Stoichiometry of 4-Methyl Sterol Oxidase of Rat Liver Microsomes*

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The stoichiometry of 4-methyl sterol oxidase has been investigated by concurrent assays of rates of oxygen consumption, oxidation of reduced pyridine nucleotide, and formation of steroid 4α-oic acid, which is the oxidized product of attack of 4α-methyl sterol precursors of cholesterol. The basal, steroid-independent rates of oxidation of α-NADH and α-NADH-dependent oxygen consumption by rat liver microsomes are about 10 to 15% of the rates observed with β-NADH. Thus, α-NADH is substituted for β-NADH; α-NADH oxidation is observed spectrophotometrically. The slow rate of oxygen consumption is measured accurately with a galvanic oxygen electrode that is attached to an offset amplifier. For maximal velocity, 4α-hydroxymethyl-5α-cholest-7-en-3β-ol is the steroid substrate, and oxidase activity is induced 2-fold with a dietary bile acid sequestant. Under these conditions, accurate measurements are obtained for substrate-dependent increments, which are equal to or greater than basal, substrate-independent rates.

For each equivalent of hydroxymethyl group oxidized to carboxylic acid, 2 eq each of oxygen and α-NADH are consumed. Thus, the stoichiometry is consistent with that expected for two sequential attacks of the 4α-hydroxymethyl group by an external mixed function oxidase. In addition to establishing the stoichiometry of the 4-methyl sterol oxidase, these results further demonstrate that the steroidal 4α-carboxylic acid is formed from the hydroxymethyl intermediate by catalysis of a mixed function oxidase rather than dehydrogenases.

4-Methyl sterol oxidase of rat liver microsomes catalyzes oxidation of the 4α-methyl group of sterols during the biosynthesis of cholesterol from lanosterol (1). Oxidative attack occurs when either a methyl group or a hydrogen occupies the 4β position (Fig. 1, Equation 1) (2). Thus, attack of the 4α-methyl group is stereospecific. Furthermore, oxidation of a 4α-methyl group occurs twice during the complete demethylation of both the 4α- and 4β-methyl groups of lanosterol (3).

In addition to the oxidative attack of the methyl group (Reaction A of Equation 1), reduced pyridine nucleotide and oxygen are also required for the further metabolism of the resulting 4α-hydroxymethyl sterol to 4α-oic acid (Reactions B and C of Equation 1) (4). A significant rate of metabolism of the 4α-hydroxymethyl group has not been observed under conditions in which alcohol and aldehyde dehydrogenases would be active, i.e., during an anaerobic incubation with oxidized pyridine nucleotide (4). This evidence led to the suggestion that, in addition to the initial hydroxylation of the methyl group (Reaction A), a microsomal oxidase also catalyzes the sequential process of stepwise oxidative formation of carboxylic acid (3, 4). However, although carboxylic acid is formed under aerobic conditions, and alterations in the rates of mixed function oxidative attack of methyl sterol substrates yield similar alterations in the rates of mixed function oxidative attack of hydroxymethyl sterol substrates, substantiation of this suggestion has been delayed because measurement of the stoichiometry of consumption of oxygen and reduced pyridine nucleotide by this relatively slow microsomal oxidase has been impossible until this time.

4-Methyl sterol oxidase is a mixed function oxidase (5). According to the nomenclature of Mason (6), oxidation by an external mixed function oxidase requires an electron donor, such as reduced pyridine nucleotide. Ultimately, oxygen is reduced to water and steroid alcohol groups. Accordingly, stoichiometry of the two-step oxidation of the steroidal 4α-hydroxymethyl group to the steroidal 4α-oic acid would require 2 eq each of reduced pyridine nucleotide and oxygen, as shown in Equation 2 (Fig. 1).

The rate of oxidation of 4-methyl sterols by rat liver microsomes is quite slow compared to other microsomal oxidases (7). Indeed, the calculated rate of oxidation of the 4α-hydroxymethyl sterol by microsomes from adult, male rats...
is less than 1 nmol/min/mg of protein (4). The rate of aerobic oxidation of β-NADH at 37° (i.e., a basal, steroid-independent rate) is greater than 10 nmol/min/mg of protein (7). Therefore, increments of steroid-dependent changes in concentrations of oxygen and reduced pyridine nucleotide could not be observed accurately with the use of β-NADH as the source of reducing equivalents for the external mixed function oxidase (Equation 2).

Recently, we demonstrated that the basal rate of oxidation of α-NADH by rat liver microsomes is only about 10 to 15% of the rate that is observed with β-NADH (7). Furthermore, α-NADH supports maximal rates of 4-methyl steroid oxidation. In addition, the rate of pyridine nucleotide-dependent consumption of oxygen is also proportionally slower when α-NADH is substituted for β-NADH (7). Accordingly, measurements of stoichiometry of 4-methyl steroid oxidase may now be carried out with α-NADH as the source of reducing equivalents, since the basal and steroid-dependent rates are more nearly equal. The slow rate of oxygen uptake has been measured with a very sensitive galvanic oxygen electrode, which has been designed for use with biological fluids (8). Oxidation of α-NADH has been measured spectrophotometrically. Steroid oxidation has been measured accurately with the sensitive assay of release of 14CO₂ from the 4α-hydroxymethyl group of 4α-hydroxysterol (8C)-methyl-5α-cholest-7-en-3β-ol by coupling of the 4-methyl steroid oxidase to the NAD⁺-dependent 4α-oic acid deacylxylase of rat liver microsomes (9).

