Intracellular Localization of the 3-Hydroxy-3-methylglutaryl Coenzyme A Cycle Enzymes in Liver

SEPARATE CYTOPLASMIC AND MITOCHONDRIAL 3-HYDROXY-3-METHYLGLUTARYL COENZYME A GENERATING SYSTEMS FOR CHOLESTERGENESIS AND KETOGENESIS*

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SUMMARY

Acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl coenzyme synthase which comprise the 3-hydroxy-3-methylglutaryl-CoA-generating system(s) for hepatic cholesterogenesis and ketogenesis exhibit dual mitochondrial and cytoplasmic localization. Twenty to forty per cent of the thiolase and synthase of avian and rat liver are localized in the cytoplasmic compartment, the remainder residing in the mitochondria. In contrast, 3-hydroxy-3-methylglutaryl-CoA lyase, an enzyme unique to the "3-hydroxy-3-methylglutaryl-CoA cycle" of ketogenesis, appears to be localized in the mitochondrion. The small proportion, 4 to 8%, of this enzyme found in the cytoplasmic fraction appears to arise via leakage from the mitochondria during cell fractionation in that its properties, pH and stability, are identical to those of the mitochondrial lyase. These results are consistent with the view that ketogenesis which involves all three enzymes, acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA synthase and 3-hydroxy-3-methylglutaryl-CoA lyase, occurs exclusively in the mitochondrion, whereas cholesterogenesis, a pathway which involves only the 3-hydroxy-3-methylglutaryl-CoA synthesizing enzymes, is restricted to the cytoplasm.

Further fractionation of isolated mitochondria from chicken and rat liver showed that all three of the 3-hydroxy-3-methylglutaryl-CoA cycle enzymes are soluble and are localized within the matrix compartment of the mitochondrion. Likewise, cytoplasmic acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl-CoA synthase are soluble cytosolic enzymes, no thiolase or synthase activity being detectable in the microsomal fraction.

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Chicken liver mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase activity consists of a single enzymic species with a pI of 7.2, whereas the cytoplasmic activity is composed of at least two species with pI values of 4.8 and 6.7. Thus it is evident that the mitochondrial and cytoplasmic species are molecularly distinct as has been shown to be the case for the mitochondrial and cytoplasmic acetoacetyl-CoA thiolases from avian liver (CLINKENBEARD, K. D., SUGIYAMA, T., MOSS, J., REED, W. D., AND LANE, M. D. (1973) J. Biol. Chem. 248, 2275).

Substantial mitochondrial 3-hydroxy-3-methylglutaryl-CoA lyase activity is present in all tissues surveyed, while only liver and kidney possess significant mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase activity. Therefore, it is proposed that tissues other than liver and kidney are unable to generate acetoacetate because they lack the mitochondrial synthase.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), is synthesized from acetyl-CoA by a two-step reaction sequence (Scheme 1, Reactions 1 and 2) catalyzed by acetoacetyl-CoA (AcAc-CoA)thiolase and HMG-CoA synthase (1–4). By 1958 Rudney and Lynen and their collaborators had determined that HMG-CoA is an intermediate in two major pathways in animal liver, the synthesis of cholesterol and acetoacetate (1, 2). Based on these facts, Gould and Popjak proposed (5) that HMG-CoA is synthesized in common for both cholesterogenesis and ketogenesis, and that a branch point occurs between these two pathways at the first reaction unique to each pathway, i.e. the reduction of HMG-CoA to mevalonate and the cleavage of HMG-CoA to acetoacetate as illustrated in Scheme 1 (Reactions 3 and 4, respectively). This concept of a branched pathway for cholesterogenesis and ketogenesis has been generally accepted (6–9).

The work of Bucher et al. (10) on the subcellular distribution

The abbreviations used are: HMG, 3-hydroxy-3-methylglutaryl; Hepes, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid.
Unlabeled and [1-14C]acetetyl-CoA were prepared from the anhydride by the method of Simon and Shemin (21); acetoycetyl-CoA was similarly prepared; however, diketene replaced the anhydride. Unlabeled and (R, S) [3-14C]HMG-CoA were synthesized as described by Goldfarb and Pitot (22). The [1-14C]acetetyl-CoA and (R, S)-[3-14C]HMG-CoA were purified by DEAE-cellulose chromatography (20). Lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37), adenylate kinase (EC 2.7.4.3), monoamine oxidase (EC 1.4.3.4), succinate dehydrogenase (EC 1.3.99.1), citrate synthase (EC 4.1.3.7), and acetoycetyl-CoA thiolase (EC 2.3.1.9) were assayed as described elsewhere (13, 24-28). Assays were conducted at 30° and reaction mixtures contained 0.2% (w/v) Triton X-100 where indicated; Triton X-100 had no inhibitory effect on the enzymes assayed. Protein was determined by the methods of Lowry et al. (29) or Warburg and Christian (30) or by the biuret method employing deoxycholate (31) as indicated.

