Oxidative Demethylation in Sterol Metabolism

INHIBITION BY NADH, A REQUIRED COFACTOR*

Donald R. Brady

From the Center for Nuclear Studies, Memphis State University, Memphis, Tennessee 38152

Oxidative demethylation of 4-methyl sterols by rat liver microsomes has been shown to be inhibited by low levels of NADH. Using snake venom-treated microsomes to remove endogenous NADH-utilizing activities, we observed inhibition of overall demethylation at concentrations of NADH as low as 2.5 μM. At 20 μM NADH, overall demethylation had been reduced to 60% of optimal activity.

In the presence of NADH, oxidative metabolism of [30,31-14C]4,4-dimethyl-5a-cholest-7-en-3β-ol resulted in formation of an intermediate which was metabolized anaerobically to 14CO2 in the presence of NAD+. This intermediate and its acetylated derivative had coincident mobility on silicic acid thin layer chromatograms with 3β-hydroxy-[14C]4β-methyl-5a-cholest-7-en-[14C] 4α-carboxylic acid sterol and its acetylated derivative.

The inhibition by NADH is transitory. Normal rates return after ~5 min if NADH/NAD+ = 0.2, and after ~15 min if NADH/NAD+ = 2. At an NADH/NAD+ ratio of 10, the return to normal rates is even slower, exceeding 30 min. Further, the inhibition, though transitory, occurs again upon introduction of additional NADH to the assay mix.

Pyridine nucleotides play an important role in oxidative demethylation of the 4-methyl sterols which occur as metabolites in cholesterol biosynthesis. As shown in Fig. 1, NAD+, NADH, and NADPH serve as cofactors for oxidative removal of the methyl group at position 4 of the sterol nucleus. Thus, oxidative attack initiated by molecular oxygen in the presence of either NADH or NADPH (Fig. 1, Reaction A) results in formation of a 4α-carboxylic acid sterol (1-3). Subsequent metabolism requires NAD+ (Fig. 1, Reaction B) resulting in decarboxylation, producing a 3-keto steroid. Further metabolism requires NADPH (Fig. 1, Reaction C) which completes the overall sequence referred to as demethylation (4). Thus, a mixed-function oxidase using either NADH or NADPH functions initially in the pathway followed by an NAD+-requiring dehydrogenase which effects decarboxylation. Finally, an NADPH-specific reductase completes the process of demethylation. Because of the frequency with which pyridine nucleotides occur along the metabolic pathway (Fig. 1), it would be reasonable to expect that changes in the oxidation-reduction state of NADH and NADPH (Fig. 1) might influence the overall rate of demethylation.

In all studies concerning oxidative demethylation of 4-methyl sterols, the cofactors NADH, NADPH, and NAD+ have been introduced as exogenous reactants, generally at saturating concentrations. The implication from this would be that the microsome-associated proteins which participate in oxidative demethylation are sufficiently exposed so as to allow the polar cofactors to bind and transfer the necessary reducing equivalents. Since exogenous cofactors can provide electrons for this oxidative process, it would also be assumed that in vivo, both NADH and NADPH might be derived from enzyme-catalyzed reactions associated with the cytosol, making oxidative demethylation potentially subject to changes in cytosolic concentrations of these cofactors. However, we have recently shown that reactions associated with the microsomes can also provide either NADH or NADPH for oxidative demethylation of 4-methyl sterols (5), thus making this oxidative system nondependent on cytosolic reactions for these two cofactors. Indeed, the need for these microsomal reactions becomes more apparent when the reports of others (6-9) are considered, from which concentrations for cytosolic NADPH and NADH can be estimated (see "Discussion"). NADPH has been reported to be present at 0.38 mM (8, 9). This concentration appears not to be saturating for oxidative demethylation of 4-methyl sterols (see Table IV of Ref. 5). Further, NADH can be estimated to be present at about 1.9 μM in the cytosol (see "Discussion"). The concentration is below the reported Km for NADH of 1.7 μM (5). Thus, if oxidative demethylation were solely dependent on cytosolic NADH or NADPH, the rate of demethylation would vary as NADH and NADPH concentrations varied in the cytosol. This is prevented from happening by the availability of NADH and NADPH from microsome-associated reactions (5).

