Oxidation of 7-dehydrocholesterol by a mouse liver microsomal system dependent on reduced pyridine nucleotides

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ABSTRACT Aerobic incubation of 7-dehydrocholesterol with mouse liver microsomes in the presence of a detergent, an iron salt, and NADH or NADPH resulted in the conversion of the sterol to more polar products. In the presence of Fe^{3+} or low levels of Fe^{2+} the reaction was dependent upon reduced pyridine nucleotide and a microsomal enzyme system.

At high levels of Fe^{2+} or in the presence of Fe^{2+} or Fe^{3+} and ascorbic acid, nonenzymatic oxidation of 7-dehydrocholesterol occurred in the absence of NADH or NADPH. Chromatograms of products resulting from the enzyme-dependent and enzyme-independent reactions were similar.

The enzymatic reaction was inhibited by certain chelating agents, by antioxidants, and by menadione, phenazine methosulfate, and ferricyanide. Low concentrations of EDTA stimulated the reaction and high concentrations inhibited it. In the complete system sterol oxidation was correlated with the peroxidation of microsomal lipids, but peroxidation of microsomal lipids proceeded more rapidly when either the sterol, the detergent, or both were omitted. Ergosterol was resistant to oxidation under conditions that caused extensive loss of 7-dehydrocholesterol. Microsomes from tissues other than liver were relatively inactive.

KEY WORDS 7-dehydrocholesterol · enzymatic oxidation · pyridine nucleotide dependence · mouse liver · autoxidation · microsomal lipids

In the course of experiments designed to test whether 7-dehydrocholesterol might be metabolized to cholesta-4,7-dien-3-one as was suggested in a previous publication (1), it was found that aerobic incubation of 7-dehydrocholesterol with liver microsomes in the presence of NADH or NADPH, a detergent, and an iron salt resulted in the rapid conversion of the sterol to more polar products. The reaction differed in its requirements from that in which 7-dehydrocholesterol is converted to cholesterol, and resembled reactions catalyzed by steroid hydroxylases in its dependence upon oxygen and a reduced pyridine nucleotide, its apparent substrate specificity, and the increased polarity of the resulting products. However, oxidation of the sterol was always accompanied by peroxidation of microsomal lipids and the results obtained do not eliminate the possibility that the oxidation of 7-dehydrocholesterol was accomplished indirectly by a pyridine nucleotide-linked enzyme system (or systems) that also causes peroxidation of microsomal lipids (2–4).

Abbreviation: TBA, thiobarbituric acid.

EXPERIMENTAL METHODS

7-Dehydrocholesterol and ergosterol, obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, were crystallized from acetone before use. 7-Dehydrocholesterol-4-^{14}C was synthesized from cholesterol-4-^{14}C and purified by column chromatography, followed by crystallization to constant specific activity as described previously (5): $E_{281.5m\mu}$ (ethanol), 11,990; cpm/mg, 4.03×10^5. 7-Dehydrocholesterol 5,8-peroxide was synthesized from 1 g of 7-dehydrocholesterol (6) and crystallized from methanol to a constant mp, 153–155°C; $[\alpha]_D = -3.8°$ ($CHCl_3$). Pyridine nucleotide cofactors were obtained from the California Biochemical Corporation, Los Angeles, Calif., or from Nutritional Biochemicals Corporation. Triton X 100, a nonionic detergent, mixture of [*p*, *t*-octyl poly(phenoxyethoxy) ethanols], was a gift of Rohm & Haas Co., Philadelphia, Pa. Bovine serum albumin (amorphous) was purchased from Mann Research Laboratories, Inc., New York, N.Y. Tissues

were obtained from strain A/J mice, 3–6 months of age, that were fed Old Guilford Laboratory Chow (Emory-Morse Co., Guilford, Conn.). Livers or other tissues were homogenized with 2 volumes of 0.25 M sucrose. After centrifugation at 13,000 × *g* for 10 min microsomes were sedimented from the supernatant fraction by centrifugation for 1 hr at 100,000 × *g*. The microsomes were washed once with 0.25 M sucrose and stored as pellets at −20°C. Microsomes stored under these conditions were active for at least 3 weeks. Before use, microsomes from 2 gm of liver were suspended in 1 ml of 0.14 M saline. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (7).

Incubation Conditions

7-Dehydrocholesterol was dissolved in 0.1 ml of dimethyl formamide and homogenized with 0.9 ml of 5% aqueous bovine serum albumin. Aerobic incubations of the sterol with microsomes and other components were carried out in the dark in a Dubnoff incubator. Beakers containing all components except the sterol were included as blanks for spectrophotometric measurements.

After incubation, an aliquot (0.2 ml) was removed from each beaker for measurement of TBA-reacting compounds by the method of Ottolenghi (8). Ethanol (2 ml), 1 ml of saturated NaCl, and 5 ml of cyclohexane were added to the remainder of the reaction mixture in each beaker. The mixture was shaken mechanically in a glass-stoppered centrifuge tube for 5 min and centrifuged, and the absorption spectrum (220–320 mμ) of the cyclohexane layer was recorded with a Beckman DB spectrophotometer. The amount of 7-dehydrocholesterol lost was determined from the difference between the spectra of extracts from incubated and unincubated mixtures. Recoveries of 7-dehydrocholesterol-4-^{14}C from unincubated samples ranged between 92 and 96%.