The initial part of this report describes selection of optimal conditions needed for observation of maximal rates of steroid oxidation under conditions of minimal substrate-independent rates of consumption of oxygen and α-NADH. Then, substrate-dependent changes in concentration of oxygen and α-NADH have been optimized. Finally, stoichiometry is measured by concurrent assays with the same samples of microsomes. These results not only describe methods suitable for the measurement of very slow microsomal mixed function oxidases and confirm that methyl steroid oxidase is an external mixed function oxidase, but this evidence demonstrates that metabolism of the 4α-hydroxymethyl steroid intermediate of cholesterol biosynthesis is, indeed, catalyzed by a mixed function oxidase and not by microsomal dehydrogenases.

**EXPERIMENTAL PROCEDURE**

Male, adult rats of the Wistar strain were purchased from the Kyoei Animal Co., Ltd. of Osaka and maintained on stock ration unless otherwise indicated. The rats were killed by decapitation, liver was perfused in situ with 100 to 150 ml of cold 0.25 M sucrose. Microsomes were prepared with 0.1 M potassium phosphate buffer (pH 7.4 and containing 10 mM nicotinamide and 2 mM glutathione) as described previously (7). In most experiments, assays were completed within a few hours after the preparation of fresh microsomes. All operations were carried out at 4°, and until use, the microsomal pellets were stored at 4° under a layer of fresh buffer.

Oxidation of reduced pyridine nucleotide was measured spectrophotometrically in phosphate buffer at 37° by following the change in absorbance at either 340 or 344 nm as described previously (7). Briefly, cuvettes contained 2.50 ml of phosphate buffer; 2 to 5 mg of microsomal protein; 140 nmol of reduced pyridine nucleotide; and, if added, from 20 to 50 µl of a 1 mM steroid substrate suspension that was prepared with the aid of Triton WR-1339 as described earlier (1). 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 suspensions were added after steroid-independent rates of α-NADH had been recorded.

Oxygen uptake was measured at 37° in 2.40 ml of phosphate buffer. Oxygen concentrations were measured with a galvanic oxygen electrode (8), which was supplied by the Kyusui Kagaku Co., Tokyo. The electrode was attached to a modified, offset amplifier similar to that described by Mitani and Horie (10) for sensitive measurement between 9.0 and 10.0 mv. Generally, the measurement of oxygen concentration was performed in a continuous (steroid) stabilized, then α-NADH was added, and recording was continued to establish the substrate-independent rate of oxygen uptake. To measure the steroid-dependent changes, from 20 to 50 µl of a 1 mM suspension of steroid substrate generally were added before the injection of the solution of α-NADH. Continuous recording was carried out for periods up to 5 minutes.

Optimal rates of 4-methyl steroid oxidase were established with 4,4-dil[C]methyl-5α-cholest-7-en-3β-ol (165 dpm/nmol) that was synthesized as described previously (2). The more rapidly metabolized 4α-hydroxy[C]methyl-5α-cholest-7-en-3β-ol (78.37 dpm/nmol) was synthesized by combining enzymic and nonenzymic procedures that have been described in detail (4).

Precision of assay of 4-methyl steroid oxidase was measured with two protocols in which the oxidase is coupled to steroid 4α- and carboxylases (3). In the one-step assay (3), 4α-hydroxy[C]methyl-5α-cholest-7-en-3β-ol (70.37 dpm/nmol) was synthesized as combining enzymic and nonenzymic procedures that have been described in detail (4).

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Condition for Measurement of Steroid-independent or Basal Microsomal Rates—The very slow rates of microsomal sterol oxidase by rat liver microsomes is only about 10 to 15% of the rate that is observed with β-NADH (7). Furthermore, α-NADH supports maximal rates of 4-methyl steroid oxidation. In addition, the rate of pyridine nucleotide-dependent consumption of oxygen is also proportionally slower when α-NADH is substituted for β-NADH (7). Accordingly, measurements of stoichiometry of 4-methyl steroid oxidase may now be carried out with α-NADH as the source of reducing equivalents, since the basal and steroid-dependent rates are more nearly equal.

The slow rate of oxygen uptake has been measured with a very sensitive galvanic oxygen electrode, which has been designed for use with biological fluids (8). Oxidation of α-NADH has been measured spectrophotometrically. Steroid oxidation has been measured accurately with the sensitive assay of release of 14CO₂ from the 4α-hydroxymethyl group of 4α-hydroxysterol (8C)-methyl-5α-cholest-7-en-3β-ol by coupling of the 4-methyl steroid oxidase to the NAD⁺-dependent 4α-oic acid deacylxylase of rat liver microsomes (9).

