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Adenosine Triphosphatase of Rat Liver Mitochondria: Detergent Solubilization of an Oligomycin- and Dicyclohexylcarbodiimide-Sensitive Form of the Enzyme†

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ABSTRACT: The hydrolytic activity of the ATPase bound to purified inner membrane vesicles of rat liver mitochondria can be increased threefold by washing extensively with a high ionic strength phosphate buffer. The specific ATPase activities of such phosphate-washed membranes are the highest reported to date for a mitochondrial membrane preparation (21–24 μmol of ATP hydrolyzed min^{-1} mg^{-1} in bicarbonate buffer at 37 °C). Deoxycholate (0.1 mg/mg of protein) extracts from these membranes a soluble, cold-stable ATPase complex which exhibits a specific activity under optimal assay conditions of 12 μmol of ATP hydrolyzed min^{-1} mg^{-1}. This complex is not sedimented by centrifugation at 201 000*g* for 90 min, and readily passes through a 250-Å Millipore filter. The ATPase activity of the soluble complex is inhibited 95% by 2.4 μM oligomycin. In addition, inhibitions of 60% or better are obtained in the presence of 1–8 μM dicyclohexylcarbodiimide, *p*-chloromercuribenzoate, venturicidin, and aurovertin. While a similar complex may be extracted with Triton X-100, this preparation is always lower in both specific activity and in inhibitor sensitivities than the complex extracted with deoxycholate. Detergents of the Tween and Brij series and other detergents of the Triton series are also much less effective than deoxycholate in solubilizing the oligomycin-sensitive ATPase complex of rat liver. It is concluded that deoxycholate is superior to other detergents as an extractant of the oligomycin-sensitive ATPase complex of rat liver mitochondria, and that the complex extracted with deoxycholate possesses a closer similarity to the membrane-associated ATPase than does the complex extracted with Triton X-100. These studies document the first report of a detergent-solubilized, oligomycin-sensitive ATPase preparation from rat liver mitochondria.

Lardy et al. (1958) first discovered that the antibiotic oligomycin can simultaneously block the terminal step in oxidative phosphorylation (phosphoryl transfer to ADP) and inhibit ATPase activity in submitochondrial particles. Since this time a number of workers have described conditions for extraction of a particulate form of the oligomycin-sensitive ATPase (OS-ATPase)[1] from a variety of sources. The bile salts, deoxycholate (Tzagoloff et al., 1968; Yamamoto, 1970) and cholate (Kopaczyk et al., 1968; Kagawa and Racker, 1966) have been used to carry out the majority of these extractions. Additional methods used to obtain insoluble preparations have included acetone extraction of mitochondria (Vallejos et al., 1968) and treatment of submitochondrial particles with lysolecithin (Sadler et al., 1974).

Tzagoloff and Meagher (1971) have solubilized from yeast mitochondria a dispersed OS-ATPase of high specific activity. The nonionic detergent Triton X-100 (TX-100) was used for this extraction, while a similar preparation has been described also by Ryrie (1975). TX-100 extractions of beef heart mitochondria have been carried out by Linnett et al. (1975) and by Swanljung et al. (1973).

Although the oligomycin-insensitive ATPase (F_1) has been solubilized and purified from rat liver (Catterall and Pedersen, 1971; Lambeth and Lardy, 1971), no reports have yet appeared on the properties of an oligomycin-sensitive preparation from

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[1] Abbreviations used are: OS-ATPase, oligomycin-sensitive adenosine triphosphatase; F_1, oligomycin-insensitive ATPase; DCCD, dicyclohexylcarbodiimide; ClHgBzO, *p*-chloromercuribenzoate; TX-100, Triton X-100; cmc, critical micelle concentration; Cl_3CCOOH, trichloroacetic acid; mitoplast, inner membrane + matrix; H medium, PA buffer and P buffer, see Methods and Results; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

this source. The work described here was undertaken with the objective of defining optimal conditions for extraction of such an ATPase complex, in soluble form, from rat liver. We have sought to obtain a complex which bears the closest possible resemblance to the original membranes in terms of specific activity, kinetic characteristics, and sensitivity to inhibitors.

By focusing our attention on the acquisition of a soluble OS-ATPase complex which retains the above characteristics, as extracted, we may greatly simplify its later purification. Subsequent steps will then be concerned with the task of removing extraneous material, rather than the more difficult job of reconstituting activities lost during extraction.

Materials and Methods

Materials

Pyruvate kinase, lactic dehydrogenase, sodium deoxycholate, mannitol, phosphoenolpyruvate, oligomycin, and *p*-chloromercuribenzoate were all obtained from Sigma. ATP and NADH were purchased from P-L Biochemicals, while Hepes, digitonin, and dithiothreitol were obtained from Calbiochem. Lubrol WX was supplied by ICI and dicyclohexylcarbodiimide by Schwarz/Mann. Aurovertin and venturicidin were generous gifts of Dr. E. Carafoli, University of Zurich, and Dr. J. Mattoon of this department. Triton X-100 was of scintillation grade and was purchased from Research Products International. Other Triton compounds and sucrose were obtained from the J. T. Baker Chemical Company, while the nonionic detergents Tween and Brij were supplied by the Atlas Chemical Company. Bovine serum albumin (fraction V) was purchased from ICN Pharmaceuticals.

Sodium deoxycholate was precipitated with HCl, washed with water, and recrystallized twice from 70% methanol. The acid form was then dissolved at a concentration of 10 mg/ml by addition of Tris base until a pH of 8.0 had been reached. Triton X-100 was made up as a 10% (w/w) stock solution in water. Both detergent solutions were stored frozen to prevent microbial growth.

Methods

Preparation of Mitochondria. Mitochondria were prepared by a triplicate nuclear extraction procedure. Briefly stated, this technique involves the homogenization of liver in a 2.5 mM Hepes-buffered solution (*H medium*), pH 7.4, containing 70 mM sucrose, 220 mM mannitol, and 0.5 mg/ml defatted bovine serum albumin (Schnaitman and Greenawalt, 1968). A 30% (w/v) suspension of liver in the above medium was prepared by use of a Teflon–glass homogenizer and centrifuged in a Sorvall SS-34 rotor at 1100*g* for 3 min. The sediment was then resuspended, with gentle homogenization, to the original volume and recentrifuged as above. This process was repeated once more, saving all supernatant fractions and discarding the nuclear fraction after the third centrifugation. Mitochondria were separated from the pooled supernatants by centrifugation at 6800*g* for 15 min. Finally, the mitochondria were washed four times in H medium and resuspended at a concentration of approximately 100 mg/ml. All steps were carried out at 0–4 °C.