This report presents additional data which further emphasizes that these microsome-associated reactions should be considered as possible sources to provide NADH and NADPH for oxidative demethylation of 4-methyl sterols. The observation we report that NADH, at concentrations not much greater than the Km for NADH, can be inhibitory to overall demethylation points again to the need for a source of NADH other than cytosolic NADH.

Thus, it seems that these microsome-associated reactions are important for two reasons. First, normal levels of cytosolic NADH and NADPH are rate-limiting for oxidative demethylation of 4-methyl sterols (see "Discussion"), and second, an increase in NADH concentration to allow an increased rate of demethylation would result in inhibition of demethylation, as shown in this report.

EXPERIMENTAL PROCEDURES

Isolation of Microsomes—Microsomes were isolated from rat liver, frozen, and stored at ~28 °C as previously described (10). Unless indicated otherwise, 0.1 M potassium phosphate buffer, pH 7.4, containing 10 mM nicotinamide and 2 mM glutathione was used for all operations and assays. Frozen microsomes were suspended in phosphate buffer to a concentration of 25-30 mg of protein/ml and used...
Mixed Function Oxidation of 4-Methyl Sterols

10443

directly. These are referred to as normal microsomes. Normal microsomes were also washed by suspension in phosphate buffer containing 10 mM nicotinamide and 2 mM glutathione. The procedure has been reported (11). This preparation is referred to as washed microsomes.

Other studies involved treatment of the microsomes with snake venom (Crotalus adamanteus) (12). Frozen microsomes were suspended in 1 ml Tris-acetate buffer, pH 7.4, containing 2 concentrations of glutathione and 10 mM nicotinamide at a protein concentration of >25 mg/ml. 100 μg of snake venom and 100 μmol of CaCl₂ were added to 6 ml of microsomes. The microsomes were heated at 37 °C for 10 min, after which 200 μmol of EDTA were added, and the microsomal suspension was diluted to 25 ml with ice-cold Tris-acetate buffer. Following centrifugation at 180,000 × g for 30 min, the microsomal pellet was resuspended in 25 ml of Tris-acetate buffer and centrifuged as before. Spin time included the approximately 20 min required to achieve maximal velocity. The resulting microsomal pellet was suspended in the potassium phosphate buffer to a concentration of 10-15 mg of protein/ml. This preparation will be referred to as snake venom-treated microsomes. Protein concentration for all preparations was determined by the biuret assay (13).

Assay for Demethylation—Demethylation was assayed in one of two ways. The first method, herein referred to as the one-step assay, was essentially the same as that reported earlier for demethylation of a formyl sterol (10). This procedure was based on that published by others (1-3, 14). In the one-step assay, reactants were incubated aerobically at 37 °C for indicated times, and upon termination of the reaction by addition of acid, 14CO₂ was collected in the usual manner (10).

The second method for measuring demethylation is referred to as the two-step assay and is the same as that described by Miller et al. (14). For this assay, all reactants were incubated at 37 °C for indicated times, and the reaction was terminated with heat for 5 min at steam bath temperature. Any 14CO₂ which may have accumulated up to that point was expelled and not counted. The second step of the assay was performed anerobically at 37 °C for 10 min. Fresh reactants were added for this step, which was terminated by addition of acid, and 14CO₂ was collected in the usual manner (10). Thus, the one-step assay measured the actual rate of demethylation occurring under the experimental conditions, whereas the two-step assay measured the amounts of any intermediate which accumulated during the first step and which yielded 14CO₂ during the anaerobic second step of the assay. Sterol concentration was 50 μM for all assays.

