BIOCHEMICAL AND ULTRASTRUCTURAL PROPERTIES OF A MITOCHONDRIAL INNER MEMBRANE FRACTION DEFICIENT IN OUTER MEMBRANE AND MATRIX ACTIVITIES

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ABSTRACT

Treatment of the inner membrane matrix fraction of rat liver mitochondria with the nonionic detergent Lubrol WX solubilized about 70% of the total protein and 90% or more of the following matrix activities: malate dehydrogenase, glutamate dehydrogenase, and isocitrate dehydrogenase (NADP). The Lubrol-insoluble fraction was enriched in cytochromes, phospholipids, and a Mg^{++}-stimulated ATPase activity. Less than 2% of the total mitochondrial activity of monoamine oxidase, an outer membrane marker, or adenylate kinase, an intracristal space marker could be detected in this inner membrane fraction. Electron micrographs of negatively stained preparations showed vesicles ($\leq 0.4\ \mu$ diameter) literally saturated on the periphery with the 90 A ATPase particles. These inner membrane vesicles, which appeared for the most part to be inverted with respect to the normal inner membrane configuration in intact mitochondria, retained the succinicoxidase portion of the electron-transport chain, an intact phosphorylation site II with a high affinity for ADP, and the capacity to accumulate Ca^{++}. A number of biochemical properties characteristic of intact mitochondria and the inner membrane matrix fraction, however, were either absent or markedly deficient in the inner membrane vesicles. These included stimulation of respiration by either ADP or 2,4-dinitrophenol, oligomycin-sensitive ADP-ATP exchange activity, atractyloside sensitivity of adenine nucleotide requiring reactions, and a stimulation of the Mg^{++}-ATPase by 2,4-dinitrophenol.

INTRODUCTION

Treatment of intact rat liver mitochondria with a buffered solution of digitonin-containing sucrose, mannitol, and bovine serum albumin (BSA), has been shown by Schnaitman and Greenawalt (1) to result in removal of the outer mitochondrial membrane without seriously impairing the structural integrity of the inner membrane matrix (IMM) fraction. In the electron microscope, fixed, sectioned, preparations appear to be structurally intact, containing a relatively electron-opaque matrix enclosed by a single continuous membrane (1). The freshly isolated IMM preparation assumes an unusual "pseudopodial" morphology. When assayed in a sucrose-mannitol-ethylenediaminetetraacetic acid (EDTA)-BSA medium in the absence of added Mg^{++}, this fraction exhibits acceptor control ratios as high as 3.0 with sodium D-β-hydroxybutyrate as substrate

and as high as 2.0 with sodium succinate as substrate. Subsequent studies carried out by Pedersen and Schnaitman (2, 3) have shown that in the absence of added Mg^{++} the IMM fraction catalyzes oligomycin-sensitive ADP-ATP and P_i-ATP exchange reactions, a dinitrophenol-stimulated ATPase activity, and oxidative phosphorylation. Moreover, the exchange reactions, as well as oxidative phosphorylation in this fraction, are markedly inhibited by low concentrations of atractyloside, indicating that permeability to adenine nucleotides is still dependent on the adenine nucleotide carrier system (4, 5). The IMM fraction prepared by the digitonin procedure has been shown, therefore, to exhibit a high degree of morphological and biochemical integrity independent of the presence of an intact outer membrane.

As will be described in detail below, the IMM fraction can be resolved with the nonionic detergent Lubrol WX into a soluble fraction enriched in the enzymatic activities associated with the matrix, and a membrane fraction enriched in cytochromes, phospholipids, and a Mg^{++}-stimulated ATPase activity. Experiments summarized in this report have been designed to establish the biochemical and morphological characteristics of this inner membrane fraction as an approach to better understanding the structure-functional relationships underlying the mechanism of energy coupling and ion translocation in rat liver mitochondria.

EXPERIMENTAL METHODS

Isolation of Mitochondria and Preparation of the IMM Fraction

Mitochondria were isolated as previously described by Schnaitman and Greenawalt (1) in a medium containing 220 mM D-mannitol, 70 mM sucrose. 2.0 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, and 0.5 mg/ml crystalline BSA, pH 7.4. Mitochondria prepared in this manner exhibited acceptor control ratios of at least 5.0 with either succinate or D-β-hydroxybutyrate as substrate, when these measurements were made in the respiration medium described by Schnaitman and Greenawalt (1). Mitochondria with acceptor control ratios of less than these minimal values were discarded.

The isolated mitochondria were resuspended in the isolation medium to give a concentration of 100 mg of mitochondrial protein/ml. An equal volume of digitonin solution (12 mg digitonin/ml), freshly prepared as previously described (1, 2), was added, and the resultant medium was stirred slowly at 0°C for 15 min. The suspension was diluted with 3 volumes of isolation medium and centrifuged at 9,000 *g* for 10 min. The sediment was again resuspended in 3 volumes of isolation medium and centrifuged a second time. The final sediment contained essentially all of the inner membrane and matrix as characterized by Schnaitman and Greenawalt (1).

Preparation of the Lubrol-Insoluble Fraction

The IMM fraction containing less than 2% of the outer membrane marker monoamine oxidase was suspended in the isolation medium at a concentration of 30–35 mg/ml and then treated with a volume of Lubrol WX (obtained from I. C. I. Organics Inc., Providence, Rhode Island), 19 mg/ml, such that the final suspension contained 0.16 mg Lubrol WX/mg of IMM protein. After standing at 0°C for 15 min, the suspension was diluted with isolation medium so that the final volume was two times the original volume of the IMM fraction. Centrifugation was then carried out at 144,000 *g* for 1 hr in the Spinco Model L No. 40 rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). The supernatant (Lubrol-soluble fraction) was carefully removed with a Pasteur pipette. The sediment or "Lubrol-insoluble" fraction was rinsed twice with a small amount of isolation medium and then suspended in this medium at a concentration of 10.0 mg/ml. A diagram outlining the preparative procedure is shown in Fig. 1.

Enzyme Assays

Monoamine oxidase, cytochrome oxidase, malate dehydrogenase, glutamate dehydrogenase, isocitrate dehydrogenase (NAD), and isocitrate dehydrogenase (NADP) were assayed as described by Schnaitman et al. (1, 6). Adenylate kinase and nucleoside diphosphate kinase were assayed electrophoretically as described by Pedersen and Schnaitman (3). The ADP-UTP substrate pair was used in the nucleoside diphosphate kinase assay.

Two different procedures were employed to assay ATPase activity. In the first, 10 mM $MgCl_2$, 8.3 mM imidazole buffer, pH 6.9, and 0.5 mg membrane protein were incubated for 4 min at 30°C in a total volume of 0.45 ml. ATP or another nucleoside-5'-triphosphate was then added in a volume of 0.150 ml to give a final concentration of 10.0 mM. Where indicated in the Tables, 83 μM 2,4-dinitrophenol (DNP), 30 μM atractyloside, 1.0 μg oligomycin, and 1.0 μg aurovertin were included in the assay. Incubation was carried out for 10 min before stopping the reaction with 0.10 ml 2.5 M $HClO_4$. After neutralizing with 0.10 ml 2.5 M KOH, 0.20 ml aliquots were removed and analyzed for orthophosphate by the colorimetric method of Gomori (7).

