Microsomal Enzymes of Cholesterol Biosynthesis from Lanosterol

PURIFICATION AND CHARACTERIZATION OF Δ7-STEROL 5-DESATURASE OF RAT LIVER MITOCHONDRIA

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Microsomal Δ7-sterol 5-desaturase of cholesterol biosynthesis is a multi-enzyme system which catalyzes the introduction of the Δ5-bond into Δ7-cholesterol to form 7-dehydrocholesterol. The detergent-solubilized 5-desaturase has been purified more than 70-fold and resolved from electron carriers and other rat liver microsomal enzymes of sterol biosynthesis by chromatography on DEAE-Sephacel, CM-Sepharose, and immobilized cytochrome b5; the 5-desaturase had not been fully resolved from cytochrome b5 reductase in earlier work. A functional electron transport system for the 5-desaturase has been reconstituted by combining the purified 5-desaturase and electron carriers with egg phosphatidylycholine liposomes. Optimization of conditions for reconstitution have been obtained; both cytochrome b5 and NADH-cytochrome b5 reductase serve as electron carriers. A pyridine nucleotide-dependent flavoprotein is required and the requirement can be satisfied with either purified cytochrome b5 reductase or cytochrome P-450 reductase. Cyanide and iron-chelators strikingly inhibit the 5-desaturase activity, thus suggesting that 5-desaturase is a metalloenzyme as are other well-characterized cytochrome b5-dependent oxidases.

5-Desaturase is resolved from 4-methyl sterol oxidase activity of cholesterol biosynthesis by chromatography on the immobilized cytochrome b5. This resolution of the two oxidases not only indicates that introduction of the Δ5-bond and oxidation of 4a-methyl groups are catalyzed by different terminal oxidases, but resolution affords enzymes of sufficient purity to carry out reconstitution experiments.

A novel assay based on substrate-dependent increments of oxidation of α-NADH has been developed for measurement of 5-desaturase activity. Measurement of stoichiometry of 5-desaturase demonstrates that for each equivalent of cis-desaturation of Δ7-cholesterol, 1 eq of NADH is consumed. Along with strict dependence upon oxygen, this observation confirms, as suggested by previous workers, that the 5-desaturation is catalyzed by a mixed function oxidase rather than a dehydrogenase.

The biotransformation of lanosterol to cholesterol proceeds in a multiple process involving reduction of the Δ5-bond in the side chain, removal of three methyl groups from C-4 and C-14, and movement of the Δ5-bond of lanosterol to the Δ5-bond location of cholesterol. Δ7-Sterol 5-desaturase of the latter process in a multi-enzyme system which catalyzes oxidative desaturation of Δ7-cholesterol to 7-dehydrocholesterol in the migration of the B-ring double bond (1), see Fig. 1. 4α-Methyl sterol oxidase is a multi-enzyme system which catalyzes oxidation of the 4α-methyl group to a steroid 4α-oxo acid in the process of the demethylation (2-4). Δ5-Sterol 5-desaturase and 4-methyl sterol oxidase have very similar properties: both enzymes require molecular oxygen (1, 5) and reduced pyridine nucleotide (5, 6) for activity; each is inhibited by the same concentration of cyanide, but neither is sensitive to inhibition by carbon monoxide (7, 8); cytochrome b5 participates as an electron carrier in the reactions catalyzed by both the 5-desaturase and methyl sterol oxidase systems (5-11).

Recently, both 5-desaturase and methyl sterol oxidase have been solubilized from rat liver microsomal membranes, resolved from cytochrome b5 as an electron carrier, and reconstituted by Grinstead and Gaylor (12) and Fukushima et al. (13), respectively. These studies demonstrated that cytochrome b5 is an electron carrier for 5-desaturase and methyl sterol oxidase. These and other properties of the enzymes such as membrane location, sensitivity to inhibitor, etc., indicate that 5-desaturase and methyl sterol oxidase are analogous to other cytochrome b5-dependent oxidases such as the fatty acyl coenzyme A desaturases (14-17), phospholipid desaturase (18), and a terminal oxidase in the microsomal fatty acid elongation system (19).

Although readily solubilized with detergents and resolved from electron carriers, neither 5-desaturase nor methyl sterol oxidase has been purified. Moreover, in the previous studies (12, 13), no resolution of 5-desaturase from methyl sterol oxidase could be observed even after three different chromatographic treatments. For further characterization of 5-desaturase, reconstitution of the enzymes of cholesterol synthesis, and identification of each discrete oxidase, isolation of both enzymes is required. Dependence upon cytochrome b5 for 5-desaturase led us to use cytochrome b5 affinity gel chromatography (20) for purification of the 5-desaturase. With this procedure, 5-desaturase and methyl sterol oxidase have been adsorbed onto columns of immobilized cytochrome b5, and fully resolved from each other with high levels of enrichment (70-fold over microsomes). Some properties of the resolved and purified 5-desaturase obtained from the column of im-

1 The abbreviations and trivial names used are: lanosterol, 4, 4, 4α-trimethyl-5α-cholesta-8,24-dien-3β-ol; Δ7-cholesterol, 5α-cholesta-7-en-3α-ol; 7-dehydrocholesterol, 5α-cholesta-5,7-dien-3α-ol; GSH, glutathione; HPLC, high pressure liquid chromatography; PEG, polyethylene glycol.
mobilized cytochrome b5 are now described in this report.

