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Microsomal Enzymes of Cholesterol Biosynthesis from Lanosterol

PURIFICATION AND CHARACTERIZATION OF Δ^7-STEROL 5-DESATURASE OF RAT LIVER MICROSOMES*

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Microsomal Δ^7-sterol 5-desaturase of cholesterol biosynthesis is a multienzyme system which catalyzes the introduction of the Δ^5-bond into Δ^7-cholestenol 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 b_5; the 5-desaturase had not been fully resolved from cytochrom b_5 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 phosphatidylcholine liposomes. Optimizations of conditions for reconsitution have been obtained; both cytochrome b_5 and NADH-cytochrome b_5 reductase serve as electron carriers. A pyridine nucleotide-dependent flavoprotein is required and the requirement can be satisfied with either purified cytochrome b_5 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 b_5-dependent oxidases.

5-Desaturase is resolved from 4-methyl sterol oxidase activity of cholesterol biosynthesis by chromatography on the immobilized cytochrome b_5. This resolution of the two oxidases not only indicates that introduction of the Δ^5-bond and oxidation of 4α-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 Δ^7-cholestenol, 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 Δ^{24}-bond in the side chain, removal of three methyl groups from C-4 and C-14, and movement of the Δ^8-bond of lanosterol[1] to the Δ^5-bond location of cholesterol. Δ^7-Sterol 5-desaturase of the latter process in a multienzyme system which catalyzes oxidative desaturation of Δ^7-cholestenol to 7-dehydrocholesterol in the migration of the B-ring double bond (1), see Fig. 1. 4α-Methyl sterol oxidase is a multienzyme system which catalyzes oxidation of the 4α-methyl group to a steroid 4α-oic acid in the process of the demethylation (2–4). Δ^7-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 b_5 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 b_5 as an electron carrier, and reconsituted by Grinstead and Gaylor (12) and Fukushima *et al.* (13), respectively. These studies demonstrated that cytochrome b_5 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 methy sterol oxidase are analogous to other cytochrome b_5-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, reconsitution of the enzymes of cholesterol synthesis, and identification of each discrete oxidase, isolation of both enzymes is required. Dependence upon cytochrome b_5 for 5-desaturase led us to use cytochrome b_5 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 b_5 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-

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[1] The abbreviations and trivial names used are: lanosterol, 4, 4, 14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; Δ^7-cholestenol, 5α-cholest-7-en-3α-ol; 7-dehydrocholesterol, cholesta-5,7-dien-3β-ol; GSH, glutathione; HPLC, high pressure liquid chromatography; PEG, polyethylene glycol.

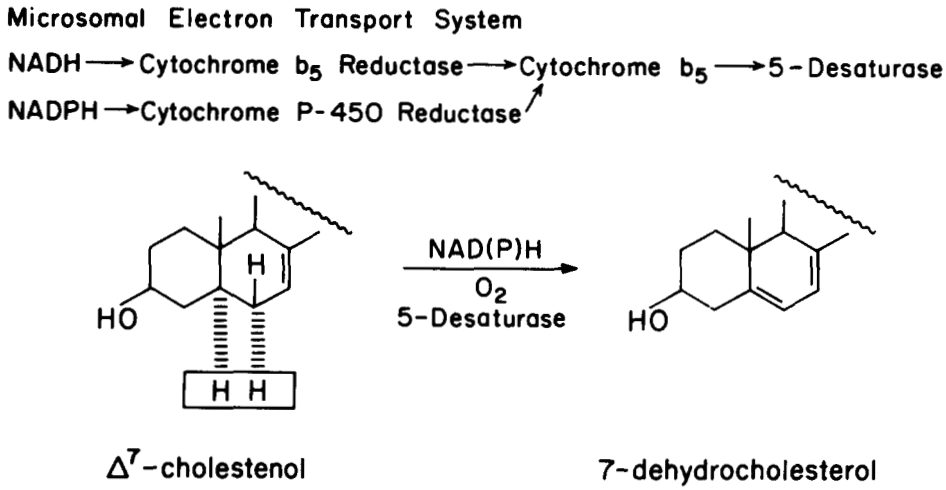


FIG. 1. **Oxidative *cis*-desaturation of Δ^7-cholestenol at C-5 to form 7-dehydrocholesterol as catalyzed by Δ^7-sterol 5-desaturase.** Suggested electron transport to the 5-desaturase.

mobilized cytochrome b_5 are now described in this report.

Several lines of evidence suggest that Δ^7-sterol 5-desaturase is a mixed function oxidase (1, 6) that consumes both oxygen and reduced pyridine nucleotide. Stoichiometry of Δ^6-desaturation of Δ^7-cholestenol requires 1 eq of reduced pyridine nucleotide, but stoichiometry of microsomal-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 the 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-b_5-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 microsomal methyl sterol oxidase (22). In this report, stoichiometry of *cis*-elimination (6, 23, 24) and reduced pyridine nucleotide consumption of 5-desaturase is measured by concurrent assays of 7-dehydrocholesterol formation and oxidation of α-NADH. The study not only affords a simple measurement of 5-desaturase, but the results of stoichiometry measurement show that 5-desaturation of Δ^7-cholestenol 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 Δ^7-sterol 5-desaturase activity.

