

# LOCALIZATION OF THE ENZYMES OF KETOGENESIS IN RAT LIVER MITOCHONDRIA

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## ABSTRACT

The localization of the enzymes of ketogenesis in isolated rat liver mitochondria has been investigated. Mitochondrial subfractions were isolated after disruption of this subcellular organelle by (a) hypotonic lysis in water, which permitted the ultracentrifugal separation of the soluble and membranous compartments of the mitochondrion, or by (b) a procedure involving swelling, contraction, and ultrasonic treatment, which permitted the isolation from discontinuous sucrose gradients of subfractions rich in intermembrane space protein, outer membrane, and inner membrane-matrix particles. Two membrane subfractions were invariably present as distinct bands at the lower interface of the discontinuous gradient. The upper of these two bands was found to be a highly purified preparation of outer mitochondrial membrane. Subfractions rich in matrix and in inner membrane were isolated from inner membrane-matrix particles after hypotonic treatment. The content of the various mitochondrial compartments in all subfractions was assessed from their enzymic and electron microscopic characteristics. The ketogenic activity of each subfraction was determined by measuring its capacity to form ketone bodies from acetyl CoA. The activity of this process was markedly enhanced by dithiothreitol. These measurements of ketone body formation, together with assays of individual enzymes of the ketogenic pathway, show that thiolase, HMGCoA synthase, and HMGCoA cleavage enzyme are localized in the matrix of the inner membrane-matrix particles. The rates of ketone body formation indicate that the HMGCoA synthase is the rate-limiting enzyme of the pathway in subfractions of high matrix content. Studies with sodium chloride indicate that a large portion of the HMGCoA synthase, which remains present in membrane subfractions derived from water-treated mitochondria, is bound by ionic interaction to component(s) of the membrane.

## INTRODUCTION

The liver was identified as the principal source of the ketone bodies, acetoacetic and  $\beta$ -hydroxybutyric acids, by the perfused organ studies in the Embden laboratories over 60 yr ago (1, 2). In support and extension of the work of Geelmuyden (3), who first associated the production of

ketone bodies with fat metabolism, the Embden group observed that the addition of fatty acids to the liver perfusion medium increased the production of ketone bodies (4, 5). With the application of isotopic methods to studies of fatty acid oxidation, Weinhouse and co-workers (6) and Crandall and

Gurin (7) established that fatty acids were converted to ketone bodies via the formation and condensation of two-carbon intermediates.

The earliest demonstrations of ketogenesis in cell-free preparations of liver were those of Munoz and Leloir (8) and of Lehninger (9). Upon the development of methods to separate cellular organelles from tissue homogenates, it was shown that the mitochondrial fraction possessed essentially all of the hepatic capacity for the oxidation of fatty acids to ketone bodies (10, 11).

The enzymatic studies of Lynen et al. (12) elucidated the pathway by which ketone bodies are produced from acetyl CoA in the liver. This process of ketogenesis was found to involve three enzymes, acetoacetyl-CoA thiolase,  $\beta$ -hydroxy- $\beta$ -methylglutaryl CoA (HMGCoA) synthase, and the HMGCoA cleavage enzyme, which together constitute the HMGCoA cycle. The acetoacetate produced in this cycle may be reduced to D- $\beta$ -hydroxybutyrate by the action of D- $\beta$ -hydroxybutyrate dehydrogenase (13, 14).

Further evidence for a principal role of the mitochondrion in the production of ketone bodies in the liver was provided by Williamson et al. (15), who found about 90% of the hepatic ketogenic activity in the particulate fraction. The outer membrane of liver mitochondria is permeable to acetyl CoA, whereas the inner membrane is not (16). If the enzymes of ketogenesis are located on the outer surface of, or external to, the inner mitochondrial membrane, intact mitochondria should produce ketone bodies from acetyl CoA as readily as disrupted mitochondria. The increased ketogenesis from acetyl CoA consequent to freezing and thawing (17, 18) and sonication (15) of cell particles is therefore indicative of localization of the enzymes of ketogenesis in the inner membrane and/or the matrix of mitochondria. This has been substantiated by the observation that ketogenesis is associated with inner membrane-matrix particles (19).

To investigate further the intramitochondrial localization of the enzymes of ketogenesis, we have studied the formation of ketone bodies and the activity of the enzymes of the HMGCoA cycle in each of the soluble and membranous compartments of rat liver mitochondria. The mitochondrial subfractions, which correspond with these compartments, have been characterized by the activities of "marker" enzymes and by electron microscopy.

## MATERIALS AND METHODS

### *Materials*

Citrate synthase,  $\beta$ -hydroxybutyrate dehydrogenase, hexokinase, glucose-6-phosphate dehydrogenase, and malate dehydrogenase were obtained from Boehringer Mannheim Corp. (New York). Phosphotransacetylase, coenzyme A, acetyl phosphate, AMP, ADP, ATP, EGTA, succinic acid, diketene, dithiothreitol (DTT), triethanolamine HCl, and Tris (Trizma base) were obtained from Sigma Chemical Co. (St. Louis, Mo.). L-malic acid and  $\alpha$ -ketoglutaric acid were purchased from Calbiochem (San Diego, Calif.). NADH, NAD, and NADP were supplied by P-L Biochemicals, Inc. (Milwaukee, Wis.). Crystalline sucrose, Ultrapure, was purchased from Schwarz/Mann Div. Becton, Dickinson & Co. (Orangeburg, N. Y.) and Pentex crystallized bovine albumin from Miles Laboratories, Inc., Miles Research Div. (Kankakee, Ill.). HMGCoA was synthesized according to the method of Louw et al. (20), and acetoacetyl CoA was prepared with diketene under the conditions described by Simon and Shemin (21).

### *Animals*

Adult male Holtzman rats (Holtzman Co., Madison, Wis.), 300-400 g, were used. These animals were maintained on Rockland rat diet and water ad lib. All animals were fasted for 24 h before sacrifice; food was removed between 9 and 11 a.m. on the preceding day.

### *Preparation of Mitochondria*

The rats (two to three) were stunned by a blow on the head, exsanguinated, and the livers quickly placed in ice-cold 0.9% NaCl. After the removal of any extraneous tissue, these livers (about 20 g total weight) were placed in 20-30 ml of ice-cold isolation medium (0.25 M sucrose, 3 mM Tris chloride, and 1 mM EGTA, pH 7.4) and cut into small pieces with scissors. 2-g portions of the chopped tissue were homogenized by hand (four to five strokes) in a glass Potter-Elvehjem homogenizer with a loose Teflon pestle (Kontes Glass Co., Vineland, N. J.) after the volume of each portion was adjusted to 20 ml with isolation medium. On completion of homogenization, the pooled homogenate was centrifuged at 500 *g* for 7 min. Mitochondria were isolated from the resulting supernate by centrifugation at 9,000 *g* for 10 min. The supernate was discarded. The mitochondrial pellet was resuspended with a cold finger (test tube containing ice) in the original volume of isolation medium, and recentrifuged under the same conditions.

After discarding any fluffy layer, the mitochondria were resuspended in one-half of the original volume of isolation medium. In order to discard erythrocytes in the base of the tube, the mitochondria adjacent to these erythrocytes were not resuspended. The mitochondrial suspension was transferred to other tubes and recentrifuged under the same conditions. After discarding any fluffy layer, the mitochondria were resuspended and washed twice in one-fourth of the original volume of isolation medium; centrifugation in each case was at 9,000  $g$  for 10 min. After removal of the fluffy layer, the final mitochondrial pellet was resuspended as outlined below. All procedures involved in the isolation of the mitochondria were carried out at 0–3°C.

### Preparation and Subfractionation of Water-Suspended Mitochondria

The final mitochondrial pellet, obtained from approximately 20 g of liver, was resuspended in 40 ml of distilled water (0–3°C) with a Pasteur pipette. After the observation that DTT stimulated ketogenesis, the mitochondria were resuspended in 1 mM DTT. Under these conditions the protein concentration of the resulting suspension was 2–4 mg/ml. Any remaining clumps of mitochondria were dispersed by drawing the suspension into, and allowing it to drain by gravity from, a 25 ml large bore pipette several times. This procedure was carried out at 0–3°C. After standing for 15 min at 0°C, 11-ml aliquots of the mitochondrial suspension in water were centrifuged at 47,000 rpm in the 50 Ti rotor of the Beckman L2-50 ultracentrifuge for 1 h at 0–1°C (146,000  $g_{av}$ ). On completion of ultracentrifugation, the supernate was quantitatively removed with a Pasteur pipette. The pellet of mitochondrial membranes was then resuspended in ice-cold distilled water (3 ml per tube) by light hand homogenization (three strokes) in a glass homogenizer (Kontes Glass Co.) and maintained at 0°C in an ice bath.

### Preparation of Mitochondrial Subfractions

The procedure adopted for the subfractionation of rat liver mitochondria was based on that of Sottocasa et al. (22). The final pellet of sucrose-washed mitochondria (containing 90–100 mg protein) was suspended in 30 ml of 10 mM Tris-phosphate, pH 7.5, with the use of a cold finger and a 10 ml large bore pipette. After standing for 5 min at 0°C, 10 ml of a solution containing 1.8 M sucrose, 2 mM ATP, and 2 mM  $MgSO_4$  was added to the suspension. After 5 min at 0°C, 13-ml samples of this suspension were subjected to ultrasonic treatment in 20-ml beakers at 0°C with a Branson sonifier (model W140). The suspension was sonicated for 15 s at

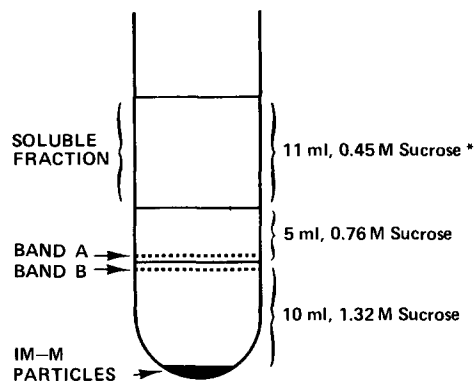


FIGURE 1 The distribution of mitochondrial subfractions in the discontinuous sucrose gradient after ultracentrifugation. The inner membrane-matrix (IM-M) particles are completely sedimented at the base of the tube. (\*) The mitochondrial suspension was applied to the gradient in a 0.45 M sucrose solution containing 0.5 mM  $MgSO_4$ , 0.5 mM ATP, and 7.5 mM Tris-phosphate, pH 7.5. The densities of the sucrose solutions were; 0.45 M, 1.057 g/ml; 0.76 M, 1.125 g/ml; and 1.32 M, 1.178 g/ml, respectively.

setting 4 with the standard  $\frac{1}{2}$  inch disruptor horn and  $\frac{1}{2}$  inch tip. Under these conditions, the instrument reading during sonication was 33. After ultrasonic treatment, an 11 ml aliquot of the combined suspension was layered upon a discontinuous sucrose gradient, consisting of 5 ml of 0.76 M sucrose and 10 ml of 1.32 M sucrose (Fig. 1). The three resulting layers gave a stepwise gradient of densities from 1.057 g/ml (11 ml containing the mitochondria) to 1.125 g/ml (5 ml) to 1.178 g/ml (10 ml). Three such discontinuous gradients were centrifuged in the SW 25.1 rotor of the Beckman L2-50 ultracentrifuge at 24,000 rpm for 3 h at 0–1°C (58,600  $g_{av}$ ).

