ENZYMATIC PROPERTIES OF THE INNER AND OUTER MEMBRANES OF RAT LIVER MITOCHONDRIA

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ABSTRACT

Treatment of rat liver mitochondria with digitonin followed by differential centrifugation was used to resolve the intramitochondrial localization of both soluble and particulate enzymes. Rat liver mitochondria were separated into three fractions: inner membrane plus matrix, outer membrane, and a soluble fraction containing enzymes localized between the membranes plus some solublized outer membrane. Monoamine oxidase, kynurenine hydroxylase, and rotenone-insensitive NADH–cytochrome *c* reductase were found primarily in the outer membrane fraction. Succinate–cytochrome *c* reductase, succinate dehydrogenase, cytochrome oxidase, β-hydroxybutyrate dehydrogenase, α-ketoglutarate dehydrogenase, lipoamide dehydrogenase, NAD– and NADH–isocitrate dehydrogenase, glutamate dehydrogenase, aspartate aminotransferase, and ornithine transcarbamoylase were found in the inner membrane-matrix fraction. Nucleoside diphosphokinase was found in both the outer membrane and soluble fractions; this suggests a dual localization. Adenylate kinase was found entirely in the soluble fraction and was released at a lower digitonin concentration than was the outer membrane; this suggests that this enzyme is localized between the two membranes. The inner membrane-matrix fraction was separated into inner membrane and matrix by treatment with the nonionic detergent Lubrol, and this separation was used as a basis for calculating the relative protein content of the mitochondrial components. The inner membrane-matrix fraction retained a high degree of morphological and biochemical integrity and exhibited a high respiratory rate and respiratory control when assayed in a sucrose-mannitol medium containing EDTA.

INTRODUCTION

The recent development of techniques for separating the outer and inner membranes of liver mitochondria and the concurrent establishment of enzymatic markers for these membranes have permitted the systematic study of submitochondrial enzyme localization. The techniques for separating the two membrane systems include density-gradient centrifugation following mitochondrial swelling and contraction (1, 2), gradient centrifugation following controlled osmotic lysis (3, 4), and treatment with digitonin in isotonic media followed by differential centrifugation (3, 5). Of these techniques the digitonin fractionation, described first by Levy et al. (5) and subsequently by Schnaitman et al. (3), is often the most advantageous. This procedure does not employ hypertonic density gradients and does not markedly affect the integrity of the inner mitochondrial

membrane. Hence soluble matrix proteins are retained within the isolated inner membrane and this inner membrane-matrix fraction retains some of the integrated functions characteristic of whole mitochondria. Included among these functions are the ability to incorporate amino acids into acid-precipitable protein (6) and the exhibition of oscillatory conformational states similar to those of intact mitochondria (7). In addition, the concentration of dititonin and the techniques of differential centrifugation employed in the fractionation procedure may be varied to satisfy different experimental needs, and the fractionation procedures may easily be scaled up for enzyme purification purposes.

The present paper describes improved techniques for the digitonin fractionation of rat liver mitochondria and the application of these techniques to the localization of soluble mitochondrial enzymes. These improvements have permitted for the first time the isolation of a morphologically and biochemically intact preparation of the inner membrane-matrix fraction which exhibits respiratory control.

EXPERIMENTAL METHODS

Isolation of Mitochondria and Microsomes

Adult male albino rats weighing 250–350 g were used (Sprague-Dawley strain obtained from Longacre Farms, Glen Arm, Maryland). The animals were sacrificed by a blow on the head and exsanguinated, and the livers were quickly removed and placed in ice-cold medium containing 220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) buffer, and 0.5 mg/ml crystalline bovine serum albumin (BSA). The pH of the medium was adjusted to 7.4 with KOH just prior to use. This medium will be referred to as "isolation medium." The livers were minced, washed twice with isolation medium, and suspended in 2 volumes of isolation medium. The mince was homogenized with four passes of a motor-driven Potter-Elvehjem tissue grinder and diluted with isolation medium to give a 10% homogenate. The homogenate was centrifuged at 560 *g* for 15 min, and the pellet was discarded. The supernatant was centrifuged at 7,000 *g* for 15 min. The fluffy layer was carefully discarded, and the pellet was washed twice by resuspension in one-half and one-fourth of the original volume of isolation medium followed by centrifugation at 7,000 *g* for 15 min. Microsomes were prepared by centrifuging the supernatant from the first 7,000 *g* centrifugation at 12,000 *g* for 15 min. The pellet and the floating lipid were discarded, and the supernatant was centrifuged at 144,000 *g* for 1 hr for sedimentation of the microsomes. The final mitochondrial and microsomal pellets were suspended in isolation medium. All operations of preparation and subsequent fractionation were carried out at 0°C.

Digitonin Fractionation of Mitochondria

Stock 2% digitonin solutions were prepared just prior to use by dissolving digitonin in isolation medium as previously described (3) or by heating. The BSA was added to the isolation medium after the digitonin had been dissolved, and the stock solution was diluted with isolation medium as required to give the desired ratio of digitonin to mitochondrial protein. Aliquots of ice-cold digitonin solution were added with continuous stirring to equal aliquots of suspensions containing 100 mg mitochondrial protein per milliliter. The resulting suspension was gently stirred for 15 min and then diluted with 3 volumes of isolation medium. The diluted suspension was homogenized gently by hand and centrifuged at 8,000–12,000 *g* (see Results for exact details). The supernatant was carefully drawn off, and the pellet was gently resuspended in the same volume of isolation medium. This suspension was centrifuged again at the same speed for 10 min. The pellet from the second centrifugation is subsequently designated as the "low-speed pellet." The supernatants from the first and second centrifugation were pooled, and are subsequently designated as the "low speed supernatant." In some cases the low speed supernatant was fractionated further by centrifugation at 144,000 *g* for 1 hr. The pellet from this centrifugation is designated as the "high speed pellet," and the supernatant is designated as the "high speed supernatant." The exact ratios of digitonin to protein and the speeds employed for sedimentation of the low speed pellet were varied, as described in Results, so that particularly clean preparations of inner or outer membrane or optimum recovery of both membranes could be obtained. With the improved isolation medium and procedures described above, the previously described fluffy layer (3) consisting of damaged inner membranes was eliminated.

Assays

USE OF LUBROL IN ENZYMATIC ASSAYS: The nonionic detergent Lubrol WX (obtained from I.C.I. Organics, Inc., Providence, R. I.) has proved to be a useful alternative to sonication for activating many mitochondrial enzymes. Concentrations of 0.1 mg of Lubrol per milligram protein generally produced maximal activation of cytochrome oxidase and soluble mitochondrial enzymes, and concentrations as high as 1 mg/mg protein caused no inhibition of these enzymes. With some enzymes a several fold increase in activity was noted when activation with

Lubrol was used instead of sonication. In addition, in spectrophotometric assays the clearing of mitochondrial suspensions with Lubrol eliminates many of the problems caused by mitochondrial swelling. Lubrol activation, as specified in many of the assays below, consists of pretreatment of the mitochondrial samples with 0.3 mg Lubrol per milligram protein at 0°C for at least 15 min prior to assay. The samples were suspended in isolation medium or in buffers appropriate for specific assays. Lubrol inhibited β-hydroxybutyrate dehydrogenase, succinate dehydrogenase, and succinate- and NADH-cytochrome *c* reductase. The effect of Lubrol on glucose-6-phosphatase was not tested.

Monoamine oxidase was assayed as previously described (3). Samples were activated with Lubrol for minimization of changes in optical density that are associated with mitochondrial swelling.

NADH- and succinate-cytochrome c reductase were assayed exactly as described by Sottocasa et al. (2). Rotenone (5 μM) was added to all assays of NADH-cytochrome *c* reductase, and the data presented in this paper refer only to the rotenone-insensitive activity.

Cytochrome oxidase was assayed as previously described (3) except that samples were activated with Lubrol. Lubrol activation eliminated the additional activation by digitonin which was previously observed.

Succinate dehydrogenase was assayed polarographically by measuring oxygen consumption at 23°C with a Clark electrode. Samples were activated by brief sonication and added to a medium 2 ml of which contained 0.5 M phosphate buffer, pH 7.6, 25 mM succinate, and 0.6 mM KCN. The samples were incubated for 10 min, and the reaction was initiated by the addition of 0.05 ml of 40 mM phenazine methosulfate.