Assay of Δ^7-Sterol 5-Desaturase Activity—The substrate, Δ^7-cholestenol, was prepared as previously described (12). Δ^7-Cholestenol was suspended with Tween 80 at a 50-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 Δ^7-cholestenol 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 b_5; NADH-cytochrome b_5 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 Δ^7-cholestenol for 5 min at 30 °C before addition of 2.0 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 N_2 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 Ultrashere 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-dien-3-one that was added prior to processing and chromatography. Recovery consistently exceeded 90%

Assay of Δ^7-Sterol 5-Desaturase and 4-Methyl Sterol Oxidase Activities by Measuring Substrate-dependent Oxidation of α-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 mg of protein, 2.0 μmol of pyruvate, 0.02 mg of lactic dehydrogenase (to prevent formation and metabolism of β-NADH), 56 nmol of α-NADH, and, if added, 100 nmol of either Δ^7-cholestenol or 4,4-dimethyl-5α-cholest-7-en-3β-ol as substrate for 5-desaturase and 4α-methyl sterol oxidase, respectively, in a final volume of 1.0 ml of the buffer. Δ^7-Cholestenol was suspended with Tween 80 (12), and 4,4-dimethyl-5α-cholest-7-en-3β-ol was suspended with Triton WR-1339 as previously described (2, 22). An equal volume of buffer containing the same amount of either Tween 80 or Triton WR-1339 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 α-NADH oxidation had been recorded as previously described (22). Rate of substrate-dependent oxidation of α-NADH was calculated by subtracting 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 185 cm^{-1} mM^{-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 μl of 20 mM NADH solution was added to 0.1 M potassium phosphate buffer (pH 7.4) which contained 0.2 μmol of cytochrome *c*, a source of enzyme, and 1.0 nmol of purified 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 μmol of the acceptor/min at 25 °C. Unless otherwise indicated as α-NADH, NADH refers to β-NADH.

NADPH-cytochrome P-450 reductase activity was measured by monitoring the rate of cytochrome *c* reduction at 25 °C. 20 μl of 20 mM NADPH solution was added to 50 mM potassium phosphate buffer 9pH 7.7) which contained 0.2 μmol of cytochrome *c*, 0.1 μmol of EDTA, and a source of enzyme in a final volume of 1.0 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 μmol 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 Δ^7-Sterol 5-Desaturase—All procedures were carried out at 0–4 °C. Solubilization of Δ^7-sterol 5-desaturase was performed according to the procedure initially described by Grinstead and Gaylor (12). Microsomes were treated with a 10% solution of Triton WR-1339 for 10 min as described previously to remove loosely bound protein (2, 5). The Triton-treated microsomes were collected by centrifugation, and the resulting microsomal pellet was suspended in fresh 40 mM potassium phosphate buffer (pH 7.8 and containing 1 mM GSH and 20% glycerol) to a protein concentration of 15 mg/ml. A solution of octylglucoside and sodium taurodeoxycholate in the same buffer was added so that the final octylglucoside-to-protein and sodium taurodeoxycholate-to-protein ratios were 1.3-to-1 and 0.4-to-1 (w/w), respectively. The mixture of microsomes and detergents was gently stirred for 20 min and then centrifuged at 105,000 × *g* for 50 min. An appropriate volume of 50% solution of polyethylene glycol 3000 (PEG, M_r = 3,000–3,700) was added dropwise to the resulting supernatant fraction, and the suspension was mixed well with gentle stirring to give a final concentration of 7%. After addition of the PEG solution, the suspension was stirred for 2 h and then centrifuged to sediment the precipitated protein at 105,000 × *g* for 50 min. The concentration of PEG in the supernatant fraction was carefully raised to 16% and, after stirring for an additional 2 h, the suspension was centrifuged at 105,000 × *g* for 50 min. The precipitated protein was dissolved in 10 mM potassium phosphate buffer (pH 7.8 and containing 0.2% octylglucoside, 20% glycerol, and 1 mM GSH) to give a protein concentration of 8 to 10 mg/ml; the solution was then applied to a DEAE-Sephacel column (2.6 × 7.5 cm) that had been equilibrated with 10 mM potassium phosphate buffer (pH 7.8 with 20% glycerol and 1 mM GSH). The column was eluted with the same buffer. The unbound protein fraction was adjusted to pH 7.0 with 0.1 N HCl and passed over a CM-Sepharose column (1.6 × 5.0 cm) that had been equilibrated with 10 mM potassium phosphate buffer (pH 7.0 and containing 0.2% octylglucoside, 20% glycerol, and 1 mM GSH). The unbound fraction from the CM-Sepharose column was adjusted to pH 7.8 with a 20 mM dibasic potassium phosphate solution. In the next step, chromatography of the unbound fraction was carried out on a column of trypsin-solubilized cytochrome b_5-Sepharose as described below.