Several lines of evidence suggest that Δ7-stearol 5-desaturase is an oxidative desaturase (1, 6) that consumes both oxygen and reduced pyridine nucleotide. Stoichiometry of Δ7-desaturation of Δ7-cholesterol requires 1 eq of reduced pyridine nucleotide, but stoichiometry of micromolar-bound enzyme cannot be studied because the rate of 5-desaturation by rat liver microsomes is quite slow relative to basal rates of oxidation of NADH. For example, the average rate of desaturation reaction is approximately 0.13 nmol/min/mg of protein at 37 °C (12) whereas a basal rate of aerobic oxidation of β-NADH is in excess of 10-fold of that of the desaturation reaction (21). Miyake and Gaylor (21) demonstrated that the basal rate of oxidation of α-NADH epimer by rat liver microsomes is only about 10% of the rate that is observed with β-NADH and, furthermore, α-NADH fully supports cytochrome-b5-dependent methyl sterol oxidase. A novel spectrophotometric assay based on substrate-dependent oxidation of α-NADH has been developed for determination of 5-desaturase activity as previously described for measuring the rate of micromolar methyl sterol oxidase (22). In this report, stoichiometry of cis-elimination (6, 23, 24) and reduced pyridine nucleotide consumption of 5-desaturation is measured by concurrent assays of 7-dehydrocholesterol formation and oxidation of α-NADH. The study not only affords a simple measurement of 5-desaturation, but the results of stoichiometry measurement show that 5-desaturation of Δ7-cholesterol is catalyzed by a mixed function oxidase rather than a dehydrogenase.

**EXPERIMENTAL PROCEDURES**

*Preparation of Microsomes from Rat Liver—Male, Sprague-Dawley rats (150 to 250 g) were used in this study. Animals were maintained on a diet of cholestyramine and on a light cycle of alternating periods of 12-h light and 12-h dark with killing at the midpoint of the dark cycle. Cholestyramine diet was prepared as described previously (12, 25). Rats were killed by decapitation, and livers were perfused in situ with 100 ml of cold 0.25 M sucrose solution. Microsomes were obtained from cell-free homogenate by centrifugation at 105,000 × g for 1 h as described earlier (12, 13) except 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM reduced glutathione (GSH) and 1 mM EDTA was employed as the homogenization buffer. The microsomal pellet thus obtained was washed once by suspension in fresh buffer and centrifugation. Isolated microsomes could be stored at -80 °C for several weeks without loss of Δ7-sterol 5-desaturase activity.

*Assay of Δ7-Sterol 5-Desaturase Activity—The substrate, Δ7-cholesterol, was prepared as previously described (12). Δ7-Cholesterol was suspended with Tween 80 at a 50:1 to-1 (w/w) ratio of detergent to-sterol as also previously described (12). Assays were conducted by incubating the source of enzyme with 0.1 mM Δ7-cholesterol in 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM GSH) for 5 min at 30 or 37 °C in air, prior to starting the reactions by adding 2.0 μmol of NAD(P)H to the final volume of 2.0 ml. Incubation was continued for 10–20 min as indicated in the tables and figures. In assays for the reconstituted system containing partially purified 5-desaturase, the reaction mixture contained in a final volume of 2.0 ml 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM GSH) unless otherwise indicated; detergent-solubilized cytochrome b5, cytochrome b5 reductase or NADPH-cytochrome P-450 reductase; egg phosphatidylcholine (lecithin) liposomes; and a preparation of 5-desaturase at concentrations indicated in the legends of the tables and figures. The mixture was incubated at 4 °C for 20 min. Assays were conducted by incubating the reaction mixture with 0.1 mM Δ7-cholesterol for 5 min at 30 °C before addition of 5.0 μmol of NAD(P)H to start the reaction. Incubation was continued for 10 min at 30 °C unless otherwise indicated. Reaction was stopped by the addition of an equal volume of 10% potassium hydroxide (w/w) in 95% ethanol. Sterols were extracted three times with a total of 15 ml of petroleum ether. The extracts were evaporated under N2 gas at 40 °C.

The resulting residue was suspended in 250 μl of absolute ethanol for assay by HPLC with a minor modification of the procedure described by Grinstead and Gaylor (12). 50 μl of each sample was injected by a WISP autoinjector (Waters Associates, Milford, MA) onto a 5-μm Ultrasphere Octyl column (4.6 mm × 25 cm) equipped with a 3-cm guard column (Brownlee Laboratories, Santa Clara, CA). Chromatography was conducted at a flow rate of 1 ml/min at 45 °C using a Beckman Model 344 liquid chromatograph with a mobile phase of acetonitrile/methanol/water (45:45:10, v/v/v). The product sterol, 7-dehydrocholesterol, was detected by recording absorbance of the conjugated double bond at 280 nm (1040A HPLC-Detection System, Hewlett-Packard). The amounts of product formed were calculated by comparison to integrated peak areas obtained with varying amounts of authentic 7-dehydrocholesterol. Correction for recovery was measured with an internal standard of 4,4-dimethylcholesta-5,7,14-triene-3-one that was added prior to processing and chromatography. Recovery consistently exceeded 90%.