β-hydroxybutyrate dehydrogenase was assayed spectrophotometrically by following the reduction of NAD at 25°C as described by Gotterer (8). Samples were activated by brief sonication and preincubated with NAD, thioglycerol, and an extract of mitochondrial lipids as described by this author.

Lipoamide dehydrogenase was assayed spectrophotometrically by following the oxidation of NADH at 30°C. The following assay mixture was contained in 2.5 ml: 0.12 mM NADH, 0.4 mM KCN, and 40 mM phosphate buffer, pH 7.6. The reaction was initiated by the addition of 0.5 ml of 0.5 M phosphate buffer, pH 7.6, saturated with lipoamide at 30°C. The samples were activated with Lubrol.

α-ketoglutarate dehydrogenase was assayed spectrophotometrically by following the reduction of NAD at 25°C. Samples were incubated for 3 min in a medium containing the following in 1.5 ml: 2.5 mg BSA, 0.6 mM $MgCl_2$, 0.15 mM EDTA, 0.6 mM KCN, 1.4 mM thiamine pyrophosphate, 66 mM HEPES buffer, pH 7.4, and 0.3 mg Lubrol per milligram mitochondrial protein. To this was added 1.5 ml containing the following: 6 mM cysteine, 0.86 mM coenzyme A, 5 mM $MgCl_2$, and 133 mM HEPES buffer. The reaction was initiated by the addition of 0.05 ml of 0.1 M α-ketoglutarate. This enzyme was quite labile and was assayed immediately after fractionation of the mitochondria.

Malate dehydrogenase was assayed as previously described (3). Samples were activated with Lubrol.

NAD- and NADP-isocitric dehydrogenase were assayed spectrophotometrically at 25°C as described by Plaut and Sung (9). A final substrate concentration of 12.5 mM DL-isocitrate was employed in both assays, and 0.3 mM KCN was added to both assays. Samples were activated with Lubrol. The NAD-linked enzyme is quite labile, and was assayed immediately after fractionation of the mitochondria.

Glutamate dehydrogenase was assayed at 25°C according to the method of Beaufay et al. (10). BSA (0.8 mg/ml) and 0.4 mM KCN were added to the assay medium. Samples were activated with Lubrol.

Aspartate aminotransferase was assayed spectrophotometrically at 25°C by the method of Karmen (11) in which the formation of oxalacetate is coupled to the oxidation of NADH with malate dehydrogenase. The following assay mixture was contained in 2.8 ml: 33 mM aspartate, 0.1 mM NADH, 0.4 mM KCN, 10 μg crystalline malate dehydrogenase, and 66 mM HEPES buffer, pH 7.4. The reaction was initiated by the addition of 0.2 ml of 0.1 M α-ketoglutarate. Samples were activated with Lubrol.

Ornithine transcarbamoylase was assayed according to the method of Schimke (12). Triton X-100 (0.1%) was included in the assay mixture. The citrulline formed was assayed by the method of Ratner (13).

Adenylate kinase was assayed spectrophotometrically at 25°C by following the conversion of ADP to ATP + AMP and coupling the formation of ATP to the reduction of NADP with hexokinase and glucose-6-phosphate dehydrogenase. The assay mixture contained the following in 1 ml: 0.75 mM NADP, 15 mM glucose, 10 IU of hexokinase, 0.4 IU of glucose-6-phosphate dehydrogenase, 0.45 mM KCN, 3 mM ADP, 5 mM $MgCl_2$, and 70 mM glycylglycine buffer, pH 8.0. The assay mixture was allowed to incubate for about 5 min for consumation of trace amounts of ATP present in the ADP, and the reaction was initiated by the addition of Lubrol-activated enzyme. This enzyme was quite labile at low protein concentrations and was assayed immediately after fractionation of the mitochondria.

Nucleoside diphosphokinase was assayed spectrophotometrically at 25°C by a modification of the assay described by Goffeau et al. (14). This assay employs a coupled assay system similar to that used for adenylate kinase, but measures the formation of

ATP from ADP + deoxycytidine-5-triphosphate (dCTP). An important modification was the inclusion of AMP in the assay system for minimization of interfering adenylate kinase activity. The assay system contained the following in 1 ml: 0.5 mM NADP, 10 mM glucose, 7.5 IU hexokinase, 0.4 IU of glucose-6-phosphate dehydrogenase, 0.45 mM KCN, 5 mM $MgCl_2$, 40 μM ADP, 100 μM AMP, 3 mM dCTP, and 70 mM glycylglycine, pH 8.0. All samples were activated with Lubrol. The enzyme sample was added to the assay solution minus dCTP, and the change in absorbance was followed for 1-2 min before addition of dCTP to correct for any change in absorbance due to adenylate kinase activity. The difference between the rates before and after the addition of dCTP was also corrected for the slow change in absorbance due to the reaction of dCTP with glucose and hexokinase. The validity of these corrections was established by the addition to the assay of purified, adenylate kinase-free nucleoside diphosphokinase isolated from beef liver mitochondria.[1] This assay method was selected because it is unaffected by the ATPase activity present in mitochondrial preparations.

Kynurenine hydroxylase was assayed spectrophotometrically at 25°C by the procedure of Hayaishi (15). Identical cuvettes were prepared which contained the following in 1 ml: 0.1 M potassium phosphate buffer, pH 8.0, 0.2 mM NADPH, 10 mM KCl, 0.45 mM KCN, and identical amounts of Lubrol-activated samples. The cuvettes were placed in a Beckman DBG ratio-recording spectrophotometer, and the change in absorbance at 340 mμ was followed after the addition of 0.1 ml of water to the reference side, and 0.1 ml of 1 mM kynurenine to the sample cuvette.

Glucose-6-phophatase was measured exactly as described by Swanson (16). The phosphate liberated was estimated by the method of Gomori (17).

Protein was estimated by the method of Lowry et al. (18). Crystalline BSA was used as a standard.

Electron Microscopy

Glutaraldehyde stock solutions (25%) used for preparing fixatives were stored at 4°C in the presence of excess $BaCO_3$ and centrifuged just prior to use. The centrifuged stock solution was diluted 1/10 with isolation medium minus BSA. The pH of this solution was 7.4. The diluted glutaraldehyde was placed in the bottom of Beckman microfuge tubes, and small aliquots of samples were placed in the tops of the tubes and centrifuged into the fixative as previously described (3). All fixation steps were carried out at 0°-4°C. The remaining steps of fixation, embedding, and sectioning were carried out as previously described. Sections were stained for 30 min at 60°C with 1% sodium borate saturated with uranyl acetate, and then with lead citrate according to the procedure of Reynolds (19).

Negative staining was accomplished by diluting thick suspensions of mitochondrial samples suspended in isolation medium with 2% phosphotungstic acid adjusted to pH 6.5. A drop of the diluted suspension was placed on a grid covered with a thin film of Formvar reinforced with evaporated carbon. After 1 min the drop was blotted off with filter paper, and the grid was allowed to air-dry. All specimens were examined in a Siemens Elmiskop I operated at 80 kv and photographed at plate magnifications from 4,000 to 40,000.

RESULTS

Distribution of Mitochondrial and Microsomal Enzymes after Digitonin Fractionation

Table I shows the distribution and specific activity of a number of mitochondrial enzymes in fractions obtained by treating mitochondria with 1.1 mg digitonin per milligram mitochondrial protein followed by differential centrifugation. The low speed pellet was obtained by centrifuging the digitonin-treated suspension at 9,500 *g*. Monoamine oxidase and rotenone-insensitive NADH–cytochrome *c* reductase were found primarily in the high speed pellet; lesser amounts were found in the high speed supernatant and low speed pellet. This result is in accord with previous results and thus indicates that these enzymes are localized in the outer mitochondrial membrane (1–3) which is found primarily in the high speed pellet (3). All of the other enzymes listed in this table were found predominantly in the low speed pellet which contains the inner membrane and matrix. Cytochrome oxidase, succinate dehydrogenase, succinate–cytochrome *c* reductase, and β-hydroxybutyrate dehydrogenase are firmly membrane-bound and hence are presumed to be localized on the inner mitochondrial membrane. The remainder of the enzymes listed in this table are released from the mitochondria by sonication, osmotic shock, or mild treatment with Lubrol and are presumed to be localized in the mitochondrial matrix. Recoveries approaching 100% were obtained with all of these enzymes.

Table II shows an identical experiment in which the distribution of nucleoside diphospho-

[1] Kindly supplied by Dr. P. Pedersen, Johns Hopkins University School of Medicine.