Preparation of Sonicated Mitochondria. Mitochondria prepared by the above techniques were stored at −20 °C for 1 week and then washed with 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5 (Tzagoloff, 1969). The resultant sediment was resuspended to approximately 50 mg/ml in the above buffer and subjected to sonic oscillation at 80% of maximum power from a Bronwill sonicator (Biosonik II) for 3 min in 1-min intervals. Mitochondrial membranes were separated from this suspension at a centrifugal force of 201 000*g* for 60 min in the Spinco Type 65 rotor. The resultant sediment was then washed in an equivalent volume of the above buffer and resuspended in 0.25 M sucrose, 5.4 mM Tris-acetate, pH 7.5 (Tzagoloff and Meagher, 1971).

ATPase Extraction Procedure. P_i-Washed membranes, as prepared in Results section, were thawed and diluted to 1–2 mg/ml with *PA buffer* (300 mM K_2HPO_4, 2 mM ATP, 10% ethylene glycol, 5 mM EDTA, and 0.5 mM dithiothreitol, pH 7.9). Membranes were extracted with detergents for 15 min at 0 °C and then subjected to centrifugation at 201 000*g* for 90 min in the Spinco Type 65 rotor. Supernatant fractions were passed through a 250-Å Millipore filter to remove residual membrane contamination. Samples extracted with deoxycholate were passed through Millipore filters which had been pretreated with bovine serum albumin and deoxycholate to prevent nonspecific adsorption of ATPase to filters. This treatment was not required for samples containing TX-100.

Sonicated mitochondrial membranes in sucrose–Tris-acetate were extracted at the protein and TX-100 concentrations described elsewhere (Tzagoloff and Meagher, 1971). After a 15-min incubation with TX-100, the membranes from sonicated mitochondria were subjected to the same centrifugation and filtration procedures as described above for Triton extracts of P_i-washed membranes.

ATPase Assays. ATPase assays were carried out by coupling the hydrolysis of ATP to the loss of absorbance of NADH at 340 nm in a Gilford spectrophotometer (Model 222) equipped with a Sargent SRL recorder. The assay buffer was composed of 40 mM Tris-HCO_3, 5 mM $MgSO_4$, 5 mM KCN, pH 7.9. Tris-HCO_3 has been reported to increase the activity of membrane-bound ATPase about twofold when compared with buffers in which Cl^- or SO_4^- are counterions to $Tris^+$ (Ebel and Lardy, 1975a; Pedersen, 1976). The maximal velocities of the membrane-bound and soluble OS-ATPase described here were also activated approximately twofold by Tris-HCO_3. Prior to assay, the following components, dissolved in the assay buffer, were added at the indicated final concentrations: ATP (4 mM), NADH (0.3 mM), and phosphoenolpyruvate (0.4 mM). One microliter of lactic dehydrogenase (6.2 IU) and 2 μl of pyruvate kinase (8 IU), used as commercially obtained, were then added per assay. The final volume of the complete assay medium was 1.0 ml. Components sufficient to carry out ten assays were routinely added to 8.2 ml of assay buffer and these solutions were usually used within 30 min. Conversion of small amounts (~5% of total nucleotides) of exogenous ADP to ATP was always allowed to proceed before beginning assays. Assays were begun by addition of a quantity of ATPase activity sufficient to hydrolyze approximately 0.5 nmol of ATP/min. In the case of detergent/supernatants, these volumes were always less than 10 μl. Linear initial rates during the first 3–5 min of assay were used in calculating activity. Unless otherwise noted, all assays were carried out at 37 °C.

Inhibitor Assays. Alcoholic solutions of oligomycin, venturicidin, and dicyclohexylcarbodiimide (DCCD) were diluted in methanol to the desired final concentrations. *p*-Chloromercuribenzoate (ClHgBzO), at a final concentration of 100 μM, was dissolved in 40 mM Tris-HCO_3 at pH 7.9. Aurovertin was dissolved in ethanol at a concentration of 100 μg/ml.

In all inhibitor assays a quantity of ATPase sufficient to hydrolyze approximately 2 nmol of ATP/min was added to 1.25 ml of 40 mM Tris-HCO_3, pH 7.9. In the case of assays

Table I: Purification of Inner Membrane Vesicles with High ATPase Activity.

Fraction	ATPase Spec. Act. (μmol min^{-1} mg^{-1})	Recovery of Act. (%)	Recovery of Protein (%)
Mitochondria[a]	1.3	100	100
Mitoplasts	2.1	69	44
H-Medium membranes[b]	4.6	40	11
P_i-Washed membranes	14.3	68	6

[a] Preparation originally contained 2.1 g of mitochondria. All procedures were carried out exactly as described under Methods and Results. Assays were carried out at room temperature. [b] H-Medium membranes are purified inner membrane vesicles which were prepared identically to P_i-washed membranes, except that they were washed with H medium rather than P buffer. Their preparation terminates with the initial freezing in Step 3 of Results. Specific activity values were calculated from results obtained from triplicate assays.

containing ClHgBzO, this amount of ATPase was always added to the Tris-HCO_3 buffer in a total volume of 25 μl of PA buffer. Exactly 0.25 ml of the ATPase-HCO_3 mixture was added to each of four tubes. The equivalent of 50 μl of the various methanolic inhibitor solutions, or of 100 μl of ClHgBzO stock solution, was then added to the above Tris-HCO_3 buffer to a final equivalent volume of 0.25 ml. Additions of 0.25 ml of these inhibitor solutions were immediately made to the above enzyme solutions, and the enzyme–inhibitor mixes were allowed to preincubate for 20 min. An enzyme-control tube containing only the appropriate quantity of methanol, or of additional buffer, was included in every set of assays. The removal of KCN from these assays was necessary because of its interaction with ClHgBzO. Assay rates in the absence of KCN were never more than 5% in excess of those in its presence.

At the end of the preincubation period, 0.5 ml of a solution containing a twofold concentration of all ATPase assay components in 40 mM Tris-HCO_3, 10 mM $MgSO_4$, 10 mM KCl, pH 7.9, was added to each tube and incubated for 1 min. All procedures to this point were carried out on ice. Tubes were then incubated at 37 °C for 1 min and immediately assayed at 37 °C in multiple cuvettes. In the case of aurovertin, ATPase was added to the assay buffer mix immediately prior to the addition of aurovertin, and assays were then rapidly carried out without any preincubation. In all cases a small absorbance change in the absence of ATPase (approximately 5% of control rates) was subtracted from the initial rates.

