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Sr^{2+} UPTAKE BY BEAN (*PHASEOLUS VULGARIS*) MITOCHONDRIA

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SUMMARY

1. The properties of Sr^{2+} uptake in bean mitochondria are examined and compared with selected properties of Sr^{2+} uptake in rat liver mitochondria. Uptake with plant mitochondria was dependent on the presence of substrate and P_i and is largely inhibited by 2,4-dinitrophenol or an ATP-generating system.

2. Uptake in bean mitochondria lacks the concomitant extrusion of H^+ or transient stimulation of respiration seen in rat-liver mitochondria. Under appropriate conditions both Sr^{2+} and Ca^{2+} induce a stimulated respiration, but this stimulation is not associated with the uptake of salt.

3. Of a series of anions tested including P_i, arsenate, acetate, nitrate, malate, oxalate and carbonate, only P_i supports divalent cation uptake. Yet of the anions examined, including P_i, arsenate, chloride, acetate and nitrate, all induced metabolically independent mitochondrial swelling indicating that the membrane is permeable to these anions. In addition, it was shown that P_i, arsenate, and acetate induce an active or metabolically dependent swelling. Thus the specificity of P_i in supporting divalent cation uptake does not reside in its permeability.

4. It is concluded that both the specificity as well as stoichiometry of divalent cations and P_i in uptake is consistent with a transport mechanism involving the formation of a metal:P_i complex at the transport site. The formation of the complex triggers the delivery of salt into the mitochondrial interior.

INTRODUCTION

Studies with both plant and animal mitochondria have established that oxidative phosphorylation supports the accumulation of a large number of ionic species. While detailed studies with rat liver or beef heart mitochondria have been reported for divalent cation transport systems involving Ca^{2+}, Mg^{2+}, Sr^{2+}, Mn^{2+} and Zn^{2+} (refs 1–6), plant mitochondrial studies have largely been confined to Mg^{2+} and Ca^{2+} (refs 7–10).

With mammalian mitochondria it has been concluded that an active transport mechanism exists for the accumulation of divalent cations. This conclusion is based primarily on the studies of Ca^{2+} transport, which demonstrate that the presence of Ca^{2+} induces a transient stimulation of respiration with a concomitant accumulation of divalent cations in the absence of any specifically required

anion[2,5,11–13]. When P_i or other so-called permeant anions are added, they are thought to move passively in response to the distribution of cations[14,15]. Under selected conditions of pH and salt-content accumulation of Ca^{2+} results in H^+ ejection with a ratio of Ca^{2+}/H^+ of approximately 1.0[2,13,16]. Studies with Sr^{2+} uptake in rat liver mitochondria show several characteristics similar to Ca^{2+} uptake including a respiratory jump and H^+ ejection under limited loading conditions[1,17].

With plants, beet root mitochondria have the capacity to accumulate large amounts of Mg^{2+} and P_i which has been interpreted to suggest the existence of an active Mg^{2+} transport system perhaps in exchange with H^+ similar to animal mitochondria[8,18]. P_i uptake was considered of secondary importance[18]. In comparison, the accumulation of Ca^{2+} in maize mitochondria was found to be critically dependent on the presence of P_i which has been interpreted to indicate that P_i is actively transported and that Ca^{2+} uptake follows P_i to deliver Ca^{2+} and P_i into the mitochondria[11,19,20].

In this study we have examined the uptake of Sr^{2+} and other divalent cations in bean mitochondria. The results show no transient respiratory jump or detectable H^+ efflux on the addition of Sr^{2+}. Moreover, with the exception of P_i, a series of anions which passively penetrate mitochondrial membranes did not support either Ca^{2+} or Sr^{2+} accumulation. The results are consistent with the studies reported with Ca^{2+} uptake in maize mitochondria and are interpreted to support the conclusion that salt transport is specifically dependent on the formation of a complex composed of a divalent cation and P_i (M^{2+}:P_i) which is the actively accumulated species.

