Investigation of the Rate-determining Microsomal Reaction of Cholesterol Biosynthesis from Lanosterol in Morris Hepatomas and Liver

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SUMMARY

Previously, we reported that the properties of microsomal 4-methyl sterol demethylase isolated from liver and Morris hepatomas 5123C and 7777 are grossly similar. The individual enzymic steps of this multicomponent system have now been studied, and the rate-determining step has been determined and shown to be identical for liver and these hepatomas.

The rates of microsomal oxidative attacks of the 4α-methyl, 4α-hydroxymethyl, and 4 α-aldehydic groups are similar for microsomes prepared from rat liver and hepatoma 7777. The rates of mixed-function oxidative attack appear to increase in the order: —CH3 < —CH2OH < —CHO. Furthermore, the hepatic and hepatoma NAD-dependent decarboxylase, which catalyzes the reaction following the third oxidative attack, is similar in properties and velocity. The fifth step, an NADPH-dependent reduction of the 3-ketosteroid that is produced by decarboxylation, is also similar. For both tissues, the latter two reactions, under in vitro conditions, proceed at rates that exceed the initial oxidative process. Thus, for elimination of both of the 4-methyl groups of lanosterol, the 10 individual reactions catalyzed in this multicomponent system are identical in liver and hepatoma 7777 microsomes, and the rate-determining step for both liver and hepatoma is the initial oxidative attack on the 4α-methyl group of cholesterol precursors.

When the rate-determining reaction of both liver and hepatoma 7777 microsomes is assayed at different temperatures, the same activation energies and the same characteristic breaks in the Arrhenius plots are observed. Thus, for both liver and hepatoma, both the nature and the site of rate determination in this multienzymic system must be similar.

Since the microsomal enzymes of liver and hepatoma appear to be catalytically similar and rate determination appears to be similar, too, the characteristic lack of response of tumor microsomes to treatments in vivo that alter host liver microsomal demethylation activity suggests that the insensitivity of these tumors to dietary cholesterol should not be ascribed to alterations in the catalytic proteins. Evidence in this report suggests that the postmicrosomal supernatant fraction of both liver and hepatoma contains a cytosolic protein that may participate in the regulation of the rate-determining attack of 4α-methyl sterol substrates. Thus, either qualitative or quantitative differences between the postmicrosomal supernatant fractions obtained from liver and hepatomas may account for the observed differences in rates of cholesterol biosynthesis.

INTRODUCTION

Rat liver microsomes contain enzymes that catalyze the oxidative demethylation of lanosterol and other 4-methyl sterols (e.g., 4α-methyl-5α-cholest-7-en-3β-ol and 4,4-dimethyl-5α-cholest-7-en-3β-ol) to yield carbon dioxide from the 4α- and 4β-methyl groups (10, 23, 26, 27). The sequence of reactions of 4-methyl sterol demethylase3 has been established as an initial attack on the 4α-methyl group by a microsomal mixed-function oxidase, followed sequentially by 2 more mixed-function oxidations to yield the 4α-aldehydic and 4α-carboxylic acid products (25–27). After decarboxylation (31) and reduction (38) of the resulting 3-ketosteroid (23), the sterol biosynthetic intermediate is returned to the 3β-hydroxyl configuration that exists in lanosterol, cholesterol, and other 4α-substituted substrates. Although the nature of the supporting microsomal electron transport of the mixed-function oxidase has not been fully characterized, the stoichiometry of the mixed-function oxidase has been established (14).

In an earlier communication, Miyake et al. (28) reported that the rate of 4-methyl sterol oxidase in broken-cell preparations of Morris hepatoma 7777 microsomes was very low. These findings seemed somewhat paradoxical because intact hepatoma 7777 carries out rapid and efficient cholesterol biosynthesis (15); therefore, a systematic investigation of these terminal reactions of cholesterol biosynthesis in

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3 "Demethylase" is used in this report to describe the 10 reactions in the collective processes of monoxygenation, decarboxylation, and reduction, which, together, account for the conversion of each of the 2 4-methyl groups of lanosterol to carbon dioxide.
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Morris hepatomas was undertaken. Williams et al. (39) were able to obtain an active preparation of microsomal 4-methyl sterol demethylase when hepatoma 7777 was homogenized in 0.1 M PP₆ buffer. Grossly, the active hepatoma demethylase was found to be quite similar to the demethylases of control and host liver microsomes. In this report, we describe the assays of the individual enzymes of the hepatoma 4-methyl sterol demethylase and the determination and nature of the rate-limiting step of methyl sterol demethylase in both hepatic and tumor microsomes. In addition, this report contains results from initial studies on regulation of the rate of methyl sterol demethylase of liver and Morris hepatoma, as well as further study on the effect of PP₆ buffer substitution on Morris hepatoma 7777 (39) as it may relate to regulation.