*Assay of Δ7-Sterol 5-Desaturase and 4-Methyl Sterol Oxidase Activities by Measuring Substrate-dependent Oxidation of a-NADH—Substrate-dependent oxidation of α-NADH was measured spectrophotometrically in 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM GSH) at 30 or 37 °C by following the change in absorbance at 344 nm with a Perkin-Elmer 557 Double Beam Dual Wavelength Spectrophotometer as described previously (22), using an extinction coefficient of 5.6 cm-1 mM-1 (21). Cuvettes contained 0.5-2.0 μg of protein, 2.0 μmol of pyruvate, 0.02 mg of lactic dehydrogenase (to prevent formation and metabolism of α-NADH), 0.02 mg of lactic dehydrogenase, and, if added, 100 nmol of either Δ7-cholesterol or 4,4-dimethyl-5a-cholesterol-7-en-3-ol as substrate for 5-desaturase and 4α-methyl sterol oxidase, respectively, in a final volume of 1.0 ml of the buffer. Δ7-Cholesterol was suspended with Tween 80 (12) and, 4,4-dimethyl-5α-cholesterol-7-en-3-ol was suspended with Triton WR-1539 as previously described (2, 22). An equal volume of buffer containing the same amount of either Tween 80 or Triton WR-1539 was added to the cuvette for measurements of α-NADH oxidation without substrate. Rates of α-NADH oxidation in the reconstituted 5-desaturase system were assayed similarly in cuvettes containing the enzyme preparation, appropriate amounts of purified electron carriers, egg
phosphatidylcholine liposomes, and substrate. The onset of reaction was measured with equal accuracy by the final addition of either steroid substrate or pyridine nucleotide. However, for ease of balancing the double beam instrument, substrate suspension was added after substrate-independent rates of \( \alpha \)-NADPH oxidation had been recorded as previously described (22). Rate of substrate-dependent oxidation was calculated by subtracting the initial velocity of substrate-independent oxidation in the cuvette without substrate from that of the substrate-dependent oxidation in the cuvette with substrate.

**Assay of Other Microsomal Enzymes—**Cytochrome \( b_5 \) was determined spectrophotometrically by the method described by Omura and Sato (26), using a reduced versus oxidized difference extinction coefficient of 186.6 cm\(^{-1}\) M\(^{-1}\) at 424 and 409 nm, respectively. In the assay, cytochrome \( b_5 \) was reduced by addition of a few grains of crystalline sodium dithionite, and the cytochrome content was determined from the resulting reduced-minus-oxidized difference spectrum.

NADH-cytochrome \( b_5 \) reductase activity was assayed by measuring NADH-cytochrome \( c \) reduction at 25 °C. 20 \( \mu l \) of 20 mM NADH solution was added to 0.1 mM potassium phosphate buffer (pH 7.4) which contained 0.2 \( \mu l \) of cytochrome \( b_5 \), a source of enzyme, and 1.0 \( \mu l \) of NADH-cytochrome \( b_5 \) in a final volume of 1.0 ml. Changes in absorbance at 550 nm were recorded with time. The molar extinction coefficient for cytochrome \( c \) was taken as 19.6 mm\(^{-1}\) cm\(^{-1}\) (27). One unit of activity is equivalent to the reduction of 1 \( \mu l \) of the acceptor/min at 25 °C. Unless otherwise indicated as \( \alpha \)-NADH, NADPH is used as the substrate.

NADPH-cytochrome \( P-450 \) reductase activity was measured by monitoring the rate of cytochrome \( c \) reduction at 25 °C. 20 \( \mu l \) of 20 mM NADPH solution was added to 50 mM potassium phosphate buffer (pH 7.7) which contained 0.2 \( \mu l \) of cytochrome \( c \), 0.1 \( \mu l \) of EDTA, and a source of enzyme in a final volume of 1.9 ml. Changes in absorbance at 550 nm were recorded with time. The extinction coefficient for cytochrome \( c \) was taken as 19.6 mm\(^{-1}\) cm\(^{-1}\) (27). One unit of activity is equivalent to the reduction of 1 \( \mu l \) of the acceptor/min at 25 °C.

**Protein concentration** was determined by the method of Lowry et al. (28) with bovine serum albumin used as standard.

**Solubilization and Chromatography of \( \Delta^\text{A} \)-Sterol 5-Desaturase—**All procedures were carried out at 0-4 °C. Solubilization of \( \Delta^\text{A} \)-sterol 5-desaturase was performed according to the procedure initially described by Grinstein and Gaylor (12). Microsomes were treated with a 10% solution of Triton WR-1339 for 10 min as described previously (21). After addition of the PEG solution, the suspension was stirred for 2 h, the suspension was centrifuged at 105,000 \( \times g \) for 50 min. An appropriate volume of 50% ammonium sulfate was added to the supernatant to yield 16% saturation, and the mixture was stirred for 0.5 h. The suspension was centrifuged at 105,000 \( \times g \) for 50 min. The supernatant was collected and the precipitated protein was dissolved in 10 mM potassium phosphate buffer (pH 7.5) containing 0.1 M KCl, 0.5% Triton X-100, and 0.1 mM EDTA (pH 8.3). The amount of cytochrome \( b_5 \) immobilized on the Sepharose gel was estimated, by subtraction of the amount unbound from the amount of cytochrome \( b_5 \) used. Approximately, 100 nmol of cytochrome \( b_5 \) was immobilized on each 1.0-ml volume of settled Sepharose. The affinity gel could be used repeatedly after washing with 0.1 M potassium phosphate buffer containing 0.5 M KCl, 0.05 M Trition X-100, and 0.1 mM EDTA (pH 7.4).

