Properties of Purified Rat Hepatic 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase and Regulation of Enzyme Activity*

(Received for publication, August 6, 1979, and in revised form, November 16, 1979)

Peter A. Edwards,† Donna Lemongello, John Kane,§ Ishaiahu Shechter,¶ and Alan M. Fogelman ||

From the Division of Cardiology, Department of Medicine, University of California, Los Angeles, Los Angeles, California 90024 and the §Department of Medicine, University of California, San Francisco, San Francisco, California 94143

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase from rat liver microsomes has been purified to apparent homogeneity with recoveries of approximately 50%. The enzyme obtained from rats fed a diet supplemented with cholestyramine had specific activities of approximately 21,500 nmol of NADPH oxidized/min/mg of protein. After amino acid analysis a specific activity of 31,000 nmol of NADPH oxidized/min/mg of amino acid mass was obtained. The s20,w for HMG-CoA reductase was 6.14 S and the Stokes radius was .39 nm. The molecular weight of the enzyme was 104,900 and the enzyme subunit after sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 52,000. Antibodies prepared against the homogeneous enzyme specifically precipitated HMG-CoA reductase from crude and pure fractions of the enzyme.

Incubation of rat hepatocytes for 3 h in the presence of lecithin dispersions, compactin, or rat serum resulted in significant increases in the specific activity of the microsomal bound reductase. Immunotitrations indicated that in all cases these increases were associated with an activated form of the reductase. However activation of the enzyme accounted for only a small percentage of the total increase in enzyme activity; the vast majority of the increase was apparently due to an increase in the number of enzyme molecules. In contrast, when hepatocytes were incubated with mevalonolactone the lower enzyme activity which resulted was primarily due to inactivation of the enzyme with little change in the number of enzyme molecules.

Immunotitrations of microsomes obtained from rats killed at the nadir or peak of the diurnal rhythm of 3-hydroxy-3-methylglutaryl-CoA reductase indicated that the rhythm results both from enzyme activation and an increased number of reductase molecules.

The regulation of rat hepatic microsomal 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis (1), has been studied extensively by measuring changes in activity of the enzyme in intact rats (1-8) and in isolated hepatocytes (6, 8-10).

Such measurements do not indicate whether changes in enzyme activity result from alterations in the rates of synthesis and/or degradation of HMG-CoA reductase and/or from alterations in the catalytic activity of the enzyme. Detailed studies on the regulation of this enzyme are dependent on the availability of homogeneous preparations of HMG-CoA reductase and on antibody specific for the enzyme. A number of investigators have reported purification of the rat liver enzyme (4, 11-16); however, the specific activities of the pure enzyme varied from 6.0 to 19,600 nmol of NADPH oxidized/min/mg of protein and the molecular weight of the subunit is reported to be between 47,000 and 120,000.

We report here a simple method of purifying rat liver HMG-CoA reductase and the amino acid composition of the enzyme. We have prepared antibody in rabbits against the pure enzyme and have used immunotitrations to determine whether different catalytic forms of the enzyme can exist in the microsomal membrane of isolated rat hepatocytes. Using these techniques we have also re-investigated the nature of the circadian rhythm of HMG-CoA reductase in intact rats.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were obtained from the sources indicated: HMG-CoA, NADPH, CoASH, diithiothreitol, Triton X-100, phenylmethylsulfonyl fluoride, yeast glucose-6-phosphate dehydrogenase, Sigma; agarose-HMG-CoA (V), P-L Biochemicals. Compacctin (ML-236-B) was a generous gift from Dr. R. Fears (Beechams, United Kingdom) and Dr. A. Endo (Tokyo N6k6 University, Japan). Compacctin was converted to the anion as described by Endo et al. (17) and stored at pH 12.0 at -20°C. Cholesteryamine (Querat), Mead Johnson; sodium dodecyl sulfate, acrylamide, Bio-Rad; Ouchterlony plates, Hyland; formic acid, Fluka, A.G., Sepharose 6B, Pharmacia. The sources of all other materials have been previously given (4).

Animals—Rats were housed under a reverse illumination cycle (8) and had free access to food and water, and were, where noted, fed powdered rat food supplemented with 5% cholestyramine for 4 days before they were killed. Rat hepatocytes were prepared as previously described (6).

Solubilization and Assay of HMG-CoA Reductase—Unless otherwise stated, the activity of the microsomal bound reductase and the solubilized enzyme were determined by the radioassay and spectrophotometric assay, respectively, as previously described (18). Microsomes were routinely prepared by two centrifugations at 100,000 X g (19) and were routinely preincubated for 20 min at 37°C before assay. Under these conditions we found no evidence of the ¹⁴C compound that has been reported by Ness and Moffler (20) to elute as a contaminant with mevalonolactone from the AG1-X3 formate columns. Background values were the same for blanks assayed in the absence of either cofactor, glucose-6-phosphate dehydrogenase, or enzyme.

Spectrophotometric assays were carried out in Buffer A (0.2 M KCl, 0.16 M potassium phosphate, 0.004 M EDTA, and 0.01 M diithiothreitol, pH 6.8) together with 0.2 nmol NADPH and 0.1 nmol RS-HMG-CoA. The solubilization of the enzyme was as previously described (18) except that the microsomes were homogenized with

* The abbreviation used is: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase.
Buffer B (0.1 M KCl, 0.08 M potassium phosphate, 2 mM EDTA, 10 mM KH2PO4, pH 7.2) was diluted with an equal volume of glycerol. Subsequent dilutions were as previously described (18), except that Buffer B was used as the diluent.

