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ION TRANSPORT IN HEART MITOCHONDRIA

XIII. THE EFFECT OF ETHYLENEDIAMINETETRAACETATE ON MONOVALENT ION UPTAKE

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SUMMARY

1. Ethylenediaminetetraacetate (EDTA) markedly activates the accumulation of Na^+ and Li^+ and the swelling which accompanies the ion uptake by isolated heart mitochondria. This activation is reflected in the removal of limited amounts of endogenous Mg^{2+} and extensive loss of K^+. The removal of these cations requires the presence of Na^+, a source of energy, and a permeant anion as well as EDTA. The effects of EDTA on the activation of Na^+ uptake and cation removal are duplicated by chelators with a high affinity for Mg^{2+}, but not by ethyleneglycol-bis-(β-aminoethylether)-*N*, *N'*-tetraacetic acid. Mg^{2+} at concentrations 5 to 6 times less than EDTA prevents both activation of Na^+ uptake and cation removal.

2. EDTA does not appear to be bound by heart mitochondria. At neutral pH the chelator penetrates into the mitochondrial water volume to the same extent as sucrose and mannitol. At pH 8.1 where the removal of mitochondrial Mg^{2+} by EDTA is more effective, EDTA penetrates virtually the entire water volume. This penetration requires the presence of a source of energy, a transported cation such as Na^+, and a permeant anion. It appears possible that the oscillations in ion uptake and swelling observed in the presence of EDTA at pH 8.1 may be related to the presence of the chelator in the interior compartment under these conditions.

INTRODUCTION

Work reported from a number of laboratories suggests that bound Mg^{2+} may be an important factor in controlling the permeability of the mitochondrial membrane[1-4]. AZZONE AND AZZI[1] have noted that treatment of liver mitochondria with EDTA results in changes which are similar to those induced by valinomycin and gramicidin. The addition of EDTA resulted in an increased permeability of liver mitochondria to univalent ions and cycles of swelling and shrinking which were

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*, *N'*-tetraacetic acid; TMPD, tetramethyl-*p*-phenylenediamine.

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coupled to ion movement[2]. The effects of EDTA were antagonized by low concentrations of added Mg^{2+} (ref. 2). PACKER, UTSUMI AND MUSTAFA[3] have emphasized the oscillatory nature of this response in EDTA-treated rat liver mitochondria when the reaction is carried out at pH 8.0–8.3. It has been reported that parathyroid hormone which is known to affect the permeability of the mitochondrion to Mg^{2+} and K^+ also promotes the loss of mitochondrial Mg^{2+} under the proper conditions[5]. In addition, the removal of Mg^{2+} by parathyroid hormone and histones has been related to increases in $^{42}K^+$ flux across the mitochondrial membrane[4].

Mitochondrial cation transport can be activated by the interaction of the membrane with Zn^{2+} and other heavy metal ions[6–8] and with thiol-group reagents[9], such as *p*-chloromercuriphenyl sulfonate, as well as by the 'ionophorous' antibiotics[10]. The reported interaction of EDTA with the membrane appears to represent still another chemically distinct reaction which can bring about the activation of mitochondrial ion transport. In the course of a survey of changes in the intrinsic permeability of isolated beef heart mitochondria which result from treatment with the above classes of reagents, we became aware that changes as a result of EDTA treatment required much more specific conditions than did those resulting from the addition of heavy metals, thiol-group reagents, or the antibiotics. The present communication examines the conditions necessary to obtain EDTA-dependent changes in ion uptake and swelling in heart mitochondria and the effect of EDTA under these conditions on the level of the endogenous Mg^{2+} and K^+ of the mitochondrion. In addition, the uptake of EDTA by the mitochondrion has been investigated and related to the cation accumulation reaction.

METHODS

Beef heart mitochondria were prepared by a modification of the Nagarse procedure of HATEFI, JURTSHUK AND HAAVIK[11]. Ethyleneglycol-bis-(*β*-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) (1 mM) was used in place of EDTA in order to keep the endogenous Ca^{2+} concentration low without removing bound Mg^{2+}. Otherwise the isolation conditions were identical to those previously described[6,12]. Yields of mitochondria were improved considerably when 600 g of washed muscle mince was suspended in 3 l of sucrose–Tris–EGTA rather than the smaller volume used in the procedure as published[6].

Mitochondrial swelling was measured by the decrease in absorbance at 546 mμ and was monitored either in a circular cuvette (0.75 inch in diameter) mounted in the light path of the Eppendorf photometer or in a standard 1-cm cuvette.

The cation content of the mitochondria was determined from acid extracts by use of atomic absorbtion spectroscopy[8]. The mitochondria were removed from the incubations by rapid centrifugation using a Sorvall SE-12 rotor operated at 40000 × *g*. The resulting pellets were extracted with 0.5 M $HClO_4$ and diluted with 0.1 M HCl. A Perkin–Elmer Model 303 Atomic Absorbtion Spectrophotometer was used to determine Mg^{2+}, Ca^{2+}, Na^+, and K^+.