Subcellular Fractionation of Liver—Animals were killed by decapitation and livers were removed and washed with ice-cold homogenizing buffer containing 0.25 M sucrose, 0.1 M EDTA, and 2.0 mM Hepes, pH 7.2. All operations were conducted at 4°. Two grams of cubed liver suspended in 10 ml of homogenizing buffer were disrupted by three passes of a motor-driven Teflon pestle in a loose fitting glass homogenizer. The homogenate was separated into particulate and cytoplasmic fractions by centrifugation at 11,300 X g for 15 min and the supernatant, i.e., cytoplasmic fraction (Table I, Fraction I), was used directly, while the pellet was gently resuspended in 10 ml of homogenizing buffer using a test tube filled with ice as a pestle, after which the suspension was resedimented at 11,300 X g for 15 min. This pellet which contained mitochondria and nuclei was resuspended as above and used directly (Table I, Fraction I). Subcellular fractionation by this procedure gave reasonably good resolution of cytoplasm and mitochondria as judged by the localization of lactate dehydrogenase and citrate synthase, marker enzymes for the cytoplasmic and mitochondrial fractions, respectively (Table I). Since the usual low speed centrifugation (600 X g) was omitted to avoid the loss of mitochondria with the nuclei, essentially quantitative recovery of mitochondrial enzyme activities was achieved. This is important, particularly with avian liver because of the tendency of these mitochondria to clump. A final concentration of 0.2% (w/v) of Triton X-100 was employed in all enzyme assays in Table I, and in other experiments as indicated in tables and figure legends, to release membrane-occluded enzyme activities.

The cytoplasmic fraction (Table I, Fraction II) was fractionated further by centrifugation at 100,000 X g for 1 hour. The supernatant from this centrifugation, i.e., the cytosolic fraction (Table IV, Fraction I), while the pellet was resuspended in 10 ml of homogenizing buffer and resedimented at 106,000 X g for 1 hour. The pellet, resuspended as above, is referred to as the washed microsomal fraction (Table IV, Fraction II).

For intramitochondrial fractionation, livers were homogenized as described above except that the homogenizing buffer also contained 0.1% (w/v) bovine serum albumin. The homogenate was centrifuged at 160 X g for 10 min to remove nuclei and the supernatant from this centrifugation was resedimented at 6700 X g to collect the mitochondria. This mitochondrial pellet was gently resuspended in 3 ml of the homogenizing buffer (containing 0.1% bovine serum albumin) and resedimented at 9700 X g for 15 min. The washed mitochondria were resuspended in 2 ml of homogenizing buffer (containing 0.1% bovine serum albumin) and further fractionated as indicated in Table IV and III.

Isoelectric Focusing—Isoelectric points of HMG-CoA synthase and HMG-CoA lyase preparations were determined using an LKB 8101 electrofocusing column. The dense electrode solution consisted of 0.4 ml of monochloroaniline, 12 g of sucrose, and 14 ml of water, while the light electrode solution contained 0.1 ml of phosphoric acid and 10 ml of water. For the column gradient, the dense solution contained 28 g of sucrose, 50 ml of 2-mercaptoethanol, and 75% of the appropriate Ampholine, in some cases the sample, and water to bring the final volume to 42 ml; the light...
Intracellular distribution of acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA lyase activity in chicken liver

Livers from 20 8-week-old white Leghorn cockerels fed ad libitum were fractionated into mitochondria (Fraction I, also containing nuclei and some unbroken cells) and cytoplasm (Fraction II) after which enzyme assays were conducted in the presence of 0.2% Triton (w/v) as described under "Experimental Procedure." HMG-CoA synthase and HMG-CoA lyase were assayed spectrophotometrically; 100 mm glycylglycine buffer, pH 5.8, was employed for HMG-CoA synthase assays. Protein was determined by the biuret method (31).

"Homogenate" refers to enzymatic activity in the homogenate prior to the separation of the subcellular fractions, and this activity is taken as 100% for the calculation of percentages shown in parentheses. Enzyme activity is expressed as units (1 μmol of substrate converted/min)/g wet weight of liver ± the standard deviation of the mean.

| TABLE I | Intramitochondrial distribution of acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA lyase of chicken liver |
|---------|-------------------------------------------------------------------------------------------------|
| Homogenate | 93 ± 14 (100%) 1.9 ± 0.4 (100%) 7.3 ± 1.4 (100%) 6.3 ± 1.0 (100%) 240 ± 40 (100%) |
| Fraction | |
| I. Mitochondria (plus nuclei) | 47 ± 12 (51%) 1.6 ± 0.1 (84%) 6.6 ± 1.0 (99%) 5.5 ± 0.9 (87%) 26 ± 5 (10%) |
| II. Cytoplasm | 41 ± 8 (44%) 0.4 ± 0.1 (21%) 0.3 ± 0.2 (4%) 0.1 ± 0.1 (2%) 210 ± 30 (88%) |
| Total recovery in Fractions I + II | 88 (95%) 2.0 (105%) 6.9 (94%) 5.6 (89%) 235 (98%) |

Intramitochondrial distribution of acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA lyase of chicken liver

Isolated mitochondria from chicken liver were prepared as described under "Experimental Procedure" except that the washed mitochondria were resuspended in 50 mm potassium phosphate buffer, pH 7.0, instead of homogenizing buffer. This mitochondrial suspension (20 mg of protein/ml) was sonicated for three 20-s periods at 4°C with a Bronwill Biosonik sonicator (model Bio II) at a setting of 20 using the small probe. Sonicated mitochondria were separated into a soluble and particulate fraction by centrifugation at 120,000 × g for 1 h at 4°C, after which the pellet was suspended in 50 mm potassium phosphate, pH 7.0. HMG-CoA synthase and HMG-CoA lyase were assayed spectrophotometrically; Tris(Cl-) buffer, pH 8.2, was used for the synthase assays. Acetoacetyl-CoA thiolase and malate dehydrogenase assays were as described under "Experimental Procedure.