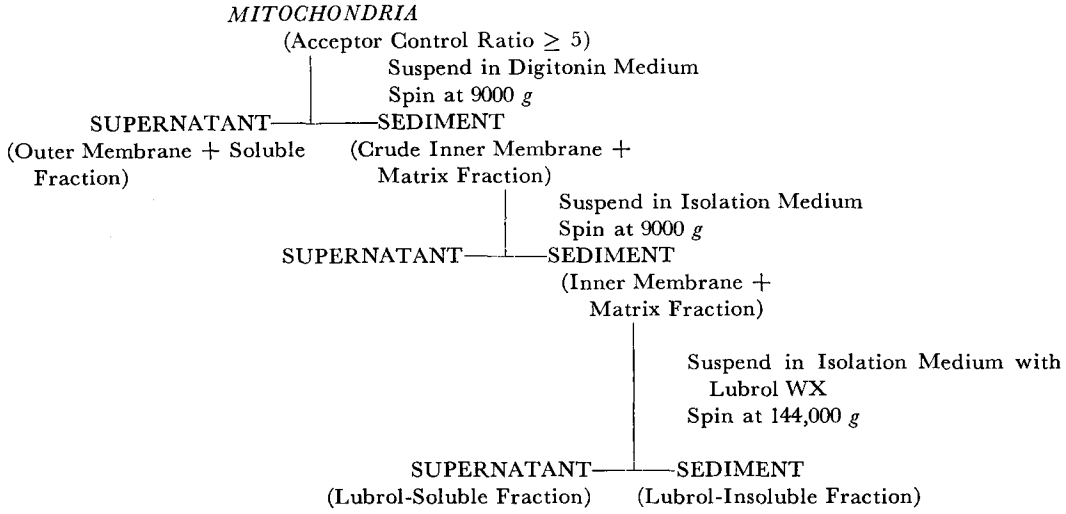

FIGURE 1 Fractionation scheme employed in the preparation of the Lubrol-insoluble fraction.

In the second method, ATPase activity was assayed by a modification of the method of Pullman et al. (8). ADP formed in the ATPase assay was coupled to a pyruvate kinase-lactate dehydrogenase system, and the oxidation of NADH was followed at 340 mμ. The assay mixture contained the following in a final volume of 1.0 ml; 250 mM sucrose, 4.5 mM KCN, 4.0 mM $MgCl_2$, 3.0 mM ATP, 3.0 mM phosphoenolpyruvate, 0.3 mM NADH, 1.0 IU lactate dehydrogenase, 1.0 IU pyruvate kinase, and 50 mM Tris-chloride, pH 7.4. Prior to addition of the membrane suspension, the assay mixture devoid of lactic dehydrogenase, pyruvate kinase, and NADH, was incubated for 5 min at 30°C in a volume of 0.95 ml. The remaining assay components were then added in a volume of 0.040 ml and the decrease in NADH absorbance, resulting from a small contamination of the commercial preparation of ATP by ADP, was followed until the rate decreased to zero. A 0.01 ml aliquot of the membrane suspension (10–12 mg/ml) was added and the decrease in absorbance of NADH at 340 mμ was followed. A molar extinction coefficient of 6.22×10^3 cm^2 M^{-1} was used for NADH in calculations of specific activities.

ADP-ATP exchange activity was assayed in a manner similar to that described previously by Pedersen and Schnaitman (3). The preliminary incubation mixture contained in a volume of 0.45 ml: 294 mM D-mannitol, 93 mM sucrose, 2.7 mM HEPES, 3.3 mM potassium phosphate, 5.4 mM ADP, 2.0 mg defatted BSA, and 1.0 mg membrane protein. Incubation was carried out for 4.0 min at 30°C. ATP, 6.0 μmoles, and ^{14}C-labeled ADP, 0.10 μCi were then added in a total volume of 0.150 ml. Where indicated, 1.0 μg oligomycin and 1.0 μg aurovertin were included in the assay. Incubation was carried out at 30°C for 10 min before stopping the reaction with 0.10 ml 2.5 M $HClO_4$. Separation of nucleotides, counting procedures, and calculations of exchange rates were performed exactly as previously described (3).

P_i-ATP exchange activity was assayed exactly as the ADP-ATP exchange reaction, with 1.6 μCi ^{32}P-labeled orthophosphate replacing the ^{14}C-labeled ADP. This activity was also assayed at 30°C in a 0.60 ml system containing 42 mM sucrose, 10 mM ATP, 4.0 mM/ADP, 1.6 μCi ^{32}P-labeled orthophosphate, 8.3 mM imidazole, pH 6.9, and phosphate as indicated. Incubation times, nucleotide separations, counting procedures, and calculations were performed exactly as previously described (3).

Respiration and Phosphorylation

Respiration studies and oxidative phosphorylation measurements were conducted at 25°C in a closed 3.0 ml reaction vessel equipped with a Clark oxygen electrode. The basic medium referred to as "Respiration System I" contained the following in a total volume of 2.8 ml: 220 mM D-mannitol, 70 mM sucrose, 0.5 mM EDTA, 20 mM sodium succinate, 2.5 mM potassium phosphate, and 2.0 mM HEPES, pH 7.4. "Respiration System II" was identical to Respiration System I, except that 5.4 mM ascorbate and 0.11 mM TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) were used in place of succinate. Where indicated, 2 mg defatted BSA, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 152 μM ADP, 89 μM DNP, 0.14 μM antimycin A, and 0.45 mM KCN were included.

In oxidative phosphorylation experiments, the basic medium referred to as "Phosphorylation System

I" was identical to Respiration System I, with the succinate concentration reduced to 5.0 mM. In addition, the system contained 2.5 mM $MgCl_2$, 0.8 mg defatted BSA, 10.6 mM glucose, 30.0 IU hexokinase, 1.0 μCi ^{32}P-labeled orthophosphate, and ADP at concentrations given in legends to Tables and figures. Phosphorylation System II was identical to Phosphorylation System I, except that 5.4 mM ascorbate and 0.11 mM TMPD were used in place of succinate. Extraction and counting of ^{32}P-labeled glucose-6-phosphate was performed exactly as previously described.

Calcium Uptake

Ca^{++} uptake experiments were conducted at 25°C in two different systems. "Ca^{++} Uptake System I" contained in a total volume of 2.0 ml: 5.0 mM sodium succinate, 80 mM NaCl, and 0.20 mM $CaCl_2$, 0.1 μCi $^{45}CaCl_2$, and Tris-chloride, pH 7.4. "Ca^{++} Uptake System II" contained in a total volume of 2.0 ml Phosphorylation System I without the glucose, hexokinase, and ADP. In addition, the system contained 0.2 mM $CaCl_2$ and 0.1 μCi $^{45}CaCl_2$. Where indicated, 5.0 mM ATP, 5.0 mM ADP, and 75.0 μM DNP were included in the assays. After a 2.0 min incubation period, the polycarbonate tubes containing the reaction medium were centrifuged immediately at 28,000 *g* for 8.0 min in a refrigerated centrifuge (0–4°C). The sediment was dissolved in 2.0 ml concentrated formic acid and the radioactivity was estimated in a Beckman liquid scintillation counter.

Determination of Cytochrome Content

Two quartz cuvettes of 1.0 cm light path, which contained, in a total volume of 2.5 ml, 220 mM mannitol, 70 mM sucrose, 2.0 mM HEPES, and 11.0 mg of mitochondrial protein or 4.6 mg of membrane protein (Lubrol-insoluble fraction), were placed in a split beam spectrophotometer. An absorbancy base line was obtained by scanning the spectrum between 630 and 400 mμ. A small amount of sodium hydrosulfite was then added to one cuvette and potassium ferricyanide to the other, and the same wavelength region was again scanned to obtain the oxidized/reduced difference spectrum. Cytochrome extinction coefficients used in calculations were those summarized by Estabrook and Holowinsky (9).

Phospholipid Analysis

Phospholipids were extracted from aqueous suspensions of the submitochondrial fractions by the method of Folch et al. (10). The chloroform-methanol extract was washed once with 0.20 volume of 0.9% KCl, dried by filtration through anhydrous sodium sulfate, and evaporated with a stream of N_2. The phospholipid was digested with H_2SO_4 in the presence of H_2O_2. Total phosphate was determined by the method of Gomori (7).

Mg^{++} and Ca^{++} Determinations

Mitochondria or membranes (5–10 mg) were suspended in 2.0 ml deionized H_2O and 2.0 ml 1.0 N HCl and boiled for 10 min. After centrifugation at 10,000 *g* for 15 min, the supernatant was removed for Mg^{++} and Ca^{++} determinations, which were carried out by atomic absorption spectrometry.