Chromatography of Reaction Products

Samples to be chromatographed were incubated under conditions similar to those described above except that 7-dehydrocholesterol-4-^{14}C was included with the unlabeled sterol added to the reaction mixture. After incubation, the contents of each of six to eight beakers were extracted with 5 ml of chloroform–methanol 2:1 and then with a further 3 ml of the same solvent mixture.

The extracts were pooled, washed once with water, dried over anhydrous sodium sulfate, and evaporated to dryness in an amber flask on a rotary evaporator at room temperature. The residue was dissolved in benzene and chromatographed on a 1.5 × 20 cm column of alumina (Fisher Scientific Company) partially deactivated as described by Schneider, Clayton, and Bloch (9). Radioactive bands were eluted with solvents in the following order: benzene (200 ml); ethyl acetate–benzene 1:3 (100 ml); ethyl acetate (75 ml); methanol–ethyl acetate 1:1 (75 ml); methanol (75 ml). Fractions (4 ml) were collected and 0.2 ml aliquots were plated for ^{14}C analysis with a thin-window, gas-flow Geiger counter.

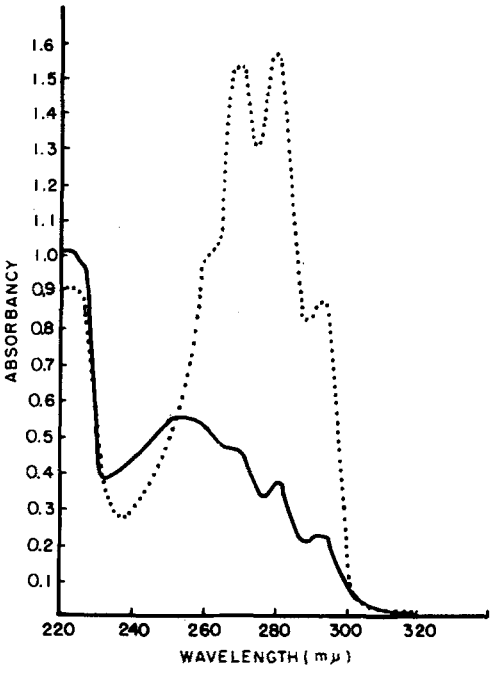

FIG. 1. Absorption spectra before and after incubation. The reaction mixture contained in a total volume of 1 ml: 60 μmoles of Tris–maleate buffer pH 6.8, 30 μmoles of nicotinamide, 1.5 μmoles of NADH, 0.4 μmole of $FeCl_3$, microsomes equivalent to 200 mg of liver (2.5 mg of protein), 0.1 ml of a 5% solution of Triton X 100, and 0.75 μmole of 7-dehydrocholesterol in 0.1 ml of suspending medium. Incubation was for 20 min at 37 °C in air. The absorption spectrum before incubation is designated by the dotted line, that after incubation by the solid line.

RESULTS

As shown in Fig. 1, incubation of 7-dehydrocholesterol resulted in a diminution of the absorbancy due to 7-dehydrocholesterol, accompanied by the appearance of a new absorption band with a maximum at 255 mμ. Since the reaction products did not absorb appreciably at 293 mμ, amounts of 7-dehydrocholesterol lost were estimated from the change in absorbancy at this wavelength. Under the conditions shown in Fig. 1, 76% of added 7-dehydrocholesterol was lost after 20 min of incubation.

Requirements for the oxidation of the sterol are shown in Table 1. Appreciable conversion of 7-dehydrocholesterol to other products in the presence of unboiled microsomes did not occur when incubations were anaerobic or when a reduced pyridine nucleotide cofactor was omitted. Triton X 100 markedly stimulated the rate at which the sterol was oxidized, but partially inhibited the production of TBA-reactive products. When sodium deoxycholate (0.1 ml of a 5% solution) was substituted for

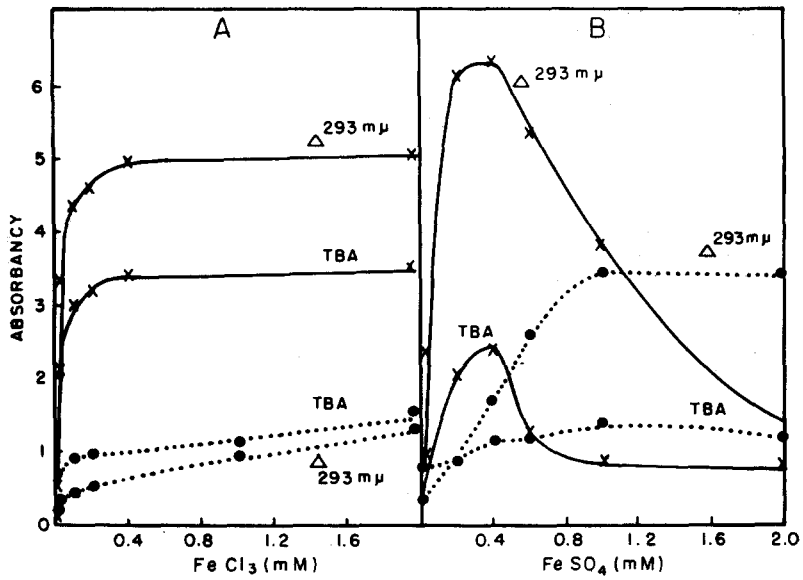

FIG. 2. Effects of $FeCl_3$ and $FeSO_4$ on the rate of disappearance of 7-dehydrocholesterol and on the production of TBA-reactive products. Conditions were similar to those in Fig. 1 except that concentration of $FeCl_3$ (A) or $FeSO_4$ (B) were varied and microsomes were either unboiled (X——X) or boiled (●···●).

