Metabolism of cholesta-4,7-dien-3-one and cholesta-4,6-dien-3-one by mouse liver microsomes*†

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SUMMARY

Incubation of cholesta-4,7-dien-3-one with mouse liver microsomes and TPN or TPNH resulted in the production of two new metabolites. The ultraviolet absorption spectra, chromatographic mobilities, and reactions of the two products with the Zimmermann and Liebermann-Burchard reagents were identical with those of cholesta-4,6-dien-3-one and cholest-4-en-3-one. Incubation of cholesta-4,6-dien-3-one with liver microsomes and TPNH resulted in the production of cholest-4-en-3-one. The results indicate that cholesta-4,7-dien-3-one is converted to cholest-4-en-3-one by two steps: isomerization of the Δ^7-bond to the 6-position, followed by reduction of the Δ^6-bond. The effects of pH, substrate concentration, and other factors, on the reactions were examined. Conversion of either cholesta-4,7-dien-3-one or cholesta-4,6-dien-3-one to the metabolic products described was inhibited by *p*-chloromercuribenzoate, by certain detergents, and by triparanol.

Reduction of the Δ^7-bond of cholesta-5,7-dien-3β-ol (7-dehydrocholesterol), catalyzed by a TPNH-linked enzyme present in microsomes (2), may be the final step in a pathway of cholesterol biogenesis, which would include as intermediates 4,4,14-trimethyl-cholest-8-en-3β-ol, (24,25-dihydrolanosterol), 4α-methylcholest-8-en-3β-ol, and cholest-7-en-3β-ol (3, 4). In view of the existence in animal tissues of dehydrogenases capable of oxidizing the 3-hydroxyl groups of a number of steroids, including cholesterol (5), oxidation of the 3-hydroxyl group of 7-dehydrocholesterol may be considered as a possible reaction alternative to reduction of the Δ^7-bond. If oxidation of the 3-hydroxyl function were accompanied by migration of the Δ^5-bond to the 4-position through enzymic or nonenzymic mechanisms, the product, cholesta-4,7-dien-3-one, could be detected by means of its absorption band in the region of 232 mμ (in cyclohexane). Our attempts to demonstrate conversion of 7-dehydrocholesterol by tissue homogenates to a product with such an absorption band

have, so far, been unsuccessful. However, it was found that incubation of the expected product, cholesta-4,7-dien-3-one, with liver microsomes in the presence of added cofactor, resulted in the production of two metabolites with ultraviolet absorption spectra and chromatographic properties identical with those of cholesta-4,6-dien-3-one and cholest-4-en-3-one. Under similar conditions of incubation, cholesta-4,6-dien-3-one was converted to cholest-4-en-3-one. Some properties of the enzyme systems that catalyze the reactions were examined.

METHODS

Cholesta-4,7-dien-3-one (ϵ^{238}(ethanol) = 15,691; mp 87.5–88°) was synthesized from 7-dehydrocholesterol (6). Ergosta-4,7,22-trien-3-one (mp 125–126°) and cholesta-4,6-dien-3-one (mp 79–80°) were purchased from Elite Chemical Co., Newark, N.J. Cholest-4-en-3-one (mp 81°) was purchased from Mann Research Laboratories, New York, N.Y. Cholesta-4,6-dien-3β-ol was synthesized from 500 mg of cholesta-4,6-dien-3-one by reduction with LiAlH$_4$. The reduced mixture was crystallized from methanol and the

3β-hydroxy isomer was separated by precipitation with digitonin. The regenerated sterol was recrystallized from methanol to constant mp: yield, 36%; mp 114–115°; $\epsilon^{239.5}$(ethanol) = 24,615. Triton × 100 was a gift from Rohm and Haas, Philadelphia, Pa. Triton WR-1339 was purchased from Winthrop Laboratories, New York, N.Y. Triparanol[1] and triparanol succinate were gifts from the W. S. Merrell Co., Cincinnati, Ohio. Cofactors were purchased from Sigma Chemical Co., St. Louis, Mo., and from the California Corporation for Biomedical Research, Los Angeles, Calif.

Livers were obtained from male or female hybrid LAF₁/J (C57L/J × A/HeJ) or BAF₁ (C57BL/6J × A/J) mice or from strain B10.D2 (a C57BL/10 subline) 4–12 months of age. All mice were obtained from the stocks of the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The livers were homogenized in a glass homogenizer with 2 vol 0.14 M NaCl containing 0.003 M reduced glutathione. The homogenates were centrifuged at 13,000 × g for 10 min, the sediment was discarded, and microsomes were sedimented from the supernatant fraction by further centrifugation for 1 hr at 100,000 × g. The microsomes were suspended in a volume of 0.14 M NaCl equivalent to one-half the weight of the liver used.

Incubation of Steroids with Liver Microsomes. Incubation conditions were similar to those used previously in studies of 7-dehydrocholesterol metabolism (2). Cholesta-4,7-dien-3-one and cholesta-4,6-dien-3-one were emulsified in 5% bovine serum albumin containing 10% ethanol. Incubations were carried out in an atmosphere of nitrogen at 37° in a Dubnoff incubator with microsomes and other additions in a total volume of 1 ml in a 10-ml beaker.