The initial part of this report describes selection of optimal conditions needed for observation of maximal rates of steroid oxidation under conditions of minimal substrate-independent rates of consumption of oxygen and α-NADH. Then, substrate-dependent changes in concentration of oxygen and α-NADH have been optimized. Finally, stoichiometry is measured by concurrent assays with the same samples of microsomes. These results not only describe methods suitable for the measurement of very slow microsomal mixed function oxidases and confirm that methyl steroid oxidase is an external mixed function oxidase, but this evidence demonstrates that metabolism of the 4α-hydroxymethyl steroid intermediate of cholesterol biosynthesis is, indeed, catalyzed by a mixed function oxidase and not by microsomal dehydrogenases.
The average rate of α-NADH oxidation by rat liver microsomes has been reported to be 0.88 nmol/min/mg of microsomal protein at 25°C (7). Similar rates of α-NADH-dependent oxygen consumption have been observed at 35°C. When the incubation temperature was increased to 37°C, the temperature at which methyl sterol oxidase is measured, the average rate of oxygen consumption was increased about 3-fold over the rate observed at 25°C, 2.50 ± 0.95 (S.D.) nmol/min/mg of protein for nine experiments. Also, the rate of α-NADH-dependent oxygen consumption was constant for several minutes of incubation at this temperature, and the increment produced by addition of α-NADH was quite significant and easily measured (Fig. 2). Ten-fold scale expansion by offset amplification markedly enhanced the accuracy. Thus, rates of oxygen uptake of about 2 nmol/min/mg of protein could be measured accurately. However, when measurements were carried out at 37°C on the high sensitive recorder setting, the duration of accurate measurement was limited to less than 5 min when values of 9.0 mv were reached on the offset scale of 9 to 10 mv (Fig. 2). Therefore, if increments of steroid-dependent consumption of oxygen would be equal to the steroid-independent rate, experiments would be limited to durations of about 2 min for measurement of initial velocities. Clearly, the substrate-independent rates of oxygen uptake in the presence of β-NADH are too rapid to use β-NADH in the assay of stoichiometry of methyl sterol oxidase. For example, the rate observed with the β-isomer was very rapid, even when the protein concentration was reduced to 40% of that used for measurement of α-NADH-dependent oxygen uptake (Fig. 2).

The average rate of aerobic oxidation of α-NADH at 37°C, measured spectrophotometrically with the same suspensions of microsomes, was 1.74 ± 0.43 (S.D.) nmol/min/mg of protein. Thus, within the limits of observed deviation, which were somewhat smaller for the spectrophotometric measurement of α-NADH oxidation than for α-NADH-dependent oxygen uptake (see above), the rates of oxygen consumption and α-NADH oxidation were equal.

For maximal velocity of methyl sterol oxidase, the substrates have been added as stable suspensions that are prepared with the aid of Triton WR-1339 (13). Because relatively high concentrations of substrate were needed for maximal velocities during the necessarily short incubations (Fig. 2) and the ratio of Triton to steroid in the substrate suspensions was about 50/1 (w/w), the effects of Triton detergent on the rates of methyl sterol oxidase, α-NADH oxidation, and α-NADH-dependent oxygen consumption were measured to determine whether the detergent produced adverse effects. The rates of α-NADH-dependent oxygen uptake and α-NADH oxidation were not altered when the Triton concentrations were less than 1.5 mg/ml (Fig. 3). Oxidation of steroid substrate was inhibited somewhat when the detergent concentration exceeded 1 mg/ml. Accordingly, all subsequent measurements in this study were carried out with a final concentration of Triton that was less than 1 mg/ml. Under these conditions, measurement of stoichiometry was not restricted by the concentration of detergent because a final concentration of 1 mg/ml of Triton would be equivalent to steroid concentrations of about 50 μM; 𝐾ₚ for the 4α-hydroxymethyl-5α-cholest-7-en-3β-ol substrate that was used for stoichiometry measurements reported below has been established in earlier studies as 12 μM (4).

Earlier, we reported that oxidations of β-NADH and NADPH at 25°C by liver microsomes were inhibited by nonionic detergents, such as Triton WR-1339 and Lubrol (7, 14). At 37°C, inhibition of these oxidations by Triton WR-1339 was quite pronounced; oxidation of NADPH was somewhat less sensitive (Fig. 3). Use of either β-NADH or NADPH for the present study of stoichiometry thus was eliminated by the necessary

![Fig. 2. Oxygen uptake in the presence of α-NADH and β-NADH. The change in millivolts is indicated for an incubation of 6.0 and 2.4 mg of rat liver microsomal protein with α-NADH and β-NADH, respectively, at the low sensitive setting on the amplifier (top lines, full scale = 0 to 10 mv). With the offset between 9 and 10, the α-NADH-dependent rate was measured again (expanded scale, full scale = 1 mv). Substitution of β-NADH under the high sensitive setting yielded extremely rapid changes between 9 and 10 mv, which were too rapid to measure accurately when velocities in excess of about 25 nmol/min were studied. The 1.0 mv between 9 and 10 mv is equivalent to approximately 58 nmol of oxygen.](http://www.jbc.org/)

![Fig. 3. Effect of Triton concentration on 4-methyl sterol oxidase, oxygen uptake, and pyridine nucleotide oxidation rates. The indicated amounts of Triton WR-1339 were added to the assays of 4-methyl sterol oxidase (O—O, 4,4-dimethyl sterol substrate), α-NADH-dependent oxygen uptake (●), and oxidations of β-NADH (Δ—Δ), α-NADH (■—■), and NADPH (▲—▲). Each value is the average of data from four separate experiments. For calculation of velocities, representative original specific activities may be obtained from Table I. The final concentration of Triton in the assay of methyl sterol oxidase included detergent in the substrate suspension, as well as that added in this experiment.](http://www.jbc.org/)
addition of substrates in Triton suspension, as well as by the
much greater basal rates of oxidation of reduced pyridine
nucleotide in the absence of steroid substrate (Fig. 2).

The substrate-independent rates of a-NADH oxidation and
oxygen uptake were about 2 nmol/min/mg of protein (see above).
For relatively accurate measurement of substrate-depen-
dent rates, increments produced by addition of substrate
should be equal to or greater than those rates observed in
the absence of substrate, i.e., about 2 nmol/min. However, the rate
of oxidation of 4a-hydroxymethyl-5a-cholest-7-en-3\-ol by
control rat liver microsomes is only about one-half of the above
rate (4). Thus, stimulation of steroid oxidation rates was
investigated.