**Purification of HMG-CoA Reductase—**The method is modified from that previously reported by Edwards et al. (4). However, the present method required only one affinity column and gave yields of approximately 50% and enzyme of specific activity similar to those already reported by Edwards et al. (4, 18) and Kleineke et al. (13). The reductase was purified by room temperature except where otherwise noted.

The solubilized enzyme was fractionated with solid ammonium sulfate; the protein precipitating between 35% and 50% ammonium sulfate was dissolved in Buffer B (pH 6.8), containing 30% glycerol and 1 M KCl at a protein concentration between 8 and 12 mg/ml. Aliquots (4 ml) were heated for 20 min at 55°C, diluted with an equal volume of Buffer B (pH 6.8), and centrifuged at 100,000 x g for 30 min. The supernatant was removed and the protein precipitating between 0% and 60% ammonium sulfate collected by centrifugation. The pellet was dissolved in a small volume of Buffer B (pH 6.8) and was routinely stored overnight at 4°C under nitrogen with no loss of activity. The enzyme was warmed at 37°C for 30 min, centrifuged at 100,000 x g for 45 min, and the supernatant containing all the enzyme activity was removed and diluted 1:1 with 5 mM dithiothreitol.

This solution was applied to an Agarose-Hexane-HMG-CoA affinity column (0.25 to 1.5 ml), the column washed with Buffer C (0.05 M KCl, 0.04 M potassium phosphate, 4 mM EDTA, 5 mM dithiothreitol, pH 6.8) until the A280 = 0 (approximately 12 column volumes), and the enzyme eluted in 5 column volumes of Buffer C containing 1 mM CoASH. The 50% and enzyme of specific activity (21,500) obtained at 65°C but in loss of 30% of enzyme activity within 8 min. Consequently, the CoASH concentration in the assay was 0.01 to 0.02 mM. Such concentrations of CoASH resulted in less than 5% inhibition of the reductase (Fig. 3). In contrast, inclusion of 10 mM compactin in the assay inhibited activity by greater than 98% (Fig. 3). Mevalonic acid, the natural product of HMG-CoA reductase, or its lactone derivative had no significant effect on reductase active at concentrations as high as 2 mM (Fig. 3) even though compactin is structurally

**Antibody Production—**Rat liver HMG-CoA reductase was purified to apparent homogeneity by the method described above. A total of 0.2 mg of purified protein was injected into each of two New Zealand rabbits; the first intradermal and subcutaneous injections (0.07 mg of protein) were carried out using constant amounts of enzyme activity and 1.0 ml of crude extract, then focal areas were injected with 1.0 ml of antibody at focal concentrations of 0.5% and 1.0% of antibody. Each 1.0 ml of crude extract was diluted 1:1-fold with 5 mM dithiothreitol. The reductase was purified at room temperature except where otherwise noted.

**Immunotitrations—**Unless otherwise stated, immunotitrations were carried out using constant amounts of enzyme activity and increasing amounts of antibody in the presence of 0.5% Triton X-100. This mixture was preincubated at 37°C for 30 min and the residual reductase activity was determined after addition of cofactors and [3H]HMG-CoA (18). The antibody used in the immunotitration studies was prepared against a purified enzyme previously published method (4). One enzyme unit is defined as the biosynthesis of 1 nmol of mevalonate/min. The equivalence point is defined as the number of enzyme units inactivated by 1.0 l of antibody. Each 1.0 ml of crude extract antibody contained 4.5 mg of protein. Polycrylamide gel electrophoresis and protein determinations were performed as previously described (4).

**Determination of Sc, and Stokes Radius of HMG-CoA Reductase—**The Sc, and Stokes radius was determined as described by Martin and Ames (22), using sucrose gradients of 5 to 20% in Buffer D or Buffer D (0.05 M KCl, 0.04 M KH2PO4, 0.03 M EDTA, 5 mM dithiothreitol, pH 7.2). The Stokes radius was determined by the method of Ackers (23) on Sepharose 6B in the presence of Buffer E (Buffer D containing 0.1 M sucrose).

**RESULTS**

**Purification of HMG-CoA Reductase—**Purification of HMG-CoA reductase by the method described is absolutely dependent on both the time and the concentration of the protein during the 65°C heat treatment (Fig. 1). If the 20-min heat treatment was carried out at protein concentrations between 5 and 12 mg of protein/ml, the enzyme in the supernatant could be purified to apparent homogeneity on an HMG-CoA affinity column (Table I; Fig. 2). The recovery of purified enzyme from the affinity column was 53 ± 3% (n = 8). This method of purification routinely gave enzyme of high specific activity (21,500 ± 2,800 nmol of NADPH oxidized/min/mg of protein, n = 5) and yields of approximately 50% from the microsomal fraction.

Purified enzyme was eluted from the HMG-CoA affinity column in a buffer containing 1.0 mM CoASH (Table I) and it was possible that the enzyme activity was partially inhibited by CoASH since inclusion of 1 mM CoASH in the assay inhibited enzyme activity by approximately 55% (Fig. 3). However, 5 to 10 µl of eluted pure enzyme were routinely assayed spectrophotometrically in a total volume of 0.5 ml. Consequently, the CoASH concentration in the assay was 0.01 to 0.02 mM. Such concentrations of CoASH resulted in less than 5% inhibition of the reductase (Fig. 3). In contrast, inclusion of 10 µM compactin in the assay inhibited activity by greater than 98% (Fig. 3). Mevalonic acid, the natural product of HMG-CoA reductase, or its lactone derivative had no significant effect on reductase active at concentrations as high as 2 mM (Fig. 3) even though compactin is structurally