Total mitochondrial water, dextran-permeable water, mannitol-permeable water, and EDTA-permeable water were determined by a dual isotope method developed in this laboratory which employs a distribution of the solute similar to that of MALAMED AND RECKNAGLE[13]. The details of this procedure will be presented elsewhere[14].

The composition of the reaction mixtures and other experimental conditions are described with the individual experiments.

RESULTS

EDTA markedly enhances the energy-linked swelling of isolated heart mitochondria suspended in 100 mM sucrose and 60 mM Na^+ acetate at pH 7.1 (Fig. 1A). Swelling under these conditions is closely related to ion uptake by mitochondria[1,4,5,7,10,12].

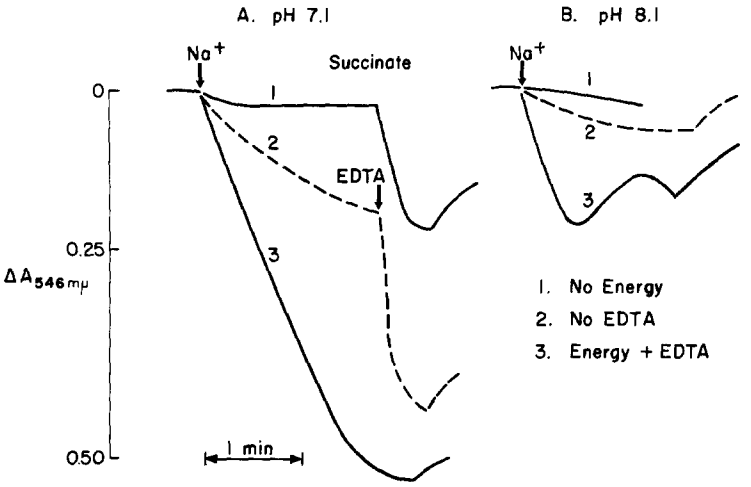

Fig. 1. EDTA-dependent swelling of isolated heart mitochondria. Mitochondria (2.5 mg of protein) were added to 3 ml of a medium of sucrose (100 mM), Tris chloride (10 mM, pH 7.1 or 8.1 as indicated) and rotenone (7 μM). The absorbance at 546 mμ was recorded with an Eppendorf photometer. At the designated point, the reaction was started by the addition of Na^+ acetate to a final concentration of 60 mM. The experiments labeled 1 and 3 were obtained in the presence of EDTA (1.3 mM). In Expt. 2 the EDTA was omitted and added at the indicated point. Expts. 2 and 3 contained Na^+ succinate (3 mM) as the source of energy. Succinate was omitted from Expt. 1 and added where indicated. In each case the sharp inflection point corresponds to the point at which the suspension became anaerobic.

EDTA induces no swelling in the absence of respiration, but increases the spontaneous rate of respiration-dependent swelling substantially. At pH 8 and above the rate of EDTA-induced swelling is greater than at neutral pH, but the extent of swelling is less and there is a decided tendency for an oscillation in the trace of absorbance at 546 mμ (Fig. 1B). These observations are in agreement with earlier reports from other laboratories[1-3]. The observed effects of EDTA on mitochondrial swelling require the presence of rather high concentrations of Na^+ and of a permeant anion such as acetate. Li^+ responds like Na^+ but the effect of EDTA in a medium in which Na^+ is replaced by K^+ is usually difficult to observe. A corresponding swelling is also not seen in media containing 60 mM Tris acetate, or 60 mM choline$^+$ acetate in place of the Na^+ salt when EDTA is added. Since it has been suggested[1-3] that the effects of EDTA on mitochondrial ion uptake and swelling are dependent on the removal of Mg^{2+} from the membrane, we felt it would be of interest to obtain analytical data on the mitochondrial cation content under several of these conditions.

Effect of EDTA on mitochondrial cations

The data of Table I, Expt. 1 were obtained with the same preparation of mitochondria and under the identical conditions as the swelling experiments presented in Fig. 1. It is apparent that in the absence of energy EDTA has little, if any, effect on the endogenous levels of Mg^{2+} and K^+ in the high Na^+ medium. In the presence of respiration, however, the mitochondrial Mg^{2+} is reduced from 23 to 17 mμmoles per mg of protein by addition of 1.3 mM EDTA at pH 7.0. Mitochondrial K^+ drops from 97 to 68 mμmoles per mg of protein under these conditions and there is a marked increase in the Na^+ content of the pellets. The Na^+ values should be regarded only as a qualitative estimate of ion uptake in this case since the concentration gradient favors loss of accumulated Na^+ to the medium during centrifugation. At pH 8.1 there is a greater spontaneous loss of K^+ as compared to neutral pH. Addition of EDTA at this

TABLE I

CATION CONTENT OF ISOLATED HEART MITOCHONDRIA TREATED WITH EDTA

Mitochondria (5 mg of protein) were suspended for 3 min at 25° in 3 ml of a medium containing 60 mM sodium acetate, 100 mM sucrose, 10 mM Tris chloride (pH 7.0 or 8.1) and 7 μM rotenone. Where indicated Tris succinate (3 mM), Tris–EDTA (1.3 mM), dinitrophenol (100 μM), and gramicidin (10 μM) were also present. The tubes were centrifuged and the resulting pellets prepared for metal analysis as described in the text. Expt. 1 represents a centrifuge experiment conducted with the same mitochondrial preparation used for the study presented in Fig. 1. Expts. 2 and 3 represent studies with different preparations of mitochondria.