Protein Determinations

Protein was estimated by the biuret procedure (11). Crystalline BSA served as standard.

Electron Microscopy

Samples of the submitochondrial fractions were rapidly fixed, dehydrated, and embedded as previously described (6). Small volumes (5–20 μl) were placed on top of a Beckman Microfuge tube containing 200 μl of glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 and were centrifuged immediately. The supernatant fluid was decanted, and fresh glutaraldehyde was added; fixation was continued at 0°C for 2 hr. Fixed pellets were stored overnight in 0.2 M sucrose in 0.1 M phosphate buffer. The micropellets were then postfixed with 2% OsO_4, pH 7.2 in 0.1 M phosphate buffer plus 0.005% $CaCl_2$. Following dehydration by rapid passage through a cold ethanol series, the pellets were embedded in Epon 812 as described by Luft (12). Thin sections were cut with a diamond knife on an LKB Ultrotome and stained with saturated uranyl acetate in 1% sodium borate for 15 min at 60°C, and then with saturated lead citrate for 10 min at room temperature (13).

Fractions, unfixed, were negatively stained with 2% potassium phosphotungstate, pH 6.5, by the drop method. Thick suspensions were diluted directly with the negative stain before applying the drop to the Formvar-carbon-coated grids. The excess drop suspension was removed with filter paper, and the remaining thin film was air-dried.

Samples were examined in Siemens-Halske Elmiskop I operated at 80 kv; plate magnification ranged from 4,000 to 40,000.

RESULTS

Removal of Malate Dehydrogenase with Lubrol WX

In a previous study (1), Lubrol WX was found to be a useful alternative to sonication for activating many mitochondrial enzymes. For this reason Lubrol WX was also tested for its effectiveness in removing activities associated with the

mitochondrial matrix. Results presented in Fig. 2 *A* show that titration of the IMM fraction with Lubrol WX results in maximal solubilization of the matrix marker enzyme malate dehydrogenase

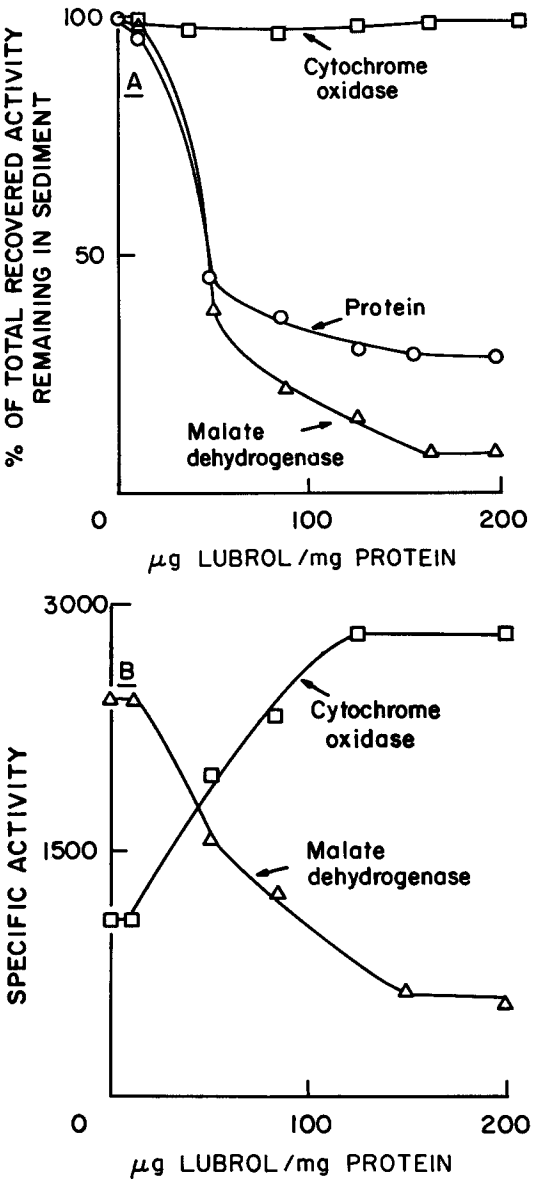

FIGURE 2 (*A*) The release of protein and malate dehydrogenase activity from the inner membrane matrix fraction by titration with Lubrol WX. (*B*) Change in specific activity of malate dehydrogenase and cytochrome oxidase in the Lubrol-insoluble fraction at various concentrations of Lubrol WX. Specific activities are expressed in mμmoles product formed/min per mg protein.

(4, 6) at a Lubrol to protein ratio of 0.16 (mg/mg). Approximately 90% of the total recoverable malate dehydrogenase activity and 70% of the total starting protein are solubilized by Lubrol, whereas all of the cytochrome oxidase activity, an inner membrane marker (1–3, 6), remains with the Lubrol-insoluble fraction. About 5–10% of the total malate dehydrogenase activity of the IMM fraction is not removed with Lubrol concentrations as high as 200 μg/mg protein and thus remains with the Lubrol-insoluble fraction.

As shown in Fig. 2 *B*, the maximal specific activity of cytochrome oxidase attained in the Lubrol-insoluble fraction (2, 880 mμmoles/mg) is about three times that in the IMM fraction (1,080 mμmoles/mg). Conversely, the specific activity of malate dehydrogenase in the Lubrol-insoluble fraction (556 mμmoles/mg) is about five times less than the IMM value (2,415 mμmoles/mg). Thus the Lubrol-insoluble fraction is greatly enriched in the inner membrane marker cytochrome oxidase, and highly deficient in the matrix marker malate dehydrogenase.

Enzymatic, Ionic, and Phospholipid Composition of the Lubrol-Insoluble Fraction

Data summarized in Table I show that the Lubrol-insoluble fraction contains less than 2% of the total mitochondrial activity of monoamine oxidase, an outer membrane marker (6), and adenylate kinase, a marker for the intracristal space (1). Nucleoside diphosphate kinase, which appears to be dually localized in the outer membrane, as well as in the intracristal space (1–3), is also deficient in this fraction. Glutamate dehydrogenase, isocitrate dehydrogenase (NAD), and isocitrate dehydrogenase (NADP), which are considered along with malate dehydrogenase to be closely associated with the matrix (4, 14), are present at activity levels in this fraction amounting to less than 10% of the total mitochondrial activity. In contrast, 86% of the Mg^{++}-stimulated ATPase, a well-recognized inner membrane marker, is recovered in the Lubrol-insoluble fraction.

Data tabulated in Table I show also that all of the mitochondrial cytochromes (*a*, *b*, *c*, and c_1) are present in the Lubrol-insoluble fraction at a concentration three to four times greater than that found in the control mitochondria. About 89% of the cytochrome *a*, 53% of the cytochrome *b*, and 68% of the cytochromes *c* and c_1 remain in

TABLE I

Enzymatic, Cytochrome, and Metal Ion Composition of the Lubrol-Insoluble Fraction

Prior to assay the mitochondrial fraction was pretreated with 0.16 mg Lubrol WX/mg protein

Enzyme, cytochrome, or metal Ion	Specific activity or composition*		% recovered in Lubrol-insoluble fraction‡§
	Mitochondria	Lubrol-insoluble fraction	
Monoamine oxidase	15.5	1.9	1.9
Adenylate kinase	264	3.8	0.2
Nucleoside diphosphokinase	80	9.0	1.8
Glutamate dehydrogenase	116	45.5	9.8
Isocitrate dehydrogenase (NADP)	115	18.2	2.4
Isocitrate dehydrogenase (NAD)	11.0	6.7	9.9
Mg^{++}-stimulated ATPase	398	1160	86
Cytochrome *a*	238	975	89
Cytochrome *b*	216	530	53
Cytochrome *c*	193	605	68
Cytochrome c_1	96	301	68
Mg^{++}	38	56	32
Ca^{++}	15	46	66

* Enzyme specific activities are expressed as mμmoles product formed/min per mg; cytochromes as picomoles/mg; metal ions as mμmoles/mg.
‡ Based on total mitochondrial activity. Of the total mitochondrial protein, 19.5–21.7% was recovered in the Lubrol-insoluble fraction.
§ Recoveries of all enzyme activities were 85% or more with the exception of NAD-isocitrate dehydrogenase. Although 85% of the total mitochondrial activity of this enzyme was recovered in the IMM fraction, only 50% of this was subsequently recovered after Lubrol fractionation.

the Lubrol-insoluble fraction. Similarly, the Lubrol-insoluble fraction is enriched in the divalent cations Mg^{++} and Ca^{++}.

