Kinetics of ATP-Dependent Mg^{2+} Flux in Mitochondria†

Ernest Kun‡

ABSTRACT: ATP-dependent Mg^{2+} accumulation in isolated mitochondria occurs predominantly in the matrix and inner membrane compartments. In mitochondria contaminated with lysosomes, the time course and magnitude of ATP-dependent Mg^{2+} accumulation are influenced by various cytoplasmic substances, besides substrates of the citric acid cycle. Removal of lysosomes by treatment of the mitochondrial preparation with low concentrations of digitonin, which does not damage the mitoplast, eliminates the modifying influence of cytoplasmic components on Mg^{2+} flux. In lysosome-free mitochondria, the kinetics of Mg^{2+} flux is dependent only on the concentration of ATP, of Mg^{2+}, and on the availability of site specific reducing substrates of the electron transport system. Oligomycin at concentrations sufficient to inhibit phosphorylation coupled electron transport and ATP synthesis does not modify Mg^{2+} flux, which is dependent on added ATP. Site specific inhibitors of the electron transport system inhibit the augmenting effect of oxidizable substrates on Mg^{2+} uptake, even when electron transfer is inhibited by oligomycin. Atractyloside, by inhibiting the action of externally added ATP, diminishes Mg^{2+} flux. Ruthenium red is a powerful inhibitor of ATP dependent Mg^{2+} flux. Uncouplers not only inhibit Mg^{2+} uptake, but induce Mg^{2+} efflux. From the time course of Mg^{2+} flux, a first-order rate constant of egress of Mg^{2+} and other kinetic constants were calculated and a kinetic model was derived which describes the bi-directional movement of Mg^{2+} in mitoplasts.

Magnesium, besides being an essential component of numerous enzymatic reactions, plays a critical role in cellular metabolism by controlling intracellular concentrations of adenine nucleotides (Rose, 1968), thus regulating energy charge (Atkinson, 1968). More recently the regulatory function of intracellular Mg^{2+} on macromolecular biosyntheses in fibroblast cultures has been emphasized (Rubin, 1975). It follows that cellular and subcellular transports of Mg^{2+} are significant cellular processes; yet none of these translocation mechanisms is presently understood. It was observed by Brierley et al. (1963), Carafoli et al. (1964), Lehninger (1964), Judah et al. (1965), and Johnson and Pressman (1969) that isolated respiring mitochondria can accumulate externally added Mg^{2+}. Schuster and Olson (1973) observed the association of Mg^{2+} with frozen (−80 °C) heart mitochondria. As already pointed out by Johnson and Pressman (1969), it is unknown what portion of added Mg^{2+} is adsorbed to isolated mitochondria and how much actually penetrates the inner membrane. This question is far from being trivial since it is known that a part of cellular Mg^{2+} tends to be associated with cellular membranes (Sanui, 1970). For this reason, analysis of mitochondrial Mg^{2+} content alone, without the knowledge of its submitochondrial distribution, is an inadequate test for the uptake of Mg^{2+} in these particles.

Direct analyses of Mg^{2+} content of isolated liver mitochondria by Thiers and Vallee (1957), Thiers et al. (1960), Pfaff et al. (1968), Kun et al. (1969), Bogucka and Wojtczak (1971), and Reed and Lardy (1973) indicated that there is about 10–20 times higher concentration of Mg^{2+} in mitochondria (25–65 mM on the basis of total protein) than in the cytosol; therefore it is reasonable to assume that some form of active transfer of Mg^{2+} into mitochondria takes place in the cell. The submitochondrial distribution of Mg^{2+} is not uniform and, depending on the techniques of fractionation, 41% (Bogucka and Wojtczak, 1971) or 50–60% (Kun, 1972) of mitochondrial Mg^{2+} can be found in the mitoplast compartment, which consists of the matrix surrounded by the inner membrane. A small fraction (less than 0.1%) of mitochondrial Mg^{2+} is tightly associated with the inner membrane and, among other unknown functions, appears to regulate the $NADP^+$ specificity of mitochondrial glutamate (EC 1.4.1.3) and isocitrate (EC 1.1.1.42) dehydrogenases (Lin and Kun, 1973a,b).

A study of Mg^{2+} translocation in isolated mitochondria is complicated by the fact that particles prepared by conventional techniques invariably contain lysosomes (Baudhuin et al., 1969). Release of highly active degradative lysosomal enzymes was shown to damage mitochondrial membranes (Mellors et al., 1967; Scholte, 1969; Kadenbach, 1969; Donaldson et al., 1970) and can produce a large array of artefacts in vitro. The seriousness of this problem was recently demonstrated by Wieland (1975), who found that, in mitochondria isolated by conventional differential centrifugation techniques, the pyruvate dehydrogenase activity decayed within 3–6 min unless contaminating lysosomes were removed. It is evident that the degree of interference by variable contamination of lysosomes with in vitro studies of mitochondrial functions depends on the time constants and stabilities of particular mitochondrial reactions and rapid processes are less likely to be obscured by artifacts caused by mitochondrial decay. The relatively slow event of Mg^{2+} flux through the inner mitochondrial membrane is especially vulnerable to contamination by lysosomal enzymes. As shown in this paper, decay of mitochondria and partial prevention of this process by various substances can indirectly modify Mg^{2+} flux.

The present paper is concerned with the kinetics of bi-directional Mg^{2+} flux in isolated mitochondria. Kinetics was studied with lysosome-free mitochondria which retained their functionally intact mitoplast compartment, as estimated by

† From the Cardiovascular Research Institute and the Departments of Pharmacology and Biochemistry and Biophysics, School of Medicine, University of California, San Francisco, San Francisco, California 94143. *Received July 23, 1975.* This work was supported by grants from the United States Public Health Service (HL-6285, GM-20552) and a grant from the Dean of the School of Medicine (Clough Fund) at the University of California, San Francisco, California.

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several criteria. It will be shown that Mg^{2+} uptake is an ATP-dependent process, but is independent from oxidative phosphorylation coupled electron transfer. A kinetic model was derived which permits the calculation of the first-order rate constant of egress and other constants from the analysis of net flow of Mg^{2+} over a period of 40–60 min. Kinetics formally complies with an active uptake and passive exit (cf. Stein, 1967) or a bi-directional carrier mechanism. A similar, but not identical, kinetic model was previously proposed for the translocation of glycine into Erhlich ascites tumor cells by Heinz (1954).

Experimental Section

Experimental animals used were male Sprague Dawley rats which were deprived of food for 16 h prior to the experiment. The experiments reported in this paper were performed with liver mitochondria. Similar results were found with heart and kidney mitochondria.