On completion of ultracentrifugation, it was possible to distinguish two narrow yellow-brown bands separated by a clear zone 2–3 mm wide in each gradient (Fig. 1); the interface of the 0.76 M and 1.32 M sucrose solutions bisected this clear zone. In addition, a brown pellet was visible at the base of each tube. All other zones of the gradients were clear and colorless, with the exception of the 0.45 M sucrose layer, which was clear but faintly yellow. This layer, which will be referred to as the soluble fraction, was quantitatively removed with a Pasteur pipette. The upper portion of the 0.76 M sucrose layer was removed similarly. Band A (Fig. 1), the upper of the two bands at the 0.76 M/1.32 M sucrose interface, was then removed with a syringe, fitted with a needle bent at 90° halfway along its length. Band A was normally removed in a volume of about 3 ml; this volume included the upper half of the clear zone. Band B was then removed in a similar

volume from each gradient and included the lower half of the clear zone. The remainder of the 1.32 M sucrose layer was then removed and the pellet of inner membrane-matrix (IM-M) particles (Fig. 1) was resuspended in 5 ml of isolation medium. Resuspension was effected by light hand homogenization (two strokes) of the pellet in a glass homogenizer.

Having combined the volumes of each A band and of each B band from the three gradients, their total volumes were increased to 11.0 ml with isolation medium and centrifuged in the 50 Ti rotor of the Beckman L2-50 ultracentrifuge at 47,000 rpm for 1 h at 0–1°C (146,000  $g_{av}$ ). In this way the material from each of the two bands was sedimented. Each membrane fraction was resuspended in isolation medium (2 ml) with a Pasteur pipette.

#### *Subfractionation of Inner Membrane and Matrix Particles*

For the preparation of inner membrane and of matrix subfractions from the inner membrane-matrix particles (IM-M), each IM-M pellet was resuspended in an ice-cold solution of 1 mM dithiothreitol (DTT) in water (11 ml) by hand homogenization (six strokes) in a glass homogenizer. After standing for 15 min at 0°C, the suspension was centrifuged in the 50 Ti rotor of the Beckman L2-50 ultracentrifuge for 1 h at 0–1°C (146,000  $g_{av}$ ). After ultracentrifugation, the supernate (matrix fraction) was removed, and the pellet of inner membrane was resuspended in 1 mM DTT (5 ml).

#### *Ultrasonic Treatment of Mitochondrial Fractions*

All of the mitochondrial subfractions which contained membranous elements, i.e. the material in bands A and B, the inner membrane-matrix particles, and the inner membrane in addition to samples of the original mitochondrial suspension, were subjected to ultrasonic treatment before assay for marker enzyme activities. A Branson sonifier (Branson Sonic Power Co., Danbury, Conn.), model W140 with the standard ½ inch disruptor horn and ½ inch tip, was used. Each subfraction was sonicated in a 20 ml beaker in an ice bath at setting 8 for 15 s. The instrument meter reading under these conditions was between 40 and 46.

#### *Electron Microscopy*

The various mitochondrial preparations were fixed with cold (4°C) 2% glutaraldehyde in 0.08 M cacodylate buffer, pH 7.2, for 15 min. Fixation was carried out either in suspension or as a pellet. The suspended material was centrifuged following fixation in order to form a pellet. The pellets were washed

with isosmotic wash solution (23) for 24 h. Post-fixation was accomplished with osmium tetroxide (24). The osmicated material was cut into small blocks, dehydrated and embedded in Maraglas (25). Silver to grey sections were made on a Porter-Blum ultramicrotome. These sections were mounted on naked grids, and subsequently stained with uranyl acetate and lead citrate (26, 27). The sections were viewed with an RCA EMU 4f electron microscope.

#### *Enzymatic and Chemical Assays*

Monoamine oxidase was assayed spectrophotometrically by the method of Tabor et al. (28), as modified by Schnaitman et al. (29). Adenylate kinase activity was determined by the method of Sottocasa et al. (22) except that KCN (final concentration 0.45 mM) was added. Glutamic dehydrogenase was assayed by the method of Schmidt (30).  $\beta$ -Hydroxybutyrate dehydrogenase was measured at 340 nm in cuvettes containing 0.4 mM NADH and 33 mM phosphate, pH 7.0. The reaction was initiated by adding 50  $\mu$ l of 0.5 mM sodium acetoacetate. HMGCoA cleavage enzyme activity was determined by recording the reaction rate, after the addition of HMGCoA, as described in the assay of HMGCoA by Hamprecht and Lynen (31). To decrease oxidation of NADH by NADH dehydrogenases, 1  $\mu$ M rotenone was added. The thiolase assay of Stewart and Rudney (32) was used. Protein was estimated by the method of Lowry et al. (33) with bovine serum albumin as a standard.

#### *Determination of Ketone Body Formation by Mitochondrial Fractions*

Duplicate samples of each mitochondrial fraction (0.5–1.5 mg of protein) were incubated at 37°C for 10 min with an acetyl CoA-generating system (12) in a final volume of 2.0 ml. This system contained 25 units of phosphotransacetylase and the following components at the final concentrations indicated: 10 mM sodium phosphate (pH 7.4), 4 mM ATP, 30 mM lithium acetyl phosphate, 1 mM CoA, 35 mM KCl, and 3 mM MgCl<sub>2</sub>. The pH was adjusted to 7.4 before use. Although ATP was present in these experiments, it was subsequently observed that its omission did not diminish ketogenesis. After the stimulation of ketogenesis by DTT was observed (see Results), a final concentration of 0.5 mM DTT was employed. It was found in separate experiments that maximum stimulation was provided by 0.25 mM DTT. A constant rate of ketogenesis was observed during the incubation period. At the end of the incubation 2 ml of cold 30% perchloric acid was added. After removing the precipitate by centrifugation, the pH of the super-

nate was adjusted to 6-7 as described previously (34). The resulting precipitate was again removed by centrifugation. The supernates were analyzed for acetoacetate (35) and  $\beta$ -hydroxybutyrate (36).  $\beta$ -Hydroxybutyrate was not produced by any of the mitochondrial preparations incubated in these experiments.

## RESULTS

### *Water-Suspended Mitochondria*

Suspension of rat liver mitochondria in water, at a final protein concentration of 2-4 mg/ml, resulted in the release of 53% of the total mitochondrial protein into the 146,000 g supernatant fraction (Table I). The proportion of mitochondrial protein present in the supernatant fraction ranged from 46.0 to 59.0% in a series of 17 experiments in which the protein concentration of the mitochondrial suspension varied from 2.09 to 3.75 mg/ml. The activities of the marker enzymes for the outer and inner mitochondrial membranes, monoamine oxidase (29), and  $\beta$ -hydroxybutyrate dehydrogenase (37, 38) respectively, were located exclusively in the 146,000 g

pellet (Table I). In contrast, the activity of adenylate kinase, a marker for the intermembrane space compartment (39, 40), was present solely in the supernatant fraction. Moreover, a high proportion (86%) of the activity of glutamic dehydrogenase, a marker for the matrix compartment (27, 37, 39), was similarly located in the supernatant fraction.

These data show that the supernatant fraction is derived from both soluble compartments of the mitochondrion, i.e., the intermembrane space and matrix. In comparison, the pellet is composed primarily of the two mitochondrial membrane systems. However, about 13% of the activity of the matrix marker, glutamic dehydrogenase, was detected in this membrane pellet. Since the pellet was not sonicated before assay, the possibility existed that additional matrix remained inside membrane vesicles and remained undetected due to diffusion barriers. The glutamic dehydrogenase activity in the pellet relative to the supernate was increased by sonication from 14.9% to 26.5% (Table II). Thus, about 75% of the matrix protein was released

TABLE I  
*Distribution of Protein and Enzyme Activities in Fractions Derived from Water-Suspended Mitochondria*

|  | Water-suspended mitochondria | 146,000 g Supernate  | 146,000 g Pellet    |
|--|------------------------------|----------------------|---------------------|
| Protein content                        |                              |                      |                     |
| mg/ml                                  | 2.95 $\pm$ 0.58* (17)†       |                      |                     |
| Percent of total                       |                              | 52.6 $\pm$ 3.3 (17)  | 47.4 $\pm$ 3.3 (17) |
| Monoamine oxidase                      |                              |                      |                     |
| Percent of total                       | 100 (5)                      | 0 (5)                | 96.9 $\pm$ 8.6 (5)  |
| Specific activity§                     | 3.8 $\pm$ 1.5 (5)            | 0 (5)                | 7.8 $\pm$ 0.7 (5)   |
| Adenylate kinase                       |                              |                      |                     |
| Percent of total                       | 100 (3)                      | 102.2 $\pm$ 4.7 (3)  | 0 (3)               |
| Specific activity                      | 38.5 $\pm$ 13.0 (3)          | 82.5 $\pm$ 26.1 (3)  | 0 (3)               |
| $\beta$ -Hydroxybutyrate dehydrogenase |                              |                      |                     |
| Percent of total                       | 100 (5)                      | 0 (5)                | 94.7 $\pm$ 7.0 (5)  |
| Specific activity                      | 50.2 $\pm$ 7.5 (5)           | 0 (5)                | 108.0 $\pm$ 8.3 (5) |
| Glutamic dehydrogenase                 |                              |                      |                     |
| Percent of total                       | 100 (5)                      | 85.8 $\pm$ 7.6 (5)   | 12.7 $\pm$ 5.8 (5)  |
| Specific activity                      | 151.7 $\pm$ 27.3 (5)         | 257.0 $\pm$ 38.1 (5) | 41.4 $\pm$ 21.2 (5) |

\* All values are means  $\pm$  SD.

† Figures in parentheses indicate the number of preparations analyzed.

§ Units are nanomoles of benzaldehyde formed per minute per milligram of protein.

|| Units are nanomoles of pyridine nucleotide oxidized or reduced per minute per milligram protein. The supernatant and pellet subfractions were derived from the same suspension of mitochondria in each experiment; the enzyme activity in each subfraction is expressed as a percentage of the activity in the mitochondrial suspension (taken as 100%) from which it was derived. Samples of the mitochondrial suspension and 146,000 g pellet were sonicated (setting 8, 15 s) before assay of  $\beta$ -hydroxybutyrate dehydrogenase activity.

