

Characterization of the microsomal steroid-8-ene isomerase of cholesterol biosynthesis

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Abstract Rat liver microsomes contain an enzyme that catalyzes the isomerization of the nuclear double bond of steroids from the 8(9) position to the 7(8) position. The enzyme is most active with zymosterol, 5 α -cholesta-8,24-dien-3 β -ol, which is a precursor of cholesterol. Properties of the microsomal isomerase have now been studied, and preliminary data are reported on both regulation of enzymic activity and first steps in the solubilization of the enzyme from membranes. After a brief lag period, the velocity of isomerase is relatively constant for about 5 min of incubation, and then isomerization subsides. The apparent Michaelis constant (52–70 μ M) is difficult to determine accurately, due to these complex kinetic changes. V_{max} is 4.0–4.7 nmol/min per mg of microsomal protein. The apparent specific activity is more than ten times that of liver microsomal methyl sterol oxidase. The maximal specific activity of microsomal isomerase is approximately doubled when rats are fed an intestinal bile acid sequestrant, cholestyramine. Changes in specific activity of isomerase parallel changes in activities of other microsomal enzymes of cholesterol biosynthesis, such as 3-hydroxy-3-methylglutaryl-CoA reductase and 4-methyl sterol oxidase. Isomerase activity is destroyed by phospholipase A digestion, high concentration of bile salts, and solvent extraction, all of which are known either to remove phospholipid or to alter microsomal membrane integrity. On the other hand, isomerase remains active in the presence of a mild, nonionic detergent, Triton WR-1339; thus, solubilization with nonionic detergents is under study.

Supplementary key words microsomes · steroid isomerase · cholestyramine

In the biological synthesis of cholesterol from lanosterol, a series of approximately 20 reactions is catalyzed by particulate-bound enzymes that may be isolated attached to microsomal membranes. The types of reactions catalyzed by these microsomal enzymes can be grouped within three categories: reduction of isoocentenyl side chain; removal of three "extra" methyl groups in positions 4 α , 4 β , and 14 α ; and transition of the Δ^8 bond to the 5C-position (1, 2). Extensive work in several laboratories has led to identification of steroidal intermediates and postulation of a tentative sequence of reactions, both

within and between each of the three types of processes. In addition, the enzymology of the process has been studied more recently, and several workers are attempting to study discrete enzymic steps (3). For each enzyme of the multienzymic system, generally the presence of the enzyme is established first, the properties of the microsomally bound enzyme are studied, the properties of solubilized and purified enzyme are compared to the membrane-bound form, and then the enzymes are reassembled, or reconstituted, with phospholipid, noncatalytic proteins (when needed), and other supporting enzymes of multienzymic systems, e.g., the enzymes of microsomal electron transport that are needed for mixed-function oxidations of intermediates (3).

Several years ago, we confirmed the presence of a microsomal enzyme that catalyzes the isomerization of the double bond of zymosterol (5 α -cholesta-8,24-dien-3 β -ol) and other Δ^8 -steroids to the Δ^7 -position (4). Although the nature of the reaction catalyzed has been studied somewhat, e.g., the demonstrated loss of the 7 β -hydrogen (5) during $\Delta^8 \rightarrow \Delta^7$ -isomerization, the properties of the enzyme have not been characterized sufficiently to either proceed with attempted solubilization or compare properties of solubilized and partially purified enzyme with those exhibited by the microsomally bound enzyme. We have now obtained rat liver microsomes that contain an isomerase of high specific activity. Accordingly, we carried out an extensive study of the properties of the membrane-bound enzyme. In addition, results from initial attempts to solubilize the enzyme, as well as preliminary information on

Abbreviations: Δ^8 , Δ^7 , steroids with double bonds in the 8(9) or 7(8) positions, respectively; zymosterol, 5 α -cholesta-8,24-dien-3 β -ol, would be a Δ^8 -steroid; K'_m , apparent Michaelis constant.

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regulation of the rate of isomerization, are reported below.