Extraction and Measurement of Metabolic Products. After incubation, 1.5 ml of absolute ethanol and 0.5 ml of a saturated solution of NaCl were added to each beaker. The mixtures were shaken gently for approximately 1 min; then 0.5 ml of ethanol, and 0.5 ml of water or 0.5 ml of a 50% w/v solution of KOH were added, and the contents were poured into 15-ml glass-stoppered centrifuge tubes. When KOH was omitted, 5 ml of cyclohexane was added to each tube; the tubes were stoppered, shaken for 5 min and centrifuged at low speed to clarify the cyclohexane layer. When KOH was added, the mixtures were allowed to stand for 15 min at room temperature before adding cyclohexane and extracting. After centrifugation, the cyclohexane layers were removed and absorption spectra were

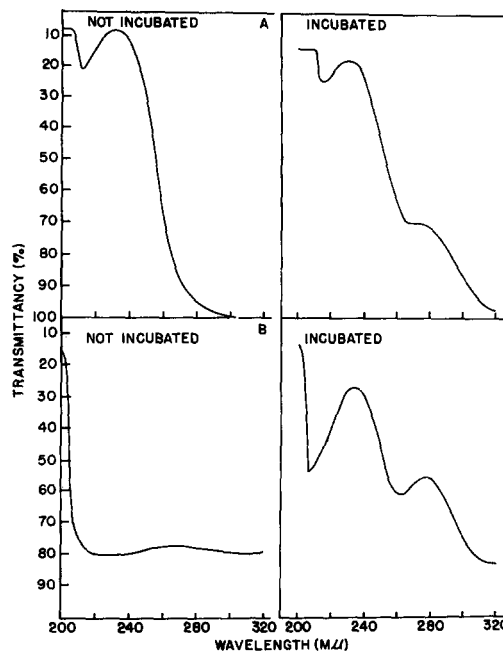[1] 1-[p-(β-diethylamino)ethoxyphenyl]-1-(p-tolyl)-2-(p-chlorophenyl) ethanol.

FIG. 1. Effects of KOH on absorption spectra of cholesta-4,7-dien-3-one and its reaction products. Reaction mixtures contained in a total volume of 1 ml: 60 μmoles of Tris-maleate buffer, pH 6.7; 1.6 μmoles of TPN; 30 μmoles of nicotinamide; 0.52 μmole of cholesta-4,7-dien-3-one in 0.1 ml of emulsion; and 0.3 ml of microsomes equivalent to 0.6 g of liver. Incubation was for 75 min under N₂. Spectra shown in A were obtained when treatment with KOH was omitted. Those shown in B were obtained with extracts of mixtures treated with KOH.

examined over the range of 220–320 mμ in a Beckman D. B. recording spectrophotometer, using cyclohexane extracts of mixtures containing all additions except the substrate as blanks. Maxima of absorption bands and absorbancy values between 1 and 2 were determined with a Beckman D. U. spectrophotometer. Maximum absorbancies for cholesta-4,7-dien-3-one, cholesta-4,6-dien-3-one, and cholest-4-en-3-one in cyclohexane were at 232.5, 277.5, and 232.5 mμ, respectively. (Maximum absorbancies of the three steroids in alcohol were at 240, 285, and 240 mμ, respectively.)

The effect of treatment with KOH on the absorption spectra, obtained when cholesta-4,7-dien-3-one was used as substrate, is shown in Fig. 1. When KOH was omitted (Fig. 1A), recoveries of cholesta-4,7-dien-3-one from nonincubated mixtures were quantitative, while, in incubated samples, a shoulder, with maximum absorbancy at 277.5 mμ corresponding to that for cholesta-4,6-dien-3-one, appeared. Recoveries of added cholesta-4,6-dien-3-one and cholest-4-en-3-one in the absence of KOH were quantitative. Treatment with KOH of nonincubated mixtures, or of an incubated mixture containing boiled microsomes, resulted in complete elimination of the specific absorption band of

cholesta-4,7-dien-3-one, but resulted in the production of material that absorbed relatively evenly over the entire range of wavelengths examined (Fig. 1B). In contrast, after incubation with fresh microsomes, two alkali-stable products, with absorbancy maxima at 232.5 and 277.5 mμ corresponding to those of cholest-4-en-3-one and cholesta-4,6-dien-3-one, were found. Under these conditions, recoveries of added cholesta-4,6-dien-3-one and cholest-4-en-3-one ranged between 90 and 100% and 65 and 100%, respectively. When lower concentrations of KOH were used, destruction of cholesta-4,7-dien-3-one was incomplete; at higher concentrations, recoveries of added cholest-4-en-3-one were diminished to values as low as 50%. In view of the variable recoveries, particularly of cholest-4-en-3-one, recoveries of cholest-4-en-3-one and cholesta-4,6-dien-3-one were determined for each experiment. Completeness of destruction of substrate cholesta-4,7-dien-3-one was also verified in unincubated mixtures included with each experiment. After treatment with KOH, estimates of amounts of cholesta-4,6-dien-3-one and cholest-4-en-3-one were made from absorbancy values at 232.5 and 277.5 mμ. Corrections for the absorbancy due to decomposition products of cholesta-4,7-dien-3-one were made by subtracting absorbancy at 310 mμ from absorbancies found at 232.5 and 277.5 mμ. Corrections were also made for percentage recoveries determined from analyses of the standard samples and for the slight overlap of the cholesta-4,6-dien-3-one absorbancy band with that of cholest-4-en-3-one.

When cholesta-4,6-dien-3-one was added as substrate, similar results were obtained when incubated mixtures were treated with KOH and when KOH was omitted. Two absorption bands were found, one with the maximum at 277.5 mμ corresponding to that of the substrate, the other with the maximum at 232.5 mμ corresponding to that of cholest-4-en-3-one. Treatment with KOH was included regularly so that any cholesta-4,7-dien-3-one formed from cholesta-4,6-dien-3-one would not be measured as cholest-4-en-3-one. The amount of substrate cholesta-4,6-dien-3-one metabolized was determined from the difference in absorbancy at 277.5 mμ between incubated and nonincubated mixtures. Cholest-4-en-3-one was determined from absorbancy measurements at 232.5 mμ. Values were corrected for recoveries on the basis of values determined for standard samples and for overlap of the cholesta-4,6-dien-3-one absorbance band with that of cholest-4-en-3-one.