Generating systems for NADH and NADPH used in the assays are described in the figures and tables. For some assays using snake venom-treated microsomes in which demethylation activity was measured at various NAD'/NADH concentration ratios, the concentrations were maintained by including 0.5 mM NAD', varying concentrations of NADH, 1.5 mM acetocacetate, 0.31 units of 5-hydroxybutyrate dehydrogenase (Type II); 5-hydroxybutyrate, sodium salt (98%); acetocetate, sodium salt (90-95%); pyruvate, sodium salt (Type II); lactate dehydrogenase (Type III); glucose oxidase (Type II); NADP-isocitrate, trisodium salt (Type I); isocitrate dehydrogenase (Type IV); glutathione (reduced); nicotinamide; NAD⁺ (grade III); NAD⁺, disodium salt (Grade IV); NADH; disodium salt (Grade II); and snake venom (C. adamanteus) were purchased from Sigma. Triton WR-1339 was purchased from the Reger Chemical Co.

![Fig. 1. Reactions of oxidative demethylation. Methyl sterol oxidase catalyzes a series of reactions designated as Reaction A and requires molecular oxygen and either NADH or NADPH. Reaction B is catalyzed by 4a-carboxylic acid sterol decarboxylase and requires NAD⁺. A 3-keto sterol reductase catalyzes Reaction C and requires NADPH.](image)

**TABLE I**

Effect of NADH on NAD⁺-supported demethylation of [30,31-'4C]-4,4-dimethyl-5α-cholest-7-en-3β-ol by normal microsomes

<table>
<thead>
<tr>
<th>Cofactor addition</th>
<th>One-step assay</th>
<th>Two-step assay</th>
<th>One-step + two-step</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD⁺</td>
<td>1.27 ± 0.05</td>
<td>0.131 ± 0.029</td>
<td>1.40 ± 0.08</td>
</tr>
<tr>
<td>NAD⁺ + NADPH</td>
<td>1.17 ± 0.11</td>
<td>0.147 ± 0.028</td>
<td>1.32 ± 0.14</td>
</tr>
</tbody>
</table>

**RESULTS**

Inhibition of Demethylation by NADH—Although both NAD⁺(P)H and NAD⁺ are required in order for demethylation to occur (Fig. 1 and Ref. 14), demethylation will result when only NAD⁺ is added to a microsomal preparation (14). This has been shown to be due to the presence of a microsomal dehydrogenase which catalyzes formation of the small amounts of NADH sufficient to support high rates of oxidative demethylation (5, 12). Since a requirement for either NADPH or NADH along with NAD⁺ has been known for some time, we were surprised to observe that oxidative demethylation was inhibited when high concentrations of both NADH and NAD⁺ were included in the reaction vessel (Table I). When NADH and NAD⁺ were added in equimolar amounts, demethylation was inhibited to about 55% of that observed in the absence of NADH.

Since oxidative demethylation occurs in the presence of NADPH as well as NADH, it was of interest to determine whether NADPH could relieve the inhibition caused by NADH. NADPH can either be added to the reaction vessel or generated from NADP⁺ present in the microsomes (5). However, regardless of the source of NADPH, NADH still inhibited oxidative demethylation (Fig. 2).

The possibility that the observed inhibition in the presence of

*The expression of variation in all tables and graphs is average error.
Studies of others on demethylation of 4-methyl sterols by NADH would be that decarboxylation of the sterol present, so the 4α-carboxylic acid sterol was presumed to be this may be the case. Several lines of evidence suggest that which has been shown to accumulate in the presence of which is metabolized to CO₂ in the second step. As seen in Table I, an intermediate appears to accumulate in increasing amounts as NADH concentration increases. That the radioactivity not being released as CO₂ during the first step of the assay (one-step assay) is present as an intermediate which is metabolized to CO₂ in the second step (two-step assay) is suggested by the constant values obtained when nanomoles of ¹⁴CO₂/mg of protein are added together for the one- and two-step assay procedures (Table I).