Earlier, we demonstrated that methyl sterol oxidase is
enhanced more than 100% when rats are fed a diet containing
the nonabsorbed bile acid sequestrant, cholestyramine resin
(15). Furthermore, cholestyramine stimulation of 4-methyl
sterol oxidative attack has been demonstrated directly with the
4a-hydroxymethyl sterol substrates, as well as with the 4a-
methyl sterols (4). Therefore, rats were fed cholestyramine
resin for periods of one week or more to determine whether or
not the substrate-independent rates of consumption of oxygen
and a-NADH would remain unaltered under conditions of
doubled rates of 4a-substituted sterol metabolism.

As reported earlier (15), administration of cholestyramine
resin doubled the rate of 4-methyl sterol oxidase (Table I).
Furthermore, the basal rates of steroid-independent a-NADH
oxidation and a-NADH-dependent oxygen uptake were not
elevated when the rats were fed cholestyramine resin (Table I).
For comparison, data are also included in Table I for rates of
oxidation of a-NADH and NADPH and rates of a-NADH- and
NADPH-dependent oxygen uptake. Very interestingly, chole-

| TABLE I
| Effect of cholestyramine feeding on methyl sterol oxidase, pyridine nucleotide oxidation, and oxygen consumption |

<table>
<thead>
<tr>
<th>Assay</th>
<th>Number of samples</th>
<th>Control</th>
<th>Cholestyramine-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl sterol oxidase^b</td>
<td>6</td>
<td>0.079</td>
<td>0.160</td>
</tr>
<tr>
<td>a-NADH oxidation</td>
<td>5</td>
<td>1.66</td>
<td>1.69</td>
</tr>
<tr>
<td>a-NADH-dependent O2 uptake</td>
<td>7</td>
<td>2.50</td>
<td>1.90</td>
</tr>
<tr>
<td>5a-NADH oxidation</td>
<td>5</td>
<td>10.7</td>
<td>9.80</td>
</tr>
<tr>
<td>B-NADH-dependent O2 uptake</td>
<td>4</td>
<td>6.50</td>
<td>4.80</td>
</tr>
<tr>
<td>NADPH oxidation</td>
<td>3</td>
<td>17.9</td>
<td>22.6</td>
</tr>
<tr>
<td>NADPH-dependent O2 uptake</td>
<td>3</td>
<td>24.0</td>
<td>30.7</td>
</tr>
<tr>
<td>Aminopyrine demethylation^b</td>
<td>3</td>
<td>4.7</td>
<td>4.7</td>
</tr>
</tbody>
</table>

^b Assayed with 4,4-di\[^14\]C\]methyl-5\-cholest-7-en-3\-ol.
These rates observed with the 4a-methyl sterol substrate are
considerably slower than rates observed with the 4a-hydroxymethyl
sterol that was used in the study of stoichiometry below.
However, the 4,4-dimethyl sterol substrate is more readily synthesized,
and in previous work, parallel effects of cholestyramine treatment have been observed with these two
sterol substrates (4,15).
^b Corrected for the formation of formaldehyde in the ab-

ence of either substrate or NADPH. The procedure of Kupfer
and Levin (16) was used.

Thus, under these conditions the increments produced by
addition of steroid substrate should be equal to the increments
produced by addition of a-NADH in the absence of oxidizable
steroid substrate. Furthermore, if the stoichiometry described
in Equation 2 of Fig. 1 is observed for metabolism of the
4a-hydroxymethyl sterol substrate, upon addition of 4a-
hydroxymethyl-5a-cholest-7-en-3\-ol, 2 eq each of a-NADH
and oxygen should be consumed for each equivalent of steroid
oxidized. Thus, under the conditions described very accurate
assays of steroid-dependent changes may be obtained.

Conditions for Measurement of Steroid-dependent Changes
—Substrate-dependent increments of oxygen consumption and
a-NADH oxidation were measured after the addition of small
amounts (about 50 \(\mu\)l) of a 1 mM suspension of steroid
substrate. In the spectrophotometric assay of a-NADH oxida-
tion, an equal volume of buffer containing an equal amount of
Triton WR-1339 was added to the reference cell.

When a-NADH was added to microsomal suspensions that
contain steroid substrate, the initial rate of oxygen uptake was
quite rapid. In agreement with previous studies of oxidation of
\[^14\]C-labeled steroid substrates (2, 4), the increment in velocity
of oxygen consumption under addition of 4a-hydroxymethyl
5a-cholest-7-en-3\-ol was markedly greater than that observed
with the addition of a 4a-methyl sterol substrate (Curves III
versus II, Fig. 4). Furthermore, as shown in this experiment,
 omission of steroid substrate yielded a relatively slow basal
rate of a-NADH-dependent oxygen uptake (approximately 2.3
nmol/min/mg of protein). Similar results were obtained with the
spectrophotometric assay of a-NADH oxidation rates (an
example of an original tracing is not shown).