MATERIALS AND METHODS

Isolation of zymosterol

Initially, zymosterol was purchased from a commercial source (Steraloids, Inc., Wilton, NH), but that proved too costly and, upon storage, partially decomposed samples could not be avoided. Accordingly, zymosterol was isolated in good yield from baker's yeast by a modified method that does not require removing ergosterol as the maleic anhydride adduct on a large scale (4). Moist yeast was exhaustively extracted with hot $\text{CH}_3\text{OH}-\text{CHCl}_3$ 1:1 for 3 hr; a large fraction of the ergosterol was extracted into the solvent, and most of the zymosterol remained in the extracted yeast. The dry yeast residue was suspended in methanol and saponified with 15% KOH (w/v) for 3 hr under reflux. The crude hydrolysate was filtered through glass wool while still hot. The solution was cooled, water was added, and the crude, nonsaponifiable fraction was extracted with diethyl ether. The ether solution was washed with water, desiccated over anhydrous sodium sulfate, and evaporated. The resulting residue was easily crystallized with petroleum ether (bp 30–60°C) in which contaminating ergosterol was only slightly soluble. The resulting mother liquor was reduced to dryness, and portions of the residue were chromatographed on a silica gel preparative plate (500 nm) to which 5% AgNO_3 had been added; the developing solvent was benzene–ethyl acetate 10:3.5. The gel containing zymosterol was removed from the plate, and the eluted sterol was crystallized from methanol (mp 107–109°C; reported, 108–109°C (6)). The crystalline product was free of significant absorbance of ultraviolet light at 280 nm due to ergosterol, and the steroid exhibited the gas–liquid chromatographic and mass spectral properties of authentic zymosterol (see Reference 4 and below).

Enzyme preparation

Livers from adult male rats (200–300 g) were minced after perfusion *in situ* with 0.25 M sucrose; the suspension, in two volumes of 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM glutathione and 10 mM nicotinamide), was homogenized in a tightly fitting glass homogenizer. The resulting suspension was centrifuged at 500 g for 10 min, and the precipitate was discarded. The resulting supernatant fraction was centrifuged at 9,000

for 20 min, and the supernatant fraction was decanted and filtered through glass wool. The supernatant fraction was centrifuged at 105,000 g for 90 min. The resulting precipitate, the microsomal pellet, was collected and suspended with fresh buffer to a concentration of about 20 mg of microsomal protein/ml. Microsomal pellets, when frozen rapidly, could be stored at -20°C for several weeks without significant loss of isomerase activity.

Assay of isomerase

For all experiments, zymosterol (200 nmol) was suspended in 0.1 ml of 0.1 M potassium phosphate buffer (see above) with the aid of 5.8 mg of Triton WR-1339. The substrate suspension was added to a mixture of 5 mg of microsomal protein, 60 mg of glucose, and 15 units of glucose oxidase (to remove dissolved oxygen), which had been preincubated for 3 min at 37°C , in a final volume of 2.0 ml. Unless otherwise specified, all buffers used for incubation had been equilibrated with nitrogen, and nitrogen was exchanged for air in all flasks. Incubation with substrate was carried out anaerobically for 10 min at 37°C unless otherwise indicated. After incubation, the samples were saponified by the addition of 0.5 g of KOH in 2.0 ml of ethanol by heating under reflux for 15 min. Sterol was extracted three times with 8 ml of pentane. The extract was washed with water, dried over sodium sulfate, and evaporated to dryness under a stream of nitrogen gas.

The extent of isomerization of zymosterol to 5α -cholesta-7,24-dien- 3β -ol was measured by gas–liquid chromatography as modified from a procedure that was described earlier (4). Briefly, the steroid-containing residue, which could not be stored without decomposition, was dissolved in 0.1 ml of ethyl acetate, and 4 μl of the ethyl acetate solution was injected onto a gas–liquid chromatographic column (0.4×122 cm) of 1% SE-30 (7) that was maintained at 230°C . Zymosterol and $\Delta^{7,24}$ -cholestadien- 3β -ol were well resolved from microsomal cholesterol, which was minimized in this study by reducing the microsomal protein concentration from 20 mg/ml used earlier (4) to 2.5 mg/ml in this study. Minor contaminants of zymosterol were not present in the pure substrate (see chromatographic peaks labeled A and B in Reference 4). Thus, with several recorder attenuation changes after elution of cholesterol from the column, essentially full-scale recorder responses could be used for accuracy and precision. The mass of each isomer was estimated manually by three different methods and shown to be reproducible within a few percent on replicated samples.

Protein was determined by the method of Lowry et al. (8). All chemicals were of the highest grade available commercially. Triton WR-1339 was obtained from Ruger Chemical Co., Hillside, NJ. Cholestyramine, a nonabsorbable bile salt sequestrant, was obtained from Mead Johnson Co., Evansville, IN. Phospholipase A and cofactors were purchased from Sigma Chemical Co., St. Louis, MO.

