Mechanism of C-5 Double Bond Introduction in the Biosynthesis of Cholesterol by Rat Liver Microsomes

EVIDENCE FOR THE PARTICIPATION OF MICROSOMAL CYTOCHROME b5

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The dehydrogenation reaction of cholest-7-en-3β-ol (I) to cholesta-5,7-dien-3β-ol (II) in the presence of NADH was studied in rat liver microsomes and in microsomal acetone powder preparations, using [5α-3H]cholest-7-en-3β-ol. It was found that the reaction was inhibited by menadione, adenosine diphosphate, potassium ferricyanide, and cytochrome c while p-cresol had no effect. These results indicated the participation of a microsomal electron transport system in the dehydrogenation of cholest-7-en-3β-ol. The conversion of cholest-7-en-3β-ol to cholesta-5,7-dien-3β-ol was also observed in the absence of NADH when ascorbic acid was included in the incubation mixture. However, the ascorbic acid-catalyzed dehydrogenation was not inhibited by potassium ferricyanide.

Immunological evidence that microsomal cytochrome b5 is involved in the dehydrogenation of (I) to (II) was obtained. Antibodies specific for rat liver microsomal cytochrome b5 were elicited in rabbits. The anti-cytochrome b5 immunoglobulin fraction inhibited rat liver microsomal NADH-cytochrome c reductase but not NADPH-cytochrome c reductase. Also, the extent of reduction of cytochrome b5 was not affected by the antibodies. The conversion of (I) to (II) by rat liver microsomes was inhibited (73%) by anticytochrome b5 immunoglobulin at a ratio of microsomal protein:immunoglobulin of 1:5.6.

These results are consistent with the participation of microsomal cytochrome b5 in the introduction of the C-5 double bond in cholesterol biosynthesis. A close analogy of the microsomal dehydrogenation of fatty acids and of cholest-7-en-3β-ol is apparent and this suggests a possible similarity in the mechanisms of the two reactions.

The transformation of lanosterol to cholesterol by rat liver enzymes is a multistep process involving the removal of three methyl groups (from C-4 and -14), reduction of the C-24 double bond, and transposition of the C-8(9) double bond to C-5(6) position (1). An intermediary step of the process is the desaturation of a 5α-C-27 sterol having a 7(8) double bond to a 5,7-dien-ol (2). It is considered likely that the 7(8)-olefinic intermediate is 5α-cholesta-7,9,14-trien-3β-ol, which is desaturated to cholesta-5,7-dien-3β-ol. The introduction of the 5(6) double bond is stereospecific, and involves the abstraction of the cis-5α- and 6α-hydrogen atoms (3). We have noted that the two abstracted hydrogen (tritium) atoms were located in the water of the incubation medium (4-6). On the basis of these observations, we have considered the possibility that the 5(6)-desaturation may proceed via a mechanism similar to that operating in the oxidation of o-diphenols (6).

The requirements of liver microsomal desaturase systems of fatty acyl-CoAs and of the 5α-cholesta-7-en-3β-ol are very similar. This suggested that the two desaturation mechanisms may also be similar. The fatty acyl-CoA desaturase system was shown to involve a microsomal electron transport system (7). The possibility was therefore considered that the Δ5-sterol desaturase may also involve an electron transport. It was noted that both the fatty acyl-CoA desaturase and the Δ5-sterol desaturase require pyridine nucleotides (8, 9), molecular oxygen (8, 10), cyanide-sensitive factors (11, 12), and are inhibited by cyanide and sulfhydryl blocking agents (6, 13). Both enzyme systems abstract the cis-oriented hydrogens (3, 14). Moreover, both enzymatic activities are localized in the microsomal fraction (8, 10), are insensitive to carbon monoxide (8, 13), and are stimulated by a soluble protein factor (15-17). It has been shown that fatty acid desaturation (18-21) and a desaturation in plasmalogen synthesis (22) involve cytochrome b5 as an electron carrier between the flavoprotein and the cyanide-sensitive factor. However, until now, evidence for the participation of cytochrome b5 in the desaturation of Δ5- to Δ5-7-sterols has not been obtained.

