Cytoplasmic 3-Hydroxy-3-methylglutaryl Coenzyme A Synthase from Liver

PURIFICATION, PROPERTIES, AND ROLE IN CHOLESTEROL SYNTHESIS*

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SUMMARY

The cytoplasmic fraction of avian liver contains multiple forms of 3-hydroxy-3-methylglutaryl coenzyme A synthase which are distinct from their mitochondrial counterpart. Of the four cytoplasmic species, synthases I and II predominate, constituting 22 and 62%, respectively, of the total cytoplasmic synthase activity; synthases III and IV are minor forms comprising the remaining 16%. Each of the cytoplasmic synthases was purified to homogeneity from chicken liver and found to be a dimeric protein of approximately 100,000 daltons composed of identical molecular weight subunits. Despite this similarity the enzymes differ with respect to isoelectric point, elution behavior upon ion exchange chromatography, and migration upon disc gel electrophoresis. Synthase I, although not identical to the mitochondrial synthase, possesses several properties in common with it. These include immunochemical cross-reactivity, inhibition by MgCl₂, and dissociation to monomeric form by KCl. Cytoplasmic synthases II, III, and IV are immunochemically related but, unlike synthase I, do not cross-react with antisera against the mitochondrial synthase. Moreover, synthases II, III, and IV are activated by MgCl₂ and are not dissociated by KCl. Cytoplasmic synthase II is converted to the related species, synthases III and IV, by a nondialyzable cytosolic component. The livers of other avian species, e.g., pigeon and turkey, also possess multiple cytoplasmic 3-hydroxy-3-methylglutaryl-CoA synthases, as indicated by their cross-reactivity with antibodies against the chicken liver cytoplasmic synthases. All tissues of the chicken examined were found to contain cytoplasmic 3-hydroxy-3-methylglutaryl-CoA synthase; however, only liver and kidney possessed mitochondrial synthase. The tissue distributions of the cytoplasmic and mitochondrial synthases are compatible with a role of the cytoplasmic enzyme in cholesterogenesis and of the mitochondrial enzyme in ketogenesis, a pathway restricted to liver and kidney. Moreover, the cytoplasmic 3-hydroxy-3-methylglutaryl-CoA synthase activity of chicken liver is responsive to cholesterol feedback control, synthase activity being depressed by feeding cholesterol and elevated by cholestyramine feeding.

Unlike avian liver which contains multiple cytoplasmic forms of 3-hydroxy-3-methylglutaryl-CoA synthase, rat liver possesses a single cytoplasmic synthase species. Like hepatic cholesterogenesis in the rat, both the cytosolic thiolase and synthase of liver are subject to negative feedback control by cholesterol. Feeding cholesterol caused marked decreases in cytosolic thiolase and synthase activities which declined to levels 25 and 15% of normal, respectively. Feedback control by cholesterol appears to be specific for the cytoplasmic enzymes of 3-hydroxy-3-methylglutaryl-CoA synthesis, mitochondrial acetoacetyl-CoA thiolase activity being unaffected by cholesterol feeding. Dietary cholestyramine, which sequesters bile salts, preventing their intestinal reabsorption, and induces hepatic cholesterogenesis, promotes increases in both the cytoplasmic thiolase and synthase activities of liver. Fasting which also suppresses hepatic cholesterogenesis causes a substantial decrease in hepatic cytoplasmic synthase activity. Taken together, these results implicate cytosolic acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl-CoA synthase in hepatic cholesterogenesis in the chicken and rat.

Although cholesterogenesis and ketogenesis are diverse functions of liver requiring independent regulation, the first two steps of both pathways (Reactions 1 and 2) are chemically identical and lead to HMG-coenzyme A, a common intermediate (1-4).}

1 The abbreviations used are: HMG, 3-hydroxy-3-methylglutaryl; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

[The text continues with additional details and experiments related to the purification, properties, and role of cytoplasmic 3-hydroxy-3-methylglutaryl coenzyme A synthase in avian and rat liver.]
Acetoacetyl CoA + acetyl CoA

\[
\text{HMG-CoA} + \text{CoA} \quad \text{synthase}
\]

Thus, autonomy of cholesterol and acetoacetate synthesis might be expected to require control at a step in each pathway beyond the HMG-CoA branch point (5). Recently, however, it was reported (6-9) that liver possesses dual HMG-CoA generating systems, one cytoplasmic, to supply cholesterologenesis and another, mitochondrial, for ketogenesis. This, coupled with the fact that the enzymatic steps from HMG-CoA to cholesterol take place in the cytoplasm (10) while the conversion of HMG-CoA to acetoacetate appears to occur primarily, if not exclusively, in the mitochondrion (9), suggests that the two pathways function independently.

Compelling evidence now exists for the dual localization of acetoacetyl-CoA thiolase and HMG-CoA synthase in liver (9, 11, 12) and in yeast (13). In liver these enzymes comprise the HMG-CoA generating systems for cholesterologenesis and ketogenesis; both the cytoplasmic and mitochondrial forms of each enzyme can be distinguished by isoelectric focusing, as well as by other means (7, 8). The isolation, purification, and characterization of the mitochondrial HMG-CoA synthase in a recent report from this laboratory (9) and of the cytoplasmic synthase(s) in this communication provide rigorous proof for the existence of distinct mitochondrial and cytoplasmic forms in liver. In the present paper a number of factors known to alter hepatic cholesterologenesis also are shown to promote similar changes in cytoplasmic HMG-CoA synthase activity of avian and rat liver.