TABLE II  
*Effect of Ultrasonic Treatment on the Glutamic Dehydrogenase Activity of Subfractions  
 Derived from Water-Suspended Mitochondria*

| Mitochondrial subfraction | Percent of total glutamic dehydrogenase activity |                             |
|---------------------------|--|-----------------------------|
|                           | Control (4)                                      | Ultrasonically-treated* (4) |
| 146,000 g supernate       | 85.1 ± 0.4                                       | 73.5 ± 2.4                  |
| 146,000 g pellet          | 14.9 ± 0.4                                       | 26.5 ± 2.1                  |

Figures in parentheses indicate the number of different mitochondrial preparations analyzed. In each experiment, the subfractions were derived from the same original suspension of mitochondria in water. All values are means ± SD.

\* A sample of each subfraction was subjected to ultrasonic treatment at 0°C for 15 s at setting 8, using a Branson sonifier (model W140). The average increase in total glutamic dehydrogenase activity of the membrane subfraction resulting from this treatment was 106%.

into the supernate upon water treatment of the mitochondria and 25% remained associated with the mitochondrial membranes.

#### *Electron Microscopic Observations on the Water-Suspended Mitochondria*

Minimal ergastoplasmic contamination was present in the original mitochondrial preparations (Fig. 2). While the mitochondria were well preserved, separation and evagination of the outer mitochondrial membrane were occasionally observed.

The typical appearance of the water-suspended mitochondrial preparation, 15 min after the suspension of the mitochondria in water, is shown in Fig. 3. The mitochondria are swollen and irregular in shape, and contain little residual matrix. Most are devoid of outer membrane. Vesicular structures may be seen interspersed with the partially lysed and completely disrupted mitochondrial ghosts. A number of extra-mitochondrial strands of electron-opaque matrix substance were consistently observed in the water-suspended mitochondrial preparations. The mitochondrial ghosts resemble those described by Schnaitman et al. (29), which were obtained by sequential water washing, although the concentration of matrix in the ghosts in the present preparation was lower.

A section from the 146,000 g pellet of the water-suspended mitochondrial preparation is seen in Fig. 4. While all of the membranous structures observed in the mitochondrial suspension (Fig. 3) were present in the pelleted material, the electron-opaque matrix substance was absent.

#### *Ketone Body Production by Subfractions of Water-Suspended Mitochondria*

The formation of ketone bodies by water-suspended rat liver mitochondria and by the supernate and pellet derived from this suspension after ultracentrifugation at 146,000 g is presented in Table III. The water-suspended mitochondria and the membrane subfraction were not subjected to ultrasonic treatment before incubation with the acetyl CoA-generating system, since it was initially established that such treatment did not significantly increase the ketogenic activity of these preparations.

Of the subfractions prepared from the water-suspended mitochondria, only the 146,000 g supernate produced acetoacetate in significant amounts. This soluble fraction contained 30% of the ketogenic activity of the original mitochondrial suspension, while in contrast, only 4% of this original activity was present in the 146,000 g membrane fraction. Upon reconstitution of the original suspension of water-treated mitochondria, represented by fraction I + II, the production of acetoacetate increased to a level comparable with that of the mitochondrial suspension. Synergism therefore occurred upon recombination of the mitochondrial subfractions, suggesting a differential distribution of the enzymes and/or a cofactor(s) of ketone body formation between the mitochondrial membrane and supernatant fractions.

A marked increase in the production of acetoacetate by the water-suspended mitochondria and by each subfraction resulted when water lysis was carried out in the presence of 1 mM dithio-

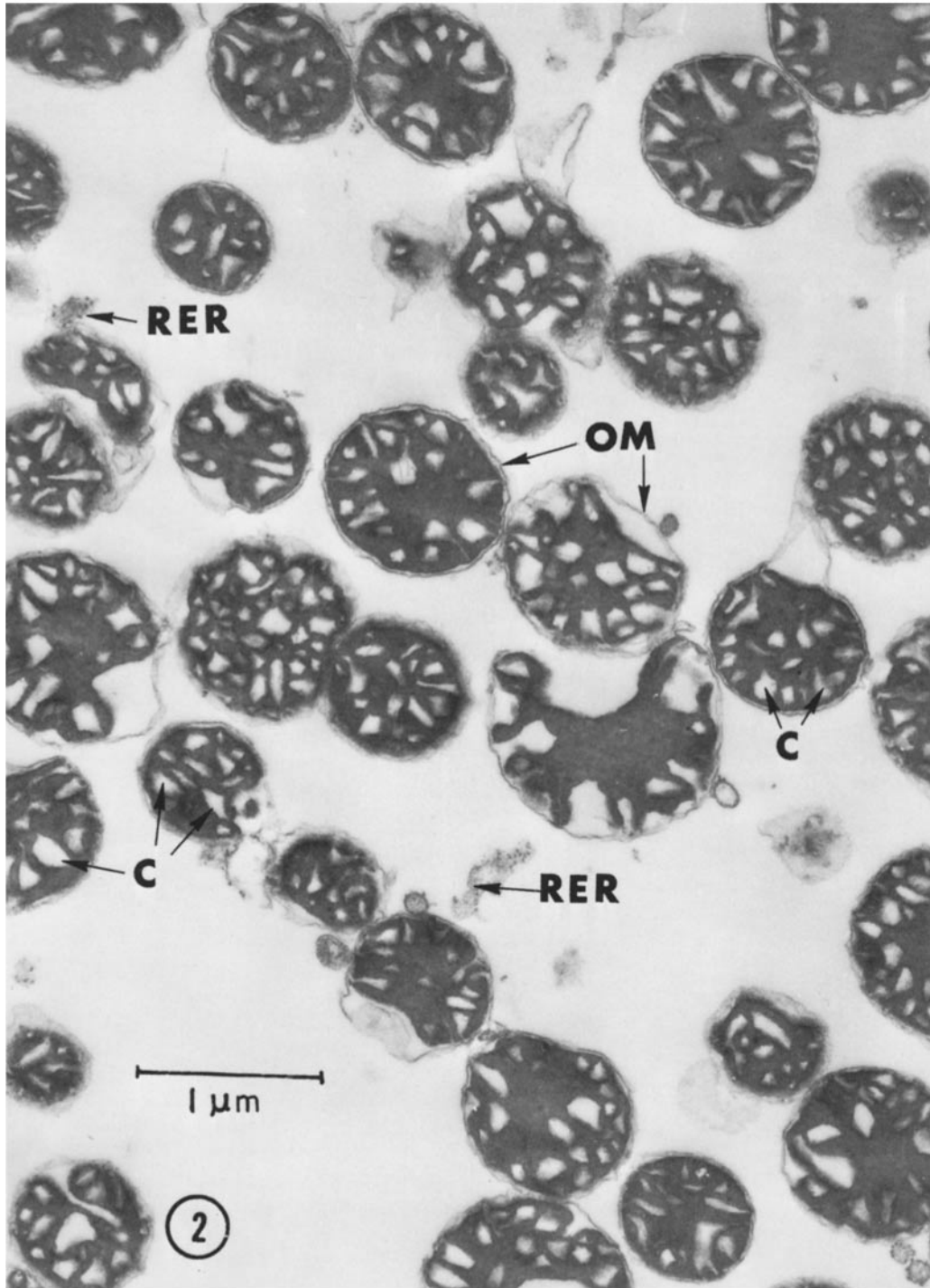


FIGURE 2 Electron micrograph of freshly isolated rat liver mitochondria, fixed while suspended in isolation medium. Swollen cristae (*C*) are prominent within the electron-opaque matrices. The outer membranes (*OM*) may be clearly seen. Traces of rough endoplasmic reticulum (*RER*) are present. The bar represents 1  $\mu\text{m}$ .  $\times 27,000$ .

threitol (DTT) (Table III). The membrane pellet derived therefrom was similarly suspended in this solution. DTT more markedly enhanced the ketogenic activity of the membrane subfraction

than that of the soluble fraction. The synergism in the reconstituted fraction I + II in the absence of DTT was also observed in its presence. It was later found that DTT was equally effective

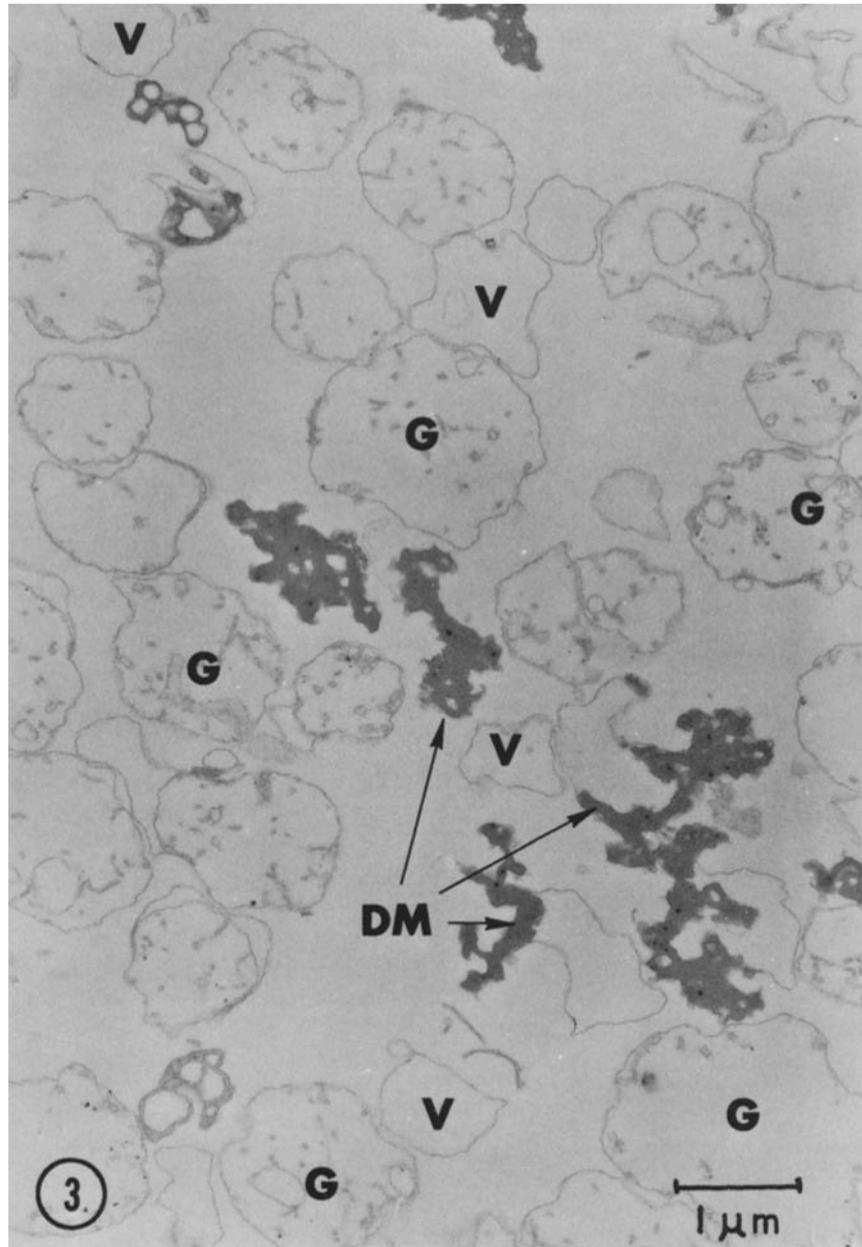


FIGURE 3 Electron micrograph of water-suspended mitochondria, fixed in suspension with glutaraldehyde. The mitochondria appear as ghosts (*G*) and possess a single membrane. Small, round membrane profiles are recognizable within the ghosts. Amorphous material may be seen associated with these profiles. Numerous vesicular structures (*V*) are also present, in addition to extra-mitochondrial strands of dense matrix substance (*DM*). The bar represents 1  $\mu\text{m}$ .  $\times 16,000$ .