RESULTS

Determination of optimal assay conditions

As shown earlier (4), isomerization occurred under anaerobic conditions. Furthermore, addition of either NAD^+ or NADP^+ did not affect the apparent rate of isomerization. On the other hand, addition of NADPH led to net loss of sterols that possessed the isooctenyl side chain (e.g., zymosterol); apparently, NADPH -dependent reduction occurred concomitantly with isomerization (7). Thus, for assay of isomerase activity, no pyridine nucleotide cofactors were added. Addition of as much as 30 mM Ca^{2+} or Mg^{2+} was without effect. Inhibition by EDTA was observed only when incubations were carried out with >5 mM EDTA.

Incubation of microsomes in phosphate buffer adjusted to pH 5.5–9.0 was examined. The maximal rate of isomerization was observed at pH 7.4 (Fig. 1).

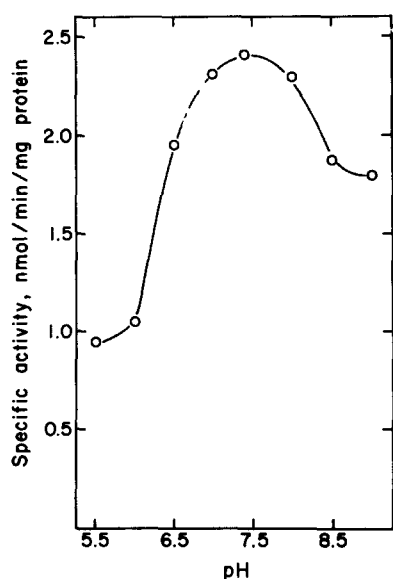


Fig. 1. Effect of pH. Each incubation flask contained 200 nmol of substrate, 5 mg of protein, 60 mg of glucose, and 15 units of glucose oxidase in a total volume of 2 ml of 0.1 M potassium phosphate buffer of each pH. Incubation was for 10 min at 37°C, anaerobically. Values are means of samples from at least three separate experiments.

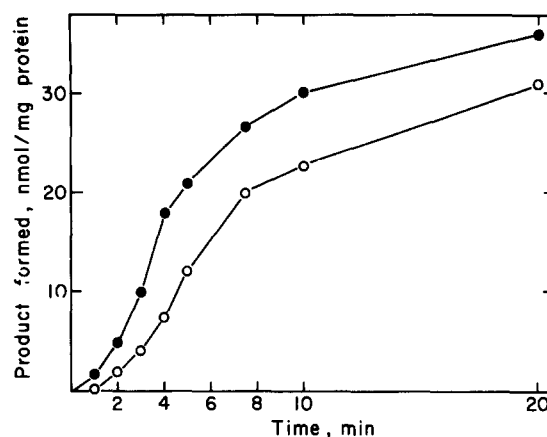


Fig. 2. Time course of $\Delta^8 \rightarrow \Delta^7$ -steroid isomerization. Samples were incubated as described in Fig. 1. Cholestyramine microsomes (●); control microsomes (○).

A markedly slower rate was observed when the medium was somewhat more acidic. At a more alkaline pH, the loss of isomerase activity was less pronounced. At pH 7.4, substitution of either Tris-HCl or Tris-acetate buffer decreased activity by about 50%.

After a lag phase of about 2 min, increasing lengths of incubation yielded increasing amounts of the $\Delta^{7,24}$ -isomer (Fig. 2). The extent of the lag was minimized by a short anaerobic preincubation of flask contents without substrate at 37°C. After about 20 min of incubation, the rate of isomerization was negligible. By 20 min, the calculated extent of isomerization had reached approximately 75%. Although the rate was considerably slower after approximately 5 min of incubation, a 10-min interval was chosen as a compromise to minimize the 1–2 min lag period and to maximize the accuracy of the determination.

Incubation of increasing amounts of microsomal protein yielded greater extents of isomerization during 10-min incubations (Fig. 3). The response upon varying the amount of microsomes was approximately linear from 0.5 to about 5 mg of protein. Addition of greater amounts of protein probably yielded diminished increments of isomerization as substrate became limiting. Subsequent experiments were limited to no more than 5 mg of microsomal protein.