**Fig. 1. Effect of heat on HMG-CoA reductase.** HMG-CoA reductase was solubilized from microsomal membranes obtained from cholesteryamine-fed rats, purified through the 35 to 50% ammonium sulfate step, resuspended in Buffer B, pH 6.8, containing 30% glycerol and 1.0 M KCl at 1.17 (A), 2.07 (B), 8.14 (C), and 12.14 (D) mg of protein/ml and placed in a water bath at 65°C. At the indicated times aliquots were removed, diluted with an equal volume of Buffer B, centrifuged at 100,000 x g for 30 min, and the specific activity of the soluble enzyme determined. The specific activity is given in nanomoles of NADPH oxidized/min/mg of protein. In each case the recovery of active enzyme was between 50 and 100%. Subsequent purification of the reductase to apparent homogeneity on HMG-CoA affinity columns was dependent on the protein concentration at the time of heating; when enzyme was heated at 4.07 mg of protein/ml, it was not possible to purify the enzyme on HMG-CoA affinity columns as judged by a low enzyme specific activity and multiple protein bands after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Heat treatment at 70°C of samples initially containing 8.14 mg of protein/ml resulted in no higher enzyme activities than obtained at 65°C but in loss of 30% of enzyme activity within 8 min and a 60% loss at 20 min.
Regulation of HMG-CoA Reductase

Purification of HMG-CoA reductase from cholestyramine-fed rats killed at the mid-dark point

Hepatic microsomes were prepared from 13 rats fed 5% cholestyramine for 4 days and killed at the midpoint of the 12-h dark cycle. The solubilized reductase activity was measured spectrophotometrically. The microsomal enzyme activity was determined by a radioassay and gave 3.4 nmol of mevalonic acid synthesized/min/mg of protein. This latter value was multiplied by the theoretical value 6.8 nmol of NADPH oxidized/min/mg of protein.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>Total reductase activity</th>
<th>Reductase specific activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>nmol NADPH oxidized/min</td>
<td>nmol NADPH oxidized/min/mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal suspension</td>
<td>2,728</td>
<td>18,500</td>
<td>6.8</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Soluble extract</td>
<td>520</td>
<td>24,480</td>
<td>47</td>
<td>132</td>
<td>6.9</td>
</tr>
<tr>
<td>35-50% (NH₄)₂SO₄</td>
<td>138.5</td>
<td>26,539</td>
<td>191</td>
<td>143</td>
<td>28</td>
</tr>
<tr>
<td>Heat 65°C</td>
<td>13.95</td>
<td>23,625</td>
<td>1,683</td>
<td>127</td>
<td>247</td>
</tr>
<tr>
<td>Agarose-HMG-CoA</td>
<td>0.483</td>
<td>10,037</td>
<td>20,778</td>
<td>54</td>
<td>3,056</td>
</tr>
</tbody>
</table>

FIG. 2. Analysis of HMG-CoA reductase purified from rat liver microsomes from animals fed the cholestyramine-supplemented diet. Fractions were analyzed on 5% acrylamide gels in the presence of sodium dodecyl sulfate: A, proteins solubilized from the microsomes; B, proteins applied to the agarose-HMG-CoA affinity column; C, purified HMG-CoA reductase. The bromophenol blue front is shown (F).

Phosphorylase on gels containing stacking gels and each protein bands were also observed with a second purified enzyme preparation of specific activity 22,100 nmol of NADPH oxidized/min/mg of protein. MVA, mevalonate.

FIG. 3. In vitro modulation of HMG-CoA reductase activity. HMG-CoA reductase obtained from rats fed the cholestyramine diet was purified through the heat step (open symbols) or through the affinity column (closed symbols). Standard spectrophotometric enzyme assays were carried out in the presence of CoASH (○), 3-hydroxy-3-methylglutaryl acid (●), mevalonic acid (■), mevalonolactone (△), or compactin (□). The 10-µl samples of purified enzyme assayed in this study contained 10 nmol of CoASH in the absence of any additions. The compactin concentration was 10 µM.

FIG. 4. Polyacrylamide gel electrophoresis of purified HMG-CoA reductase. The reductase was purified from animals fed the cholestyramine diet to a final specific activity of 24,750 nmol of NADPH oxidized/min/mg of protein and 40 µl (4.8 µg of protein) were mixed with 40 µl of 5 mM dithiothreitol and applied to a 5% acrylamide gel plus stacking gel as described by Maurer (24) for gel system No. 6 except that the buffers contained 25 mM β-mercaptoethanol and the gels were polymerized with riboflavin. After electrophoresis one gel was stained with Coomassie Blue R-250 and its companion gel cut into 0.3-cm lengths and assayed for HMG-CoA reductase activity with [3-¹⁴C]HMG-CoA as previously described (4) except that the assay was for 2.5 min. F represents the bromophenol blue front of the gel. The stacking gel is not shown. Two protein bands and two activity peaks were also observed with a second purified enzyme preparation of specific activity 22,100 nmol of NADPH oxidized/min/mg of protein. MVA, mevalonate.

Homogeneity of Purified Reductase—The purified protein gave a single band after sodium dodecyl sulfate-electrophoresis with an apparent molecular weight of 52,000 (Fig. 2). Purified HMG-CoA reductase was analyzed for homogeneity on 5% acrylamide gels in the absence or presence of stacking gels. Two discrete protein bands were observed after electrophoresis on gels containing stacking gels and each protein band was associated with HMG-CoA reductase activity (Fig. 4). In the absence of a stacking gel, the protein bands were more diffuse although enzyme activity was again associated with each band. If the amount of protein applied to these latter gels was low, only the major protein band, with the higher RF, was observed, although analysis of the gel for reductase activity showed two bands of enzyme activity.

aggregation of the 104,000-dalton form of the enzyme to a larger, but still active enzyme form, or to the presence of isoenzymes.