MATERIALS AND METHODS

Male Buffalo rats (200 to 275 g) were inoculated with tumors as described previously (30). Unless otherwise stated, animals were fed ad libitum a stock diet of laboratory chow. Animals bearing the 7777 hepatoma were sacrificed 25 to 35 days after inoculation. All 5123C tumor-bearing animals were sacrificed 2 to 3 months after inoculation. Grossly visible hemorrhagic and necrotic material was removed before homogenization. Microsomal preparations were the same as those described previously (39). Since the choice of buffer was important for some assays, specific references to buffers will be made as needed; unless otherwise specified, 0.1 M potassium phosphate buffer and 0.1 M potassium PP₆ buffer (both pH 7.4 and containing 1 mM glutathione and 10 mM nicotinamide) were used for liver and tumors, respectively. Except where indicated, the assay procedure for methyl sterol demethylase was the same as that used previously (39). Anaerobic conditions were used to assay the decarboxylase (31) and 3-ketosteroid reductase (3, 38) to eliminate oxidative attacks of either substrates or products; stopped incubation flasks were flushed with nitrogen, and solutions of glucose (30 mg) and glucose oxidase (15 IU) were injected into the stoppered flasks.

4α-Hydroxyethyl-5α-cholest-7-en-3β-ol was incubated with 0.15 to 0.2 mg of microsomal protein and cofactors: NAD⁺ (0.71 μmole) and an NADPH generator (containing 19.3 μmoles of isocitrate, 0.65 μmole of NADP⁺, and 2 mg of isocitrate dehydrogenase). The final volume was adjusted to 0.5 ml. 4-Hydroxymethyl-5α-cholest-7-en-3-one (4) was incubated with 2 to 3 mg of microsomal protein and 0.34 μmole of NADPH (with generator) in a final volume of 0.5 ml. Assay of the 4α-carboxylic acid decarboxylase (31) was carried out with 1.51 μmoles of NAD⁺ and 50 μg of microsomal protein in a final volume of 0.5 ml. Assay of 3-ketosteroid reductase was carried out with NADPH was described earlier (38).

Substrate Preparation. 4,4-Di[30,31-14C]methyl-5α-cholest-7-en-3β-ol (240 dpm/nmole) was synthesized by the method described by Gautschi and Bloch (10, 27). The 4α-hydroxy[30-14C]methyl-5α-cholest-7-en-3β-ol (73 dpm/nmole) was synthesized by combined biosynthetic and non-enzymic steps, in which 3β-hydroxy-5α-cholest-7-en-4α[30-14C]oic acid (770 dpm/nmole) was produced from 4α-methyl-5α-cholest-7-en-3β-ol biosynthetically, and then the carboxylic acid was reduced with lithium aluminum hydride (25). For assay of the decarboxylase, the 3β-hydroxy-5α-cholest-7-en-4α[30-14C]oic acid was obtained and purified as described earlier (26, 31). The 4-hydroxy[30-14C]methylene-5α-cholest-7-en-3-one (117 dpm/nmole) was synthesized, purified, and characterized by Dr. T. W. Mattingly in this laboratory (4). Purity of all substrates was established by thin-layer and gas chromatography. All substrates were judged to be greater than 98% pure at the time of synthesis; after storage, purification by thin-layer chromatography was repeated before use. All of the above substrates (with the exception of the water-soluble carboxylic acid) were suspended in buffer with the aid of Triton WR-1339 (26) unless otherwise stated.