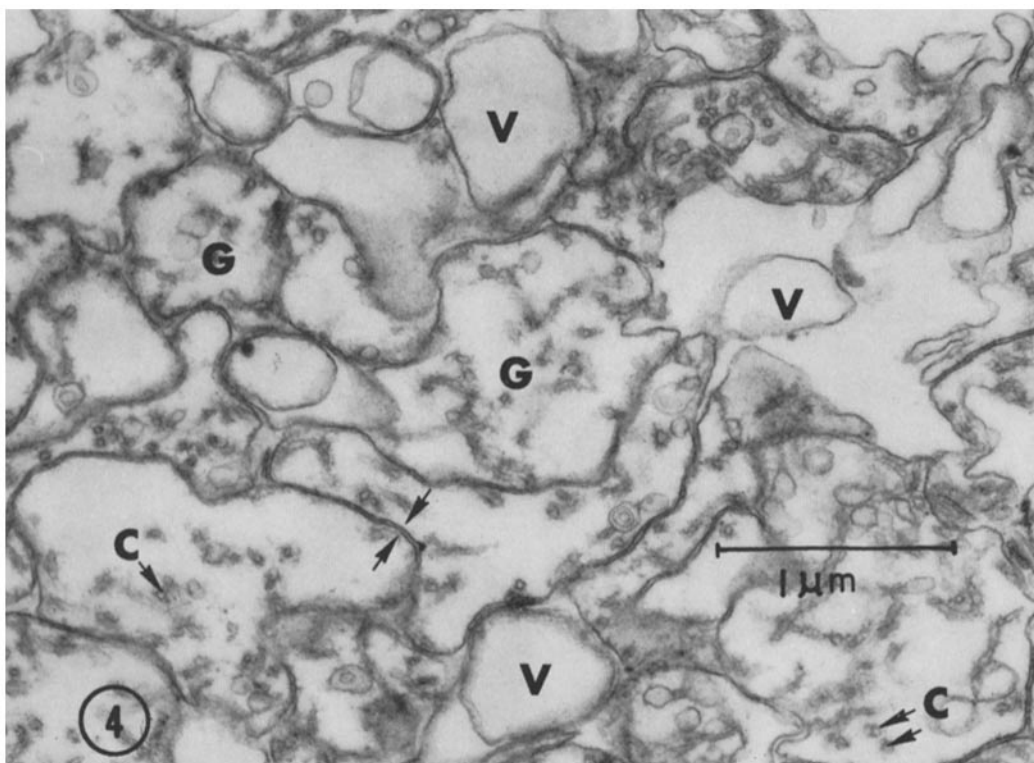


FIGURE 4 Electron micrograph of a section from the 146,000 *g* pellet of water-suspended mitochondria. Several ghosts (*G*) may be seen, together with large numbers of empty vesicular structures (*V*). The ghosts possess a single limiting membrane, which appear as a double membrane (arrows) when adjacent ghosts are in close proximity. Remnants of the cristal membranes (*C*) were seen within the ghosts. The bar represents 1  $\mu\text{m}$ .  $\times 30,000$ .

when added to the incubation medium as when added at the time of lysis. These observations suggest that the decreased ketogenic activity of the mitochondrial subfractions in the absence of DTT (Table III) resulted from the inactivation of a participating component(s) of the system during the 10 min of incubation at 37°C. This component could be an enzyme(s) and/or coenzyme A, which is protected from inactivation by DTT. The synergism in acetoacetate formation in fraction I + II in the presence of DTT again suggests that the enzymes of ketogenesis and/or a cofactor(s) are differentially distributed between the supernatant and membrane subfractions. Subsequently, water treatment of the original mitochondrial pellet was carried out with water containing 1 mM DTT, and the membrane fractions derived therefrom were suspended in 1 mM DTT. A final concentration of 0.5 mM DTT in

the incubation medium was employed (see Materials and Methods).

When the osmolarity of the water-suspended mitochondria was raised by the addition of sodium chloride (to a final concentration of 0.15 M before centrifugation at 146,000 *g*), the ketogenic activity of the supernatant fraction was markedly increased (Table IV). Thus, the total amount of acetoacetate formed by the supernate was only 13% less than that of the mitochondrial suspension from which it was derived. The activity of the membrane pellet was concurrently decreased by the salt treatment. The presence of sodium chloride did not alter the ketogenic activity of the reconstituted fraction (I + II). Furthermore, it was found in other experiments that the addition of sodium chloride to the supernatant and membrane subfractions after their isolation from the mitochondrial suspension did not alter

TABLE III  
Effect of Dithiothreitol on the Formation of Ketone Bodies by Subfractions of Water-Suspended Rat Liver Mitochondria

| Fraction                     | Control*               |                  |                    | Dithiothreitol-treated* |                  |                    |
|------------------------------|------------------------|------------------|--------------------|-------------------------|------------------|--------------------|
|                              | Acetoacetate Formation |                  |                    |                         |                  |                    |
|                              | Total‡                 | Percent of total | Specific activity§ | Total‡                  | Percent of total | Specific activity§ |
|                              | $\mu\text{mol}$        |                  |                    | $\mu\text{mol}$         |                  |                    |
| Water-suspended mitochondria | 11.9                   | 100.0            | 33.7               | 19.9                    | 100.0            | 56.4               |
| 146,000 g supernate (I)      | 3.6                    | 30.3             | 22.8               | 6.8                     | 34.2             | 43.0               |
| 146,000 g pellet (II)        | 0.5                    | 4.2              | 2.7                | 5.6                     | 28.1             | 30.8               |
| I + II                       | 10.8                   | 90.8             | 31.8               | 20.0                    | 100.5            | 58.8               |

\* The control and dithiothreitol-treated water-suspended mitochondria were obtained by the lysis of equal amounts of intact mitochondria with water and with 1 mM DTT respectively. These suspensions were then centrifuged at 146,000 g to separate the soluble (supernatant) and membrane (pellet) subfractions (see Materials and Methods).

‡ Total acetoacetate produced by each fraction in 10 min incubation at 37°C, calculated on the basis of a total volume of each fraction equivalent to that present in 11 ml of water-suspended mitochondria from which it was derived. The water-suspended mitochondria contained 3.53 mg protein/ml. 44.8% of the protein was recovered in the supernate and 51.5% in the membrane fraction (pellet) in both the control and the DTT-treated preparations.

§ Nanomoles of acetoacetate formed per milligram of protein per minute at 37°C.

|| Fraction I + II was obtained by the addition of fractions I and II in amounts proportionate to that present in the water-suspended mitochondria.

TABLE IV  
The Effect of 0.15 M Sodium Chloride Pretreatment on the Formation of Ketone Bodies by Subfractions of Water-Suspended Rat Liver Mitochondria

| Fraction                     | Control                |                  |                    | Sodium chloride-treated* |                  |                    |
|------------------------------|------------------------|------------------|--------------------|--------------------------|------------------|--------------------|
|                              | Acetoacetate formation |                  |                    |                          |                  |                    |
|                              | Total‡                 | Percent of total | Specific activity§ | Total‡                   | Percent of total | Specific activity§ |
|                              | $\mu\text{mol}$        |                  |                    | $\mu\text{mol}$          |                  |                    |
| Water-suspended mitochondria | 14.0                   | 100.0            | 53.4               | 13.5                     | 100.0            | 51.5               |
| 146,000 g supernate (I)      | 3.9                    | 27.9             | 29.5               | 11.7                     | 86.7             | 88.0               |
| 146,000 g pellet (II)        | 3.4                    | 24.3             | 27.4               | 0.6                      | 4.4              | 4.9                |
| I + II                       | 12.8                   | 91.4             | 50.0               | 12.9                     | 95.6             | 50.6               |

Conditions are described in Table III.

\* Sodium chloride, at a final concentration of 0.15 M, was added to the mitochondrial suspension immediately before ultracentrifugation.

‡ Total acetoacetate produced by each fraction in 10 min incubation at 37°C, calculated on the basis of a total volume of each fraction equivalent to that present in 11 ml of water-suspended mitochondria from which it was derived. All fractions were incubated in the presence of 0.5 mM DTT. The water-suspended mitochondria contained 2.62 mg protein/ml. 50.3% of the protein was recovered in the supernatant and 47.3% in the membrane fraction (pellet). After salt treatment 50.6% of the protein was found in the supernatant and 46.4% in the membrane fraction.

§ The units of specific activity are nanomoles of acetoacetate formed per milligram of protein per minute at 37°C.

**TABLE V**  
*Synergistic Enhancement of Ketogenesis by a Sodium Chloride-Extractable Substance in the Membrane Fraction*

| Fraction  | Acetoacetate formation |                  |                    |
|---|------------------------|------------------|--------------------|
|   | Total*                 | Percent of total | Specific activity‡ |
|   | <i>μmol</i>            |                  |                    |
| Water-suspended mitochondria                          | 16.0                   | 100.0            | 46.9               |
| 146,000 g supernate (I)                               | 5.3                    | 33.1             | 36.0               |
| 146,000 g pellet (II)                                 | 3.7                    | 23.1             | 22.4               |
| I + II  | 14.2                   | 88.8             | 46.8               |
| 146,000 g supernate after NaCl treatment of II (III)§ | 2.5                    | 15.6             | 63.1               |
| 146,000 g pellet after NaCl treatment of II (IV)§     | 1.4                    | 8.8              | 10.6               |
| III + IV  | 4.2                    | 26.3             | 28.7               |
| I + III   | 12.7                   | 79.4             | 67.9               |
| I + IV  | 7.8                    | 48.8             | 27.9               |
| I + III + IV  | 13.7                   | 85.6             | 44.9               |

Conditions as in Table III.