Triton (not shown), the rate of the reaction was comparable to that obtained in the presence of Triton only if the amount of microsomes added to the reaction mixture was increased twofold. The rate of oxidation of the sterol in the presence of boiled microsomes was much lower than that when unboiled microsomes were used, and Triton and NADH inhibited the reaction under these conditions.

The activating effects of ferric and ferrous ions are compared in Fig. 2. In the presence of unboiled microsomes, activation by Fe^{3+} increased up to a concentration of 0.4 mM and further increases in concentration to 2 mM did not alter the rate. The rate of oxidation in the presence of boiled microsomes was only slightly accelerated by Fe^{3+}. In contrast, the activating effect of Fe^{2+} with unboiled microsomes was maximal at concentrations between 0.2 and 0.4 mM and diminished at higher concentrations. When boiled microsomes were used, Fe^{2+} had little effect at 0.2 mM and had its greatest effect at concentrations of 1.0 mM or higher. In the presence of unboiled microsomes and low levels of Fe^{2+} (0.2 mM), NADH or NADPH caused an increase in the rate of the reaction similar to that found when NADH or NADPH was added to reaction mixtures containing either high or low concentrations of Fe^{3+} (Table 2). However, at high concentrations of Fe^{2+} (2.0 mM), the reaction was inhibited by NADH.

Although the experiments shown in Table 1 and Fig. 2 indicate a nearly absolute requirement for iron ions, in earlier experiments, rates of oxidation similar to those shown were obtained in the absence of added iron. An attempt to eliminate the requirement for iron by feeding a vitamin E-free diet for a period of 2 months was unsuccessful. A possible explanation for the absence of a requirement for added iron in earlier experiments may be contamination by iron of one of the reagents added to the reaction mixture (3, 10).

TABLE 1 REQUIREMENTS FOR OXIDATION OF 7-DEHYDROCHOLESTEROL

Reaction Mixture	Unboiled Microsomes		Boiled Microsomes	
	Δ 293 mμ	TBA	Δ 293 mμ	TBA
	A units	*A, 550 mμ*	*A units*	*A, 550 mμ*
Complete	4.90	4.30	0.70	0.94
−O_2	0.44	0.54	0.32	0.51
−Microsomes	0.00	0.00	—	—
−Triton X 100	1.12	7.1	1.20	3.50
−NADH	0.15	0.46	1.04	0.87
−$FeCl_3$	0.55	0.43	0.58	0.87
−NADH, + NAD^+	1.42	0.57	—	—
−NADH, + $NADP^+$	0.43	0.44	—	—
−NADH, + NADPH	3.00	1.84	—	—
−Nicotinamide	4.13	2.69	—	—

The complete reaction mixture and conditions of incubation were similar to those shown in Fig. 1. NAD^+, $NADP^+$, and NADPH were added in the same concentration as that of NADH. A, absorbancy (=OD).

Table 3 illustrates the effects of adding ascorbic acid or EDTA to reaction mixtures containing Fe^{3+} or Fe^{2+}. At 10 μM, EDTA increased the rate of the reaction in the presence of Fe^{2+} or Fe^{3+} and did not affect the requirement for a reduced pyridine nucleotide. At a higher concentration (1 mM), EDTA was inhibitory. Addition of ascorbic acid to the reaction mixture in the absence of iron did not stimulate the reaction. However, when

TABLE 2 PYRIDINE NUCLEOTIDE DEPENDENCY IN THE PRESENCE OF HIGH AND LOW CONCENTRATIONS OF Fe^{2+} AND Fe^{3+}

Iron Concentration	Cofactor	Fe^{2+} Δ 293 mμ	Fe^{2+} TBA	Fe^{3+} Δ 293 mμ	Fe^{3+} TBA
mM		*A units*	*A, 550 mμ*	*A units*	*A, 550 mμ*
0.2	—	1.07	1.26	0.00	0.29
0.2	NADH	3.97	1.74	4.40	2.55
0.2	NADPH	2.73	1.34	3.00	1.84
2.0	—	3.72	1.11	0.27	0.38
2.0	NADH	1.87	0.82	4.73	3.72

Conditions were similar to those in Fig. 1 except that the iron salt and pyridine nucleotide cofactor were varied as indicated.

Fe^{3+} or Fe^{2+} was present with ascorbate, the reaction occurred in the absence of a pyridine nucleotide cofactor. The addition of NADH to reaction mixtures containing Fe^{3+} and ascorbic acid stimulated the reaction approximately twofold, while NADH inhibited the reaction that occurred in the presence of Fe^{2+} and ascorbic acid. Combinations of ascorbic acid or EDTA and Fe^{2+} or Fe^{3+}, under the conditions shown in Table 3, caused no significant loss of 7-dehydrocholesterol in the absence of microsomes. However, when 7-dehydrocholesterol was incubated with boiled microsomes, in the presence of ascorbic acid and Fe^{2+} or Fe^{3+}, the sterol was oxidized and TBA-reactive products were formed at rates similar to those found when unboiled microsomes were used under conditions similar to those shown in Table 3, line 5; NADH did not stimulate this reaction.

Activities of Microsomes from Other Tissues

As shown in Table 4, microsomes from liver were much more effective than microsomes from brain, spleen, testes, or kidney. Microsomes from a differentiated, transplantable hepatoma (BW 7756), indigenous to strain C57L/J, were less than one-third as active as microsomes prepared from livers of mice bearing the tumor.