Results of phospholipid analyses presented in Table II show that the ratio of lipid phosphate (μmole P_i) to milligram protein in the Lubrol-insoluble fraction (0.314) is more than twice that of intact mitochondria (6) or the IMM fraction. Less than 10% of the phospholipid in the IMM fraction is solubilized by Lubrol.

Thus, these analyses indicate that the Lubrol-insoluble fraction is a highly pure inner membrane fraction. It is markedly deficient in activities associated with the outer membrane, the matrix, and the intracristal space, and is greatly enriched in cytochromes, phospholipids, and the Mg^{++}-stimulated ATPase.

Ultrastructural and Functional Properties of the Lubrol-Insoluble Fraction

ELECTRON MICROSCOPY: The morphology and ultrastructural characteristics of the freshly isolated IMM fraction are shown in Fig. 3. It can be seen that this fraction is extremely homogeneous in appearance and is essentially free of outer membrane (some small empty vesicles, presumably derived from the outer membrane, can be seen) or intact mitochondria. At least 80% of the IMM profiles show the unusual ("pseudopodial") morphology previously reported by Schnaitman and Greenawalt (1) and Greenawalt (15).

Fig. 4 shows the appearance of a fresh preparation of the Lubrol-insoluble fraction, processed for electron microscopy in precisely the same way as the IMM fraction. This fraction consists entirely of vesicles appearing to contain varying amounts of electron-opaque material; these vesicles range in size from about 0.06–0.4 μ in diameter. At higher magnification (*inset*) it can be seen that much of the apparent electron opacity results from tangential cuts across membranes and does not reflect retention of stainable material within the vesicles. A small percentage of the total number of vesicles, on the other hand, do have about the same electron opacity of the IMM fraction and probably are derived through pinching off of the

TABLE II

Comparison of Phospholipid Composition of Submitochondrial Fractions

Fraction	Phospholipid: protein	Total phospholipid*	Recovery‡
	μmole P_i/mg	*μmole P_i*	%
Inner membrane matrix	0.147	14.7	(100.0)
Lubrol-insoluble	0.314	11.9	80.0
Lubrol-soluble	0.018	1.2	8.3

* Based on 100 mg of inner membrane matrix protein.
‡ Relative to the total phospholipid of the inner membrane matrix fraction.

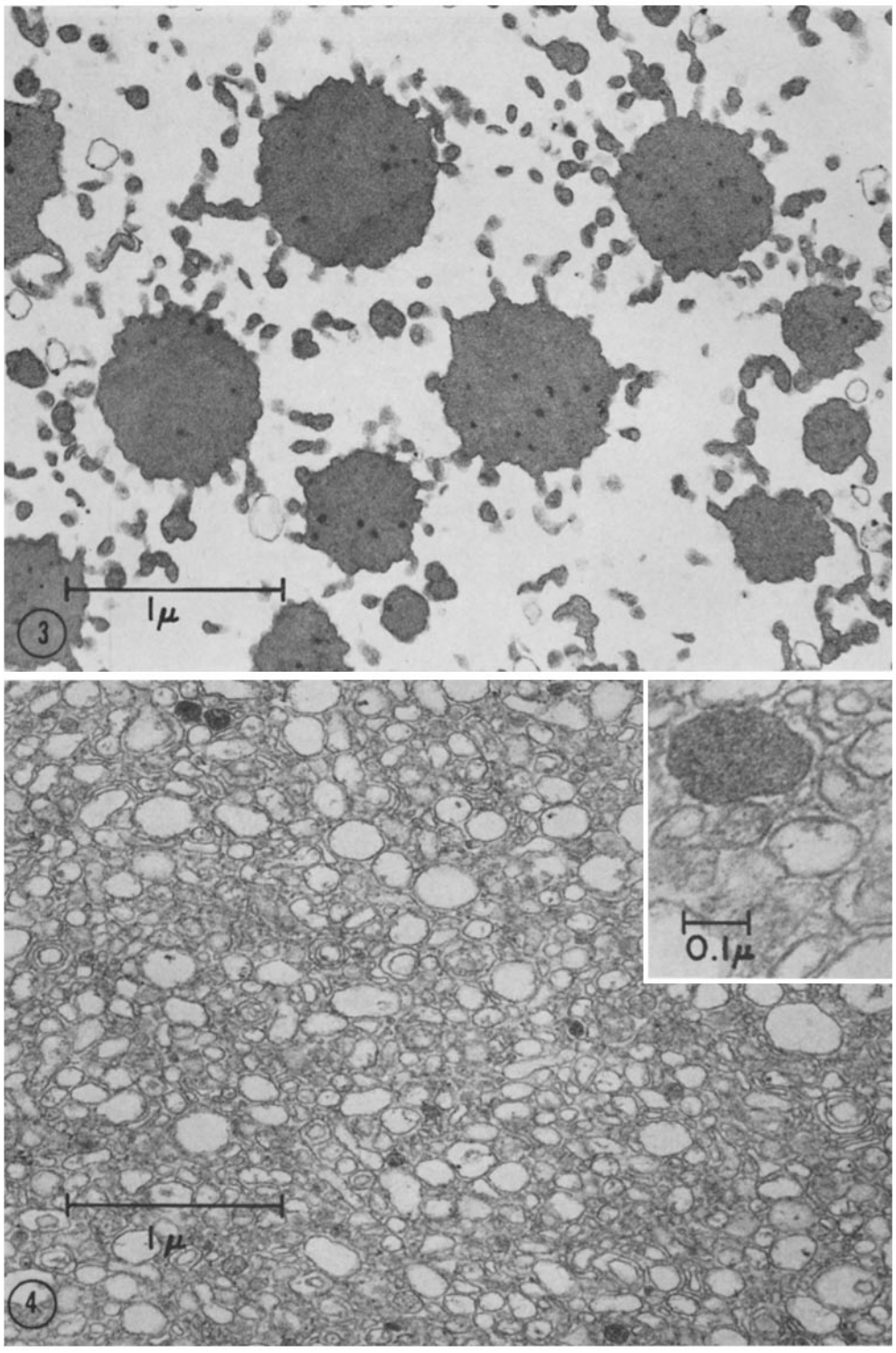

FIGURE 3 The freshly isolated inner membrane matrix fraction. The samples were fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 32,500.

FIGURE 4 The freshly isolated Lubrol-insoluble fraction fixed and stained exactly as the inner membrane matrix. The inner membrane vesicles range in diameter from 0.06 to 0.4 μ. At higher magnification (*inset*) it can be seen that much of the apparent electron opacity results from tangential cuts across membranes. × 32,500; *inset* × 97,500.