Expt.	*Conditions*	*EDTA (mM)*	*pH*	*Cation content (mμmoles/mg)*			
				Mg^{2+}	K^+	Na^+	Ca^{2+}
1	No energy	1.3	7.0	23	99	270	—
	Succinate	0	7.0	23	97	256	
	Succinate	1.3	7.0	17	68	490	
	No energy	1.3	8.1	22	61	264	—
	Succinate	0	8.1	23	67	302	
	Succinate	1.3	0.1	11	13	310	
2	No energy	0	7.0	34	110	—	—
	No energy	1.3	7.0	30	94	—	—
	Succinate	0	7.0	31	97		
	Succinate	1.3	7.0	21	66		
	Succinate + dinitrophenol	1.3	7.0	31	98		
	Succinate + Mg^{2+} (250 μM)	1.3	7.0	38	94		
	Succinate + dinitrophenol + gramicidin	1.3	7.0	29	8		
3	No energy	1.3	7.0	27	82	—	14
	Endogenous respiration	1.3	7.0	22	67		11
	Succinate	1.3	7.0	11	29		7
	TMPD–ascorbate	1.3	7.0	22	64		11
	ATP	1.3	7.0	22	70		12
	Succinate + dinitrophenol	1.3	7.0	25	85		8
	No energy	1.3	8.1	26	51	—	17
	Endogenous	1.3	8.1	19	17		13
	TMPD–ascorbate	1.3	8.1	21	23		11
	ATP	1.3	8.1	20	13		16
	Succinate	1.3	8.1	16	20		10

pH causes no loss of Mg^{2+} or K^+ in the absence of energy, but results in much more extensive loss of both of these cations in the presence of succinate respiration.

The amount of Mg^{2+} and K^+ found in isolated heart mitochondria is subject to some variation. In preparations which contain larger amounts of endogenous Mg^{2+} initially (*cf.* Table I, Expt. 2), addition of EDTA in the presence of energy removes considerably more Mg^{2+} (13 *vs.* 6 mμmoles per mg protein for the preparation of Expt. 1). More Mg^{2+} is also retained by these mitochondria under these conditions, however. The removal of Mg^{2+} by EDTA is blocked by the addition of dinitrophenol and other uncouplers. In addition, the uncoupler prevents the loss of endogenous K^+ which normally occurs in the presence of EDTA and succinate. The loss of K^+ is also prevented by the addition of low levels of Mg^{2+}. In the experiment shown in Table I, 250 μM Mg^{2+} prevented the loss of mitochondrial K^+ in the presence of about five times the molar concentration of EDTA. A similar antagonism of the Na^+-dependent swelling (such as that shown in Fig. 1) by Mg^{2+} in the presence of a large molar excess of EDTA has also been observed (*cf.* ref. 2). Addition of EDTA to uncoupled respiring mitochondria does not result in extensive loss of Mg^{2+} even when gramicidin is added to cause the loss of endogenous K^+. The requirement for a supply of energy in addition to EDTA in order to remove even a portion of the mitochondrial Mg^{2+} can be met by a number of substrates other than succinate (Table I, Expt. 3). Endogenous respiration and tetramethyl-*p*-phenylenediamine (TMPD)–ascorbate cause the loss of limited amounts of Mg^{2+} and some decrease in the internal K^+ content. Succinate is more effective in both respects, however, in the experiment shown. As mentioned above, the effect of substrate on the removal of cations is blocked by dinitrophenol. This result is consistent with the participation of an energy-linked step at some point in the reaction. At pH 8.1 all of the energy sources tested were more effective at lowering Mg^{2+} and K^+ levels. It is of interest that in preparations such as that used for Expt. 3 of Table I which contain higher endogenous levels of Ca^{2+}, there is a tendency to lose Ca^{2+} with the combination of EDTA and a source of energy. However, in the case of Ca^{2+} removal the loss is not prevented by addition of dinitrophenol.

Effect of other Mg^{2+} chelators

The correlation between the removal of limited amounts of Mg^{2+} by the chelator and the increase in Na^+ uptake and K^+ loss is further substantiated by the results obtained with two other chelators with a high affinity for Mg^{2+}. The experiments presented in Table II show that *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid and uramil diacetic acid are both more effective than EDTA in stimulating the Na^+-dependent energy-linked swelling just described. Both of these chelators also lower the endogenous Mg^{2+} of the mitochondrion only in the presence of respiration and when the Mg^{2+} is lowered there is a decrease in mitochondrial K^+. In contrast, EGTA which has a high affinity for Ca^{2+} as opposed to Mg^{2+} does not remove Mg^{2+} even in the presence of respiration, and does not result in the loss of endogenous K^+.