**EXPERIMENTAL PROCEDURE**

**Materials and Methods**

Coenzyme A (lithium salt) was purchased from P-L Biochemicals, Cuemid (cholesteramine resin) from Merck Sharp & Dohme, [1-14C]acetate anhydride from New England Nuclear, Hypatite C (hydroxylapatite) from Clarkson Chemicals, and tolune sulfonfyl fluoride from Eastman Kodak Co. Oleic acid and cholesterol were obtained from Nutritional Biochemicals, Amphocell using 1% sodium dodecyl sulfate-0.1, 2-mercaptoethanol was prepared through Step 6B of Table I; specific activity, 0.75 unit/mg of protein.

Unlabeled and [1-14C]acetate-CoA were prepared from the anhydride by the method of Simon and Shemin (14); acetoacetyl-CoA was similarly prepared except that diketene replaced the thiolase.

**Preparation of Rabbit Antiserum against Purified HMG-CoA Synthase—HMG-CoA synthase, 0.6 mg, (synthase III and IV phosphocellulose from Schleicher and Schuell.��性大鼠 (100 to 150 g) 被从 Charles River Breeding Laboratories 购买的 Purina Rat Chow (0.6 x 8.0 cm) of 7.5% acrylamide was conducted using a continuous buffering system of 50 mm Tris (KPO4), pH 8.5, or 50 mm Tris (glycine), pH 8.9. Samples were subjected to electrophoresis for 2 to 4 hours at 10° and 4 to 5 ma/column. Gels were stained with 0.3% Amido black in 7% acetic acid and destained by diffusion against 7% acetic acid-5% methanol solution. Disc gel electrophoresis in 1% sodium dodecyl sulfate-1% 2-mercaptoethanol was carried out as described by Weber and Osborn (19).

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Purification of HMG-CoA Synthase Species I to IV from Chicken Liver Cytoplasm

A single purification scheme is employed for the isolation of HMG-CoA synthases I to IV. Steps 1 to 7, outlined below and summarized in Table I, are those for the preparation of HMG-CoA synthases III and IV, the enzyme forms obtained in the highest yield by this procedure; Steps 8 and 9 are used for the purification of synthases I and II, respectively. To precipitate the enzyme from solutions having >1 mg of protein/ml, solid ammonium sulfate was added to 70% saturation at equilibrium. All solutions contained 0.1 mM EDTA and 2 mM mercaptoethanol unless specified otherwise and all operations were conducted at 4°C.

**Step 1 and 2: Cell-free Extract and Cytoplasmic Fraction**—Approximately 1.2 kg of chicken liver from laying hens were homogenized batchwise in 2 liters of buffer containing 0.25 M sucrose, 0.1 mM EDTA, and 20 mM Heps, pH 7.3, using a loose-fitting Potter-Elvehjem homogenizer with two passes of the motor-driven Teflon pestle. After dilution to 4 liters with the same buffer, the homogenate was passed through two layers of cheesecloth and the cellular debris, nuclei, and mitochondria were removed by centrifugation at 20,000 × g for 30 min; mitochondrial HMG-CoA synthase can be isolated from this loosely packed pellet (9). Following the addition of 50 ml of 40 mM toluene sulfonylfluoride (in 95% ethanol) to the supernatant to minimize proteolysis, a second centrifugation at 20,000 × g for 60 min was conducted to remove additional debris.

**Step 3: 30 to 45% Saturated Ammonium Sulfate Fractionation**—The cytoplasmic fraction was brought to 45% saturation with solid ammonium sulfate (277 g/liter) and was stirred for 30 min. Precipitated protein was collected by centrifugation and suspended in 2.5 liters of 30% saturated ammonium sulfate containing 50 mM potassium phosphate, pH 7.0, and 1 mM toluene sulfonylfluoride. After stirring for 30 min, the suspension was centrifuged and the supernatant solution was decanted. The supernatant solution (30 to 45% saturated ammonium sulfate fraction) was brought to 65% saturation with solid ammonium sulfate (250 g/liter), and the precipitated protein was recovered by centrifugation.

**Step 4: Calcium Phosphate Gel Fractionation**—The pellet from the preceding step was dissolved in 1 liter of 10 mM potassium phosphate, pH 7.0, containing 1 mM toluene sulfonylfluoride, and then was dialyzed against 50 liters of 10 mM potassium phosphate, pH 7.0. To the dialyzed enzyme was added 1.47 liter of calcium phosphate gel suspension (2.9 mg of gel dry weight/mg of protein), and the concentrations of potassium phosphate, pH 7.0, and toluene sulfonylfluoride were brought to 10 and 1 mM, respectively. After stirring for 30 min, the gel was recovered by centrifugation, and the supernatant solution was retained. The gel was eluted twice with 2.0 liters of 50 mM potassium phosphate, pH 7.0, and dialyzed overnight against 4 liters of the same buffer. The dialyzed enzyme (330 mg of protein) was applied to a DEAE-cellulose column (1.6 × 85 cm) and the column was eluted with 0.25 liters of 20 mM potassium phosphate, pH 7.0, followed by a 1 liter linear 20 to 250 mM potassium phosphate gradient; a typical elution profile is shown in Fig. 2A. Synthase I is eluted with 20 mM potassium phosphate and resolved from HMG-CoA synthases III and IV, which appear as a broad activity peak at 120 mM phosphate. Both activity peaks were pooled separately, and synthase activity was recovered by precipitation with ammonium sulfate. At this point synthase I is only partially purified; its further purification is described in Step 8. Although synthases III and IV which co-purify through the calcium phosphate gel filtration step (Fig. 3), the two forms can be resolved by phosphocellulose chromatography (Step 7).