Under these conditions established for optimal activity measured with 200 nmol of substrate, from 40 to 400 nmol of zymosterol were incubated for 10 min with 2 mg of microsomal protein. The isomerase exhibited substrate saturation, and nearly maximal extents of conversion were observed at 0.2 mM substrate (Fig. 4). Conventional double reciprocal expression of measured velocity and substrate concentra-

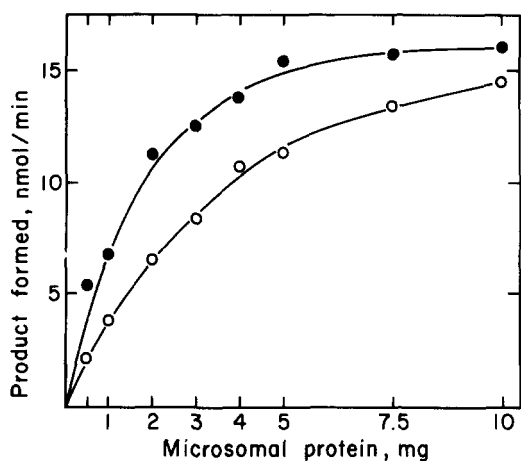


Fig. 3. Effect of microsomal concentration. Incubation was for 10 min as described in Fig. 1. Cholestyramine microsomes (●); control microsomes (○).

tion was linear, and the average maximal velocity from four separate assays was 4.7 ± 0.2 nmol/mg protein per min (for a 10-min incubation). The calculated value for K'_m was $52 \pm 3 \mu\text{M}$ under these conditions.

Finally, a series of experiments was carried out with a large number of samples in which the same microsomal preparation was incubated, both for various times up to 5 min and with various concentrations of substrate up to 0.2 mM (Fig. 5). At the relatively low substrate concentrations, the time course of isomerization was constant. With only 4 min of incubation, the calculated value of K'_m was somewhat larger ($70 \mu\text{M}$), although V_{max} was approxi-

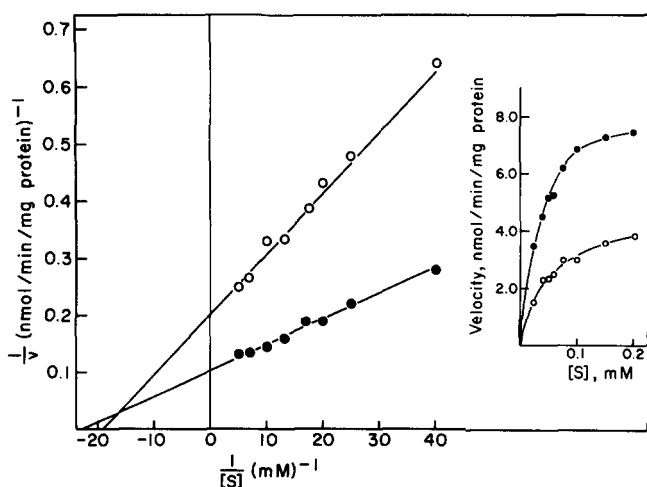


Fig. 4. Effect of variable substrate concentrations. Each incubation flask contained the indicated amount of zymosterol; incubations were as described in Fig. 1. Cholestyramine microsomes (●); control microsomes (○). Both hyperbolic saturation curves and double reciprocal graphs are shown for representative data from an average of three experiments.

mately the same, 4 nmol/min per mg protein. After the initial lag of about 1 min, addition of greater amounts of substrate up to 0.1 mM yielded proportional increases in the extent of isomerization. However, with 0.15 and, especially, with 0.2 mM zymosterol, evidence of substrate inhibition was suggested. Since increases in substrate concentration also necessitate increases in detergent concentration, part of the apparent substrate inhibition may be ascribed to a less specific detergent effect (see below). Additional experimentation on the lag and mechanism should await the more precise investigation that can be carried out with solubilized enzyme.

Sensitivity of isomerase to lipid changes

Microsomal isomerase was assayed with various concentrations of both free and taurine-conjugated bile salts. At concentrations less than 1 mM, no inhibition of isomerase activity was observed (Fig. 6). However, when the concentrations of bile salts were elevated above 1 mM, pronounced inhibition was observed with chenodeoxycholic acid and cholic acid. The extent of inhibition was much greater with the taurine conjugates. Indeed, in the conjugated form, even deoxycholate was inhibitory (Fig. 6).