\[ \bar{s}_{20,w} \text{ Stokes Radius, Partial Specific Volume, and Molecular Weight of HMG-CoA Reductase—Sedimentation velocity experiments were performed at pH 6.8 using impure and purified preparations of enzyme obtained from animals fed either a normal diet or a diet supplemented with cholestyramine; the } \bar{s}_{20,w} \text{ value was 6.14 S for each preparation (Fig. 5) and corresponded to an apparent molecular weight of 104,000 for the nondissociated enzyme (Fig. 5A). Analysis of enzyme solubilized either by the method of Heller and Shrewsbury (12) or by the method of Edwards et al. (18), except that in the latter method the step involving treatment at 37°C for 60 min was omitted, also gave a } \bar{s}_{20,w} \text{ of 6.14, indicating that the method of solubilization and the treatment at 37°C did not significantly affect the } \bar{s}_{20,w} \text{ value or the apparent molecular weight of the active enzyme. In addition, the } \bar{s}_{20,w} \text{ value was unchanged when Buffer B in the sucrose gradient was replaced by a buffer of lower ionic strength (Buffer D), or if the enzyme was solubilized in the presence of 2 M phenylmethylsulfonyl fluoride. Taken together, these results demonstrate that under a variety of conditions of enzyme solubilization and for sucrose gradient centrifugation the } \bar{s}_{20,w} \text{ value remained unchanged at 6.14 S and corresponded to an apparent molecular weight of 104,000.}

The Stokes radius of HMG-CoA reductase was determined from chromatography on Sepharose 6 B by the method of Ackers (23). When yeast glucose-6-phosphate dehydrogenase was added to the sample of reductase applied to the column, the enzyme activities co-eluted (Fig. 6). The Stokes radius of yeast glucose-6-phosphate dehydrogenase was calculated from the data of Yue et al. (25, 26) and Cohn and Edsall (27) and shown to be 3.39 nm. The Stokes radius of HMG-CoA reductase calculated by the methods of Ackers (23) and Fish et al. (28) was 3.39 nm. The apparent molecular weight of HMG-CoA reductase calculated from the calibrated Sepharose 6B column was 104,000 (Fig. 6B).

The partial specific volume (\( \bar{v} \)) of HMG-CoA reductase was calculated from the chemical equation: \( \bar{v} = \frac{\text{mass of protein}}{\text{volume of protein}} \). The partial specific volume of cholestyramine-fed enzyme was calculated from the classical equation: \( \bar{v} = \frac{\text{mass of protein}}{\text{volume of protein}} \), where \( M \) is the molecular weight, \( \eta \), the viscosity of the medium (0.0106 poise); s, the sedimentation coefficient (6.14 \( \times 10^{-14} \) s); \( A \), the Stokes radius 3.39 nm; N, Avogadro's number; \( v \), the partial specific volume (0.738); and \( \rho \), the density of the buffer (1.0245). The molecular weight of HMG-CoA reductase was calculated to be 101,400.

Amino Acid Composition—The amino acid composition of homogeneous HMG-CoA reductase is given in Table II.

Cholesterol Content of Purified HMG-CoA Reductase—Analysis of the lipid content of purified reductase (19,000 nmol of NADPH oxidized/min/mg of protein) obtained from cholestyramine-fed animals showed the presence of 0.05 \( \mu \text{g} \) of cholesterol/mg of protein. No increase in reductase activity was observed after preincubation of purified or crude fractions of the reductase obtained from cholestyramine-fed rats with dispersions of phosphatidylcholine, phosphatidylserine, or phosphatidylglycerol at concentrations below 400 \( \mu \text{g} \) of phospholipid/0.6 ml assay (data not shown).

Inhibition of HMG-CoA Reductase by Nucleotides—The reaction catalyzed by HMG-CoA reductase results in the release of CoASH, NADP, and mevalonic acid. Neither mevalonic acid nor mevalonolactone were inhibitors of reductase activity (Fig. 3). However, CoASH and NADP inhibited enzyme activity at concentrations as low as 40 \( \mu \text{M} \) with \( K_i \) of 175 \( \mu \text{M} \) and 260 \( \mu \text{M} \), respectively (Fig. 7). In addition to the data

![Figure 5](image-url)

**Fig. 5.** Analysis of HMG-CoA reductase on sucrose gradients. Rats were fed a normal diet or a diet supplemented with cholesterol and killed at the peak of the diurnal rhythm. The reductase was solubilized from the microsomes (18) and either a 0 to 50% ammonium sulfate fraction collected and the pellet dissolved in a minimal volume of Buffer B (pH 6.8) or the enzyme was purified to homogeneity by the method described in Table I. Enzyme (100 \( \mu \text{l} \)) was applied to a 4.2 ml sucrose gradient (5 to 2%) in Buffer B (pH 6.8) and centrifuged for 12.5 h (A) or 17 h (B) at 42,500 rpm (235,500 \( \times g \) at 20°C) in a SW 56 rotor. Aliquots (98 \( \mu \text{l} \)) were removed after piercing the tube and assayed for reductase activity or protein. Aldolase (\( M = 161,000 \)), glucose-6-phosphate dehydrogenase (\( M = 100,000 \)), bovine serum albumin (BSA) (\( M = 68,000 \)), and ovalbumin (\( M = 43,500 \)) were used as standards. \( \bar{s}_{20,w} \text{ for glucose-6-phosphate dehydrogenase was 6.14 S (25, 26) and the value } \bar{s}_{20,w} \text{ for HMG-CoA reductase was determined by the method of Martin and Ames (22). The data from the 12.5-h centrifugation has been plotted as described by Martin and Ames (22). It represents the ratio of distance travelled from meniscus by standardistance travelled from meniscus by unknown. We have arbitrarily set the ratio for glucose-6-phosphate dehydrogenase (\( R^{2+} \)) to 1.0. The sample of reductase (0 to 50% (NH\(_4\))\text{SO}_4 fraction of enzyme solubilized from cholestyramine-fed rats) had a molecular weight of 104,000. B shows the activity peaks obtained after a 17-h centrifugation of HMG-CoA reductase. @, 0 to 50% (NH\(_4\))\text{SO}_4 fraction of enzyme from cholestyramine-fed rats; C, purified HMG-CoA reductase from cholestyramine-fed rats; X, 0 to 50% (NH\(_4\))\text{SO}_4 fraction obtained from rats fed a normal diet. The arrows indicate the position of the protein standards. Tube 1 represents the fraction at the top of the tube (meniscus).
shown in Fig. 7, CoASH was shown to be a noncompetitive inhibitor with respect to NADPH ($K_I = 1.05 \text{ mm}$) and NADP was a noncompetitive inhibitor with respect to HMG-CoA ($K_I = 1.72 \text{ mm}$). Preliminary results also indicated that NADH at 200 $\mu \text{M}$ was a noncompetitive inhibitor with respect to NADPH with a $K_I$ of approximately 2.05 mm. 2. Reductase activity was not affected by addition of 200 $\mu \text{M}$ NAD (data not shown).