**Step 5: Phosphocellulose Chromatography**—The precipitated protein from the preceding step was dissolved in 200 ml of 20 mM potassium phosphate, pH 7.0, containing 0.5 mM EGTA and 2 mM toluene sulfonylfluoride and then was dialyzed overnight against 12 liters of 20 mM potassium phosphate, pH 7.0. After removal of insoluble material by centrifugation, the enzyme solution (4.3 g of protein) was applied to a column (2.3 × 26 cm) of phosphocellulose, and the column was washed with 1.5 liters of 20 mM potassium phosphate, pH 7.0, prior to initiation of a 5-liter linear 20 to 300 mM gradient of the same buffer. Fig. 1 shows a typical elution profile in which two peaks of HMG-CoA synthase activity were resolved; the activity peaks were eluted separately, and the enzyme was recovered by ammonium sulfate precipitation. The “breakthrough” peak, i.e. that eluted at 20 mM phosphate, is synthase II, the further purification of which is described in Step 9, while the activity peak which appears at 120 mM phosphate is a composite of three synthase species, HMG-CoA synthases I, III, and IV.

**Step 6: Further Purification of HMG-CoA Synthases I, III, and IV by DEAE-cellulose Chromatography**—The precipitate containing synthases I, III, and IV from Step 5 was dissolved in 20 ml of 20 mM potassium phosphate, pH 7.0, and dialyzed overnight against 4 liters of the same buffer. The dialyzed enzyme (41 g of protein) was applied to a DEAE-cellulose column (1.6 × 85 cm) and the column was eluted with 0.25 liters of 20 mM potassium phosphate, pH 7.0, followed by a 1 liter linear 20 to 250 mM potassium phosphate gradient; a typical elution profile is shown in Fig. 2B. Synthase I is eluted with 20 mM potassium phosphate and resolved from HMG-CoA synthase III and IV, which appear as a broad activity peak at 120 mM phosphate. Both activity peaks were pooled separately, and synthase activity was recovered by precipitation with ammonium sulfate. At this point synthases I, III, and IV are resolved; each activity peak was pooled separately, and the “breakthrough” peak, i.e. that eluted at 20 mM phosphate, is synthase II, the further purification of which is described in Step 9. Following the addition of 50 ml of 40 mM toluene sulfonylfluoride (in 95% ethanol) to the supernatant to minimize proteolysis, a second centrifugation at 20,000 × g for 60 min was conducted to remove additional debris.

**Step 7: Resolution of HMG-CoA Synthases I, III, and IV by Phosphocellulose Chromatography**—The precipitated synthase III–IV fraction from Step 6 was dissolved in 10 ml of 20 mM potassium phosphate, pH 7.0, and dialyzed overnight against 4 liters of the same buffer. The dialyzed enzyme (40 mg of protein) was applied to a column (0.9 × 50 cm) of phosphocellulose and eluted with 100 ml of 20 mM potassium phosphate, pH 7.0, followed by a 1.0-liter linear 20 to 300 mM gradient of the same buffer. Synthase activity was eluted in two activity peaks, synthase IV at 80 mM phosphate, and synthase III at 110 mM phosphate. The yields for synthases III and IV were 2.7 and 3.2%, respectively, and the final specific activities were 1.0 and 0.69 unit/mg of protein, respectively (Table I).

**Step 8: Further Purification of HMG-CoA Synthase I by Hydroxyapatite Chromatography and Gel Filtration**—When 1.2 kg of liver were processed through Step 6 of the purification procedure, approximately 5 units of HMG-CoA synthase I with a specific activity of 0.65 unit/mg of protein were obtained. The precipitated synthase I from Step 6 was dissolved in 10 ml of 20 mM potassium phosphate, pH 7.0, and dialyzed overnight against the same buffer. The enzyme was applied to a hydroxylapatite column (2.3 × 26 cm) and eluted with a 0.6-liter linear 20 to 300 mM gradient of potassium phosphate, pH 7.0. The enzyme was eluted at 130 mM phosphate and recovered from the pooled fractions by inward dialysis of ammonium sulfate; synthase I, recovered from hydroxylapatite chromatography, had a specific activity of 0.19 unit/mg of protein.
RESULTS

Ion exchange chromatography (Figs. 1 and 2) and isoelectric focusing (results not shown) revealed a multiplicity of HMG-CoA synthase species in the cytoplasmic compartment of avian liver more complex than previously recognized (6). Thus, four species of HMG-CoA synthase, i.e. synthases I, II, III, and IV, were identified in the cytoplasmic fraction of chicken liver (Table II).

Using the purification scheme outlined above, the four synthases have been resolved and purified. The results are summarized in Table I.