METHODS

Isolation

Mitochondria were isolated from 50 g of 6–7-day-old etiolated bean hypocotyls (*Phaseolus vulgaris* L. var. Kentucky Wonder Pole) by grinding in a cold mortar with 100 ml of medium containing: 0.4 M mannitol, 50 mM Tricine buffer (pH 7.5), 4.0 mM $MgCl_2$ and 1.0 mg/ml bovine serum albumin. The homogenate was strained through a 45-μm nylon mesh and centrifuged at 1100 $\times$ g for 5 min. The supernatant was decanted into cold tubes and centrifuged at 7700 $\times$ g for 10 min. The resulting pellet was resuspended in 10 ml of grinding medium and centrifuged at 1100 $\times$ g for 5 min. The supernatant was decanted into cold tubes and centrifuged at 17 300 $\times$ g for 5 min. The final pellets were suspended in 2.0 ml of the grinding medium and stored at ice temperature.

Rat liver mitochondria were isolated by differential centrifugation according to the procedure of Johnson and Lardy[21] in a medium of 0.25 M sucrose *plus* 10 mM Tris buffer (pH 7.2). Corn mitochondria were isolated by the procedure of Kenefick and Hanson[20] without the use of a wash layer on the terminal centrifugation. Protein was determined by the method of Lowry *et al.*[22] with albumin as a standard.

Respiration and ion determination

The standard procedure used for studies of ion uptake involved incubating mitochondria for 10 min in a medium containing: 0.4 M mannitol, 50 mM Tricine buffer (pH 7.5) and 1 mg/ml albumin with modification as indicated in the legends. Samples were collected on a Swinnex-25 filter unit with 0.45-μm Millipore filters,

and washed with three 5.0-ml aliquots of 0.6 M mannitol. The mitochondria were broken open by resuspending in 4.0 ml of 5.0% trichloroacetic acid containing 1.0% La_2O_3 and 5.0% HCl. The mitochondrial fragments were removed by centrifugation at 17300 × *g* for 15 min. Divalent cations were determined with a Perkin–Elmer Model 303 atomic absorption spectrophotometer. P_i was measured by the Fiske–SubbaRow[23] method. Oxygen measurements were made with a Beckman oxygen electrode, Beckman Model 160 gas analyzer and a Sargent recorder using a 3.2-ml constant temperature glass cuvette. Measurements of pH changes were made with a Sargent Model DR pH meter and Sargent recorder Model DSRG. Absorbance changes were followed on a Beckman DU spectrophotometer with Gilford attachments.

RESULTS

General properties of Sr^{2+} accumulation

Table I shows a series of conditions which affect the uptake of Sr^{2+} with freshly isolated bean mitochondria. The uptake supported by succinate was between 250 and 300 nmoles/mg of protein which is comparable with previously reported values for Ca^{2+} uptake with peas[24] and Mg^{2+} uptake with beet root mitochondria[8], but lower than those recorded for rat liver[5] and maize mitochondria[9]. The uptake was reduced by dinitrophenol and an ATP-generating system but not oligomycin. Sr^{2+} uptake was largely inhibited in the absence of P_i, although a small net accumulation (less than 20 nmoles/mg of protein) was consistently observed. Since low levels of divalent cations are accumulated in rat liver mitochondria in the absence of P_i, we examined further the properties of the uptake observed here.

Fig. 1 shows the uptake of Sr^{2+} increases with the concentration of Sr^{2+} up to about 20–30 μM $SrCl_2$. This uptake is sensitive to cyanide and antimycin A and

TABLE I

GENERAL CHARACTERISTICS OF Sr^{2+} UPTAKE INTO BEAN MITOCHONDRIA

The complete reaction mixture of 6.4 ml contained 0.4 M mannitol, 50 mM Tris–Tricine (pH 7.5), 1 mg/ml albumin, 5 μM rotenone, 2.0 mM $SrCl_2$, 2.0 mM Tris phosphate and 8 mM Tris succinate. Protein was 0.80 mg.