Mitochondrial Preparations. Two types of mitochondrial preparations were used. In experiments concerned with intramitochondrial distribution of Mg^{2+}, the procedure of Schnaitman and Greenawalt (1968) was followed, using mannitol (0.225 M), sucrose (0.07 M), Hepes-Tris[1] (0.002 M, pH 7.15), and 0.5 mg of bovine serum albumin (crystalline, A grade, Calbiochem) per ml as a homogenizing medium. These mitochondria contained the usual variable contamination by lysosomes (Baudhuin et al., 1969). The second type of mitochondria were prepared in bovine serum albumin free mannitol–sucrose–Hepes (cf. Schnaitman and Greenawalt, 1968). Omission of bovine serum albumin was of importance since it was found that lysosomal enzymes, liberated by their disruption, tend to associate with mitochondrial particles in the presence of bovine serum albumin. Preparation of lysosome-free mitochondria from homogenates was carried out as follows. After removal of the nuclear fraction (at 2500 rpm for 15 min) and separation of mitochondria (at 7500 rpm in a Sorvall RC-2B refrigerated centrifuge at 4 °C for 15 min), the mitochondrial pellet, prepared from three rat livers (body weight 180–200 g), was resuspended in 15–25 ml of mannitol–sucrose–Hepes medium and placed in an ice bath. Following the method of Lowenstein et al. (1970), digitonin (A grade, $[\alpha]_D$ −54.6°, Calbiochem), dissolved in 50% ethanol and the suspending medium, was added with stirring in a volume of 0.5 ml to a total volume of 25–40 ml. The amount of digitonin was 0.25 mg/1 g of liver (wet weight). Since the yield of mitochondria was reasonably constant (i.e., 9–11 mg of biuret protein per g of liver weight), this method of dosage proved to be highly reproducible. The mitochondrial suspension was kept at 0 °C for 15 min with frequent stirring and then diluted with 10 volumes of the suspending medium (at 0 °C) and mitochondria were sedimented at 7500 rpm for 15 min (at 4 °C). Incubation with the given concentration of digitonin disrupts lysosomes and solubilizes lysosomal enzymes (cf. Lowenstein et al., 1970), which are subsequently removed by two successive high speed (7500 rpm) centrifugal washings for 10 min. Large granular contaminating fragments are further removed by one low-speed spin (2000 rpm for 10 min). Mitochondria are then re-sedimented at (7500 rpm for 10 min) and taken up in an appropriate volume of mannitol–sucrose–Hepes medium. The entire procedure starting with three rat livers (wet weight 26–29 g) lasts 2.5 h. Mitochondria prepared in this manner have lost about 30–40% of adsorbed or loosely bound Mg^{2+} present in the outer membrane and in the intermembrane compartment. The Mg^{2+} content of lysosome-free mitochondria varies between 25 and 35 mM, which is the Mg^{2+} present in the mitoplast.

Since the method of removal of lysosomes from mitochondria requires digitonin which is known to disrupt the outer mitochondrial membrane at certain concentrations (Pederson and Schnaitman, 1969), the functional intactness of the mitoplast was specifically investigated. It should be noted that the concentration of digitonin (on a protein basis) used in the present method is 15–20 times smaller than employed in the technique of submitochondrial fractionation (Pederson and Schnaitman, 1969). Electron microscopic examination of mitochondria exposed to the treatment with digitonin, as described here, showed some irregular discontinuities of the outer membrane and an enlargement of the intermembrane space, but the structure of the inner membrane and matrix was indistinguishable from equivalent structures of mitochondria, isolated without digitonin. Lysosome-free mitochondria exhibited normal phosphate induced swelling and contraction cycles by ATP (Lin et al., 1973). Acceptor control index of 6–10 with glutamate as substrate and P/O ratios of 2.7–2.9 were identical in digitonin-treated and untreated liver mitochondria. Lysosome-free mitochondria contained 160–180 mM K^+, the same value found in untreated mitochondria; therefore no apparent leakiness of the mitoplast was detectable for K^+. There is, however, a significant difference in the magnitude and stability of the ATP synthetase activity of conventionally prepared and digitonin-treated mitochondria. The rate of ^{32}P incorporation into ADP in the presence of glutamate or citrate was 300–400 nmol per min per mg of mitochondrial protein at 30 °C with lysosome-free mitochondria (Kun, 1976) and this was maintained at a nearly linear rate over a period of 50–80 min. In contrast to digitonin-treated mitochondria, we found that particles prepared according to Schnaitman and Greenawalt (1968) had an ATP synthetase activity of 100–110 nmol per min per mg of mitochondrial protein at 30 °C and the $t_{1/2}$ of ATP synthetase varied between 6 and 12 min (Lin and Kun, 1973a,b). In addition, lysosome-free mitochondria accumulated externally added citrate by an ATP-dependent process, just as mitochondria which have not been exposed to digitonin (unpublished results). On the basis of these experimental results, it is highly probable that digitonin treatment, while disrupting the outer membrane and rendering the intermembrane compartment freely accessible to the cytosol, does not alter the permeability and energy coupling properties of the mitoplast. Electron microscopic examination of lysosome-free mitochondria, after an accumulation of 250 mM Mg^{2+} (see Figure 1), showed a two- to threefold enlargement of the mitoplast and an increased density of cristae but no visible disruption of the inner membrane.

Cytoplasmic extracts were prepared from rat livers according to Lee et al. (1971). The in vitro technique of Mg^{2+} uptake by mitochondria was a micromodification of the sieve method as described by Brawand and Walter (1974). The refrigerated filter funnel was made of stainless steel with an outside width of 3 in. (diameter) and an inside diameter of 1.85 in. The height of the funnel was 2 in. with a depth of 1 and $\frac{3}{8}$ in. of the filter cavity. Precise fitting of Type DA Millipore filters (pore size 0.65 μm) was accomplished by a stainless steel filter seal held in place by a vertical ring fitted into the cavity

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Calbiochem); Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (Calbiochem); FCCP, *p*-trifluoromethoxyphenylhydrazone of carbonyl cyanide; Tris, tris(hydroxymethyl)aminomethane (Calbiochem); EDTA, ethylenediaminetetraacetic acid.

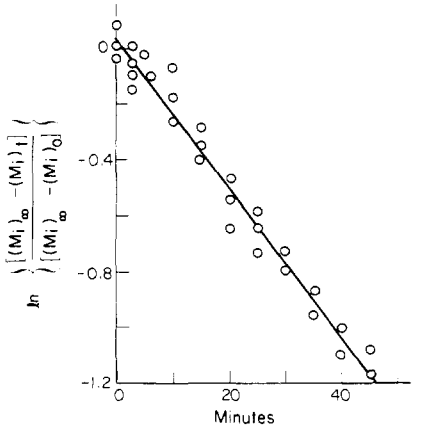

FIGURE 1: Experimental determination of k_i (see eq 5a). Experimental conditions are the same as described in Table II, experiments 9–13. In Figure 1, ln instead of log is plotted against time in order to obtain k_i directly.

and tightened by stainless steel wedges. The filter was supported by a tight-fitting metal screen. This device was constructed by Mr. Bernard Dignam (Research and Development, University of California, San Francisco). The temperature of the filter funnel was kept at 2–4 °C with circulating cooling liquid.