Characterization of the Accumulating Intermediate—Through the studies of others on demethylation of 4-methyl sterols, it has been shown that an intermediate accumulates in the presence of oxygen and either NADPH or NADH alone (1–3, 14). This intermediate has been identified as a carboxylic acid sterol (1, 2) which is decarboxylated in the presence of NAD⁺ (17). In our studies, both NADH and NAD⁺ were present, so the 4α-carboxylic acid sterol was presumed to be present. Thus, one possible explanation for the inhibition caused by NADH would be that decarboxylation of the sterol acid was being inhibited. Inhibition at this step would result in the accumulation of 3β-hydroxy-4β-methyl-5α-cholest-7-en-4α-carboxylic acid. Several lines of evidence suggest that this may be the case.

The contents of the reaction vessel were extracted with acetone in an attempt to isolate an intermediate. The properties of this intermediate were then compared with those of 3β-hydroxy-4β-methyl-5α-cholest-7-en-4α-carboxylic acid, which has been shown to accumulate in the presence of NADPH and oxygen (2). Microsomes were assayed using NADPH, oxygen, and the labeled dimethyl sterol substrate in order to produce the 4α-carboxylic acid sterol as described by Miller and Gaylor (2). This preparation was then treated with acetone and chromatographed on a silicic acid column (Fig. 3). The preparations assayed in the presence of inhibiting amounts of NADH were treated in the same manner. As shown in Fig. 3, the elution pattern from a silicic acid column is very similar for the 4α-carboxylic acid (Peak II) produced using NADPH as well as the intermediate (Peak II) formed when NADH is present. This elution pattern is also very similar to that reported by Miller and Gaylor (1) for another sterol acid, 3β-hydroxy-5α-cholest-7-en-4α-carboxylic acid.

Peak I was shown to be due to (30,31,⁴¹C)4,4-dimethyl-5α-cholest-7-β-ol by comparing mobilities in thin layer chromatography, using a known standard.

The retention of the compound present in Peak II was 3.1 times that of the compound present in Peak I for the preparation performed using NADPH, and 2.9 times that for the preparation using NADH (Fig. 3). At least 95% of the radioactivity originally present in the assay vessels was recovered from the column. Further, in the experiment reported in Fig. 3, the disintegrations/min of ¹⁴CO₂ released in the absence of NADH was reduced in the presence of NADH by an amount consistent with the disintegrations/min of ¹⁴C found in Peak II (Table II).

If the intermediate was chromatographed on thin layer sheets of silicic acid using two different solvent systems (petroleum ether/diethyl ether/acetic acid, 40:60:10, and chloroform/methanol/water/acetic acid, 66:34:1:0.5), mobility was coincident with 4α-carboxylic acid sterol made by the method of Miller and Gaylor (2) using NADPH (Fig. 4). Finally, if the intermediate (=1500 dpm) was acetylated with acetic anhydride, it was shown that it was identical with 3α-acetoxy-4β-methyl-5α-cholest-7-en-4α-carboxylic acid (Peak II) produced using NADPH as well as the intermediate (Peak II) formed when NADH is present. This elution pattern is also very similar to that reported by Miller and Gaylor (1) for another sterol acid, 3β-hydroxy-5α-cholest-7-en-4α-carboxylic acid.

Table II

**Comparison of ¹⁴CO₂ released with ¹⁴C-sterol intermediate produced in the presence and absence of NADH**

<table>
<thead>
<tr>
<th>Product</th>
<th>Cofactor</th>
<th>Dpm/flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹⁴CO₂</td>
<td>Without NADH</td>
<td>513</td>
</tr>
<tr>
<td></td>
<td>With 0.1 mM NADH</td>
<td>159</td>
</tr>
<tr>
<td>¹⁴C-sterol intermediate</td>
<td>(Fig. 3, Peak II)</td>
<td>397</td>
</tr>
</tbody>
</table>
dride/pyridine (50 μl/25 μl) overnight at room temperature, and chromatographed on thin layer sheets using petroleum ether/diethyl ether/acetic acid, 40:60:10, as the solvent system. mobility was again coincident with the acetylated derivative of the 4α-carboxylic acid sterol isolated by the procedure of Miller and Gaylor (Fig. 4 and Ref. 2). At least 80-90% of the applied radioactivity was found in the spot corresponding to acetylated sterol. The remaining 10-20% was present at the origin and was unidentified. Overall, ~80% recovery of radioactivity was obtained during acetylation and chromatography.