In a preliminary communication Dempsey et al. (13) reported that a rat liver microsomal fraction which contained cytochrome b5, but no cytochrome P450, was active in the dehydrogenation of 5α-cholesta-7-en-3β-ol. Ritter and Dempsey (23) have also reported that the reaction was inhibited by cyanide, but not by carbon monoxide. However, no convincing evidence has been offered for the participation of cytochrome b5 in the Δ5-sterol dehydrogenation.

We present evidence that microsomal cytochrome b5 is involved in the dehydrogenation of 5α-cholesta-7-en-3β-ol. Also,
we show that the pathway of the electron flow in the Δ-sterol dehydrogenation is apparently similar to that in stearyl-CoA desaturase system (18) and in other microsomal dehydrogenations (22, 24).

**MATERIALS AND METHODS**

NAD, NADH, NADP, NADPH, ADP, menadione, and cytochrome c were purchased from Sigma Chemical Co. The inhibitor of the reduction of C-7 double bond of cholesta-5,7-dien-3β-ol AY9944 (25) was a gift from Dr. I. Doornick (Ayerst Laboratories, Montreal). Thin layer chromatography was carried out on plates coated with silica gel (HF. 254—356). Recording spectrophotometer (Cary model 14 equipped with a light scattering accessory and a 0.1 absorbance slide wire) was used for cytochrome b₆ estimations. Female Sprague-Dawley rats (Charles River Breeding Laboratories) and male albino rabbits (Margaretts Home Farm) were used [3αH]-Cholesta-7-en-3β-ol was prepared as previously described (5). The radioactivity was determined (5) in a liquid scintillation counter (Mark II, Nuclear Chicago Co.).

**Enzyme Preparation and Incubations—** Female rat liver microsomal acetone powder was prepared as described earlier (5, 9, 17). Acetone powder (250 mg) was suspended in 24 ml of Tris buffer, pH 7.4 containing MgCl₂ (4 mM), EDTA (0.1 mM), and AY9944 (0.05 mM) and distributed equally in eight flasks. NADH (3 mg in buffer (0.5 ml)) and (2αH)-cholesterol 7 en 3β-ol (2.5 μCi, 75 μg) were added to each flask (0.5 ml) with Tween 80) were added to each flask as described earlier (5). The indicated amounts of inhibitors were dissolved in 0.5 ml of the above buffer and added to the incubation flasks (Table I). The final volume was adjusted to 5 ml with the same buffer and the flasks were agitated (2 h at 37°C) in an atmosphere of air. The obtained [3αH]-cholesta-5,7-dien-3β-ol was isolated, purified, and its H content determined (5). The dehydrogenation activity was calculated from the amounts of radioactivity found in the recovered [3αH]-5,7-dien-3β-ol and that of the incubated [3αH]-7-en. The 2.3% conversion of the [3αH]-7-en to [3αH]-5,7-diene in control experiments was considered to correspond to 100% of the dehydrogenation activity (Table I).

**Purification of Cytochrome b₆ from Female Rat Liver Microsomes—** The cytochrome b₆ from female rat livers was prepared by the method of Oma and Takeda (20). From 000 g of liver, 20 mg of cytochrome b₆, with a specific activity of 66 nmol of cytochrome b₆/mg of protein, were obtained. The cytochrome b₆ was subjected to cytochrome b₆ and incomplete cytochrome b₆ by the method of reporting (8). The spectral properties of the purified cytochrome b₆ were similar to those reported (26). The reduced form showed absorption peaks at 423, 526, and 550 nm with a shoulder at 599 nm. The oxidized form had absorption maxima at 415, 540, and 560 nm. The ratio of the optical density at 526 nm (a peak) to the reduced cytochrome b₆ to that at 290 nm of the oxidized form was 1.25 to 1.30.