In the previous experiment with the higher concentrations of bile salts, a time-dependent clarification of turbidity ascribed to the particulate nature of microsomes could be observed when substrate and inhibitor were incubated simultaneously. Thus, microsomes were exposed to 5 mM taurodeoxycholate for various intervals prior to the addition of sub-

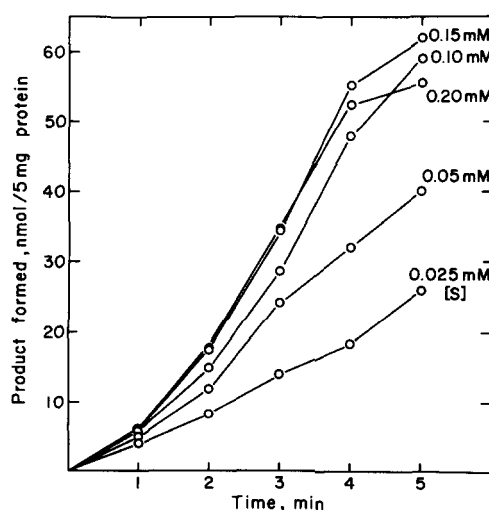


Fig. 5. Relationship between substrate concentration and length of incubation. Each incubation flask contained the indicated concentration of substrate plus 5 mg of protein, and incubation was for the designated period. Results are the means of duplicate values from two separate experiments.

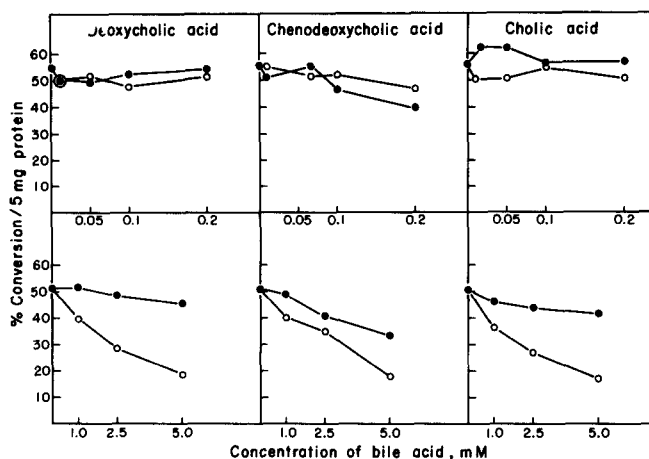


Fig. 6. Effect of various bile acids. Each incubation flask contained 200 nmol of substrate, 60 mg of glucose, 15 units of glucose oxidase, 5 mg of control microsomal protein, and the designated concentration of bile acids in a total volume of 2.0 ml of 0.1 M potassium phosphate buffer (pH 7.4). Incubation was for 10 min at 37°C. Taurine-conjugated bile acid (○); free bile salt (sodium) (●).

strate and for two intervals of incubation after addition of substrate. There was a marked time dependence in loss of activity (Table 1). Under all experimental conditions studied, greater durations of preincubation yielded diminishing rates of isomerization after an initial loss of >50% of isomerase activity (Table 1). When the lengths of preincubation without substrate were studied for two intervals of incubation with substrate, 5 and 10 min, the time-dependent loss of activity was comparable.

Since loss of activity occurred with high concentrations of bile salts that destroy membranes, the effect of membrane destruction produced alternatively by partial digestion of microsomal membranes with phospholipase A (9) was investigated. With as little as 1–2 μ g of phospholipase A incubated with 50 mg of microsomal protein, loss of 25% of activity was observed after 10 min, and activity was completely lost by 20 min. Activity could not be restored by the addition of phosphatidylcholine (0.5 mg/mg protein) in which the lipid was supplied either in methanol solution or as liposomes.

Delipidation of microsomes by solvent extraction according to either Jones and Wakil (10) or Cham and Knowles (11) led to similar losses of isomerase activity, and activity could not be restored by the addition of either phosphatidylcholine or phosphatidylethanolamine to the extracted microsomes. It is possible that loss of activity could have been associated with extraction of an essential protein; however, digestion of rat liver microsomes at 5°C for 2 hr with Subtilisin VII (10 μ g/mg microsomal protein)

removed >50% of the total microsomal protein. Several enzymes were lost completely (12), but the total isomerase activity remained unchanged. Thus, the specific activity repeatedly doubled as a result of Subtilisin treatment.

Stimulation of isomerase activity

Several hepatic microsomal enzymes of cholesterol biosynthesis are stimulated two- to sixfold by dietary cholestyramine. Notably, in this laboratory, we have demonstrated that two rate-determining enzymes are markedly stimulated, methyl sterol oxidase and 3-hydroxy-3-methylglutaryl-CoA reductase (13, 14).