Chromatography and Tests for Identity. Cholesta-4,7-dien-3-one and cholesta-4,6-dien-3-one were incubated with liver microsomes as described above except that the amount of incubation mixture in each beaker was increased to 2 ml. After incubation for 2 hr in a nitrogen atmosphere, 0.7 ml of saturated NaCl solution and 2 ml of absolute alcohol were added to each flask. The contents of 16 flasks containing approximately 10 mg of substrate and products were pooled and extracted with petroleum ether. The ether extract was washed with water, dried with anhydrous Na$_2$SO$_4$, and evaporated in a vacuum. Extraction of the products of cholesta-4,7-dien-3-one metabolism after destruction of excess substrate with KOH was similar except that after incubation, additions to the flasks were 4 ml of alcohol, 1 ml of saturated NaCl solution, and 1 ml of 50% KOH. Extraction with petroleum ether was begun 15 min after the addition of KOH. In each experiment, spectrophotometric analyses by the procedure described above were carried out on additional flasks. The extracts were chromatographed on 1 x 23-cm columns of silicic acid–Celite 3:1. Before use, the silicic acid (Mallinkrodt) was activated by heating for 30 min at 105° and stored in a desiccator. Gradient elution from petroleum ether (bp 88–100°, Skellysolve-C) to benzene, with 250 ml of petroleum ether in the mixing flask, was employed. Fractions of approximately 1.8 ml were collected and evaporated in a vacuum. Alternate fractions were dissolved in alcohol and absorbancy at 240 and 285 mμ was determined. Chromatograms were plotted and fractions corresponding to the nonoverlapping portions of the peaks were pooled. The ultraviolet absorption spectra of the isolated steroids and their reactions with the Zimmermann and Liebermann-Burchard reagents were determined. The material in the band corresponding to cholest-4-en-3-one produced from cholesta-4,6-dien-3-one was crystallized from methanol and its infrared absorption spectrum in a pressed KBr wafer was determined.

RESULTS

Conversion of cholesta-4,7-dien-3-one to cholesta-4,6-dien-3-one or cholest-4-en-3-one, or of cholesta-4,6-dien-3-one to cholest-4-en-3-one, did not occur when the steroids were incubated at pH 5.3 or pH 7.0 with microsomes heated at 70° for 5 min or with the 100,000 $\times$ *g* supernatant fraction. Microsomes were active after storage for several weeks at −20°, but fresh preparations were used in most of the experiments.

Pyridine Nucleotide Requirements. In the absence of added cofactors, the amount of cholesta-4,7-dien-3-one converted to cholesta-4,6-dien-3-one and cholest-4-en-3-one was small (Table 1). The addition of DPN or DPNH increased the amounts of the two products

TABLE 1. Pyridine Nucleotide Requirements*

Substrate	Pyridine Nucleotide	Cholesta-4,6-dien-3-one		Cholest-4-en-3-one Accumulated
		Accumulated	Metabolized	
		$\mu moles \times 10^2$	$\mu moles \times 10^2$	$\mu moles \times 10^2$
Cholesta-4,7-dien-3-one	None	0.78		3.29
	DPN	1.14		3.41
	DPNH	1.12		4.19
	TPN	2.96		21.13
	TPNH	2.56		20.14
Cholesta-4,6-dien-3-one	None		0	.94
	DPN		0	1.67
	DPNH		0	1.05
	TPN		4.45	4.52
	TPNH		25.2	19.9

* Reaction mixtures contained in a total volume of 1 ml: 60 μmoles Tris-maleate buffer, 1.6 μmoles pyridine nucleotide, 30 μmoles nicotinamide, 0.52 μmole cholesta-4,7-dien-3-one or 0.26 μmole of cholesta-4,6-dien-3-one in 0.1 ml of emulsion, and 0.3 ml microsomes equivalent to 0.6 g of liver. When cholesta-4,7-dien-3-one was used as substrate, the pH of the buffer was 6.7. When cholesta-4,6-dien-3-one was used as substrate, the pH of the buffer was 6.4. Incubation was for 75 min under N₂. All mixtures were treated with KOH before extraction.

only slightly, while the addition of TPN or TPNH resulted in marked increases in the two products. Reduction of cholesta-4,6-dien-3-one to cholest-4-en-3-one was markedly increased by the addition of TPNH and only slightly by TPN, while DPN or DPNH were ineffective. The small amounts of cholest-4-en-3-one measured, wher there was no measurable disappearance of cholesta-4,6-dien-3-one, are probably due to slight variations in extraneous absorbancy at 232.5 mμ.

As shown in Fig. 2A, TPN or TPNH in concentrations higher than 0.6 mM did not increase the amount of cholesta-4,6-dien-3-one found in the extracts when cholesta-4,7-dien-3-one was used as substrate. Amounts of cholest-4-en-3-one formed increased with increasing TPN or TPNH concentration to levels of about 1.3 mM, declining at higher concentrations. The curve indicating amounts of cholest-4-en-3-one accumulated with increasing concentrations of TPNH when cholesta-4,6-dien-3-one was used as substrate (Fig. 2B) was similar to that in Figure 2A. However, the rate at which cholesta-4,6-dien-3-one was metabolized did not decline in the presence of high concentrations of TPNH. Interpretation of these results requires that two other reactions, which have been only partially studied, be considered. One reaction, catalyzed by a 3β-hydroxysteroid dehydrogenase, present in microsomes, results in the oxidation of cholesta-4,6-dien-3β-ol to cholesta-4,6-dien-3-one. Cholesta-4,6-dien-3β-ol has an absorption spectrum with a maximum in cyclohexane at 240 mμ and prominent shoulders at 232 and 248 mμ. Oxidation of the 3-hydroxyl group of cholesta-4,6-dien-3β-ol by liver microsomes in the presence of DPN