Kinetics of Mg^{2+} uptake was measured by incubating mitochondria in appropriate media (see Results) in 50-ml Erlenmeyer flasks at 37 °C in a shaking water bath. The volume of the mitochondrial solution varied (from 6 to 10 ml) according to the anticipated number of samples. The concentration of mitochondrial suspensions was adjusted so that 0.5-ml aliquot corresponded to 1 mg of mitochondrial protein (biuret). When the effects of inhibitors were studied, these inhibitors were added first to mitochondrial suspensions at 0 °C prior to incubation with substrates and ATP. At zero time and at given time intervals, 0.5-ml aliquots were withdrawn with an Eppendorf pipet and instantly delivered into pre-chilled (0 °C) tubes containing 3 ml of 0.15 M NaCl and 6.5 mM Tris-HCl (pH 7.4), and rotenone or antimycin (0.2 μM) or both. This inhibitor-stop method gave highly reproducible results (±10%) suitable for kinetic analyses. The mitochondrial suspension was rapidly (within 10 s) filtered through the Millipore filter (suction was maintained by a vacuum pump) and washed with 10 ml of buffered solution of cold (0 °C) 0.15 M NaCl containing respiratory inhibitors. The filters, which retained the plated mitochondria, were dried, and then dissolved in 0.25 ml of concentrated HCl at 90 °C during a period of 45 min. Analyses for Mg^{2+} were performed on these solutions after appropriate dilutions (10–20 fold) in a Perkin-Elmer 403 atomic absorption spectrophotometer. An average of 100 readings was taken for each experimental value. In experiments dealing with the submitochondrial distribution of Mg^{2+} (Table I), the mitochondria were sedimented by centrifugation (at 8000 rpm at 4 °C for 10 min) and washed with ice-cold sucrose–mannitol–Hepes-Tris. After resuspension, submitochondrial fractionation was carried out by the method of Pederson and Schnaitman (1969) as reproduced in our laboratory (Skilleter and Kun, 1972). Metabolites and adenine nucleotides were analyzed by enzymatic methods used previously (Kun et al., 1969).

Results

The submitochondrial localization of Mg^{2+} was first investigated with mitochondria prepared by conventional techniques. These mitochondria were incubated with externally added Mg^{2+}, ATP alone, or with added cytoplasmic constituents in the presence of 3 μg of oligomycin per mg of mitochondrial protein. This quantity of oligomycin completely inhibited oxidative phosphorylation and mitochondrial O_2 uptake was reduced to 0–5% of the controls.

Incubation of liver mitochondria, prepared by conventional techniques (Schnaitman and Greenawalt, 1968) with 2 mM ATP, 30 mM $MgCl_2$ at 30 °C for 25–30 min, resulted in a significant accumulation of Mg^{2+} in the particles (Table I, No. 1). Addition of a cytoplasmic extract (Lee et al., 1971) containing mainly glutamate and sugar derivatives (see footnote *d*, Table I), or of a mixture of glutamate and components identified in the cytoplasmic extract, increased Mg^{2+} uptake above the concentration reached in the presence of ATP alone (Table I, No. 2 and 3). No matter what conditions were used, 77–84% of mitochondrial Mg^{2+} was found in the mitoplast (matrix and inner membrane). Subfractionation of the mitoplast showed that 53–59% of Mg^{2+} in the mitoplast was localized in the matrix fraction and about 25% was either in the inner membrane or adsorbed to it. These results are consistent with an active concentration of Mg^{2+} into the mitoplast. There was some accumulation of Mg^{2+} in the intermembrane compartment (38.4% in experiment 2; 9.2 and 12.7% in experiments 1 and 3).

The time course of Mg^{2+} uptake with mitochondria contaminated by lysosomes exhibited great variability. Oxidizable mitochondrial substrates consistently increased Mg^{2+} uptake even under conditions when phosphorylation coupled O_2 uptake was completely inhibited by oligomycin. Additional augmenting effects were occasionally observed by hexose 6-phosphates and biosynthetic precursors of sialic acid (Kun et al., 1974); however, these effects were difficult to reproduce. It was also noted that, during prolonged (30–50 min) incubation of mitochondria, there was a variable decay of the sedimentable particles (20–40%); thus no kinetically interpretable results could be obtained with these preparations.

In contrast to conventionally prepared mitochondria, elimination of lysosomes by the technique described in Experimental Section abolished the variability of results. Rates of Mg^{2+} uptake by lysosome-free mitochondria were influenced only by ATP, Mg^{2+}, and by the presence of oxidizable citric acid cycle intermediates, despite the fact that their oxidation was nearly completely inhibited by oligomycin (by 95–100%). It was therefore evident that, with the exception of site specific reducing substrates, the apparent augmenting effects of various cytoplasmic components were directly related to the decay of mitochondria by lysosomal enzymes. Increased mitochondrial stability of lysosome-free mitochondria permitted the use of higher temperatures (37 °C), which were detrimental to mitochondria prepared by conventional techniques. Accurate values of the time course of Mg^{2+} flux enabled us to derive a kinetic model from which kinetic constants of both influx and egress can be calculated. This is shown below.

Kinetic Model Let: (M_i) = "internal" concentration of Mg^{2+}, $(M_i)_0$ = "internal" concentration of Mg^{2+} at zero time, $(M_i)_\infty$ = "internal" concentration of Mg^{2+} at steady state, (M_e) = "external" concentration of Mg^{2+} which is a constant during the entire time course. The concentration of matrix Mg^{2+} is determined by

$$d(M_i)/dt = J_e - J_i, \quad (1)$$

where J_e and J_i are the contributions to the internal concen-

Table I: Submitochondrial Distribution of Mg^{2+} Resulting from Mg^{2+} Uptake in the Presence of Oligomycin.