**Effect of NADH on Demethylation of Low Concentrations**—Most of the studies to this point were done using NADH at 100 μM or higher concentrations. We were interested in determining the lowest NADH concentration at which inhibition could be seen. This was done using a oxidation-reduction couple in order to maintain the NAD+/NADH concentration ratio. Snake venom-treated microsomes, as well as washed microsomes were used, since treatment with snake venom removed a significant amount of activity responsible for microsome-catalyzed loss of NADH. The rate of loss of NADH for snake venom-treated microsomes was 0.688 ± 0.093 nmol/mg of microsomal protein/min, and for untreated microsomes, 12.8 ± 4.7 nmol/mg of microsomal protein/min. In the presence of snake venom-treated microsomes, NADH was varied from 2.5 μM to 500 μM and demethylation activity was assayed in the presence of 0.5 μM NAD⁺. The activity increased as a function of NADH concentration and reached a maximum at 5-10 μM NADH. Thereafter, the activity decreased as NADH concentration increased. 50% inhibition was observed at about 100 μM NADH. Inhibition was present at concentrations as low as 10 μM NADH. Thus, demethylation appears to be inhibited at Rather low NADH concentrations. Similar results were observed using washed microsomes and a oxidation-reduction couple to control NADH concentration (Table III). Thus, snake venom-treated microsomes did not behave differently from untreated microsomes in this respect.

Although overall demethylation did not appear to decrease significantly until the concentration of NADH was above 10 μM, a more quantitative measure of the effect of the increasing NADH concentration is obtained if levels of ¹⁴CO₂ released during the two-step procedure are determined at these low NADH concentrations (Fig. 5, inset). The two-step assay gives an accurate measurement of the accumulation of a sterol intermediate in the presence of NADH. This sterol intermediate can be measured directly, as was done previously (Fig. 3) by acetone extraction or by ¹⁴CO₂ release in the two-step assay. The actual amount of (30,31-¹⁴C)4,4-dimethyl-5α-cholest-7-en-3β-ol being oxidized by methyl sterol oxidase would be the sum of ¹⁴CO₂ measured in the one-step and two-step assay. When this is considered, ¹⁴CO₂ being released in the one-step assay at 10 μM NADH is only 75% of the total amount of methyl sterol substrate being metabolized (Fig. 5, inset).

**Time Course of NADH Inhibition**—The data to this point clearly imply that NADH, even at low concentrations, can inhibit overall demethylation by affecting the step catalyzed by 4α-carboxylic acid sterol decarboxylase. It is not clear, however, whether this represents a true inhibition, in which the activity of the enzyme would be reduced and remain reduced as long as the inhibitor is present. If the inhibition were due to reversal of the metabolic step, there would be no loss in enzyme activity, only a momentary drop in the overall.

![Fig. 4. Chromatography of sterol intermediate and sterol intermediate acetate. The sterol intermediate (squares) and 3β-hydroxy-4α-methyl-5α-cholestan-7-en-3β-carboxylic acid (circles), as well as the acetates of each were chromatographed on thin layer sheets of silicic acid (20 x 20 cm) using petroleum ether/diethyl ether/acetic acid, 40:60:10 (open symbols). Also, the sterol intermediate and 4α-carboxylic acid sterol were chromatographed using chloroform/methanol/water/acetic acid, 96:3:4:1:0.5 (closed symbols).](image-url)
forward rate. This would be due to a temporary reversal of the forward reaction until a new steady state condition had been achieved and the concentration of the substrate for the forward reaction had increased to a point that a new forward rate of reaction was achieved.