TABLE I

Distribution and Specific Activity of Mitochondrial Enzymes after Digitonin Fractionation

Enzyme	Total activity*				Specific activity			
	Low speed pellet	High speed pellet	High speed supernatant	Recovery	Low speed pellet	High speed pellet	High speed supernatant	Untreated mitochondria
	%	%	%	%	mμmoles/min/mg protein			
Monoamine oxidase	19.3	63.6	16.0	98.9	6.08	183	30.0	30.0
Rotenone-insensitive NADH-cytochrome *c* reductase	20.1	57.5	15.7	93.3	53	1380	244	220
Cytochrome oxidase	87.2	8.0	0.8	96.0	2380	2000	177	2272
Succinate dehydrogenase	83.3	7.1	3.8	94.2	956	750	265	955
Succinate-cytochrome *c* reductase	97.7	2.3	0	100.0	424	90	0	362
β-hydroxybutyrate dehydrogenase	83.3	6.2	2.1	91.6	623	260	70	623
Lipoamide dehydrogenase	86.7	0	4.4	91.1	68.5	0	21.3	65.6
β-ketoglutarate dehydrogenase	97.9	0	0	97.9	62.8	0	0	53.1
Malate dehydrogenase	96.0	1.1	8.0	104.1	2220	242	1067	1922
NAD-isocitrate dehydrogenase	98.1	1.4	0	99.5	13.4	1.8	0	11.3
NADP-isocitrate dehydrogenase	90.4	1.6	10.4	102.4	99.0	15.5	46.2	91.2
Glutamate dehydrogenase	94.5	0	11.0	105.5	136	0	51	119
Aspartate aminotransferase	92.6	4.4	1.1	98.1	3300	1450	213	2970
Ornithine transcarbamoylase	83.3	3.3	5.5	90.3	456	167	213	455

* Compared to untreated, unfractionated mitochondria.

kinase and adenylate kinase was examined. In contrast to the enzymes described above, these phosphotransferases were found primarily in the high speed supernatant. A significant amount of a nucleoside diphosphokinase activity was also associated with the high speed pellet. This distribution suggests a dual localization of this enzyme or a loose association of this enzyme with the outer mitochondrial membrane. All of the adenylate kinase activity was found in the high speed supernatant. This finding indicates, as suggested by Klingenberg and Pfaff (20), that this enzyme is localized in the space between the inner and outer membranes, referred to in this paper as the intracristate space.

The localization of adenylate kinase was examined in more detail in the experiment shown in Fig. 1. In this experiment mitochondria were treated with varying amounts of digitonin and fractionated as described above. It was predicted that any enzyme localized between the membranes would be removed by lower concentrations of digitonin than were required for removal of the outer membrane, since previous results (3) indicated that low concentrations of digitonin ruptured the outer membrane without removing it. Fig. 1 shows the recovery of adenylate kinase and other mitochondrial enzymes in the low speed pellet after treatment with varying amounts of digitonin. In this experiment the distribution of kynurenine hydroxylase was also determined, since Okamoto et al. (21) have shown that this enzyme is also associated with the outer mitochondrial membrane. Monoamine oxidase and kynurenine hydroxylase were released from the mitochondria at the same digitonin concentration,

TABLE II

Distribution of Adenylate Kinase and Nucleoside Diphosphokinase after Digitonin Fractionation

Sample	Protein*	Monoamine oxidase		Adenylate kinase		Nucleoside diphosphokinase	
		Specific activity	Total activity*	Specific activity	Total activity*	Specific activity	Total activity*
	%		%		%		%
Whole mitochondria	100	14.6	100	308	100	17.0	100
Low speed pellet	75.8	2.2	11.3	0	0	1.3	5.7
High speed pellet	6.9	140	66.3	159	3.1	75.0	30.9
High speed supernatant	13.4	30.1	27.5	2910	121.5	78.5	62.4
Recovery*	96.1		105.1		124.6		99.0

* Based on whole mitochondria.

All specific activities are expressed as mμmoles/min/mg protein.

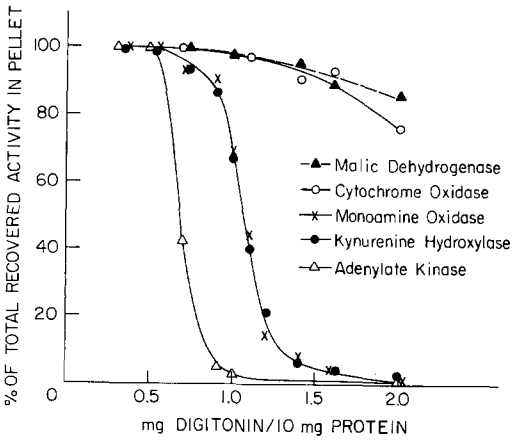

FIGURE 1 The release of mitochondrial enzymes by various digitonin concentrations. The percentage of the total recovered enzymatic activity sedimented by centrifugation at 9,500 *g* for 10 min is plotted versus the digitonin concentration.

while adenylate kinase was released at a significantly lower digitonin concentration. Malate dehydrogenase and cytochrome oxidase were retained in the low speed pellet. These results support the conclusion that adenylate kinase is localized in the intracristate space.

Parsons et al. (1) have suggested that glucose-6-phosphatase is associated with both the outer mitochondrial membrane and microsomal membranes. Since glucose-6-phosphatase is often used as an indicator of microsomal contamination, the distribution of this enzyme was compared to the distribution of monoamine oxidase, kynurenine hydroxylase, and rotenone-insensitive NADH–cytochrome *c* reductase. The specific activities of these four enzymes in the high speed supernatant, high speed pellet, and low speed pellet obtained after treatment with varying digitonin concentrations are shown in Fig. 2. These data were obtained from the same experiment shown in Fig. 1. In the high speed pellet, monoamine oxidase, kynurenine hydroxylase, and NADH–cytochrome *c* reductase reached a peak activity at 1.1 mg digitonin per 10 mg protein. This result is similar to previous results obtained with monoamine oxidase (3). The glucose-6-phosphatase specific activity in this fraction reached a peak at much lower digitonin concentration, and the specific activity decreased over the range of digitonin concentrations which produced the greatest increase in the specific activity of the other enzymes. The specific activity of all four enzymes decreased in the low speed pellet at digitonin concentrations greater than 1.0 mg digitonin per 10 mg mitochondrial protein. However, the specific activity of glucose-6-phosphatase decreased by only 50%, whereas that of the other enzymes decreased almost to zero. At high digitonin concentrations much of the activity of monoamine oxidase, kynurenine hydroxylase, and NADH–cytochrome *c* reductase was found in the high speed supernatant; this indicates the solubilization of the outer membrane. No glucose-6-phosphatase could be detected in this fraction; this finding does not appear to be due to enzyme inactivation, since recoveries approaching 100% of the enzyme present in unfractionated mitochondrial preparations were obtained at all digitonin concentrations tested. These results indicate that glucose-6-phosphatase is bound to membranes other than the outer mitochondrial