Averages of time course data from repeated assays of oxygen and
a-NADH consumption by the same preparation of micro-
somes (e.g., as shown in Fig. 4) were used to minimize experi-
mental variations (Fig. 5, A and B). The initial, rapid
velocity of oxygen consumption could be observed for less than
2 min of incubation (Fig. 5A, Curve I). The substrate-depend-
ent rates of a-NADH oxidation were very similar, as shown in
Fig. 5B, Curve 1. Average rates of 4.23 and 4.01 nmol/min/mg
of protein were calculated for the substrate-dependent oxygen
uptake and a-NADH oxidation, respectively, after correction

![Fig. 4. Tracing of changes in oxygen consumption after addition of steroids and a-NADH. Steroids and microsomes were preincubated before addition of a-NADH (arrow). Microsomes were obtained from control rats (0.96 mg of protein/ml). Each sample was incubated with 50 nmol of: I, no steroid; II, 4,4-dimethyl-5a-cholest-7-en-3\-ol; III, 4a-hydroxymethyl-5a-cholest-7-en-3\-ol added before a-NADH.](http://www.jbc.org/)
oxygen concentration was measured in 2.40 ml of final volume with 5a-cholest-7-en-3-one, 0.96; and 4-hydroxymethylene-5cu-4a-methyl-5cu-cholest-7-en-3-one, 1.2; 4,4,14a-trimethyl-dimethyl-5a-cholest-7-en-3@-ol also markedly produced a ste-

same preparations of rat liver microsomes. Addition of 4,4-

Several different steroids were incubated similarly with the observed with the 4,4-dimethyl sterol was constant for several

(Fig. 1) or 4,4-dimethyl-5a-cholest-7-en-3@-ol (Curue 2) were added. B, a-NADH oxidation. The velocity of a-NADH oxidation was measured in 2.50 ml of final volume with 1.67 mg of protein and 58 to 80 nmol of steroid. The concentration of steroid in the spectrophotometric assay generally was slightly lower to prevent changes in light scattering, which resulted when larger amounts of the Triton-containing suspension were added to the cuvette containing microsomal particles. Each value is the mean of results obtained from at least three assays.

for substrate-independent changes (i.e. Curues III minus I of Fig. 4).

These rates somewhat exceeded the expected rate of change for 4a-hydroxymethyl sterol metabolism, which would be approximately 1.5 to 2.0 nmol/min/mg of protein, and if the stoichiometry of Fig. 1 is assumed (4), these rates would be equivalent to 3 to 4 nmol of oxygen and a-NADH consumed. Several different steroids were incubated similarly with the 4,4-dimethyl sterol substrate, the steroid-dependent rate observed with the 4,4-dimethyl sterol was constant for several minutes of incubation. However, with the 4,4-dimethyl sterol substrate, the observed rates of oxygen and a-NADH consumption, relative to calculated rates of metabolism, were even greater than those observed with the 4a-hydroxymethyl sterol substrate (for 4,4-dimethyl sterol, about 0.16 nmol/min/mg of protein x 3, from Table I; see Refs. 1 and 2, and Fig. 1, also). Other known steroidal intermediates between lanosterol and cholesterol, as well as steroids that are not attacked by 4-methyl sterol oxidase (3, 13), were studied, too. For example, 4,4-dimethyl-5a-cholest-7-en-3-one and the corresponding 4C-unsubstituted-3-ketosteroid, 5a-cholest-7-en-3-one, which is the product of decarboxylation of the 4a-oic acid (9), were investigated. Addition of these 3-ketosteroids also elevated the rates of consumption of oxygen and a-NADH to between 0.7 and 1.0 nmol/min/mg of protein. With saturating amounts of various other steroids, constant rates of oxygen uptake and a-NADH oxidation were observed: 5a-cholest-7-en-3@-ol, 1.2; 4a-methyl-5a-cholest-7-en-3-one, 1.2; 4,4,14a-trimethyl-5a-cholest-7-en-3-one, 0.96; and 4-hydroxymethylene-5a-cholest-7-en-3-one, 1.1 nmol/min/mg of protein.

From these data, it was concluded that stimulation of oxygen and a-NADH consumption may be produced upon addition of either substrates or pseudosubstrates of methyl sterol oxidase. Within limits of experimental variation, the extent of stimulation was similar for all compounds tested, i.e. about 1 nmol/min/mg of protein measured under these conditions.

The nature of the stimulatory effect produced by nonspecific steroid addition has not been elucidated. Furthermore, selec-
tive inhibition of the enzymes responsible for this effect has not been possible. However, for the purpose of measuring stoichiometry of methyl sterol oxidase, several properties distinguished this process from methyl sterol oxidase. First, as pointed out above, the 3-ketosteroids that are not attacked by methyl sterol oxidase also produce the stimulation. Second, the K_m for sterol oxidase was consistently smaller for this nonspecific than for sterol oxidase by methyl sterol oxidase (Table II). As measured by oxidation of 14C-labeled substrate, the K_m for metabolism of the 4a-hydroxymethyl sterol by methyl sterol oxidase is about 12 µM (4), and the K_m for oxidative attack of the 4a-methyl sterol substrates is somewhat greater, about 35 µM (17). When the initial rates were measured for 4a-hydroxymethyl sterol, as shown in Figs. 4, 5, 6, and 7, K_m for sterol, when either oxygen uptake (16 µM) or 3,5-NADH oxidation (13 µM) were measured, was very similar to the value cited above for 14C-sterol metabolism (Table II). On the other hand, when the K_m of the effect was measured for the nonspecific, constant effect produced by addition of the 4,4-dimethyl sterol, the calculated values for K_m were as small as 10% of the K_m calculated for sterol metabolism, 3.4 versus 35 µM (Table II). Although K_m measurements for this effect were not conducted with all of the steroids studied above, this effect was maximal when about 5 µM sterol suspensions of any compound, such as the slowly metabolized 4a-methyl sterols with 3β-hydroxyl groups, were used. The K_m of the nonspecific effect produced by the ketosteroid, 4,4-dimethyl-5a-cholest-7-en-3-one, was approximately 40 µM, which was consistent with the very poor binding of this steroid to methyl sterol oxidase as a competitive inhibition of attack of the corresponding 3β-hydroxy sterol substrate (13).