Protein Determinations. All determinations of protein for the purpose of calculating protein concentrations during the treatment of mitochondria with digitonin and lubrol were based on the biuret procedure, with bovine serum albumin as a standard. All protein determinations for calculation of specific activity were based on a micro-modification of the Lowry procedure (Lowry et al., 1951). Potentially interfering materials were removed by precipitation of protein prior to Lowry determinations. Membranous samples were precipitated with ice-cold 10% Cl_3CCOOH and then centrifuged at 23 000g for 30 min. Detergent supernatants were first precipitated with ice-cold acetone, followed by dissolution in 50 μl of 1 N NaOH and reprecipitation with 1.5 ml of ice-cold 10% Cl_3CCOOH. All Lowry determinations were standardized against solutions of bovine serum albumin taken through identical precipitation procedures.

Results

Preparation of Inner Membrane Vesicles with a High ATPase Activity. In an effort to achieve the highest possible specific activity for detergent-soluble ATPase preparations, it was desirable to start with membrane preparations of high ATPase activity. Early experiments indicated that inner membrane vesicles prepared by the digitonin–lubrol procedure of Chan et al. (1970) have a consistently higher specific ATPase activity than membrane vesicles prepared by sonic oscillation of mitochondria. Subsequent washing of inner membrane vesicles with P_i yields membrane preparations with ATPase specific activities as high as 24 μmol of ATP hydrolyzed min^{-1} mg^{-1} protein. The exact procedure for preparing these membranes is described below and is summarized in Table I.

Step 1. Freshly isolated mitochondria, prepared as described under Methods, were treated with a solution of digitonin in H medium equivalent to 0.12 mg/mg of mitochondrial protein (Chan et al., 1970). Immediately after addition of digitonin, the protein concentration was adjusted to 50 mg/ml and the suspension was incubated at 0 °C for 20 min. At the end of this time the reaction mixture was quickly diluted fivefold with H medium and centrifuged at 23 600g for 20 min in a Sorvall SS-34 rotor. The *mitoplast* (inner membrane + matrix) fraction was resuspended and washed twice in H medium. A low-speed fraction sedimenting at 3000g was discarded prior to the final wash.

Step 2. Lubrol WX sufficient to give 0.16 mg/mg protein was added to the mitoplast suspension from a 4% stock solution in H_2O and the final protein concentration was immediately adjusted to 35 mg/ml (Chan et al., 1970). After 20 min of incubation on ice, the mitoplast suspension was diluted with an equal volume of *P buffer* (300 mM K_2HPO_4, 50 mM EDTA, pH 7.9). The diluted suspension was then centrifuged at 201 000g for 90 min in a Spinco Type 65 rotor. The resultant sediment, resuspended in 40 ml of P buffer, was centrifuged in the Sorvall SS-34 rotor at 4300g for 10 min. The low-speed sediment was discarded.

Step 3. The above supernatant fraction containing purified *inner membrane vesicles* was centrifuged at 201 000g for 60 min and resuspended in a minimal volume of PA buffer (see Methods). Membranes were then rapidly frozen in a dry ice–acetone bath and stored at −20 °C for approximately 1 week.

Step 4. Membranes were thawed, diluted to approximately 80 ml with P buffer, and re-centrifuged at 4300g for 10 min. The low-speed sediment was discarded, while the supernatant membranes were washed three times in P buffer by centrifuging at 201 000g for 60 min. The resultant membrane sediment was resuspended in PA buffer and material sedimenting after centrifugation for 20 min at 12 300g in the Sorvall SS-24 rotor was discarded. Finally, the membranes remaining in the supernatant were adjusted to a protein concentration of 5 mg/ml and 2-ml aliquots were frozen in dry ice–acetone and stored at −20 °C until use. These membranes are referred to in the remaining part of this manuscript as *P_i-washed membranes*.

ATPase activities of a typical P_i-washed membrane preparation are seen in Table I. These membranes have high ATPase activities, about 14 μmol of ATP hydrolyzed min^{-1}

Table II: Effect of Detergent:Protein Ratio on the Amount of Total and Oligomycin-Sensitive ATPase Activity Extracted from P_i-Washed Membranes.[a]

TX-100:Protein Ratio (mg/mg. of protein)	Final Concn Triton (%)	Amount of Total ATPase Act. Extracted (%)	Inhibition by Oligomycin (%)
1.0	0.1	10	80
1.0	0.2	10	70
1.3	0.2	16	59
2.0	0.2	16	61
2.0	0.3	20	61
2.0	0.4	20	59

[a] P_i-Washed membranes were extracted in PA buffer at protein concentrations of 0.5, 1.5, or 2.0 mg/ml, as described under Methods. ATPase assays were carried out at room temperature as described under Methods. Specific activity of membranes was 20 μmol of ATP hydrolyzed min^{-1} mg^{-1}. Oligomycin sensitivity was determined in the presence of 30 μM oligomycin added without preincubation. The maximum concentration of TX-100 in assays for ATPase activity was 5×10^{-4} %.

Table III: Specific Activities of Fresh and Aged OS-ATPase Preparations.

Fraction[a]	Spec Act. of Fresh Preparation (μmol min^{-1} mg^{-1})	Spec Act. of Preparation Aged 4 Days[b] (μmol min^{-1} mg^{-1})	Retention of Act. (%)
P_i-Washed membranes	21.6	19.8	91.5
Deoxycholate extract	12	9.1	75.6
TX-100 extract	8.5	6.1	72.4

[a] All fractions were prepared exactly as described under Methods and Results. Assays were carried out at 37 °C as described under Methods. [b] Preparations were aged on ice in PA buffer (see Methods). Specific activity values represent averages of duplicate determinations.

mg^{-1} when assayed in bicarbonate buffer at room temperature (Table I) and about 21–24 μmol of ATP hydrolyzed $min^{-1}mg^{-1}$ when assayed in the same buffer at 37 °C (Table III, Legend to Figure 1). These specific activity values are about threefold higher than values of inner membrane preparations prior to washing with P buffer. Such increases are generally higher than can be accounted for solely by the removal of extraneous protein. The possibility that some component of PA buffer, when diluted into the assay medium, might produce an artifactually high ATPase activity was investigated. This possibility was discounted by the addition of two- and fivefold excesses of PA buffer to assays containing membrane-bound or soluble ATPase, respectively. The total concentration range of PA buffer was 50-fold and produced no change in the activities of either type of ATPase.