"Mitochondria" refers to the sonicated, but uncentrifuged mitochondria. Values given in parentheses are total units (micromoles converted/min) per g wet weight of liver. The chicken liver and rat liver (referred to in the text) mitochondria had accpetor control ratios of 5.7 and 7.5 respectively, with succinate as substrate.

| TABLE II | Intracellular distribution of acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA lyase activity in chicken liver |
|---------|-------------------------------------------------------------------------------------------------|
| Acetoacetyl-CoA thiolase | HMG-CoA synthase | HMG-CoA lyase | Malate dehydrogenase |
| Homogenate | 100 | 100 | 100 | |
| Mitochondria | 100 | 100 | 100 | 100 |
| Fraction | |
| I. Matrix plus intracrystal space | 78 | 87 | 97 | 87 |
| II. Inner plus outer membrane | 17 | 6 | 0 | 10 |
| Total recovery in Fractions I + II | 95 | 93 | 97 | 106 |

* This value and that reported earlier (13) are low relative to the activity shown in Table I. The difference is attributable to partial inactivation of thiolase by sonication.
Isolated mitochondria were prepared as described under "Experimental Procedure" and subsequently fractionated into outer membrane plus intracristal space (Fraction I) and inner membrane-matrix vesicles (Fraction II) by the method of Schnaitman and Greenwald (26). HMG-CoA synthase and HMG-CoA lyase were assayed as described in Table II, while acetoacetyl-CoA (AAc-CoA) thiolase, succinate dehydrogenase (Succ. dehydr.), citrate synthase, monoamine oxidase, and adenylyl kinase were assayed as described under "Experimental Procedure". Triton X-100 (0.2%, w/v) was used in all enzyme assays. Mitochondria had acceptor control ratios as in Table II prior to digitonin treatment. "Mitochondria" refers to isolated digitonin-treated mitochondria. Distribution is expressed in percentages.

| Fraction | A. Chicken | B. Rat |
|----------|------------|--------|
| I. Intracristal space + outer membrane | 100 | 100 |
| II. Matrix + inner membrane | 90 | 90 |
| Total recovery in Fractions I + II | 104 | 104 |

a Protein was determined by the biuret method (31). The yield of mitochondrial protein was 30 and 17 mg/g wet weight of liver for the chicken and rat, respectively. Since the objective was to obtain clean mitochondrial preparations, yield was sacrificed. It is estimated that the total content of mitochondrial protein is 60 to 70 mg/g wet weight of liver.

b Milliunits (nanomoles of substrate converted/min)/mg of mitochondrial protein.

c Microunits (micromoles of substrate converted/min)/mg of mitochondrial protein.

Acetoacetyl-CoA has an $K_m = 16.1 \times 10^{-8}$ M$^{-1}$ in this buffer. Mitochondria HMG-CoA synthase is inhibited by MgCl$_2$ (19) and is assayed in the absence of MgCl$_2$ in Tris (Cl$^-$), pH 8.2, where acetoacetyl-CoA has an $K_m = 3.6 \times 10^{-6}$ M$^{-1}$. In experiments in which both cytoplasmic and mitochondrial HMG-CoA synthase were assayed, glycylglycine (Na$^+$), pH 8.8, was used without MgCl$_2$ where acetoacetyl-CoA has an $K_m = 7.5 \times 10^{-6}$ M$^{-1}$

HMG-CoA synthase activity is equal to one-half the rate of acetoacetyl-CoA consumption for preparations, e.g. crude mitochondrial or cytoplasmic extracts, in which acetoacetyl-CoA thiolase activity > HMG-CoA synthase activity. This correction is made to account for the CoA-dependent consumption of acetoacetyl-CoA catalyzed by thiolase, the extent of which is governed by the amount of CoA generated by HMG-CoA synthase. As illustrated in Fig. 1, A and B, the activities determined by the radiochemical and spectrophotometric assays are in good agreement.

**HMG-CoA Lyase Assays**—A new radiochemical assay for HMG-CoA lyase was developed which has the advantage of increased sensitivity over the spectrophotometric assay (36). The lyase-catalyzed conversion of [3-14C]HMG-CoA (not volatile when taken to dryness in 6 N HCl and 40 nmol of (R,S)-[3-14C]HMG-CoA (specific activity, 5 to $2 \times 10^4$ cpm/mmol) in a total volume of 0.2 ml. After 2, 4, 6, and 8 min of incubation at 30°, 0.04 ml aliquots are transferred to glass vials containing 0.1 ml of 6 N HCl and nonvolatile 14C activity determined as described for the radiochemical HMG-CoA synthase assay.