Special Incubation Conditions with Added Cytosolic Protein. When assays were carried out with protein obtained from the postmicrosomal supernatant fraction added to the usual microsomal incubation, labeled 4,4-dimethyl-5α-cholest-7-en-3β-ol of high specific activity (842 dpm/nmole) was dissolved in propylene glycol:acetone (1:1, v/v). Approximately 50 nmole of substrate were incubated for 20 min at 37° in a final volume of 2 ml with the indicated amounts of soluble protein from the postmicrosomal supernatant fraction. The flask contents were cooled, and between 1 and 2 mg of microsomal protein, 1.51 μmoles of NAD⁺, and 0.6 μmole of NADPH (with generator) were added. A 2nd incubation at 37° lasted for 30 min. Crude, soluble protein was prepared from approximately 100 ml of the postmicrosomal supernatant fraction of liver and tumors and homogenized in phosphate buffer by ammonium sulfate fractionation and differential heat treatment as follows. A fraction precipitating between 0 to 40% of saturation with ammonium sulfate was removed by centrifugation for 10 min at 10,000 × g and discarded; the resulting supernatant fraction was adjusted to 85% of saturation by the addition of solid ammonium sulfate (pH 7.4 was maintained by the addition of NH₄OH). The resulting precipitate was collected and suspended in 0.02 M phosphate buffer (pH 7.4); the solution was dialyzed overnight against 4 liters of the same buffer. For preparations from liver, the dialyzed fraction was heated rapidly to 80°, and the temperature was maintained for 1 min. After rapid cooling on ice, the suspension was centrifuged at 20,000 × g to remove the precipitated protein. The resulting clear, yellow supernatant fraction was concentrated by ultrafiltration (PM-10 membrane; Amicon Corp., Lexington, Mass.) to a final protein concentration of approximately 8 to 8 mg/ml. For similar preparations of crude, soluble proteins from tumor tissue, the heat treatment was carried out at 70° for 10 min; the resulting mixture required centrifugation at 105,000 × g for 30 min to achieve clarification. In a limited number of experiments, the crude, postmicrosomal supernatant fraction of tumors was treated with phospholipase A (1) to decrease the excessive amount of endogenous lipid before the addition of ammonium sulfate.

RESULTS

4-Methyl Sterol Oxidase. The rate of attack of the 4α-methyl sterol substrate was approximately equal for micro-
sommes obtained from host liver, hepatoma 7777, and hepatoma 5123C (Table 1). Although the rates of oxidation compare well with data previously reported for assays in which oxidase was coupled to decarboxylase (39), the measured specific activities of methyl sterol oxidase generally were slightly slower due to the necessary detergent extraction of endogenous pyridine nucleotide from microsomes (26). Methyl sterol attack was fully dependent upon the supply of cofactor (either NADH or NADPH) and oxygen (Table 1), as expected for mixed-function oxidative attack (24); furthermore, as has been reported for liver microsomes (13), the rate of oxidative attack was not depressed when incubations were carried out in a 9:1 (v/v) mixture of carbon monoxide and oxygen (data not shown).

Miller et al. (25) showed that the rate of oxidation was approximately 10-fold greater when the 4α-hydroxymethyl sterol substrate replaced the 4α-methyl sterol substrate. To establish appropriate conditions for initial velocity measurements, Chart 1A shows the time-dependent metabolism of the 4α-hydroxymethyl substrate by tumor 7777 microsomes, which were incubated with and without additional amounts of NADPH. There was no significant difference in activity if either NAD+ or NADH plus NADPH was used as cofactor. Thus, as was observed with liver microsomes (1), the endogenous rate of reduction of NAD+ by tumor microsomes was sufficient to support the attack of the 4α-hydroxymethyl sterol. Because the same oxygenase catalyzes the slower attack of the 4α-methyl group, rate determination could not be associated with electron flux to the terminal oxygenase, as has been suggested for mixed-solvent oxidation of drugs and other xenobiotics (15). Chart 1B shows the protein concentration curves for mixed-function oxidation of the hydroxymethyl substrate by tumor 7777 microsomes. Oxidative activity was proportional to protein concentration up to approximately 0.6 mg of microsomal protein per ml.

The rate of attack of the 4α-hydroxymethyl sterol substrate, measured under the optimal conditions established in Chart 1, was considerably faster than the rate of attack of the 4α-methyl sterol (Table 1). Furthermore, as reported earlier for methyl sterol substrates (28, 39), tumor 7777 microsomes prepared in 0.1 M phosphate buffer exhibited much less activity toward 4α-hydroxymethyl sterol, when compared to microsomes collected from homogenates prepared in PP buffer (1.6 versus 6.8; Table 1).

Microsomal oxidation by both tumors 7777 and 5123C yielded the same calculated Km for the hydroxymethyl substrate, 3.3 μM (Chart 2). The Km for the liver system was somewhat greater, 12 μM; however, Km for the liver system is in excellent agreement with that reported earlier (25).