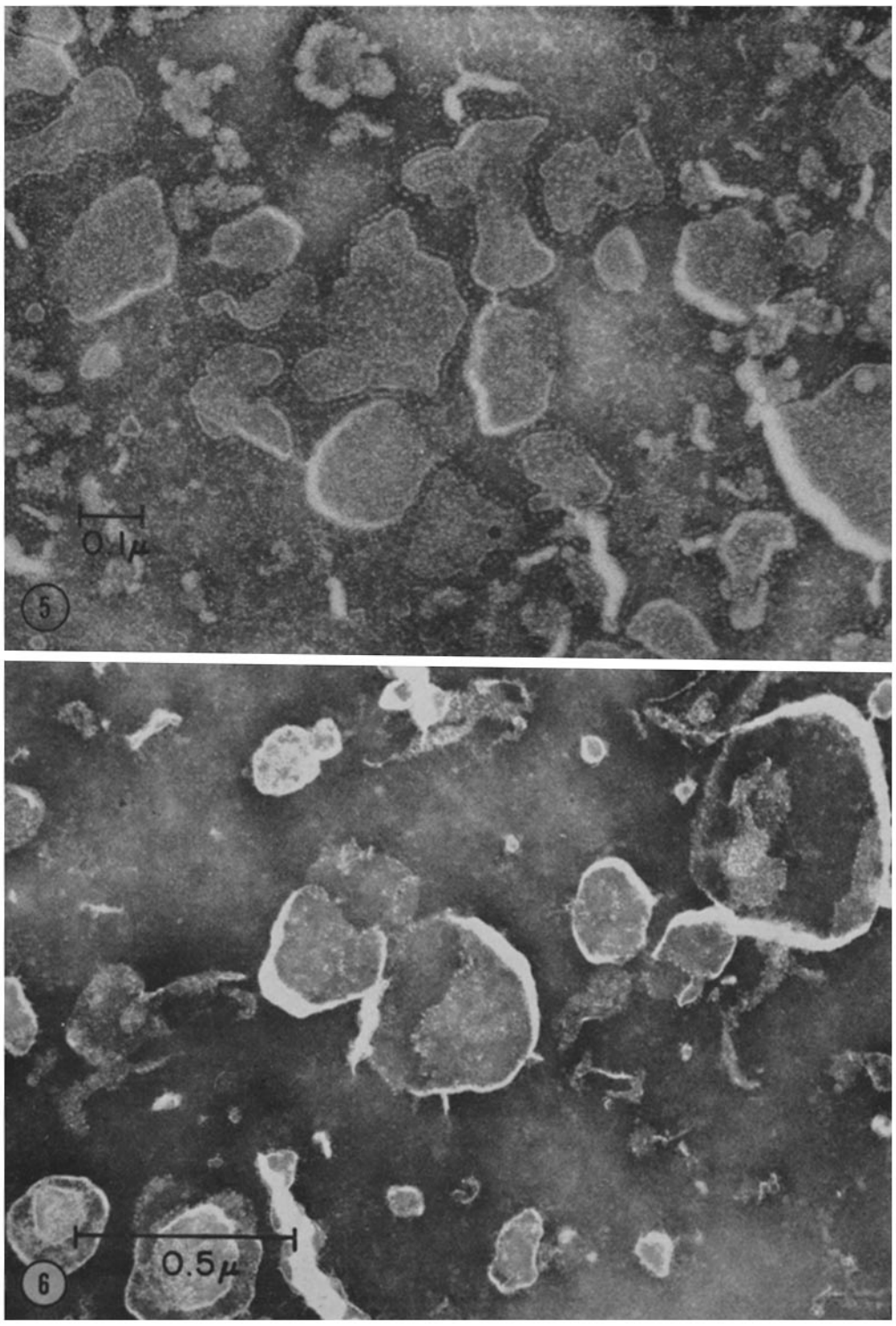

FIGURE 5 An unfixed freshly isolated preparation of the Lubrol-insoluble fraction negatively stained with phosphotungstic acid. The vesicles are lined on the periphery with 90 A stalked particles, presumably the mitochondrial ATPase. × 97,500.

FIGURE 6 An aged preparation of the Lubrol-insoluble fraction negatively stained as in Fig. 5. The preparation was aged 48 hr at 0°C in the isolation medium at a protein concentration of 10 mg/ml. Note the apparent deficiency of 90 A particles on the surface of the vesicles. × 65,000.

long pseudopodial extensions of the IMM fraction. It should be emphasized, however, that these "filled" vesicles constitute a very small fraction of the total.

An unfixed preparation of the Lubrol-insoluble fraction, negatively stained with phosphotungstic acid, reveals the presence of 90 A ATPase particles projected outward from the surface of the vesicles. In many cases the periphery of the vesicular membranes are literally saturated with the particles (Fig. 5). In contrast, the Lubrol-insoluble fraction, after aging at 0°C for 48 hr, is markedly deficient in the 90 A particles (Fig. 6). This obvious loss of particles at 0°C is correlated with the loss of Mg^{++}-stimulated ATPase activity. See below.

ATPase ACTIVITY: It is well established that the Mg^{++}-stimulated ATPase activity of intact mitochondria is low and can be enhanced by DNP, aging, or mechanical disruption (16–18). Data tabulated in Table III show that, in contrast to intact mitochondria (18, 19), the Lubrol-insoluble fraction catalyzes a high Mg^{++}-stimulated ATPase activity (915 mμmoles/min per mg), which is only slightly stimulated (~10%) by 83 μM DNP. This hydrolytic activity is not completely specific for ATP, but also catalyzes hydrolysis of ITP, GTP, UTP, and CTP. All of these hydrolytic reactions are insensitive to 30 μM atractyloside but are markedly inhibited by low concentrations of oligomycin as well as aurovertin, which presumably binds directly to the ATPase molecule (19, 20).

Also in contrast to intact mitochondria (16–18), aging the Lubrol-insoluble fraction at 0°C for 48 hr results in a two-fold reduction in the specific activity of the Mg^{++}-stimulated ATPase activity (Fig. 7). This loss is not due simply to a reordering of the Lubrol-insoluble fraction to a more mitochondrial-like state, since DNP does not reactivate the lost activity. On the contrary, this loss appears to be related to a loss of 90 A particles from the membrane surface (cf. Fig. 6).

RESPIRATORY ACTIVITY: Table IV shows that without ADP the Lubrol-insoluble fraction catalyzes the oxidation of succinate at state 4 rates in the range of 60–70 mμatoms O_2/min per mg. State 4 rates for the IMM fraction and mitochondria are 42 and 32 mμatoms/O_2 per mg, respectively (1), when measured under identical conditions. Phosphate is not required for maximal rates and can be omitted from the assay medium. Similarly, ADP, DNP, Ca^{++}, Mg^{++}, EDTA, and defatted BSA at the concentrations employed have no effect on the respiratory rate. Both antimycin and cyanide inhibit succinate oxidation, but only cyanide inhibits when ascorbate and TMPD replace succinate. In experiments not shown, negligible rates of respiration were obtained with β-hydroxybutyrate as substrate. Thus, the succinic oxidase portion of the respiratory chain appears to remain intact in the Lubrol-insoluble fraction.

PHOSPHORYLATION CAPACITY: Results presented in Table V summarize the basic requirements for oxidative phosphorylation in the Lubrol-

TABLE III

Specificity and Inhibitor Sensitivity of the ATPase Activity Catalyzed by the Lubrol-Insoluble Fraction

Substrate	Activity*				
	No inhibitor	Oligomycin	Aurovertin	Atractyloside	DNP
ATP	915	99	307	910	1032
ITP	562	41	110	591	649
GTP	434	23	180	440	423
UTP	128	12	81	128	99
CTP	41	12	29	41	41

* mμmoles ATP hydrolyzed/min per mg.
ATPase activity was estimated by the colorimetric procedure (see Methods).

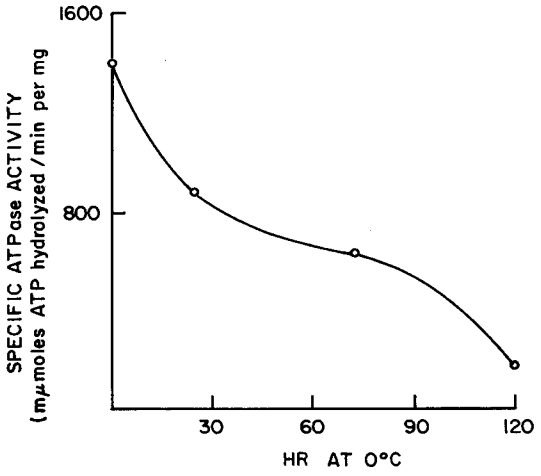

FIGURE 7 Loss of ATPase activity on aging the Lubrol-insoluble fraction. Aging conditions are given in Fig. 6. ATPase was estimated by the spectrophotometric procedure (see Methods).