As shown in Fig. 2, A and B, the disappearance of nonvolatile 14C activity, i.e. [3-14C]HMG-CoA, is linear with time and the rate of the reaction is proportional to enzyme concentration within the limits specified above. Moreover, the end point of the reaction is reached when approximately 90% of the (R,S)-[3-14C]HMG-CoA has been cleaved which is consistent with the fact that the lyase acts only on (S)-HMG-CoA (37). That this assay in fact measures
chondrion. Only 4% of the cellular lyase activity is found in cytoplasm, and this small percentage is probably the result of lyase, appears to be exclusively localized within the mitochondria of the origin. In contrast to this dual localization of the HMG-CoA lyase in Fraction I is of mitochondrial, rather than nuclear, origin. In chicken liver, a substantial fraction, 44 and 21%, respectively, is present in the mitochondrial-nuclear fraction of chicken liver. Table I shows that although most of the cellular fractionation studies on the HMG-CoA cycle enzymes in compartments of liver, prompted us to undertake careful intramitochondrial distribution studies to be discussed subsequently. Contamination of the cytoplasmic fraction with mitochondrial components was minimal (Table I) as estimated by the presence of the mitochondrial matrix marker enzyme, citrate synthase. Cytoplasmic contamination of the mitochondrial fraction was about 10% as judged by the distribution of the cytosolic marker, lactate dehydrogenase (Table I). A similar intracellular enzyme distribution was obtained with rat liver (results not shown) where 30 and 20% of the total cellular acetoacetyl-CoA thiolase and HMG-CoA synthase activities, respectively, were present in the cytoplasmic fraction, the remainder being mitochondrial.

Does HMG-CoA Lyase Activity Detected in Cytoplasmic Fraction Arise by Leakage from Mitochondria during Subcellular Fractionation?—It has been reported that a significant fraction of the HMG-CoA lyase activity of rat and avian liver resides in the cytoplasm (41, 42). However, there is disagreement on this point since the present investigation (Table I) and others (10, 43) indicate that the lyase activity of the cytoplasm constitutes only a minor fraction of the total hepatic activity, an amount readily accounted for by mitochondrial leakage during cell fractionation. In our studies the percentage of hepatic lyase in the cytoplasm ranged from 4% in the fed state (Table I) to 7 to 8% in the fasted state (results not shown). Nonetheless, it was important to ascertain whether a cytoplasmic lyase exists because its presence in the cytoplasm would require that the flux of HMG-CoA into acetoacetate versus cholesterol be regulated. To determine whether HMG-CoA lyase found in the cytoplasmic fraction could have arisen by leakage from mitochondria, the isoelectric points and heat inactivation profiles of lyase activities were compared for the enzyme from the mitochondrial and cytoplasmic fractions of liver (from 48-hour fasted chickens). As shown in Fig. 3, both HMG-CoA lyase activities present in the mitochondrial and cytoplasmic fractions focus at precisely the same point, i.e. at pH 6.1. Although the identical isoelectric points of these activities could be fortuitous, this result supports the view that the lyase found in the cytoplasmic fraction is the mitochondrial enzyme. Additional evidence for this contention was obtained by comparing the heat lability of the HMG-CoA lyase activity in the cytoplasmic and mitochondrial fractions. As illustrated in Fig. 4, both activities exhibit identical exponential activity decay at 48°, the apparent half-life for inactivation being 1.4 min. Thus, several lines of evidence indicate that “cytoplasmic” HMG-CoA lyase activity and hence cytoplasmic ketogenesis are artifacts resulting from the leakage of the lyase from mitochondria. It appears that HMG-CoA lyase, an exclusively ketogenic enzyme, is localized solely within the mitochondrial.

Intracellular Distribution of Acetoacetyl-CoA Thiolase, 3-Hydroxy-3-methylglutaryl-CoA Synthase, and 3-Hydroxy-3-methylglutaryl-CoA Lyase—It has been assumed and earlier studies points of these activities could be fortuitous, this result supports the view that the lyase found in the cytoplasmic fraction is the mitochondrial enzyme. Additional evidence for this contention was obtained by comparing the heat lability of the HMG-CoA lyase activity in the cytoplasmic and mitochondrial fractions. As illustrated in Fig. 4, both activities exhibit identical exponential activity decay at 48°, the apparent half-life for inactivation being 1.4 min. Thus, several lines of evidence indicate that “cytoplasmic” HMG-CoA lyase activity and hence cytoplasmic ketogenesis are artifacts resulting from the leakage of the lyase from mitochondria. It appears that HMG-CoA lyase, an exclusively ketogenic enzyme, is localized solely within the mitochondrial.