\* Total acetoacetate produced by each fraction in 10 min incubation at 37°C, calculated on the basis of a total volume of each fraction equivalent to that present in 11 ml of water-suspended mitochondria from which it was derived. The water-suspended mitochondria contained 3.10 mg protein/ml. Fractions I, II, III, and IV contained 1.34, 1.50, 0.36, and 1.20 mg protein/ml, respectively.

‡ The units of specific activity are nanomoles of acetoacetate formed per milligram of protein per minute at 37°C.

§ The membrane fraction (II) was brought to 0.15 M NaCl and recentrifuged to obtain the sodium chloride extract (III) and the extracted membrane fraction (IV).

their ketogenic activities. These data suggest that one or more of the enzymes of ketogenesis and/or a cofactor(s) was released from the mitochondrial membrane pellet into the soluble (supernatant) fraction in the presence of sodium chloride and that, in all probability, this component is present in the supernate in rate-limiting quantities when disruption and separation of the mitochondria are effected in the presence of 1 mM DTT alone.

The possibility that salt treatment of the mitochondrial suspension released an enzyme(s) or cofactor(s) from the mitochondrial membranes was subsequently examined. Thus, the ketogenic activities of subfractions and reconstituted fractions of water-suspended mitochondria and of mitochondrial membranes treated with sodium chloride were compared (Table V). The reconstituted fraction I + II again exhibited the synergistic effect characteristic of the recombined supernatant and membrane subfractions when derived from mitochondria suspended in 1 mM DTT solution. The addition of supernatant frac-

tion III, derived from salt-treated mitochondrial membranes, to supernate I displayed the synergistic effect whereas the addition of the salt-extracted membranes (IV) to the supernate I did not. Therefore, synergism in the formation of acetoacetate was associated with mitochondrial membranes prepared in the absence of salt and the component(s) responsible for this synergism was readily extracted from this membrane fraction by 0.15 M sodium chloride.

#### *Subfractionation of Rat Liver Mitochondria*

To investigate further the intramitochondrial localization of the enzymes of ketogenesis, the activities of the constituent enzymes of the HMGCoA cycle were determined in mitochondrial subfractions of more precise derivation. The various mitochondrial subfractions, isolated from the discontinuous sucrose gradient (Fig. 1), were characterized by their content of specific marker enzymes and by electron microscopy.

The soluble fraction, representing about 20%

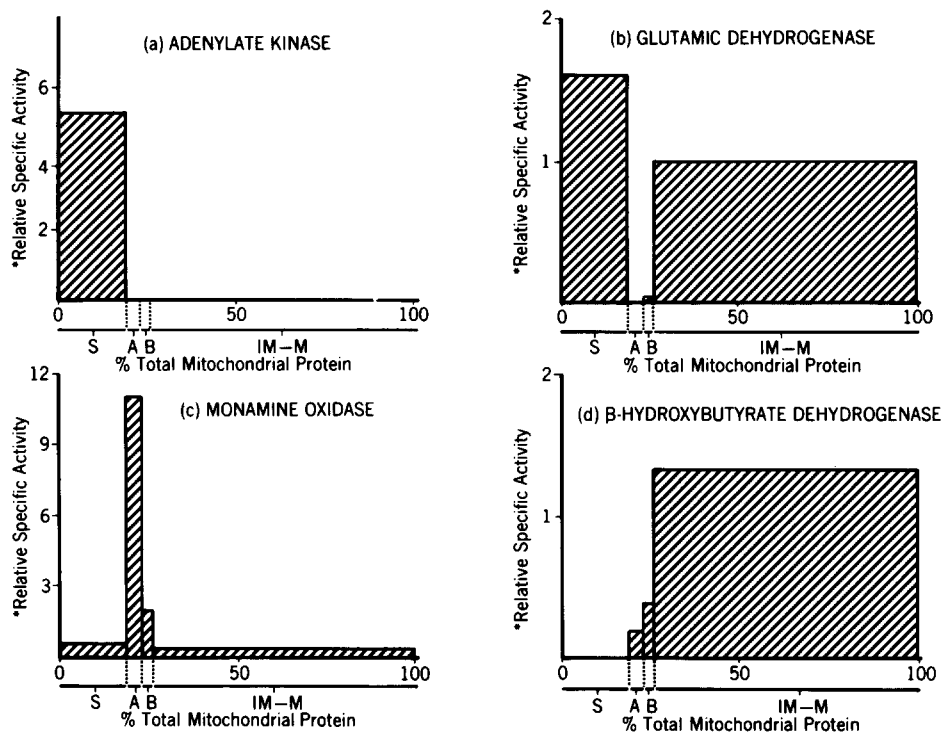


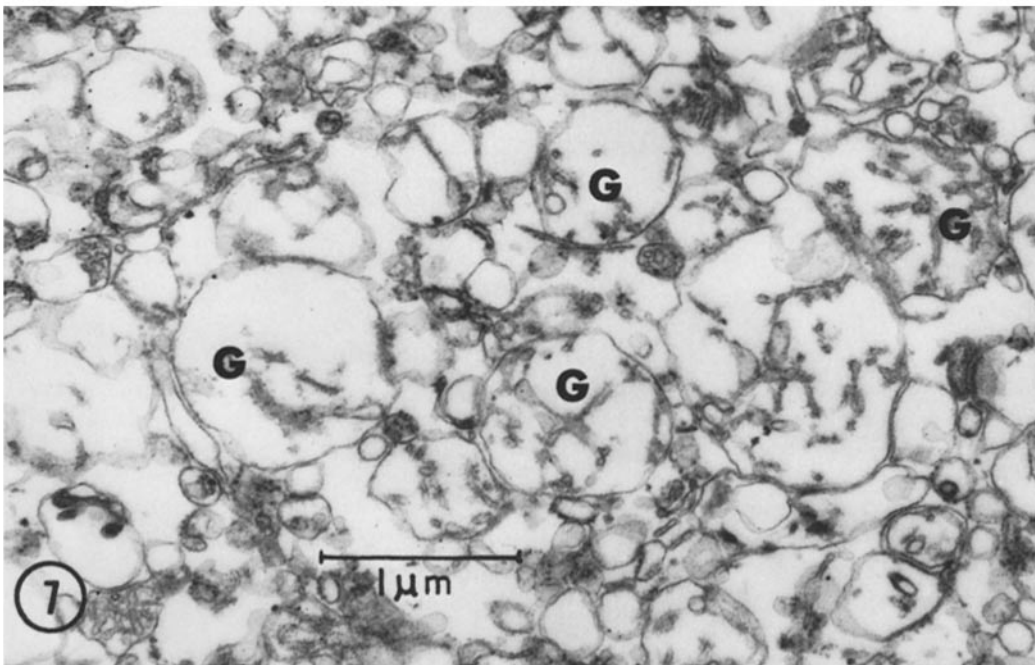
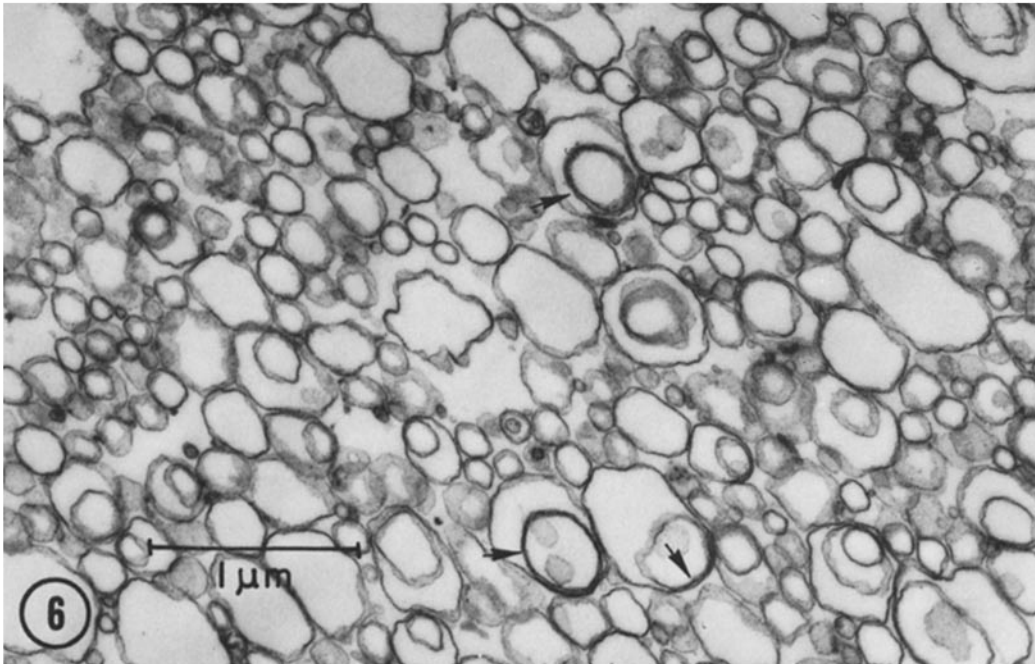
FIGURE 5 Distribution of marker enzyme activities in subfractions of rat liver mitochondria separated by discontinuous density gradient centrifugation: (a) adenylate kinase, (b) glutamic dehydrogenase, (c) monoamine oxidase, (d)  $\beta$ -hydroxybutyrate dehydrogenase. The data shown are from a representative experiment. (\*) Relative specific activity is the specific activity of each subfraction divided by that of the mitochondrial suspension applied to the gradient. The total protein present in the four fractions was taken as 100%. The recovery of protein applied to the gradient was 96%. The percentages of total mitochondrial protein in the subfractions are denoted by: S, soluble fraction (18.8%); band A (3.7%); band B (3.0%); and IM-M, inner membrane-matrix particles (74.5%).

of the total protein applied to the gradient and corresponding to its uppermost layer, contained all of the detectable adenylate kinase activity (Fig. 5 a). This fraction also contained a significant proportion (about 25%) of the total glutamic dehydrogenase activity (Fig. 5 b), in addition to 10% of the total monoamine oxidase activity (Fig. 5 c). The soluble fraction therefore contained all of the intermembrane space compartment of the mitochondrion and one-quarter of the mitochondrial matrix. In addition, the soluble fraction also contained one-tenth of the outer membrane, which is probably the result of incomplete sedimentation of small vesicles under the centrifugation conditions employed.