Each of the cytoplasmic synthases was purified between 100- and 300-fold by the procedures outlined, the final specific activities ranging from 0.36 to 1.0 unit/mg of protein. Moreover, each enzyme was judged homogeneous by polyacrylamide gel electrophoresis, sedimentation velocity, and equilibrium centrifugation. Disc gel electrophoresis of each purified enzyme species using either Tris-glycine buffer, pH 8.9, or Tris-phosphate buffer, pH 8.5, yielded a single Amido black staining band. Shown in Fig. 3 are representative acrylamide gel patterns of preparations of synthase I, synthase II, and synthase III-IV mixture purified through Steps 8, 9, and 0B of the purification procedure (Table I). Measurement of HMG-CoA synthase activity on companion gels showed that enzymatic activity migrated coincidently with the stained protein bands. The results of such experiments with HMG-CoA synthases I and II were described in a preliminary communication (6). Furthermore, dodecyl sulfate acrylamide gel electrophoresis of purified HMG-CoA synthases I to IV by the method of Weber and Osborn (19) gave rise to a single Coomassie blue staining band in each case.

Equilibrium centrifugation of purified synthase II and III yielded linear plots (20) of natural logarithm of Rayleigh fringe displacement versus $r_2$ indicative of weight homogeneity of the preparations. Sedimentation velocity analysis with purified HMG-CoA synthase III revealed a single sedimenting boundary with $s_{20w} = 6.2$ S.

Properties of Purified Synthases

Molecular and Subunit Weights—In an earlier communication (6), we reported that cytoplasmic HMG-CoA synthases I and II had molecular weights of 52,000 and approximately 100,000, respectively, and subunit weights of 52,000 and 55,000, respectively. Thus, synthase I appeared to be a monomeric protein and synthase II, a dimer composed of identical or similar weight subunits. Thus, synthase I appeared to be a monomeric protein and synthase II, a dimer composed of identical or similar weight subunits; the molecular weight of synthase I was reassessed at a lower salt concentration (20 mM potassium phosphate, pH 7.0) and can be stored up to 3 months at -90° without loss of activity as pellets following precipitation by 90% saturated ammonium sulfate.

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Experiments conducted with resolved HMG-CoA synthase III and IV preparations also yielded single stained protein bands; however, the results are not shown since they were not photographically recorded.

Stability—Purified HMG-CoA synthase I is stable for 3 to 4 months when stored frozen in 40% glycerol containing 20 mM potassium phosphate, pH 7.0, and 5 mM diethiothreitol but is unstable at 4° in the same buffer without glycerol. HMG-CoA synthase II to IV are stable for several weeks at 4° in 20 mM potassium phosphate, pH 7.0, containing 5 mM diethiothreitol and can be stored up to 3 months at -90° without loss of activity as pellets following precipitation by 90% saturated ammonium sulfate.

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Table II

Molecular and catalytic properties of cytoplasmic and mitochondrial HMG-CoA synthases from chicken liver

$K_m$ values were determined by Lineweaver-Burk analysis at pH 8.0 and 50 μM acetyl-CoA and 200 μM acetoacetil-CoA when acetoacetil-CoA and acetyl-CoA concentrations, respectively, were varied. Other properties were determined as described in the text. N.D., not determined.

| Property | Cytoplasmic HMG-CoA synthases | Mitochondrial HMG-CoA synthase (from Reed et al. 0)
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<td>A. Similar properties</td>
<td>N.D.</td>
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| 1. $M_r$ | 90,000* | 94-100,000* | 100,000* | N.D. | 96-105,000*
| 2. Molecular weight | 52,000 | 55,000 | 58,000 | N.D. | 52,000 |
| 4. pH optimum | 9 3 | 9 2 | 9 4 | N D | N D |
| 5. $K_m$ acetyl-CoA | 290 μM | 310 μM | 310 μM | N.D. | 1000 |
| 6. $K_m$ acetoacetyl-CoA | <2 μM | <2 μM | <2 μM | N.D. | <5 μM |
| B. Distinguishing properties | | | | |
| 1. Elution (mU) from: Phosphocellulose | 100 | 20 | 110 | 80 | 50 |
| DEAE-cellulose | 20 | 80 | 120 | 100 | 20 |
| 2. Isoelectric point | 6.6 | 5.4 | 5.2 | N.D. | 7.2 |
| 3. Electrophoretic mobility on 7.5% acrylamide disc gels 50 mM Tris(KPO4) pH 8.5 | N.D. | 0.21 | 0.31 | 0.29 | 0.10 |
| 50 mM Tris(glycine) pH 8.9 | 0.37 | 0.91 | N.D. | N.D. | 0.30 |
| 4. Presence in chicken brain | + | + | + | N.D. | - |
| C. Unifying properties | | | | |
| 1. Antigenic titer | | | | |
| Antiserum C | 0 | 1.0 | 2.4 | 2.4 | 0 |
| Antiserum M | 3.0 | 0 | 0 | 0 | 2.6 |
| 2. Dissociation by KCl | + | - | - | N.D. | + |
| 3. Effect of MgCl2 on activity | ↓ | 1 | 1 | N.D. | ↓ |

* $M_m$ determined in 20 mM phosphate (K+), pH 7.0.

* $M_m$ determined in 50 mM phosphate (K+), pH 7.0, containing 0.1 M KCl.

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* Molecular weight determined by gel filtration under the conditions described in Footnote d.

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Unless otherwise noted, $K_m$ values for acetyl-CoA of all three enzymes are extremely low, <2 μM, and could not be assessed accurately because of the limited sensitivity of the assay below 2 μM substrate. A saturating acetocetyl-CoA concentration is reached below 2 μM and levels greater than this are somewhat inhibitory. Thus, it was not possible to differentiate the cytoplasmic synthases based on the dependence of initial velocity on pH or substrate concentration.