Ionic environment and the removal of Mg^{2+} by EDTA

As noted before EDTA induces little change in mitochondrial swelling in media in which Na^+ is replaced by Tris, or choline$^+$, and the changes are less striking in K^+ media. There is also no swelling in a Na^+ medium when the impermeant Cl^- is used in place of acetate. The data summarized in Table III show that EDTA in the presence

TABLE II

EFFECT OF VARIOUS CHELATORS ON SWELLING RATE AND ION CONTENT OF MITOCHONDRIA

Mitochondria were incubated at pH 7 under the conditions described in Table I. Energy was provided as 3 mM Tris succinate where indicated. Chelators were present at a concentration of 670 μM. Swelling was followed at 546 mμ with an Eppendorf photometer in the presence of succinate respiration. The reaction was started by the addition of mitochondria.

Chelator	*Rate of swelling ΔA for first min after Na^+ addition*	*Mg^{2+} (mμmoles/mg)*		*K^+ (mμmoles/mg)*	
		No energy	*Energy*	*No energy*	*Energy*
EDTA	0.290	34.1	21.0	84	35
EGTA	0.035	34.3	34.5	94	96
Trans-1,2,-diamino-cyclohexane-*N,N,N',N'*-tetraacetic acid*	0.364	34.2	17.7	96	44
Uramil diaacetic acid**	0.326	32.0	17.0	87	45
No chelator	0.056	31.0	31.0	94	87

* Aldrich Chemical Co., Inc., Milwaukee, Wisc.
** Geigy Chemical Corporation, Ardsley, N.Y.

of succinate respiration lowers the Mg^{2+} level from 19 to 10 mμmoles per mg of protein when the incubation is carried out in the Na^+ acetate medium already described. This removal is accompanied by increased swelling as monitored by absorbance at 546 mμ in the photometer and also by an increased tritiated water content. As shown before, Na^+ increases under these conditions and there is an extensive loss of mitochondrial K^+. When the same experiment is carried out in a NaCl medium there is no loss of endogenous K^+ at pH 7, and no increase in the loss of K^+ as a function of EDTA treatment at pH 8.1, no increase in Na^+ uptake, and no increase in swelling by the criterion of tritiated water uptake. A similar lack of effect of EDTA on Mg^{2+} removal, K^+ loss, Na^+ uptake, and water content is seen when Tris acetate is used in place of Na^+ acetate. When K^+ replaces Na^+ in this experiment some Mg^{2+} is removed (3 mμmoles/mg of protein) by the combination of EDTA and respiration. In this case there appears to be no corresponding increase in swelling, water content, or K^+ content as a function of EDTA addition, however. It appears, therefore, that the amount of mitochondrial Mg^{2+} removed by EDTA depends on the ionic environment as well as a supply of energy.

When mitochondria are suspended in isotonic Na^+ acetate a spontaneous swelling is observed in the absence of a source of energy[12]. If the energy requirement for Mg^{2+} removal by EDTA were simply a matter of increasing the permeability of the membrane to the chelator by salt uptake and osmotic swelling, then larger amounts of Mg^{2+} might be expected to be removed by EDTA in isotonic Na^+ acetate. Such is not the case (*cf.* Table III). Small decreases in endogenous Mg^{2+} and K^+ are observed only in the presence of respiration in isotonic Na^+ acetate. In this medium the tendency of centrifuged pellets to lose accumulated Na^+ to the medium during centrifugation is minimal since the concentration gradient favors entrance of Na^+ into the mitochondrion. Under these conditions analytical values for accumulated cations and for water content probably reflect Na^+ uptake and swelling quantitatively[12,14].

TABLE III

CHANGES IN MITOCHONDRIAL WATER VOLUME, CATION AND EDTA CONTENT AS A FUNCTION OF EDTA TREATMENT

Mitochondria (10 mg of protein) were suspended for 3 min at 25° in 6 ml of the incubation medium. The isotonic salt studies contained 0.12 M sodium acetate, 10 mM Tris chloride (pH 7.0 or 8.1) and 7 μM rotenone. The remaining studies contained the indicated salt (66 mM), sucrose (100 mM), 10 mM Tris chloride (pH 7.0 or 8.1) and 7 μM rotenone. Energy was provided as 3 mM Tris succinate and chelator as 1.3 mM Tris–EDTA where incidated. EDTA and mitochondrial water content were determined by use of [^{14}C]EDTA and tritiated water by the procedure of HUNTER AND BRIERLEY[14]. Mitochondrial ion content was determined from acid extracts as described in METHODS. Swelling rates represent changes in absorbance as obtained with the Eppendorf photometer at 546 mμ.

