direct bile salt–enzyme interaction. The effects of bile salts on the 7-hydroxylation of sterols may, therefore, play only a minor role in the regulation of bile acid production in vivo.

[3] When acetone was used to solubilize cholesterol (22) no 7α-hydroxylation was observed.

The cholestyramine used in this study was a gift from the Mead Johnson Research Center.

This work was supported in part by Research Grants AM-05222 and HE-10894 from the National Institutes of Health, United States Public Health Service.

Manuscript received 27 November 1967; accepted 12 January 1968.

References

1. Haslewood, G. A. D. 1967. Bile Salts. Methuen & Co., Ltd. London. 7–10.
2. Hofmann, A. F., V. Bokkenheuser, R. L. Hirsch, and E. H. Mosbach. 1968. *J. Lipid Res.* **9:** 244.
3. Hoshita, T., S. Shefer, and E. H. Mosbach. 1968. *J. Lipid Res.* **9:** 237.
4. Kallner, A. 1967. *Acta Chem. Scand.* **21:** 87.
5. Karavolas, H. J., W. H. Elliott, S. L. Hsia, E. A. Doisy, Jr., J. T. Matschiner, S. A. Thayer, and E. A. Doisy. 1965. *J. Biol. Chem.* **240:** 1568.
6. Hofmann, A. F., and E. H. Mosbach. 1964. *J. Biol. Chem.* **239:** 2813.
7. Burger, P. C., and P. B. Herdson. 1966. *Am. J. Pathol.* **48:** 793.
8. Bevans, M., and E. H. Mosbach. 1956. *Arch. Pathol.* **62:** 112.
9. Shefer, S., S. Hauser, and E. H. Mosbach. 1966. *J. Lipid Res.* **7:** 763.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. *J. Biol. Chem.* **193:** 265.
11. Hais, I. M., and N. B. Myant. 1965. *Biochem. J.* **94:** 85.
12. Shefer, S., S. Milch, and E. H. Mosbach. 1964. *J. Biol. Chem.* **239:** 1731.
13. Hirsch, J., and E. H. Ahrens, Jr. 1958. *J. Biol. Chem.* **233:** 311.
14. Fieser, L. F., J. E. Herz, M. W. Klohs, M. A. Romero, and T. Utne. 1952. *J. Am. Chem. Soc.* **74:** 3309.
15. Wintersteiner, O., and M. Moore. 1943. *J. Am. Chem. Soc.* **65:** 1503.
16. Sato, Y., and N. Ikekawa. 1959. *J. Org. Chem.* **24:** 1367.
17. Danielsson, H., and K. Einarsson. 1964. *Acta Chem. Scand.* **18:** 831.
18. Mendelsohn, D., L. Mendelsohn, and E. Staple. 1965. *Biochim. Biophys. Acta.* **97:** 379.

19. Hoshita, T. 1964. *Steroids*. **3:** 523.
20. Hoshita, T. 1967. *Biochem. J.* (*Tokyo*). **61:** 440.
21. Mosbach, E. H., J. Blum, E. Arroyo, and S. Milch. 1963. *Anal. Biochem*. **5:** 158.
22. Danielsson, H., K. Einarsson, and G. Johansson. 1967. *European J. Biochem*. **2:** 44.
23. Myant, N. B., and H. A. Eder. 1961. *J. Lipid Res*. **2:** 363.
24. Dietschy, J. M. *J. Clin. Invest*. In press.
25. Shefer, S., S. Hauser, and E. H. Mosbach. 1967. *Circulation*. **36** (Suppl. II): 37. (Abstr.)
26. Lee, M. J., and M. W. Whitehouse. 1963. *Biochem. J*. **89:** 189.
27. Miller, W. L., and J. L. Gaylor. 1967. *Biochim. Biophys. Acta*. **137:** 400.