Third, the extent of the nonspecific effect was not different when rats were fed cholestyramine; however, cholestyramine treatment consistently enhanced sterol metabolism about 100% (Table II). Fourth, these differences between nonspecific stimulation and metabolism could be observed with in the same experiments by allowing the incubation of the 4a-hydroxymethyl sterol to proceed beyond the initial period. For example, the rate of oxidation of a-NADH was measured over periods of 10 to 15 min after the addition of 4a-hydroxymethyl-5a-cholest-7-en-3β-ol substrate. After the initial velocity subsided, the rate of oxidation of a-NADH became constant and only slightly less than the rate observed when the 4,4-dimethyl

FIG. 5. Concurrent measurement of substrate-dependent oxygen uptake and a-NADH oxidation. A, oxygen uptake. The change in oxygen concentration was measured in 2.40 ml of final volume with 1.75 mg of microsomal protein from cholestyramine-induced rats. Eighty nanomoles of either 4a-hydroxymethyl-5a-cholest-7-en-3β-ol (Curue 1) or 4,4-dimethyl-5a-cholest-7-en-3β-ol (Curue 2) were added. B, a-NADH oxidation. The velocity of a-NADH oxidation was measured in 2.50 ml of final volume with 1.67 mg of protein and 58 to 80 nmol of steroid. The concentration of steroid in the spectrophotometric assay generally was slightly lower to prevent changes in light scattering, which resulted when larger amounts of the Triton-containing suspension were added to the cuvette containing microsomal particles. Each value is the mean of results obtained from at least three assays.
TABLE II

Steroid substrate-dependent changes in consumption of oxygen and a-NADH

Each value represents the mean of the number of samples indicated in parentheses. Values of $K_m$ were calculated from measurements with at least four concentrations of steroid substrate. Also indicated are standard deviations of means for four or more samples.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conc. of</th>
<th>Source of</th>
<th>Calc'd</th>
<th>Assay</th>
<th>a-NADH oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>steroid</td>
<td>microsomes</td>
<td>value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta$-Hydroxymethyl</td>
<td>4 to 25</td>
<td>cholestyramine</td>
<td>$K_m$</td>
<td>16 $\mu M$ (3)</td>
<td>13 $\mu M$ (3)</td>
</tr>
<tr>
<td>4,4-Dimethyl</td>
<td>1 to 10</td>
<td>cholestyramine</td>
<td>$K_m$</td>
<td>&lt;5 $\mu M$ (2)$^a$</td>
<td>3.4 $\mu M$ (2)</td>
</tr>
<tr>
<td>$\Delta$-Hydroxymethyl</td>
<td>20</td>
<td>control</td>
<td>v</td>
<td>3.62 ± 0.80 (4)</td>
<td>4.02 ± 1.20 (4)</td>
</tr>
<tr>
<td>$\Delta$-Hydroxymethyl</td>
<td>20</td>
<td>cholestyramine</td>
<td>v</td>
<td>6.25 ± 1.13 (4)</td>
<td>8.13 ± 1.93 (4)</td>
</tr>
<tr>
<td>4,4-Dimethyl</td>
<td>20</td>
<td>control</td>
<td>v</td>
<td>2.30 (2)</td>
<td>2.43 (3)</td>
</tr>
<tr>
<td>4,4-Dimethyl</td>
<td>20</td>
<td>cholestyramine</td>
<td>v</td>
<td>1.96 (3)</td>
<td>2.27 (3)</td>
</tr>
</tbody>
</table>

$^a$One value of $K_m$ was calculated from each of two separate experiments; however, in one sample, low concentrations of the $\Delta$-hydroxymethyl sterol substrate were used rather than the 4,4-dimethyl sterol substrate. In each case, however, the nonspecific effect of steroid addition was saturated with low concentrations of substrate: 2.1 and 5.0 $\mu M$ were obtained for values of $K_m$ for the $\Delta$-hydroxymethyl sterol and 4,4-dimethyl sterol, respectively.

$^b$Velocity measurements were calculated as nmoles/min/mg protein.

Sterol substrate was added initially. A similar measurement of oxygen consumption was achieved by delaying scale expansion for recording of changes in oxygen uptake until the initial, rapid metabolism of the 4a-hydroxymethyl sterol ceased.

In addition to these distinguishing features, the nonspecific effect was constant throughout the entire period of incubation, whereas rapid initial rates of metabolism were observed with the 4a-hydroxymethyl sterol.

Because the nonspecific effect was maximal when low concentrations of steroid were incubated (Table II) and the nonspecific effect was constant, the apparent rates of consumption of $\alpha$-NADH and oxygen could be corrected accordingly (Fig. 5, A and B, Curve 1 minus 2). The corrected rates of $\alpha$-NADH oxidation and oxygen uptake were stoichiometrically equal. The extent of correction was never more than that shown in Fig. 5, A and B for 2 min of incubation. Finally, the observed and expected rates of $\alpha$-NADH oxidation and $\alpha$-NADH-dependent oxygen uptake were identical.

Several attempts were made to eliminate selectively the nonspecific effect while maintaining maximal rates of methyl sterol oxidation. Partial proteolysis (18), partial lipolysis (19), and extraction of microsomes with strong solutions of Triton (17) have been used to eliminate pyridine nucleotide-dependent microsomal processes that accompany methyl sterol oxidation. Elimination was not achieved. Selective inhibition of the nonspecific effect was attempted with equally unsuccessful results.