Suspending medium	*EDTA added (mM)*	*pH*	*Succinate added (mM)*	*Cation content (mμmoles/mg)*			*EDTA content (mμmoles/mg)*	*Swelling ($\Delta A_{546\ m\mu}$/min)*	*Total pellet water (μl/mg)*	*EDTA-permeable water (μl/mg)*	*EDTA-impermeable water (μl/mg)*
				Mg^{2+}	K^+	Na^+					
Sodium acetate (66 mM)	0	7.0	3	18	69	225	—	0.113	4.55	—	—
Sodium acetate (66 mM)	1.3	7.0	0	19	79	124	1.90	0.020	4.24	2.84	1.40
Sodium acetate (66 mM)	1.3	7.0	3	10	25	443	2.65	0.263	5.13	3.85	1.28
Sodium acetate (66 mM)	0	8.1	3	16	36	229	—	0.106	4.67	—	—
Sodium acetate (66 mM)	1.3	8.1	0	19	36	200	3.08	0.015	4.74	3.60	1.04
Sodium acetate (66 mM)	1.3	8.1	3	11	10	250	3.70	0.200	5.16	5.43	0
NaCl (66 mM)	1.3	7.0	0	17	60	230	2.36	—	4.43	3.52	0.91
NaCl (66 mM)	1.3	7.0	3	17	63	200	2.28	—	4.29	3.41	0.88
NaCl (66 mM)	1.3	8.1	0	18	32	290	2.42	—	4.45	3.61	0.84
NaCl (66 mM)	1.3	8.1	3	18	33	380	2.39	—	4.78	3.56	1.22
Tris acetate (66 mM)	1.3	7.0	0	18	54	7	1.81	0.001	4.21	2.70	1.51
Tris acetate (66 mM)	1.3	7.0	3	17	53	7	1.81	0.038	4.42	2.70	1.72
Tris acetate (66 mM)	1.3	8.1	0	16	38	10	1.83	0.001	4.31	2.73	1.58
Tris acetate (66 mM)	1.3	8.1	3	15	30	10	2.01	0.004	4.41	3.00	1.41
Potassium acetate (66 mM)	0	7.0	3	16	411	—	—	0.111	5.10	—	—
Potassium acetate (66 mM)	1.3	7.0	0	16	235	—	1.85	0.003	4.27	2.76	1.51
Potassium acetate (66 mM)	1.3	7.0	3	13	412	—	2.74	0.113	5.94	4.09	1.85
Potassium acetate (66 mM)	0	8.1	3	17	332	—	—	0.085	4.88	—	—
Potassium acetate (66 mM)	1.3	8.1	3	14	281	—	2.98	0.081	4.49	4.45	0.04
Isotonic sodium acetate	1.3	7.1	0	16	43	700	2.39	0.065	7.40	3.57	3.83
Isotonic sodium acetate	1.3	7.1	3	14	25	1100	3.32	0.350	10.75	4.97	5.78
Isotonic sodium acetate	1.3	8.1	0	16	30	1210	3.42	0.075	7.31	5.12	2.19
Isotonic sodium acetate	1.3	8.1	3	12	16	1600	5.65	0.375	9.35	8.43	0.92

The data of Table III establish that the uptake of Na^+ is increased by EDTA at both pH 7.1 and 8.1 and that in both cases swelling as monitored both by absorbance at 546 mμ and by tritiated water distribution is increased.

Penetration of EDTA into mitochondria

Since the amounts of Mg^{2+} removed from the mitochondrion by EDTA were seldom large and seemed to have rather specific requirements, the possibility that the observed effects of EDTA on mitochondrial cation uptake were the result of a binding of EDTA to the membrane was investigated. The penetration of ^{14}C-labeled EDTA into mitochondrial water in a medium of 0.12 M Na^+ acetate at pH 7.0 is shown in Fig. 2A. Under these conditions in the absence of respiration there is a slow passive penetration of Na^+ and acetate into the mitochondrion[12,14] and an osmotic swelling. The bar graph shows that the total water content of a mitochondrial pellet under these conditions is 5.0 μl per mg of protein. Of this volume 1.4 μl/mg is penetrated by [^{14}C-]-