In the case of the step catalyzed by 4α-carboxylic acid sterol decarboxylase, NAD' and the 3β-hydroxy-4α-carboxylic acid sterol are substrates for the forward reaction. Since the mechanism of this step is not known, we do not know the substrates of the reverse reaction, but for the purposes of this argument we might assume that NADH and the 3-keto-4α-carboxylic acid sterol would be the substrates for the reverse reaction. In the absence of added NADH, but in the presence of added NAD', a steady state condition would be established between NAD', NADH, and both sterol acids during the course of a demethylation assay. If NADH were also added, however, this steady state condition would be disturbed and appropriate concentration changes would occur in NAD', NADH, and both sterol acids until a new steady state condition was reached. During this time the overall forward rate of the decarboxylase-catalyzed reaction would be temporarily impeded due to the law of mass action, resulting in a temporary enhancement of the reverse reaction caused by the addition of NADH. Once the new steady state condition was reached, a new overall rate of reaction in the forward direction would be achieved. Thus, by observing a time course of the inhibition, one might be able to see a change in the slope of the line indicative of a change in inhibition with time.

Before considering the data, however, one more point needs to be considered concerning oxidative demethylation. That is, that the assay involves more than just the decarboxylase step. The $^{13}$CO$_2$ released is a measure not only of decarboxylase activity but also of a mixed-function oxidase activity, since the substrate used is a 4-methyl sterol. In this sequence of reactions, the rate-limiting step is the initial oxidation, not the step catalyzed by the decarboxylase (18). The inhibition of demethylation caused by NADH is therefore due to the diminution of the rate of the decarboxylase reaction to a value below that of the rate of the initial oxidation step. If the inhibition is temporary for the reasons already presented in previous arguments, the rate of the decarboxylase step could, with time, again become greater than that of the initial oxidation step, and the overall rate of demethylation would return to the initial, uninhibited rate.

Overall demethylation was measured in the presence and absence of NADH (Fig. 6). As seen, the initial inhibition does not rema인, but rather, disappears with time. Note that the extent of inhibition as well as the length of time required for the rate to return to uninhibited values is dependent not upon NADH concentration alone, but rather upon the ratio of NAD' and NADH concentrations. Thus, if NAD' is maintained at 500 μM and NADH is introduced at 100 μM or 1 mM, the rates after 20 min of assay time are reduced, respectively, to 82 and 38% of control. If, however, NADH is included at a concentration of 1 mM and NAD' is introduced at 500 or 100 μM, the rates of demethylation after 20 min of assay time are reduced to 38 and 20% of control, respectively. Thus, as the ratio of NAD'/NADH concentrations increases, the extent of inhibition increases, and this holds true for any time point, not just 20 min. Also, it should be noted that the length of time required to return to uninhibited rates also increases as the ratio of NADH/NAD' concentrations increases. When the ratio is 0.2, the uninhibited rate returns in 5–10 min; when the ratio is 10, the uninhibited rate does not return even after 30 min of assay, although the rate is still steadily increasing.

**Effect of a Subsequent Addition of NADH**—If the reversible nature of the inhibition of demethylation caused by NADH is due to a return to a new steady state condition as suggested earlier, then readdition of NADH should cause an additional inhibitory response, followed by a return to normal rates. Such seems to be the case (Fig. 7). The initial inhibition caused by 100 μM NADH is transitory, and after normal rates have returned, additional NADH (200 μM) causes inhibition again, which also slowly disappears.

**Loss of NADH Due to Extraneous Microsomal Reaction**—The reversible nature of the inhibition might also be explained by losses in NADH due to microsomal reactions other than those involved in sterol demethylation. This could cause NADH to become limiting for inhibition. Although we observed significant loss of NADH at a concentration of 100 μM in the presence of microsomes alone (12.8 ± 4.7 nmol/mg of protein), these losses did not affect the results, since the initial NADH concentration was always higher than the rate-limiting amount of NADH at any time.
Mixed-function Oxidation of 4-Methyl Sterols

10447

Microsomal protein/ml), this rate was significantly reduced in the presence of Triton WR 1339, the detergent concentration equivalent to that used in routine assays. The rate dropped to 1.808 nmol/mg of microsomal protein/min. This effect has been observed by others also (15). At this rate, only 13.5 nmol of NADH would be lost within 5 min in an assay blank containing ~1.5 mg of protein, an average amount for most of our assays. In the case of the experiment shown in Fig. 6, in which NADH was present initially at 100 µM, the concentration would have diminished to only 86.5 µM in 5 min, and yet the rate of demethylation had returned to the uninhibited rate by this time.