The material isolated as band A represented about 3% of the total mitochondrial protein and was markedly enriched in monoamine oxi-

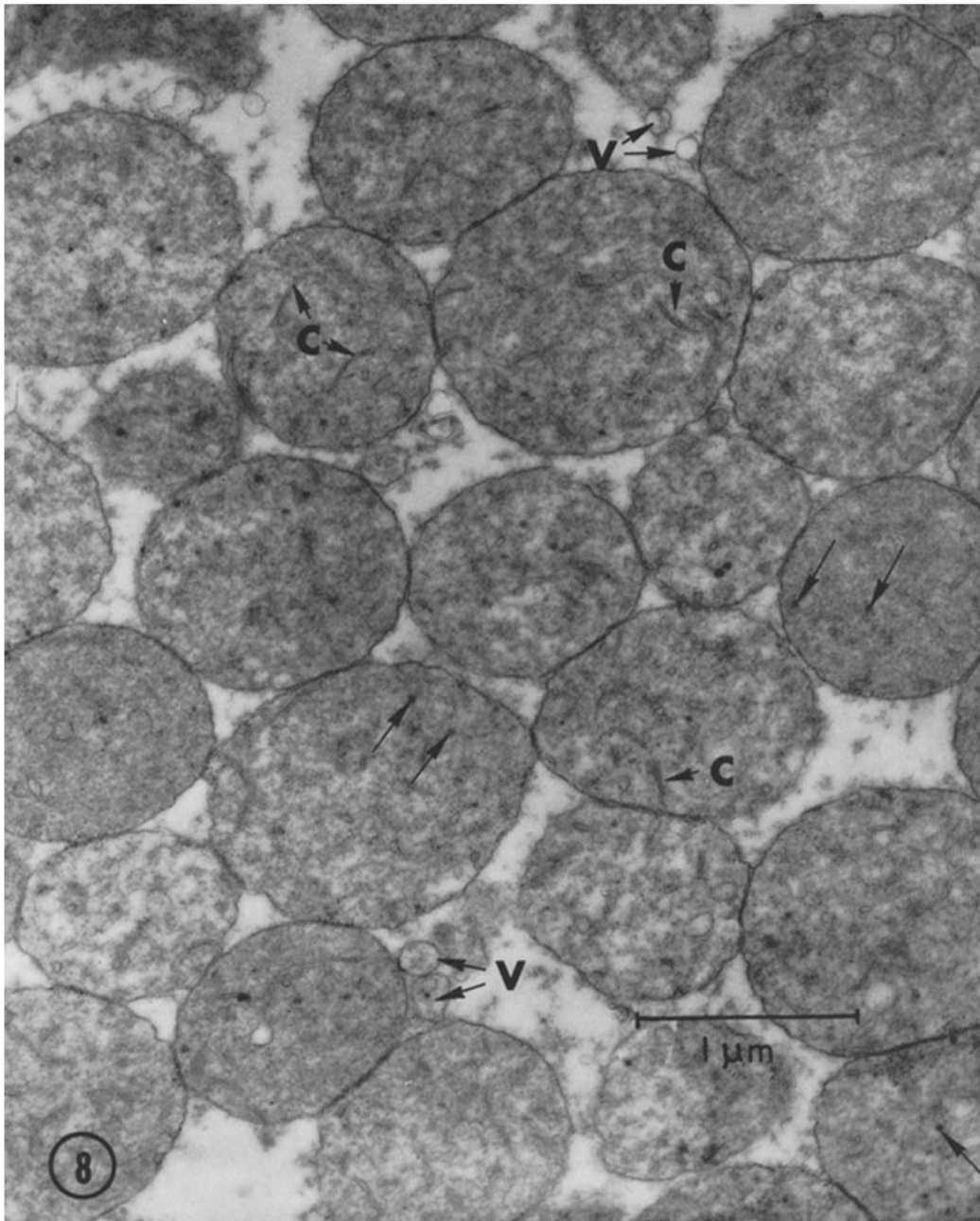
dase activity (Fig. 5 c). The monoamine oxidase specific activity was over ten times greater than that of the original mitochondrial suspension. This fraction contained only a trace of  $\beta$ -hydroxybutyrate dehydrogenase activity (Fig. 5 d). These enzymatic data support the conclusion that the material in band A is a highly purified preparation of outer mitochondrial membrane. Electron microscopic examination of the fraction was consistent with this conclusion. Thus myriad pleomorphic vesicular arrays were routinely observed (Fig. 6). Moreover, the membrane profiles were either single or partially laminated; mitochondrial cristae were not seen.

Small proportions of both monoamine oxidase and  $\beta$ -hydroxybutyrate dehydrogenase were present in band B (Fig. 5 c and d), which contained about 3% of the total mitochondrial pro-



**FIGURE 6** Electron micrograph of a section from the material isolated as band A by discontinuous density gradient centrifugation and fixed as a pellet in glutaraldehyde. Large numbers of vesicular structures, varying widely in size, are evident. Many of these vesicles exhibited laminations (arrows). The bar represents 1  $\mu$ m.  $\times 27,000$ .

**FIGURE 7** Electron micrograph of a section from the material isolated as band B by discontinuous density gradient centrifugation. The material was fixed as a pellet. This fraction was heterogenous in nature, containing numerous mitochondrial ghosts (G) and many membrane profiles. The bar represents 1  $\mu$ m.  $\times 27,000$ .



**FIGURE 8** Electron micrograph of a section from the pellet at the base of the discontinuous density gradient. The mitochondria possessed a single membrane and exhibited electron-opaque matrices. Within these matrices, the cristae (*C*) and matrix granules (arrows) were clearly visible. Smooth-surfaced vesicles (*V*) were occasionally seen in this pellet. Amorphous material of moderate electron density was noted at the periphery and in the interstices between the mitochondria. The bar represents 1  $\mu$ m.  $\times 30,000$ .

tein. A trace of glutamic dehydrogenase was generally detectable in this fraction (Fig. 5 *b*). Band B was shown by electron microscopy to consist of mitochondria in various stages of disruption, together with numerous membrane profiles of mitochondrial origin (Fig. 7). These enzymatic and electron microscopic observations indicate that the material in band B consisted of elements of both inner and outer mitochondrial membranes.

The pellet formed at the base of the centrifuge tube contained about 70% of the total mitochondrial protein applied to the gradient. This subfraction possessed 98% of the  $\beta$ -hydroxybutyrate dehydrogenase activity (Fig. 5 *d*) and the major portion (about 75%) of the glutamic dehydrogenase (Fig. 5 *b*). Monamine oxidase activity was detectable in the fraction (Fig. 5 *c*), but at a substantially diminished specific activity in comparison with the original mitochondrial preparation. Sections of this pellet were found by electron microscopy to contain swollen mitochondria, the vast majority of which lacked an outer membrane (Fig. 8). In comparison with the freshly isolated mitochondria (Fig. 2), these mitochondrial particles contained narrowed intracrystal spaces and less electron-opaque, expanded matrices. A small amount of amorphous material of moderate electron density and a few smoothly surfaced vesicles were observed between the mitochondrial particles.

Since the electron microscopic and biochemical data show the pelleted material to consist of mitochondria depleted of outer membrane and devoid of adenylate kinase, this subfraction may be accurately described as mitochondrial inner membrane-matrix particles.

The inner membrane-matrix particles (IM-M) were subsequently separated into inner membrane and matrix subfractions by disruption of the IM-M preparation by homogenization in water containing 1 mM DTT (see Materials and Methods). The typical distribution of marker enzymes between the matrix and inner membrane subfractions thus derived from the IM-M particles is presented in Fig. 9. Adenylate kinase activity was not detectable in either the matrix or inner membrane subfractions.

The matrix subfraction contained about 43% of the protein present in the original IM-M preparation (Fig. 9, Table VII), 74% of its glutamic dehydrogenase activity (Fig. 9 *b*) and did not exhibit detectable  $\beta$ -hydroxybutyrate dehydrogenase (Fig. 9 *a*) or monoamine oxidase activities. These results are consistent with the conclusion that this subfraction is derived exclusively from the matrix of the inner membrane-matrix particles.

The membrane fraction which sedimented at 146,000 *g* contained about 57% of the total protein of the IM-M particles (Fig. 9, Table VII). The  $\beta$ -hydroxybutyrate dehydrogenase

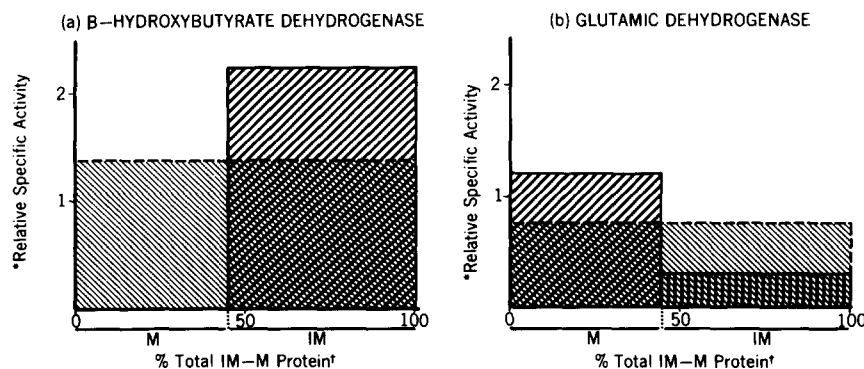


FIGURE 9 The distribution of marker enzyme activities in subfractions of inner membrane-matrix particles; (a)  $\beta$ -hydroxybutyrate dehydrogenase, and (b) glutamic dehydrogenase. (\*) Relative specific activity is the specific activity of each subfraction expressed relative to that of the mitochondrial suspension (taken as 1) from which it was derived. (†) Percentage of total IM-M protein indicates the percentage of protein in each subfraction expressed relative to that of the IM-M preparation (taken as 100%) from which it was derived. The dashed line indicates the relative specific activity of the IM-M preparation. *M* represents the matrix subfraction, and *IM* the inner membrane subfraction, which were derived from the IM-M preparation after suspension in water.