or TPN was found to occur. However, in none of the experiments wherein cholesta-4,6-dien-3-one was used as substrate was there any evidence for the formation of a product with the characteristic absorption spectrum of cholesta-4,6-dien-3β-ol, indicating that this reaction was essentially nonreversible under the conditions used. The second reaction to be considered results in the reduction of cholest-4-en-3-one to a product (or products), as yet unidentified, which has no absorption in the region examined. Studies using cholest-4-en-3-one as substrate indicated that microsome preparations in the presence of added TPNH catalyze the conversion of this steroid to an unidentified product or products. No such conversion occurred in the presence of DPN, DPNH, or TPN. The observed difference in the amount of cholest-4,6-dien-3-one metabolized and the amount of cholest-4-en-3-one formed at elevated concentrations of TPNH (Fig. 2B) may be explained by a lower affinity for TPNH of the enzyme (or enzymes) that catalyzes the reduction of cholest-4-en-3-one as compared with that of the Δ⁶-reductase. In agreement with this explanation, the rate of disappearance of cholest-4-en-3-one did not become optimal until the concentration of TPNH reached 2.2 mM.

Effects of pH. When cholesta-4,7-dien-3-one was used as substrate, maximal amounts of cholesta-4,6-dien-3-one were found when the pH of the buffer used was approximately 5.6 (Fig. 3A). Elevation of the pH resulted in a decline in the amounts of this steroid formed to a relatively constant level over the pH range of 6–8. Amounts of cholest-4-en-3-one found
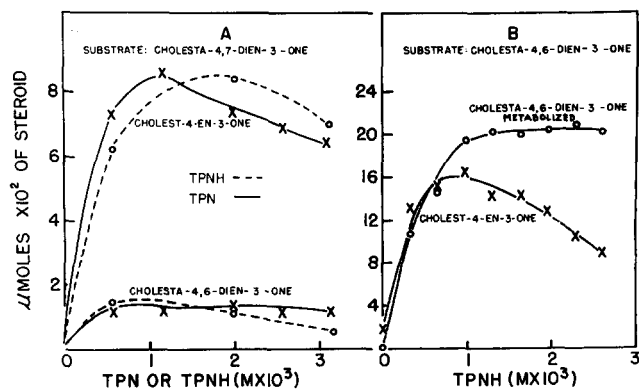

Fig. 2 Effects of varying concentrations of cofactors. Conditions were similar to those in Table 1 except that the amount of cholesta-4,7-dien-3-one added as substrate was 0.26 μmole and amounts of TPN or TPNH added were varied. In A, the substrate was cholesta-4,7-dien-3-one and the cofactor was TPNH (- - - - -) or TPN (———). The lower two curves represent cholesta-4,6-dien-3-one found. The uppermost two curves represent cholest-4-en-3-one found. In B, the substrate was cholesta-4,6-dien-3-one: (O———O) represents cholesta-4,6-dien-3-one metabolized; (X———X) represents cholest-4-en-3-one found.

reached a maximum at a pH of approximately 6.7, a pH at which the amount of cholesta-4,6-dien-3-one measured was relatively low. Maximum amounts of cholesta-4,6-dien-3-one were metabolized in the pH range 6–6.5 (Fig. 3B), while, in general agreement with the curve in Fig. 3A, the greatest amounts of cholest-4-en-3-one were found in the pH range 6.5–7.5. The dissimilarity between the two curves can be explained by a more rapid rate of reduction of cholest-4-en-3-one in the lower pH range. In agreement with this interpretation, the pH range found to be optimal for the reduction of cholest-4-en-3-one by the TPNH-linked microsomal enzyme(s) extended from 6.2–7.

Kinetics. The effects of substrate concentrations on the reactions are shown in Fig. 4. In accordance with results shown in Fig. 3A, at pH 7.0, amounts of cholest-4-en-3-one, measured when cholesta-4,7-dien-3-one served as substrate, were greater than amounts of cholesta-4,6-dien-3-one. The amounts of both products increased in a nearly linear fashion with increasing substrate concentrations to 0.53 mM and more slowly at higher concentrations. At pH 5.5, the ratio of the two products was approximately 1, amounts of cholesta-4,6-dien-3-one increasing slightly over those of cholestenone at higher substrate concentrations. The curve indicating accumulation of cholest-4-en-3-one when cholesta-4,6-dien-3-one was used as substrate showed an initial lag at low substrate concentrations, falling off again at the highest substrate concentrations; while the curve indicating amounts of cholesta-4,6-dien-3-one metabolized was essentially linear over the range examined (Fig. 4B). Since the evidence indicates that the only enzyme acting upon cholesta-4,6-dien-3-one in this system was the Δ^6-reductase, Km values were calculated for this enzyme from a Lineweaver-Burk plot of data on the metabolism of cholesta-4,6-dien-3-one from two experiments similar to that shown in Fig. 4B. The values obtained, 1.54 and 1.61 mM, were of the same order as those found for 7-dehydrocholesterol Δ^7-reductase (2). The extent to which the calculated Km values may be influenced by the insolubility of the substrate is not clear.

Curves obtained by varying the incubation time are shown in Fig. 5. The cholesta-4,6-dien-3-one curve shown in Fig. 5A and the cholest-4-en-3-one curves shown in both A and B reflect the difference between the rates at which they are produced and the rates at which they are converted to other products. Since the concentrations of the substrates in these experiments were less than those required for optimal rates of conversion, the curves are influenced by the decreasing amounts of the substrates available with increasing time.
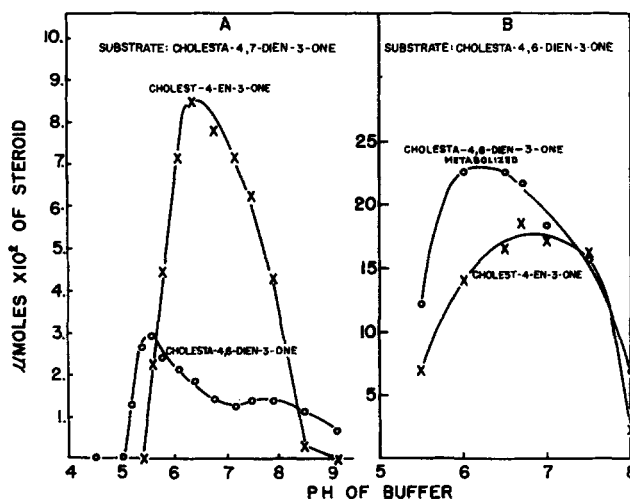