TABLE 3 EFFECTS OF EDTA AND ASCORBATE ON THE REACTION

Reaction Mixture	Fe^{2+} Δ 293 mμ	Fe^{2+} TBA	Fe^{3+} Δ 293 mμ	Fe^{3+} TBA
	A units	*A, 550 mμ*	*A units*	*A, 550 mμ*
Complete	4.02	1.74	4.60	2.55
+ EDTA (10 μM)	5.50	4.43	5.75	4.61
+EDTA (10 μM), − NADH	1.34	1.34	0.17	0.49
+EDTA (1 mM)	0.17	0.30	0.17	0.46
+Ascorbate (1 mM)	1.82	0.74	4.96	3.85
+Ascorbate (1 mM), − NADH	3.34	1.01	2.67	1.55

Conditions, and the complete mixture, were similar to those in Fig. 1, except that Fe^{2+} (0.2 μmole) or Fe^{3+} (0.2 μmole) were added as indicated.

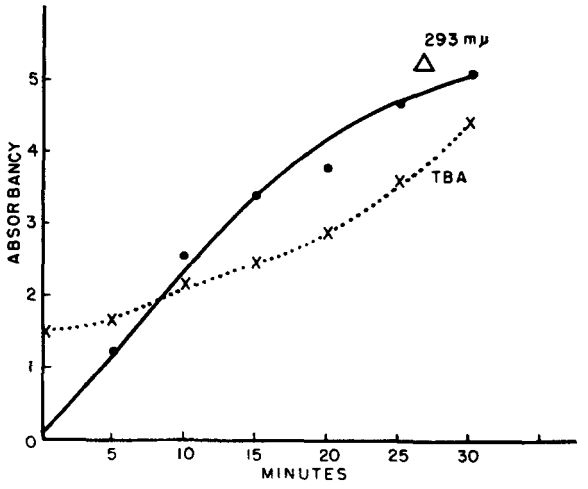

FIG. 3. Effect of incubation time. Conditions were similar to those in Fig. 1 except that the period of incubation was varied.

Kinetics

Nonlinearity with time in the rates of sterol oxidation and formation of TBA-reactive products is indicated by Fig. 3. As shown in Fig. 4, the rate of sterol oxidation increased with increasing amounts of microsomes over the range 0–2.5 mg of protein and declined at higher levels, whereas production of TBA-reactive products increased to a plateau at 5.0 mg of protein. The effects of various concentrations of NADH and NADPH on the rates of the two reactions are shown in Fig. 5. At concentrations up to 0.75 mM, NADH was more effective than NADPH. At higher concentrations, NADH was somewhat inhibitory and NADPH was equally effective. Fig. 6 shows a linear increase in the rate of sterol oxidation with

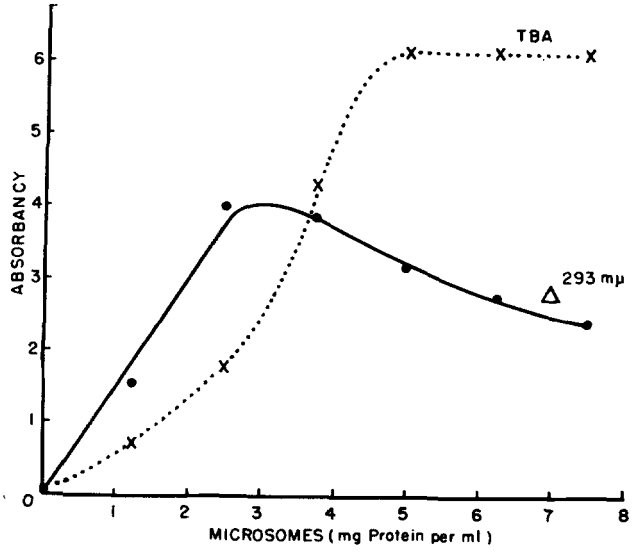

FIG. 4. Effect of microsome concentrations. Conditions were similar to those in Fig. 1 except that the concentration of microsomes was varied.

TABLE 4 OXIDATION OF 7-DEHYDROCHOLESTEROL BY MICROSOMES FROM VARIOUS TISSUES

Tissue	Fe^{2+}		Fe^{3+}	
	Δ293 mμ	TBA	Δ293 mμ	TBA
	A units	*A, 550 mμ*	*A units*	*A, 550 mμ*
Liver	4.25	1.93	4.36	4.30
Brain	0.17	1.46	0.49	0.33
Spleen	—	—	0.33	0.10
Testes	—	—	0.49	0.33
Kidney	0 85	1.00	0.09	0.10
Liver*	4 50	2.82	5.55	4.60
Hepatoma*	1.45	1.68	1.57	1.5

Conditions were similar to those in Fig. 1 except that, where indicated, Fe^{2+} (0.2 μmole) was substituted for Fe^{3+}. Concentrations of microsomes were adjusted to 2.5 mg of protein per ml of reaction mixture.

* Tissues harvested from C57L/J mice bearing a transplantable hepatoma (BW 7756) 21 days after inoculation of the tumor.