Medium	*Sr^{2+} uptake (μmoles/mg protein)*		*% of maximum*
	Total uptake	*Net uptake**	
Complete	0.510	0.267	100
Complete + 3 mM ATP	0.502	0.259	97
–8 mM succinate	0.243	—	0
–8 mM succinate, $-P_i$	0.243	0.000	0
$-P_i$	0.258	0.015	6
+ 100 μM 2,4-dinitrophenol	0.278	0.035	13
+ 2 μg/ml oligomycin	0.498	0.255	95
+ 196 μM ADP *plus* hexokinase trap**	0.300	0.057	22

* Net Sr^{2+} uptake is the difference between uptake in the presence *versus* absence of substrate.
** 0.5 mg hexokinase (Sigma) + 25 mM glucose.

TABLE II

Sr^{2+} UPTAKE IN THE ABSENCE OF ADDED P_i

The complete reaction mixture is identical to that given in Table I with the exception of the 50 μM $SrCl_2$ and the absence of Tris phosphate. Volume was 12.4 ml. Protein was about 2.0 mg. The results are the average of duplicate runs.

Medium	*Sr^{2+} uptake (μmoles/mg protein)*	
	Total uptake	*Net uptake*
Expt I		
Complete	0.047	0.013
-succinate	0.034	—
Expt II		
Complete + ADP preincubation *	0.039	−0.001
-succinate + ADP preincubation *	0.040	—
Complete + 20.0 mM acetate ADP preincubation *	0.035	−0.001
-succinate	0.036	—
Expt III		
Complete + 2.0 mM KCN	0.026	0.000
-succinate + 2.0 mM KCN	0.026	—
Expt IV		
Complete + 2 μM antimycin A	0.043	−0.015
-succinate + 2 μM antimycin A	0.058	—

* The mitochondria were incubated for 12 min with 324 nmoles ADP and 270 nmoles NADH prior to adding Sr^{2+} and succinate where indicated.

is eliminated by preincubation with ADP and an exhaustible supply of NADH added previous to Sr^{2+}. These results implicate endogenous P_i as a possible source of accompanying anion for Sr^{2+} uptake. The endogenous P_i level was measured as 125 nmoles/mg of protein (Table II).

Fig. 2 shows that maximum Sr^{2+} uptake occurred at alkaline pH. It was found that ATP in concentrations from 1.0 to 15.0 mM did not support uptake. It has been observed that high concentrations of sucrose[25,26] or mannitol inhibit ATPase activity of plant mitochondria. 5 mM ATP supported between 10 and 20 % of the maximum uptake observed with oxidizable substrates when the concentration of mannitol was lowered to 50 mM, but not when the osmoticant was 100 mM mannitol or 100 mM KCl. While it is unclear why ATP did not support Sr^{2+} uptake at levels comparable with those obtained with respiratory substrates, it is interesting that water-lysed rat liver mitochondria lose their capacity for ATP supported Sr^{2+} uptake, but not Ca^{2+} uptake[35].

Respiration and proton gradients

With rat liver mitochondria Carafoli[17] has shown that Sr^{2+} uptake in the absence of P_i results in a transient stimulation of respiration which is proportional in duration to the amount of Sr^{2+} added. The uptake is associated with the ejection of H^+ (Fig. 3A). Bean mitochondria lack both a transient stimulation of respiration