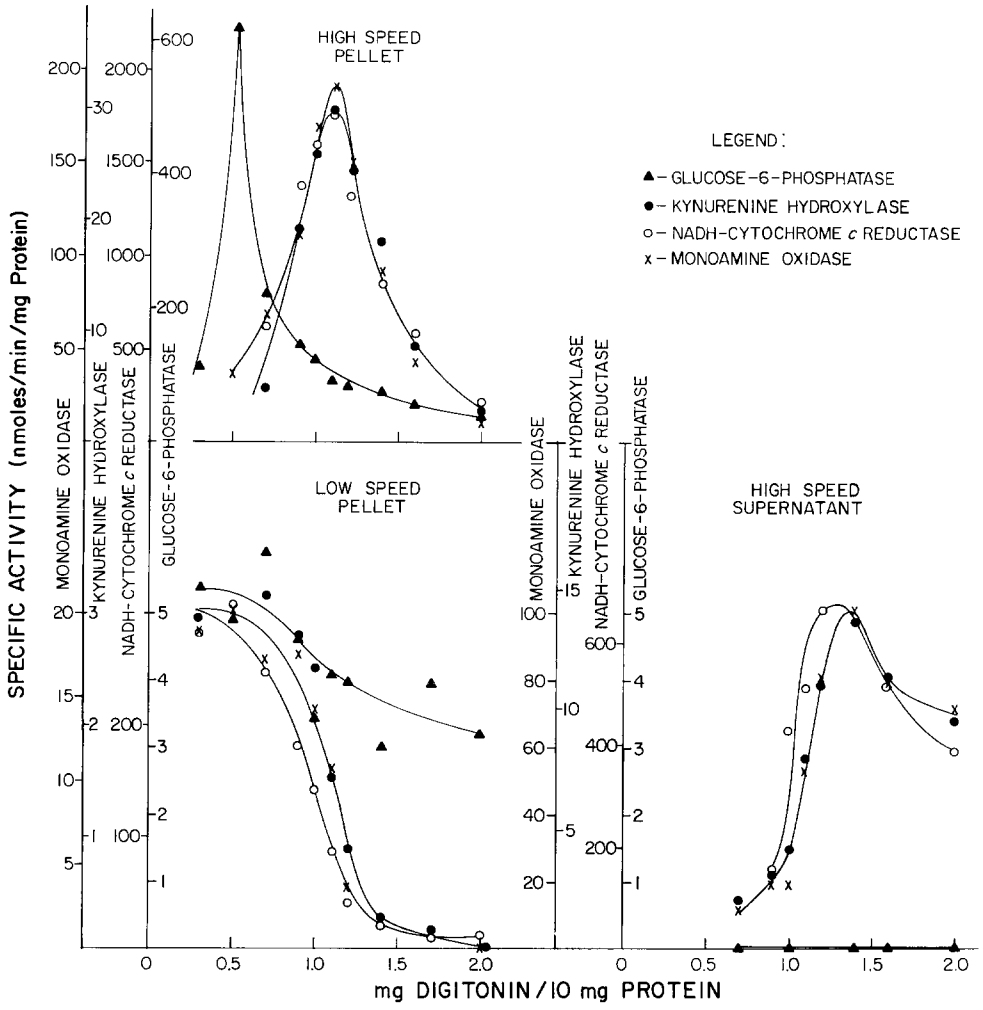

FIGURE 2 A comparison of the localization of glucose-6-phosphatase to the localization of kynurenine hydroxylase, rotenone-insensitive NADH-cytochrome *c* reductase, and monoamine oxidase. The specific activity of these enzymes in each of the three fractions are plotted versus the digitonin concentration used in fractionating the mitochondria. The details of the fractionation procedure are given in the text.

membrane, presumably to microsomal membranes of various sizes adsorbed to the outer mitochondrial membrane. The smaller microsomal membranes are removed by digitonin and recovered in the high speed pellet. Larger pieces of microsomal membrane continue to sediment with the low speed pellet at all digitonin concentrations.

Use of Enzymatic Markers to Calculate the Protein Content of Mitochondrial Components

Since it is not possible by these techniques to obtain absolutely pure, uncontaminated preparations of inner and outer mitochondrial membranes, it is not possible to calculate directly the protein content of these components. However, it is possible to calculate the protein content of these components from the specific activity of marker enzymes in these fractions and in whole mitochondria. These calculations depend upon the following basic assumptions: first, that each of the enzymatic markers is localized in only one component of the mitochondria; second, that the enzymatic markers are neither activated nor inactivated by the fractionation procedures. These assumptions appear to be valid in view of the results presented previously in this paper.

The following enzymatic markers were used for the respective components: malate dehydrogenase for the matrix; cytochrome oxidase for the inner membrane; adenylate kinase for the intracristate protein; monoamine oxidase for the outer membrane; and glucose-6-phosphatase for microsomal contamination. A preparation of inner membrane plus matrix which was relatively free of outer membrane and intracristate protein was prepared by treating mitochondria with 1.2 mg digitonin per 10 mg protein. The low speed pellet (inner membrane plus matrix) was obtained by centrifuging twice at 8,000 *g* for 10 min. The enzyme content of this preparation is shown in Table III. This preparation was fractionated further by treatment with 1 mg Lubrol per 10 mg protein. The Lubrol-treated preparation was then fractionated by centrifuging at 144,000 *g* for 1 hr. Preliminary experiments showed that the pellet obtained after Lubrol treatment contained almost all of the cytochrome oxidase and phospholipid of the unfractionated, low speed pellet, whereas much of the malate dehydrogenase activity was release into the supernatant. The high speed pellet (primarily outer membrane) was prepared by treating mitochondria with 1.05 mg digitonin per 10 mg protein. The low speed pellet was removed by its being centrifuged twice at 12,000 *g* for 10 min, and the high speed pellet was obtained by centrifugation at 144,000 *g* for 1 hr. The intracristate protein was obtained by treating mitochondria with 0.7 mg digitonin per 10 mg protein. The low speed pellet and high speed pellet were removed as above. The high speed supernatant contained about 60% of the total adenylate kinase of the mitochondria, but contained no significant amounts of malate dehydrogenase, cytochrome oxidase, or monoamine oxidase. The intracristate protein fraction is presumed to contain proteins, which are loosely bound to the outer membrane and/or to the outside of the inner membrane, as well as soluble proteins localized between the two membranes. Microsomes were prepared as described in the methods section.

The specific activity of microsomal and mitochondrial enzymes in these fractions is given in Table III. These specific activities were used for calculation of the specific activities of the enzymatic markers in their respective components by the following procedures. First, the specific activity of malate dehydrogenase in the supernatant from the Lubrol-treated, low speed pellet was assumed to represent the specific activity of malate dehydrogenase in the mitochondrial matrix. This assumption is based upon the observations that the low speed pellet was almost free of outer membrane and intracristate protein and that the Lubrol treatment did not solublize appreciable amounts of the inner membrane. The pellet obtained from the Lubrol-treated, low speed pellet is enriched with respect to inner membrane, but still contains some matrix as indicated by the remaining malate dehydrogenase activity. The specific activity of cytochrome oxidase in the inner membrane was obtained by correcting the specific activity observed in the Lubrol pellet for matrix contamination by the following formula:

$$\frac{\text{Specific activity of cytochrome oxidase in Lubrol pellet}}{1 - \left(\dfrac{\text{Specific activity of malate dehydrogenase in Lubrol pellet}}{\text{Specific activity of malate dehydrogenase in matrix}}\right)} = \text{Specific activity of cytochrome oxidase in inner membrane}$$

The outer membrane preparation (high speed pellet) shown in Table III is contaminated by inner membrane, as indicated by the cytochrome oxidase activity, and by microsomal membrane, as indicated by glucose-6-phosphatase activity. The specific activity of monoamine oxidase in the outer membrane can be obtained by correcting for these contaminating components as follows:

$$\frac{\text{Specific activity of monoamine oxidase in high speed pellet}}{1 - \left(\dfrac{\text{Specific activity of cytochrome oxidase in high speed pellet}}{\text{Specific activity of cytochrome oxidase in inner membrane}}\right) - \left(\dfrac{\text{Specific activity of glucose-6-phosphatase in high speed pellet}}{\text{Specific activity of glucose-6-phosphatase in microsomes}}\right)} = \text{Specific activity of monoamine oxidase in outer membrane}$$

The specific activity of adenylate kinase obtained from the high speed supernatant from mitochondria treated with a low concentration of digitonin did not require correction, since this fraction was not contaminated with enzymes from other components.

TABLE III

Specific Activity of Enzymes in Various Mitochondrial Fractions and in Microsomes

Fraction	Specific activity					
	Cytochrome oxidase	Malate dehydrogenase	NADH-cytochrome *c* reductase	Adenylate kinase	Monamine oxidase	Glucose-6-phosphatase
	mμmoles/min/mg protein					
Whole mitochondria	1980	2608	298	421	22.2	5.3
Inner membrane + matrix: (low-speed pellet)						
Unfractionated	2230	2681	39	0	1.6	0.9
Lubrol supernatant	55	3895	—	—	—	—
Lubrol pellet	6880	1018	—	—	—	—
Outer membrane (high-speed pellet)	1890	0	2800	0	340	45.8
Intracristate protein (soluble material released at low digitonin conc.)	0	0	—	6690	0	0
Microsomes	218	—	549	—	6.3	252

Table IV gives the specific activity of each of the enzymatic markers in whole mitochondria and in each respective component as calculated from the data in Table III. The percentage of total mitochondrial protein comprised by each component is also given in Table IV, and was calculated by using the following formula:

$$\frac{\text{Specific activity of marker enzyme in whole mitochondria}}{\text{Specific activity of marker enzyme in component}} \times 100\% = \text{Percentage of total mitochondrial protein present in component}$$

The sum of the percentages of the protein present in each component calculated in this fashion is almost equal to 100% (Table IV). This supports the validity of the assumptions used in making these calculations.