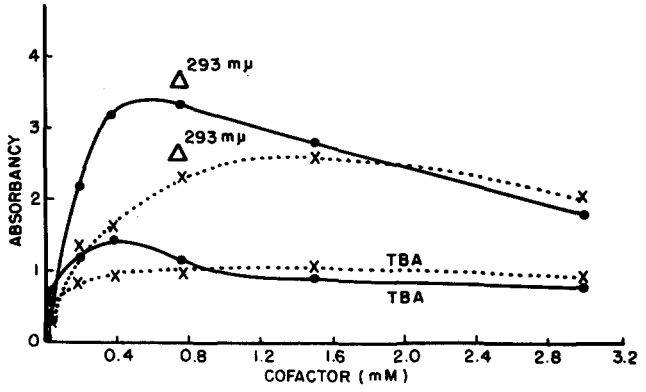

FIG. 5. Effects of NADH and NADPH. Conditions were similar to those in Fig. 1 except that concentrations of NADH (●——●), or NADPH (X····X), were varied.

increasing concentrations of 7-dehydrocholesterol, accompanied by a diminution in the rate of production of TBA-reactive products. An inverse relationship between oxidation of the sterol and the production of TBA-reactive products was also apparent when the rates of the two reactions were plotted against the concentration of Triton (Fig. 7).

Specificity of the Reaction

Ergosterol was not oxidized to any appreciable extent under conditions similar to those that resulted in the rapid and nearly complete oxidation of 7-dehydrocholesterol, and that resulted in the production of considerable amounts of TBA-reactive substances (Table 5). Although the failure of ergosterol to undergo oxidation under these conditions is suggestive of the high degree of specificity to be expected of an enzyme system, the slow rate of ergosterol oxidation under nonenzymatic conditions, i.e. in the presence of boiled microsomes, indicates that ergosterol is relatively resistant to autoxidation under these conditions. Since ergosterol is less soluble than

TABLE 5 OXIDATION OF ERGOSTEROL

Incubation Time	$FeSO_4$	$FeCl_3$	Δ 293 mμ	TBA
min	*mM*	*mM*	*A units*	*A, 550 mμ*
		Unboiled microsomes		
20	—	0.4	0.35	2.92
20	0.2	—	0.62	4.26
60	—	—	0.55	4.20
60	2.0	—	0.00	0.43
60	—	2.0	0.43	2.77
		Boiled microsomes		
60	0.4	—	0.25	3.68
60	1.0	—	1.03	0.98
60	2.0	—	0.05	0.65

Conditions were similar to those in Fig. 1 except that ergosterol was substituted for 7-dehydrocholesterol and concentrations of Fe^{2+} or Fe^{3+} and the period of incubation were varied.

7-dehydrocholesterol in polar solvents, the effects of various concentrations of Triton and of deoxycholate on the oxidation of ergosterol were examined. Concentrations of these detergents between one-half and twice the amounts found to be optimal for the oxidation of 7-dehydrocholesterol did not increase the rate at which ergosterol was oxidized. Incubation of two other steroids possessing nuclear conjugated double bonds, cholesta-4,6-dien-3-one and cholesta-4,7-dien-3-one, with microsomes under conditions that resulted in the rapid oxidation of 7-dehydrocholesterol did not significantly alter the absorbancy due to these steroids.

Inhibitors of the Reaction

The enzymic reaction was inhibited by a number of chelating agents, by CN^-, α-tocopherol, menadione, phenazine methosulfate, and $K_3Fe(CN)_6$ as well as by a number of divalent cations (Table 6). None of the metal

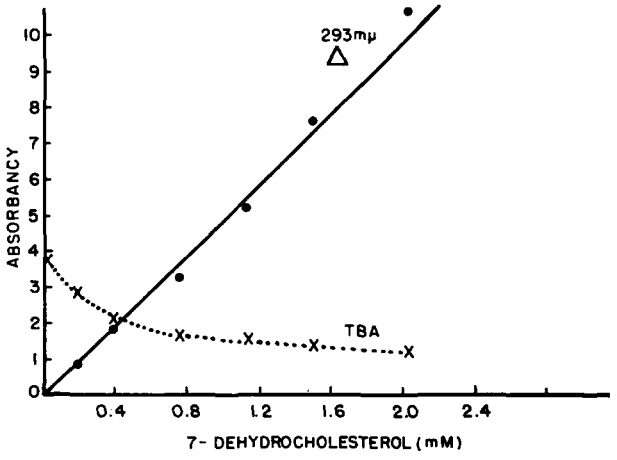

FIG. 6. Effect of varying the concentration of 7-dehydrocholesterol. Conditions were similar to those in Fig. 1 except that the concentration of 7-dehydrocholesterol was varied.

ions tested activated the reaction in the absence of iron. Without exception, agents that inhibited oxidation of the sterol also inhibited the production of TBA-reactive products. ADP increases NADPH-dependent lipid peroxidation under some conditions (3, 4), but in the present experiments addition of ADP (1 mM) in the presence or absence of iron salts did not alter the rate of the reaction. Incubation in an atmosphere containing 80% carbon monoxide did not alter the rate of the reaction, which indicates that a CO-sensitive cytochrome was not involved.