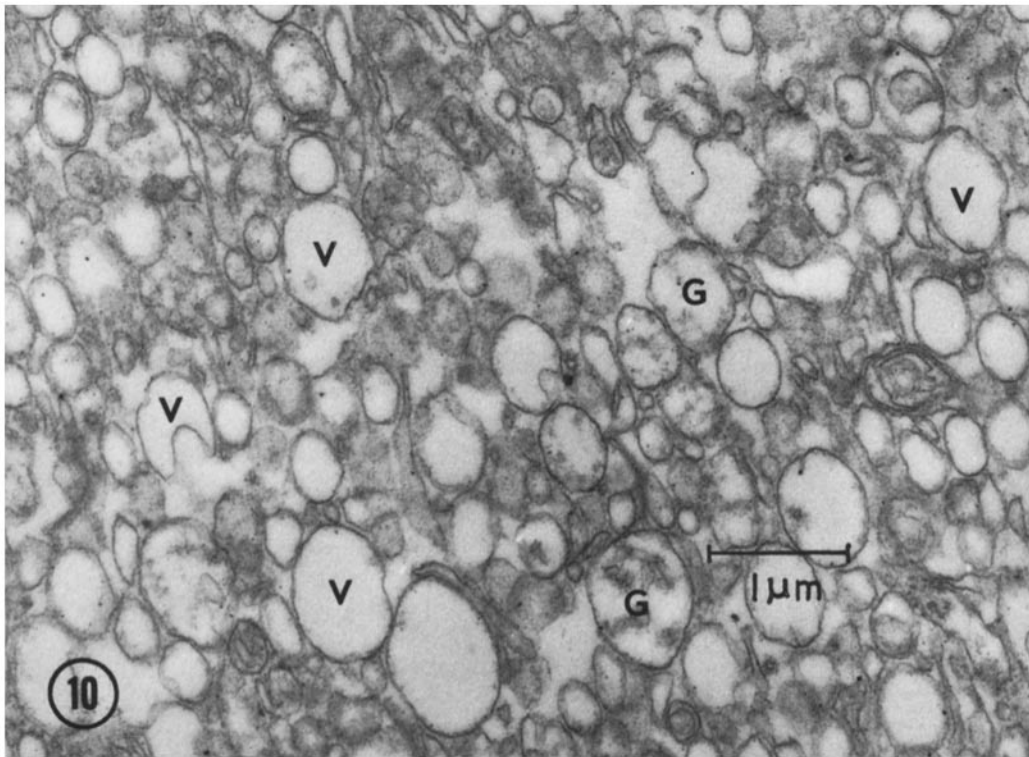


FIGURE 10 Section of the 146,000 *g* mitochondrial membrane pellet derived from water-suspended inner membrane-matrix particles. This pellet contained numerous vesicular profiles (*V*), many of which lacked electron-opaque contents. Occasionally mitochondrial ghosts (*G*) were seen which contained small amounts of material of moderate electron density. The bar represents 1  $\mu\text{m}$ .  $\times 18,000$ .

activity of the original IM-M preparation was localized entirely in this membrane fraction (Fig. 9 *a*). In addition, 26% of the glutamic dehydrogenase activity of the IM-M particles remained in association with the membranes (Fig. 9 *b*). The low monoamine oxidase activity detectable in the IM-M particles (Fig. 5 *c*) was recovered in this membrane fraction. These data indicate that this membrane fraction is composed principally of inner membrane, but also contains one-quarter of the matrix and a small amount of outer membrane.

Electron microscopic examination of the pelleted inner membrane preparation showed it to contain large numbers of vesicular profiles which lacked electron-opaque matrices (Fig. 10). Thus water-suspension and homogenization of the IM-M particles caused fragmentation and vesiculation of the inner membrane and the release of the major portion of the matrix. The resulting vesicular membrane fraction contained

less highly organized membrane structures than the membrane fraction derived from water-treated mitochondria (Fig. 4).

When the inner membrane and matrix subfractions were prepared from IM-M particles in the presence of 0.15 M NaCl, the proportion of protein in the matrix increased to 50% of the total (Table VII), although electron microscopy did not reveal any differences in the structure of the membranes after NaCl treatment. The matrix subfraction obtained in the presence of NaCl was again devoid of  $\beta$ -hydroxybutyrate dehydrogenase activity.

#### *Localization of the Enzymes of Ketogenesis in Mitochondrial Subfractions*

The formation of ketone bodies by subfractions of rat liver mitochondria, isolated by discontinuous sucrose gradient centrifugation, was compared with that of a sample of the mito-



chondrial preparation applied to the gradient (Table VI). In order to provide a measure of the total ketogenic activity applied, this sample was sonicated at setting 8 for 15 s before incubation with the acetyl CoA-generating system.

Neither the interzone, band A, nor band B subfractions produced ketone bodies in detectable amounts after incubation with the acetyl CoA-generating system. The inner membrane-matrix particles produced as much acetoacetate as did the reference mitochondrial preparation applied to the gradient. The resuspension of the IM-M particles in 0.25 M sucrose, before incubation with the acetyl CoA-generating system, required extensive homogenization which caused partial disruption of the particles as revealed by electron microscopy. The permeability barrier of the mitochondrial inner membrane to acetyl CoA (16) would thereby be removed. These data indicate that the enzymes of ketone body formation are associated with the inner membrane and/or matrix, in agreement with the results of Lee and Fritz (19). The soluble fraction contained about 20% of the ketogenic activity applied to the gradient (Table VI). This suggests the presence of the enzymes of ketogenesis in the matrix fraction of

the IM-M particles, since the soluble fraction also contained about 20% of the matrix marker glutamic dehydrogenase (Fig. 9 b).

In order to establish the location of the enzymes of ketogenesis within the inner membrane-matrix particles, these particles were separated into inner membrane and matrix subfractions after suspension and homogenization in 1 mM DTT (see Materials and Methods). The production of ketone bodies by these submitochondrial fractions was then determined and compared with that of the inner membrane-matrix particles from which they were derived (Table VII).

The major portion (68%) of the ketogenic activity of the original IM-M preparation was recovered in the matrix subfraction (I). A substantially smaller amount (26%) remained associated with the inner membrane (I). The ratio of ketogenic activity of the matrix to that of the membrane fraction was 2.6. The corresponding ratio of glutamic dehydrogenase activity was 2.9 (Fig. 9 b). The combined inner membrane and matrix subfractions produced acetoacetate equivalent to the sum of that formed by the matrix and inner membrane subfractions separately. No synergistic effect occurred, in contrast to that

TABLE VI  
*Formation of Ketone Bodies by Rat Liver Mitochondrial Membrane Subfractions Separated by Discontinuous Sucrose Density Gradient*

| Fraction                        | Percent protein | Acetoacetate formation |                  |                    |
|---------------------------------|-----------------|------------------------|------------------|--------------------|
|                                 |                 | Total*                 | Percent of total | Specific activity† |
|                                 |                 | <i>μmol</i>            |                  |                    |
| Mitochondrial suspension‡       |                 | 12.2                   | 100.0            | 34.7               |
| Soluble                         | 19.2 ± 3.7 (6)  | 2.1                    | 17.2             | 29.8               |
| Band A                          | 3.3 ± 0.6 (6)   | 0.0                    | 0.0              | 0.0                |
| Band B                          | 2.6 ± 0.7 (6)   | 0.0                    | 0.0              | 0.0                |
| Interzones                      | 6.2 ± 1.8 (6)   | 0.0                    | 0.0              | 0.0                |
| Inner membrane-matrix particles | 68.7 ± 4.7 (6)  | 12.5                   | 102.5            | 43.3               |

Values for acetoacetate formation are from a representative experiment. The total protein content of the subfractions isolated from the sucrose density gradient was taken as 100%. The recovery of protein applied to the gradient was >98%. Figures in parentheses indicate the number of experiments from which the mean values ± standard deviation were calculated.

\* Total acetoacetate formation in a representative experiment by each fraction, derived from 11 ml of the mitochondrial preparation applied to the gradient, in 10 min incubation at 37°C in the presence of 0.5 mM DTT.

† Nanomoles of acetoacetate formed per milligram protein per minute at 37°C.

‡ An aliquot of the mitochondrial suspension applied to the gradient was sonicated at setting 8 for 15 s (see Materials and Methods) before incubation as a measure of total activity.

|| The zone between the soluble fraction and band A was combined with the zone between band B and the inner membrane-matrix particles (Fig. 1) for analysis.

TABLE VII  
Ketogenic, Thiolase, and HMGCoA Cleavage Enzyme Activities in Subfractions of Mitochondrial Inner Membrane-Matrix Particles

| Fraction                                 | Protein (5), percent of total* | Acetoacetate formation (5) |                    | Thiolase (3)      |                   | HMGCoA cleavage enzyme (3) |                    |
|--|--------------------------------|----------------------------|--------------------|-------------------|-------------------|----------------------------|--------------------|
|  |                                | Percent of total†          | Specific activity‡ | Percent of total* | Specific activity | Percent of total*          | Specific activity¶ |
| Inner membrane-matrix particles          |                                | 100                        | 55.4 ± 6.5         |                   |                   |                            |                    |
| Matrix I                                 | 43.4 ± 3.2                     | 67.7 ± 6.0                 | 86.4 ± 9.6         | 73.5              | 1007              | 79.7                       | 118                |
| Inner membrane I                         | 56.6 ± 3.3                     | 25.7 ± 4.0                 | 25.1 ± 3.0         | 26.5              | 318               | 20.3                       | 26                 |
| Combined inner membrane I and matrix I   |                                | 95.6 ± 3.3                 | 53.0 ± 10.1        |                   |                   |                            |                    |
| Matrix II                                | 50.2 ± 3.6                     | 100.3 ± 8.8                | 110.6 ± 16.4       | 82.5              | 1104              | 87.3                       | 125                |
| Inner membrane II                        | 49.8 ± 3.6                     | 9.0 ± 2.2                  | 10.0 ± 1.5         | 17.5              | 245               | 12.7                       | 19                 |
| Combined inner membrane II and matrix II |                                | 98.7 ± 6.5                 | 54.7 ± 7.9         |                   |                   |                            |                    |

Figures in parentheses indicate the number of experiments from which means were calculated ( $\pm$ SD when five experiments were averaged). I indicates fractions derived from inner membrane-matrix particles after lysis in 1 mM DTT and ultracentrifugation at 146,000 *g* (1 h). II indicates fractions derived after lysis in 1 mM DTT followed by the addition of 0.15 M NaCl before ultracentrifugation as above. All fractions were incubated in duplicate in the presence of 0.5 mM DTT.

\* The total protein contents and enzyme activities of the matrix and inner membrane subfractions were taken as 100% in each instance.

† The amount of acetoacetate formed by the total IM-M particle preparation (derived from 11 ml of mitochondrial preparation applied to the gradient) after 10 min incubation at 37°C was taken as 100%.

‡ Units are nanomoles acetoacetate formed per milligram protein per minute at 37°C.

|| Units are nanomoles acetoacetyl CoA removed per minute per milligram protein at 30°C.

¶ Units are nanomoles HMGCoA cleaved per minute per milligram protein at 30°C.

observed in fractions derived from water-suspended mitochondria (Tables III-V).

When sodium chloride (0.15 M) was added after water lysis, before the separation of the matrix and inner membrane subfractions by centrifugation, the ketogenic activity of the matrix (II) increased and that of the inner membrane (II) preparation decreased (Table VII). The total ketogenic activity of the original IM-M particles was recovered in the matrix (II) subfraction. Only a small amount of ketogenic activity remained in the inner membrane (II) subfraction. The specific activity of acetoacetate formation in the matrix II subfraction was twice that of the original IM-M preparation. In contrast, that of the inner membrane subfraction was markedly lower.