Respiration of Digitonin-Fractionated Mitochondria

Table V lists the components of the medium used to assay the respiration and respiratory control of intact mitochondria and the low speed pellet obtained after digitonin fractionation. The low speed pellet was prepared by treating mitochondria with 1.2 mg digitonin per 10 mg protein followed by centrifugation at 8,000 *g* for 10 min. The final low speed pellet contained about 90% of the malate dehydrogenase of intact mitochondria, but contained no detectable adenylate kinase and less than 3% of the monoamine oxidase activity. Respiration was assayed by measuring oxygen uptake with a Clark electrode in a 2 ml open system at 23° C.

Fig. 3 shows the respiration of the low speed pellet and intact mitochondria with succinate as a substrate. In the absence of added Mg^{++} a stimulation of respiration was observed with the low speed pellet after addition of ADP. This stimulated state corresponds to the respiratory state 3 described by Chance and Williams (22). The rate of respiration decreased after exhaustion of the ADP and this corresponded to state 4 of Chance and Williams (22). This transition from state 4 to state 3 upon addition of ADP could be repeated several times before the assay system became anaerobic. The low speed pellet exhibited a respiratory control index (state 3 rate/state 4 rate) of 2.0 or better in the absence of Mg^{++}. The addition of Mg^{++} stimulated the respiratory rate of the low speed pellet but abolished all respiratory control. The intact mitochondria exhibited a similar state 3 rate in the absence of added Mg^{++}, but the state 4 rate was slower and thereby resulted in an increase in the respiratory control index to about 3.0. When intact mitochondria were assayed in the presence of added Mg^{++}, there was a stimulation of the state 3 rate and a decrease in the state 4 rate; this resulted in a marked increase in the respiratory control index to almost 7.0.

TABLE IV

Amounts of Various Mitochondrial Components Calculated from Specific Activities of Enzymatic Markers

Component Enzymatic marker	Inner membrane Cytochrome oxidase	Outer membrane Monamine oxidase	Matrix Malate dehydrogenase	Intracristate space Adenylate kinase	Microsomes contaminant Glucose-6-phosphatase	Total
Specific activity of enzymatic marker in whole mitochondria	1980	22.2	2608	421	5.3	
Specific activity of enzymatic marker in component	9315	551	3895	6690	252	
Total mitochondrial protein in component, %	21.3	4.0	66.9	6.3	2.1	100.6

TABLE V

Respiration Assay Medium

	+EDTA	+Mg^{++}
	mM	*mM*
Sucrose	70	same
Mannitol	220	same
HEPES buffer	2	same
BSA	1*	same
Phosphate	2.5	same
Substrate (succinate)	5	same
(OH-butyrate)	10	same
EDTA	1	0.5
$MgCl_2$	none	2.5

pH to 7.4 at 20°C.

* mg/ml.

Fig. 4 shows an identical experiment with β-hydroxybutyrate as a substrate. The results were essentially the same as with succinate, except that the effect of added Mg^{++} on intact mitochondria was much more striking. When β-hydroxybutyrate was used as substrate the rate of respiration of the low speed pellet was substantially lower than with intact mitochondria, and there was a gradual decrease in the respiratory rate during the course of the assay. This finding may reflect a leakage of pyridine nucleotides or other cofactors. With both substrates the stimulation of respiration of the low speed pellet was specific for ADP. The addition of equal amounts of AMP and/or ATP had no effect on either the rate of respiration or the respiratory control index observed after subsequent addition of ADP. In the absence of Mg^{++}, both the low speed pellet and intact mitochondria gave ADP/O ratios approaching 2.0 with succinate and 3.0 with β-hydroxybutyrate.

The low speed pellet preparation failed to respire in the KCl-containing medium described by Weinbach (23), although intact mitochondria gave a similar respiratory rate and respiratory control index in this medium. Respiration of the low speed pellet was restored by the addition of 30 μM cytochrome *c*, but no respiratory control was observed even when Mg^{++} was omitted from the medium.

Electron Microscopy

The low speed pellet preparation shown in Figs. 6–8 is the same preparation which was used in the respiratory control experiments described in Figs. 3 and 4. The high speed pellet shown in Fig. 9 is the same preparation as shown in Table III.

Fig. 5 is an electron micrograph of a fixed, sectioned preparation of the intact mitochondria prior to fractionation. Fig. 6 shows the appearance of a similar preparation of the low speed pellet. This preparation appears to consist of intact inner membrane plus matrix and is free of any significant amount of outer membrane or intact mitochondria. The diameter of the majority of these forms is about the same as that of intact mitochondria, and the matrix appears relatively dense with little evidence of swelling. However, the inner membrane appears to be evaginated and thus results in numerous finger-like projections. Fig. 7, taken at higher magnification, more clearly demonstrates the integrity of the inner membrane and the details of the finger-like projections. The inset shows the trilamellar nature of the inner membrane.

Fig. 8 shows the appearance of an unfixed preparation of the low speed pellet, negatively stained with phosphotungstic acid. The prepara-

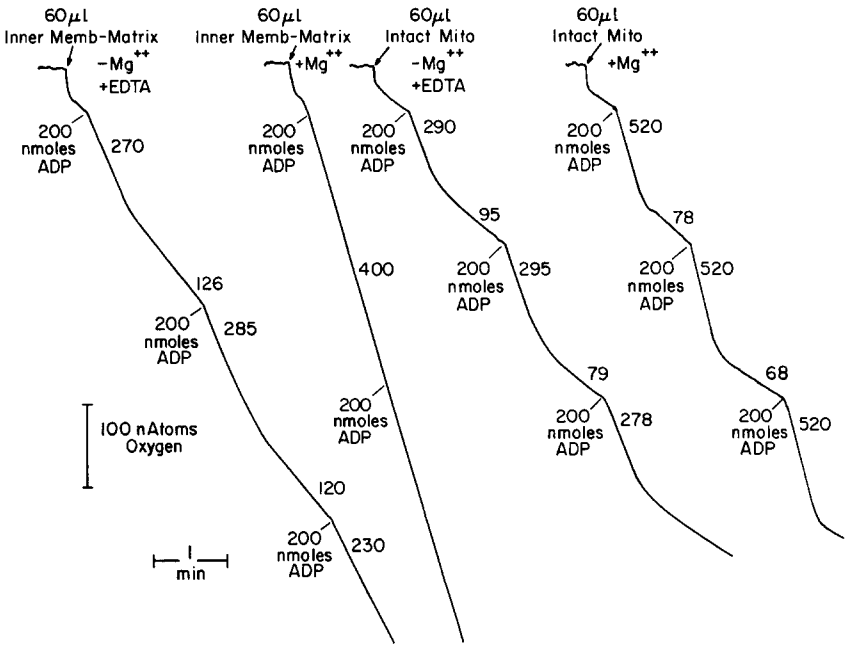

FIGURE 3 Respiration and respiratory control of the inner membrane-matrix fraction and intact mitochondria with succinate as substrate. Both the inner membrane-matrix fraction and the intact mitochondria contained the same amount of starting mitochondrial protein (50 mg/ml). Details of the medium are given in Table V.

tion consists of membranous bags in various stages of disruption. Some of the disrupted material appears in the form of strips or strands of fairly uniform width. These strands shown at higher magnification in the inset of Fig. 8 illustrate the presence of the 90-A particles first described by Fernández-Morán (24). The appearance of negatively stained preparations of the low speed pellet is similar to that described by Parsons et al. (1) and Sottocasa et al. (2) for inner membrane preparations obtained by the swelling contraction method. The strands are presumed to arise from the inner membrane, since they have been observed in negatively stained preparations of osmotically lysed mitochondria which were almost devoid of matrix enzymes (Greenawalt, J. W. Unpublished data). Some of the more intact membranous bodies observed in these preparations also have finger-like projections similar to those observed in sectioned preparations of the inner membrane-matrix (Figs. 6 and 7).