Inhibition by phenazine methosulfate and by $K_3Fe(CN)_6$ is of special interest since both of these compounds may act as electron acceptors for microsomal NADH oxidase. Measurements of oxidation of NADH were attempted under conditions similar to those required for pyridine nucleotide-dependent oxidation of 7-dehydrocholesterol. NADH (0.15 μmole) was incubated in a reaction mixture containing Triton (0.5%), nicotinamide (30 μmoles), Tris [tris(hydroxymethyl)-aminomethane]–maleate buffer pH 6.8 (60 μmoles), and microsomes equivalent to 60 mg of liver in a total volume of 1 ml in a 1 ml cuvette at room temperature (25°C). Difference spectra between 580 and 320 mμ were recorded with a Beckman DB spectrophotometer and measurements of NADH oxidation were made at 340 mμ against a blank containing all components except NADH. Addition of NADH to the reaction mixture resulted in a difference spectrum characteristic of cytochrome b_5 (11), with a prominent absorption band at 423 mμ and a minor band at 555 mμ, but NADH was not appreciably oxidized over a 30 min incubation period. Addition of 7-dehydrocholesterol, Fe^{2+} (2 μmoles) or Fe^{3+} (2 μmoles) caused no appreciable increase in the rate of NADH oxidation. However, in the presence of phenazine methosulfate (0.002 μmole) or $K_3Fe(CN)_6$ (1 μmole) oxidation of NADH was essentially complete within 5 min. The difference spectrum after oxidation of NADH was complete indicated the absence of any distinctive absorption bands.

TABLE 6 INHIBITORS OF 7-DEHYDROCHOLESTEROL OXIDATION

Inhibitor	Concentration	Inhibition	
		Fe^{3+}	Fe^{2+}
	mM	% of control	
Diethyldithiocarbamate	2.0	2.5	0.0
Tiron*	2.0	0.0	0.0
α-Tocopherol	2.0	8.5	5.5
α,α-Dipyridyl	1.0	0.0	7.0
NaCN	10.0	10.2	3.5
Menadione	0.2	31.2	23.8
Phenazine methosulfate	0.02	4.4	8.5
$Fe(CN)_6$	0.2	5.6	3.2
$HgCl_2$	2.0	5.0	22.2
$CoCl_2$	2.0	7.8	33.4
$CuCl_2$	2.0	26.0	34.0
$MnCl_2$	2.0	22.0	4.2
$MgCl_2$	2.0	100.0	100.0
$CaCl_2$	2.0	100.0	100.0

Conditions were similar to those in Fig. 1 except that concentrations of Fe^{2+} or Fe^{3+} were 0.2 mM or 0.4 mM, respectively, and inhibitors were added as indicated.

* Pyrocatechol disulfonate, K & K Laboratories, Inc., Plainview, N.Y.

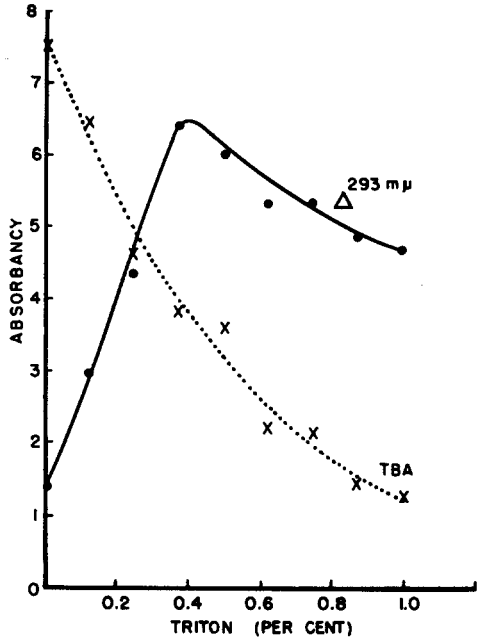

FIG. 7. Effect of Triton concentration. Conditions were similar to those in Fig. 1 except that the concentration of Triton X 100 varied.

Products of the Oxidation of 7-Dehydrocholesterol

The enzymatic conversion of 7-dehydrocholesterol to cholesterol in vitro is dependent upon NADPH and is independent of oxygen (5, 6). The rate is relatively slow and it is not significantly increased by the addition of Triton or deoxycholate (unpublished observations). The conditions used in the present experiments are, therefore, not favorable for extensive conversion of 7-dehydrocholesterol to cholesterol. The extent to which this reaction did occur under conditions similar to those shown in Fig. 1 was determined by measuring the incorporation of 7-dehydrocholesterol-4-^{14}C into cholesterol. After incubation of 7-dehydrocholesterol-4-^{14}C (24,000 cpm per flask) with the other components of the reaction mixture for 20 min, carrier cholesterol (25 mg) was added to the pooled contents of six flasks. The mixture was saponified and sterols were extracted and precipitated with digitonin as described previously (5). After regeneration of sterols from the digitonide, followed by bromination, regeneration of the sterol from the dibromide derivative, and crystallization from methanol to constant specific activity, 2.8% of the ^{14}C added as 7-dehydrocholesterol-4-^{14}C was recovered in cholesterol whereas spectro-