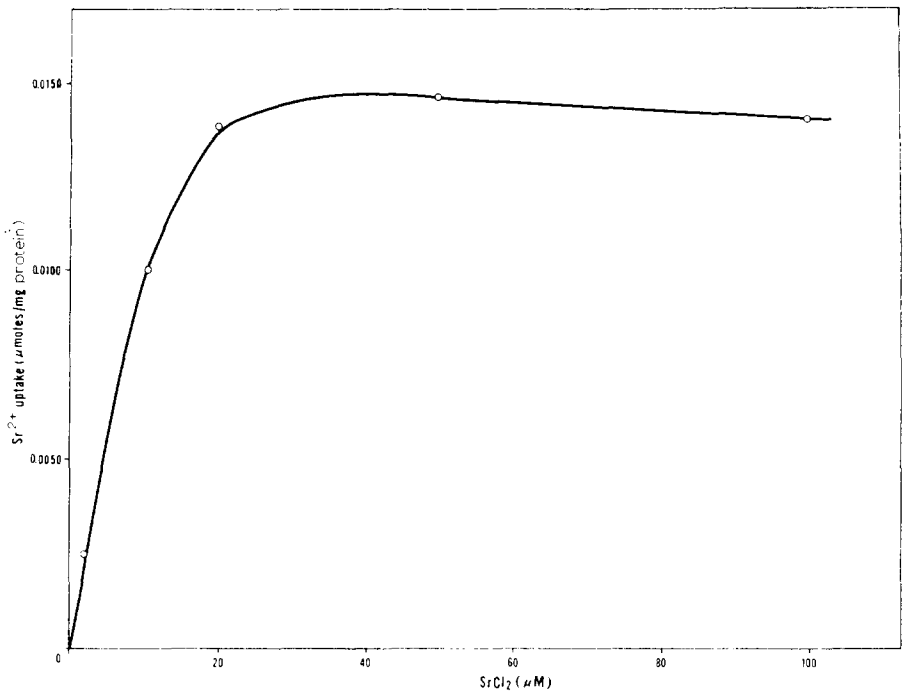

Fig. 1. The uptake of Sr^{2+} in the absence of added P_i. The conditions were identical with those in Table I with the exception that the concentration of $SrCl_2$ was 50 μM and there was no Tris phosphate added. Volume was 12.4 ml. Protein was 1.6 mg.

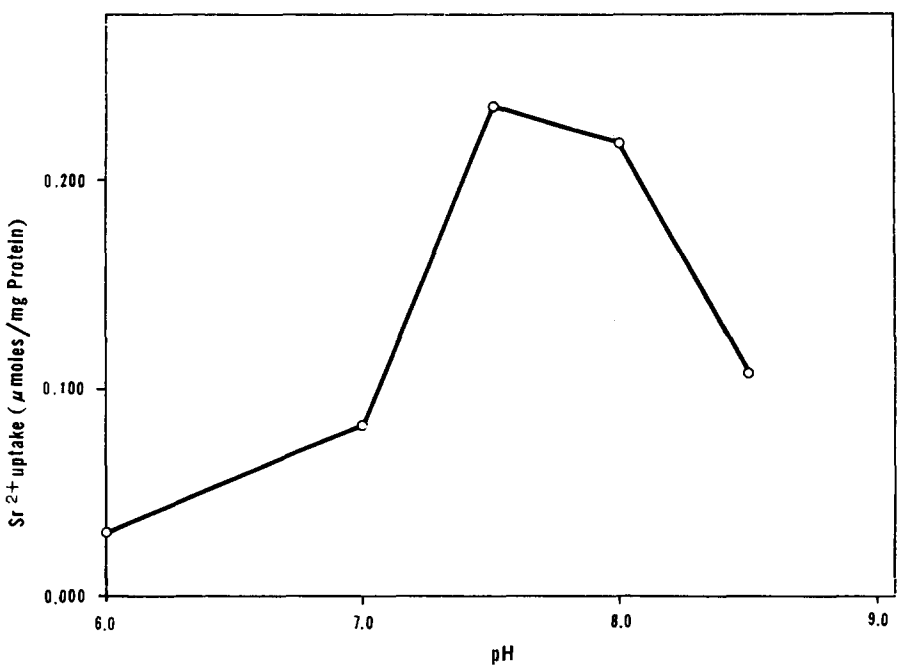

Fig. 2. Effect of pH on Sr^{2+} uptake. The conditions were identical with those in Table I with the exception that Tris and Tricine concentrations were varied to obtain the desired pH. Protein was 1.2 mg.