Purification of Microsomal Electron Transport Components—Detergent-solubilized cytochrome cytochrome b_5 from rabbit liver microsomes was purified by a modification of the method of Spatz and Strittmatter (29). The specific content was 53 nmol/mg of protein for the final detergent-solubilized cytochrome b_5 preparation. Trypsin-solubilized cytochrome b_5 from rabbit liver microsomes was purified by the method of Kajihara and Hagihara (30). The absorbance ratio of A_{556} to A_{280} was 1.34 for the final trypsin-solubilized cytochrome b_5 preparation.

NADH-cytochrome b_5 reductase from rabbit liver microsomes was purified by the method of Mihara and Sato (31) to a specific activity of 591 units/mg of protein measured as NADH-cytochrome c reductase activity. NADPH-cytochrome P-450 reductase was purified by the method of Yasukochi and Masters (32) to a specific activity of 42 units/mg of protein measured as NADPH-cytochrome *c* reductase. All preparations of these electron transport components did not contain detergent. All of these purified proteins were stored at −80 °C without loss of activity.

Preparation of Egg Phosphatidylcholine (Lecithin) Liposomes—Egg phosphatidylcholine liposomes were prepared as described previously (12). A solution of egg phosphatidylcholine in ethanol was evaporated to dryness under N_2 and the resulting lipid layer was resuspended by vortexing in 0.1 M potassium phosphate buffer (pH 7.4) to a concentration of 3 mg/ml. The milky suspension was then sonicated in a bath sonicator (Model G112SP1G, Laboratory Supplies Co., Inc., Hicksville, NY) under an atmosphere of N_2 until clear.

Preparation of Cytochrome b_5-Sepharose—Cytochrome b_5-Sepharose was prepared according to the method described by Miki *et al.* (20). 100 ml of well-washed, decanted Sepharose 6B was suspended in 0.1 M sodium bicarbonate (pH 8.3) and activated with cyanogen bromide (100–200 mg/ml of settled gel) by the method of Axen and Ernback (33). The gel thus activated was washed with cold 0.1 M sodium bicarbonate buffer (pH 8.3) and suspended in 100 ml of 0.1 M sodium bicarbonate buffer containing 11 μmol of purified trypsin-solubilized cytochrome b_5 and 0.5 M KCl. The mixture was gently stirred overnight at room temperature in the dark. After extensive washing, residual reactive groups were blocked with 1 M glycine in 0.1 M sodium bicarbonate buffer (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, 0.5% Triton X-100, and 0.1 mM EDTA (pH 7.4).

Chemicals and Reagents—The following were purchased from Sigma: α-NADH, disodium salt, grade II; β-NADH, disodium salt, grade III: NADPH, tetrasodium salt, type I; cytochrome *c*, horse heart, type VI; L-lactic dehydrogenase, porcine muscle, type XXIX; bathophenanthroline; orthophenanthroline; and 4,5-dihydroxy-1,3-benzene-disulfonic acid. Octylglucoside was obtained from Calbiochem-Behring, and polyethylene glycol was from EM Science. Δ^7-Cholestenol was purchased from Steraloids, Inc., and 7-dehydrocholesterol from Aldrich, and each was crystallized to homogeneity. The observed melting point and optical rotation of Δ^7-cholesterol preparation were 123–123.5 °C and $[\alpha]_D$ 0°, respectively; reported (34), 123–125 °C and $[\alpha]_D$ + 4°. The melting point of 7-dehydrocholesterol was 143–145 °C; reported (35), 142–143.5 °C. 4,4-Dimethyl-5α-cholest-7-en-3β-ol was prepared as described previously (36). The melting point and optical rotation were 140–142 °C and $[\alpha]_D$ −2.4°, respectively; reported (37), 145–147 °C and +5°. 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 analyzed by gas-liquid chromatography. (N^6-hexane)-adenosine-5′-diphosphate-agarose (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 α-NADH Oxidation and the Stoichiometry of Δ^7-Sterol 5-Desaturase—An assay based on substrate-dependent oxidation of α-NADH has been developed for determination of Δ^7-sterol 5-desaturase activity as described previously for 4 α-methyl sterol oxidase (22). The

rate of 5-desaturation of Δ^7-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.