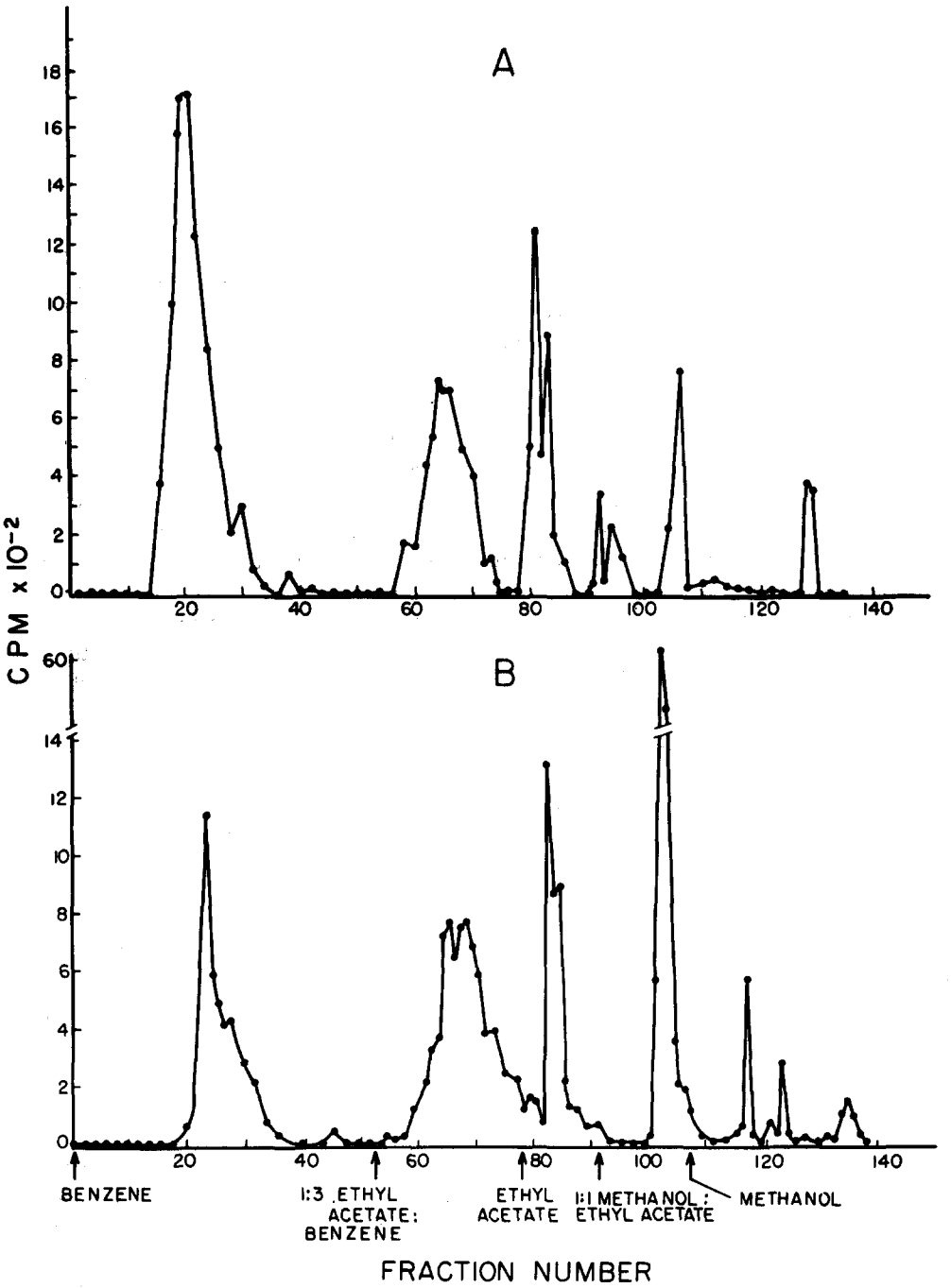

FIG. 8. Chromatography on alumina of products resulting from the oxidation of 7-dehydrocholesterol. In A, incubation conditions were similar to those in Fig. 1 except that 7-dehydrocholesterol-4-^{14}C (6000 cpm) was included in each flask. Pooled extracts from six flasks were chromatographed as described in the text. In B, conditions of incubation and chromatography were similar to those in A except that boiled microsomes were used; incubation was for 60 min and the pooled extracts from eight flasks were chromatographed.

photometric assays carried out concomitantly by the usual method indicated that 60% of the 7-dehydrocholesterol had been lost.

The results obtained by chromatographing the products resulting when 7-dehydrocholesterol-4-^{14}C was oxidized in the presence of unboiled microsomes and Fe^{3+} (enzyme-dependent reaction) and in the presence of boiled microsomes and Fe^{2+} (nonenzymatic reaction) are shown in Fig. 8. Recovery of ^{14}C from the reaction mixtures after incubation was approximately 80% and

recoveries of ^{14}C from the column were 80 and 78%, respectively. Comparison of the two chromatograms indicates a close similarity between the products produced by the enzymatic and nonenzymatic systems. 7-Dehydrocholesterol and cholesterol were eluted together in the first radioactive band. A chromatogram of an unincubated sample indicated the recovery of 82% of the ^{14}C as unchanged 7-dehydrocholesterol and 9% of the ^{14}C in the band eluted by methanol. After evaporation of the solvents from radioactive bands other than that containing 7-dehydrocholesterol, the residues were tan-colored oils that resisted crystallization, and further information regarding their identities was not obtained.

Since the 5,8-peroxide of 7-dehydrocholesterol has been isolated from mammalian liver (12), the production of 7-dehydrocholesterol 5,8-peroxide was investigated in an experiment similar to that shown in Fig. 1 but modified by the addition of 48 mg of 7-dehydrocholesterol peroxide to the pooled reaction mixtures after incubation was completed. The peroxide was eluted from the column with the second major radioactive band.

After evaporation to dryness and crystallization to constant specific activity (mp 152–154°C), the total counts in the added peroxide amounted to 11.7% of the counts in the band and 1.3% of the total counts recovered from the column in radioactive bands more polar than 7-dehydrocholesterol, which indicated that the peroxide was not a major product.