**Chemicals and Reagents—**The following were purchased from Sigma: \( \alpha \)-NADH, disodium salt, grade II; \( \beta \)-NADH, disodium salt, grade III; NADPH, tetrasodium salt, type I; cytochrome \( c \), horse heart, type VII; L-leucine, octyl glucoside, polystyrene-850 Sepharose, avidin-Sepharose, polyethylene glycol. Cholesterol was from Sigma; \( \Delta^\text{A} \)-cholesterol was from CalbiochemBehring, and polyethylene glycol was from EM Science. \( \Delta^\text{A} \)-cholesterol was purchased from Steraloids, Inc., and 7-dehydrocholesterol from Aldrich, and each was crystallized to homogeneity. The observed melting point and optical rotation of \( \Delta^\text{A} \)-cholesterol was 123-123.5 °C and \([\alpha]_D +5^\text{O}\). (N6-hexane)-adenosine-5'-diphosphate-7-y 1 was prepared as previously described (9). The melting point and optical rotation were 140-142 °C and \([\alpha]_D -2.4^\text{O}\). 4,4-Dimethyl-7-dehydrocholesterol-7-en-3-ol was prepared as described previously (30). The observed melting point and optical rotation were 145-145 °C and \([\alpha]_D +5^\text{O}\). 4,4-Dimethylcholesta-5,7-dien-3-one was prepared with the same physical constants as previously described (36). Purity of these sterols used were at least more than 98% when analysed by gas-liquid chromatography. \( (N^6\text{-hexane}) \)-adenosine-5'-diphosphate-angase (type II) was obtained from P-L Biochemicals. Egg phosphatidylcholine was provided by Avanti Polar Lipids, Inc., and cholestyramine was from the Mead Johnson Company. All other chemicals were of the best grade available commercially.

**RESULTS**

**Measurement of Substrate-dependent \( \alpha \)-NADH Oxidation and the Stoichiometry of \( \Delta^\text{A} \)-Sterol 5-Desaturase—**An assay based on substrate-dependent oxidation of \( \alpha \)-NADH has been developed for determination of \( \Delta^\text{A} \)-sterol 5-desaturase activity as described previously for \( 2 \)-methyl sterol oxidase (22). The
rate of 5-desaturation of Δ7-cholestenol by rat liver microsomes is quite slow, and the average rate of desaturation was 0.13 nmol/min/mg of microsomal protein when measured at 37 °C with an HPLC assay (12), whereas the average rate of substrate-independent oxidation of α-NADH at 37 °C was more than 1 nmol/min/mg of microsomal protein as described previously (22). Therefore, although an increment of activity is consistently observed, 5-desaturase activity in the microsomes could not be accurately assayed even with α-NADH oxidation. However, 5-desaturase activity in solubilized preparations could be assayed directly by measuring the rate of substrate-dependent oxidation of α-NADH since the rate of substrate-independent oxidation of α-NADH in solubilized preparations was much less than the rate of substrate-dependent oxidation. On the other hand, rates of substrate-independent oxidation of β-NADH were still 5- to 10-fold greater than that of α-NADH even in the solubilized preparations.

The time-dependent consumption of α-NADH was readily measured under conditions of assay of initial rate changes for either methyl sterol oxidase or 5-desaturase. Incubations were carried out for specified periods of time. Continuous tracings are difficult because you must continuously correct for substrate-independent oxidation of α-NADH (See Ref. 22). The rate of Δ7-cholesterol-dependent oxidation of α-NADH by the DEAE-Sephacel unbound fraction was constant during incubation from 30 s to 2 min (Fig. 2). The rate of 4,4-dimethyl-5α-cholestan-7-en-3β-ol-dependent oxidation of α-NADH was also constant from 30 s to 2 min. In addition, initial velocity measurements with various amounts of protein showed that the velocity was proportional to the amount of protein added (Fig. 3). The rate of Δ7-cholesterol-dependent oxidation of α-NADH by the DEAE-Sephacel unbound fraction was proportional to the concentrations of protein to 2.0 mg, and the 4,4-dimethyl-5α-cholestan-7-en-3β-ol-dependent oxidation was similarly proportional to the concentrations of protein to 2.0 mg. Finally, 5-desaturase and methyl sterol oxidase could be saturated by the substrates (Fig. 4). Appropriate conditions for assay of initial velocity changes were established within these limits of assay, and this assay based on substrate-dependent oxidation of α-NADH was used for determination of the activities of 5-desaturase and methyl sterol oxidase.

**Fig. 2.** Time course of substrate-dependent oxidation of α-NADH by Δ7-sterol 5-desaturase and 4-methyl sterol oxidase in the DEAE-Sephacel unbound fraction. Measurements of substrate-dependent oxidation of α-NADH were spectrophotometrically assayed at 37 °C as described under "Experimental Procedures." 5 nmol of purified cytochrome b5 was added to the reaction mixture containing 2 mg of protein of the unbound fraction. •—•, Δ7-cholestenol-dependent oxidation; O—O, 4,4-dimethyl-5α-cholestan-7-en-3β-ol-dependent oxidation. Each assay was carried out twice in duplicate. Three equivalents of α-NADH are consumed by methyl sterol oxidase (22); thus, the actual rates of the steroid transformations are comparable.

**Fig. 3.** Effect of protein concentration on substrate-dependent oxidation of α-NADH by Δ7-sterol 5-desaturase and 4-methyl sterol oxidase in the DEAE-Sephacel unbound fraction. Measurements of substrate-dependent oxidation of α-NADH were spectrophotometrically assayed at 37 °C as described under "Experimental Procedures." Purified cytochrome b5 was added to the reaction mixture and ratio of cytochrome b5 to amount of protein of the fraction was 2.5 nmol/mg of protein. Detergent concentration was held constant in each assay. •—•, Δ7-cholestenol-dependent oxidation; O—O, 4,4-dimethyl-5α-cholestan-7-en-3β-ol-dependent oxidation. Each assay was carried out twice in duplicate. Results given are averages of four values obtained.