Extraction of OS-ATPase from P_i-Washed Membranes. Variation of Detergent Type. The best previously reported preparations of a soluble, oligomycin-sensitive ATPase (Tzagoloff and Meagher, 1971) were obtained by using TX-100 as a dispersing agent. In preliminary studies we

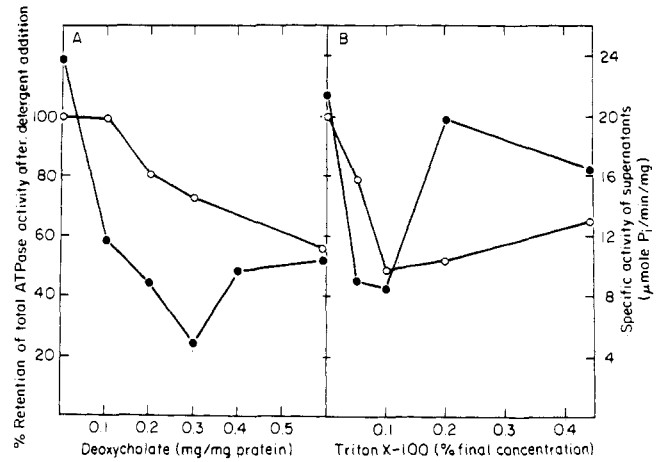

FIGURE 1: Retention of total ATPase activity after detergent addition to P_i-washed membranes and specific activities of solubilized ATPase. P_i-Washed membranes were extracted as described under Methods with deoxycholate (A) or TX-100 (B) at the indicated concentrations. Membrane protein concentrations were 1 mg/ml. Two different membrane preparations with an average specific activity of 24 μmol min^{-1} mg^{-1} were used for extractions with deoxycholate, while membranes used for extractions with TX-100 had a specific activity of 21. Activity retained in detergent–membrane mixes (○) represents the total ATPase activity after addition of detergent and prior to centrifugation. Specific activity in μmol of P_i formed min^{-1} mg^{-1} (●) refers to that of the centrifuged, and filtered supernatants prepared as described under Methods. All assays were carried out at 37 °C.

compared the relative effectiveness of this compound with a number of other nonionic detergents, used either singly, or in combination with each other. The detergents so tested were Triton compounds X-100, X-114, X-165; Brij compounds 35, 58, 78, 98; and Tween compounds 20, 21, 40. In terms of the total amount of enzymatic activity extracted, TX-100 proved to be superior to all other detergents tested. There was no obvious correlation between amount of oligomycin-sensitive ATPase extracted and detergent HLB, "hydrophilic–lipophilic balance", number (Helenius and Simons, 1975). The relative abilities of TX-100 and deoxycholate to extract OS-ATPase are described in detail below.

Variation of Detergent:Protein Ratio. The effect of increasing the ratio of detergent:protein has a complex effect on the amount of ATPase activity extracted and on its sensitivity to oligomycin, as demonstrated in Table II. At all concentrations of detergent tested there is an increase in the apparent amount of ATPase extracted as the detergent:protein ratio varies from 1 to 2. There is, however, a concomitant drop in the sensitivity of the ATPase to oligomycin. Somlo and Krupa (1974) found exactly analogous results with pig heart mitochondria when TX-100 was included in the assay medium. Similarly, Tzagoloff and Meagher (1971) found that a progressive increase of TX-100 resulted in an increased loss of oligomycin sensitivity.

Extractions using TX-100 are compared in Figure 1 with extractions using deoxycholate. In both cases, the OS-ATPase extracted, even at the lowest concentrations of detergent, has a lower specific activity than the original P_i-washed membranes. (About 12 μmol of ATP hydrolyzed min^{-1} mg^{-1} in the case of deoxycholate and about 10 μmol in the case of TX-100.) As the concentrations of deoxycholate and TX-100 used to extract OS-ATPase are progressively increased, the specific activity of the solubilized enzyme decreases and then undergoes an apparent increase. Although the data points on oligomycin-sensitivity are not included in Figure 1, it should be noted that, as in Table II, the sensitivity to oligomycin continues to

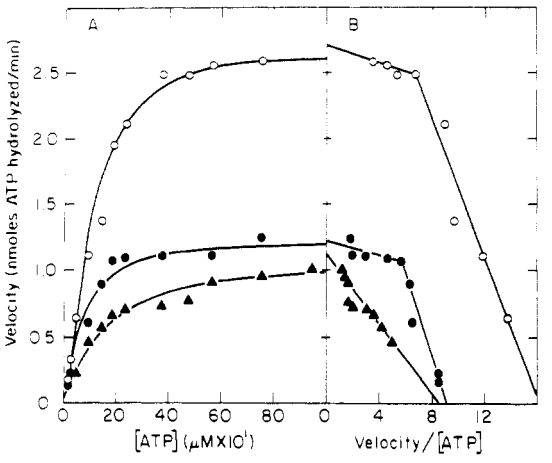

FIGURE 2: Kinetic properties of OS-ATPase bound to P_i-washed membranes and of OS-ATPase extracted with deoxycholate or TX-100. P_i-Washed membranes at a concentration of 1 mg/ml were freshly extracted with either 0.1 mg of deoxycholate/mg of protein or 0.05% TX-100, as described under Methods. Various aliquots of a 9.4 mM ATP solution were added to a solution containing all other ATPase assay components, except Mg^{2+}. The Mg^{2+} necessary for assay was provided in the stock solution of ATP as a 1:1 Mg–ATP complex. Assays were initiated by the addition of 0.26, 0.29, or 0.42 μg of membranes (○), deoxycholate extracts (●), or TX-100 extracts (▲), respectively. Panel A represents a plot of velocity vs. [ATP] while panel B represents an Eadie–Hofstee plot of velocity vs. velocity/[ATP]. Assays were carried out at 37 °C. Each point represents an average of duplicate determinations.

decrease at higher concentrations of detergent. For example, at an oligomycin concentration of 1.25 μM, the percent inhibition by oligomycin of ATPase activity extracted at 0.1 and 0.6 mg deoxycholate/mg of protein was 93 and 65%, respectively. Corresponding inhibition values for ATPase activity extracted at final concentrations of 0.05 and 0.2% TX-100 were 87 and 29%, respectively.