DISCUSSION

Interpretation of these results is hindered by a lack of understanding of the relationship of the enzymatic oxidation to autoxidation of the sterol in the nonenzymatic system. If the enzymatic reaction fills a metabolic role, the relationship of the reaction to any known or postulated metabolic pathways is not evident. In addition to the established role of 7-dehydrocholesterol as an immediate precursor of cholesterol, it has been suggested that 7-dehydrocholesterol may be converted to cholestanol by a pathway that excludes cholesterol (1). Any relationship between this proposed pathway and the present reaction appears to be excluded by my failure to detect, among the reaction products, cholestanol or the sterones that are intermediates in this pathway. In view of the relatively high polarity of the reaction products and the requirements of the enzymatic and nonenzymatic systems capable of oxidizing 7-dehydrocholesterol, the occurrence of a hydroxylation reaction may be suspected. Hydroxylation of cholesterol appears to be an initial step in its conversion to bile acids (13, 14) and it is possible that the substrate specificities of enzymes catalyzing these or other steroid hydroxylation reactions are broad enough to allow acceptance of 7-dehydrocholesterol as a substrate. The existence of hydroxylases with low substrate specificities may also be an explanation for the conversion in vitro of steroid hormones and cholesterol to hydroxylated or unidentified derivatives of unknown metabolic significance (13–17).

An alternative explanation for the oxidation of 7-dehydrocholesterol in vitro, that excludes a metabolic role for the reaction, is that oxidation of the steroid is caused indirectly by reduced pyridine nucleotide-linked enzyme systems that also cause peroxidation of microsomal lipids. NADH- and NADPH-dependent peroxidation of microsomal lipids has been described in several recent reports (2–4) and the probable nature of these reactions has been discussed (3, 4, 18). In the present study, peroxidation of microsomal lipids occurred under all conditions that resulted in oxidation of 7-dehydrocholesterol. Metal ions, electron acceptors, and antioxidants had similar effects on both reactions. The dissimilar activating effects of Fe^{3+} and Fe^{2+} at various concentrations and under enzymatic and nonenzymatic conditions are generally consistent with a suggestion that the role of the enzyme system involved in peroxidation of microsomal lipids is to transport electrons from NADPH to Fe^{3+}, giving Fe^{2+} which initiates the peroxidation reaction (4). Inhibition of 7-dehydrocholesterol oxidation and of peroxidation of microsomal lipids by phenazine methosulfate and $K_3Fe(CN)_6$ observed in the present studies indicates that maintenance of a pyridine nucleotide oxidase system in a reduced state may be required by both reactions. Inhibition of microsomal lipid peroxidation and acceleration of sterol oxidation with increasing sterol concentrations is not inconsistent with a single mechanism for both reactions if 7-dehydrocholesterol is oxidized preferentially to microsomal lipids. On the other hand, the contrasting effects of detergents upon the two reactions are unexplained and may argue against a common mechanism.

Whereas the nature of the observed enzymatic oxidation of 7-dehydrocholesterol is not yet clear, the results obtained illustrate the susceptibility of this sterol to autoxidation under conditions that may occur in tissues—in particular, when tissues are deficient in antioxidants or are exposed to conditions that result in the formation of free radicals.

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References

1. Kandutsch, A. A. *J. Lipid Res.* **4:** 179, 1963.

2. Thiele, E. H., and J. W. Huff. *Arch. Biochem. Biophys.* **104:** 468, 1964.
3. Hochstein, P., K. Nordenbrand, and L. Ernster. *Biochem. Biophys. Res. Commun.* **14:** 323, 1964.
4. Beloff-Chain, A., G. Serlupi-Crescenzi, R. Catanzaro, D. Venettacci, and M. Balliano. *Biochim. Biophys. Acta* **97:** 416, 1965.
5. Kandutsch, A. A. *J. Biol. Chem.* **237:** 358, 1962.
6. Dempsey, M. E., J. D. Seaton, G. J. Schroepfer, Jr., and R. W. Trockman. *J. Biol. Chem.* **239:** 1381, 1964.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. *J. Biol. Chem.* **193:** 265, 1951.
8. Ottolenghi, A. *Arch. Biochem. Biophys.* **79:** 355, 1959.
9. Schneider, P. B., R. B. Clayton, and K. Bloch. *J. Biol. Chem.* **224:** 175, 1957.
10. Engel, F. L., M. F. Ball, and W. G. Blackard. *J. Lipid Res.* **6:** 21, 1965.
11. Modirzadeh, J., and H. Kamin. *Biochim. Biophys. Acta* **99:** 205, 1965.
12. Dvornik, D., M. Kraml, J. Dubuc, M. Givner, and R. Gaudry. *J. Am. Chem. Soc.* **85:** 3309, 1963.
13. Danielsson, H. *Acta Chem. Scand.* **14:** 846, 1960; **18:** 831, 1964.
14. Mendelsohn, D., and E. Staple. *Biochemistry* **2:** 577, 1963.
15. Lazier, C., and P. H. Jellinck. *Can. J. Biochem.* **43:** 281, 1965.
16. Kuntzman, R., M. Jacobson, K. Schneidman, and A. H. Conney. *J. Pharmacol. Exptl. Therap.* **146:** 280, 1964.
17. Chamberlain, J., N. Jagarinec, and P. Ofner. *Steroids* **Suppl. 2:** 1, 1965.
18. Orrenius, S. *J. Cell Biol.* **26:** 713, 1965.