The time-dependent consumption of oxygen and $\alpha$-NADH were readily measured under conditions of assay of initial rate changes (Figs. 4 and 5, A and B). In addition, initial velocity measurement with various amounts of protein demonstrated that velocity was directly proportional to the amount of protein added (Fig. 6). Velocities were proportional for both the metabolism of 4a-hydroxy methyl sterol substrate (metabolism) and the 4a-methyl sterols (nonspecific effect), as shown in Fig. 6. Finally, the methyl sterol oxidase was readily saturated by sterol substrate, and lower concentrations (Fig. 7) yielded linear relationships for calculation of kinetic constants reported above (Table II), and treatment with bile acid sequestrant produced only changes in velocity (Fig. 7). Thus, within these limits of assay, appropriate conditions for assay of initial velocity changes were established, and stoichiometry was studied.

Concurrent Measurements of Oxygen Uptake, $\alpha$-NADH Oxidation, and Steroid Oxidation—All measurements for calculation of stoichiometry were carried out concurrently with superimposition of at least one independent variable, such as either the length of incubation, the amount of protein, or cholestyramine resin pretreatment conditions, to minimize fortuitous selection of conditions. All measurements were completed within a few hours of preparation of microsomes generally obtained from cholestyramine-fed rats. Stoichiometry of formation of CO$_2$ and 3-ketosteroid from the 4a-oic acid intermediate has been established (9, 13), and absence of other steroidal transformations within the short period of incubation.
under these conditions has been demonstrated (4, 13). Changes in the rates of oxygen and α-NADH consumption were corrected for the nonspecific effect produced by addition of the steroid, as shown in Figs. 4 and 5. Unless otherwise stated, unlabeled 4,4-dimethyl-5α-cholest-7-cholest-7-en-3β-ol was used as the steroid for these corrections.

Variation of the length of incubation from 30 s to 2 min yielded very similar time-dependent changes in concentrations of α-NADH and oxygen (Fig. 8). Furthermore, the time course of α-NADH oxidation was measured at two different concentrations of protein, and oxygen consumption was measured both by final addition of either α-NADH or steroid. In addition, formation of 14CO2 from steroid was measured with both the one-step and two-step incubation procedures described under “Experimental Procedure.” The rates of consumption of both oxygen and α-NADH were essentially 2 times the rate of steroid oxidized after all intervals of incubation.

From 0.5 to 2.4 mg of protein were incubated with substrate for 2 min. Compared to either steroid oxidized or α-NADH consumed, somewhat greater variation in the measurement of oxygen consumption was found (Table III). The same stoichiometric relationship of approximately 2 eq each of oxygen and α-NADH consumed for each hydroxymethyl group of steroid oxidized was observed.

Measurement of stoichiometry was carried out in a third set of experiments, in which the microsomes were washed with Triton as described previously to eliminate endogenous β-NAD+ (17) and any possible β-NADH-dependent variation in oxygen uptake rates, which, of course, is quite rapid (19). The amount of protein incubated was adjusted as shown in Table IV. In addition, 4,4-di[30,31-14C]methyl-5α-cholest-7-ene-3β-ol was substituted for the unlabeled 4,4-dimethyl sterol, which had been used to measure the extent of the nonspecific stimulation. Thus, in this experiment, simultaneous correction for the slow rate of metabolism of the 4α-methyl group of the 4,4-dimethyl sterol, as well as the nonspecific effect, was attempted. Once again, 2 eq each of α-NADH and oxygen were consumed for each equivalent of hydroxymethyl group oxidized (Table IV). Experimental variation was reduced somewhat by the Triton treatment. The same rates were obtained when pyruvate and lactic dehydrogenase were added (Table IV; Ref. 7). The additional correction in the rates of observed oxygen consumption and α-NADH oxidation due to metabolism of the 4α-methyl group of the “control” sterol was very small during the short incubation; <0.4 nmol/min/mg of protein of the stimulation produced by adding the steroid was associated with metabolism if consumption of 2 eq each of α-NADH and oxygen was assumed, as shown in Fig. 1 (see also Table I).

Accordingly, maximal correction for metabolism of the 4α-methyl sterol used as control thus reduced oxygen uptake and α-NADH oxidation to 4.0 and 3.6 nmol/min/mg of protein, respectively.

Methyl sterol oxidase is inhibited by cyanide (1). When 1.25 mM cyanide was added either to 1.2 or 2.4 mg of microsomal protein incubated as described in Table IV, complete inhibi-

![Fig. 7. Effect of 4α-hydroxymethyl-5α-cholest-7-en-3β-ol concentration on steroid dependent uptake of oxygen by microsomes from control (O—O) and cholestyramine-fed (●—●) rats.](http://www.jbc.org/content/258/15/7165/F7.large.jpg)

**Fig. 7.** Effect of 4α-hydroxymethyl-5α-cholest-7-en-3β-ol concentration on steroid dependent uptake of oxygen by microsomes from control (O—O) and cholestyramine-fed (●—●) rats.

![Fig. 8. Time course of measurements of stoichiometry. Measurements included oxygen uptake when α-NADH was added last (O—O) and steroid was added last (●—●); α-NADH oxidation with 2.43 mg of protein (▲—▲) and 1.82 mg of protein (△—△); and formation of 3β-hydroxy-5α-cholest-7-ene-3α-ol from 4α-hydroxymethyl-5α-cholest-7-en-3β-ol assayed by the one-step (□—□) and two-step (■—■) incubation procedures. For comparison, all values have been normalized to 2.0 mg of protein and corrected for the nonspecific effect as described in Fig. 5. Fifty nanomoles of steroid substrate were used throughout.](http://www.jbc.org/content/258/15/7165/F8.large.jpg)

**Fig. 8.** Time course of measurements of stoichiometry. Measurements included oxygen uptake when α-NADH was added last (O—O) and steroid was added last (●—●); α-NADH oxidation with 2.43 mg of protein (▲—▲) and 1.82 mg of protein (△—△); and formation of 3β-hydroxy-5α-cholest-7-ene-3α-ol from 4α-hydroxymethyl-5α-cholest-7-en-3β-ol assayed by the one-step (□—□) and two-step (■—■) incubation procedures. For comparison, all values have been normalized to 2.0 mg of protein and corrected for the nonspecific effect as described in Fig. 5. Fifty nanomoles of steroid substrate were used throughout.