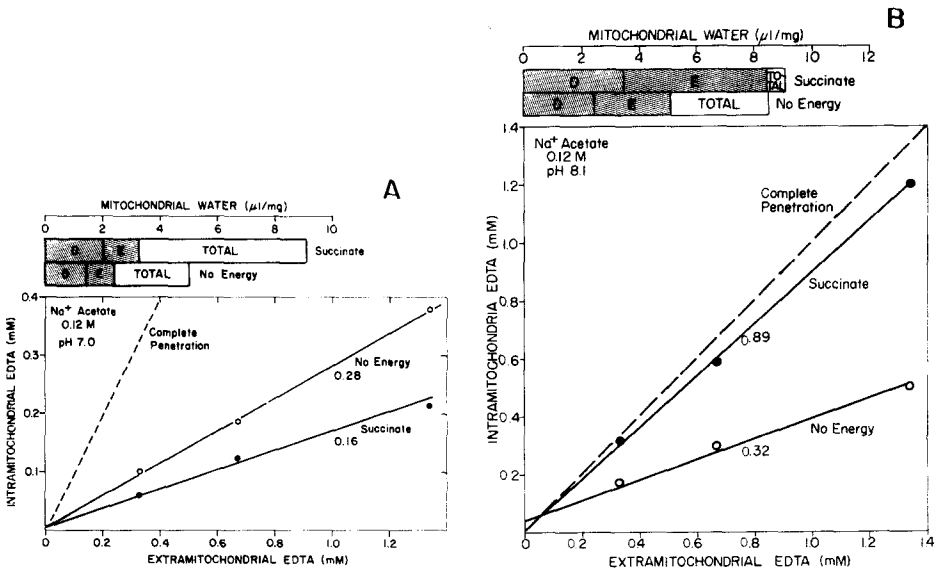

Fig. 2. A. Penetration of EDTA into heart mitochondrion at pH 7.0. Mitochondria (10 mg of protein) were treated with rotenone (4 mμmoles/mg) and suspended for 3 min at 25° in 6 ml of a medium of Na^+ acetate (0.12 M) and Tris chloride (10 mM, pH 7.0) containing tritiated water and the indicated concentrations of ^{14}C-labeled EDTA. The mitochondria were isolated by centrifugation (Sorvall SE-12 rotor, 20000 rev./min for 5 min), extracted with $HClO_4$ and the ^3H and ^{14}C radioactivity of the residue and the supernatant was determined with a liquid scintillation counter[14]. In parallel incubations unlabeled EDTA was used and ^{14}C-labeled carboxydextran (New England Nuclear, mol. wt. 60000–90000) was included to estimate the extraparticulate water space of the pellets. The total water, dextran-permeable water, and EDTA-permeable water were calculated by comparing the specific activities (disint./min per μl) of the supernatants with those of the residues as described by HUNTER AND BRIERLEY[14]. The bar graph compares the values found in mitochondria isolated under these conditions in the absence of a source of energy with the corresponding volumes obtained in the presence of succinate (3 mM) respiration. Also plotted is the intramitochondrial EDTA concentration (corrected for the EDTA of the dextran-permeable volume[15]) as a function of the concentration in the suspending medium. The slope of such a plot gives an indication of the percent of intramitochondrial water in equilibrium with the suspending medium and the intercept may be taken as a value for bound EDTA[15]. B. Penetration of EDTA into heart mitochondria at pH 8.1. The experiment was carried out as described for A except at pH 8.1.

carboxydextran of 60 000–90 000 molecular weight. This volume has been taken to be the value of the extramitochondrial solvent occluded in the centrifuged pellet[15]. [^{14}C]EDTA penetrates a total of 2.4 μl/mg under these conditions. Therefore about 1.0 μl/mg of the intramitochondrial water volume is penetrated by EDTA and about 2.6 μl/mg is not penetrated by this solute. When a plot of the intramitochondrial concentration of EDTA (corrected for EDTA and water in the dextran-permeable space) is made against the EDTA concentration of the suspending medium (Fig. 2A) under these conditions, a straight line is obtained which extrapolates very nearly to the origin. This result is consistent with EDTA penetration as a strict function of the concentration in the external medium into a compartment within the mitochondrion. The slope of the line would indicate that about 28 % of the intramitochondrial water is penetrated by EDTA under these conditions. Parallel evaluation of the sucrose and mannitol-permeable space under these conditions established that EDTA penetrates to the same extent as these solutes. The fact that the line of Fig. 2A extrapolates to the origin indicates that little, if any, EDTA is bound by the membrane. In the case of solutes such as Mg^{2+} a similar plot intersects the ordinate at a value very close to the amount of Mg^{2+} adsorbed to washed mitochondria[15]. In addition virtually all of the [^{14}C]EDTA is removed by a single wash in isotonic sucrose.

In the presence of succinate respiration the accumulation of Na^+ and acetate is accelerated and is accompanied by more extensive swelling[12]. Under these conditions the water volume of the packed pellets increases to 9.1 μl/mg, the dextran space to 2.0 μl/mg and the amount of water permeable to EDTA is 3.3 μl/mg (Fig. 2A). This further emphasizes that EDTA is excluded from the compartment within the mitochondrion which is expanding due to the uptake of salt and water. The plot of intramitochondrial EDTA concentration *vs.* the concentration of chelator in the suspending medium now shows that only 16 % of the intramitochondrial water is penetrated by EDTA. Again, as in the absence of energy, there is no indication of adsorption of the chelator and the degree of penetration corresponds well to that of mannitol.

When the corresponding experiment is carried out at pH 8.1 in the absence of energy there is considerably more swelling and uptake of Na^+ and acetate than is seen at pH 7.0. About 30 to 40 % of the intramitochondrial water is penetrated by EDTA under these conditions. The plot of Fig. 2B does not extrapolate exactly to zero but still indicates very little, if any, bound EDTA. A maximum value for bound EDTA from the plot shown would be about 0.3 mμmole/mg of protein. In the presence of respiration at pH 8.1 there is only a slight additional increase in total water but a striking increase in EDTA penetration. Under these conditions 90 % or more of the intramitochondrial water is penetrated by the chelator. This more complete penetration of EDTA into the mitochondrion corresponds well with the increased ability of EDTA to remove Mg^{2+} from the mitochondrion under these conditions (*cf.* Tables I and III).

A similar situation prevails in the 100 mM sucrose, 60 mM Na^+ acetate medium described previously. The volume of water which remains impermeable to EDTA decreases from about 1.4 μl/mg at pH 7.0 to nearly zero at pH 8.1 in this medium in the presence of respiration (Table III). No such change is seen in the Na^+ acetate medium in the absence of energy, or in the NaCl or Tris acetate media, however. In the case of the latter two media the amount of water which is inaccessible to EDTA remains between 0.9 and 1.5 μl per mg and does not increase in the presence of respi-

ration at high pH. Increased EDTA penetration occurs at high pH in the K^+ medium, however, and under these conditions the mitochondrial Mg^{2+} is lowered from 17 to 14 mμmoles per mg of protein. It appears therefore that the accumulation of ions and osmotic swelling at high pH are conducive to the penetration of EDTA into the interior (probably into the matrix[14,16]) of the mitochondrion and that this penetration results in the removal of an additional increment of Mg^{2+} from the membrane.