**Antibodies to Cytochrome b₆—** A mixture of the purified rat cytochrome b₆ (15 mg), saline (9.9% NaCl solution) (3 ml), and the complete Freund’s adjuvant (Difco Co.) (3 ml) was thoroughly stirred and injected subcutaneously into four places on the backs of two male albino rabbits, each weighing 5 to 6 kg. Three weeks later, 5 mg of cytochrome b₆ (5 mg) and saline (3 ml) were mixed with incomplete adjuvant (3 ml) and injected into the rabbits near the sites of the earlier injections. After 2 weeks, a third injection of 2 mg of cytochrome b₆ in incomplete adjuvant was similarly injected into the rabbit. A week later, the blood was collected from an ear vein and the presence of antibodies was determined by a precipitin test (28). Ten days after the third injection, blood was collected from the rabbits by heart puncture. The sera were obtained and the immunoglobulin fractions from 0 ml of sera were precipitated with 40% ammonium sulfate. The precipitated globulin fraction (anticytochrome b₆ Ig) was dissolved in 0.3% saline (10 ml) containing phosphate buffer (10 mM, pH 7.4) and dialyzed overnight against the same saline buffer. The dialyzed solution was stored at 4°C and used for experiments. Control immunoglobulin (Control Ig) was prepared similarly from an untreated rabbit.

**Estimation of Reduction of Cytochrome b₆—** Rat liver microsomes (29) were suspended in 0.1 M KCl (8 mg of protein/ml). A mixture of the microsomal suspension (0.25 ml; 2 mg of protein) in sodium phosphate buffer (0.05 M, 0.7 ml, pH 7.6) containing KCN (1.5 mM), nucitoniamide (30 mM), and EDTA (1 mM) was prepared. Aliquots (0.95 ml) of the mixture were placed in two semimicrocuvettes. The reaction was initiated by the addition of a solution of NADH (0.10 μg in 0.05 ml of buffer) to the sample cuvette. Only buffer (0.05 ml) was added to the reference cuvette. The amount of the reduced cytochrome b₆ was calculated from the changes in optical density (50) at 423 nm using ε₄₅₂ = 171.

**Determination of NADH-Cytochrome c Reductase (30)—** Aliquots (0.7 ml) of a rat liver microsomal suspension (25 μg of protein/0.7 ml of buffer) were placed in two semimicrocuvettes. The cytochrome c (0.17 ml, 0.51 mg) was added to each cuvette and the final volume of the reaction mixtures was adjusted to 1 ml. The reaction was started by the addition of NADH (0.05 ml, 40 μg/mg) to the sample cuvette and the reference cuvette received the same volume of buffer lacking NADH. The reduction of cytochrome c was recorded at 550 nm. Similar incubations were carried out in presence of control IgG (0.5 mg of protein) and anticytochrome b₆ IgG (0.5 mg of protein).

The estimation of NADH-cytochrome c reductase was carried out essentially as described above, except that NADPH was used instead of NADH. Protein was estimated by the method of Lowry et al. (31) using bovine serum albumin as standard.

**Inhibition of Dehydrogenation of 5α-[3αH]-Cholesta-7-en-3β-ol by Anticytochrome b₆ Immunoglobulin—** In Experiment 1, rat liver microsomal acetone powder (200 mg) was suspended in 25 ml of Tris buffer (0.1 M, pH 7.4) containing MgCl₂ (4 mM), EDTA (0.1 mM), and AY9944 (0.05 mM) and distributed equally into ten Erlenmeyer flasks. To each flask was added 3 mg of NADH and 5α-[3αH]-cholesta-7-en-3β-ol (2.5 μCi, 75 μg). To evaluate the effect of dilution on the dehydrogenation, 1 ml and 4 ml of phosphate-buffered saline containing AY9944 (0.00 mM) were added to two flasks. The remaining eight flasks were divided into two equal groups. One group of flasks was incubated with increasing amounts of control sera and the other group with increasing amounts of anticytochrome b₆ sera. Different amounts of AY9944 were supplemented to achieve 0.05 mM concentration in all the flasks. The incubation procedure, isolation of (enols), and the calculation of dehydrogenation activity were the same as described above.

In Experiment 2, microsomal pellets (5) (12 mg of protein/pellet) were mixed with increasing amounts of control Ig or anticytochrome b₆ Ig in a phosphate buffer (0.1 M, pH 7.4) containing MgCl₂ (4 mM), EDTA (0.1 mM), and AY9944 (0.05 mM). The mixture was incubated at 25°C for 15 min and then centrifuged at 105,000 × g for 1 h. The supernatant fraction which contained unbound globulin was discarded. For the determination of the dehydrogenation activity, the microsomal pellets were resuspended in the above phosphate buffer (5 mg) and added to the incubation flasks (Table I). The reaction mixtures were adjusted to 1 ml. The reaction was initiated by the addition of a solution of NADH (3 mg) and 5α-[3αH]-cholesta-7-en-3β-ol (2.5 μCi, 75 μg) were added, and the mixture was incubated as described above. The interassay variability was about 5%; in controls Ig product formation varied from 15.1 to 15.8 nmol. Suitable control incubations without added Ig were performed.