Thus, it would appear that the reversible inhibition cannot be explained by a loss of NADH during the assay.

Discussion

It seems apparent from the results of this paper that oxidative demethylation of 4-methyl sterols supported by extramicrosomal NADH and NAD⁺ will proceed at optimal rates only when NADH concentrations are maintained within specific limits which are determined by the Km of NADH for methyl sterol oxidase and the optimal NADH concentrations which allow adequate rates of overall demethylation. The Km for NADH has been reported to be 1.7 x 10⁻⁶ M (5). Thus, at 20 µM NADH, the rate of methyl sterol oxidase should be approaching Vmax. At this concentration, however, demethylation is inhibited to 85% of the observed rate and to 60% of the optimal rate (Fig. 5, inset). In other words, overall demethylation in the presence of saturating concentrations of NAD⁺ and NADH does not reach Vmax for methyl sterol oxidase, the rate-limiting step for overall demethylation (18). Attempts to increase overall demethylation by increasing NADH concentrations further above the Km for methyl sterol oxidase resulted in increased inhibition (Fig. 5).

That this inhibition occurs at the step catalyzed by sterol 4a-carboxylic acid decarboxylase is suggested by the data in Figs. 3 and 4 and Table I. In the presence of NADH, an intermediate appears to accumulate which can be metabolized to produce 14CO₂ during the second step of the two-step assay (Table I). Evidence for the identity of the accumulated intermediate was obtained by isolating the intermediate and comparing its chromatographic properties with a sterol product isolated under conditions proven to produce a 4a-carboxylic acid sterol (1, 2). Coincident mobility is obtained for the 4a-carboxylic acid and the isolated intermediate (Fig. 3), and for the acetylated derivatives of each (Fig. 4).

The reversible nature of the inhibition (Figs. 6 and 7) is also consistent with the conclusion that the step catalyzed by 4a-carboxylic acid sterol decarboxylase is the one affected by NADH, assuming the decarboxylase is a dehydrogenase. Since dehydrogenases are, in general, readily reversible, it is not surprising that the inhibition caused by NADH disappears with time (Fig. 6) and can be caused to reoccur by addition of more NADH (Fig. 7).

The concentration of NADH needed to cause accumulation of the sterol acid is quite low since the acid can be detected by the two-step assay at ~2 µM NADH (Fig. 5, inset). In order to avoid accumulation of acid, the concentration of NADH would have to be below 2 µM. This apparently is not a problem in vivo since the intracellular concentration of NADH is within this range (6, 7) and thus would not be inhibitory to oxidative demethylation of 4-methyl sterols. The concentration in the cytosol of both NAD⁺ and NADH appears to be compatible with these observed properties of the demethylase system.

Cytosolic NAD⁺ is reported at a concentration of 331 µg/g of fresh liver tissue (19). Since living tissue is 70-80% water, this would be 0.6-0.7 mM NAD⁺, very close to the concentration of NAD⁺ (0.5 mM) used in these studies. Cytosolic NADH is reported to be present at a concentration of 35 µg/g of fresh liver tissue (19) or 0.06-0.07 mM, about 10-fold higher than the 5-10 µM concentration of NADH which gives optimal rates of demethylation (Fig. 5). This concentration of NADH would be inhibitory to demethylation. However, this concentration of NADH includes bound and unbound cofactor. If we consider the free, unbound concentrations of cytosolic NAD⁺ and NADH, we find that NADH concentrations are more compatible with the properties of the demethylase system. For cytosolic NAD⁺/NADH concentration ratios, values from 700-1000 have been reported (6, 7). Thus, we can calculate an NADH concentration using 0.6-0.7 mM as the NAD⁺ concentration and 700 as the ratio for NAD⁺/NADH. This gives 0.8-1.0 µM as the concentration for free, unbound NADH. NADH would not inhibit demethylation at this concentration (Fig. 5). However, it would be below KmNADH (1.7 x 10⁻⁶ M, see Ref. 5) and therefore limiting for methyl sterol oxidase.