Osmotic response of mitochondria suspended in EDTA

Mitochondria respond as osmometers[17–19] when suspended in a medium containing non-permeable solutes. The data of Fig. 3 establish that a typical osmometer response is obtained when mitochondria are suspended in varying concentrations of Na^+ EDTA both at pH 7.0 and 8.1. The plots are not altered by the addition of

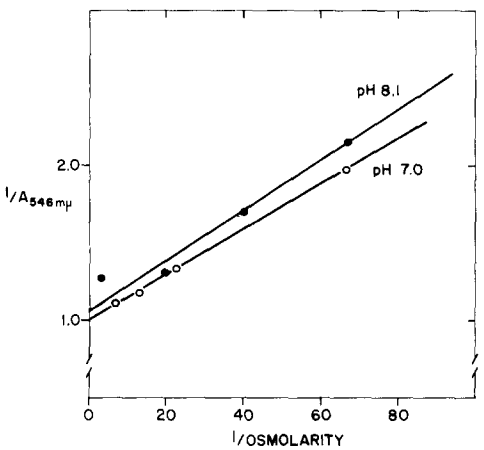

Fig. 3. Osmotic response of heart mitochondria suspended in Na^+ EDTA. Mitochondria (1.2 mg of protein) were added to 3 ml of Na^+ EDTA of the indicated osmolarity (calculated) and the absorbance at 546 mμ was recorded using the Eppendorf photometer.

succinate as a source of energy. Since Na^+ appears to penetrate isolated heart mitochondria extensively[12] these results suggest that EDTA is acting as a non-permeant anion under all of these conditions. They further suggest that the extensive penetration of the mitochondrion by EDTA which occurs in the presence of respiration at pH 8 depends on the presence of the permeant acetate ion. The low permeability of EDTA at pH 8 in the presence of respiration in a NaCl medium (Table III) also supports this suggestion.

Passive permeability of mitochondria which have been depleted of Mg^{2+}

A number of experiments were carried out to determine whether mitochondria which had been treated with Na^+ acetate, EDTA, and a source of energy so as to lower the endogenous levels of Mg^{2+} and K^+ showed altered permeability in a second incubation. No consistent increase in permeability to Na^+ in the absence of a source of energy could be detected either by the criterion of passive swelling in Na^+ acetate or by direct analysis of centrifuged pellets. Particles which had been treated at pH 8.1 to remove Mg^{2+}, washed, and then resuspended were very refractory and showed little indication of energy-linked swelling and ion uptake in the second incubation. It should

be noted that these mitochondria respired poorly with most substrates with the exception of ascorbate–TMPD. Control particles treated in the same way but with the omission of EDTA also respired poorly but responded to EDTA in the expected way during the second incubation. It appears, therefore, that the passive permeability to Na^+ in subsequent incubations is not affected by the removal of Mg^{2+} under the specific conditions described. The rate of oxidation of most of the substrates employed is activated by EDTA and it appears possible that the major effect of Mg^{2+} removal is not on permeability *per se* but on the coupling of respiration to ion transport.

DISCUSSION

Isolated heart mitochondria accumulate Na^+ and acetate spontaneously and swell when supplied with a source of energy at neutral pH. In addition to a supply of energy the reaction requires the presence of a rather high concentration of Na^+ and a permeant anion such as acetate. In agreement with previous reports[1–3] we find that addition of EDTA under these conditions markedly enhances the swelling and ion uptake and activates respiration. The present study establishes that this enhanced Na^+ uptake and swelling are closely related to the Mg^{2+} content of the membrane. The conditions for the removal of significant amounts of Mg^{2+} from the membrane parallel the requirements for the energy-linked uptake of Na^+ remarkably. In addition to the presence of rather high concentrations (about 0.5 mM) of a chelator such as EDTA with a high affinity for Mg^{2+}, the removal of Mg^{2+} from the mitochondrion at pH 7.0 requires the presence of a source of energy, high concentrations of Na^+, and a permeant anion. The Na^+ requirement for Mg^{2+} removal can be met by Li^+ and to a variable degree by K^+ but not by Tris, or choline$^+$. Li^+ and K^+ are accumulated by mitochondria by an energy-linked reaction under these conditions and the uptake of Li^+ is activated by EDTA. Tris and choline$^+$ do not appear to be accumulated under these conditions and the uptake of K^+ is not consistently affected by the addition of EDTA. It appears, therefore, that when EDTA is effective in removing a portion of the membrane Mg^{2+} it also brings about an activation of ion uptake. It must be emphasized, however, that Mg^{2+} is removed only under the conditions of energy-linked ion uptake.