***Table III***

<table>
<thead>
<tr>
<th>Conc. of protein</th>
<th>Oxygen consumption</th>
<th>α-NADH oxidation</th>
<th>Steroid oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>nmoles/min/mg protein</td>
<td>nmoles/min/mg protein</td>
<td>mg2 nmoles/min/mg protein</td>
</tr>
<tr>
<td>2.00</td>
<td>4.7 (b)</td>
<td>4.9 (b)</td>
<td>2.42</td>
</tr>
<tr>
<td>2.00</td>
<td>4.7</td>
<td>4.3</td>
<td>2.42</td>
</tr>
<tr>
<td>1.50</td>
<td>5.9</td>
<td>4.5</td>
<td>1.82</td>
</tr>
<tr>
<td>1.00</td>
<td>3.8</td>
<td>4.4</td>
<td>1.31</td>
</tr>
<tr>
<td>0.50</td>
<td>4.6</td>
<td>4.6</td>
<td>0.60</td>
</tr>
<tr>
<td>Average</td>
<td>4.7 (5)</td>
<td>4.5 (5)</td>
<td>2.1 (8)d</td>
</tr>
</tbody>
</table>

a The same protein suspensions were used for measurement of α-NADH oxidation and steroid metabolism.

b Duplicate rates were measured with samples of unlabeled substrate, as well as samples of the same labeled substrate that was used for measurement of steroid oxidation.

c Average of rate data from time-course experiments in which conversion to the 4α-ocic acid was measured with the two-step incubation procedure (see "Experimental Procedure").

d When α-NADH was omitted in the coupled assay, the average rate observed was 0.05 nmoles/min/mg of protein.
The effect of variation in protein concentration was measured with microsomes from cholestyramine-fed rats as described in Table III, except that the microsomes were first washed with Triton as described earlier (17).

Table IV

<table>
<thead>
<tr>
<th>Oxygen consumption</th>
<th>a-NADH oxidation</th>
<th>Steroid oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. of protein</td>
<td>Velocity</td>
<td>Conc. of protein</td>
</tr>
<tr>
<td>mg</td>
<td>nmoles/min/mg protein</td>
<td>mg</td>
</tr>
<tr>
<td>1.26</td>
<td>4.27 (2)</td>
<td>3.91</td>
</tr>
<tr>
<td>2.56</td>
<td>4.46 (2)</td>
<td>3.79</td>
</tr>
<tr>
<td>Average</td>
<td>4.36 (5)</td>
<td>4.02</td>
</tr>
</tbody>
</table>

The same protein suspensions were used for measurements of a-NADH oxidation and steroid metabolism.

One sample was incubated with the one-step procedure and one with the two-step procedure. When a-NADH was omitted, the average rate was <0.02 nmoles/min/mg of protein. When NADH was omitted during the second step of the two-step procedure, the calculated rate was 0.06 nmoles/min/mg of protein. Thus, the endogenous NADH had been removed from these samples of microsomes by the Triton treatment, as demonstrated earlier (17).

The nonspecific effect may be the result of an unrelated microsomal process, such as lipid peroxidation, which is known to occur in rat liver microsomes (21). Alternatively, the observations are somewhat parallel to those published earlier in this laboratory (22), from which it was concluded that certain microsomal oxidases may function both as hydroxylases and oxidases. Indeed, others have suggested that the addition of substrate may affect the balances between these two activities (23), and, as shown in the present results, Kᵢ constant velocities, nonstimulation by cholestyramine, and other properties may be used to distinguish soluble oxygenases (24, 25). It is interesting that with all steroids tested in the present study, the nonspecific stimulation of oxygen consumption and a-NADH oxidation were stoichiometrically 1/1 (e.g. see Fig. 5).

Alternatively, a very slow rate of formation of steroid hydroperoxide (26) may occur under these incubation conditions. Since all steroids tested had Δ¹-double bonds, and essentially identical rates of nonspecific oxidation were observed for all steroids tested, steroid radical formation, air oxidation (26), and a-NADH-dependent reduction of resulting hydroperoxide must remain as one possibility to explain the 1/1 consumption of oxygen and a-NADH. It is obvious that heretofore this slow, nonspecific effect has not been observed, since substitution of either β-NADII or NADPII for a-NADH completely masks the very small increments observed in the nonspecific effect.

With the stoichiometry established, further work on the nature of the oxidase is now very greatly facilitated by applica-

A preliminary report of some of these results has been made (27).
tion of fast and precise assays of 4α-hydroxymethyl sterol-dependent changes in either α-NADH or oxygen concentrations.

Acknowledgments—The authors acknowledge the very careful preparation of the steroid substrates by Dr. Thomas Mattingly who also synthesized 4α-hydroxymethyl-5α-cholestan-3β-ol nonenzymically and compared the properties of this synthetic 4α-hydroxymethyl steroid to the 4α-hydroxymethyl steroid of combined enzymic and nonenzymic synthesis that was used in this study. Details of the nonenzymic synthesis will be reported elsewhere. During the course of these studies (3), cholestyramine resin has been the generous gift of the Mead Johnson Co.

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