Hill plots, obtained from enzyme assays in the absence or presence of 200 $\mu \text{M}$ NADP, gave a slope of 1, indicating the absence of negative cooperativity (data not shown).

Specificity of Antibody to HMG-CoA Reductase—Antibody raised in rabbits against purified HMG-CoA reductase gave precipitin lines against rat serum. Immunoelectrophoresis at pH 8.6 gave one precipitin line (Fig. 8A). No precipitin lines were observed against rat serum. Immunoelectrophoresis was carried out with CoA concentrations of 200 $\mu \text{M}$ of the circadian rhythm. The soluble enzyme was assayed spectrophotometrically without further treatment. In A the CoA concentrations were zero (A), 40 $\mu \text{M}$ (A), 100 $\mu \text{M}$ (B), or 200 $\mu \text{M}$ (C). The $K_m$ for

**Table II**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues/1000 residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>54.3</td>
</tr>
<tr>
<td>Histidine</td>
<td>16.4</td>
</tr>
<tr>
<td>Arginine</td>
<td>51.7</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>88.8</td>
</tr>
<tr>
<td>Threonine</td>
<td>53.2</td>
</tr>
<tr>
<td>Serine</td>
<td>68.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>113.4</td>
</tr>
<tr>
<td>Proline</td>
<td>53.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>99.5</td>
</tr>
<tr>
<td>Alanine</td>
<td>111.4</td>
</tr>
<tr>
<td>Half cystine</td>
<td>7.4</td>
</tr>
<tr>
<td>Valine</td>
<td>64.7</td>
</tr>
<tr>
<td>Methionine</td>
<td>22.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>49.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>101.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>26.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>16.4</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* ND, not determined.

In other preliminary experiments, isolated rat hepatocytes were incubated in the presence of [3H]leucine and the microsomes isolated. HMG-CoA reductase was solubilized, precipitated with antibody, and the precipitate analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Analysis of the gel for radioactivity indicated a single radioactive peak at approximately 52,000 (data not shown). Determination of the rates of synthesis and degradation of the reductase under different experimental conditions require specific antibody that will precipitate only the enzyme under investigation. We are currently utilizing the antibody characterized in this study to investigate the turnover of the reductase.

**Effect of Triton X-100 on Enzyme Activity and Immunotitration**—Interpretation of results obtained from immunotitration of the microsomal bound enzyme could be complicated if changes in membrane conformation affected the availability of the reductase for antibody. Such effects would be minimized if all the reductase enzyme were solubilized. However, routine solubilization of all the reductase is not presently possible; maximal solubilization occurs only after a concentrated suspension of microsomes are frozen and thawed and then warmed at 37°C for 90 min in a buffer containing 50% glycerol (18). Such a method was considered impractical for solubilizing all the enzyme present in the microsomal fraction obtained from 1.1 $\times 10^7$ hepatocytes. We have therefore determined conditions in which most of the other microsomal proteins are solubilized, since such conditions should minimize steric constraints on the antigen-antibody interaction.

Exposure of hepatic microsomes to a buffer containing 0.1%, 0.5%, or 1.0% Triton X-100 resulted in solubilization of up to 73% of the microsomal protein and less than 1.5% of the reductase (Table III). The activity of the microsomal enzyme assayed in the absence or presence of antibody was not affected by Triton X-100 at concentrations below 1.0% (Fig. 10; Table III). In other experiments HMG-CoA reductase was solubilized, after exposing microsomes to a buffer containing 50% glycerol (18). This latter method solubilizes approximately 10% of the membrane proteins and a high percentage of the reductase (18). Immunotitration of the glycerol-solubilized enzyme was not affected by inclusion of 0.5% Triton X-100 in the assay (data not shown).

We concluded that both the reductase activity and the reaction of the antibody with the antigen were not affected by
...the reductase. The percent inhibition of HMG-CoA reductase under "Experimental Procedures" and 20-pl aliquots were added to each well. Well 1, glycerol-solubilized enzyme, 3.1 units; well 2, 35 to 50% (NH4)2SO4 fraction, 87 units; well 3, 0 to 60% (NH4)2SO4 fraction, 58 units; well 4 and 5, pure HMG-CoA reductase, 32 and 16 units, respectively. One unit is defined here as the oxidation of 1 nmol of NADPH/min.

Immunoelectrophoresis of pure HMG-CoA reductase (well 1) and enzyme in the 35 to 50% (NH4)2SO4 fraction (well 2). Immunoelectrophoresis was performed at pH 8.6 by the method of Garvey et al. (29). The position of the cathode was at the right of the slide.