TABLE IV

Respiratory Properties of the Lubrol-Insoluble Fraction

Experiment	Conditions	Respiratory rate*
1	System I	70.7
	Phosphate omitted	69.0
	EDTA omitted	69.0
2	System I	60.0
	ADP added	60.0
	Antimycin added	0
	Cyanide added	0
3	System I	58.7
	Ca^{++} added	56.2
	Mg^{++} added	57.0
	BSA added	56.5
	DNP added	58.0
4	System II	130
	ADP added	130
	Antimycin added	130
	Cyanide added	0

In both Respiration Systems I and II, 1.58 mg of the Lubrol-insoluble fraction was used. System I contained succinate; System II, ascorbate + TMPD.

* mμatoms O_2 consumed/min per mg.

insoluble fraction. With succinate as substrate, maximal P/O ratios (0.70–0.85) are obtained only at low ionic strength (<10 mM), low protein concentration (<1 mg/ml), and in the presence of defatted BSA (~1 mg/mg protein). These requirements are similar to those reported by Gregg (21) and Gregson et al. (Submitted for publication) for membrane fragments of intact mitochondria prepared by sonication and Lubrol WX treatment, respectively. When ascorbate replaces succinate as substrate P/O ratios of 0.10 or less are obtained, suggesting that the energy-coupling mechanism is completely intact only at the second phosphorylation site.

The effects of ADP on the rate of oxidative phosphorylation catalyzed by the Lubrol-insoluble fraction, by the IMM fraction, and by intact mitochondria are compared in Fig. 8 *A*. In order to obtain maximal rates with the Lubrol-insoluble fraction, the protein concentration was kept as low as experimentally possible (0.3 mg/ml). The rate of phosphate esterification is seen to be much lower in the Lubrol-insoluble fraction than in either intact mitochondria or the IMM fraction; however, the affinity of all three fractions for phosphate acceptor ($Km \sim 10$ μM ADP) is not remarkably different. As can be seen in Fig. 8 *B*, increasing the protein concentration of the Lubrol-insoluble fraction in the assay is accompanied by a sharp reduction of the rate of oxidative phosphorylation and the P/O ratio.

ADP-ATP AND P_i-ATP EXCHANGE ACTIVITY In intact mitochondria and in the IMM fraction the ADP-ATP exchange reaction proceeds at optimal rates in the absence of Mg^{++} (2, 3, 22, 23). The P_i-ATP exchange reaction also proceeds at an optimal rate in the IMM fraction in the absence of Mg^{++} (3). Both exchanges are inhibited by oligomycin and presumably are associated closely with the terminal stages of oxidative phosphorylation (2, 3, 22, 23). Results tabulated in Table VI show that these exchange reactions proceed at very low rates in the Lubrol-insoluble fraction (~3.0 mμmoles labeled ATP formed/min per mg) when assayed in the absence of added Mg^{++} in an assay system similar to that used in oxidative phosphorylation experiments. Both exchange reactions, however, retain their sensitivity to oligomycin and in addtion are inhibited by aurovertin. Addition of Mg^{++} and phosphate markedly enhances the rate of P_i-ATP exchange reaction (Fig. 9) but does not unmask any oligomycin sensitive ADP-ATP exchange activity.

CALCIUM ACCUMULATION: It is well documented that intact mitochondria accumulate Ca^{++} in a medium containing only succinate, NaCl and Tris-chloride, pH 7.4 (24, 27). Results summarized in Table VII show that the IMM

TABLE V

Inhibition of Oxidative Phosphorylation in the Lubrol-Insoluble Fraction

Conditions	P/O
System I (succinate)	0.73
BSA omitted	0.37
DNP added	0.03
Oligomycin added	0.03
Atractyloside added	0.59
KCl added	0.37
NaCl added	0.37
System II (ascorbate + TMPD)	0.10

In both Phosphorylation Systems I and II, 0.85 mg of the Lubrol-insoluble fraction and 0.83 mM ADP were used.

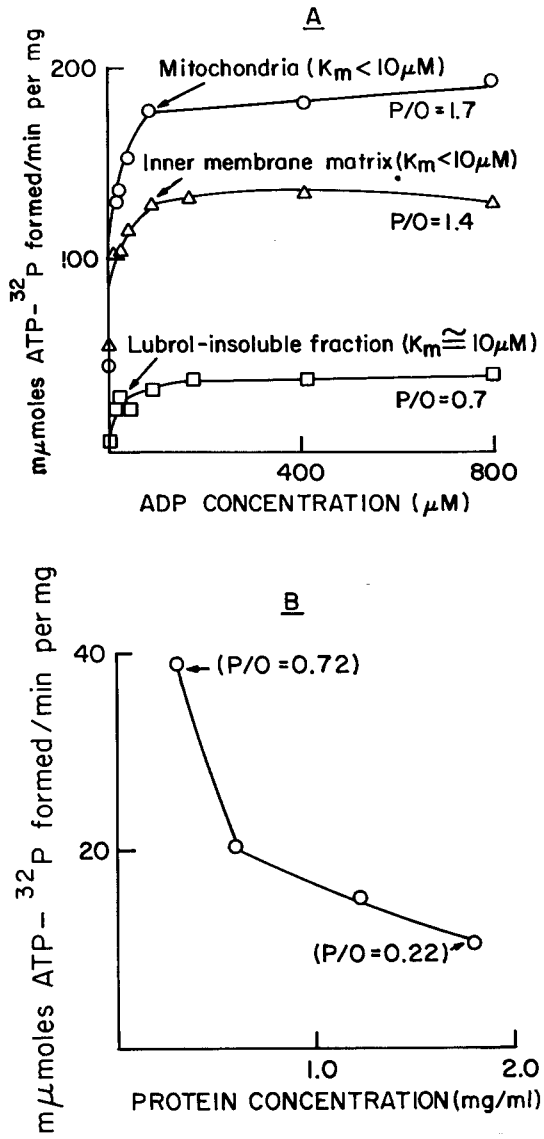

FIGURE 8 (*A*) Effect of ADP concentration on the rate of oxidative phosphorylation in mitochondria, the inner membrane matrix fraction and in the Lubrol-insoluble fraction. Phosphorylation System I was used for assaying the Lubrol-Insoluble fraction (see Methods). The medium used for assaying the other two fractions was exactly the same as Phosphorylation System I except BSA was omitted and 10 mM potassium phosphate was employed in place of the usual 2.5 mM. Mitochondria, 3.75 mg, inner membrane matrix, 3.30 mg, and Lubrol-insoluble fraction, 0.85 mg were used in the assays. (*B*) Effect of protein concentration on the rate of oxidative phosphorylation in the Lubrol-insoluble fraction. Phosphorylation System I was used in the assay.

fraction also accumulates significant amounts of Ca^{++} in this NaCl- containing medium. In contrast to intact mitochondria and the IMM fraction, however, the Lubrol-insoluble fraction accumulates Ca^{++} only at a low rate in this medium. For this reason Ca^{++} uptake was also studied in a medium almost identical to Phosphorylation System I containing sucrose, mannitol, HEPES, succinate, phosphate, $MgCl_2$, and defatted BSA. Results presented in Table VII show that in the complete medium the Lubrol-insoluble fraction has about the same rate of Ca^{++}accumulation as intact mitochondria. The IMM fraction has a very low Ca^{++} accumulation capacity under these conditions. For optimal Ca^{++} uptake in the Lubrol-

TABLE VI

ADP-ATP And P_i-ATP Exchange Activity in the Lubrol-Insoluble Fraction

Additions	Exchange rate*	
	ADP-ATP	P_i-ATP
None	2.8	2.6
Oligomycin	0	0
Aurovertin	0	0

In the assays 1.0 mg of the Lubrol-insoluble fraction was used.

* mμmoles labeled ATP formed/min per mg.