**RESULTS AND DISCUSSION**

**Inhibitory Studies on Dehydrogenation of Cholest-7-en-3β-ol—** The following experiments were designed to examine whether cytochrome b₆ is involved in Δ-sterol dehydrogenation by rat liver microsomes. Menadione and potassium ferricyanide, known to interfere with electron transport at the site of flavoproteins e.g. NADH-cytochrome b₆, reductase (32, 33), inhibited the dehydrogenation of cholest-7-en-3β-ol by the rat liver microsomal acetone powder (Table I). This finding indicated that a flavoprotein, presumably microsomal NADH-cytochrome b₆ reductase, may participate in the dehydrogenation reaction. In addition, adenosine diphosphate at 10 mM concentration was also inhibitory to the reaction. It has been reported that ADP and ADP-ribose inhibit NADH oxidation in the presence of ferricyanide as the electron acceptor (34). The inhibition is presumably due to competition by the ADP derivatives for the NAD sites on the NADH-cytochrome b₆ reductase. It could, therefore, be assumed that in our system, ADP acts similarly. These observations suggested that NADH-cytochrome b₆ reductase is involved in the dehydrogenation of 5α-cholesta-7-en-3β-ol to cholesta-5,7-dien-3β-ol.
We explored the utility of Paltauf's method of treating microsomes for the possible elimination of the nonspecific inhibition of the 5α-cholesterol dehydrogenation. For this purpose, we used freshly prepared microsomes to which the pretreatment with anticytochrome b Ig, showed inhibition of the dehydrogenation reaction, while the control Ig microsomes did not inhibit the process (Fig. 1). The dehydrogenation was inhibited by approximately 73% when 3.6 mg of the anticytochrome b Ig protein were used per 1 mg of microsomal proteins. The present evidence is consistent with the participation of cytochrome b in the conversion of 5α-cholesterol-7-en-3β-ol to cholesterol-5,7-dien-3β-ol by rat liver microsomes.

**Site of Inhibition of Anticytochrome b Ig in Microsomal Electron Transport** — We have established that the anti-b Ig inhibits the dehydrogenation reaction by approximately 73%.

To further study the role of microsomal cytochrome b, in the Δ-sterol dehydrogenation, the effect of anticytochrome b Ig on the pyridine nucleotide-mediated reduction of cytochrome c by liver microsomes was examined. We observed that anticytochrome b Ig inhibited microsomal NADH-cytochrome c reductase (Fig. 2) but not that of NADPH-cytochrome c reductase (Fig. 3). This indicates the specificity of the antibodies and excludes the possibility of a cross reaction with a flavoprotein not participating in the b mediated electron transport. There was about 70% inhibition of the reduction of cytochrome c by NADH in the presence of anticytochrome b Ig. A mixture of microsomal protein and Ig protein at a ratio of 1:20 was used in these incubations (Figs. 2 and 3). The participation of cytochrome b, in the reduction of added cytochrome c by NADH in microsomal preparations is well established (35). Hence, these observations indicate that the inhibition of NADH-cytochrome c reductase by anticytochrome b Ig was at the level of cytochrome b.

Attempts were then made to study the site of interaction of anticytochrome b Ig with cytochrome b. The accepted mode of reduction of cytochrome c by NADH in liver microsomes is presented in Scheme 1. We have explored whether the reduction of cytochrome b, (Site 1) or the reoxidation of reduced cytochrome b, (Site 2) are affected by anticytochrome b Ig.