Triton X-100 under conditions where up to 60 to 75% of the membrane proteins were solubilized. Consequently, all subsequent immunotitration of the microsomal-bound enzyme were performed in the absence of 0.5% Triton X-100.

Effect of Hepatocyte Incubation Conditions on the Catalytic Activity of HMG-CoA Reductase—Incubation of rat hepatocytes for 3 h either under standard conditions or in the presence of compactin, lecithin dispersions, or rat serum resulted in increased specific activities of microsomal HMG-CoA reductase (Table IV, Fig. 11) (30, 31). The effect of compactin was maximal at 1.66 μM and at this concentration the microsomal reductase specific activity increased 8-fold after a 3-h cell incubation, compared to a 2.8-fold increase in controls (Fig. 11). In other experiments, preincubation of hepatocytes for 2% h with 0.1 to 20 μM compactin inhibited the incorporation of [2-14C]acetate into nonsaponifiable lipids during a subsequent 30-min incubation by over 95% (data not shown).

In agreement with earlier studies (8), the microsomal enzyme specific activity decreased after cells were incubated with high concentrations of mevalonolactone (Table IV).

In order to determine whether these changes in enzyme specific activity resulted from changes in either the amount and/or catalytic activity of the reductase, quantitative immunotitration were conducted with microsomes isolated from the cells. The data indicate that the equivalence point (units of enzyme activity inactivated/1.0 μl of antibody) increased after cells were incubated for 3 h under standard conditions or in the presence of compactin, lecithin dispersions, or rat serum (Table IV, Fig. 12).

![Fig. 8 (left). Immunodiffusion and immunoelectrophoresis of HMG-CoA reductase. A. Ouchterlony double immunodiffusion of the reductase. The center well contained 20 μl of anti-reductase IgG. Enzyme was purified from rats fed cholestyramine as described under "Experimental Procedures" and 20-pl aliquots were added to each well. Well 1, glycerol-solubilized enzyme, 3.1 units; well 2, 35 to 50% (NH4)2SO4 fraction, 87 units; well 3, 0 to 60% (NH4)2SO4 fraction, 58 units; well 4 and 5, pure HMG-CoA reductase, 32 and 16 units, respectively. One unit is defined here as the oxidation of 1 nmol of NADPH/min. Immunoelectrophoresis of pure HMG-CoA reductase (well 1) and enzyme in the 35 to 50% (NH4)2SO4 fraction (well 2). Immunoelectrophoresis was performed at pH 8.6 by the method of Garvey et al. (29). The position of the cathode was at the right of the slide.](image)

![Fig. 9 (right). Analysis of the antigen-antibody complex. An impure fraction of solubilized reductase (45 nmol of NADPH oxidized/min/mg of protein) containing 200 units of enzyme activity was precipitated with a 1.5-fold excess of antibody in the presence of 1% Triton X-100 and the washed precipitate was analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The relative positions and molecular weights of protein standards and the bromophenol blue front (F) are shown. We have previously demonstrated that immunotitrations of glycerol-solubilized reductase resulted in a linear decay of enzyme activity during inactivation of approximately 90% of the enzyme (4). However, addition of increasing amounts of antibody to enzyme bound to the microsomal membrane resulted in a linear decay of activity until approximately 50% of the enzyme had been inactivated (Fig. 12). The nonlinear decay after approximately 50% inhibition may be due to steric hindrance of enzyme activity by antibody which does not bind to the enzyme's active site. No evidence for two different decay curves was observed and the results are therefore not consistent with two or more populations of enzyme with different affinities for the antibody in the same microsomal membrane at one time. An increased equivalence point is consistent with the presence of an activated form of HMG-CoA reductase. However, in no incubation could the enzyme activation account for the increase in enzyme specific activity; for example, when cells were incubated in the presence of compactin, microsomal reductase specific activity increased 349% but the enzyme was activated only 157% (Table IV). Hence the major increase](image)
TABLE III
Effect of Triton X-100 on microsomal HMG-CoA reductase and microsomal proteins

Hepatic microsomes were prepared from animals fed a normal diet and killed at 12 noon. The reductase was assayed in the presence of antibody and in the absence of antibody to the reductase as described under "Experimental Procedures." Duplicate samples were incubated with Triton X-100 for 30 min at 37°C in the absence of antibody, centrifuged at 100,000 × g for 70 min, and the amount of microsomal protein and HMG-CoA reductase solubilized determined. The reductase specific activity (nanomoles of mevalonate synthesized/min/mg of protein) is given as a percentage of controls which were not treated with Triton X-100.

<table>
<thead>
<tr>
<th>Treatment of microsomes</th>
<th>Reductase specific activity</th>
<th>Reductase solubilized</th>
<th>Microsomal proteins solubilized</th>
<th>Equivalence point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
<td>100</td>
<td>0.4</td>
<td>&lt;2</td>
<td>0.14</td>
</tr>
<tr>
<td>0.5% Triton X-100</td>
<td>105</td>
<td>1.0</td>
<td>29</td>
<td>0.14</td>
</tr>
<tr>
<td>1.0% Triton X-100</td>
<td>105</td>
<td>1.0</td>
<td>57</td>
<td>0.14</td>
</tr>
<tr>
<td>2.5% Triton X-100</td>
<td>102</td>
<td>1.5</td>
<td>73</td>
<td>0.14</td>
</tr>
<tr>
<td>ND</td>
<td>91</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* ND, not determined.