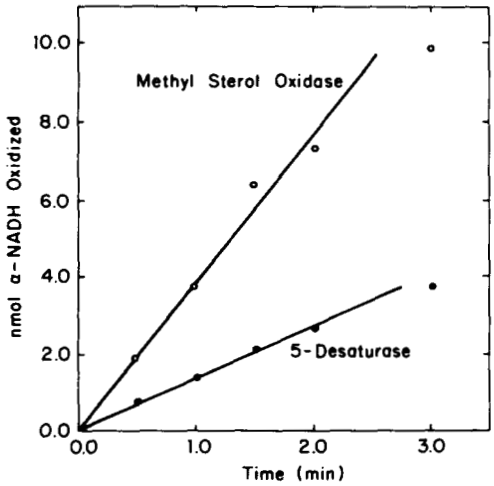


FIG. 2. **Time course of substrate-dependent oxidation of α-NADH by Δ^7-sterol 5-desaturase and 4-methyl sterol oxidase in the DEAE-Sephacel unbound fraction.** Measurements of substrate-dependent oxidation of α-NADH were spectrophotometrically assayed at 37 °C as described under "Experimental Procedures." 5 nmol of purified cytochrome b_5 was added to the reaction mixture containing 2 mg of protein of the unbound fraction. ●——●, Δ^7-cholestenol-dependent oxidation; ○——○, 4,4-dimethyl-5α-cholest-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.

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 Δ^7-cholestenol-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-cholest-7-en-3β-ol-dependent oxidation of α-NADH was also constant from 30 s to 2 min. In addition, initial velocity measurement with various amounts of protein showed that the velocity was proportional to the amount of protein added (Fig. 3). The rate of Δ^7-cholestenol-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-cholest-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

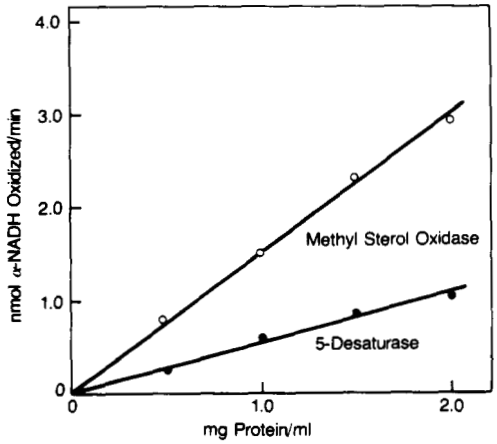


FIG. 3. **Effect of protein concentration on substrate-dependent oxidation of α-NADH by Δ^7-sterol 5-desaturase and 4-methyl sterol oxidase in the DEAE-Sephacel unbound fraction.** Measurements of substrate-dependent oxidation of α-NADH were spectrophotometrically assayed at 37 °C as described under "Experimental Procedures." Purified cytochrome b_5 was added to the reaction mixture and ratio of cytochrome b_5 to amount of protein of the fraction was 2.5 nmol/mg of protein. Detergent concentration was held constant in each assay. ●——●, Δ^7-cholestenol-dependent oxidation; ○——○, 4,4-dimethyl-5α-cholest-7-en-3β-ol-dependent oxidation. Each assay was carried out twice in duplicate. Results given are averages of four values obtained.

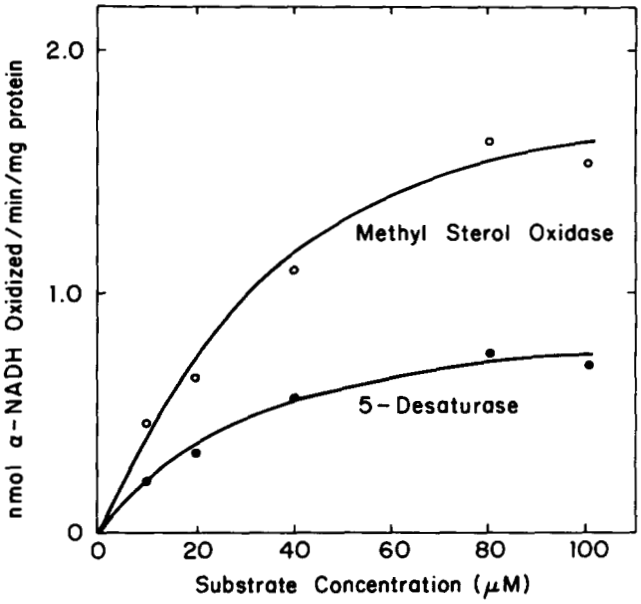


FIG. 4. **Effect of substrate concentration on substrate-dependent oxidation of α-NADH by Δ^7-sterol 5-desaturase and 4-methyl sterol oxidase in the DEAE-Sephacel unbound fraction.** Substrate-dependent oxidation of α-NADH was spectrophotometrically assayed at 37 °C as described under "Experimental Procedures" except that substrate concentrations were varied from 10 to 100 μM. 5.0 nmol of purified cytochrome b_5 was added to the reaction mixture containing 2.0 mg of protein of the fraction. Detergent concentration was held constant in each assay. ●——●, Δ^7-cholestenol-dependent oxidation; ○——○, 4,4-dimethyl-5α-cholest-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-cholestenol-dependent oxidation of α-NADH was essentially equal to the rate of 7-dehydrocholesterol formation. Thus, the stoichiometry of the consumption of reducing equivalents and steroid oxidation for the reaction showed that, for each equivalent of *cis*-desaturation (6, 23, 24) of Δ^7-cholestenol to 7-dehydrocholesterol, 1 eq of α-NADH was consumed.