The addition of TX-100 to P_i-washed membrane preparations was found consistently to result in a loss of total ATPase activity. At a final concentration of 0.1% TX-100, Cosson and Spiridakis (1974) report an activity loss of 14% for yeast mitochondria, while for P_i-washed membranes of rat liver mitochondria there is a loss of 52% at the same concentration of TX-100 (Figure 1). Although deoxycholate, when added to P_i-washed membrane at relatively high concentrations, also causes a loss of total ATPase activity this loss is much less abrupt than with TX-100. At the lowest concentrations of detergents tested in these experiments (0.1 mg of deoxycholate/mg of protein or 0.05% TX-100), the recoveries of total ATPase activity were 99 and 79%, respectively. As noted above these concentrations of detergent also extract OS-ATPase with the highest oligomycin sensitivity. For these reasons all subsequent experiments described in this manuscript were carried out on OS-ATPase preparations obtained from P_i-washed membranes by extraction with 0.1 mg of deoxycholate/mg of protein or 0.05% TX-100. Under these conditions approximately 10 and 6% of the membrane-bound ATPase was removed by deoxycholate and TX-100, respectively. Prior to assay, the detergent-soluble extract was centrifuged at 201 000*g* for 90 min and filtered through 250-Å Millipore filters to remove possible membrane contamination (see Methods).

Stability of OS-ATPase When Bound to P_i-Washed Membranes and When Extracted with Deoxycholate or TX-100. A considerable amount of time was spent in these studies designing a buffer system which would stabilize both the ATPase activity of P_i-washed membranes and the ATPase activity of detergent solubilized extracts of these membranes. Low ionic strength buffers and buffers containing sucrose or sucrose–Tris-acetate (Tzagoloff and Meagher, 1971) were ineffective. The buffer cocktail found to be optimal for stabilizing ATPase activity of all preparations used in these studies contained P_i, ATP, ethylene glycol, EDTA, and dithiothreitol (PA buffer; see Methods). When stored in PA buffer at −20 °C P_i-washed membranes (5 mg/ml) retain about 95% of their original ATPase activity after 1 week. The half-life of the ATPase activity of such preparations is about 85 days. When stored on ice, P_i-washed membranes in PA buffer retain about 91% of their original ATPase activity after 4 days, whereas OS-ATPase preparations extracted from these membranes with deoxycholate or TX-100 retain 75 and 72%, respectively of their original ATPase activity in the same time period (Table III). When stored on ice for 6 days in PA buffer OS-ATPase extracted with deoxycholate retained greater than 90% of its inhibitor sensitivities. Since, even in PA buffer, there is some loss of ATPase activity after several days, the kinetic studies described below were carried out on OS-ATPase complexes immediately after extraction.

Kinetic Properties of Membrane-Bound OS-ATPase and of OS-ATPase Solubilized with Deoxycholate or TX-100. Eadie–Hofstee plots of kinetic data obtained for both the ATPase of P_i-washed membranes and the ATPase of deoxycholate extracts therefrom are biphasic (Figure 2B). The apparent K_m values for ATP, as determined from these plots, are 33 and 260 μM for membrane-bound ATPase, with corresponding values of 29 and 313 μM for deoxycholate extracted ATPase. As seen in Figure 2A, the ATPase of TX-100 extracts appears to more nearly approximate simple Michaelis–Menten kinetics with a single K_m of 133 μM. These data suggest that TX-100 may damage an ATP-binding site which is present in the membrane-bound enzyme and that this site is extracted intact with deoxycholate. It is important to emphasize here that comparative extractions with TX-100 and deoxycholate were always carried out at the same time from a common membrane preparation.

Inhibitor Sensitivities of Membrane-Bound OS-ATPase and OS-ATPase Solubilized with Deoxycholate or TX-100. Oligomycin. As indicated in Figure 3, a plot of percent inhibition of ATPase activity extracted with TX-100 vs. the log of oligomycin concentration reveals an exponential, rather than a linear increase in inhibition, as oligomycin concentration is increased. Tzagoloff and Meagher (1971) also found a partially exponential[2] relationship for the OS-ATPase of yeast mitochondria after extraction with TX-100. The activity of the OS-ATPase extracted with deoxycholate shows a more complex oligomycin inhibition pattern (Figure 3). Instead of the simple exponential function noted above, OS-ATPase preparations extracted with deoxycholate exhibit a sigmoidal plot of inhibition vs. the log of oligomycin concentration. Although much less pronounced, a similar plot for the membrane-bound OS-ATPase is also sigmoidal over a 10 000-fold concentration range of oligomycin.

Venturicidin. In contrast to oligomycin, inhibition of the membrane-bound OS-ATPase by venturicidin becomes essentially maximal when the inhibitor concentration is increased over only two orders of magnitude. The inhibitions of the

[2] Figure 2 of this reference indicates that the percent inhibition of purified ATPase undergoes a 40% increase in the fivefold range of 15–75 μg of rutamycin/mg of protein, but undergoes an additional increase of only 30% in the eightfold range from 75 to 600 μg of rutamycin/mg of protein.

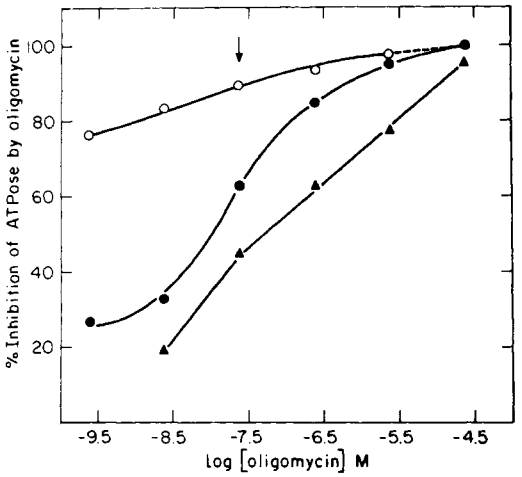

FIGURE 3: Effect of oligomycin concentration on ATPase activity of P_i-washed membranes and on ATPase activity of deoxycholate and TX-100 extracts therefrom. Assays were carried out at varying concentrations of oligomycin exactly as described under Methods. Assays contained 0.26 μg of P_i-washed membranes (○), 0.34 μg of deoxycholate extracts (●), or 0.50 μg of TX-100 extracts (▲). Points for oligomycin sensitivities of deoxycholate extracts represent average values from a total of three different extractions of two different membrane preparations. Other points represent an average from duplicate extractions of a common membrane preparation. Arrow represents concentration chosen for inhibition values summarized in Table IV.