For purposes of comparison with the other synthases, the molecular weight of native cytoplasmic synthase III and its subunits was determined. Sedimentation equilibrium runs with the native enzyme were carried out in 50 mM potassium phosphate buffer containing 100 mM KCl at 17,000 rpm and 2°C (7) with a Spinco model E analytical ultracentrifuge. A molecular weight for cytoplasmic synthase III of 100,000 ± 4,000 was calculated (20, 21) from linear plots of log Rayleigh fringe displacement versus $r^2$ and an estimated partial specific volume of 0.73. The subunit weight of purified HMG-CoA synthase III was determined by the sodium dodecyl sulfate disc gel electrophoresis method of Weber and Osborn (19) using the molecular weight markers previously described (7). The mobility of the single Coomassie blue staining band corresponded to a subunit weight of 58,000. Hence, HMG-CoA synthase III, like synthases I and II, is a dimeric protein composed of two identical or similar weight subunits. Purified HMG-CoA synthase IV also was found to have a subunit weight of 58,000 by the sodium dodecyl sulfate gel electrophoresis method.

Catalytic Properties—Despite their distinctive electrophoretic and chromatographic properties, cytoplasmic HMG-CoA synthases I, II, and III exhibit similar catalytic properties (Table II). The three enzymes exhibit nearly identical activity-pH dependence profiles with optima at pH 9.2 to 9.4. Synthases I, II, and III have $K_m$ values for acetyl-CoA of about 300 μM, at saturating acetocetyl-CoA concentration. The $K_m$ values for acetocetyl-CoA of all three enzymes are extremely low, <2 μM, and could not be assessed accurately because of the limited sensitivity of the assay below 2 μM substrate. A saturating acetocetyl-CoA concentration is reached below 2 μM and levels greater than this are somewhat inhibitory. Thus, it was not possible to differentiate the cytoplasmic synthases based on the dependence of initial velocity on pH or substrate concentration.

However, synthase I can be distinguished from synthases II and III through the effect of MgCl2 on catalytic activity. Like mitochondrial HMG-CoA synthase (9), synthase I is inhibited by MgCl2 (Fig. 4a). That Mg2+ and not Cl-, is responsible for the inhibition is indicated by the fact that 400 μM KCl has no effect on activity. In contrast, MgCl2 has a small activating effect on cytoplasmic synthases II and III (Fig. 4, B and C). Possibly the effect of Mg2+ is mediated indirectly through effects on substrate...
activity of unfractionated avian liver cytosol, leaving no synthase II (results not shown), III, and IV, as well as the synthase antibody against cytoplasmic HMG-CoA synthase (antiserum C, presence of Mg2+.

Thus, inhibition could be due to the enolate of acetoacetyl-CoA rather than by a direct interaction with the enzyme. Mg2+ is known to complex the enolate form of acetoacetyl-CoA displacing the equilibrium from the keto toward the enol tautomer (7). Thus, inhibition could be due to the enolate of acetoacetyl-CoA or decreased inhibition by the keto form in the presence of Mg2+.

**Immunochemoal Properties**—As illustrated in Fig. 5 rabbit antibody against cytoplasmic HMG-CoA synthase (antiserum C, "Experimental Procedure") quantitatively precipitates synthases II (results not shown), III, and IV, as well as the synthase activity of unfractionated avian liver cytosol, leaving no synthase activity in the supernatant. Nevertheless, synthase precipitated by antiserum C retains full enzymatic activity. Neither normal serum nor 0.9% NaCl solution (saline) affected enzymatic activity or caused enzyme precipitation. The effect of antiserum C on HMG-CoA synthase I or the mitochondrial HMG-CoA synthase could not be ascertained by this procedure since both enzymes are inactivated by incubation at 4°C with normal serum.8 Fortunately, it was possible to assess the cross-reactivity of HMG-CoA synthase I and mitochondrial HMG-CoA synthase with antiserum C by the Ouchterlony double diffusion technique. As shown in Fig. 6, a single precipitin line is formed between antiserum C (Well 1) and chicken liver cytosol (Wells 2 and 10). This precipitin line must arise from HMG-CoA synthases II, III, and/or IV present in the cytosol, since a precipitin line is not formed with purified cytosolic synthase I (Well 4). Thus, synthase I is immunochemically distinct from synthases II, III, and IV. Moreover, precipitin lines were not generated (Fig. 6) between antiserum C and chicken liver mitochondrial matrix (Wells 3, 5, and 9), purified mitochondrial HMG-CoA synthase (Well 6), or rat liver mitochondrial matrix or cytosol (Wells 7 and 8, respectively). However, antiserum C does react with pigeon and turkey liver cytosols (data not shown), indicating that these species possess a cytoplasmic form of HMG-CoA synthase which is antigenically similar to synthases II to IV of the chicken.

In view of the apparent immunological nonidentity of synthase I and the other cytoplasmic synthases, i.e. II to IV, their antigenic relationship was explored further with antibody against the avian liver mitochondrial HMG-CoA synthase (antiserum M, see accompanying paper (9)). Unlike the effect of antiserum C on cytoplasmic synthases II to IV, antiserum M markedly inactivates (~80%) purified mitochondrial HMG-CoA synthase.