Intramitochondrial Localization of Acetoacetyl-CoA Thiolase, HMG-CoA Synthase, and HMG-CoA Lyase—Mitochondria from chicken liver after brief sonication were separated into soluble and particulate fractions by centrifugation at 120,000 × g for 1 hour. As shown in Table II, the small percentages of activity found in the particulate fraction (Table II, Fraction II) are probably due to disrupted mitochondria. This is supported by the fact that 10% of the soluble mitochondrial matrix marker enzyme, malate dehydrogenase, is also found in this membrane fraction (Table II). Experiments conducted in an identical manner, but with rat liver mitochondria (results not shown), revealed that the HMG-CoA cycle enzymes of rat liver also appear in the soluble fraction of the mitochondria. These results indicate that mitochondrial acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA

FIG. 2. Radiochemical assay for HMG-CoA lyase. Mitochondria were isolated and the matrix fraction was prepared as described in Table II. A, 0, 14, and 28 µg of mitochondrial matrix protein were assayed for HMG-CoA lyase activity using the radiochemical assay described under "Experimental Procedure." R,S-[3-14C]-HMG-CoA removed refers to the loss of 14C activity not volatile upon drying at 95°. The endpoint of the assay is reached when one of the two enantiomers of (R,S) HMG-CoA is exhausted. B, the rate of HMG-CoA lyase activity was determined as a function of mitochondrial matrix protein added.
fraction (Fraction I, Table III) external to the inner membrane of chicken liver mitochondria (Table III) is not understood, this matrix vesicle. Although the basis for the apparent activation phenomenon was observed consistently.

The method of Schnaitman and Greenwalt (26) to separate the mitoplast (inner membrane-matrix vesicle) from the outer membrane and intracristal space components. The ability of this procedure to resolve these submitochondrial fractions from both chicken and rat liver mitochondrial preparations were fractionated by the method of Schnaitman and Greenwalt (26) to separate the mitoplast (inner membrane-matrix vesicle) from the outer membrane and intracristal space components. The ability of this procedure to resolve these submitochondrial fractions from both chicken and rat liver is verified by the distribution of appropriate marker enzymes (Table III). Essentially all of the succinate dehydrogenase and citrate synthase, inner membrane, and matrix enzymes (26, 44), respectively, was found to be associated with the mitoplast. Monoamine oxidase, an accepted outer membrane marker (26, 44), also yielded the expected intramitochondrial distribution pattern although in chicken liver mitochondria contains the HMG-CoA cycle enzymes, chicken and rat liver mitochondrial preparations were fractionated by the method of Schnaitman and Greenwalt (26) to separate the mitoplast (inner membrane-matrix vesicle) from the outer membrane and intracristal space components. The ability of this procedure to resolve these submitochondrial fractions from both chicken and rat liver is verified by the distribution of appropriate marker enzymes (Table III). Essentially all of the succinate dehydrogenase and citrate synthase, inner membrane, and matrix enzymes (26, 44), respectively, was found to be associated with the mitoplast. Monoamine oxidase, an accepted outer membrane marker (26, 44), also yielded the expected intramitochondrial distribution pattern although in chicken liver mitochondria, the level of activity is quite low and approached the mitochondrial distribution pattern although in chicken liver mitochondria, the level of activity is quite low and approached the mitochondrial distribution pattern. Adenylate kinase which served as intramitochondrial marker enzyme (26, 44) was localized primarily in the fraction (Fraction I, Table III) external to the inner membrane-matrix vesicle. Although the basis for the apparent activation (or deinhibition) of adenylate kinase caused by fractionating chicken liver mitochondria (Table III) is not understood, this phenomenon was observed consistently.

As illustrated in Table III, the three HMG-CoA cycle enzymes, acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA lyase, were found primarily in the fraction containing the matrix and inner membrane components. Since these enzymes had already been shown to be soluble and not membrane-bound (Table II), it is evident that the ketogenic pathway is compartmentalized within the mitochondrial matrix. This is in basic agreement with a recent report of Chapman et al. (45) who found thiolase and HMG-CoA lyase to be localized within the matrix compartment. However, these investigators studied neither the distribution of these enzymes between mitochondria and cytoplasm nor the localization of HMG-CoA synthase.

Intracytoplasmic Localization of Acetoacetyl-CoA Thiolase and HMG-CoA Synthase—Cytoplasmic HMG-CoA synthase has been reported to be both a soluble and a membrane-bound enzyme in liver (15, 46). To evaluate the intracytoplasmic localization of the enzymes of the HMG-CoA synthesizing system, cytoplasm was separated into cytosol and washed microsomes as described under "Experimental Procedure," and the distribution of acetoacetyl-CoA thiolase and HMG-CoA synthase to be localized within the matrix compartment. Between these fractions was compared with that of lactate dehydrogenase, a cytosolic marker enzyme. All of the acetoacetyl-CoA thiolase and HMG-CoA synthase activity was found in the cytosolic fraction (Table IV, Fraction 1) of both chicken and rat liver; no activity of either enzyme could be detected in the washed microsomal fraction. Thus, it appears that the intracytoplasmic localization of the HMG-CoA generating system which presumably provides precursor for cholesterol synthesis, is cytosolic.