**Fig. 4.** Effect of substrate concentration on substrate-dependent oxidation of α-NADH by Δ7-sterol 5-desaturase and 4-methyl sterol oxidase in the DEAE-Sephacel unbound fraction. Substrate-dependent oxidation of α-NADH was spectrophotometrically assayed at 37 °C as described under "Experimental Procedures" except that substrate concentrations were varied from 10 to 100 μmol. 5.0 nmol of purified cytochrome b5 was added to the reaction mixture containing 2.0 mg of protein of the fraction. Detergent concentration was held constant in each assay. •—•, Δ7-cholestenol-dependent oxidation; O—O, 4,4-dimethyl-5α-cholestan-7-en-3β-ol-dependent oxidation. Each assay was carried out in duplicate. Results given are averages of the values obtained in two incubations.
sterol oxidase throughout this study.

Concurrent measurements of 7-dehydrocholesterol formation and α-NADH oxidation were carried with the DEAE-Sephacel unbound fraction (Table I). The rate of Δ^7-cholesterol-dependent oxidation of α-NADH were concurrently measured with the DEAE-sterol oxidase throughout this study.

The change in absorbance at 344 nm as described under "Experimental Procedures." Δ^7-Cholesterol-dependent oxidation of α-NADH was measured spectrophotometrically at 37 °C by following the change in absorbance at 344 nm as described under "Experimental Procedures." The sample cuvette contained 1.0 mg of protein of the fraction, 2.5 nmol of purified cytochrome bs, 56 nmol of α-NADH, 2.0 μmol of pyruvate, 0.02 mg of lactate dehydrogenase, and 0.1 mM Δ^7-cholesterol in a final volume of 0.1 ml of 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM GSH). Each assay was carried out in duplicate. Results given are averages of values obtained in duplicate assays. The difference between the values was less than 5% of the averages in each assay.

<table>
<thead>
<tr>
<th>Experiment I</th>
<th>Experiment II</th>
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<tbody>
<tr>
<td>Rate of 7-dehydrocholesterol formation (A)</td>
<td>Rate of α-NADH oxidation (B)</td>
</tr>
<tr>
<td>nmol/min/mg protein</td>
<td>nmol/min/mg protein</td>
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</table>

*Simultaneous measurements of oxygen consumption by 5-desaturase in the DEAE-Sephacel unbound fraction have been attempted with a Clark oxygen electrode (Oxygen Uptake System 102 A, Intech Laboratories Co., Fort Washington, PA). This type of electrode consumes oxygen, and the rate of consumption by the electrode was similar to that observed for 5-desaturase in the fraction. Therefore, although an increment of activity was consistently observed, an accurate rate of substrate-dependent oxygen consumption was not obtained. For experiments I and II, DEAE-Sephacel unbound fraction was obtained from different preparations of microsomes.

AΔ^7-Sterol 5-Desaturase—The initial step of purification of Δ^7-sterol 5-desaturase was PEG fractionation of solubilized proteins (Table II). This step resulted in a high recovery and about 3-fold enrichment of the 5-desaturase activity. A yellow, cloudy unbound fraction was obtained consistently from DEAE-Sephacel chromatography. In this step, the enzyme preparation consists of cytochrome bs, as previously described (12, 13). CM-Sepharose chromatography enriched the activity to about 6-fold when compared to that in the microsomes, and cytochrome P-450 was completely resolved from 5-desaturase on this column.

The unbound fraction from the CM-Sepharose column, after adjustment of pH, was applied to the trypsin-solubilized cytochrome bs-Sepharose column (20 × 15 cm) that had been equilibrated with 20 mM potassium phosphate buffer (pH 7.8 and containing 0.2% octylglucoside, 20% glycerol, and 1 mM GSH). After washing with 5 volumes of the same buffer to elute unbound proteins, the first peak of protein was eluted with 120 mM potassium phosphate buffer (pH 7.8 and containing 0.2% octylglucoside, 20% glycerol, and 1 mM GSH) and then a second peak of protein was obtained with the same buffer to which 0.2 M KCl was added (Fig. 5).

The specific activity of 5-desaturase was enriched to as much as 70-fold with a 30% yield of activity during chromatography on the immobilized cytochrome bs. Moreover, the 5-desaturase activity was fully resolved from 5α-methyl sterol oxidase activity by chromatography on trypsin-solubilized cytochrome bs-Sepharose and was devoid of cytochrome bs reductase, cytochrome P-450 reductase, and cytochrome bs.

The 5-desaturase preparation thus obtained was unstable. Approximately 30% of the activity was lost during storage at 0 °C for 24 h. Attempts to stabilize the 5-desaturase have not been successful.

Reconstitution of Δ^7-Sterol 5-Desaturase—Δ^7-Sterol 5-desaturase has been reconstituted by combining the purified 5-desaturase and electron carriers with egg phosphatidylcholine and α-NADH.

<table>
<thead>
<tr>
<th>Purification procedures</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Purification</th>
<th>Yield</th>
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<tr>
<td>I. Microsomes</td>
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<td>0.11</td>
<td>89.6</td>
<td>1.0</td>
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<td>II. Triton WR-1339-treated microsomes</td>
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<td>III. Solubilized protein</td>
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<td>0.135</td>
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<td>IV. PEG 3000 (7-16%)</td>
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<td>V. DEAE-Sephacel*</td>
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<td>VI. CM-Sepharose*</td>
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<td>VII. Trypsin-solubilized cytochrome bs-Sepharose*</td>
<td>1.2</td>
<td>7.9</td>
<td>9.8</td>
<td>72</td>
<td>11</td>
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</tbody>
</table>

*DEAE-Sephacel unbound fraction contained 1.1 units of cytochrome bs reductase/mg of protein. The reaction mixture contained 2.0 mg of protein of the fraction, 5.0 nmol of purified cytochrome bs, 0.1 mM Δ^7-cholesterol, and 1 mM NADH in a final volume of 0.1 ml of 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM GSH), and then 2.5 nmol of purified cytochrome bs and 2.0 units of purified NADH-cytochrome bs reductase were added to the reaction mixture containing 2.0 mg of protein in the CM-Sepharose unbound fraction.