Fig. 9 shows the appearance of the unfixed high speed pellet (outer membrane) negatively stained with phosphotungstic acid. This preparation is more than 60% pure outer membrane on the basis of enzymatic analysis, and consists of small, flat, somewhat ragged vesicles. There is no evidence of the strands or 90-A particles seen in Fig. 8. The size and morphology of this preparation agrees well with previous observations made on fixed, sectioned preparations (3)

DISCUSSION

From the data presented here obtained with improved methods for subfractionating mitochondria it is concluded that monoamine oxidase, rotenone-insensitive NADH–cytochrome *c* reductase, and kynurenine hydroxylase are localized in the outer membrane of rat liver mitochondria. These results agree with studies on the localization of these enzymes reported by a number of other investigators (1, 2, 6, 21, 25–27). All of the respiratory functions and the respiration-linked dehydrogenases examined in the present study are associated with the inner membrane-matrix fraction. The data presented in Fig. 1 and Table IV provide additional strong evidence that the enzyme localization scheme employed in this paper is correct. As de Duve et al. have pointed out (28) the validity of assuming that enzymes

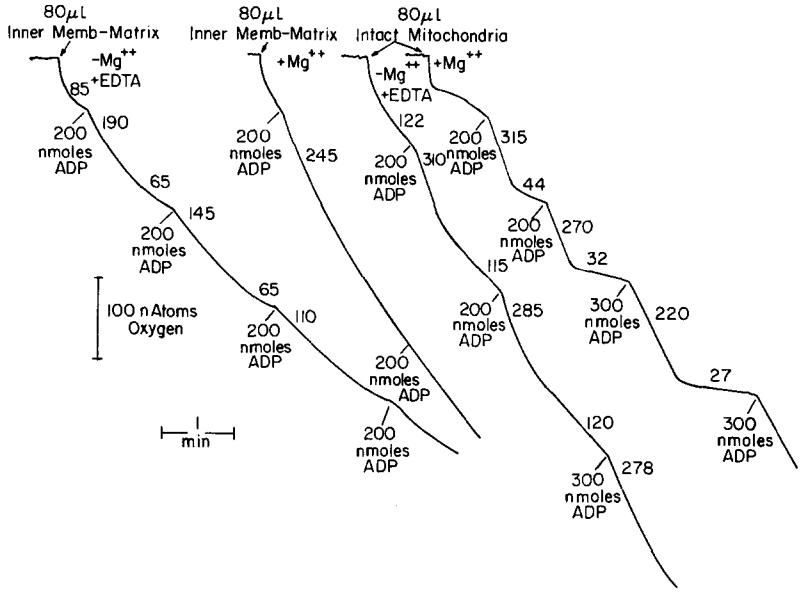

FIGURE 4 Respiration and respiratory control of the inner membrane-matrix fraction and intact mitochondria with β-hydroxybutyrate as substrate. All other conditions are the same as in Fig. 3.

have single loci in subcellular organelles is generally confirmed or rejected by subsequent fractionation studies. It is difficult to conceive of an alternative scheme of localization for monoamine oxidase, adenylate kinase, cytochrome oxidase, and malate dehydrogenase which would fit the data presented here.

It must be pointed out that the values for the protein content of the various mitochondrial components given in Table IV are based upon operational definitions of these components. The distinction between the enzymes of the inner membrane and matrix depends upon the techniques used to separate these enzymes, since *in situ* these enzymes must show a continuous spectrum of affinities for the membrane. This distinction is even more important in considering enzymes localized between the two membranes, where the ratio of membrane surface area to volume is very large in the intact mitochondrion.

The distribution of respiration-linked dehydrogenases observed in this study does not agree with the data reported by Green et al. (29–32) on the localization of these enzymes in beef heart mitochondria. These workers have suggested that β-hydroxybutyrate dehydrogenase, α-ketoglutarate dehydrogenase, and pyruvate dehydrogenase are localized in the outer membrane of beef heart mitochondria. In the present study all of the β-hydroxybutyrate dehydrogenase and α-ketoglutarate dehydrogenase activities of rat liver mitochondria were recovered in the inner membrane-matrix fraction. It was not possible to determine the activity of pyruvate dehydrogenase in the mitochondrial preparations employed in this study, because the mitochondria are slightly contaminated with lactate dehydrogenase. However, the recovery of essentially all of the lipoamide dehydrogenase, a component of both pyruvate and α-ketoglutarate dehydrogenases (33), in the inner membrane-matrix fraction suggests that pyruvate dehydrogenase is also localized in this fraction. Parsons et al. (1) have suggested that the preparation of outer membrane from beef heart mitochondria described by Allman et al. (31) is contaminated with matrix proteins, and this suggestion is supported by the observations of Beattie et al. (6) who found that a great deal of matrix protein was associated with outer membrane fractions obtained from rat liver mitochondria by the phospholipase procedure of Allman et al. (31). This contamination would explain the large discrepancy between the protein content of the outer membrane fraction from beef heart mitochondria reported by Allman et al. (31) and the protein content of the outer

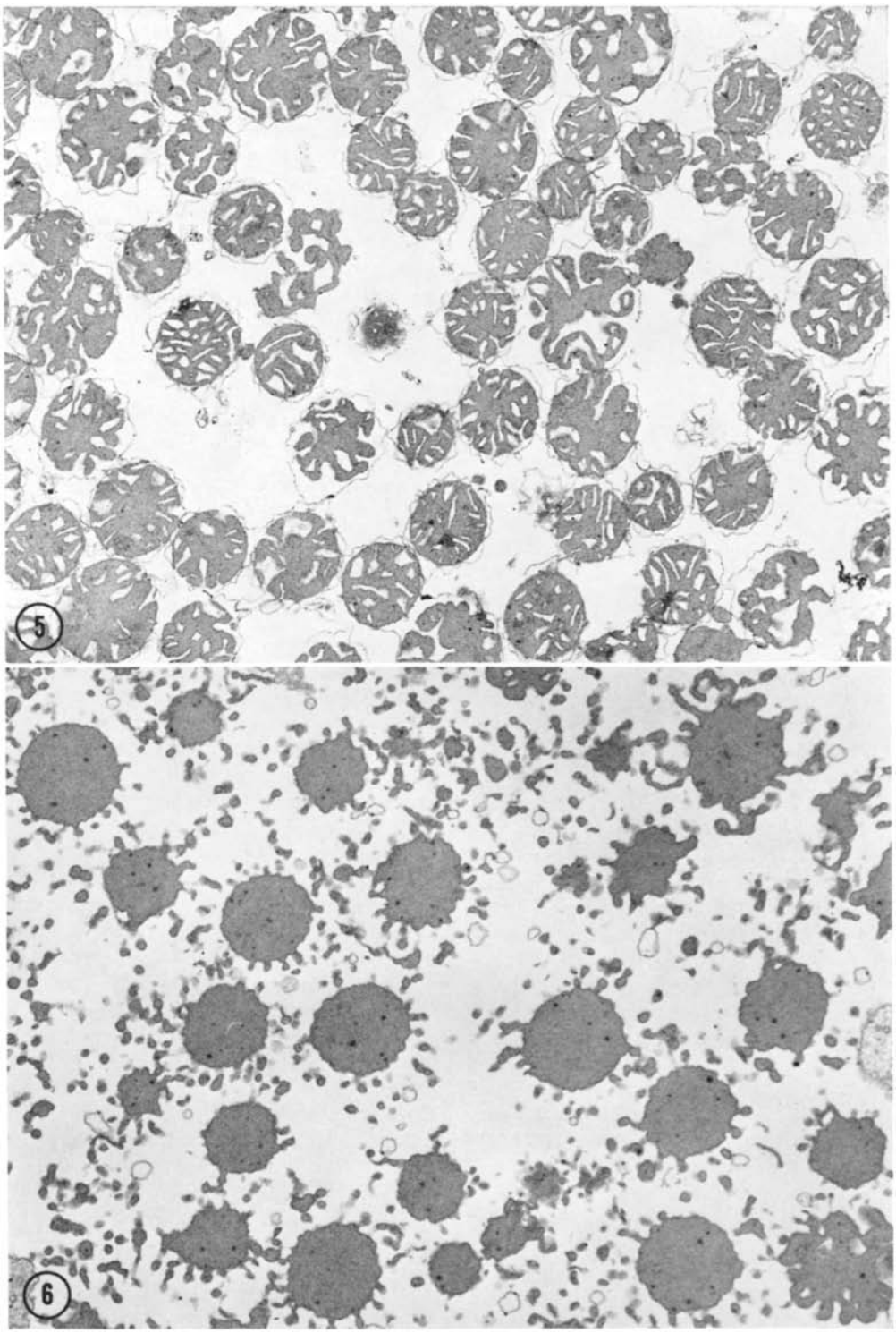

FIGURE 5 Control of freshly-isolated rat liver mitochondria. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 19,500.