No.	Incubation System[a]	Submitochondrial Fractions: Matrix ΔMg^{2} [b]	Matrix %[c]	Inner Membr ΔMg^{2+}	Inner Membr %	Outer Membr ΔMg^{2+}	Outer Membr %	Intermembr Space ΔMg^{2+}	Intermembr Space %	Inc in Tot Mitochondrial Mg^{2+} ΔMg^{2+} (nmol/mg)	Tot Mitochondria Recovery of Mg^{2+} in Fraction (%)
1	ATP, Mg^{2+}	21.9	53	10.1	24	0.2	0.4	9.8	2.4	41.5	101
2	ATP, Mg^{2+}, cytoplasmic extract	98.2	52	47.2	25	0.9	0.5	38.4	2.0	189.0	102
3	ATP, Mg^{2+}, F-6-P, glutamate, NH_4^+	52.7	59	21.3	24	0.3	0.3	12.7	1.4	88.9	102

[a] Components of the incubation systems are described in footnote *d* of this table. [b] ΔMg^{2+} is the ratio of the increase in Mg^{2+} to the total mitochondrial protein, in nmol of Mg^{2+}/mg of protein. Increase in Mg^{2+} was measured from the zero-time Mg^{2+} content of 30.7 nmol/mg of protein. [c] Percent (%) is the percent of the total Mg^{2+} uptake found in each submitochondrial compartment. [d] In all three experiments, 3 μg of oligomycin/mg of mitochondrial protein was present. Temperature; 30 °C; time of incubation was 25 min. The incubation medium in experiments 1 and 3 was 0.15 M sucrose, 50 mM Tris-Tes (pH 7.45 ± 0.05). Experiment 1: ATP, 2 mM; added Mg^{2+}, 30 mM. Experiment 2: The cytoplasmic extract (cf. Lee et al., 1971) contained: 11.5 mM glucose, 0.19 mM glutamate, 0.3 mM phosphate, 80 μM NH_4^+, 3 μM malate, 2 μM fructose 6-phosphate, 10 mM Na^+, 5 mM K^+, 0.61 mM unidentified acid-labile phosphates (liberated at 100 °C in 1 N HCl during 30 min), traces of inositol, and some unidentified carbohydrates; ATP, 2 mM; Mg^{2+}, 30 mM. Experiment 3: Fructose 6-phosphate and ammonium glutamate, 2 mM; ATP, 2 mM; Mg^{2+}, 30 mM.

tration change of Mg^{2+} due to flux from external medium (J_e) and the outward flux from the interior (J_i). Set

$$J_e = \frac{V_e(M_e)}{K_e + (M_e)} \tag{2a}$$

and

$$J_i = \frac{V_i(M_i)}{K_i + (M_i)} \tag{2b}$$

which is to assume a saturatable carrier on either side of the inner membrane with different permeability parameters for flux from exterior (V_e and K_e) than from the interior (V_i and K_i).

A similar equation was suggested by Stein (1967) for a "pump" and "leak" system. At plateau $d(M_i)/dt = J_e - J_i = 0$, and $(M_i) = (M_i)_\infty$; rearranging we obtain:

$$\frac{K_e}{V_e(M_e)} - \frac{K_i}{V_i(M_i)_\infty} = \frac{1}{V_i} - \frac{1}{V_e} \tag{3}$$

It is generally assumed (but seldom proved) that $V_i = V_e$. Then

$$(M_i)_\infty/(M_e) = K_i/K_e$$

For net accumulation, $(M_i)_\infty/(M_e) > 1$; then $K_i > K_e$. The carrier in the interior state must have a weaker affinity for Mg^{2+} than in the exterior state. If K_i is sufficiently large, then $J_i \approx V_i(M_i)/K_i = k_i(M_i)$ for the entire course of the transport process. Then with $V_i/K_i \equiv k_i$

$$\frac{d(M_i)}{dt} = J_e - \frac{V_i(M_i)}{K_i + (M_i)} \approx J_e - k_i(M_i) \tag{4}$$

integrating we obtain:

$$(M_i) = (M_i)_\infty + [(M_i)_0 - (M_i)_\infty]^{e^{-k_i t}} \tag{5a}$$

which can be rearranged to

$$\log\left\{\frac{[(M_i)_\infty - (M_i)]}{[(M_i)_\infty - (M_i)_0]}\right\} = -\frac{k_i t}{2.303} \tag{5b}$$

where

$$(M_i)_\infty = \frac{J_e}{k_i} = \frac{V_e(M_e)}{k_i[K_e + (M_e)]} \tag{6a}$$

and hence

$$\frac{1}{(M_i)_\infty} = \frac{k_i K_e}{V_e}\left[\frac{1}{(M_e)}\right] + \frac{k_i}{V_e} \tag{6b}$$

It should be noted that, according to eq 5a and 5b, an apparent first-order rate constant of *egress* is calculated from the kinetics of net flow *inward*. Double-reciprocal plots of $(M_i)_\infty^{-1}$ in $(M_e)^{-1}$ yield $k_i K_e/V_e$ and k_i/V_e. Assuming that $V_e = V_i$, then as $k_i \equiv V_i/K_i$, $K_i = V_e/k_i$. Initial velocity measurements should permit the determination of V_e and K_e when $d(M_i)/dt \approx J_e$, i.e., $J_e \gg J_i$. From initial velocity measurements, which were not so accurate as the determination of the entire time course, K_e was found to be between 12 and 15 nmol per mg of protein (compare with K_e = 17 nmol/mg of protein, see Figure 3). It follows from eq 5b that plots of

$$\log\left\{\frac{[(M_i)_\infty - (M_i)_t]}{[(M_i)_\infty - (M_i)_0]}\right\}$$

against time should yield straight lines under all experimental conditions and the slope is k_i. This is indeed illustrated in Figure 1, which was compiled from data obtained under variable conditions as shown in Table II. In the presence of ATP variation of the concentration of site I specific reducing substrates (between 136 and 830 nmol/mg of mitochondrial protein) or the presence of oligomycin had no significant effect on k_i (and on $t_{1/2}$), whereas $(M_i)_\infty$ varied significantly.

The time course of Mg^{2+} accumulation in lysosome-free mitochondria followed a highly reproducible pattern. In 85 separate kinetic experiments carried out at 37 °C, the time course of Mg^{2+} characteristically approached a plateau. This time course was modified by components of the reaction system. The effects of increasing amounts of oligomycin are shown in Figure 2. This time course was determined in the presence

Table II: The Effect of Experimental Conditions on k_i and $t_{1/2}$.