TABLE I

Measurement of stoichiometry of 5-desaturase

Rates of 7-dehydrocholesterol formation and Δ^7-cholestenol-dependent oxidation of α-NADH were concurrently measured with the same chromatographically resolved preparation from DEAE-Sephacel. This fraction contained 1.2 units of cytochrome b_5 reductase but was free from cytochrome b_5. Assay for 7-dehydrocholesterol formation was conducted by incubating 2.0 mg of protein of the fraction with 5 nmol of purified cyrochrome b_5 and 0.1 mM Δ^7-cholestenol in 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM GSH) for 5 min at 37 °C in air prior to starting the reaction by adding α-NADH (1 mM) in a final volume of 2 ml. The incubation was continued for 10 min. Measurement of 7-dehydrocholesterol formed during the incubation was assayed with HPLC as described under "Experimental Procedures." Δ^7-Cholestenol-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 b_5, 56 nmol of α-NADH, 2.0 μmol of pyruvate, 0.02 mg of lactate dehydrogenase, and 0.1 mM Δ^7-cholestenol in a final volume of 1.0 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 values obtained in duplicate assays. The difference between the values was less than 5% of the averages in each assay.

	Rate of 7-dehydrocholesterol formation (*A*)	Rate of α-NADH oxidation	Ratio (*A*/*B*)
	nmol/min/mg protein	*nmol/min/mg protein*	
Experiment I[a]	0.63	0.65	0.97
Experiment II[a]	0.72	0.74	0.97

[a] 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, Instech 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.

Purification of Δ^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 is resolved from cytochrome b_5 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 b_5-Sepharose column (2.0 × 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 b_5. Moreover, the 5-desaturase activity was fully resolved from methyl sterol oxidase activity by chromatography on trypsin-solubilized cytochrome b_5-Sepharose and was devoid of cytochrome b_5 reductase, cytochrome P-450 reductase, and cytochrome b_5.

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

TABLE II

Purification of Δ^7-sterol 5-desaturase from rat liver microsomes

Enzyme assay was 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 values obtained in duplicate.

Purification procedures	Total protein	Specific activity	Total activity	Purification	Yield
	mg	*nmol/min/mg*	*nmol/min*	*-fold*	%
I. Microsomes	896	0.11	89.6	1.0	100
II. Triton WR-1339-treated microsomes	672	0.13	87.4	1.2	98
III. Solubilized protein	638	0.135	86.1	1.2	96
IV. PEG 3000 (7–16%)	224	0.34	76.2	3.1	85
V. DEAE-Sephacel[a]	85.6	0.49	41.9	4.5	47
VI. CM-Sepharose[b]	49.8	0.66	32.9	6.0	37
VII. Trypsin-solubulized cytochrome b_5-Sepharose[c]	1.2	7.9	9.8	72	11

[a] DEAE-Sephacel unbound fraction contained 1.1 units of cytochrome b_5 reductase/mg of protein. The reaction mixture contained 2.0 mg of protein of the fraction, 5.0 nmol of purified cytochrome b_5, 0.1 mM Δ^7-cholestenol, and 1 mM NADH in a final volume of 2 ml of 0.1 M potassium phosphate buffer (pH 7.4 and containing 1 mM GSH).

[b] 5.0 nmol of purified cytochrome b_5 and 2.0 units of purified NADH-cytochrome b_5 reductase were added to the reaction mixture containing 2.0 mg of protein in the CM-Sepharose unbound fraction.

[c] The reaction mixture contained 1.0 unit of purified NADH-cytochrome b_5 reductase, 2.5 nmol of purified cytochrome b_5, 100 μg of protein of purified 5-desaturase preparation, 200 μg of egg lecithin liposomes, 200 nmol of Δ^7-cholestenol, 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).