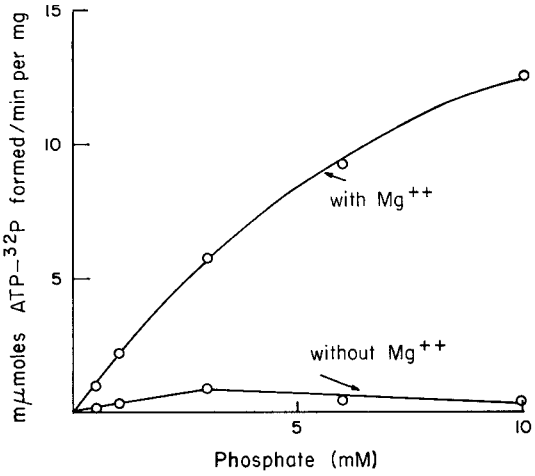

FIGURE 9 Effect of phosphate and $MgCl_2$ on the P_i-ATP exchange rate of the Lubrol-insoluble fraction. The imidazole-sucrose medium (see Methods) and 0.55 mg of the Lubrol-insoluble fraction were used in the assay. Where indicated 4.0 mM $MgCl_2$ was present.

TABLE VII

Accumulation of Ca^{++} by the Lubrol-Insoluble Fraction

Experiment	Fraction	System	Ca^{++} accumulated*
1	Mitochondria	I	79.5
		II	89.7
	Inner membrane matrix	I	45.0
		II	7.9
	Lubrol-insoluble	I	3.0
		II	53.3
2	Lubrol-insoluble	II	48.4
		Succinate omitted	1.9
		Mg^{++} omitted	37.2
		Phosphate omitted	8.9
		BSA omitted	32.3
		ATP added	19.7
		ADP added	25.9
		NaCl added	29.6
		KCl added	21.6
		DNP added	2.1

* mμmoles Ca^{++} accumulated/min per mg (Mitochondria and Imm fraction); mμmoles Ca^{++} accumulated/min per 0.5mg (Lubrol-insoluble fraction).

In the assay 1.0 mg of each fraction was used. System I contained only succinate, NaCl, Tris-chloride, and Ca^{++}. System II contained no NaCl. See Methods for complete composition.

insoluble fraction, succinate, $MgCl_2$, phosphate, and defatted BSA are required. Surprisingly, ATP, ADP, NaCl and KCl, all of which enhance Ca^{++} uptake by intact mitochondria (28, 29), markedly inhibit this process in the Lubrol-insoluble fraction. DNP (75 μM) almost completely inhibits the Ca^{++} accumulation process.

DISCUSSION

Results of experiments summarized in the present paper clearly show that the nonionic detergent Lubrol WX selectively removes the bulk of those enzymatic activities of the inner membrane matrix that are usually considered to be associated with the matrix space. The Lubrol-insoluble fraction (essentially the inner mitochondrial membrane) is enriched in phospholipids, cytochromes, divalent cations, and the Mg^{++} stimulated ATPase, while less than 10% of the total mitochondrial activity of glutamate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase (NAD), and isocitrate dehydrogenase (NADP) are present. Furthermore, the Lubrol-insoluble fraction is deficient also in outer membrane and intracristal space activities, which are common contaminants of submitochondrial systems prepared directly from intact mitochondria. With respect to composition, therefore, the Lubrol-insoluble fraction prepared from the inner membrane matrix fraction of rat liver mitochondria is perhaps one of the most highly purified inner membrane fractions studied to date.

Three different types of experimental evidence suggest that most of the inner membrane vesicles prepared with Lubrol WX are inverted with respect to the normal configuration of the inner membrane in intact mitochondria. First, electron micrographs of negatively stained samples show that the majority of the vesicles contain 90 A particles that are projected toward the external milieu, away from the surface of the vesicles (Fig. 5). Secondly, the hydrolytic activity of the Mg^{++}-stimulated ATPase is nonspecific with respect to nucleoside-5′-triphosphates (Table III). This observation is consistent with the ultrastructural data, since an ATPase on the external surface of the membrane would be independent of the ATP-specific adenine nucleotide translocase (4, 5, 30). Finally, the failure of atractyloside (30 μM) to inhibit the Mg^{++}-stimulated ATPase (Table III) and to only partially inhibit phosphorylation of ADP (Table V) supports the view that most of the inner membrane vesicles are "inside out" relative to their normal mitochondrial state.

Although the Lubrol-insoluble fraction retains

the capacity to catalyze oxidative phosphorylation and to accumulate Ca^{++}, the requirements for these processes differ significantly from those of intact mitochondria (Tables V, VII). High ionic strength, which has no effect on the efficiency of oxidative phosphorylation in intact mitochondria (31) and actually enhances Ca^{++} accumulation (29), has a deleterious effect on both of these processes in the Lubrol-insoluble fraction. These observations are thus consistent with the findings of Papa et al. (32) using beef heart submitochondrial particles, and support the suggestion that monovalent cations at relatively high concentrations (>30 mM) may have an uncoupling effect once the inner membrane barrier to their translocation is removed or damaged (33–35). Current hypotheses for energy coupling in mitochondria depict the immediate energy conservation product of respiration as either an easily hydrolyzable intermediate of a respiratory carrier C ~ I (36, 37), as a conformational state of the inner membrane (38), or as a protonmotive force (membrane potential + a pH gradient) (39). High salt concentrations are known to enhance certain hydrolytic reactions (40), to influence protein conformation (41), and to shift the potential across biological membranes (42). Therefore, the apparent uncoupling action of NaCl and KCl on the Lubrol-insoluble fraction or on other submitochondrial systems can be rationalized within the framework of all three hypothetical schemes for energy coupling.

In the presence of a respiratory substrate, Ca^{++} uptake is usually linked to H^+ ejection (23, 26). It is well documented, however, that under appropriate conditions the concentration of Lubrol used in these studies equilibrates the pH of the external and internal phases of intact mitochondria (27). Thus Ca^{++} accumulation in the Lubrol-insoluble fraction may not depend on a preexisting pH gradient, but may be driven by the membrane potential developed during electron transport. This suggestion is consistent with the chemiosmotic hypothesis (39) in which the total protonmotive force for driving ion accumulation is depicted as the sum of two terms, a pH gradient and a membrane potential.

Ca^{++} uptake has been assumed to be an unidirectional process and not to occur in inverted particles (43). Although the present data indicate that Ca^{++} uptake can take place in inner membrane vesicles that are for the most part "inside out," it is possible that the vesicles undergo a reversion to their normal geometry and hence a reversion to the normal membrane polarity under the experimental conditions employed. One should also not exclude the possibility that the negative stain, phosphotungstate, alters the sidedness of the membrane to some extent. It has recently been reported that phosphotungstic acid is not inert with respect to the mitochondrial membrane system (44). These rather important points will hopefully be clarified with the advent of more reliable methods for detecting rapid ultrastructural changes using negative staining techniques.

The finding that ATP and ADP inhibit Ca^{++} uptake in the Lubrol-insoluble fraction is subject to at least two possible interpretations. One possibility is that ATP and ADP tend to complex the Ca^{++}, and therefore compete with the active site of a Ca^{++} carrier. A second possibility is that they induce conformational changes that are unfavorable for the Ca^{++} uptake process.

With respect to the biochemical properties of submitochondrial particles prepared directly from intact rat liver mitochondria (having the outer membrane present at the time of preparation), the Lubrol-insoluble fraction resembles most closely the sonic membrane fragments described by Gregg (21) and Gregg and Lehninger (45). The Lubrol-insoluble fraction is analogous to sonic particles in that it also carries out oxidative phosphorylation only at site II by a mechanism dependent on protein concentration. Moreover, both membrane systems have low ADP-ATP and P_i-ATP exchange activity and no respiratory stimulation with ADP (21, 44). In contrast to the Lubrol-insoluble fraction, however, sonic particles exhibit respiratory control with phosphate, a very pronounced DNP-stimulated ATPase activity, and a high rate of respiration with β-hydroxybutyrate. In addtion, sonic particles are stable for long periods of time at 0°C, whereas the ATPase activity of the Lubrol-insoluble fraction is rapidly lost at 0°C. So, although the Lubrol-insoluble fraction has a number of properties similar to those of sonic particles, important differences also exist.