FIGURE 6 The inner membrane-matrix preparation isolated by the improved digitonin fractionation procedure. This preparation was the same preparation used in the respiration studies shown in Figs. 3 and 4. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 19,500.

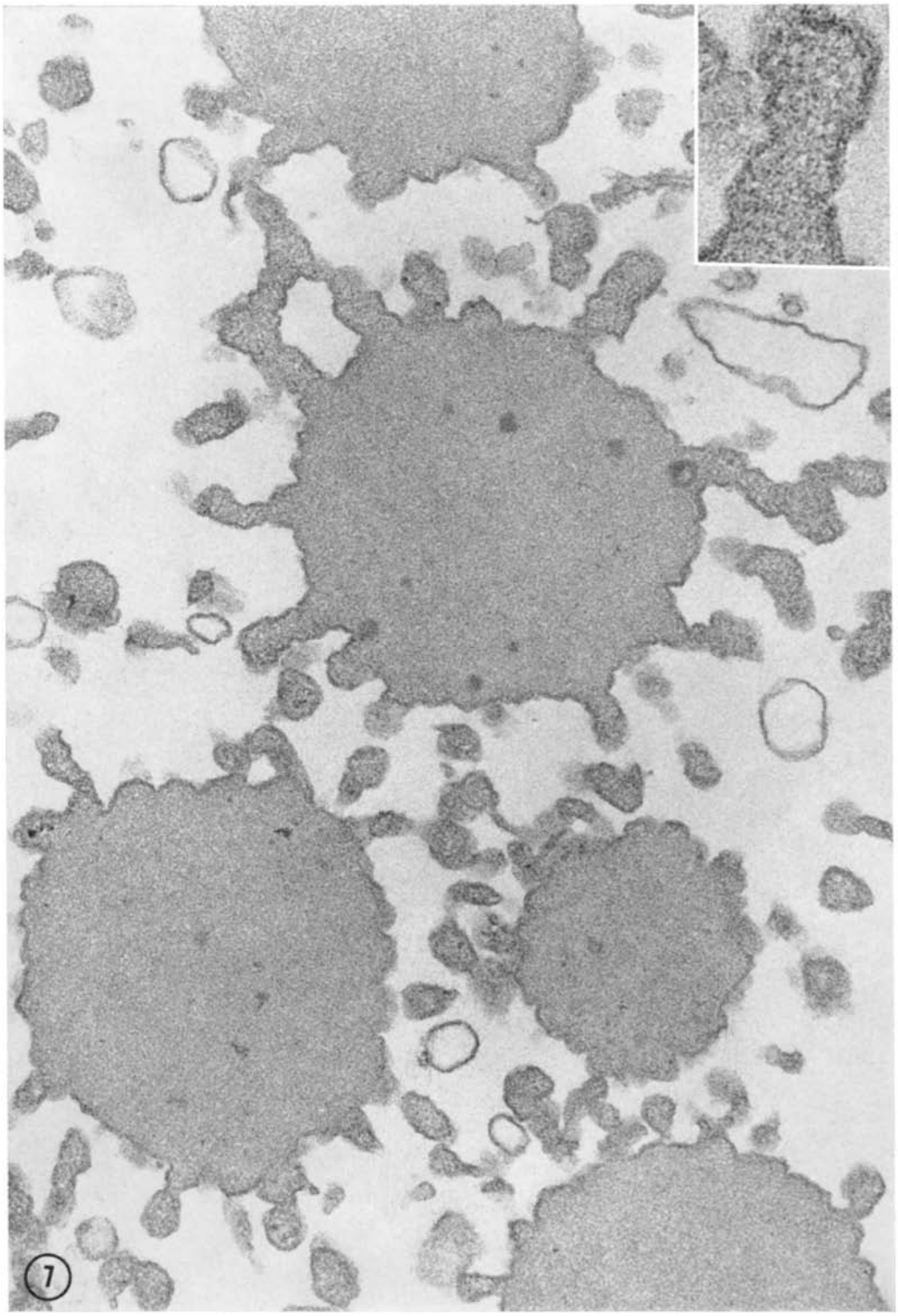

FIGURE 7 The inner membrane-matrix preparation in Fig. 6 is shown at higher magnification, showing the details of the finger-like projections and the intact appearance of the inner membrane. The inset shows the trilamellar nature of the inner membrane. × 97,500; inset × 255,000.

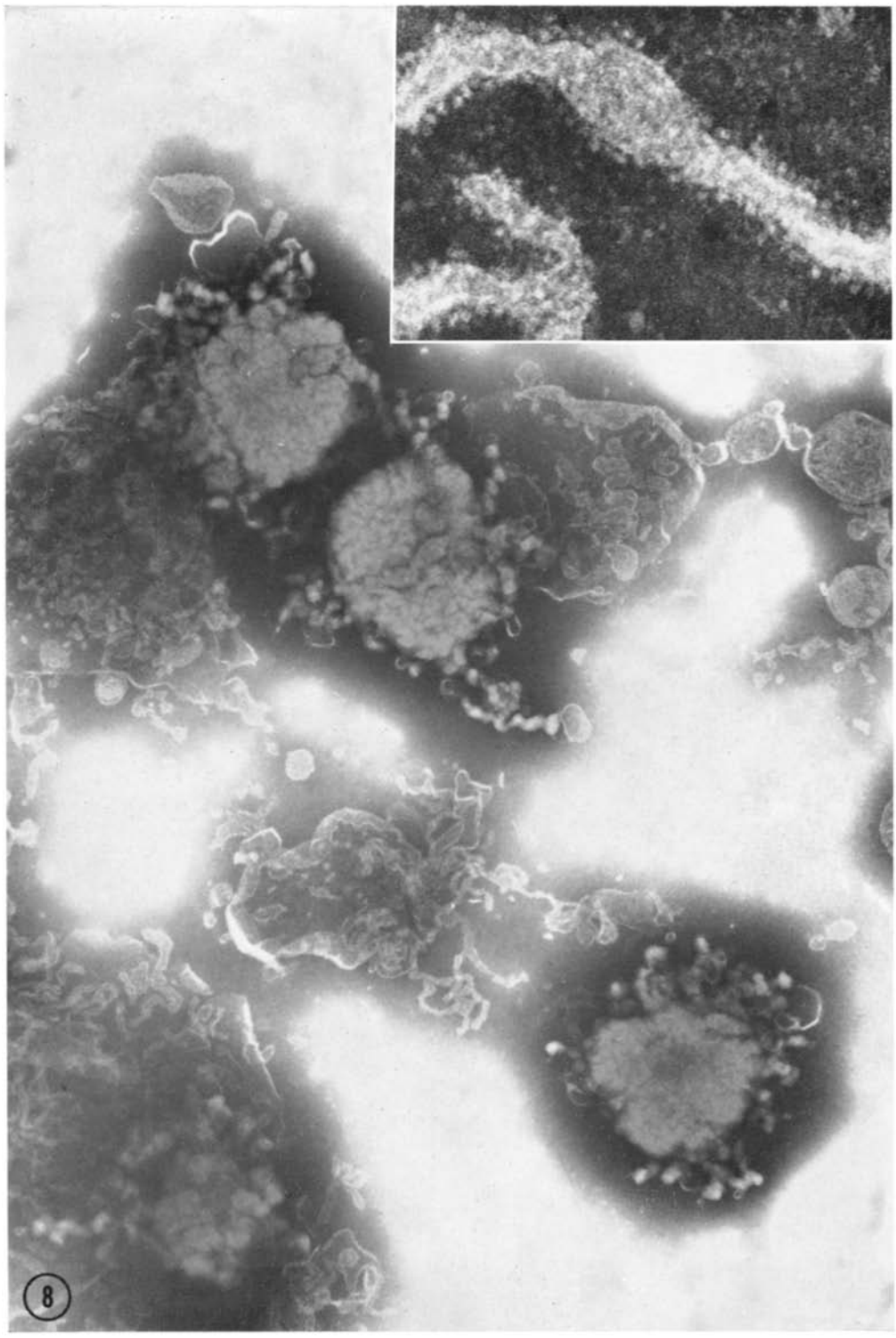

FIGURE 8 An unfixed preparation of the inner membrane-matrix negatively stained with phosphotungstic acid. This preparation consists of membranous bodies in various stages of disruption. The more intact bodies have finger-like projections similar to those seen in sectioned preparations. The disrupted membrane often appears in the form of strips or strands. The inset shows the details of these strands, which are lined with 90-A stalked particles. × 39,000; inset, × 220,000.