*The reaction mixture contained 1.0 unit of purified NADH-cytochrome bs reductase, 2.5 nmol of purified cytochrome bs, 100 μg of protein of purified 5-desaturase preparation, 200 μg of egg lecithin liposomes, 200 nmol of Δ^7-cholesterol, and 2.0 μmol of NADH in a final volume of 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM GSH).
in the presence of molecular oxygen and reduced pyridine nucleotide. The rate of 7-dehydrocholesterol formation in the reconstituted system was constant during the course of incubation through 10 min (Fig. 6), and amounts of conversion were proportional to the amounts of added 5-desaturase to 100 μg in a final volume of 2.0 ml of the reaction mixture (Fig. 7). All subsequent experiments in the reconstituted system were carried out with 100 μg of protein of the 5-desaturase preparation in 10-min incubations.

The 5-desaturase preparation was free from cytochrome b₅ reductase and cytochrome P-450 reductase. As shown in Fig. 8, NADH-supported 5-desaturase activity was proportional to the amount of detergent-solubilized cytochrome b₅ when increasing amounts of the cytochrome were employed with excesses of other components of the system. However, no enzymic activity was observed upon substitution of trypsin-solubilized cytochrome for the complete amphipathic heme protein. The 5-desaturase activity was also proportional to the amount of purified cytochrome b₅ reductase added (Fig. 9). NADPH-supported 5-desaturase activity was similarly dependent on the amount of purified cytochrome P-450 reductase when substituted for cytochrome b₅ reductase in the reconstituted system (Fig. 10). However, the maximal NADPH-supported rate of 5-desaturation of Δ⁷-cholestenol was only about 40% of that of the NADH-supported rate with the same amounts of the terminal oxidase and other components (Fig. 10 versus Fig. 9).

Optimal ratios of egg phosphatidylcholine liposomes to 5-desaturase (w/w) were determined in the reconstituted system under conditions in which the amounts of the three enzymes were held constant and the concentrations of octylglucoside and Tween 80 were 0.05 and 0.19%, respectively, in each assay. As shown in Fig. 11, the optimal ratio was 2-to-1 of phosphatidylcholine-to-protein (w/w), and the rate of 5-desaturation could be increased consistently to about 4-fold of that in the system without added lipid. The complexity of detergent-supported versus phospholipid-supported activity was not studied at this time because sterol substrate suspension required detergent for observation of maximal rates of conversion (38).

As summarized in Table III, both cytochrome b₅ and cytochrome b₅ reductase were required as electron carriers for reconstitution of the 5-desaturase system under optimal conditions. NADH and molecular oxygen were also required for the reconstitution of the activity as described by others with microsomes (1, 6). As pointed out above, NADPH could be substituted, in part, for NADH provided cytochrome P-450 reductase was also substituted for cytochrome b₅-reductase (Fig. 10).

7-Dehydrocholesterol formation in the reconstituted system followed Michaelis-Menten kinetics (Fig. 12). The apparent Kₘ, calculated from Lineweaver-Burk plots was 35.7 μM, while Vₘₐₓ of 10.5 nmol/min/mg of protein was observed.

Effect of Inhibitors on Reconstituted Δ⁷-Sterol 5-Desaturase—Effects of iron-chelators and cyanide were studied in the reconstituted system (Table IV). 5-Desaturase activity was strikingly inhibited in a concentration-dependent manner by the addition of iron-chelators: bathophenanthroline, orthophenanthroline, and 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron). Inhibition of activity by the iron-chelators suggested that 5-desaturase may be a metallo-enzyme. Attempts to restore activity following removal of chelated metal ions have only been partially successful.

Dithiothreitol mildly inhibited the activity. As reported
earlier, 5-desaturase activity of microsomes is cyanide-sensitive. In the reconstituted system, activity was almost completely inhibited by 0.5 mM KCN. As pointed out above, after purification through the affinity column step, the terminal oxidase is extremely labile, and more detailed studies must await either stabilization or more-rapid purification.

**DISCUSSION**

A novel assay based on substrate-dependent oxidation of α-NADH has been developed for mixed function oxidases of very low activity. Δ⁷-Sterol 5-desaturase activity could be assayed spectrophotometrically by measuring the rate of Δ⁷-cholestenol-dependent oxidation of α-NADH under the appropriate conditions. Concurrent measurements of 7-dehydrocholesterol formation and oxidation of Δ⁷-cholesterol showed that the rate of the substrate-dependent oxidation of α-NADH is equal to the rate of product formation. The stoichiometry indicates that for each equivalent of cis-desaturation (6, 23, 24) of Δ⁷-cholesterol to 7-dehydrocholesterol, 1 eq of α-NADH is consumed. Together with the requirement for molecular oxygen (Table III), this evidence demonstrates that 5-desaturase is a mixed function oxidase rather than a dehydrogenase. No monoxygogenated intermediate has been obtained for mammalian 5-desaturase (12). Thus, in the 4-electron reduction of molecular oxygen, 2 electrons could come from the pyridine nucleotide and 2 could be derived directly from the steroid substrate undergoing 5-desaturation.