Previous investigations in this laboratory with the mitochondrial HMG-CoA synthase (9) indicate that this enzyme is inactivated by a serum protease; cytoplasmic HMG-CoA synthase I is similarly inactivated by normal rabbit serum or antiserum.
synthase I is severely inhibited by MgCl₂ (Fig. 4), only cytosolic activity contributed by HMG-CoA synthases II to IV was measured.

photometric assay employing 20 mM MgCl₂ and since HMG-CoA synthase activity in Figs. 5A and 7B was measured by the spectroscopic method. This unexpected result indicates that, while synthase I could carry more determinants than the mitochondrial synthase, it is not cytosolic synthase II, III, and IV (Fig. 7). Moreover, the titers for synthase I and the mitochondrial synthase are similar, 3.0 (Fig. 7A) and 2.6 (Fig. 4A) milliunits, respectively.

Immunodiffusion of antiserum M (Fig. 8, Well 1) with chicken liver cytosol (Well 2) and HMG-CoA synthase I (Wells 4 and 7) gave rise to single precipitin lines in each case, while purified synthases II, III, and IV (Wells 3, 5, and 6) showed no immunoprecipitation by antiserum M. The component of cytosol precipitated by antiserum M must be synthase I since the precipitin line arising from cytosol fused completely with that from synthase I. A comparison of the relatedness of HMG-CoA synthase I and mitochondrial HMG-CoA synthase revealed (Fig. 8C) a single precipitin line arising from the immunoprecipitation of mitochondrial HMG-CoA synthase (Well 8) by antiserum M which fused with that from synthase I (Well 9); however, the reverse was not true, i.e., the synthase I precipitin line ran a spur across the precipitin line from mitochondrial HMG-CoA synthase (Fig. 8C). This unexpected result indicates that, while synthase I and mitochondrial synthase possess some common antigenic determinants, synthase I carries more determinants for antiserum M than does mitochondrial synthase. One explanation as to how synthase I could carry more determinants than the mitochondrial synthase used to prepare the antiserum is that the purified mitochondrial synthase may have been contaminated with synthase I. This is unlikely, however, since: (a) synthase I and mitochondrial synthase are completely resolved by the purification procedure described in Fig. 4.

1 It should be noted that, since the cytosolic HMG-CoA synthase activity in Figs. 5B and 7B was measured by the spectrophotometric assay employing 20 mM MgCl₂ and since HMG-CoA synthase I is severely inhibited by MgCl₂ (Fig. 4), only cytosolic activity contributed by HMG-CoA synthases II to IV was measured.
tion procedure, (b) mitochondria used for synthase purification were extensively washed to preclude contamination of the mitochondria by cytoplasmic components, and (c) the mitochondrial synthase used to prepare antisem M was subjected to rigorous tests of homogeneity and was found to be free of synthase I (9). A more likely possibility is that some mitochondrial synthase antigenic determinants were lost during immunodiffusion due to the action of protease present in antisem. Had determinants on the mitochondrial synthase, but not synthase I, been lost by a nick(s) introduced by the protease at the time of diffusion, the observed spurring pattern could have resulted. The related question of whether HMG-CoA synthase I is an authentic cytoplasmic enzyme is raised because of the apparent relationship to its mitochondrial counterpart. Although the two synthases are immunochemically related, clearly they are different proteins having dissimilar isoelectric points, chromatographic behavior, and migration upon disc gel electrophoresis. The possibility was considered that synthase I arose via leakage of the mitochondrial synthase into the cytoplasm during the cell disruption procedure, followed by conversion of the mitochondrial synthase to synthase I by a modification process occurring only in the cytoplasm. Were this the case, synthase I should be present only in tissues possessing mitochondrial HMG-CoA synthase, i.e., liver and kidney (8). Immunodiffusion of the cytosolic fractions of liver, kidney, heart, and brain against antisem M produced precipitin lines with all tissues tested (Fig. S1), indicating that brain and heart, which are devoid of mitochondrial synthase (8), as well as liver and kidney, contain HMG-CoA synthase I. Additionally, phosphocellulose and DEAE-cellulose chromatography of brain cytosol confirmed the presence of HMG-CoA synthase I in this tissue (results not shown). Thus, it can be concluded that HMG-CoA synthase I is a discrete cytoplasmic enzyme.

Role of Cytosplasmic HMG-CoA Synthase in Cholesterol Synthesis in Avian and Rat Liver

Factors Affecting Hepatic Cytosplasmic HMG-CoA Synthase Activity in Chicken—To investigate the role(s) of the multiple cytoplasmic species of HMG-CoA synthase, it was necessary to determine the fractional contribution of each species to the total cytoplasmic synthase activity of liver. This was accomplished by resolving the synthases of chicken liver cytosol using ion exchange chromatography. As shown in Table III, synthase II is the principal form in freshly prepared liver cytosol constituting 62% of the total cytosolic activity, with synthase I comprising 22 to 23% of the total. It became evident that, during "aging" of the cytosolic fraction for 24 hours at 4°C, synthase II was converted, apparently enzymatically, into the related species, synthase III-IV. Nevertheless, total and synthase I activities remained constant. This process appears to be dependent upon a cytosolic factor since homogenate preparations of synthase II are not converted to synthases III-IV under similar conditions.  