FIG. 10. Inactivation of HMG-CoA reductase by antibody. Microsomal HMG-CoA reductase activity was determined after preincubation of the microsomes at 37°C in the presence of the indicated amount of antibody and in the absence (○) or presence of Triton X-100 at 0.1% (●), 0.5% (▲), or 1.0% (△). MVA, mevalonate.

in microsomal reductase specific activity is presumed to result from an increased number of reductase molecules. In the experiment with compactin we have calculated that a 22.2-fold increase in the number of reductase molecules would account for the observed increase in reductase specific activity (Table IV). This result is in agreement with results obtained in vivo (4) and is consistent with the presence of a partially inactivated (less active) form of the reductase after mevalonolactone treatment.

Effect of Sodium Fluoride on the Immunotitration of HMG-CoA Reductase—Nordstrom et al. have recently reported that the activity of HMG-CoA reductase in hepatic microsomes isolated in the presence of sodium fluoride is only 15% of that observed in controls and they have proposed that this lower level of enzyme activity may be the physiologically active form of the enzyme (3). It is not known whether reductase kinase, which is reported to inactivate HMG-CoA reductase (3, 32, 33), is inactive during the isolation of the microsomes in the presence of sodium fluoride and EDTA. The nature of the physiologically active enzyme has yet to be determined.

We have previously demonstrated that chaotropic agents, such as sodium fluoride, increase the solubilization of the

with mevalonolactone (Table IV). This result is in agreement with results obtained in vivo (4) and is consistent with the presence of a partially inactivated (less active) form of the reductase after mevalonolactone treatment.

Effect of Sodium Fluoride on the Immunotitration of HMG-CoA Reductase—Nordstrom et al. have recently reported that the activity of HMG-CoA reductase in hepatic microsomes isolated in the presence of sodium fluoride is only 15% of that observed in controls and they have proposed that this lower level of enzyme activity may be the physiologically active form of the enzyme (3). It is not known whether reductase kinase, which is reported to inactivate HMG-CoA reductase (3, 32, 33), is inactive during the isolation of the microsomes in the presence of sodium fluoride and EDTA. The nature of the physiologically active enzyme has yet to be determined.

We have previously demonstrated that chaotropic agents, such as sodium fluoride, increase the solubilization of the

with mevalonolactone (Table IV). This result is in agreement with results obtained in vivo (4) and is consistent with the presence of a partially inactivated (less active) form of the reductase after mevalonolactone treatment.

Effect of Sodium Fluoride on the Immunotitration of HMG-CoA Reductase—Nordstrom et al. have recently reported that the activity of HMG-CoA reductase in hepatic microsomes isolated in the presence of sodium fluoride is only 15% of that observed in controls and they have proposed that this lower level of enzyme activity may be the physiologically active form of the enzyme (3). It is not known whether reductase kinase, which is reported to inactivate HMG-CoA reductase (3, 32, 33), is inactive during the isolation of the microsomes in the presence of sodium fluoride and EDTA. The nature of the physiologically active enzyme has yet to be determined.

We have previously demonstrated that chaotropic agents, such as sodium fluoride, increase the solubilization of the
We have investigated whether the lower enzyme activity observed in membrane fractions isolated in the presence of sodium fluoride was due to the presence in the microsomes of a partially inactivated enzyme. We have also investigated whether the partially inactivated reductase cross-reacts with the antibody to HMG-CoA reductase.

The ratio of enzyme specific activities or equivalence points of membrane fractions isolated in the presence of sodium fluoride were similar, 9.0 and 10.2, respectively (Fig. 13). Similar results were obtained for animals killed at the peak or nadir of the rhythm (Table V) implying that the circadian rhythm of hepatic HMG-CoA reductase activity results from the enzyme molecules, but that each molecule of the enzyme has only 11% of the activity of controls. Our data also indicate that the partially inactivated enzyme species present after sodium fluoride treatment cross-reacts with the antibody prepared against enzyme purified from rats fed cholestyramine.

When microsomes were isolated in the presence of sodium fluoride the enzyme specific activity was, on average, 12.5% of controls (Table V). We hypothesized that, if the immunotitration results were valid, the enzyme purified from these sodium fluoride-treated microsomes has only 11% of the activity of controls. Our data also indicate that the partially inactivated enzyme species present after sodium fluoride treatment cross-reacts with the antibody prepared against enzyme purified from rats fed cholestyramine and killed at the peak of the diurnal reductase rhythm. We have used the purification procedure described in this paper to purify HMG-CoA reductase to apparent homogeneity from such animals; the pure enzyme had a specific activity of 3100 nmol of NADPH oxidized/min/mg of protein, a value approximating that predicted from immunotitration of the microsomal enzyme.

Activation of HMG-CoA Reductase during the Circadian Rhythm.—It has been generally accepted that the circadian rhythm of hepatic HMG-CoA reductase activity results from changes in the rate of synthesis of the enzyme (1). We have investigated the nature of the rhythm using immunotitration of the microsomal-bound enzyme.

The findings that absolute changes in the equivalence points for HMG-CoA reductase determined with either microsomal or crude solubilized enzyme correlated with similar changes in the specific activities of enzyme purified to homogeneity from animals killed at the peak or nadir of the rhythm confirmed our expectations. We believe the results and conclusions we have drawn from immunotitration of the microsomal-bound enzyme obtained either from liver cells or from animals killed at the nadir or peak of the circadian rhythm.