Cholestyramine (3%, w/w), fed for 7–10 days, significantly enhanced the rate of microsomal conversion of zymosterol to cholesta-7,24-dien-3 β -ol (Figs. 2 and 3). The lag observed with control microsomes was less with microsomes from cholestyramine-treated animals. The maximal velocity of isomerization was approximately doubled to 9.6 ± 0.2 nmol/min per mg protein (Fig. 4). The corresponding value of K'_m could not be calculated under identical conditions due to the rapid velocity; however, K'_m was about the same as for control samples.

A noncatalytic protein of rat liver cytosol stimulates microsomal 4-methyl sterol oxidase activity of cholesterol biosynthesis (15). The effect of both crude and purified cytosolic protein on the $\Delta^8 \rightarrow \Delta^7$ -steroid isomerase was investigated. Crude cytosolic fraction from preparations of control and cholestyramine microsomes was added in amounts from 2 to 15 mg of protein/2 mg of microsomal protein. No effect of addition of cytosol was observed. Similarly, when the noncatalytic protein was purified (see Reference 15), no change in isomerase activity was observed. However, although the isomerase incubated under these

TABLE 1. Effect of various lengths of exposure to taurodeoxycholate on isomerase activity^a

Taurodeoxycholate Added	Length of Preincubation	Specific Activity of Isomerase			
		5-Min Incubation	10-Min Incubation	%	%
	min	nmol/mg protein			
0	0	15.3	23.4	100	100
+	0	6.7	10.2	43	44
+	1.0	4.1	7.8	27	33
+	2.5	2.8	5.0	18	21
+	5.0	1.7	3.0	11	13

^a Each flask contained 5 mg of microsomal protein, 60 mg of glucose, 15 units of glucose oxidase, and 5 mM taurodeoxycholate (when added) in a final volume of 2.0 ml. Samples were preincubated at 37°C for the indicated lengths of time, 200 nmol of substrate were added, and the incubation was continued at 37°C for either 5 or 10 min as shown.

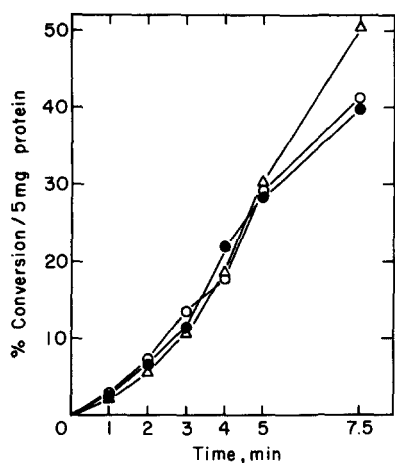


Fig. 7. Time course of isomerase activity in microsomes pre-treated with Triton WR-1339. Each incubation flask contained 200 nmol of substrate, 5 mg of microsomal protein, 60 mg of glucose, and 15 units of glucose oxidase. Control samples were incubated for the designated period without Triton (Δ). Similar samples were preincubated for 10 min at 37°C in the same medium, then substrate (200 nmol) was added (\circ). An identical incubation was carried out, except the preincubation flask contained 5.8 mg of Triton in addition (\bullet).

conditions was apparently unresponsive to changes produced by the noncatalytic protein, methyl sterol oxidase also did not respond when similarly high concentrations of detergent were used for substrate suspension (15). Thus, the effects of the cytosolic protein on isomerase activity will have to be studied later with solubilized enzyme reconstituted into the membrane-bound state and incubated with small amounts of substrate (and detergent).

Detergent stability of microsomal isomerase

Several microsomal proteins of cholesterol biosynthesis and microsomal electron transport have been solubilized by treatment of microsomes with bile salts and other detergents (3). However, with the relatively marked diminution of activity produced by bile salts (Fig. 6, Table 1), mild nonionic detergents have been examined for possible use in solubilization of the isomerase.

The isomerase was quite active in the presence of Triton WR-1339, since zymosterol had been suspended with the aid of Triton at a final incubation concentration of 2.9 mg/ml. Control microsomes (80 mg) were suspended in solutions that contained 1.3–10% Triton WR-1339 (v/v). The microsomes were collected by centrifugation and assayed for isomerase activity. The decrease in specific activity was less than 20%, even after treatment with as much as a 10% solution of Triton WR-1339.