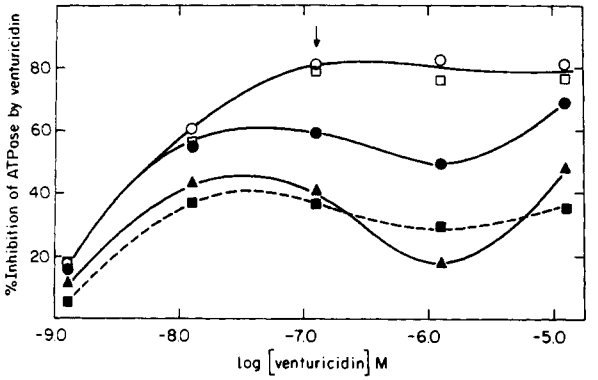

FIGURE 4: Effect of venturicidin concentration on ATPase activity of membrane fractions and on ATPase activity of deoxycholate and TX-100 extracts therefrom. Assay conditions and symbols identifying various fractions are as in Figure 4. Quantities of protein added were 0.26, 0.23, and 0.33 μg for P_i-washed membranes, deoxycholate, and TX-100 extracts of these membranes, respectively. Each of these points represents duplicate determinations of two separate extractions of a common membrane preparation. In addition, assays were carried out in duplicate containing 2.0 μg of either sonicated membranes (□) or 1.6 μg of Triton extracts of these membranes (■), prepared as described under Methods.

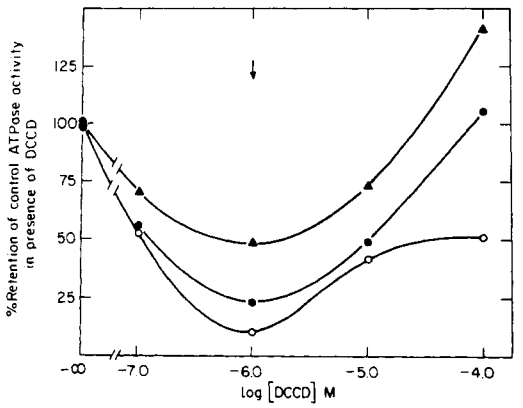

FIGURE 5: Effect of DCCD concentration on ATPase activity of P_i-washed membranes and on ATPase activity of deoxycholate and TX-100 extracts therefrom. Symbol notations and assay procedures are as in Figure 3. Averages of duplicate determinations are expressed in percent of control activity retained, rather than percent inhibition. Data must be expressed in this way because at certain concentrations of DCCD the ATPase activity of TX-100 extracts is actually activated above control membrane values. Amounts of protein per assay are 0.36, 0.35, and 0.57 μg for membranes, deoxycholate, and TX-100 extracts, respectively.

ATPase activity of both P_i-washed membranes and membranes from sonicated mitochondria are essentially identical, as seen in Figure 4. A maximal inhibition of approximately 80% is reached at a venturicidin concentration of 0.13 μM. This concentration is tenfold lower than that found by Walter et al. (1967) to bring about the same degree of maximal inhibition for valinomycin-induced ATPase activity of rat liver mitochondria.

At very low venturicidin concentrations the inhibition patterns of detergent solubilized OS-ATPase preparations are similar to those of membrane fractions. At approximately 0.03 μM ($-\log = 7.49$), however, the degree of inhibition of detergent solubilized preparations actually begins to decrease. Above 1.3 μM ($-\log = 5.9$), the percent inhibition once again increases, with the percent inhibition of the ATPase activity of deoxycholate extracts nearly reaching the inhibition level of membrane fractions. The venturicidin inhibition patterns of the OS-ATPase extracted from P_i-washed membranes and from sonicated mitochondria with TX-100 are nearly identical with each other and are qualitatively similar to the inhibition pattern observed with the deoxycholate extracted OS-ATPase.

Dicyclohexylcarbodiimide. Maximal inhibition by DCCD of membrane-bound ATPase (81%) and of detergent solubilized OS-ATPase (73 and 50% for deoxycholate and TX-100 extracts, respectively) occurs at a concentration of 1 μM as seen in Figure 5. In comparison, Sone et al. (1975) have achieved a 75% inhibition of an OS-ATPase preparation from a thermophilic bacterium at a concentration of 30 μM DCCD. After a 4-h preincubation at approximately the same concentration of DCCD, Stekhoven et al. (1972) achieved a maximal inhibition of 96% for the particulate OS-ATPase of Tzagoloff et al. (1968).

As the DCCD concentration in Figure 5 is increased above 1 μM, the degree of inhibition becomes increasingly less for all fractions. The OS-ATPase extracted with deoxycholate is not inhibited by 100 μM DCCD, while the OS-ATPase extracted with TX-100 is activated 1.4-fold above control rates. Landry and Goffeau (1973) have reported very similar effects of DCCD on the OS-ATPase extracted from submitochondrial particles of yeast with TX-100.

Other Inhibitors. A composite picture of the results obtained with oligomycin, venturicidin, and DCCD, together with those obtained with ClHgBzO and aurovertin, are summarized in Table IV. It will be noted that ClHgBzO is a very potent inhibitor of OS-ATPase preparations solubilized with detergents. At 8 μM ClHgBzO, the ATPase activities of OS-ATPase preparations extracted with deoxycholate and TX-100 are inhibited by 94 and 80%, respectively. Aurovertin is known to act at the level of the F_1 portion of the OS-ATPase complex and to inhibit the ATPase activity of rat liver F_1 preparations by 65 (Catterall and Pedersen, 1974) and 80% (Ebel and Lardy, 1975b). At aurovertin concentrations identical with those used by these investigators, the deoxycholate-extracted OS-ATPase reported here is inhibited by 71%, while that extracted with TX-100 is inhibited by 66%.

Finally, results presented in Table IV suggest that two general types of statements can be made about the relative

Table IV: Comparison of Inhibitor Sensitivities of OS-ATPase Bound to P_i-Washed Membranes and of OS-ATPase Solubilized with Deoxycholate and TX-100.[a]

Inhibitor	Concn (μM)	Inhibition of ATPase Act. (%)		
		P_i-Washed Membranes	Deoxycholate Extracts	TX-100 Extracts
Oligomycin	0.025	88 (3)[b]	61 (6)	48 (3)
Venturicidin	0.13	80 (4)	57 (6)	43 (4)
DCCD	1.0	81 (3)	73 (5)	50 (3)
ClHgBzO	8.0	66 (3)	94 (5)	80 (4)
Aurovertin	2.0	75 (1)	71 (1)	66 (1)

[a] P_i-Washed membranes and detergent extracts therefrom were prepared exactly as described under Methods and Results. Assays were carried out exactly as described under Methods, and in Figure 4. [b] The number in parentheses indicates the number of separate extractions examined for each inhibitor study.

sensitivities of membrane-bound and detergent-solubilized OS-ATPase preparations. First, it would appear that in the case of inhibition by oligomycin, venturicidin, and DCCD, detergent-solubilized preparations are somewhat less sensitive than membrane-bound preparations, whereas in the case of inhibition by ClHgBzO and aurovertin detergent-solubilized preparations are at least as sensitive as membrane-bound preparations. Secondly, in the case of all inhibitors tested, the OS-ATPase solubilized with deoxycholate is more sensitive than the OS-ATPase solubilized with TX-100.