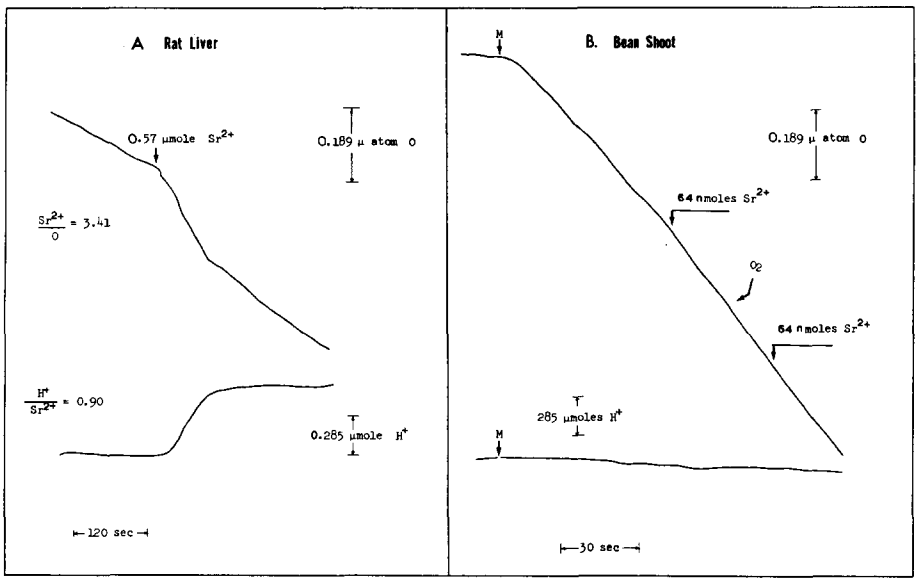

Fig. 3. Respiration and H^+ ejection with rat liver and bean shoot mitochondria. (A) The reaction mixture of 4.3 ml contained: 0.23 M sucrose, 5 mM $MgCl_2$, 10 mM Tris phosphate, 5.0 μM rotenone, 8.0 mM succinate and 9.5 mg rat liver protein. The initial pH was 6.89. (B) The reaction mixture contained 0.4 M mannitol, 5.0 mM $MgCl_2$, 5.0 μM rotenone, 8.0 mM Tris succinate, 5.0 mM Tris–Tricine (pH 6.85), 4.8 mg bean mitochondrial protein. Temp., 27 °C.

on the addition of Sr^{2+} as well as a release of H^+ (Fig. 3B). Addition of Sr^{2+} up to 1 μmole failed to stimulate respiration or the ejection of H^+ under these conditions. Recently studies with blowfly flight muscle mitochondria demonstrated a similar lack of respiratory stimulation or of H^+ ejection with Ca^{2+} uptake[27].

Table III summarizes the results of the respiratory rates observed when divalent cations are added to the reaction mixture with and without P_i present. While stimulation of respiration was observed with both bean and corn mitochondrial preparations under appropriate conditions, they were neither as extensive nor showed the transitory nature seen with rat liver[17]. Stimulation of succinate respiration by both Ca^{2+} and Sr^{2+} was dependent on the presence of P_i and the previous addition of ADP, whereas stimulation of respiration with externally added NADH occurred without either P_i or ADP present. When KCl replaced mannitol as the osmoticant, divalent cations partially inhibited respiration with bean mitochondria. It was observed that KCl resulted in lower respiratory control and coupling, in comparison with maize mitochondria[28]. The significance of the respiratory stimulations is not clear, although they do not appear to be related to the uptake of salt, since they are seen under conditions where no uptake was measured. Moreover, the addition of 2.0 mM Ca^{2+} or Sr^{2+} in the absence of P_i has no effect on volume changes as measured by light scattering under conditions where respiration is stimulated.