**Distinct Forms of HMG-CoA Synthase in Mitochondrial and Cytoplasmic Compartments of Liver**—Recently it was established (13, 14) on the basis of several criteria, including isoelectric focusing, that different forms of acetoacetyl-CoA thiolase are present in the mitochondrion and cytoplasm of liver. This and the fact that HMG-CoA synthase activity is present (Table I) in both of these cell compartments implies dual intra- and extramitochondrial localization of HMG CoA synthase from acetyl-CoA synthase activity from chicken liver. Isoelectric focusing was conducted as described under "Experimental Procedure." A, mitochondrial matrix from a chicken fasted for 3 days was prepared as described in Table I except that 20 mM sodium phosphate, pH 7.0, was substituted for potassium phosphate. Focusing of 6.9 units of mitochondrial HMG-CoA lyase activity (32 mg of protein) was carried out in 1% Ampholine 8141 for 70 hours at 300 volts. B, cytosol was prepared from a 3-day fasted chicken as described under "Experimental Procedure." Focusing of 1.0 unit of "cytoplasmic" HMG-CoA lyase activity (07 mg of protein) was carried out in 1% Ampholine 8141 for 66 hours at 300 volts. For both A and B, the column contents were collected in 2-ml fractions, and HMG-CoA lyase activity was determined by the radiochemical assay using [3-14C]HMG-CoA of specific activity 8.9 X 10^6 cpm/pmol. Recovery of HMG-CoA lyase activity after isoelectric focusing was 17% for A and 67% for B.
CoA. It became important to determine whether the synthase activities found in the mitochondrial and cytoplasmic fractions are indeed different molecular forms of the enzyme. Our earlier work (15) indicates the existence of two cytoplasmic forms of the enzyme, i.e. HMG-CoA synthase I and II. When the cytoplasmic fraction of chicken liver was subjected to isoelectric focusing, as illustrated in Fig. 5B, two distinct peaks of activity are observed. The pH values for these forms, 6.7 and 4.8, correspond to the pH values obtained for the purified cytoplasmic HMG-CoA synthases I and II, respectively (20). Isoelectric focusing of the lyed mitochondrial fraction of chicken liver yielded a single peak of HMG-CoA synthase activity with an isoelectric point at pH 7.2. Of importance is the fact that the mitochondrial and cytoplasmic synthases retain their respective pH values after purification to homogeneity (19, 20). Thus, it is evident that the mitochondrial and cytoplasmic species of HMG-CoA synthase are molecularly distinct as judged by their isoelectric points. Evidence is presented in an accompanying paper (20), indicating that the various forms of HMG-CoA synthase are also distinguished by differences in their fractionation properties and immunochemical cross-reactivity. It is concluded that liver contains both mitochondrial and cytoplasmic species of HMG-CoA synthase which presumably function in hepatic ketogenesis and cholesterogenesis, respectively.

**TABLE IV**

**Intracytoplasmic distribution of acetoacetyl-CoA thiolase and HMG-CoA synthase activity in liver**

Cytoplasm from chicken liver was prepared and fractionated, and enzyme assays were conducted as described under "Experimental Procedure." HMG-CoA synthase was assayed spectrophotometrically employing 100 mM Tris (Cl-), pH 8.0, containing 200 mM MgCl₂ as assay buffer. All enzyme assay mixtures contained 0.25% Triton X-100 (w/v). Protein was determined by the biuret method (31). Enzyme activities (values in parentheses) are expressed as total units (micromoles of substrate converted/min) in the cytoplasmic fraction/g wet weight of liver. Distribution is expressed in percentages.

|       | Acetooacyl-CoA thiolase | HMG-CoA synthases | Lactate dehydrogenase | Protein |
|-------|-------------------------|-------------------|----------------------|---------|
| **A. Chicken** | | | | |
| Cytoplasm | 100 | 100 | 100 | 100 |
| Fraction | (40.2) | (0.24) | (110) | (101)* |
| I. Cytosol | 88 | 87 | 84 | 62 |
| II. Microsomes | 0 | 0 | 3 | 36 |
| Total recovery in Fractions I + II | 88 | 87 | 87 | 98 |
| **B. Rat** | | | | |
| Cytoplasm | 100 | 100 | 100 | 100 |
| Fraction | (15.4) | (0.15) | (144) | (75)* |
| I. Cytosol | 90 | 80 | 84 | 61 |
| II. Microsomes | 0 | 0 | 8 | 24 |
| Total recovery in Fractions I + II | 99 | 80 | 92 | 85 |

* Milligrams of protein in the cytoplasmic fraction/g wet weight of liver.

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As shown in Table V, liver and kidney contain significant activities of mitochondrial HMG-CoA synthase, whereas other tissues, such as heart, ileum, brain and skeletal muscle, do not contain detectable levels of this enzyme, i.e. <0.01 unit/g wet weight of tissue. To confirm this finding, mitochondrial HMG-CoA synthase activity was estimated using antiserum prepared against purified chicken liver mitochondrial HMG-CoA synthase (19). By the Ouchterlony double diffusion technique, precipitin lines were observed for tissues such as liver and kidney which contain >0.02 unit of HMG-CoA synthase activity/g of tissue, while no precipitin line could be detected for heart, brain, ileum, or skeletal muscle.