Discussion

The primary objective of this study was to solubilize the OS-ATPase complex of rat liver mitochondria in intact form. With respect to this objective, we have shown that an ATPase complex can be extracted from P_i-washed inner membrane vesicles, and that the resultant complex catalyzes the hydrolysis of ATP at a high rate and retains the inhibitor sensitivities and kinetic properties of the membrane-bound enzyme. The best preparations of OS-ATPase previously reported (see references in Table V; Hatefi et al., 1974) have all involved the use of either TX-100 or bile salts and are in agreement with our findings that the two best choices of detergents for solubilizing the rat liver enzyme are deoxycholate and TX-100.

Perhaps the most important criterion of intactness of an OS-ATPase preparation is its degree of sensitivity to oligomycin. For this reason, we have compared the oligomycin sensitivity of the OS-ATPase solubilized from rat liver with the sensitivities of other OS-ATPase preparations (Table V). It will be noted that the OS-ATPase complex solubilized from rat liver mitochondria is inhibited to the highest extent (89%) by the lowest oligomycin concentration (0.56 μM). Moreover, the OS-ATPase of rat liver is sensitive to oligomycin as extracted. Addition of phospholipids or removal of detergent is not necessary for inhibition to occur.

Quantities of oligomycin used in experiments summarized in Table V are expressed in absolute inhibitor concentrations (μM) to emphasize the possibility that a reversible enzyme–inhibitor (ATPase–oligomycin) complex is formed. This is in agreement with the findings that [^{14}C]rutamycin is removed from the OS-ATPase of bovine heart mitochondria by washing with phospholipids (Kagawa and Racker, 1966), and that the oligomycin sensitivity of the yeast enzyme is a function only of oligomycin concentration; i.e., varying the protein concentration does not affect the degree of sensitivity (Somlo et al., 1974). The use of absolute inhibitor concentrations is further justified by the fact that five of the best six preparations of mammalian ATPase listed in Table V, despite their different degrees of purity, all undergo maximal inhibition in the narrow concentration range of 0.5–5.0 μM oligomycin.

Inhibitor studies summarized in this report (Table IV, Figures 3–5) emphasize the need of various investigators studying OS-ATPase preparations to exercise caution in their choice of inhibitor concentrations. Inhibition of ATPase activity by various inhibitors does not follow a simple linear *percent inhibition* vs. *inhibitor concentration* response pattern. Such patterns are often quite complex, as may be seen in Figure 3, and especially in Figures 4 and 5. When the inhibition of OS-ATPase by venturicidin is examined in Figure 4, it may be seen that the OS-ATPase extracted from both sonicated membranes and from P_i-washed membranes undergoes a constantly *decreasing* level of inhibition as the venturicidin concentration is increased from 0.03 to 1.3 μM. These observations are not inconsistent with the finding of Linnett et al. (1975)[3] that a final venturicidin concentration of 0.52 μM brings about an apparent increase in the ATPase activity of the OS-ATPase extracted by TX-100 from sonicated membranes of bovine heart mitochondria.

As the concentration of DCCD is increased above 1 μM (Figure 5), the inhibition of OS-ATPase activity extracted with TX-100 is decreased in a manner similar to that observed for venturicidin. This finding is in agreement with the previous observations of Landry and Goffeau (1973). While the venturicidin patterns are different from those observed with DCCD, with both inhibitors the inhibition patterns of the OS-ATPase extracted with TX-100 parallel those of the OS-ATPase extracted with deoxycholate. These findings suggest that the apparent "activating" effects observed at certain inhibitor concentrations may reflect a direct interaction of the inhibitor with the soluble ATPase, rather than an artifact involving interaction of the enzyme with a detergent–inhibitor complex.

As an extractant of OS-ATPase activity, TX-100 was found in these studies to be superior to all other nonionic detergents tested. It was, however, significantly less effective than deoxycholate. OS-ATPase preparations extracted with deoxycholate have a consistently higher ATPase specific activity than those extracted with TX-100 (Figure 1, Table III), and more closely resemble the membrane-bound enzyme in kinetic properties (Figure 2), and in inhibitor sensitivities (Table IV, Figures 3–5). As demonstrated in Table III, the percent retention of ATPase activity as a function of time in PA buffer is essentially identical for both deoxycholate and TX-100 extracts. Therefore, the lowered ability of TX-100 to extract the desired complex is probably not linked to a specific inactivation of OS-ATPase by TX-100.

The different properties of OS-ATPase preparations extracted with TX-100 and deoxycholate may be related to differences in the micellar characteristics of the two detergents. Robinson et al. (1974) find that, at the critical micelle concentration of TX-100 (0.15–0.2 mg/ml (Clarke, 1975)), the hydrophobic portion of membrane proteins can act as a nucleus for the formation of a TX-100 micelle which is at least 100 Å in diameter (Helenius and Simons, 1975). Since the original concentration of TX-100 used to extract the ATPase (Figure

[3] Figure 2 of this reference.

Table V: Comparison of Oligomycin Sensitivities of Various OS-ATPase Preparations.