Expt No.	Experimental Conditions	k_i (min^{-1})	$t_{1/2}$ (min)
1	No oligomycin, no substrates, 410 nmol of ATP/mg	0.098	7.1
2		0.070	9.9
3	No oligomycin + glutamate + malate (136–410 nmol/mg) + 415 nmol of ATP/mg	0.063	11.0
4		0.094	7.3
5		0.066	10.6
6	1.5 μg of oligomycin/mg; no substrate + 400–599 nmol of ATP/mg	0.051	13.7
7		0.049	14.2
8		0.050	13.8
9	1.5 μg of oligomycin/mg + glutamate + malate (136–830 nmol/mg) + ATP (205–415 nmol/mg)	0.050	13.8
10		0.050	13.8
11		0.049	14.2
12		0.050	13.8
13		0.070	9.9

of 1 mM ATP, 2.5 mM Tris-glutamate, 2.5 mM sodium malate, 20 mM $MgCl_2$, 150 mM sucrose, 5 mM NaOH, 2.5 mM KOH, 0.1 mM NH_4^+, and 0.1 mM Na_2KPO_4, the solution being buffered to pH 7.4 (at 37 °C) with 60 mM Tris-Tes.[2] Curve 1 is the control, while 2 shows the rate in the presence of 1.5 μg of oligomycin per mg of mitochondrial protein. This rate is indistinguishable from curve 1. This amount of oligomycin inhibited respiration of mitochondria by 90–95%. At a higher concentration of oligomycin (3 μg of oligomycin per mg of mitochondrial protein, curve 4), Mg^{2+} uptake was occasionally (about 30% of experiments) lowered to the rate obtained in the absence of added glutamate + malate (i.e., with ATP as the only energy source, curve 3). Comparison of Figure 2 with Table I shows that mitochondria prepared according to Schnaitman and Greenawalt (1968) tend to be less sensitive to the inhibitory effect of oligomycin than mitochondria further purified by digitonin to remove lysosomes. This apparent discrepancy is probably explained by the fact that the mitochondrial cell fraction prepared by conventional techniques contains variable quantities of non-mitochondrial proteins (e.g., lysosomes). The relative effectivity of oligomycin would therefore be expected to increase if nonmitochondrial contamination is removed as in the lysosome-free preparation. In subsequent experiments, oligomycin at 1.5 μg/mg of mitochondrial protein was consistently employed unless specifically stated otherwise. Omission of oligomycin did not alter the course of Mg^{2+} flux.

An almost identical time course of Mg^{2+} uptake was observed when site I specific substrates were substituted by 5 mM succinate. Whereas rotenone (2×10^{-7} M) inhibited ATP + endogenous substrate or ATP + site I specific substrate supported Mg^{2+} uptake, succinate supported Mg^{2+} accumulation was not inhibited. Antimycin A (2×10^{-7} M) inhibited succinate supported Mg^{2+} uptake. The role of site III has not been investigated at this time. The stimulatory effect of site I specific substrates or of succinate above the rate obtained by ATP alone was the same in the absence of oligomycin or in the presence of oligomycin (1.5 μg per mg of protein) sufficient to inhibit 90–95% of phosphorylative respiration. These results show that inhibition of phosphorylation coupled electron transfer does not modify the inhibitory effects of site specific inhibitors of electron transport on Mg^{2+} flux. That Mg^{2+} flux is inhibited by site specific inhibitors of the electron transport system in the *absence* of mitochondrial respiration indicates that reduction of the electron transport apparatus by site specific substrates is a necessary requirement of ATP-dependent Mg^{2+} translocation.

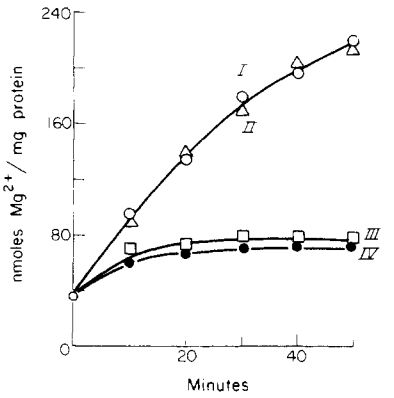

FIGURE 2: The effect of oligomycin on Mg^{2+} uptake. The incubation medium is the same as described in Results: ATP, 1 mM; glutamate, 2.5 mM; malate, 2.5 mM. (○) No oligomycin; (△) 1.5 μg of oligomycin per mg of protein; (●) 3 μg of oligomycin per mg of protein; (□) no oligomycin, no substrates, only 1 mM ATP. Temperature: 37 °C.

The Effects of Varying Concentrations of External Mg^{2+} Are Shown in Figure 3 (A and B). The rates of Mg^{2+} uptake were determined in the presence of 1 mM ATP and the components listed for the experiments are shown in Figure 2, except that the concentration of added Mg^{2+} varied from 0 to 40 mM. Oligomycin (1.5 μg/mg of mitochondrial protein) was present, but identical results were obtained without oligomycin. The Mg^{2+} concentration of mitochondria (22 mM) gradually diminished to near 0 without externally added Mg^{2+} and upon the addition of Mg^{2+} showed a gradual increase which was related to the concentration of external Mg^{2+} (M_e). This is illustrated in Figure 3A. Extrapolated maximal Mg^{2+} concentrations $(M_i)_\infty$ are as follows: for 5 mM (M_e) = 72 mM, for 10 mM (M_e) = 118 mM, for 20 mM (M_e) = 175 mM, and for 40 mM (M_e) = 220 mM. A double-reciprocal plot of $(M_i)_\infty^{-1}$ vs. $(M_e)^{-1}$ (see eq 6b) is shown in Figure 3B. It follows from the kinetic model that $V_e = k_i/\text{intercept}$ which is 0.023 mmol per l. per min or 23 nmol of Mg^{2+} per mg of mitochondrial protein per min, where $k_i = 0.074$ min^{-1}. The intercept (k_i/V_e) is 3.15 ($M_i^{-1} \times 10^3$), the slope (k_iK_e/V_e) is 0.0538; therefore

$$K_e = \left(\frac{k_iK_e}{V_e}\right) \Big/ \left(\frac{k_i}{V_e}\right)$$

is 17 nmol of Mg^{2+} per mg of mitochondrial protein.

The Effects of Various Concentrations of Added ATP. In

[2] The ionic constituents (K^+, Na^+, NH_4^+, PO_4^{3-}) of the incubation medium were chosen at given concentrations because these approximated the values found in tissue extracts (Lee et al., 1971). The effect of individual ionic species on Mg^{2+} kinetics has not been investigated in detail, except that it was found experimentally that this mixture provided optimal conditions.

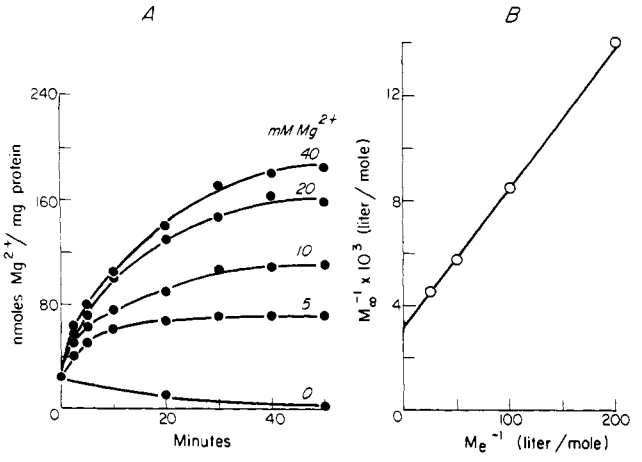

FIGURE 3: The effect of varying concentrations of externally added Mg^{2+} on the time course of Mg^{2+} uptake. ATP, 1 mM; glutamate, 2.5 mM; oligomycin, 1.5 μg/mg of mitochondrial protein were present. (A) Time course; (B) double-reciprocal plot of maximally accumulated Mg^{2+} $(M_i)_\infty$ against externally added Mg^{2+} (M_e), i.e., 0-40 mM. Temperature: 37 °C.