The specific activity of microsomal HMG-CoA reductase varied 3.7-fold between the diurnal low and diurnal high point.
The amino acid content of purified reductase differs from that previously reported by Heller and Shrewsbury (12). In the current study we have confirmed our previous finding (4) that the subunit molecular weight is approximately 52,000; a value significantly different from previous reports of 120,000 (12) or 65,000 (14) but similar to the value of 47,000 reported by Srikantaiah et al. (16). In addition we have demonstrated that after electrophoresis of purified enzyme on native acrylamide gels, enzyme activity and the protein bands co-migrated (Fig. 4). We also report a novel observation that enzyme purified by the present method could be dissociated on native gels into two active enzyme fractions. Taken together these data indicate that the present studies result in an essentially pure preparation of rat liver HMG-CoA reductase. Enzyme purified from animals fed a diet supplemented with cholestyramine contained about 0.05 µg of cholesterol/mg of protein. It is not known whether enzyme activity is regulated by enzyme-bound lipids.

A number of investigators have reported that the apparent molecular weight of impure samples HMG-CoA reductase, estimated from siev chromatography on Sephadex G-200 (4, 11, 12) or Agarose 0.5 m (34), was approximately 200,000. In agreement with Heller and Shrewsbury (12) we previously found that HMG-CoA reductase behaved in an anomalous manner on certain batches of Agarose 0.5 m chromatography (18); the nonspecific adsorption of the protein to certain batches of Agarose precludes the correct estimation of the enzyme molecular weight on these batches. In the present study we demonstrate unequivocally that the molecular weight of crude or pure HMG-CoA reductase determined from sucrose gradient centrifugation, chromatography on Sepharose 6B, or determined from the classical equation $M = 6mN/\alpha(1 - \alpha/\beta)$ was 104,000. Therefore the active enzyme appears to be a dimer containing 2 subunits of $M_r = 52,000$. We are currently investigating conditions in which enzyme aggregation may occur, to yield enzyme of molecular weight approximating 200,000. Previous estimations of the molecular weight of HMG-CoA reductase which gave values of approximately 200,000 (4, 11, 12, 34) were all determined on partially purified preparations of HMG-CoA reductase. It is possible that association of these impure samples of reductase with the other proteins originally found in the microsomal membrane would lead to the higher apparent molecular weights of the enzyme that have been observed.

Antibody raised against the pure protein gave one precipitin line against both pure and crude fractions of the reductase, after both Ouchterlony immunodiffusion and immunoelectrophoresis (Fig. 8). The protein precipitated by the antibody had a subunit of approximately 52,000 daltons (Fig. 9). These results indicate that the antibody preparation is specific for HMG-CoA reductase.

Validation of the use of immunotitration to probe for different activated forms of microsomal-bound reductase was provided both when cholestyramine feeding was shown to activate HMG-CoA reductase as judged by enzyme purification (4), immunotitration of solubilized enzyme (4), or immunotitration of microsomal bound enzyme (Table VI) and when addition of NaF to the microsomes resulted in decreased activity of the pure enzyme and corresponding changes in the immunotitration of crude enzyme. Valid interpretation of immunotitration data is dependent on two assumptions: that the affinity of different forms of the enzyme for antibody is similar and that different experimental conditions do not result in varying amounts of a totally inactive form of the reductase which continues to bind the antibody. The first assumption appears to be met for enzyme from rats fed a normal and cholestyramine-supplemented diet (Table IV) (4),

### Table VI

<table>
<thead>
<tr>
<th>Diet</th>
<th>Animals killed at</th>
<th>Reductase specific activity</th>
<th>Reductase equivalence point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol MVA/ min/mg protein</td>
<td>Microsomal enzyme</td>
<td>Soluble enzyme</td>
</tr>
<tr>
<td>Normal (5)</td>
<td>L-4</td>
<td>0.22 ± 0.08</td>
<td>0.20 ± 0.09</td>
</tr>
<tr>
<td>Normal (5)</td>
<td>D-6</td>
<td>0.81 ± 0.29</td>
<td>0.38 ± 0.08</td>
</tr>
<tr>
<td>Cholestyramine (4)</td>
<td>D-6</td>
<td>ND*</td>
<td>0.94 ± 0.10</td>
</tr>
</tbody>
</table>

* MVA, mevalonate.  
* ND, not determined.
and for enzyme isolated in the absence or presence of sodium fluoride (Fig. 13).

With these assumptions in mind the data are consistent with at least four different forms of HMG-CoA reductase: 1) the low equivalence point enzyme found after mevalonolactone administration to hepatocytes or after isolation of microsomes with sodium fluoride. We are currently investigating whether the same mechanism results in these relatively inactive forms of the enzyme. 2) The enzyme found in microsomes prepared from animals killed at the basal period of the rhythm; 3) enzyme in the livers of animals killed at the peak of the circadian rhythm or found in hepatocytes incubated under conditions which increased enzyme activity; and 4) enzyme found in microsomes isolated from animals fed cholestyramine.

As discussed above we cannot exclude the possibility that different experimental conditions result in differing percentages of totally inactive:active reductase and that the inactive enzyme is still capable of cross-reacting with the antibody.

An activated form of the reductase was present in the microsomal membrane after hepatocytes had been incubated in the presence of lecithin dispersions, or serum, or compactin (Table IV, Fig. 12). However, the mechanisms by which the latter three agents result in increased reductase activity is thought to be different. Lecithin is reported to promote the enzyme found in microsomes isolated from animals fed cholestyramine.

The findings that the reductase activity is inhibited by low (40 μM) concentrations of NADP or CoASH may indicate that such inhibition may be important in the physiological regulation of enzyme activity.

These studies emphasize that regulation of HMG-CoA reductase is a complex process and that a detailed understanding of these regulatory phenomena is dependent on the availability of homogeneous preparations of enzyme and on specific antibody that reacts only with the enzyme.

Acknowledgments—We are grateful for the excellent technical assistance of Show-Fung Lan. We express thanks to Dr. M. Haberland for useful discussions and to Dr. V. Schumaker for determination of the viscosity and density of Buffer D.

REFERENCES

Regulation of HMG-CoA Reductase