FIG. 3. Effects of pH. Conditions were similar to those in Fig. 2 except that the amount of TPN or TPNH added was 1.6 μmoles and the pH of the buffer was varied. In A, the substrate was cholesta-4,7-dien-3-one and the cofactor was TPN. (O———O) represents cholesta-4,6-dien-3-one found; (X———X) represents cholest-4-en-3-one found. In B, the substrate was cholesta-4,6-dien-3-one and the cofactor was TPNH. (O———O) represents cholesta-4,6-dien-3-one metabolized; (X———X) represents cholest-4-en-3-one found.
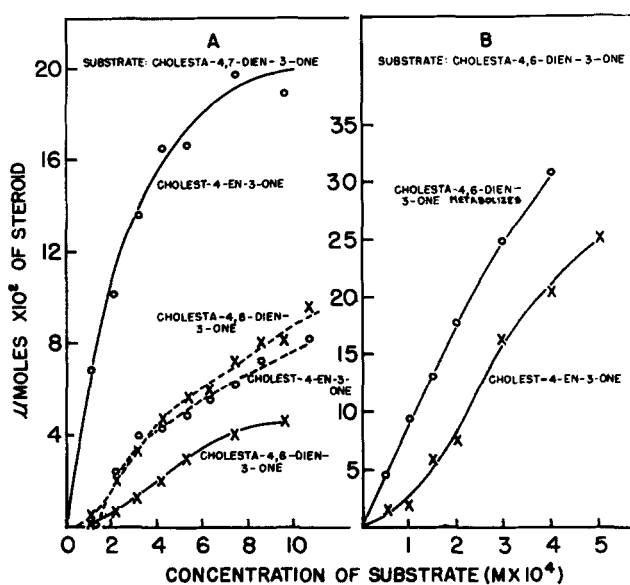

FIG. 4. Effects of substrate concentration. Conditions were similar to those in Table 1 except that the amounts of the substrates and the pH were varied. In A, the substrate was cholesta-4,7-dien-3-one and the cofactor was TPN. (X———X) and (X- - - - -X) represent cholesta-4,6-dien-3-one found at pH 7.0 and 5.5, respectively; (O———O) and (O- - - -O) represent cholest-4-en-3-one found at pH 7.0 and 5.5, respectively. In B, the substrate was cholesta-4,6-dien-3-one and the cofactor was TPNH (O———O) represents cholesta-4,6-dien-3-one metabolized; (X———X) represents cholest-4-en-3-one found.
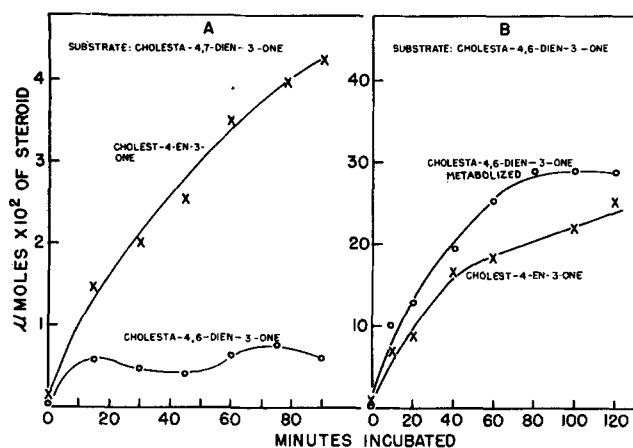
FIG. 5. Effects of variation in length of incubation period. Conditions were similar to those in Table 1 except that the amount of cholesta-4,7-dien-3-one added was 0.26 μmole and the period of incubation under nitrogen was varied. In A, the substrate was cholesta-4,7-dien-3-one and the cofactor was TPN. (O———O) represents cholesta-4,6-dien-3-one found; (X———X) represents cholest-4-en-3-one found. In B, the substrate was cholesta-4,6-dien-3-one and the cofactor was TPNH. (O———O) represents cholesta-4,6-dien-3-one metabolized; (X———X) represents cholest-4-en-3-one found.
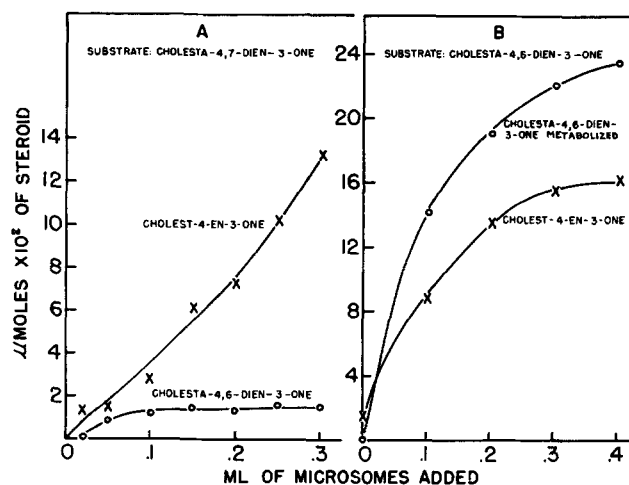

FIG. 6. Effects of microsome concentration. Conditions were similar to those in Table 1 except that the amount of microsomes added was varied. In A, the substrate was cholesta-4,7-dien-3-one and the cofactor was TPN. (O———O) represents cholesta-4,6-dien-3-one found; (X———X) represents cholest-4-en-3-one found. In B, the substrate was cholesta-4,6-dien-3-one and the cofactor was TPNH. (O———O) represents cholesta-4,6-dien-3-one metabolized; (X———X) represents cholest-4-en-3-one found.