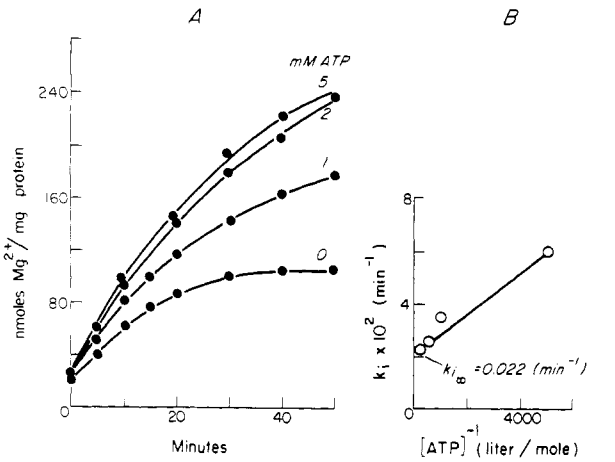

FIGURE 4: The effect of increasing concentration of ATP on the time course of Mg^{2+} uptake. Conditions are the same as described in the text, except the concentration of externally added Mg^{2+} was 40 mM and the concentration of added ATP varies from 0 to 5 mM. (A) Time course of Mg^{2+} uptake; (B) plot of k_i against $[ATP]^{-1}$. Temperature: 37 °C.

the presence of 40 mM externally added Mg^{2+} and in an incubation system identical with experiments shown in Figure 3, added ATP concentration was varied between 0 and 5 mM. Results are illustrated in Figures 4A and 4B. In the presence of site I reducing substrates alone (curve 0), a relatively small Mg^{2+} accumulation occurred. This rate was about the same as the Mg^{2+} uptake in the presence of 1 mM ATP alone (curve 3, Figure 2) or with 5 mM externally added Mg^{2+} + 1 mM ATP (Figure 3A). Increasing concentrations of ATP augmented Mg^{2+} uptake and had characteristic effects on kinetics. From Figure 4A, the calculated $(M_i)_\infty$ values were as follows: with no added ATP, 108 mM; with 1 mM ATP, 180 mM; with 2 mM ATP, 310 mM; and with 5 mM ATP, 320 mM. From a plot of $(M_i)_\infty^{-1}$ vs. $[ATP]^{-1}$, K_m(ATP) of 0.44 mM and a $[(M_i)_\infty]_{max}$ of 364 mM Mg^{2+} was calculated. From plots of

$$\ln\left\{\frac{[(M_i)_\infty - (M_i)_t]}{[(M_i)_\infty - (M_i)_0]}\right\}$$

against time (see Kinetic Model) at various ATP concentrations, varying exit velocity constants (k_i) were obtained. From a plot of k_i values against the concentration of added ATP, it is apparent that increasing concentrations of added ATP decreased the exit velocity constant (Figure 4B). Since $(V_i)_{max} = (k_i)_{max}(M_i)_\infty$, this can be calculated to be 8 nmol of Mg^{2+} per mg of protein per min.

The Effect of Inhibitors. Ruthenium red is powerful inhibitor of Mg^{2+} uptake, highly effective at a concentration of 2 nmol/mg of mitochondrial protein (Figure 5A, curve R). The uncoupler FCCP (1 nmol per mg of protein) when added at 30 min induced rapid efflux of accumulated Mg^{2+} (Figure 4B, top curve). When the uncoupler was added at the beginning, Mg^{2+} uptake was inhibited (not shown). It should be noted that the effect of the uncoupler was determined in the absence of oxidative phosphorylation (with 1.5 μg of oligomycin/mg of mitochondrial protein). The same results were obtained when oligomycin was omitted. Atractyloside also inhibited Mg^{2+} uptake as shown in Figure 5B. EDTA at 0.1 mM (or 50 nmol/mg of protein) concentration had no effect on Mg^{2+} uptake. When present in the isolation medium (2mM), EDTA interfered with the detection of Mg^{2+} uptake in a variable manner. For this reason EDTA, a commonly used reagent for the isolation of mitochondria, was omitted from the isolation

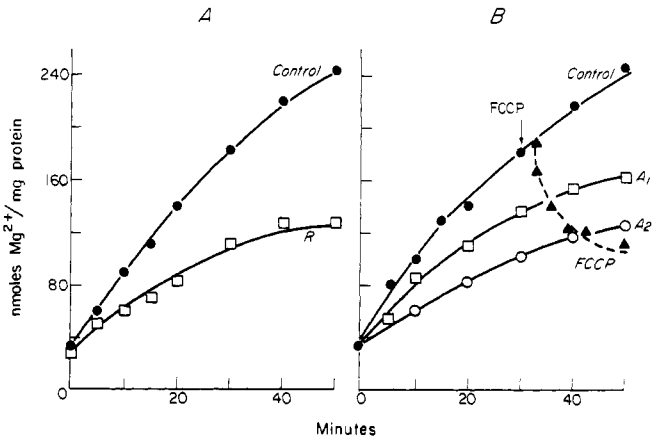

FIGURE 5: The effect of inhibitors on the time course of Mg^{2+} uptake. Conditions are the same as described in the text. In A and B, top curves are controls. (A) 2 nmol of ruthenium red per mg of protein. In B, 1 nmol of FCCP per mg of protein is added at 30-min time point: (▲) time course of Mg^{2+} efflux after FCCP; (□ A_1) 25 nmol of atractyloside per mg of protein; (○ A_2) 125 nmol of atractyloside per mg of protein. Temperature: 37 °C.

medium (see Experimental Section). Butocaine (100 uM) or La^{3+} (10 uM) had no effect on Mg^{2+} flux.

Results shown in Figure 5 were obtained under conditions when the rates of Mg^{2+} uptake were close to maximal, as predicted from Figures 3 and 4 (i.e., in the presence of 5 mM substrates, 2 mM ATP, and 40 mM Mg^{2+}). From the viewpoint of cell physiology, it was of importance to demonstrate the operation of the Mg^{2+} transport system under less artificial conditions. It would be expected that endogenous mitochondrial energy sources (substrates and ATP) should maintain a submaximal rate of Mg^{2+} uptake. We find that mitochondria contain about 5 nmol of glutamate and 5-8 μmol of ATP per mg of protein. Mitochondria without externally added ATP (1 mM) lose their selective permeability properties in 20-30 min at 37 °C, even after removal of lysosomes; therefore this concentration of external ATP (which is close to a physiological cytoplasmic concentration) had to be present. The concentration of externally added Mg^{2+} was 20 mM, which was the only nonphysiological concentration of the system.