The molecular basis of these differences is not completely apparent at this time, but it is becoming more evident that the integrity of isolated mitochondria at the time of treatment and the method of treatment are important factors in determining the activities of the derived particles.

It is not clear, for example, to what extent proteins associated with the outer mitochondrial compartment are essential for mitochondrial function or to what extent they may be trapped within inverted particles formed from intact mitochondria. It is clear, however, that little of the outer compartment is present in the Lubrol-insoluble fraction described here.

This study and previous studies from these laboratories (1–3, 6) have now made it possible to sequentially and systematically subfractionate rat liver mitochondria into the following major ultrastructural compartments: the outer membrane, soluble components of the outer compartment (intracristal space), soluble components of the inner compartment (matrix), and the inner membrane. With this systematic development it is now feasible to undertake the reconstruction of such integrated mitochondrial functions as respiratory control at a level of complexity before impossible. Then using the results from those studies one might optimistically hope to resolve the essential interacting molecular components of such systems.

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REFERENCES

1. SCHNAITMAN, C. A., and J. W. GREENAWALT. 1968. *J. Cell Biol.* **38**:158.
2. SCHNAITMAN, C. A., and P. L. PEDERSEN. 1968. *Biochem. Biophys. Res. Commun.* **30**:428.
3. PEDERSEN, P. L., and C. A. SCHNAITMAN. 1969. *J. Biol. Chem.* **244**: 5065.
4. KLINGENBERG, M., and E. PFAFF. 1966. Regulation of Metabolic Processes in Mitochondria. J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, editors. Elsevier, New York. **7**:180.
5. VIGNAIS, P. V., and E. D. DUEE. 1966. *Bull. Soc. Chim. Biol.* **48**:1169.
6. SCHNAITMAN, C. A., V. G. ERWIN, and J. W. GREENAWALT. 1967. *J. Cell Biol.* **32**:719.
7. GOMORI, G. 1962. *J. Lab. Clin. Med.* **27**:467.
8. PULLMAN, M. E., H. S. PENEFSKY, A. DATTA, and E. RACKER. 1960. *J. Biol. Chem.* **235**: 3322.
9. ESTABROOK, R. W., and A. HOLOWINSKY. 1961. *J. Biophys. Biochem. Cytol.* **9**:19.
10. FOLCH, J., I. ASCOLI, M. LEES, J. MAETH, and F. LEBARON. 1951. *J. Biol. Chem.* **191**:833.
11. JACOBS, E. E., M. JACOB, D. R. SANADI, and L. B. BRADLEY. 1956. *J. Biol. Chem.* **223**:147.
12. LUFT, J. H. 1961. *J. Biophys. Biochem. Cytol.* **9**: 409.
13. REYNOLDS, E. S. 1963. *J. Cell Biol.* **19**:58A. (Abstr.)
14. BEATTIE, D. S. 1969. *Biochem. Biophys. Res. Commun.* **35**:67.
15. GREENAWALT, J. W. 1969. *Fed. Proc.* **28**:663.
16. KIELLEY, W. W., and R. K. KIELLEY. 1951. *J. Biol. Chem.* **191**:485.
17. LARDY, H. A., and H. WELLMAN. 1953. *J. Biol. Chem.* **201**:357.
18. POTTER, V. R., P. SIEKEVITZ, and H. C. SIMONSON. 1953. *J. Biol. Chem.* **205**:893.
19. ROBERTSON, A. M., R. B. BEECHY, C. T. HOLLOWAY, and I. G. KNIGHT. 1967. *Biochem. J.* **104**:54C.
20. KAGAWA, Y., and E. RACKER. 1966. J. *Biol. Chem.* **241**:2461.
21. GREGG, C. T. 1963. *Biochim. Biophys. Acta.* **74**: 573.
22. WADKINS, C. L., and A. L. LEHNINGER. 1958. *J. Biol. Chem.* **233**:1589.
23. BYGRAVE, F. L., and A. L. LEHNINGER. 1966. *J. Biol. Chem.* **241**:3394.
24. ROSSI, C. S., and A. L. LEHNINGER. 1964. *J. Biol. Chem.* **239**:3971.
25. CARAFOLI, E., R. L. GAMBLE, and A. L. LEHNINGER. 1966. *J. Biol. Chem.* **241**:2644.
26. DRAHOTA, Z., E. CARAFOLI, C. S. ROSSI, R. L. GAMBLE, and A. L. LEHNINGER. 1965. *J. Biol. Chem.* **240**:2712.
27. ROSSI, C. S., J. BIELAWSKI, and A. L. LEHNINGER. 1966. *J. Biol. Chem.* **241**:1919.
28. VASINGTON, F. D., and J. V. MURPHY. 1961. *Fed. Proc.* **20**:146.
29. VASINGTON, F. D., and J. V. MURPHY. 1962. *J. Biol. Chem.* **237**:2670.
30. WINKLER, H. H., F. L. BYGRAVE, and A. L. LEHNINGER. 1968. *J. Biol. Chem.* **243**:20.
31. CHAPPELL, J. B., and A. R. CROFTS. 1966. Regulation of Metabolic Processes in Mitochondria. J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater, editors. Elsevier, New York. **7**:263.

32. PAPA, S., J. M. TAGER, F. GUERRIERI, and E. QUAGLIARIELLO. 1969. *Biochim. Biophys. Acta.* **172**:184.
33. MOORE, C., and B. C. PRESSMAN. 1964. *Biochem. Biophys. Res. Commun.* **15**:562.
34. GRAVEN, S. N., H. A. LARDY, D. JOHNSON, and A. RUTTER. 1966. *Biochemistry.* **5**:1729.
35. LÖW, H., and I. VALLIN. 1963. *Biochim. Biophys. Acta.* **69**:361.
36. SLATER, E. C. 1953. *Nature (London.)* **172**:975.
37. CHANCE, B., and G. R. WILLIAMS. 1956. *Advan. Enzymol.* **17**:65.
38. GREEN, D. E., J. ASAI, R. A. HARRIS, and J. T. PENNISTON. 1968. *Arch. Biochem. Biophys.* **125**: 684.
39. MITCHELL, P. 1966. Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation. Glynn Research Ltd., Bodmin, Cornwall, England. 35.
40. BATEMAN, L. C., M. G. CHURCH, E. D. HUGHES, C. K. INGOLD, and N. A. TAHER. 1940. *J. Chem. Soc. London.* **2**:979.
41. VON HIPPEL, P. H., and T. SCHLEICH. 1969. Structure and Stability of Biological Macromolecules. S. N. Timasheff and G. D. Fasman, editors. Marcel Dekker, Inc., New York. **1**:417.
42. CHANDLER, W. K., A. L. HODGKIN, and H. MEVES. 1965. *J. Physiol. (London).* **180**:121.
43. MITCHELL, P. 1966. *Biol. Rev. (Cambridge).* **41**: 445.
44. CATTERALL, W. A., and P. L. PEDERSEN. 1970. *Biochem. Biophys. Res. Commun.* **38**:400.
45. GREGG, C. T., and LEHNINGER, A. L. 1963. *Biochim. Biophys. Acta.* **78**:27.

Note Added in Proof: Since this manuscript was submitted for publication, Loyter et al. (1969. *J. Biol. Chem.* **244**:4422) and Christiansen et al. (1969. *J. Biol. Chem.* **244**:4428) reported that submitochondrial particles of beef heart mitochondria, prepared either by sonic or digitonin treatment, also catalyze the energy-linked uptake of Ca^{++}. Evidence is also presented that indicates an inverted sidedness of the beef heart particles relative to the normal inner membrane geometry. Unlike the lubrol inner membrane fraction described here, however, the submitochondrial heart particles require either ATP or ADP in addition to a respiratory substrate for maximal Ca^{++} accumulation rates. Thus, there appear to be significant differences in the Ca^{++} uptake requirements of the beef heart and rat liver submitochondrial systems.