The actual amount of Mg^{2+} removed under these conditions is variable and can be quite small (2 to 3 mμmoles of Mg^{2+} per mg of protein) in some preparations which show a large enhancement of energy-linked Na^+ uptake and swelling upon addition of EDTA. The fact that reasonably large amounts of Mg^{2+} (approx. 10 mμmoles per mg) are not removed by EDTA even under optimal conditions suggests that two or more different types of bound Mg^{2+} are present in the membrane. Studies now in progress show that submitochondrial particles also retain about 6–10 mμmoles of Mg^{2+} per mg even when treated with EDTA under optimal conditions[20]. The fact that added Mg^{2+} in quantities considerably lower than the molar concentration of EDTA can overcome the effect of EDTA on Na^+-dependent swelling and on the removal of Mg^{2+} from the membrane indicates that the Mg^{2+} which affects the rate of Na^+ uptake has a higher affinity for its binding sites in the membrane than for EDTA.

An extensive loss of endogenous K^+ from the mitochondrion is also closely related to the amount of Mg^{2+} removed by EDTA in these experiments. Since this K^+ movement is in the direction of the concentration gradient the loss of this cation may

reflect an increased passive permeability to K^+ as a function of the Mg^{2+} level of the membrane. The failure of EDTA to stimulate K^+ accumulation consistently in these experiments may be due to the diminished ability of the chelator to remove Mg^{2+} from the membrane in a medium containing K^+ as opposed to Na^+.

Studies of the distribution of ^{14}C-labeled EDTA establish that the chelator is excluded from the bulk of the intramitochondrial water at neutral pH. In contrast to earlier statements in the literature[19] we find no evidence for the binding of significant amounts of EDTA to the mitochondrial membrane. EDTA appears to be freely permeable to a portion of the mitochondrial water volume and the volume penetrated corresponds well with that penetrated by sucrose and mannitol. Recent studies indicate that the matrix of the mitochondrion is the morphological equivalent of the sucrose-impermeable compartment[14,16]. It appears, therefore, that EDTA, like many other solutes, is unable to pass the inner membrane of the mitochondrion under the conditions in which it activates the accumulation of Na^+ and swelling. The use of EDTA to evaluate extramitochondrial water has been reported[21]. However, present studies indicate that the EDTA space corresponds more closely to the sucrose-permeable space than to the extraparticulate space. The ability of EDTA to inhibit mitochondrial myokinase (see ref. 22, for example) can be readily explained by recent studies[23] which localize this enzyme activity in the space between membranes of the mitochondrion.

At pH 8.0 and above the addition of EDTA to suspensions of heart mitochondria respiring in a medium containing Na^+ and acetate induces a rapid swelling which is followed by contraction and a tendency to oscillate[3]. This aspect of the reaction has been documented extensively by PACKER and coworkers[3,24,25]. The present study establishes that the requirements for Mg^{2+} removal from the membrane at pH 8.0 and above parallel those at neutral pH. Considerably more Mg^{2+} is removed under these conditions and virtually all of the endogenous K^+ is lost when the Mg^{2+} content is lowered. The removal of Mg^{2+} by EDTA at elevated pH still requires the presence of (a) Na^+ or Li^+, (b) a permeant anion, and (c) a source of energy. The present study brings out an additional aspect of the interaction of EDTA with the membrane at high pH. At pH 8 the permeability of the mitochondrion to EDTA is enhanced considerably, and under the conditions used by PACKER, UTSUMI AND MUSTAFA[3] to obtain oscillations of various mitochondrial parameters, the chelator penetrates virtually the entire water volume of the mitochondrion. Whether the presence of EDTA in the interior compartment and the subsequent removal of Mg^{2+} from the inside of the inner membrane can provide an explanation for the induction of the oscillatory state under these conditions remains to be established. AZZI AND AZZONE[26] have shown that raising the pH of the medium increases the permeability of the mitochondrion to anions. The conditions necessary for penetration of EDTA into the matrix, however, appear identical to those which produce the oscillations. The pH must be elevated to 8 or above, there must be a permeant anion and a transported cation present, and there must be a source of energy in order for EDTA to penetrate the mitochondrion completely. These conditions correspond to those necessary to obtain the oscillatory state[3] and as noted above, result in the removal of an additional increment of Mg^{2+} and more extensive loss of K^+.

Several aspects of the present work appear perplexing. The requirement for energy-linked ion transport in order to bring about the removal of membrane-bound

Mg^{2+} by a chelator hints that the membrane or portions of the membrane may undergo conformational changes which expose the Mg^{2+} binding sites. Several alternative suggestions could be advanced but further work appears necessary in order to provide a completely satisfactory explanation. The apparent inability of the mitochondrion to retain an increased permeability to monovalent cations through a second incubation even though the Mg^{2+} content of the membrane is quite low also requires explanation. As mentioned above it appears possible that one of the major effects of Mg^{2+} removal is on the rate of respiration rather than on the permeability to cations. Further studies are in progress in an attempt to clarify this point. It should be noted that, in contrast to the results of the present study, studies of the effect of EDTA on Coliform bacteria[27] indicate a major change in passive permeability but little change in active transport as a result of the interaction. It has been suggested that the effects of Mg^{2+} removal can be reversed by steric or chemical changes requiring a source of energy[27]. Whether similar considerations apply to the mitochondrion also remains to be established.

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