As shown in Figure 6, the time course of Mg^{2+} uptake had

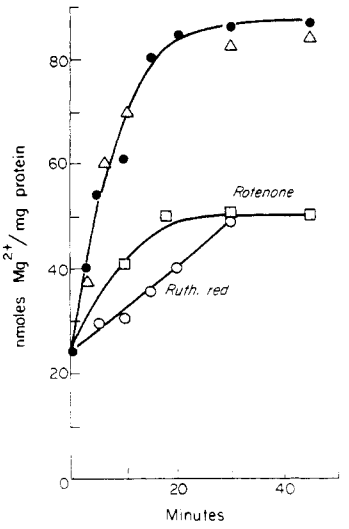

FIGURE 6: The effects of inhibitors on Mg^{2+} uptake supported by endogenous substrates + 1 mM ATP (see Results). (●) No oligomycin; (△) 2 μg of oligomycin per mg of protein; (□) 50 pmol of rotenone per mg of protein; (○) 1 nmol of ruthenium red per mg of protein; M_e = 20 mM Mg^{2+}. Temperature: 37 °C.

the same kinetic characteristics as those obtained under optimal conditions, except $(M_i)_\infty$ was lower. Oligomycin (2 μg/mg of protein) did not inhibit Mg^{2+} uptake. On the other hand, rotenone (50 pmol/mg of protein) and ruthenium red (1 nmol/mg protein) were powerful inhibitors. The same results were obtained when, instead of ATP, low concentrations of externally added glutamate + malate (0.25 mM each) were present. This low concentration of substrates approximates cellular levels and is also sufficient to stabilize mitochondria.

Discussion

Experiments reported here diminish previous uncertainties related to the nature of the association of externally added Mg^{2+} with isolated mitochondria. Two experimental advances were made. First the submitochondrial localization of Mg^{2+} during Mg^{2+} uptake was shown to be predominantly confined to the matrix compartment; therefore externally added Mg^{2+} had to be translocated through the inner membrane. The second experimental progress is the preparative technique of lysosome-free mitochondria. This enabled us to study relatively slow mitochondrial processes such as Mg^{2+} transport. As shown here (Table I), mitochondria contaminated with lysosomes—while capable of Mg^{2+} translocation—are susceptible to various cytoplasmic substances and a variable degree of augmentation of Mg^{2+} uptake can be demonstrated by metabolic precursors of sialic acid (Kun et al., 1974). These results explain previously observed phenomena produced by cytoplasmic extracts (Kun, 1972). Since removal of lysosomes abolished the effect of cytoplasmic extracts, it is apparent that these cytoplasmic substances prevented mitochondrial decay by lysosomal enzymes, and thus influenced mitochondrial integrity and mitochondrial functions which depend on structural integrity in an indirect manner. These conclusions are in variance with the claim of Binet and Volfin (1971, 1974) and Binet et al. (1971), who suggested that a specific cytoplasmic polypeptide regulates mitochondrial stability by way of controlling the Mg^{2+} content of mitochondria. Since mitochondria used by these workers were prepared by conventional techniques, they had to be contaminated by lysosomes. For this reason prolonged in vitro tests of mitochondrial stability, as used by these workers (30–40 min at 30 °C), were necessarily obscured by mitochondrial decay induced by lysosomal enzymes. Furthermore, we were unable to reproduce the method for the isolation of a cytoplasmic peptide as described by Binet et al. (1971), despite extensive efforts employing a variety of chromatographic techniques combined with mass spectrometry.

Blondin and Green (1975) proposed an interesting model of energy coupling which is based on the assumption that ATP synthesis and Mg^{2+} transport in the inner membrane are coupled reactions. As shown in this paper, the $t_{1/2}$ of Mg^{2+} translocation is 10–13 min; therefore it cannot be related to the rapid ATP synthetase reaction unless the existence of another form of Mg^{2+} transport is postulated. It is not clear from the reports of Blondin and Green how the ubiquitous interference by lysosomal contamination was ruled out in their experiments concerned with the demonstration of a postulated Mg^{2+} ionophore.

Independent criteria of functional integrity of mitoplasts of lysosome-free mitochondria (see Experimental Section) together with the submitochondrial localization of absorbed Mg^{2+} comprise the experimental basis for the proposed kinetic model. This is formally compatible with a carrier mediated transport, except without more detailed knowledge of mechanisms this model serves as a working hypothesis. Saturability with respect to the concentration of externally added Mg^{2+} exists provided ATP is present. The proposed kinetic model offers a novel and useful method of calculating both inward and outward oriented kinetic constants from the time course of Mg^{2+} uptake.

Although the present paper is primarily concerned with specific experimental conditions required for the demonstration of the observed flux of Mg^{2+}, some analysis of possible mechanisms may be proposed. The least complicated role of ATP would be that of a counterion to externally added Mg^{2+} and it may be assumed that the adenine nucleotide translocase participates indirectly in the transfer of externally added Mg^{2+} into the mitoplast. Oxidation of a substrate (e.g., succinate) would provide transmembrane potential as the energy source. This mechanism has been actually considered earlier (Kun et al., 1974) but was eventually abandoned for several reasons. It is known from the work of Pfaff and Klingenberg (1968) that in coupled mitochondria exogenous ATP enters into mitochondria at a much slower rate than ADP, and uncoupling agents raise the entry rate of ATP to the rate of ADP. The slow rate of entry of ATP could explain the relatively slow rate of Mg^{2+} uptake in the mitoplast of coupled mitochondria. As shown in Figure 5 an uncoupler, which is known to equalize the exchange rates of ATP and ADP (Klingenberg, 1970), induces rapid *efflux* of Mg^{2+} under conditions when the *influx* of ATP is clearly increased. The influx of the presumed $[ATP^{4-}$–2 $Mg^{2+}]$ complex would then be expected to depend on active mitochondrial respiration and inhibition of Mg^{2+} influx by an uncoupler could be explained by a collapse of membrane potential. This hypothesis does not explain the observed phenomena of ATP-dependent Mg^{2+} flux in the *absence* of measurable O_2 uptake which—as monitored polarographically—is sustained during the entire time course of our experiments. Other workers as well as ourselves find a partial release of the inhibitory effect of oligomycin on O_2 uptake upon prolonged incubation of mitochondria which are contaminated by lysosomes. Removal of lysosomes by the procedure given in the Experimental Section abolishes this phenomenon; thus we attribute many of the conflicting observations in this field to artifacts, induced by lysosomal degradative enzymes. It is therefore difficult to visualize a pu-