Δ⁷-Sterol 5-desaturase and 4-methyl sterol oxidase have been adsorbed onto a column of immobilized cytochrome b₅ and resolved. Until this resolution, the similar chromatographic behaviors, dependence on identical electron carriers, and strikingly similar properties of the two terminal oxidases (e.g. CN inhibition) had raised the question of whether or not a single mixed function oxidase catalyzes both reactions (12). Resolution of 5-desaturase activity from methyl sterol oxidase activity demonstrates directly that Δ⁷-desaturation and oxidation of 4α-methyl groups are catalyzed by different mixed function oxidases. Miki et al. (20) had reported that binding interaction between cytochrome b₅ and cytochrome P-450₌₃, a P-450 species with high affinity for cytochrome b₅, is also required for desaturation (14, 16) and elongation (19) of fatty acids, phospholipid desaturation (18), and plasmalogen oxidation (39) in liver microsomes. Recently, linoleoyl coenzyme A desaturase from rat liver microsomes was similarly purified by chromatography on immobilized cytochrome b₅ by Okayasu et al. (16). Further study likely will provide many new applications of this useful technique, and further study is needed to measure interactions between cytochrome b₅ and cytochrome b₅-dependent oxidases under recombination as well as resolution conditions.

The partially purified 5-desaturase preparation has been

![Figure 6](image-url)  
**FIG. 6 (left).** Effect of incubation time on the activity of Δ⁷-sterol 5-desaturase in the reconstituted system. 7-Dehydrocholesterol formation was measured with the HPLC assay as described under "Experimental Procedures." The reaction mixture contained 1.0 unit of NADH-cytochrome b₅ reductase, 2.5 nmol of cytochrome b₅, 100 μg of purified protein of 5-desaturase preparation, 200 μg of egg lecithin liposomes, 200 nmol of Δ⁷-cholestenol and 2.0 μmol of NADH in a final volume of 2.0 ml of 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM GSH). Each assay was carried out in duplicate. Results given are averages of the values obtained in duplicate.

![Figure 7](image-url)  
**FIG. 7 (center).** Effect of protein concentration on the activity of Δ⁷-sterol 5-desaturase in the reconstituted system. 7-Dehydrocholesterol formation was measured with the HPLC assay as described under "Experimental Procedures." Amount of purified 5-desaturase was varied in 2.0 ml of incubation mixture. Ratios of cytochrome b₅, NADH-cytochrome b₅ reductase, and egg lecithin liposomes to the amount of the 5-desaturase preparation were the same in each assay. The incubation time was 10 min. 7-Dehydrocholesterol formation was measured with HPLC assay as described under "Experimental Procedures." Each assay was carried out two times in duplicate. Results given are averages of four values obtained.

![Figure 8](image-url)  
**FIG. 8 (right).** Effect of cytochrome b₅ on the activity of Δ⁷-sterol 5-desaturase in the reconstituted system. 7-Dehydrocholesterol formation was measured with the HPLC assay as described under "Experimental Procedures." Various amounts of purified detergent-solubilized cytochrome b₅ (d-b₅) or trypsin-solubilized cytochrome b₅ (t-b₅) were added to the reconstituted system containing 1.0 unit of NADH-cytochrome b₅ reductase, 100 μg of purified 5-desaturase, 200 μg of egg lecithin liposomes, and 200 nmol of Δ⁷-cholesterol in a final volume of 2.0 ml. Incubation was started by adding 2.0 μmol of NADH after a 5-min preincubation at 30°C and continued for 10 min as described under "Experimental Procedures." Each assay was carried out in duplicate. Results given are averages of the values obtained in duplicate.
**A7-Sterol 5-Desaturase**

**Fig. 9 (left).** Effect of NADH-cytochrome b₅ reductase on the activity of A7-sterol 5-desaturase in the reconstituted system. 7-Dehydrocholesterol formation was measured with HPLC assay as described under "Experimental Procedures." 0.2 to 1.6 units of purified NADH-cytochrome b₅ reductase were added to the reconstituted system containing 2.5 nmol of cytochrome b₅, 100 µg of purified 5-desaturase, 200 µg of egg lecithin liposomes, and 200 nmol of Δ7-cholesterol in a final volume of 2.0 ml. Each assay was carried out in duplicate. Results given are averages of the values obtained in duplicate.

**Fig. 10 (center).** Effect of NADPH-cytochrome P-450 reductase on the activity of A7-sterol 5-desaturase in the reconstituted system. 0.23 to 2.3 units of purified NADPH-cytochrome P-450 reductase were added to the reconstituted system containing 2.5 nmol of cytochrome b₅, 100 pg of 5-desaturase, 200 pg of egg lecithin, and 200 nmol of Δ7-cholesterol in a final volume of 2.0 ml. Incubation was started by addition of 2.0 µmol of NADPH after a 5-min preincubation at 30 °C and continued for 10 min. Each assay was carried out in duplicate. Results given are averages of the values obtained in duplicate.

**Fig. 11 (right).** Effect of egg phosphatidylcholine (lecithin) liposomes on the activity of A7-sterol 5-desaturase in the reconstituted system. Various amounts of egg lecithin liposomes were added to the reconstituted system containing 1.0 unit of NADH-cytochrome b₅ reductase, 2.5 nmol of cytochrome b₅, 100 pg of purified protein of 5-desaturase preparation, 200 pg of egg lecithin liposomes, 200 nmol of Δ7-cholesterol, and 2.0 pmol of NADPH in a final volume of 2.0 ml of 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM GSH). The complete system contained 1.0 unit of NADH-cytochrome b₅ reductase, 2.5 nmol of cytochrome b₅, 100 µg of purified protein of 5-desaturase preparation, 200 µg of egg lecithin liposomes, 200 nmol of Δ7-cholesterol, and 2.0 µmol of NADH in a final volume of 2.0 ml of 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM GSH). 100% N₂ atmosphere; the buffer used for incubation was equilibrated with N₂ by bubbling with N₂ for 15 min, and N₂ was exchanged for air in all sealed reaction flasks prior to the start of incubations. Incubation of the complete mixture was carried out anaerobically in sealed flasks.