NADPH could serve in place of NADH as a source of electrons for oxidative demethylation. In fact, data from Jacobson and Kaplan (20) show for whole cell homogenate or cytosol that NAD⁺/NADH concentrations strongly favor NAD⁺ by 4- to 10-fold, and NADPH/NAD⁺ concentrations strongly favor NADPH by 4- to 5-fold. Such a relationship for NADPH and NAD⁺ would, of course, favor oxidative demethylation. One should note, however, that the concentration of intracellular NADPH may not be sufficient to provide saturating conditions for sterol demethylation. The total concentration of NADPH in rat liver has been reported to be 0.38 mM (8, 9). This includes bound and unbound cofactor. Even if we assume that most of this is free, the concentration of NADPH, although approaching saturation, would not be at saturation for methyl sterol oxidase, a principal enzyme of sterol demethylation (see Table IV of Ref. 5). Although saturating conditions are not essential for cytosolic NADPH to be the primary source of electrons for this mixed-function oxidase, the lack of such condition would, however, cause the rate of oxidation to be subject to changes in NADPH concentrations.

Since cytosolic NADH appears to be present in vivo at a concentration below KmNADH for NADH and, even if present at higher concentrations, would become inhibitory to demethylation, and since cytosolic NADPH may not be present at concentrations sufficient to saturate the enzyme systems of sterol demethylation, the finding reported earlier (5) that microsome-associated reactions are available whereby NADH and NADPH can be provided for demethylation in a manner different from that in which cytosolic NADH and NADPH are available for demethylation, becomes particularly important and significant. The dilemma of having NADH present at a high enough concentration to allow maximal rates of demethylation without causing inhibition is circumvented, since intracellular NADH is present at noninhibitory concentrations and NADH produced in the microsomes allows maximal rates of demethylation in the presence of rate-limiting concentrations of cytosolic NADH. Further, microsome-associated NADPH is also available to allow maximal rates of demethylation, although cytosolic NADPH may be rate-limiting. In short, the compartmentalization of microsome-associated NADH and NADPH suggested by our earlier report (5) may be essential for adequate rates of demethylation to persist, given the conditions associated with cytosolic NADH and NADPH.

The enzyme systems in the microsomes responsible for producing NADH and NADPH are, at present, not known. Bechtold et al. (12) have suggested that an alcohol dehydro-
Microsomal dehydrogenases have been implicated in other mixed-function oxidase systems as well. It has been suggested by others that microsomal hexose-6-phosphate dehydrogenase may be functional in providing NADPH for NADH-cytochrome P450 reductase (21-23). The role this enzyme may play in our system, as well as the role of the NADH-dependent dehydrogenase, is currently under investigation in our laboratory.

Because of the reversible nature of the inhibition of demethylation caused by NADH as well as the low concentrations of free NADH that most likely exist in the cytosol, it would seem premature at this point to propose that NADH might become regulatory would be one in which localized pools of NADH were present containing high concentrations of the cofactor. Because of the reversible nature of the inhibition, however (Fig. 6), localized pools of NADH in and of themselves would not be sufficient to cause inhibition, but rather, pools in which the NADH concentration would be changing. If this were the case, demethylation would undergo cyclic changes in activity as the NADH concentration increased and decreased within a pool affecting the sterol acid concentration of NADH and NADPH available to this mixed-function oxidase system, no definitive conclusions can be drawn presently as to whether the inhibition reported herein has any regulatory significance under physiological conditions.

REFERENCES