Liver microsomal oxidation of the model 4α-aldehyde, 4-hydroxymethylene-5α-cholest-7-en-3-one, was established recently as a valid assay of methyl sterol oxidase (4). The assay is independent of decarboxylase because the substrate is 3-ketosteroid. However, because the substrate is

![Chart 1. Optimal conditions for metabolism of 4α-hydroxymethyl-5α-cholest-7-en-3β-ol. A, each flask contained approximately 0.15 mg of tumor 7777 microsomal protein, 0.76 μmole of NAD+, and 10 nmoles of substrate, incubated without (○) and with (×) the NADPH generator, in a final volume of 0.5 ml. B, same as in A, with varying amounts of protein incubated for 10 min. Each value is the average of duplicate results from 2 separate incubations.](image-url)

<table>
<thead>
<tr>
<th>Source of microsomes</th>
<th>Experimental variables</th>
<th>nmols/10 min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4,4-CH₃⁺</td>
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<tr>
<td>Host liver</td>
<td>Complete</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>Omit either cofactors or O₂</td>
<td>0.025</td>
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<tr>
<td>Tumor 7777</td>
<td>Complete</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>Omit either cofactors or O₂</td>
<td>0.01 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Substitute phosphate buffer</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>Tumor 5123C</td>
<td>Complete</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Omit either cofactors or O₂</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Substitute phosphate buffer</td>
<td>0.28</td>
</tr>
</tbody>
</table>

* Each incubation flask contained 5 mg of microsomal protein.

* Each incubation flask contained approximately 0.2 mg of microsomal protein.

* Each incubation flask contained 3 mg of Triton-treated microsomes (4, 26).

* The number of separate samples is indicated in parentheses. When results are averages of 4 or more samples, S.D.’s are reported.
a ketone and not a 3β-hydroxy sterol, overall rates of oxidative attack are slower (38) but easily compared within any experiment, either with microsomes from different sources or with incubations under different conditions (4). Metabolism of the hydroxymethylene substrate by host liver and tumor 7777 microsomes was fully dependent on NADPH and oxygen (Table 1). The time dependence of metabolism of the aldehydic substrate was constant through about 15 min of incubation (Chart 3A). The extent of metabolism was proportional to protein over a wide range of concentrations to about 8 mg/ml (Chart 3B). As was also observed with the 4a-hydroxymethyl sterol substrate, the rates of attack of the model aldehydic substrate were approximately equal for microsomes from host liver and tumor 7777 (Table 1).

Decarboxylase and 3-Ketosteroid Reductase. Both of these nonoxygenases of methyl sterol demethylase of liver microsomes exhibit higher specific activities than methyl sterol oxidase (31, 38, 40). Table 2 shows the velocities and cofactor requirements for sterol 4a-oic acid decarboxylase in tumor microsomes. The enzyme was NAD⁺ dependent, and, as with both the microsomal-bound and solubilized liver enzymes (31), the reaction proceeded equally well under aerobic and anaerobic conditions. Also, as shown for the liver enzyme, substitution of NADP⁺ for NAD⁺ resulted in a considerably slower rate of decarboxylation (Table 2).

Under optimal incubation conditions that have been established for liver microsomes (3, 38), the rate of reduction of the 3-ketosteroid intermediate by tumor 7777 microsomes was somewhat slower than the rate observed for liver microsomes (Table 3). Furthermore, the specific activities of these 2 nonoxygenases of methyl sterol demethylation were considerably larger than specific activities observed for methyl sterol oxidative attack on the 4a-methyl group by either liver or tumor 7777 (Table 3). Indeed, the specific activities of oxygenase only approached the rapid rates of decarboxylation and reduction after the initial formation of the 4a-hydroxymethyl intermediate (Table 1 versus Table 3).

Rate Determination. Methyl sterol substrate was incubated with microsomes from either normal liver or Morris hepatoma 7777. Substrate concentrations were varied from 20 to 50 µM for Lineweaver-Burk plots (39), and incubations were carried out at 20, 25, 30, 35, and 37°. Calculated values for maximal velocity were used to construct the Arrhenius plot shown in Chart 4. Both the liver and tumor methyl sterol oxidases exhibited biphasic responses, with a departure

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**Table 2**

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Activity (nmoles 14CO₂/10 min/mg protein)</th>
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<tbody>
<tr>
<td>None</td>
<td>0.58</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>15.8</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>5.6</td>
</tr>
<tr>
<td>NAD⁺⁺</td>
<td>12.8</td>
</tr>
</tbody>
</table>

* Anaerobic incubation conditions were used.