To test if the reduction of cytochrome b, is inhibited by anticytochrome b Ig, we incubated microsomes (2 mg of protein) with NADH in a buffer solution (pH 7.6) in the presence of control Ig or anticytochrome b Ig (30 mg of protein). It was observed that the total amount of cytochrome b, which was reduced (monitored at 423 nm) was not affected by anticytochrome b Ig. As mentioned above, the experiments with cytochrome c clearly show that the anticytochrome b Ig inhibits the NADII-cytochrome c reductase (Site 2). These observations suggested that the employed anticytochrome b Ig has an affinity for a given binding site and inhibits the transfer of electrons from reduced cytochrome b to an electron acceptor, probably cytochrome c. Nevertheless, it is possible that although the overall reduction of cytochrome b, was not affected by our preparation of anticytochrome b Ig, the rate of reduction of cytochrome b, was. Unfortunately, because of rapidity of the reduction of b, our methodology could not detect whether the rate of reduction was indeed affected. This aspect will be the subject of a future investigation. In fact, the antibodies to cytochrome b, prepared by Oshima and Omura (18) were shown to inhibit both the reduction and oxidation of cytochrome b,.

**Table I**

<table>
<thead>
<tr>
<th>Inhibitor added</th>
<th>Initial activity%</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>ADP (10 mM)</td>
<td>5</td>
</tr>
<tr>
<td>NADH (2.5 μM)</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Cytochrome c (6 mg)</td>
<td>50</td>
</tr>
<tr>
<td>Ascorbate6 (8 mM)</td>
<td>40</td>
</tr>
<tr>
<td>Ascorbate4 (8 mM) + KFe(CN)6 (1 mM)</td>
<td>30</td>
</tr>
<tr>
<td>p-Cresol (6 mM)</td>
<td>120</td>
</tr>
</tbody>
</table>

*The conversion of [3α-3H]cholest-7-en-3β-ol (2.5 μCi, 75 pg) to [3α-3H]cholest-5,7-dien-3β-ol in 2.3% yield (4.6 nmol) was taken as 100% of the desaturation activity.

*No NADH was added.

Cytochrome c which can act as an electron acceptor for reduced cytochrome b, (35) also inhibited the reaction (Table I). This observation is consistent with the view that cytochrome b, may be an electron carrier in the system.

We have noted that the dehydrogenation proceeded to a lesser extent (approximately 40%) when the NADH was replaced with ascorbate in the incubation mixture (Table I). Furthermore, the ascorbate-catalyzed reaction was not inhibited by ferricyanide. It may be mentioned that similar observations were reported for the desaturation of fatty acids (8) where it was suggested that the ascorbate participates in the reduction of the terminal cyanide-sensitive factors, without involving the cytochrome b, reductase. The results on the dehydrogenation of 5α-cholesterol-7-en-3β-ol are in accord with the view that ascorbate provides a shunt in the system. However, when NADH is used as an electron donor, it appears that cytochrome b, reductase mediates the electron transport.

In spite of the similarities of the dehydrogenation reactions of fatty acids and sterols, there is an apparent difference. It was shown that p-cresol inhibits the fatty acid desaturation, but not that of Δ-sterol (Table I). In the fatty acid desaturase system, it was proposed that p-cresol interferes with the cyanide-sensitive factors. These findings raise the possibility of the existence of different cyanide-sensitive factors in the two systems.

**Inhibition of Microsomal Dehydrogenation of 5α-Cholest-7-en-3β-ol by Anticytochrome b Ig** — We now wished to secure direct immunological evidence for the participation of cytochrome b, in the Δ-cholestenol dehydrogenation. In the initial experiments, 5α-cholesterol-7-en-3β-ol was incubated with microsomal acetone powder in the presence of either cytochrome b, antiserum or control serum. In the presence of cyanide-sensitive factors. Nevertheless, it is possible that although the overall reduction of cytochrome b, was not affected by our preparation of anticytochrome b Ig, the rate of reduction of cytochrome b, was. Unfortunately, because of rapidity of the reduction of b, our methodology could not detect whether the rate of reduction was indeed affected. This aspect will be the subject of a future investigation. In fact, the antibodies to cytochrome b, prepared by Oshima and Omura (18) were shown to inhibit both the reduction and oxidation of cytochrome b.

**By contrast, Drs. I. Jansson and J. B. Schenkman (Yale University) were able to prepare anti-sera to b Ig which inhibit both the rate and extent of NADH-mediated reduction of b Ig (personal communication).
Cholesterol Biosynthesis-Cholesta-5,7-dien-3β-ol Formation

...system strongly suggests the operation of a similar pathway for electron transfer in both systems. Based on our...
34. Strittmatter, P. (1959) J. Biol. Chem. 234, 2665-2669
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