Accumulation of cholesta-4,6-dien-3-one produced from cholesta-4,7-dien-3-one reached a maximum level at low concentrations of microsomes and remained at this level as the concentration of microsomes increased (Fig. 6A). In contrast, the curve indicating amounts of cholest-4-en-3-one found rose with increasing concentrations of microsomes. Utilization of cholesta-4,6-dien-3-one and accumulation of cholest-4-en-3-one formed from this substrate increased rapidly in the low range of microsome concentrations, the rate of increase falling off as substrate concentrations became limiting (Fig. 6B). These results, and those of other experiments described above, are consistent with the production of cholest-4-en-3-one from cholesta-4,7-dien-3-one through two reactions, the first reaction—isomerization of the Δ^7-bond—being the rate-limiting step.

Identification of Products. Chromatograms of extracts of incubation mixtures where cholesta-4,6-dien-3-one was the substrate (Fig. 7B), or where the substrate was cholesta-4,7-dien-3-one and the mixtures were treated with KOH (Fig. 7D), were similar to that obtained by chromatographing a mixture of cholesta-4,6-dien-3-one and cholest-4-en-3-one (Fig. 7A). When a mixture of the three steroids was chromatographed, or when treatment with KOH of incubation mixtures containing cholesta-4,7-dien-3-one as substrate was omitted (Fig. 7C) only two bands were found, cholesta-4,7-dien-3-one being eluted with the cholest-4-en-3-one band. The presence of cholesta-4,7-dien-3-one in the
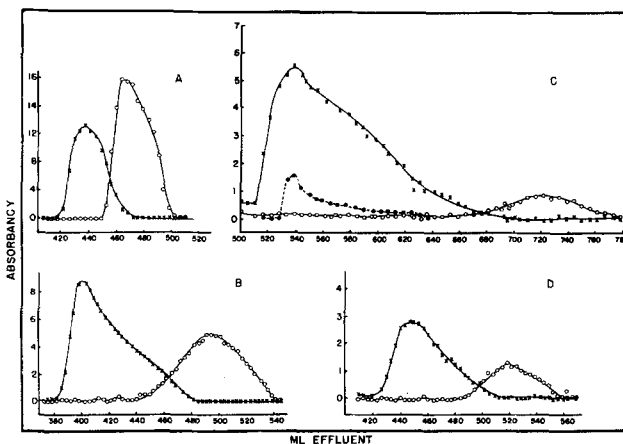
FIG. 7. Chromatograms of extracts of incubation mixtures. Absorbancy per tube at 240 mμ, X———X; absorbancy at 285 mμ, O———O; absorbancy of Liebermann-Burchard chromophore at 460 mμ, 1.25 min after addition of the reagent. ●———●. (See Table 2 for reactions with the Liebermann-Burchard reagent). (A) is a chromatogram of a mixture of authentic cholesta-4,6-dien-3-one (5 mg) and cholest-4,6-dien-3-one (5 mg); (B) an extract of incubation mixture containing cholesta-4,6-dien-3-one as substrate, treatment with KOH omitted; (C) an extract of incubation mixture containing cholesta-4,7-dien-3-one as substrate, treatment with KOH omitted. (D) was similar to (C) but treatment with KOH was included. Incubation conditions were similar to those shown in Table 1 with the following exceptions: when cholesta-4,6-dien-3-one was the substrate, the cofactor was TPNH; when cholesta-4,7-dien-3-one was the substrate, the cofactor was TPN; amounts of components other than substrate were doubled to give a final volume of 2 ml/beaker; the amount of substrate in each beaker was 1.5 μmoles; incubation was for 2 hr. The contents of 16 beakers were pooled before extraction.

initial band was indicated by the rapid production of a yellow color on reaction with the Liebermann-Burchard reagent. The reactions of the three steroids with this reagent are described below. Absorbancy at 240 mμ not accounted for by the amount of cholesta-4,7-dien-3-one found was attributed to cholest-4-en-3-one. The greater spreading of the steroid bands obtained by chromatographing extracts of incubation mixtures relative to that found when pure steroids were chromatographed may be due to the presence in the extracts of relatively large amounts of other lipids. Differences in the volume of solvents required for elution are due to variations in the packing of the columns and in the degree of activation of the silicic acid. Chromatography of cholesta-4,7-dien-3-one alone gave no indication of the presence of a band corresponding to cholesta-4,6-dien-3-one, excluding the possibility that isomerization to the 4,6-diene may have occurred as a result of chromatography. Chromatography of an extract of an incubation mixture to which no steroid had been added did not reveal any bands with maximum absorbancy at 240 or 285 mμ.

Fractions containing the band absorbing at 240 mμ, which was produced as a result of cholesta-4,6-dien-3-one metabolism, were pooled. After a single crystallization from methanol, its melting point was 77–79° and its ultraviolet and infrared absorption spectra were identical with those of authentic cholesta-4-en-3-one. Its reactions with the Zimmermann and Liebermann-Burchard reagents were those characteristic of cholest-4-en-3-one. Amounts of the two products of cholesta-4,7-dien-3-one metabolism isolated were too small to crystallize. Their ultraviolet absorption spectra and their reactions with the Zimmermann and Liebermann-Burchard reagents were those characteristic of cholest-4-en-3-one and cholesta-4,6-dien-3-one. The reactions of the three steroids with the Liebermann-Burchard reagent are indicated in Table 2. The visually distinguishable chromophores resulting from the reaction of cholesta-4,7-dien-3-one, cholesta-4,6-dien-3-one, and cholest-4-en-3-one with the Zimmermann reagent were brown-orange, intense blue, and brown-purple. Maximal absorbancy of the cholesta-4,6-dien-3-one chromophore was at 600 mμ while the two other chromophores showed no distinct maxima in the visible range.