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**Fig. 5.** Isoelectric focusing of mitochondrial and cytosolic HMG-CoA synthase activities of chicken liver. Isoelectric focusing was conducted as described under "Experimental Procedure." A, mitochondrial matrix was prepared as described in Table II and focusing of 0.33 unit of mitochondrial HMG-CoA synthase activity (90 mg of protein) was carried out in 1% Ampholine 1801 for 70 hours at 300 volts; HMG-CoA synthase was determined spectrophotometrically in 100 mM Tris (Cl-) buffer, pH 8.2, as described under "Experimental Procedure." B, cytosol was prepared as described under "Experimental Procedure" and focusing of 0.36 unit of cytosolic HMG-CoA synthase activity (102 mg of protein) conducted in 1% Ampholine 1809-101 for 69 hours at 300 volts; HMG-CoA synthase activity was measured by the radiochemical assay with [1-¹⁴C]acetyl-CoA (specific activity, 2.56 × 10⁶ cpm/mmol); fraction size was 1 ml in A and 2 ml in B. The recovery of HMG-CoA synthase activity following isoelectric focusing was 37% for A and 20% for B.
subcellular compartments, cholesterol synthesis in the cytoplasm of liver contains all of the enzymes required for the conversion of which commit acetyl-CoA to cholesterol or acetoacetate synthesis. Rather, these two biosynthetic processes occur in different subcellular compartments, cholesterol synthesis in the cytoplasm and acetoacetate formation in the mitochondria. The cytoplasm of liver contains all of the enzymes required for the conversion of HMG-CoA to cholesterol (40), while only mitochondria appear to possess the lyase needed to convert HMG-CoA to acetoacetate and acetyl-CoA (Table I, Figs. 3 and 4). Consistent with the independence of these diverse pathways is the finding that the cytoplasmic, as well as the mitochondrial, compartment of liver has the capacity to synthesize HMG-CoA, i.e. both contain acetoacetate-CoA thiolase and HMG-CoA synthase (Table I, Ref. 13). Hence, the segment common to both cholesterogenesis and ketogenesis has dual localization. Segregation of the cytoplasmic and mitochondrial HMG-CoA pools is insured by the impermeability of the inner mitochondrial membrane to CoA derivatives (11); consequently, HMG-CoA generated in the cytoplasm apparently serves as precursor for cholesterol, while mitochondrialy generated HMG-CoA serves as precursor for acetoacetate. This point was confirmed recently by the finding that the cytoplasmic acetyl-CoA pool, as precursor for cholesterol synthesis, is not in equilibrium with the mitochondrial acetyl-CoA pool from which acetoacetate is synthesized (16–18).

The dual localization of the HMG-CoA-synthesizing enzymes in liver raises the question of whether the cytoplasmic acetoacetyl-CoA thiolase and HMG-CoA synthase are different proteins or their mitochondrial counterparts. Cytoplasmic acetoacetyl-CoA thiolase which is involved in cholesterol synthesis has been shown (13) to possess molecular properties distinct from its mitochondrial, i.e. ketogenic counterpart. Compelling evidence has also been obtained which shows the mitochondrial (19) and cytoplasmic (15, 20) HMG-CoA synthases to be different proteins. Both synthases have been purified to homogeneity from chicken liver and can be distinguished immunologically as well as by other means. Also important is the fact that the cytoplasmic acetoacetyl-CoA thiolase and HMG-CoA synthase of liver appear subject to negative feedback control by dietary cholesterol, while the corresponding mitochondrial enzymes are not (15, 20).

Cholesterol synthesis occurs in most, if not all animal tissues, although in most extrahepatic tissues the process is relatively slow (50). The tissue distribution of cytoplasmic acetoacetyl-CoA thiolase, the first enzyme in cholesterogenesis, is correlated with the tissue distribution of cholesterol synthetic capacity and has been found in all tissues surveyed (14). Selected tissues of the chicken were surveyed for cytoplasmic HMG-CoA synthase. Liver was found to have the highest activity, 0.3 unit/g wet weight of tissue and extrahepatic tissues, such as kidney (0.1 unit/g) and brain (0.02 unit/g) contained significant activities. The presence of cytoplasmic HMG-CoA synthase in liver, kidney, and brain, as well as in heart and ileum, was confirmed by immunodiffusion with antiserum against homogeneous cytoplasmic HMG-CoA synthase from chicken liver. Hence, the broad tissue distribution of cytoplasmic thiolase and HMG-CoA synthase is compatible with that of cholesterogenesis.

In contrast to the ubiquity of cholesterogenesis, not all animal tissues can synthesize acetoacetate from acetyl-CoA. This pathway, i.e. the HMG-CoA cycle, appears restricted to liver and perhaps kidney (12). Moreover, liver is the only tissue known to be unable to utilize acetoacetate at a significant rate (48); thus, its export to extrahepatic tissues is facilitated. Mitochondrial acetoacetyl-CoA thiolase is involved in both the synthesis of ketones by liver, as well as the utilization of ketones by extrahepatic tissues. Consistent with these physiological roles is the fact that this enzyme has been found in all animal tissues surveyed (14). The inability of extrahepatic tissues to synthesize ketones has been attributed to the absence of mitochondrial II/M-CoA lyase or I/M-CoA synthase, or both (47). In this