Effect of anions and divalent cations on uptake

Of the anions tested only P_i supported significant levels of net Sr^{2+} uptake (Table IV). The ratio of Sr^{2+}:P_i was close to 1.4 which is comparable with a ratio

TABLE III

RESPIRATORY STIMULATION BY Ca^{2+} AND Sr^{2+}

Mitochondrial source	*Reaction media**	*Substrate***	*Respiratory control*	*ADP/O*	*Net % respiratory stimulation****			
					Without P_i		*With 2.5 mM* P_i	
					10 mM Sr^{2+}	*2.0 mM* Ca^{2+}	*10 mM* Sr^{2+}	*2.0 mM* Ca^{2+}
Bean	Mannitol	8.0 mM succinate	2.6	1.7	0	0	40§	45§
		1.5 mM NADH	2.9	1.2	50	40	140	60
	KCl	1.5 mM NADH	1.8	1.1	−30	−10	−30	−35
Corn	Mannitol	8.0 mM succinate	2.1	1.5	0	0		50§
		1.5 mM NADH	2.5	1.3	75	140	50	115
Rat liver	Sucrose	8.0 mM succinate	3.8	1.9	340§§		410§§	

* The composition of mannitol reaction media is the same as in Table I. The KCl media contained 0.2 M KCl, 1 mg/ml albumin, 5 mM $MgCl_2$ and 20 mM Tris–Tricine (pH 7.5). The sucrose media contained 0.23 M sucrose and 5.0 mM Tris–Tricine (pH 7.2). Protein was 0.80 mg. Volume was 3.2 ml.

** 2.0 μg rotenone was added with succinate.

*** Percentage respiratory stimulation based on the net increase in respiration rate over the rate in the absence of the divalent cation (State IV) times 100.

§ Required the previous addition of ADP (250 nmoles).

§§ P_i concentration equal to 1.28 mM.

TABLE IV

THE EFFECT OF A SERIES OF DIFFERENT SALTS ON Sr^{2+} UPTAKE

The complete reaction mix and volume is the same as given in Table I with the exception of the different anion of Sr^{2+} as shown. Protein was 0.87 mg.

Anion	*Concn (mM)*	*Net Sr^{2+} uptake (μmoles/mg protein)*	*Net P_i uptake (μmoles/mg protein)*	*$*Sr^{2+}/P_i$*
Phosphate	2	0.220	0.158	$1.38 \pm .12$
Acetate	2	0.007	—	
	20	0.002	—	
Arsenate	2	0.002	—	
Nitrate	2	0.002	—	
Malate	2	0.005	—	
Oxalate	2	0.006	—	
Carbonate	2	0.006	—	

* The average of five experiments.