Hepatic cholesterogenesis is primarily a cytoplasmic process (10, 22-24) and apparently is the major route of HMG-CoA metabolism in this compartment; therefore, at least one of the cytosolic species of HMG-CoA synthase must function in this pathway. It is known (25-28) that dietary cholesterol inhibits cholesterol synthesis in liver and specifically depresses the activities of various hepatic enzymes involved in its synthesis. To evaluate the effect of dietary cholesterol on cytoplasmic HMG-CoA synthase activity, a group of chickens was fed a low-cholesterol commercial diet supplemented with 2% cholesterol, while a control group was fed the same diet without added cholesterol. As shown in Table IV (Experiment 1), cholesterol feeding caused a significant reduction in the HMG-CoA synthase activity of the cytosolic fraction of liver. This decrease, although less than the extent of inhibition, i.e., ~95%, of [1-14C]acetate incorporation into cholesterol by liver slices from the same animals (results not shown), is comparable to the decreased activities of several other cholesterogenic enzymes in response to cholesterol feeding (25-27).

Bile acid synthesis is a major cholesterol-utilizing pathway of liver (29). Hence, feeding cholestyramine, an agent which binds bile salts in the intestine, thereby preventing their reabsorption, promotes an increase in hepatic cholesterogenesis (29, 30). The possibility that cytoplasmic HMG-CoA synthase might be affected similarly was tested. As shown in Table IV (Experiment 2), cholestyramine fed to chickens led to a 2-fold increase in the

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Table III

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Normal</th>
<th>Cholestyramine-fed</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthase II</td>
<td>0.24 (61%)</td>
<td>0.84 (81%)</td>
<td>3.5</td>
</tr>
<tr>
<td>Synthase I</td>
<td>0.08 (22%)</td>
<td>0.14 (14%)</td>
<td>1.5</td>
</tr>
<tr>
<td>Synthase III-IV</td>
<td>0.06 (16%)</td>
<td>0.05 (5%)</td>
<td>2.7</td>
</tr>
<tr>
<td>Total</td>
<td>0.38</td>
<td>1.02</td>
<td>2.7</td>
</tr>
</tbody>
</table>

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1 HMG-CoA synthase I activity comprises only 9% of the total cytoplasmic synthase activity of chicken brain, i.e., 1.8 milliunits of synthase 1/g of brain, compared to 22% of the cytoplasmic activity in liver, i.e., 85 milliunits of synthase 1/g of liver.

2 This accounts for the fact that synthases III and IV are the predominant species isolated by the purification procedure (Table I) which required several days to accomplish.
mine-fed animals showed a 4.3-fold increase in the rate of [l-"Cl- acetate incorporation into cholesterol (results not shown). The possibility that only one species of HMG-CoA synthase might be responsive to cholestyramine feeding is strongly indicated by the finding that cholestyramine feeding promoted a 3.5-fold increase in synthase II activity with little or no effect on the activities of

- total HMG-CoA synthase activity of liver cytosol. Consistent with this result is the fact that liver slices from the cholestyramine-fed animals showed a 4.5-fold increase in the rate of [l-"Cl-acetate incorporation into cholesterol (results not shown). The possibility that only one species of HMG-CoA synthase might be responsive to cholestyramine feeding is strongly indicated by the finding that cholestyramine feeding promoted a 3.5-fold increase in synthase II activity with little or no effect on the activities of synthases I and synthase III–IV (Table III). Thus, cholestyramine-feeding appears to preferentially elevate hepatic synthase II activity, suggesting that this synthase form is involved in hepatic cholesterogenesis. Cholesterol feeding did not lead to a comparable decrease in synthase II activity; the reason for this is not clear.

Factors Affecting Hepatic Cytoplasmic HMG-CoA Synthase and Acetoacetyl-CoA Thiolase Activities in Rat—Although the cytosol of avian liver possesses multiple forms of HMG-CoA synthase, only one synthase species occurs in the cytosol of rat liver. As shown in Fig. 9, isoelectric focusing of the cytosolic fraction of rat liver, which accounts for the total synthase activity in the cytoplasm (8), gives rise to a single peak of HMG-CoA synthase activity with a pl of 5.4. Furthermore, chromatography of rat liver cytosol on DEAE-cellulose using a 20 to 400 mM linear phosphate gradient, conditions known to resolve the major synthase forms of avian liver cytosol, results in the elution of a single, sharp HMG-CoA synthase activity peak at 120 mM phosphate (results not shown). These findings strongly indicate the existence of a single synthase species in rat liver cytoplasm.

In addition to exhibiting similar chromatographic behavior on DEAE-cellulose, rat liver cytosolic HMG-CoA synthase and the major HMG-CoA synthase species of avian liver cytosol, synthase II, possess similar kinetic properties. The reaction catalyzed by the rat liver cytosolic synthase shows a similar dependence upon acetoacetyl-CoA concentration to that of chicken liver cytosolic synthase; both synthases have low $K_m$ values for acetoacetyl-CoA (<10 $\mu$M). Rat liver cytosolic synthase is activated by MgCl$_2$ (results not shown) as is its counterpart, synthase II, in avian liver cytosol (Fig. 4).