**Table 3**

Comparison of the activities of the different enzymic reactions in methyl sterol demethylase

<table>
<thead>
<tr>
<th>Source of microsomes</th>
<th>Oxidase</th>
<th>Decarboxylase</th>
<th>Ketoreductase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.70</td>
<td>10</td>
<td>4.58 ± 0.65</td>
</tr>
<tr>
<td>Tumor 7777</td>
<td>0.60</td>
<td>16</td>
<td>1.65</td>
</tr>
</tbody>
</table>

* Data obtained by Dr. J. T. Billheimer (e.g., see Ref. 3). For liver microsomes, results and S.E. from 6 experiments are reported; 4 separate experiments have been carried out with tumor microsomes. In each case, 4α-methyl-5α-cholest-7-en-3-one was used as substrate, since slower rates (probably due to steric hindrance) are observed with the 4,4-gem-dimethyl sterol substrate. Marked stimulation of the ketoreductase by the cytosolic protein is observed with both liver and tumor microsomes.

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**Chart 2.** Effect of substrate concentration on rates of 4α-hydroxymethyl steroid oxidation. Each flask contained 0.71 µmole of NAD⁺, 0.15 mg of microsomal protein, and the indicated concentrations of substrate. Each point represents the average of duplicate results from 2 separate experiments for normal liver (△), tumor 7777 (○), and tumor 5123C (□).

**Chart 3.** Optimal conditions for metabolism of 4-hydroxymethylene-5α-cholest-7-en-3-one. A, each flask contained approximately 2 mg of Triton-treated microsomes (4) from tumor 7777, 0.34 µmole of NADPH (with generator), and 50 nmoles of substrate in a final volume of 0.5 ml. B, same as in A, with varying amounts of protein incubated for 10 min. Each point is the average of duplicate results from 2 separate incubations.
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from linearity occurring at about 30°. The calculated values for activation energy (shown in Chart 4) were remarkably similar for both sources of enzymes in both parts of the biphasic response. Thus, these observations are consistent with the suggestion that differences between the multienzyme oxidase of liver and hepatoma microsomal membranes must be minimal with respect to the nature, as well as the site, of rate determination.

In contrast to liver, the methyl sterol oxidase system of hepatoma microsomes does not generally respond to stimulation and inhibition by treatments in vivo (39), such as dietary cholesterol. However, a 3-fold variation in velocity of the tumor oxidase can be obtained by selection of either phosphate or PP, buffer (see Ref. 39 and Table 1). Accordingly, the activity of methyl sterol oxidase in tumor microsomes prepared from broken-cell preparations in each buffer was studied further. Chart 5 shows the increment of stimulation produced by the addition of different amounts of a crude, postmicrosomal fraction of protein obtained from tumor 7777 homogenized in phosphate buffer. The cytosolic protein fraction produced much greater stimulation when demethylase activity was measured with microsomes prepared in phosphate versus PP, buffer. Possibly, the lower demethylase activity observed when tumor 7777 microsomes were prepared in phosphate buffer (Table 1; Ref. 39) may have been due, in part, to the retention of a protein component in the supernatant fraction.

The effect of addition of the protein fraction was further studied with microsomes from both liver and tumor 7777, in which various concentrations of methyl sterol substrate were incubated in the presence and absence of the crude protein fraction (Chart 6). Under assay conditions employed here, the crude protein fraction significantly lowered the K_m of the reaction for both liver and tumor systems; however, in each case, the V_max of the reaction was unaffected.

The effect of addition of the crude protein fraction was negated by trypsin proteolysis, prolonged heating, and repeated freezing and thawing. Similar stimulation has been observed with a protein that has been isolated and purified from the postmicrosomal supernatant fraction of normal liver (12).

DISCUSSION

In hepatomas, cholesterol biosynthetic rates, as modulated by the 1st rate-determining enzyme, HMG-CoA* reduc-

\[ HMG-CoA \rightarrow \text{Acetyl-CoA} \]