Isolated steroid, corresponding to cholesta-4,6-dien-3-one, resulting from an experiment similar to that shown in Fig. 7C, was incubated with microsomes and TPNH for 1 hr under the usual conditions. Spectrophotometric analysis of cyclohexane extracts obtained after treatment of the mixtures with KOH indicated appreciable conversion to a product with maximal absorbancy at 232.5 mμ.

TABLE 2. REACTIONS WITH THE LIEBERMANN-BURCHARD REAGENT*

Wave-length	Time	Cholesta-4,7-dien-3-one	Cholesta-4,6-dien-3-one	Cholest-4-en-3-one
mμ	min	OD/1 mg	OD/1 mg	OD/1 mg
460	1.25	19.43	0	0
670	100	7.82	3.40	0.149
	200	6.07	4.14	0.525

* The steroids were dissolved in 1 ml of glacial acetic acid, 2 m of a 20:1 acetic anhydride–sulfuric acid mixture was added, and tubes were placed in a 25° water bath. Readings were made with a Bausch and Lomb Spectronic 20 Colorimeter.

Recoveries of authentic cholesta-4,7-dien-3-one, cholesta-4,6-dien-3-one, and cholest-4-en-3-one from the columns were 78, 86, and 94%, respectively. The amount of cholest-4-en-3-one produced from cholesta-4,6-dien-3-one, estimated from the chromatogram, was 91% of that estimated from spectrophotometric measurements of cyclohexane extracts of incubation mixtures. Amounts of cholesta-4,6-dien-3-one and cholest-4-en-3-one produced from cholesta-4,7-dien-3-one estimated from the chromatograms were 90 and 118%, respectively, of those estimated by spectrophotometric analysis when treatment with KOH was included. When treatment with KOH was omitted, the amount of cholesta-4,6-dien-3-one estimated from the chromatogram was 60% of that found by the spectrophotometric method. Since recoveries of cholest-4-en-3-one in cylcohexane extracts of KOH treated incubation mixtures were relatively low, the increased amount estimated from the chromatogram may be due to more complete extraction with petroleum ether.

Reversibility, Specificity, and Inhibitors. When cholesta-4,6-dien-3-one was used as substrate in the presence of TPNH or TPN, amounts of cholest-4-en-3-one calculated from absorbancy at 232.5 mμ were identical whether KOH was included or omitted, indicating that no detectable amount of the alkali-labile cholesta-4,7-dien-3-one was formed by reversal of the isomerization reaction. Incubation of cholest-4-en-3-one with TPN did not result in the appearance of an absorption band at 277.5 mμ indicating nonreversibility of the reaction catalyzed by the Δ^6-reductase.
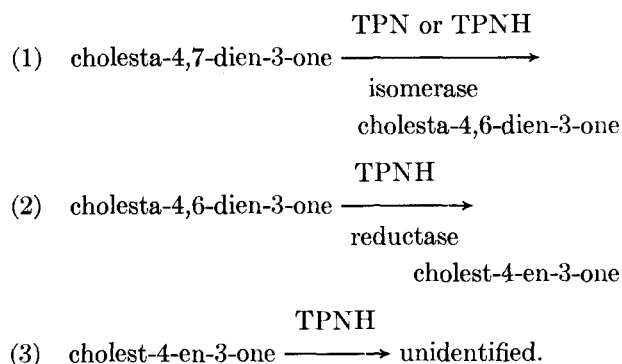
Ergosta-4,7-22-trien-3-one was the only steroid used to examine the specificities of the reactions under study. Incubation of this steroid with microsomes and TPN under the usual conditions resulted in the production of two alkali-stable products with absorbancy maxima at 277.5 and 232.5 mμ, respectively, indicating conversion to the 3-keto-$\Delta^{4,6}$ isomer and to the reduced

3-keto-Δ^4 derivative. The apparent lack of specificity of these enzyme systems contrasts with that of 7-dehydrocholesterol Δ^7-reductase, which did not catalyze the reduction of ergosterol (2).

Conversion of substrate cholesta-4,7-dien-3-one or cholesta-4,6-dien-3-one was not influenced by added EDTA or $MgCl_2$. As shown in Table 3, production of cholesta-4,6-dien-3-one and cholest-4-en-3-one from cholesta-4,7-dien-3-one, as well as conversion of cholesta 4,6-dien-3-one to cholest-4-en-3-one, was inhibited by p-chloromercuribenzoate and this inhibition was reversed by reduced glutathione. Metabolism of both substrates was inhibited by a number of surface-active agents and by triparanol succinate (Table 4). Inhibition by triparanol was of the same order as that shown for the water-soluble succinate derivative. Deoxycholate appeared to be a more effective inhibitor than the other bile acids. Triton $\times$ 100 was an effective inhibitor, while Triton WR-1339 did not inhibit over the range of concentrations examined. At intermediate concentrations, some of the inhibitors, notably deoxycholate and Triton $\times$ 100, caused an increase in the accumulation of cholesta-4,6-dien-3-one, although the amount of cholest-4-en-3-one found was markedly less. At higher concentrations of these inhibitors, the amount of cholesta-4,6-dien-3-one found was reduced below levels found in the controls.

DISCUSSION

The results obtained in these experiments are interpreted in terms of the following three reactions:

(1)   cholesta-4,7-dien-3-one $\xrightarrow[\text{isomerase}]{\text{TPN or TPNH}}$ cholesta-4,6-dien-3-one

(2)   cholesta-4,6-dien-3-one $\xrightarrow[\text{reductase}]{\text{TPNH}}$ cholest-4-en-3-one

(3)   cholest-4-en-3-one $\xrightarrow{\text{TPNH}}$ unidentified.