The role in cholesterogenesis of the coupled cytoplasmic enzyme system which synthesizes HMG-CoA, i.e. acetoacetyl-CoA thiolase and HMG-CoA synthase, was assessed by demonstrating its susceptibility to feedback control by cholesterol. Thus, it was found (Table V) that feeding 2% cholesterol to rats caused a rapid, large decrease in the cytosolic activities of acetoacetyl-CoA thiolase and HMG-CoA synthase; after 1 day of cholesterol feeding, these cytosolic activities had decreased to 61 and 52%, respectively, and after 7 days to 29 and 15%, respectively, of control values (Table V). A significant fraction of the 15 to 29% of cytoplasmic thiolase and synthase activity remaining after 7 days of cholesterol feeding is no doubt artifactual because of some leakage of mitochondrial thiolase and synthase into the cytosol during cell fractionation (8). It is interesting and perhaps significant that the lowering of cytoplasmic thiolase and synthase activities by cholesterol feeding is more pronounced in rat liver than in chicken liver (Table IV).

Moreover, feedback control by dietary cholesterol appears to be specifically directed at the cytoplasmic, rather than the mitochondrial, HMG-CoA generating system. Thus, feeding cholesterol caused a marked reduction of hepatic cytoplasmic acetoacetyl-CoA thiolase activity, while having no effect on mitochondrial thiolase activity (Table VI).

In addition, two other factors, i.e. cholestyramine feeding and fasting which are known to influence hepatic cholesterogenesis in the rat (27–31), were tested for effects on cytoplasmic thiolase and synthase activities. As illustrated in Table VII (Experiment 1), feeding cholestyramine to rats for 8 days caused a 1.8- and a 2.6-fold increase in the cytosolic activities of the thiolase and synthase in liver, respectively, results compatible with the involvement of these enzymes in cholesterogenesis. Fasting is of particular diagnostic use because of its opposing effects on the two major HMG-CoA utilizing pathways, i.e. causing a decrease

$^{3}$MgCl$_2$ inhibits the mitochondrial HMG-CoA synthase of rat liver as it does the mitochondrial synthase of avian liver (9).
in hepatic cholesterogenesis concomitant with increased ketogenesis (26-28, 32). Consistent with their apparent roles in hepatic cholesterogenesis, cytosolic acetoacetyl-CoA thiolase and HMG-CoA synthase activities (per liver) were reduced by fasting to levels 46 and 24%, respectively, of those in rats fed ad lib. Thiolase and synthase activities were compared on a per liver (or per animal) basis rather than per g of tissue since liver weight decreases significantly, i.e. to about 55% of normal, during a 48-hour fasting period (Table VII). These findings are in disagreement with those of Williamson et al. (32) who observed an increase in cytoplasmic HMG-CoA synthase activity upon fasting; the basis for this discrepancy is not clear.

Unlike hepatic cholesterogenesis and HMG-CoA reductase activity, both of which undergo diurnal variation in the rat (27, 31, 33), neither cytoplasmic thiolase nor synthase activity shows a dependence upon time of day in rats maintained on a 12-hour light and 12-hour dark cycle (results not shown). While this indicates that alterations in level of cytoplasmic thiolase or synthase are not primary factors in the control of cholesterogenesis in liver, their regulation through short term allosteric or covalent modification has not been ruled out.
part by the finding that synthase II can undergo conversion to ionic properties of synthase II, e.g. isoelectric point. Behavior upon electrophoresis, with little change in other properties of the enzyme (Table II). Purified HMG-CoA synthases II, III, and IV exhibit stable ionic properties, hence the conversion of HMG-CoA synthase II to its related species is not a spontaneous process inherent to synthase II itself. If this transition were the result of proteolysis, the peptide(s) removed from synthase II must be small (<5,000 daltons) since significant differences in subunit weight between synthases II, III, and IV were not detected (Table II). Conceivably, modification of HMG-CoA synthase II could involve removal of a group from the enzyme, e.g. a phosphoryl, adenylyl, acetyl, or amide group, which could have regulatory significance. Further study will be required to assess the significance of this process.

The presence of HMG-CoA synthase I in chicken liver cytoplasm is perplexing since its molecular, catalytic, and antigenic properties are more closely related to mitochondrial HMG-CoA synthase than to cytoplasmic synthases II, III, and IV (Table II). It is unlikely that synthase I arises via leakage of the mitochondrial synthase into the cytoplasm during subcellular fractionation since: (a) several properties of synthase I are different from those of its mitochondrial counterpart, and (b) the tissue distribution of synthase I is significantly different from that of the mitochondrial enzyme (Fig. 8D and Ref. 8). Moreover, the activity of synthase I in the cytoplasm is 10 to 15% that of the mitochondrial HMG-CoA synthase activity, and therefore, too large to be accounted for by the 2 to 8% leakage of mitochondrial enzyme activities into the cytoplasm caused by the cell disruption procedure employed (8). It is possible that synthase I is a “cytoplasmic precursor” of mitochondrial HMG-CoA synthase. The latter synthase may be synthesized on cytoplasmic ribosomes yielding a precursor molecule, synthase I, which subsequently enters the mitochondria where its properties are modified to those of mitochondrial HMG-CoA synthase. A cytoplasmic precursor of the mitochondrial enzyme, glutamate dehydrogenase, has been observed, although the properties of this precursor and mitochondrial glutamate dehydrogenases appeared to be the same (37). Preliminary experiments reveal that fasting specifically increases the activity of synthase I, as well as mitochondrial HMG-CoA synthase activity, evidence which is compatible with synthase I serving as a “cytoplasmic precursor” of the mitochondrial synthase. It is also conceivable that synthases I and II (and perhaps III and IV) arise from different cell types in liver, i.e. parenchymal versus nonparenchymal cells. This has been found to be the case with the localization of hexokinase versus glucokinase and with aldolase isozyme A versus isozyme B in liver (38).

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