The abbreviation used is: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.
tase (EC 1.1.1.34), may not be subject to feedback inhibition that is associated with dietary cholesterol suppression of sterol biosynthesis in normal liver (5, 36, 37). Three possible explanations of these observations have been advanced by Sabine (33), who has summarized both the proposals and explanations of these observations have been advanced by Sabine's 1st and 2nd listed possibilities, which have been examined in our studies, are that (a) cholesterol may be synthesized by an alternate enzyme system in tumors and (b) an alteration in the rate-controlling enzyme(s) of cholesterol biosynthesis may be associated with carcinogenesis. Results reported earlier by us (39) suggested that the overall pathway in the terminal reactions of cholesterol biosynthesis is not different, and results in the present study demonstrate clearly that the individual enzymatic steps are catalyzed similarly in microsomes from liver and Morris hepatomas 7777 and 5123C. The attack on the 4α-methyl group of lanosterol and other 4-methyl sterol precursors of cholesterol is catalyzed by the multienzymic 4-methyl sterol oxidase (11, 26). The properties of the hepatic and hepatoma monooxygenase are strikingly similar (Table 1; Charts 1 to 3). Furthermore, 2 separate assays of methyl sterol oxidase yielded comparable results (Chart 3). Properties associated with the nonoxidative enzymatic steps of cholesterol synthesis appear to be similar in microsomes from liver and these hepatomas (Tables 2 and 3). Thus, for at least one-half of the 20 reactions of cholesterol biosynthesis from lanosterol, the enzymology in liver and hepatomas is very similar. These results are consistent with analogous studies of HMG-CoA reductase, which possesses similar properties in tumors and liver (6, 21). These results argue against Sabine's 1st alternative (Point a).

Activity of hepatic microsomal 4-methyl sterol demethylase parallels changes in the activity of microsomal HMG-CoA reductase (Refs. 11 and 29; J. T. Spence, A. C. Ross, C. M. Schleide, and J. L. Gaylor, unpublished observations). Kandutsch and Hancock (21) observed similarly that, in a spontaneous hepatoma of mice, rates of sterol synthesis from mevalonate, as well as from acetate, were elevated. Accordingly, the microsomal reactions, which are subsequent to mevalonate formation, might be expected to contain a 2nd rate-determining step and to respond to physiological and nutritional variations that affect HMG-CoA reductase. Examination of the rate-determining reaction(s) of methyl sterol demethylation suggests that 4-methyl sterol oxidase sets the overall rate of this multistep process, and it is the initial attack of the 4α-methyl group that is the slowest of the 3 oxidative steps (Tables 1 and 3). Since microsomal electron transport is common to each of the 3 oxidative steps (14, 24, 25), rate determination must be associated with attack of the 4α-methyl sterol substrate and not with electron transport, which has been suggested for rate determination of liver microsomal, cytochrome P-450-dependent oxidations (15).

Rate determination was studied further by investigating the Arrhenius plots of methyl sterol oxide activity in liver and hepatoma microsomes (Chart 4). The findings of similar activation energies, both below and above the same critical temperature (see Ref. 9 for an analogous, recent study of the effect of incubation temperature on liver microsomal oxidations), suggest that the function of the membrane in rate determination at the level of 4α-methyl attack is similar in liver and hepatoma. Although not all of the approximately 35 reactions of cholesterol synthesis from acetate have been studied in tumors and liver, these results tend to eliminate Sabine's 2nd proposed mechanism (33), that hepatoma and hepatic cholesterologenesis may differ in either the sites or the nature of rate determination.

The participation of soluble proteins in the microsomal reactions of cholesterol biosynthesis has been suggested for many steps (7, 8, 32, 34, 35). Preliminary evidence now indicates that a cytosolic protein may stimulate methyl sterol oxidase in tumor microsomes (Chart 5) as it does in liver (12). The crude preparation used in this work stimulated activity by enhancing affinity (decrease in the Km') without affecting the maximal velocity (Chart 6). Thus, under normal steady-state conditions of undersaturation, the overall velocity of cholesterologenesis in the tumor would be higher. Investigation of the nature of the protein from liver and hepatomas and a possible regulatory function of the protein must await purification and characterization.

The observed effects of a cytosolic protein relate quite closely to Sabine's 3rd alternative (33), that changes in the amount of an intracellular regulatory factor may distinguish hepatoma from hepatic cholesterologenesis rates. Furthermore, conflicting results from work in several laboratories on the relationship of cholesterol uptake to cholesterologenesis in normal and altered cells suggest possible quantitative differences in concentrations of intracellular cholesterol and/or cholesterol metabolites of either endogenous or exogenous origin that may act via such a regulatory component (2, 5, 17–20, 22, 33, 36, 37). As described above for microsomal demethylation, our observations are analogous to the suggestion of Kirsten and Watson (22) for HMG-CoA reductase, that malignant cells may, indeed, possess the same phenotypic enzymes and regulation as hepatocytes but differ quantitatively in the extent of association of cholesterol and/or cholesterol metabolites with a regulatory protein, which may alter enzymic rates either allosterically or by changes in the amounts of the catalytic proteins (2).

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