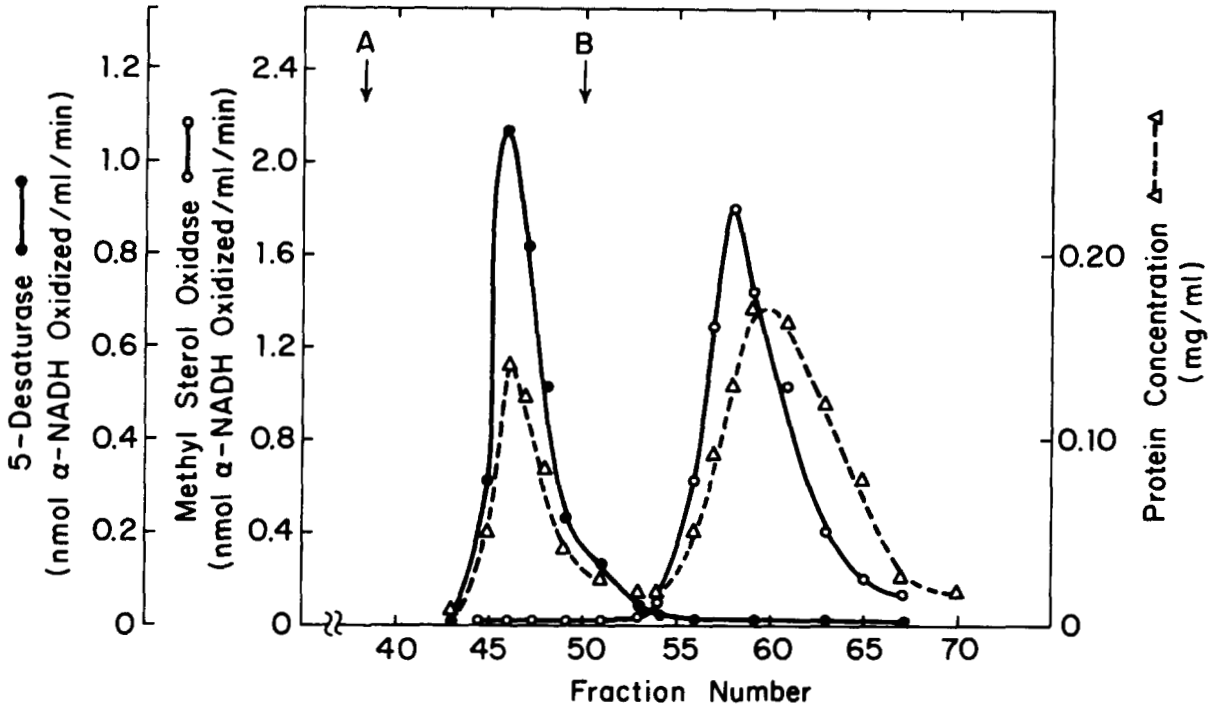


FIG. 5. **Chromatography of CM-Sepharose unbound fraction on trypsin-solubilized cytochrome b_5-Sepharose.** After washing the column, stepwise elution of adsorbed proteins was carried out as indicated by *arrows A* and *B*. *A*, 120 mM potassium phosphate buffer (pH 7.8 and containing 0.2% octylglucoside, 20% glycerol, and 1 mM GSH); *B*, *A* plus 0.2 M KCl. Fractions (3 ml) were collected from the column. Activities of 5-desaturase and methyl sterol oxidase were assayed by substrate-dependent oxidation of α-NADH as described under "Experimental Procedures." 0.4 ml of each fraction, purified cytochrome b_5, purified cytochrome b_5 reductase, and egg phosphatidylcholine liposomes were added to the reaction mixture. Ratios of cytochrome b_5, NAPH-cytochrome b_5 reductase, and liposomes to the amount of protein of each fraction were the same in each assay.

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 b_5 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 b_5 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 b_5 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 b_5 reductase in the reconstituted system (Fig. 10). However, the maximal NADPH-supported rate of 5-desaturation of Δ^7-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 b_5 and cytochrome b_5 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 b_5-reductase (Fig. 10).

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

Effect of Inhibitors on Reconstituted Δ^7-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