These results indicate that sodium chloride releases additional amounts of the enzymes of ketogenesis from the inner membrane subfraction

into the matrix subfraction. This conclusion is supported by the observation that salt also increased the proportion of protein in the matrix (II) subfraction (Table VII).

Studies on the distributions of the HMGCoA cleavage enzyme and thiolase in the IM-M preparation and its subfractions verified the results obtained in the foregoing experiments, in which the over-all activity of ketogenesis was determined (Table VII). The major proportions of both the HMGCoA cleavage enzyme (80%) and thiolase (74%) activities were present in the matrix (I) subfraction prepared by water treatment of the IM-M particles. Other studies, showing similar solubilities of mitochondrial malate dehydrogenase and thiolase, also indicate the presence of thiolase in the matrix (16). When the IM-M particles were subfractionated in the presence of sodium chloride, an increase in the proportions of both thiolase and the HMG

CoA cleavage enzyme was found in the matrix (II) subfraction (Table VII).

## DISCUSSION

The preparation of mitochondrial ghosts (Fig. 3), obtained by suspension of rat liver mitochondria in water, was similar to that resulting from sequential water washing as described by Caplan and Greenawalt (41) and Schnaitman et al. (29). The same proportion of mitochondrial protein was found in the ghosts prepared by either procedure (41, Table I). The greater extraction of matrix by a single water treatment in the present study than obtained by others after one water extraction (41) is probably a result of the use of a higher ratio of water to total mitochondrial protein. With use of glutamic dehydrogenase as a matrix marker, about 75% of the matrix was released upon hypotonic lysis of the mitochondria in water (Table II). This is somewhat higher than the extrusion of two-thirds of the matrix by sequential water extraction, estimated on the basis of malate dehydrogenase activity (41). This difference in the degree of matrix extraction may be greater since it has been reported that malate dehydrogenase is more readily extracted than glutamic dehydrogenase (42). The present ghost preparation also appears to contain somewhat less mitochondrial matrix than that obtained by others (29, 41) on the basis of its electron microscopic appearance (Fig. 3). The ghosts prepared in the present study closely resemble those obtained by Schnaitman et al. (29) after sucrose density gradient centrifugation. Whereas the 146,000 *g* supernate contained all of the intermembrane space protein and three-fourths of the matrix, the mitochondrial membranes were exclusively located in the 146,000 *g* pellet, which also contained the remaining quarter of the matrix.

Electron microscopic examination of the mitochondria after suspension in water (Fig. 3) revealed the presence of electron-opaque matrix substance. This material resembled that seen in suspensions of rat liver mitochondria which had undergone osmotically induced swelling in the range of 11–32 mosmol and subsequent shrinkage at an ambient tonicity of 250 mosmol (43). The electron-opaque matrix substance was not however observed in the membrane pellet obtained by centrifugation of water-lysed mitochondria at 146,000 *g* (Fig. 4). It would therefore appear

that this substance was extruded from the mitochondria in the course of hypotonic swelling, as suggested by Stoner and Sirak (43), and that it dispersed and dissolved during the ultracentrifugation of the suspension.

While the procedure of Sottocasa et al. (22) was employed to prepare specific mitochondrial subfractions, the separation and composition of these subfractions, isolated on discontinuous sucrose density gradients, differed from that of these authors in certain respects. The light subfraction of Sottocasa et al. (22) was seen not as a single band at the interface of the 0.76 M and 1.32 M sucrose solutions, but rather as two distinct bands, separated by a narrow clear zone (Fig. 1). Enzymic and electron microscopic characterization of the upper (A) band showed it to consist of outer membrane of high purity, exhibiting in excess of 10-fold enrichment in monoamine oxidase as compared to the original mitochondrial suspension. In contrast, the lower (B) band consisted of elements of both the inner and outer mitochondrial membranes in similar amounts. The total amount of protein in the A and B Bands was less than that obtained in the total light fraction by Sottocasa et al. (22). The amount of material present in the B band was found to be a direct result of the intensity of ultrasonic treatment applied to the swelled and contracted mitochondria, and the final conditions of ultrasonic treatment employed represent the optimum for separation of the outer mitochondrial membrane while still retaining a high proportion of matrix in the IM-M particles. The presence of two bands at the interface of the 0.76 M and 1.32 M sucrose layers is attributed to differences in the density of the vesicles derived from the two mitochondrial membranes and to the formation of a micro-continuous density gradient at the junction of the sucrose solutions.

Enzymic and electron microscopic studies showed the pellet at the bottom of the discontinuous gradient to consist of inner membrane-matrix (IM-M) particles (Fig. 8). These particles possessed a high proportion (75%) of the matrix of the original mitochondrial preparation and almost all of its inner membrane content (Fig. 5). The detectable level of monoamine oxidase activity in this subfraction indicates the presence of a small amount of outer membrane. Although the use of digitonin (40) may remove the outer membrane more completely from the mitochondrion (42), this approach to the prepa-

tion of mitochondrial subfractions was not used in the present study, since digitonin may damage the inner mitochondrial membrane (42). The amount of protein isolated in the IM-M particles subfraction was 70% of the total applied to the gradient, and was somewhat higher than reported by Sottocasa and co-workers (22, 44). This observation was consistent with the electron microscopic appearance of the IM-M particles isolated in the present study, which exhibited greater and more uniform electron density than those isolated either by Sottocasa et al. (44) or by the method of Parsons et al. (42, 45). Almost all of the IM-M particles were bounded by a single continuous membrane. Ultrastructurally, the IM-M particles were in the orthodox conformation (Fig. 8), whereas the isolated mitochondria (Fig. 2) were in the typical condensed conformation (46).

Hypotonic treatment and homogenization of the IM-M particles permitted the isolation of a matrix subfraction containing three-fourths of the total matrix of the IM-M particles on the basis of glutamic dehydrogenase activity, the remainder being associated with the mitochondrial ghosts and vesicular profiles (Fig. 10) which sedimented at 146,000 *g*. This inner membrane preparation resembled that isolated by Colbeau et al. (42), which also contained some residual matrix. This membrane fraction (Fig. 10) contained vesicles of more homogeneous size and shape than those isolated following water treatment of intact mitochondria (Fig. 3).

Our studies of ketone body formation by subfractions of water-suspended mitochondria showed that ketogenic activity was enhanced by DTT (Table III), suggesting that DTT prevents the inactivation of one or more of the enzymes of ketogenesis, and/or protects coenzyme A from undergoing oxidation. In support of the former possibility, Bachhawat et al. (47) reported enhancement of HMGCoA cleavage enzyme activity by various sulfhydryl compounds. Since the most marked effect of DTT was found in the membrane subfraction of water-suspended mitochondria, the component(s) of the ketogenic system involved in the DTT enhancement of acetoacetate formation appears more susceptible to inactivation in the membranous than in the soluble system. This may be due to a lower endogenous level of low molecular weight sulfhydryl compounds in the membrane subfraction.

The mitochondrial subfractions which con-

sisted principally of matrix protein, namely the 146,000 *g* supernate from water-suspended mitochondria and the matrix subfraction from IM-M particles, exhibited significantly higher ketogenic activities than did the corresponding membrane fractions. However, while the proportion of ketogenic activity in the matrix subfractions derived from the IM-M particles (68%) was similar to its matrix content (75%) as indicated by glutamic dehydrogenase, that present in the supernate from water-suspended mitochondria (30%) was much less than its matrix content (75%). While a synergism in acetoacetate formation was seen upon recombination of mitochondrial subfractions prepared in the absence of salt (Tables III and IV), no such effect was observed upon isolation of the subfractions in its presence (Table IV). Further studies showed that the component responsible for this synergism was readily extracted from the mitochondrial membranes by 0.15 M sodium chloride (Table V). These results suggest that at least one of the enzymes of ketogenesis is selectively retained in the membrane subfraction when release of matrix is effected by suspension of intact mitochondria in water. The absence of such a retention in the membrane subfraction of IM-M particles may be related to the procedures involved in its isolation, in particular to the homogenization step, while the absence of external mitochondrial compartments might also be involved. The physical and metabolic state of the matrix at the time of disruption of the inner mitochondrial membrane may indeed be of greater influence on the release of the enzymes of ketogenesis into the soluble fraction. When intact mitochondria (Fig. 2) are suspended in water, the matrix is in a concentrated form (46), in which a fibrous network has been revealed by freeze etching (48). In contrast, disruption of the IM-M particles by hypotonic treatment occurred when these particles (Fig. 8) were in the orthodox conformation (46) in which the matrix lattice is expanded (49). The concentrated form of the matrix, which gives rise to the dense matrix substance seen in suspensions of mitochondria in water (Fig. 3), may restrict the release of the rate-limiting component into the soluble phase in this system. The present data suggest that this component is HMGCoA synthase. This is consistent with the findings of Williamson et al. (15), who provided evidence based on indirect measurements that HMGCoA synthase is the rate-limiting enzyme of the HMGCoA cycle in rat liver. Although it is

likely that the rate-limiting component which is released by sodium chloride is the HMGCoA synthase, the possibility that it is a required cofactor cannot be excluded.

The mechanism by which sodium chloride effects the release of enzymes of ketogenesis from the membranous to the soluble subfractions of water-suspended mitochondria and IM-M particles (Tables IV, V, and VII) is not known. On the basis of protein content and enzyme activity data, it is clear that sodium chloride provides more complete release of matrix protein from IM-M particles. In addition, the portion of the rate-limiting component, apparently HMGCoA synthase, which was selectively retained in the membrane fraction when the intact mitochondria were lysed in water, was released by sodium chloride. It appears that this latter effect of sodium chloride involves disruption of ionic interactions between enzyme protein and charged components of the mitochondrial membrane (50). Whether this interaction is a physiological characteristic of rat liver mitochondria, perhaps related to the metabolic state and/or to microcompartmentation of the matrix (48, 49), or whether it is an adsorption phenomenon related to the low osmolarity during the hypotonic lysis of intact isolated mitochondria, remains to be determined.

The present results show that thiolase, HMGCoA synthase and the HMGCoA cleavage enzyme are located in the matrix of the inner membrane-matrix particles derived from rat liver mitochondria, with the residual matrix content accounting for the ketogenic activity of the membrane subfraction. The enzymes of the Krebs cycle, with the exception of succinic dehydrogenase, are also located in the mitochondrial matrix (51). These findings are therefore consistent with the accessibility of the enzymes of the Krebs cycle and the enzymes of ketogenesis to the same metabolic pool of acetyl CoA.

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