**Table III:** Reconstitution of A7-sterol 5-desaturase

<table>
<thead>
<tr>
<th>System</th>
<th>5-Desaturase activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>7.90</td>
</tr>
<tr>
<td>Minus 5-desaturase</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Minus cytochrome b₅ reductase</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Minus cytochrome b₅</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Minus liposomes</td>
<td>1.96</td>
</tr>
<tr>
<td>Minus NADH</td>
<td>0.10</td>
</tr>
<tr>
<td>Minus O₂⁶</td>
<td>0.00</td>
</tr>
</tbody>
</table>

⁶ The complete system contained 1.0 unit of NADH-cytochrome b₅ reductase, 2.5 nmol of cytochrome b₅, 100 µg of purified protein of 5-desaturase preparation, 200 µg of egg lecithin liposomes, 200 nmol of Δ7-cholesterol, and 2.0 µmol of NADH in a final volume of 2.0 ml of 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM GSH).

**Fig. 12.** Lineweaver-Burk reciprocal plot of Δ7-sterol 5-desaturase; effect of substrate concentration. 20 to 200 nmol of Δ7-cholesterol were added to the reconstituted system containing 1.0 unit of NADH-cytochrome b₅ reductase, 2.5 nmol of cytochrome b₅, 100 µg of 5-desaturase, and 200 µg of egg lecithin liposomes in a final volume of 2.0 ml. All assays contained 1 mM NADH as cofactor. Detergent concentration was held constant in each assay. The activity of 5-desaturase was determined by measuring 7-dehydrocholesterol formation with the HPLC assay as described under "Experimental Procedures." Each assay was carried out in duplicate. Results given are averages of the values obtained in duplicate.

Enzyme assays were carried out by measuring 7-dehydrocholesterol formation with HPLC as described under "Experimental Procedures." Each assay was carried out in duplicate. Results given are averages of these duplicate values obtained in two separate assays.

Enzyme assays were carried out by measuring 7-dehydrocholesterol formation with HPLC as described under "Experimental Procedures." Each assay was carried out in duplicate. Results given are averages of these duplicate values obtained in two separate assays.

completely resolved from the three microsomal electron carriers: cytochrome b₅, NADH-cytochrome b₅ reductase, and NADPH-cytochrome P-450 reductase. A previous study from this laboratory (12) demonstrated the requirement of cytochrome b₅ for activity of 5-desaturase in the solubilized enzyme system, but earlier we could not show the absolute requirement for NADH-cytochrome b₅ reductase because of minor contamination and very rapid rates of turnover of this enzyme. In the present study with the reconstituted system containing purified 5-desaturase, both cytochrome b₅ and NADH-cytochrome b₅ reductase are shown to be electron
under "Experimental Procedures." When inhibitors were added, assays were conducted by incubating the reaction mixture with the inhibitor for 5 min before addition of NADH to start the reaction. Each assay was carried out in duplicate. Results given are averages of the values obtained in duplicate, which did not vary by more than 5%.

<table>
<thead>
<tr>
<th>Inhibitor added</th>
<th>Concentration (mM)</th>
<th>Specific activity (nmol/min/mg)</th>
<th>Relative rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bathophenanthroline</td>
<td>0.5</td>
<td>2.06</td>
<td>29</td>
</tr>
<tr>
<td>Orthophenanthroline</td>
<td>0.5</td>
<td>1.86</td>
<td>26</td>
</tr>
<tr>
<td>Orthophenanthroline</td>
<td>1.0</td>
<td>1.14</td>
<td>16</td>
</tr>
<tr>
<td>4,5-Dihydroxy-1,3-benzene-disulfonic acid (Tiron)</td>
<td>0.5</td>
<td>0.98</td>
<td>14</td>
</tr>
<tr>
<td>4,5-Dihydroxy-1,3-benzene-disulfonic acid (Tiron)</td>
<td>1.0</td>
<td>1.44</td>
<td>20</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1.0</td>
<td>0.77</td>
<td>11</td>
</tr>
<tr>
<td>KCN</td>
<td>0.5</td>
<td>0.38</td>
<td>5</td>
</tr>
</tbody>
</table>

carriers for the 5-desaturase activity. Furthermore, participation of NADPH-cytochrome P-450 reductase could also be observed in the presence of cytochrome b5, as reported previously (12). However, the maximal NADPH-supported activity was only 40% of the NADH-supported activity in the reconstituted system. These observations suggest that the NADH-supported electron transport system may predominate in the Δ7-desaturation of Δ7-sterol in liver microsomes (see Fig. 1).

The addition of iron-chelators to the reconstituted 5-desaturase system strikingly inhibited the activity. This suggests that 5-desaturase is a metalloenzyme. A catalytic role for non-heme iron has been implied for other analogous cytochrome P-450 and linoleoyl coenzyme A desaturase (16). Thus, one might expect 4α-methyl sterol oxidase and 5-desaturase of cholesterol biosynthesis to be non-heme-iron-dependent enzymes.

REFERENCES

Δ7-Sterol 5-Desaturase