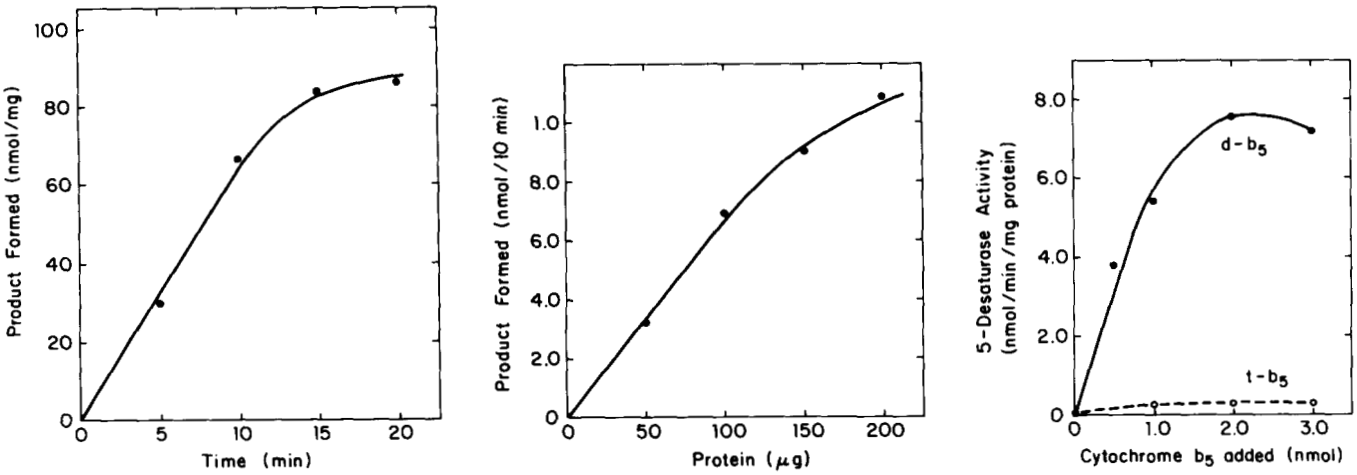


FIG. 6 (*left*). **Effect of incubation time on the activity of Δ^7-sterol 5-desaturase in the reconstituted system.** 7-Dehydrocholesterol formation was measured with the HPLC assay as described under "Experimental Procedures." The reaction mixture contained 1.0 unit of NADH-cytochrome b_5 reductase, 2.5 nmol of cytochrome b_5, 100 μg of purified protein of 5-desaturase preparation, 200 μg of egg lecithin liposomes, 200 nmol of Δ^7-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.

FIG. 7 (*center*). **Effect of protein concentration on the activity of Δ^7-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 b_5, NADH-cytochrome b_5 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.

FIG. 8 (*right*). **Effect of cytochrome b_5 on the activity of Δ^7-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 b_5 (d-b_5) or trypsin-solubilized cytochrome b_5 (t-b_5) were added to the reconstituted system containing 1.0 unit of NADH-cytochrome b_5 reductase, 100 μg of purified 5-desaturase, 200 μg of egg of lecithin liposomes, and 200 nmol of Δ^7-cholestenol 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.

earlier, 5-desaturase activity of microsomes is cyanide-sensitive. In the reconstituted sysem, 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.

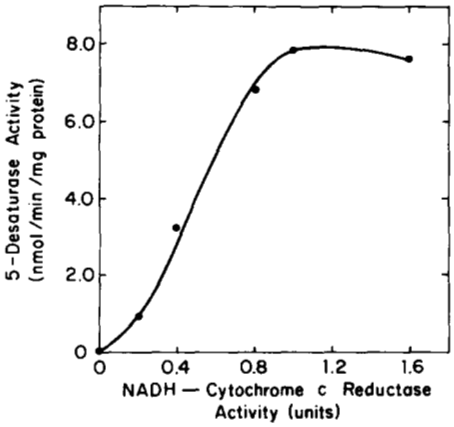
DISCUSSION

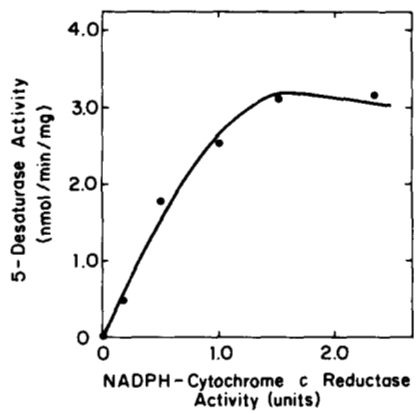
A novel assay based on substrate-dependent oxidation of α-NADH has been developed for mixed function oxidases of very low activity. Δ^7-Sterol 5-desaturase activity could be assayed spectrophotometrically by measuring the rate of Δ^7-cholestenol-dependent oxidation of α-NADH under the appropriate conditions. Concurrent measurements of 7-dehydrocholesterol formation and oxidation of α-NADH 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 Δ^7-cholestenol 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 monooxygenated 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.

Δ^7-Sterol 5-desaturase and 4-methyl sterol oxidase have been adsorbed onto a column of immobilized cytochrome b_5 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 Δ^5-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 b_5 and cytochrome P-450$_{B1}$, a P-450 species with high affinity for cytochrome b_5, are dependent on ionic strength. Resolution of 5-desaturase from methyl sterol oxidase on the immobilized cytochrome b_5 with buffers of different ionic strength suggests that binding affinity for cytochrome b_5 might also be somewhat different for these two terminal oxidases of cholesterol biosynthesis.

Cytochrome b_5 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 b_5 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 b_5 and cytochrome b_5-dependent oxidases under recombination as well as resolution conditions.

The partially purified 5-desaturase preparation has been





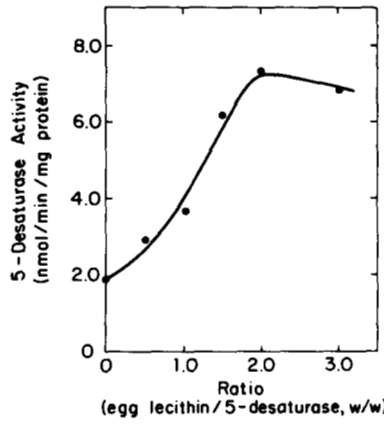


FIG. 9 (*left*). **Effect of NADH-cytochrome b_5 reductase on the activity of Δ^7-sterol 5-desaturase in the reconstituted system.** 7-Dehydrocholesterol formation was measured with HPLC assay as described under "Experimental Procedures." 0.2 to 1.6 units of purified NADH-cytochrome b_5 reductase were added to the reconstituted system containing 2.5 nmol of cytochrome b_5, 100 μg of purified 5-desaturase, 200 μg of egg lecithin liposomes, and 200 nmol of Δ^7-cholestenol 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 Δ^7-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 b_5, 100 μg of 5-desaturase, 200 μg of egg lecithin, and 200 nmol of Δ^7-cholestenol 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 Δ^7-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 b_5 reductase, 2.5 nmol of cytochrome b_5, 100 μg of 5-desaturase, and 200 nmol of Δ^7-cholestenol in a final volume of 2.0 ml. The final concentrations of octylglucoside and Tween 80 were 0.05 and 0.19% (w/v), respectively. Each assay was carried out in duplicate. Results given are averages of the values obtained in duplicate.