Source	Inhibition (%)	Oligomycin Concn (μM)	Extractant	Soluble[c]	Lipid Requirement[d]	Reference
Rat liver	89	0.56[a]	Deoxycholate	+	−	*e*
Beef heart	89	5.0[b]	Cholate	−	+	Kagawa and Racker (1966)
Beef heart	87	1.2[b]	Cholate	−	+	Kopaczyk et al. (1968)
Beef heart	87	31.0	Deoxycholate	−	+	Yamamoto (1970)
Beef heart	82	0.47	TX-100	+	−	Linnett et al. (1975)
Rat liver	82	4.0	TX-100	+	−	*e*
Yeast	80[f]	25.0[b]	TX-100	+	−	Tzagoloff and Meagher (1971)
Beef heart	59	0.62	TX-100	+	+	Swanljung et al. (1973)
Beef heart	33	3.1	Acetone	−	−	Vallejos et al. (1968)

[a] Concentration for 95% inhibition is 2.4 μM. [b] Rutamycin, a homologue of oligomycin (Lardy et al., 1975), was used instead of oligomycin. [c] (+) denotes soluble preparation. [d] (+) denotes requirement for *added* phospholipid to achieve indicated inhibition. [e] This manuscript (see Figure 4). [f] This calculation is based upon the assumption that 44 μg of protein was used in the assay. This value is derived from the fact that maximal inhibition of ATPase activity in Figure 1 of the Tzagoloff-Meagher (1971) reference is 80% (at 20 μg of rutamycin/ml) and that this is equivalent to 450 μg of rutamycin/mg of protein in Figure 2 of this reference.

2) was 0.5 mg/ml, it is extremely likely that micellar encapsulation will take place. Deoxycholate, on the other hand, has a cmc of approximately 2.3 mg/ml (Philippot and Authier, 1973), while the concentration used to extract ATPase was only 0.1 mg/ml. Therefore, there is very little chance that deoxycholate micelle formation will occur.

Despite the superiority of deoxycholate over TX-100 as an extractant of the OS-ATPase complex of rat liver mitochondria, the specific activity of the complex extracted with either detergent is less than that of the starting membranes (Figure 1). An equivalent loss of specific activity has been reported by Linnett et al. (1975) for the bovine heart enzyme. The specific activities of the OS-ATPase preparation of yeast described by Ryrie (1975) and the OS-ATPase preparation of bovine heart described by Swanljung et al. (1973) were increased by less than 2 μmol min^{-1} mg^{-1} over those of the submitochondrial particles used for extraction. Of the dispersed OS-ATPase preparations listed in Table V, only Tzagoloff and Meagher (1971) report a substantial increase in specific activity of an OS-ATPase preparation after solubilization. We do not have a satisfactory explanation for this observation. One possibility may be that the hydrophobic portion (F_0) of the membrane-bound enzyme is surrounded (in the membrane) by a special complement of phospholipid molecules, and that a proper F_0-phospholipid structure is required for maximal activity of the enzyme. Although we have not examined in detail the effect of phospholipids on the detergent-solubilized OS-ATPase preparations reported here, we have found in preliminary experiments that addition of phospholipids (mitochondrial mixture) to the TX-100-solubilized complex results in a 1.8-fold activation of ATPase activity.

A final aspect of the studies reported here that merits some discussion is the finding that the hydrolytic activity of the OS-ATPase bound to purified inner membrane vesicles of rat liver mitochondria can be increased threefold by extensive washing with a high ionic strength P_i buffer (Table I). The reason for the exceptionally high specific activity of P_i-washed membranes (~21 μmol of ATP hydrolyzed min^{-1} mg^{-1}, Figure 1, Table III) is not known. However, it is tempting to suggest that high ionic strength may dissociate from the rat liver complex a peptide inhibitor similar to that described by Pullman and Monroy (1963) for the bovine heart ATPase. In this regard we have reported recently that a peptide inhibitor can be isolated from rat liver mitochondria (Pedersen et al., 1974) by a procedure similar to that described by Horstman and Racker (1970) for bovine heart mitochondria.

At this time, however, we cannot rule out the possibility that high P_i concentrations may remove a nucleotide or some other effector that normally stabilizes a form of the OS-ATPase with low hydrolytic activity. Interestingly, Mitchell and Moyle (1970) have reported that the ATPase activity of submitochondrial particles of bovine heart can be activated as much as 6.7-fold by incubation with P_i.

Regardless of the manner in which P_i-washing enhances the ATPase activity of inner membrane vesicles of rat liver mitochondria, the studies reported here show that extraction of these membranes with deoxycholate yields a soluble ATPase complex which is equivalent or superior in inhibitor sensitivity to previously reported preparations, when tested with oligomycin, venturicidin, DCCD, aurovertin, or ClHgBzO. Efforts currently under way in this laboratory are directed at purifying to homogeneity this deoxycholate-solubilized OS-ATPase.

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Some Effects of Ionophore A23187 on Energy Utilization and the Distribution of Cations and Anions in Mitochondria†

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ABSTRACT: The effects of ionophore A23187 on the movements of Ca^{2+}, Mg^{2+}, H^+, phosphate, and succinate and its effects on energy utilization by mitochondria have been studied as a function of ionophore concentrations. At a low ratio of the compound to mitochondrial protein, below that required for maximal uncoupling, an apparent steady state is established between intra- and extramitochondrial Ca^{2+} and H^+ which is maintained until anaerobiosis or inhibition of the mitochondrial Ca^{2+} pump. Mg^{2+} is continuously lost from the mitochondria during the steady state. At higher levels of the compound, both Ca^{2+} and Mg^{2+} are released very rapidly. In the medium used, the rate of respiration produced by cyclic Ca^{2+} uptake and release is a function of the ionophore level between 0 and 0.2 nmol per mg of protein and of the extramitochondrial Ca^{2+} concentration between 1 and 15 μM. The depletion of mitochondrial Ca^{2+} by A23187 appears to result in the complete reversal of the total ion movements occurring during energy-dependent Ca^{2+} accumulation by mitochondria. The ratio of H^+ uptake to Ca^{2+} released is nearly 1. During ionophore-induced Mg^{2+} depletion, no net H^+ uptake is observed, but the mitochondria are depleted of equal molar amounts of phosphate plus succinate. It is proposed that the initial H^+ uptake produced by the ionophore is reversed through the combined actions of the mitochondrial phosphate–hydroxide and phosphate–dicarboxylate exchangers. The data are discussed in terms of factors affecting the "turnover number" of A23187, the mechanism of Ca^{2+} uptake by mitochondria, and some considerations affecting interpretation of data obtained by use of A23187 in cellular systems.

The divalent cation selective ionophorous activity of antibiotic A23187 was discovered during studies of its effects on isolated mitochondria (Reed and Lardy, 1972a). Since that time, it has been used in a number of additional studies with mitochondria, both in studies on the properties of the ionophore (Reed and Lardy, 1972b; Wong et al., 1973; Case et al., 1974; Reed et al., 1975; Pfeiffer and Lardy, 1976) and to determine effects of altered divalent cation distribution on functional parameters, including the mechanism of divalent cation accumulation by these organelles (Rottenberg and Scarpa, 1974; Wohlrad, 1974; Sordahl, 1974; Schuster and Olson, 1974;

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