Preincubation of control microsomes with 0.3% Triton WR-1339 and then continued incubation upon

addition of substrate and another 0.3% Triton did not reduce the specific activity of isomerase (Fig. 7). Clarification of microsomal turbidity was observed at these detergent concentrations at 37°C. Thus, although clarification and loss of microsomal integrity occurred, as it did with bile acids and phospholipase A treatments, the enzyme remained active, and Triton WR-1339 or another nonionic detergent may be useful in further experiments for solubilization of the $\Delta^8 \rightarrow \Delta^7$ -steroid isomerase.

DISCUSSION

Some of the enzymic properties of the $\Delta^8 \rightarrow \Delta^7$ -steroid isomerase and the conditions needed for determination of approximate values of K'_m and V_{max} have now been established. Either phospholipid or a degree of membrane integrity may be needed for isomerase activity. Neither cofactor nor metal ion requirements could be established. Oxygen is not required. Neutral pH is essential. In addition to these observations, this report contains data that suggest that the isomerase activity may be regulated in concert with other microsomal enzymes of cholesterol biosynthesis, such as 3-hydroxy-3-methylglutaryl-CoA reductase and 4-methyl sterol oxidase (14), which also respond to stimulation by dietary cholestyramine. Finally, preliminary data suggest that the isomerase may be solubilized by treatment with the mild nonionic detergents, such as Triton WR-1339.

Some properties of the enzyme, although unexplained, are particularly noteworthy. Although administration of cholestyramine in vivo resulted in a diminution of the lag of product formation (Fig. 2), a high degree of reproducible sigmoid character was apparent in the time course plot. Although the discrepancy from linearity was not too severe to prevent analysis of a first approximation of substrate saturation data (Fig. 4), the lag was always observed and not reconciled by simple relationships with product formation (Fig. 5). Wilton, Rahimtula, and Akhtar (16) have suggested that the $\Delta^8 \rightarrow \Delta^7$ -steroid isomerization may occur via a reversible two-step mechanism in which initial attack would be 9α -protonation, thus generating a C-8 carbonium ion prior to proton elimination from C-7. If the protonation reaction is relatively faster than proton elimination, a pronounced lag in product formation might be expected. It must be pointed out, though, that severe experimental limitations, in addition to complex kinetic results, limit interpretation of the basic kinetic data on this point. For example, there may be at least three phases to the apparent

time course (Fig. 2). Substrate concentration relative to protein content was necessarily held low to enhance accuracy of the gas chromatographic assay (see Materials and Methods). However, at these concentrations, most incubations had to be carried out within about twofold concentrations calculated for K'_m . Also, for maximal amounts of isomerization, a compromise of incubation length was used (10 min, Fig. 3).

The isomerase is relatively active compared to other enzymes and multi-enzyme systems of cholesterol biosynthesis from lanosterol. For example, under similar conditions of incubation, activity observed for 4-methyl sterol oxidase of liver microsomes is about 0.07 nmol/min per mg protein (17). Since 4-demethylation appears to precede isomerization (4), the rate of isomerization would be sufficiently fast to account for essentially complete formation of the Δ^7 -isomer during the course of the other nuclear transformations that occur in the conversion of lanosterol into cholesterol.

Destruction of the membrane integrity, as well as the inhibitory effects of phospholipid extraction, suggest that the isomerase may require a phospholipid for activity. No phospholipid-dependent microsomal enzyme of cholesterol formation from lanosterol has been found in our studies (3). Although the Triton treatment also destroyed the vesicles and isomerase remained active (Fig. 7), others have shown that certain nonionic detergents may be substituted for essential phospholipids in mixed-function oxidases reconstituted from liver microsomes (18). Thus, nonionic detergent substitution may have accounted for stability that was lost when ionic detergents, such as bile salts, were studied (Fig. 6, Table 1).

Since enterohepatic circulation of bile acid occurs extensively in the rat (19, 20), and 20–30 mg/kg of bile acid is secreted daily by rats (21) with about 5% of the total bile acid distributed to liver (22), significant inhibition of isomerase by bile acids may occur in vivo. Cholestyramine treatment in vivo decreases concentrations of cholesterol esters in microsomes. Although an inverse relationship between microsomal cholesterol ester concentrations and activities of other microsomal enzymes of cholesterol biosynthesis (14, 23, 24) has been demonstrated, further work on the nature of regulation of isomerase activity is needed. ■■

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