TABLE III

Reconstitution of Δ^7-sterol 5-desaturase

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.

System	5-Desaturase activity
	nmol/min/mg protein
Complete system[a]	7.90
Minus 5-desaturase	<0.05
Minus cytochrome b_5 reductase	<0.05
Minus cytochrome b_5	<0.05
Minus liposomes	1.96
Minus NADH	0.10
Minus O_2[b]	0.00

[a] The complete system contained 1.0 unit of NADH-cytochrome b_5 reductase, 2.5 nmol of cytochrome b_5, 100 μg of purified protein of 5-desaturase preparation, 200 μg of egg lecithin liposomes, 200 nmol of Δ^7-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).

[b] 100% N_2 atmosphere: the buffer used for incubation was equilibrated with N_2 by bubbling with N_2 for 15 min, and N_2 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.

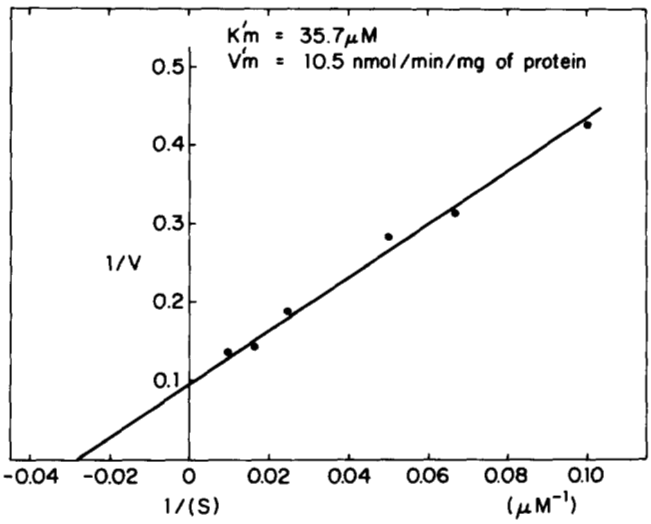


FIG. 12. **Lineweaver-Burk reciprocal plot of Δ^7-sterol 5-desaturase; effect of substrate concentration.** 20 to 200 nmol of Δ^7-cholestenol were added to the reconstituted system containing 1.0 unit of NADH-cytochrome b_5 reductase, 2.5 nmol of cytochrome b_5, 100 μg of 5-desaturase, and 200 μg of 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.

completely resolved from the three microsomal electron carriers: cytochrome b_5, NADH-cytochrome b_5 reductase, and NADPH-cytochrome P-450 reductase. A previous study from this laboratory (12) demonstrated the requirement of cytochrome b_5 for activity of 5-desaturase in the solubilized enzyme system, but earlier we could not show the absolute requirement for NADH-cytochrome b_5 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 b_5 and NADH-cytochrome b_5 reductase are shown to be electron

TABLE IV

Effect of inhibitors on the reconstituted Δ^7-sterol 5-desaturase

The 5-desaturase system was reconstituted as described in Table III except that the incubation buffer was 50 mM Tris-HCl (pH 7.4 and containing 1 mM GSH). The enzyme activity was determined by measuring 7-dehydrocholesterol formation with HPLC as described 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%.

Inhibitor added	Concentration	Specific activity	Relative rate
	mM	*nmol/min/mg*	%
None		7.10	100
Bathophenanthroline	0.5	2.06	29
Bathophenanthroline	1.0	1.14	16
Orthophenanthroline	0.5	1.86	26
Orthophenanthroline	1.0	0.98	14
4,5-Dihydroxy-1,3-benzene-disulfonic acid (Tiron)	0.5	1.44	20
4,5-Dihydroxy-1,3-benzene-disulfonic acid (Tiron)	1.0	0.77	11
Dithiothreitol	1.0	3.20	45
KCN	0.5	0.36	5

carriers for the 5-desaturase activity. Furthermore, participation of NADPH-cytochrome P-450 reductase could also be observed in the presence of cytochrome b_5 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 Δ^5-desaturation of Δ^7-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 of 5-desaturase must be shown directly in subsequent studies, since the chelators can bind various metals (40). It is noteworthy, however, that a catalytic role for a non-heme iron has been implied for other analogous cytochrome b_5-dependent oxidases: stearyl coenzyme A desaturase (14) 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.

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