tative respiration as the driving force for the translocation of Mg^{2+}-ATP^{4-} complex when this respiration cannot be demonstrated experimentally. Furthermore the rate-limiting role of adenine nucleotide translocase in Mg^{2+} flux should be easily demonstrated by the inhibitor atractyloside, which has a K_i of 10^{-7} M (Klingenberg, 1970). As shown in Figure 5, 0.25×10^{-4} M atractyloside inhibits about 50%, and even 1.25×10^{-4} M atractyloside does not completely inhibit endogenous ATP-dependent Mg^{2+} uptake. The high sensitivity of Mg^{2+} flux to ruthenium red is also incompatible with the rate-limiting role of adenine nucleotide translocase in Mg^{2+} flux since this carrier is not known to be inhibited by ruthenium red. Direct analysis of mitochondrial ATP content reveals that mitochondria (see Experimental Section) contain on a protein basis 4–6 mM ATP and this is not changed by externally added ATP at a concentration of 2 mM. Mitochondria gradually lose ATP in the absence of external ATP. It is apparent that these results are predicted by known properties of the adenine nucleotide translocase system (Klingenberg, 1970). Although the experiments aimed to demonstrate stoichiometry between Mg^{2+} uptake and adenine nucleotide content by enzymatic assays are uncertain because of the interference of adenylate kinase and various transphosphorylation reactions, we find that for each ATP hydrolyzed at least 2–3 atoms of Mg^{2+} accumulate in the matrix space under our specified experimental conditions. These results, although not conclusive, tend to indicate that the mechanism of the observed ATP-dependent Mg^{2+} flux is more complex than predicted from known information.

It is interesting that not only the influx of Mg^{2+} but also k_i is dependent on the concentration of added ATP, and k_i is decreased by increasing concentrations of this nucleotide (see Figure 4A, B). This suggests that the net flux of Mg^{2+} may be supported by ATP in the inner membrane system which in an intact state determines the observed vectorial orientation. Uncouplers which are known to cause a collapse of the H^+ gradient of the inner membrane orient Mg^{2+} flux outward. We have shown previously (Kun et al., 1970), as confirmed by others (Bogucka and Wojtczak, 1971), that added ADP (at 2–3 mM concentration) is required for 2,4-dinitrophenol-induced rapid efflux of Mg^{2+} from mitochondria. The role of ADP was found to be twofold: first to serve as a source of ATP by adenylate kinase; second to inhibit ATPase. Further experiments revealed that, in the presence of oligomycin (3 μg/mg of protein) sufficient to counteract the activation of ATPase by 2,4-dinitrophenol (50 μM), the *efflux* of Mg^{2+} becomes dependent on the concentration of added ATP with an apparent K_m for ATP of 1.4 mM. This is three times the K_m value for ATP for Mg^{2+} influx observed in the absence of an uncoupler.

Taking an average of tenfold concentration of Mg^{2+} into the matrix and assuming the standard free energy of hydrolysis of ATP to be −7.3 kcal (Lehninger, 1970), an estimated 4–5 atoms of Mg^{2+} can be transported through the inner membrane for each mole of ATP hydrolyzed. The Mg^{2+} transport associated ATPase appears to be much less sensitive to oligomycin than phosphorylation coupled O_2 uptake. The requirement for reducing substrates for the ATP-dependent Mg^{2+} transport suggests that the energy derived from the hydrolysis of ATP can only be utilized for the translocation of Mg^{2+} when the electron transport system is reduced. Alternatively carboxylate ions could also serve as counterions to Mg^{2+}, except this role alone does not explain the fact that inhibitors of the reduction of specific sites of the electron transport system (e.g., by rotenone) inhibit Mg^{2+} uptake even in the absence of detectable mitochondrial respiration (i.e., in the presence of oligomycin). A hitherto unknown existence and function of an oligomycin insensitive ATPase, serving as Mg^{2+} pump, may be postulated. This hypothesis is not without experimental foundation since it has been shown that mitochondria contain a cytochalasin B sensitive, presumably actomyosin-like mechanochemical energy transducing system (Lin et al., 1973) which may participate in translocase functions of the inner membrane. Weiner and Lardy (1974) found that inhibitors of electron transfer at specific coupling sites inhibit mitochondrial ATPase. Their results, as well as ours, could suggest that reduction of the electron transport system at specific reducing sites may be obligatory for ATP-dependent work. Our results pose new questions related to the mechanism of ATP-dependent translocations in mitochondria. The observed high sensitivity of the Mg^{2+} transport system to ruthenium red suggests the participation of a glycoprotein. On the other hand the much slower rates of Mg^{2+} flux and the insensitivity of Mg^{2+} transfer to inhibitors of Ca^{2+} translocation distinguish the Mg^{2+} transport system from the much more rapid Ca^{2+} translocation system of mitochondria.

Acknowledgments

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Retention of Enzyme Activity by Detergent-Solubilized Sarcoplasmic Ca^{2+}-ATPase†

Marc le Maire, Jesper V. Møller,‡ and Charles Tanford*

ABSTRACT: The Ca^{2+}-activated ATPase of sarcoplasmic reticulum can exist in true solution in the presence of some nonionic detergents, with retention of enzymatic activity for several days. The soluble active particles retain about 30 mol of phospholipid per mol of polypeptide chain even in the presence of a large excess of detergent, indicating the existence of relatively strong attractive forces between protein and lipid, as previous work from other laboratories has already suggested. Deoxycholate is much more effective than nonionic detergents in removing protein-bound lipid and, when used at solubilizing concentrations, completely delipidates and inactivates the ATPase. Preliminary molecular weight measurements indicate that the Ca^{2+}-ATPase exists as an oligomer in the native membrane: fully active enzyme in Tween 80 has a minimal protein molecular weight of about 400 000, corresponding to a trimer or tetramer of the ATPase polypeptide chain, and even the inactive enzyme in deoxycholate contains a substantial fraction of dimeric protein.

This paper is part of a long-term project to characterize membrane proteins by the traditional methods of protein physical chemistry, using ultracentrifugation as the primary tool. To accomplish this objective it is necessary to have the protein in true solution, i.e., dispersed in particles that contain only a single copy of the protein molecule. This solubilization must be accomplished as far as possible without disruption of the native conformation of the protein, and it is hoped that this can be done with the aid of suitable detergents. It should be noted that our criteria for what is a suitable detergent are more stringent than those that apply to "reconstitution" experiments in which detergent solutions are used for purification procedures but the detergent is ultimately replaced by lipids, and membrane-bounded vesicles each containing many copies of the protein are reformed. In such experiments the protein need not have its native conformation in the detergent solution: it is necessary only that whatever change it has undergone be reversible. For our objectives the protein must remain undenatured in the detergent solution itself, by whatever criteria are available. In the case of the sarcoplasmic Ca^{2+}-ATPase, which is the subject of this paper, we have used the Ca^{2+}-ac-

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‡ Institute of Medical Biochemistry, University of Aarhus, Denmark.
* Research Career Awardee, National Institutes of Health.