### Table V

**Distribution of mitochondrial HMG-CoA synthase and HMG-CoA lyase in various tissues of the chicken**

Mitochondrial fraction (Fraction 1) was prepared as described in Table I from chicken liver, kidney, heart, brain, ileum, and leg muscle. HMG-CoA synthase was assayed spectrophotometrically using 100 mM glycylglycine, pH 8.8, while HMG-CoA lyase was determined by the radiochemical assay using [3-14C]HMG-CoA of specific activity 8.9 X 10^6 cpm/pmol. Both assays contained 0.2% Triton X-100 (w/v) (see “Experimental Procedure” for details). Values given are means of determinations for four chickens ± the standard deviation except for brain and leg muscle where the values are the means for two chickens with the ranges indicated in parentheses.

| Tissue   | HMG-CoA synthase | HMG-CoA lyase | Ketogenic capacity (from Ref. 47) |
|----------|------------------|---------------|----------------------------------|
| Liver    | 1.6 ± 0.4        | 8.1 ± 2.2     | +                                |
| Kidney   | 0.05 ± 0.03      | 5.1 ± 0.4     | +                                |
| Heart    | <0.01            | 1.8 ± 0.6     | -                                |
| Brain    | <0.01            | 1.6 (1.5–1.7) | -                                |
| Ileum    | <0.01            | 0.8 ± 0.3     | -                                |
| Muscle   | <0.01            | 0.6 (0.5–0.7) | -                                |

* One unit equals 1.0 µmol of substrate converted/min.

In contrast, the same tissues all contained significant levels of HMG-CoA lyase activity (Table V). These results are in contradiction to similar studies conducted with the rat where HMG-CoA lyase activity was found to be absent in brain and skeletal muscle (47). Rat brain also was tested for HMG-CoA lyase activity as described in Table V with the finding that the mitochondrial activity was 0.7 unit/g wet weight of brain (average value for four rats). This level of activity also was confirmed by measuring the [3-14C]acetoacetate formed (38) instead of the [3-14C]HMG-CoA utilized.

Since kidney is the only extrahepatic tissue known to be capable of ketogenesis (47), albeit at a slow rate, it is evident that ketogenic capacity is directly correlated to the presence of mitochondrial HMG-CoA synthase activity. Ketogenic capacity is not correlated to the tissue distribution of either mitochondrial acetoacetyl-CoA thiolase or HMG-CoA lyase. This is reasonable since both mitochondrial acetoacetyl-CoA thiolase and HMG-CoA lyase serve extrahepatic functions other than ketogenesis, e.g. β oxidation of fatty acids and ketone body utilization (14) as well as leucine catabolism (36, 37).

### Discussion

Cholesterogenesis and ketogenesis are diverse functions of liver which require independent regulation, yet the first two steps of these pathways are chemically identical (Scheme 1). Upon cursory examination it might seem logical that the flux of acetyl units into these diverging pathways necessarily would be determined at the first committed step(s) beyond the HMG-CoA branch point, i.e. at the HMG-CoA reductase reaction of cholesterogenesis and at the HMG-CoA lyase reaction of acetoacetate formation (Scheme 1). However, the present study indicates that the reductase and lyase reactions are not the steps which commit acetyl-CoA to cholesterol or acetoacetate synthesis. Rather, these two biosynthetic processes occur in different subcellular compartments, cholesterol synthesis in the cytoplasm and acetoacetate formation in the mitochondria. The cytoplasm of liver contains all of the enzymes required for the conversion of HMG-CoA to cholesterol (40), while only mitochondria appear to possess the lyase needed to convert HMG-CoA to acetoacetate and acetyl-CoA (Table I, Figs. 3 and 4). Consistent with the independence of these diverse pathways is the finding that the cytoplasmic, as well as the mitochondrial, compartment of liver has the capacity to synthesize HMG-CoA, i.e. both contain acetoacetate-CoA thiolase and HMG-CoA synthase (Table I, Ref. 13). Hence, the segment common to both cholesterogenesis and ketogenesis has dual localization. Segregation of the cytoplasmic and mitochondrial HMG-CoA pools is insured by the impermeability of the inner mitochondrial membrane to CoA derivatives (11); consequently, HMG-CoA generated in the cytoplasm apparently serves as precursor for cholesterol, while mitochondrialy generated HMG-CoA serves as precursor for acetoacetate. This point was confirmed recently by the finding that the cytoplasmic acetyl-CoA pool, as precursor for cholesterogenesis, is not in equilibrium with the mitochondrial acetyl-CoA pool from which acetoacetate is synthesized (16–18).

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connection, mitochondrial HMG-CoA synthase appears to function primarily in ketogenesis, while HMG-CoA lyase participates in both hepatic ketogenesis and in the leucine catabolic pathway (37, 51). As illustrated in Table V, liver and kidney contain significant mitochondrial HMG-CoA synthase activity, whereas in other tissues, such as heart, ileum, brain, and skeletal muscle, the enzyme appears to be absent, i.e. <0.01 unit/g wet weight of tissue. In contrast, the same tissues contained significant levels of mitochondrial HMG-CoA lyase activity (Table V). These results are in disagreement with those conducted with the rat where HMG-CoA lyase activity was not detectable in brain and skeletal muscle (47, 52). Rat brain tested for HMG-CoA lyase activity as described in Table V contains 0.7 unit of the mitochondrial enzyme/g wet weight of tissue.

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