The demonstration by Tombropoulos et al. (7) that cholest-4-en-3-one is converted to 5α-cholestan-3β-ol by microsomal and mitochondrial fractions of rat liver suggests that the major product formed from cholest-4-en-3-one in the present studies may also be 5α-cholestan-3β-ol. However, since conversion of cholest-4-en-3-one to the stanol proceeds through the sequential reduction of the double bond and the ketone function, and α-and β-isomers are possible products of each reaction, the

TABLE 3. INHIBITION BY p-CHLOROMERCURIBENZOATE AND REVERSAL BY REDUCED GLUTATHIONE*

Substrate	Additions[†]	Cholesta-4,6-dien-3-one		Cholest-4-en-3-one
		Accu-mulated	Metabo-lized	Accumu-lated
		$\mu moles \times 10^2$	$\mu moles \times 10^2$	$\mu moles \times 10^2$
Cholesta-4,7-dien-3-one	None	2.99		14.98
	GSH	2.81		19.73
	PCMBA	0.70		1.45
	GSH+PCMBA	3.10		14.78
Cholesta-4,6-dien-3-one	None		18.84	16.33
	GSH		19.39	13.12
	PCMBA		1.58	0
	GSH+PCMBA		14.44	10.52

* Conditions were similar to those in Table 1 except that TPN was the cofactor added when cholesta-4,7-dien-3-one was the substrate and TPNH was the cofactor when the substrate was cholesta-4,6-dien-3-one.

† Reduced glutathione (GSH) and p-chloromercuribenzoate (PCMBA) were added to final concentrations of 0.04 M and 0.008 M, respectively.

TABLE 4. EFFECTS OF BILE ACIDS, DETERGENTS, AND TRIPARANOL*

Addition	Concentration	Substrate: cholesta-4,7-dien-3-one		Cholesta-4,6-dien-3-one
		Cholesta-4,6-dien-3-one	Cholest-4-en-3-one	3-one Metabo-lized
		% of control	% of control	% of control
Deoxycholate	5 mM	118.0	60.0	18.7
	10 mM	160.5	24.0	7.8
Cholate	5 mM	141.0	87.1	94.5
	10 mM	125.6	21.2	53.6
Dehydrocholate	5 mM	82.9	50.5	50.0
	10 mM	71.5	38.6	47.8
Triton $\times$ 100	0.1%	286.6	38.1	33.8
	0.15%	146.3	3.8	20.7
Triton WR-1339	0.5%	91.6	76.9	97.7
	0.75%			98.6
Tween-80	0.1%	83.0	101.5	76.0
	0.5%	97.1	35.8	
Triparanol succinate	0.02 mM	94.8	38.3	61.7
	0.12 mM	42.3	22.2	18.9
	0.20 mM	24.2	18.3	12.4

* Conditions were similar to those in Table 3. Bile acids were added as the sodium salts.

formation of products other than 5α-cholestan-3β-ol cannot be excluded.

The apparent requirement of cholesta-4,7-dien-3-one Δ^7-isomerase for TPNH or TPN was somewhat surprising since the only steroid isomerase that has been isolated, the Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni*, catalyzes the intramolecular transfer of hydrogen in the absence of added cofactor (8). The possibility that direct reduction of the Δ^7-bond of cholesta-4,7-dien-3-one may have occurred to some extent in the presence of the cofactor was not excluded by the present experiments. If this reaction did occur,

it must be considered as an alternative to isomerization of the Δ^7-bond. Since conversion of cholest-4-en-3-one to cholesta-4,6-dien-3-one did not occur in the system, direct reduction of the Δ^7-bond could not account for the increased synthesis of cholesta-4,6-dien-3-one in the presence of TPN or TPNH. However, isomerization of the double bond does not involve oxido-reduction and the stimulatory effect of the cofactor on this reaction may have been indirect.

The metabolic production of cholesta-4,7-dien-3-one from 7-dehydrocholesterol or some closely related steroid and its subsequent metabolism to cholest-4-en-3-one, a product known to give rise to cholestan-3β-ol, would constitute an alternate pathway along which cholesterol precursors could be diverted. The existence of enzymes capable of catalyzing some of the steps in this pathway, along with the demonstrated presence of cholesta-4,6-dien-3-one in atherosclerotic aortas and in hog spleen (9, 10), and the natural occurrence of cholestan-3β-ol in animal tissues (11, 12), suggest that the pathway may operate under physiological conditions. Since evidence for the conversion of cholesterol to cholest-4-en-3-one *in vitro* (5), and to cholestan-3β-ol *in vivo* (12) has been presented, an alternate pathway for the biosynthesis of cholestan-3β-ol might be of secondary importance. However, the activity of the pathway from cholesterol to cholestan-3β-ol appears to be weak. In earlier experiments, *in vivo* and *in vitro*, Harold et al. (13) failed to observe any conversion of cholesterol-4-C^{14} to cholestan-3-β-ol. A more recent report by Werbin et al. (12) does not exclude the possibility that the observed conversion of cholesterol-4-C^{14} to cholestan-3β-ol *in vivo* may have occurred in the gastrointestinal tract.

Viewed as a possible mechanism for the regulation of cholesterol biogenesis, the production of cholest-4-en-3-one as one of the metabolites in the suggested pathway is of significance since ingestion of cholest-4-en-3-one is known to inhibit the biosynthesis of cholesterol (14, 15, 16). Under the conditions of the present experiments, the concentration of TPNH was a factor determining the extent to which cholest-4-en-3-one accumulated. The inhibitory effects of bile acids, certain detergents, and triparanol are also of interest in this regard since at least some of these are known to affect cholesterol metabolism (17–20). However, Triton WR-1339 stimulates cholesterol biosynthesis (16, 21) but had little inhibitory effect upon the enzymes examined in the present study.

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