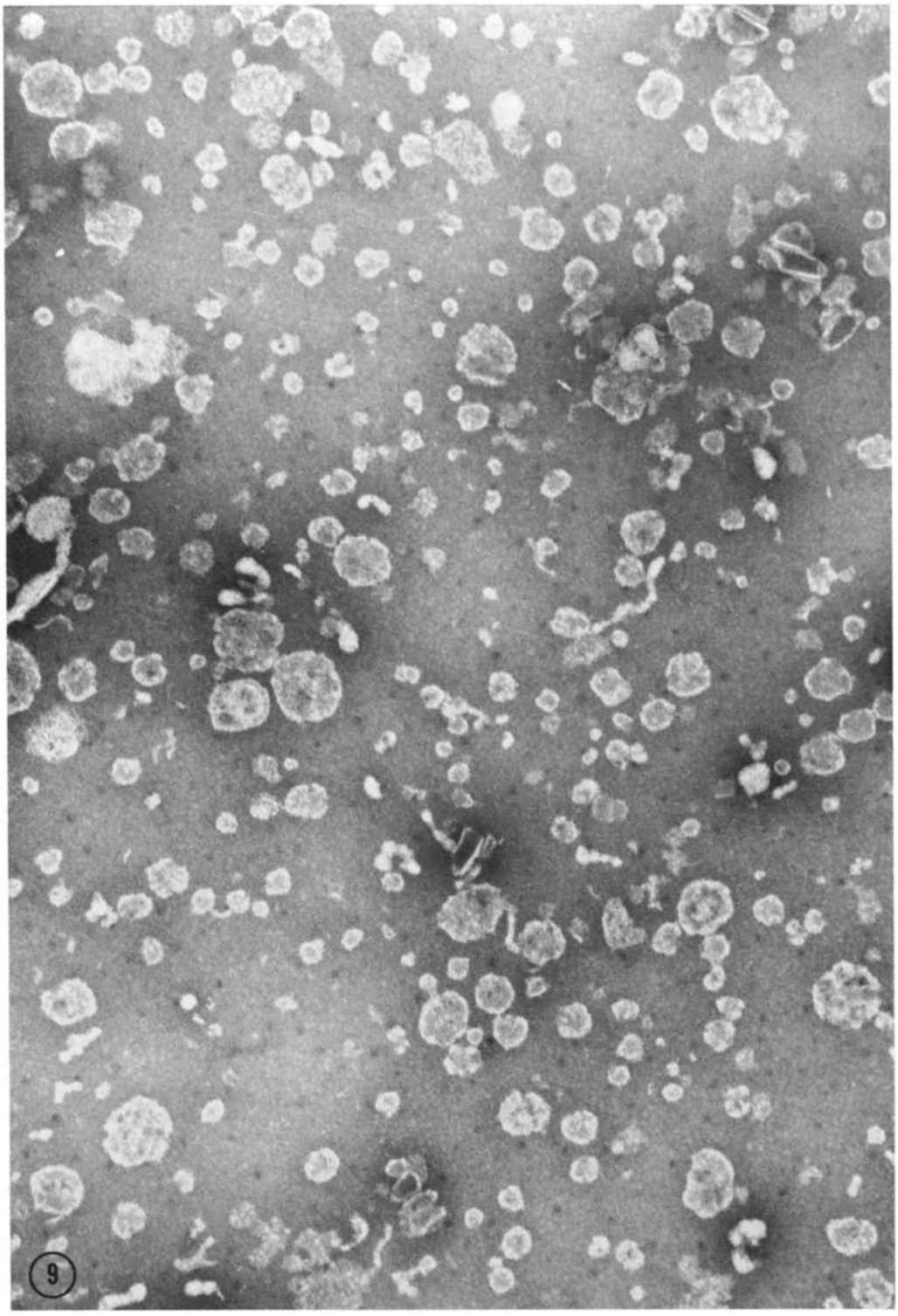

FIGURE 9 An unfixed preparation of the outer membrane negatively stained with phosphotungstic acid. This preparation consists of small, flat, somewhat ragged vesicles. × 65,000.

membrane of rat liver mitochondria observed in the present study. It is possible that these differences reflect a basic qualitative difference between mitochondria from these two sources, but this possibility appears unlikely in view of the high degree of qualitative similarity observed in mitochondria prepared from a wide variety of plant and animal sources. There is no general species difference between rat and beef mitochondria, since Erwin and Hellerman (34) have successfully applied a modified digitonin-fractionation procedure to the purification of monoamine oxidase from beef kidney mitochondria.

The appearance of the fixed, sectioned inner membrane-matrix preparation in the electron microscope supports the biochemical observations on the integrity of the inner membrane and indicates the degree of improvement of this preparation over those previously reported (3). The observation of respiratory control with this preparation indicates that this function is at least in part a function of the inner membrane, although the outer membrane and/or outer compartment is also required for full respiratory function in the presence of extramitochondrial Mg^{++}. The uncoupling effect of Mg^{++} on the inner membrane-matrix preparation is of particular interest since added Mg^{++} enhances the respiratory control of intact mitochondria. This finding may explain the beneficial effect of EDTA in the isolation of mitochondria from refractory tissues where damage to the outer membrane is likely. The lack of respiration of this preparation in KCL-containing medium is not surprising, since similar concentrations of KCL (0.1 M) may be used for extraction of cytochrome *c* from broken mitochondria (35). This fact suggests that the outer membrane may prevent the extraction of cytochrome *c* from intact mitochondria and may make possible the efficient sequestration of this protein by intact mitochondria in an environment of high ionic strength. The finding that respiratory control of the adenylate kinase-free inner membrane-matrix preparation is specific for ADP adds strength to the conclusion that ADP is the primary phosphate acceptor in oxidative phosphorylation.

The studies on the localization of glucose-6-phosphatase presented here do not support the suggestion of Parsons et al. (1) that this enzyme is localized in both the outer mitochondrial and microsomal membranes. It is appealing to think that the outer mitochondrial membrane is similar to microsomal membranes and hence to the membranes of the endoplasmic reticulum. However, some observations suggest that this is not the case. Monoamine oxidase and kynurenine hydroxylase (21) are found primarily in the outer mitochondrial membrane, whereas glucose-6-phosphatase and a large number of other enzymes are found primarily in the microsomal fraction. NADH–cytochrome *c* reductase and cytochrome b_5 (1, 2) are found in both the microsomes and the outer mitochondrial membrane, but, as Sottocasa et al. (2) have pointed out, the properties of these enzymes may be different in the two membrane fractions. How two membrane systems which are found in such close apposition *in situ* can retain such a high degree of enzyme specificity poses a challenging question for future study.

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Note Added in Proof: In the course of these experiments we have measured monoamine oxidase by following the formation of benzaldehyde from benzylamine spectrophotometrically at 250 mμ. This assay has been criticized on the basis that the benzaldehyde formed during the assay is metabolized further by aldehyde dehydrogenase, leading to an apparent loss of monoamine oxidase activity of the inner membrane-matrix fraction (Green, D. E., D. W. Allman, R. A. Harris, and W. C. Tan. Manuscript in preparation.) This was not the case, as indicated by the following experiments. First, the addition of 1 mM chloral hydrate, an inhibitor of aldehyde dehydrogenase (Erwin, V. G., and R. A. Deitrich. 1966. *J. Biol. Chem.* **241**:3533), had no effect upon the monoamine oxidase activity of either unfractionated preparations or the inner membrane-matrix fraction. This concentration of chloral hydrate produced an 80–90% inhibition of aldehyde dehydrogenase when benzaldehyde was used as a substrate. Second, no decrease in optical density at 250 mμ was observed when small amounts of benzaldehyde were incubated with the inner membrane-matrix preparations under the conditions used for the monoamine oxidase assay. Third, an identical distribution of monoamine oxidase activity was observed when the tyramine-^{14}C assay described by R. E. McCaman, M. W. McCaman, J. M. Hunt, and M. S. Smith (1965. *J. Neurochem.* **12**:15) was used in place of the spectrophotometric benzylamine assay. The tyramine-^{14}C

assay is not affected by the presence of aldehyde dehydrogenase. Fourth, a complete recovery of monoamine oxidase activity was observed when small amounts of the outer membrane fraction were assayed in the presence of the inner membrane-matrix fraction.

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