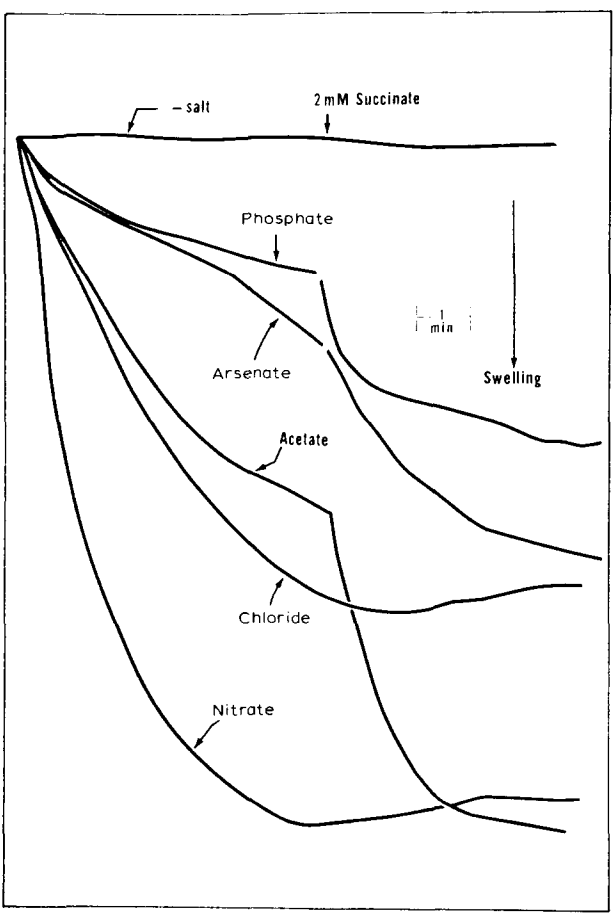

Fig. 4. Volume changes of mitochondria in different anion solutions. The *minus* salt solution contained: 0.3 M mannitol, 3 mM $MgCl_2$, 33 mM Tris–Tricine (pH 7.5), 0.66 mg/ml albumin. The *plus* salt solution contained: 0.2 M mannitol, 2 mM $MgCl_2$, 25 mM Tris–Tricine (pH 7.5), 0.5 mg/ml albumin and 128 mM potassium salts as shown. The osmolality of the above solution was between 390 and 410 mosmoles/kg. Total protein was 0.84 mg.

TABLE V

INHIBITION OF Sr^{2+} AND Ca^{2+} UPTAKE BY DIVALENT CATIONS

Media was identical with Table I except for the salt as shown. Protein was 0.75 mg.

Salt	*Concn (mM)*	*Net Sr^{2+} uptake (μmoles/mg protein)*	*Inhibition (%)*	*Net Ca^{2+} uptake (μmoles/mg protein*	*Inhibition (%)*
None		0.254	—	0.187	—
$BaCl_2$	2.0	0.096	62	0.086	54
$CaCl_2$	2.0	0.147	42	—	—
$SrCl_2$	2.0	—	—	0.125	33
$MgCl_2$	2.0	0.198	22	0.149	20

of 1.2–1.4 reported for rat liver mitochondria[1]. The K_m for P_i uptake was 0.12 mM. An examination of a series of anion salts shows that P_i was not unique in its capacity to induce either active or passive swelling (Fig. 4). Thus, the permeability of the membrane to the accompanying anion does not appear to be the critical factor in the support of Sr^{2+} uptake with plant mitochondria.

As shown in Table V, the uptake of Sr^{2+} is differentially inhibited by a series of divalent cations. Ba^{2+} inhibited most extensively at concentrations ranging from 0.5 to 2.0 mM. Moreover the background level of Sr^{2+} in the presence of Ba^{2+} was reduced 60% from the background level in the absence of Ba^{2+} whereas Ca^{2+} and Mg^{2+} reduced the background level of Sr^{2+} less than 15%. While Ba^{2+} loading has not been examined, over 0.2 μmole P_i net uptake per mg protein was measured in the absence of added Sr^{2+} and Ca^{2+} but in the presence of 2.0 mM Ba^{2+}. Further studies are in progress to examine the uptake of this divalent cation with bean mitochondria.

Ca^{2+} inhibited Sr^{2+} uptake over 40% whereas Ca^{2+} uptake was inhibited by Sr^{2+} slightly less. In comparison, rat liver mitochondria appear to show a much stronger inhibition of Sr^{2+} uptake by Ca^{2+} and no inhibition of Ca^{2+} uptake by Sr^{2+} under comparable conditions[29]. Mg^{2+} was least effective of the ions in inhibiting uptake although a slight reduction of net uptake of both Sr^{2+} and Ca^{2+} was observed. No evidence of significant uptake of Mg^{2+} with or without P_i was found.

DISCUSSION

A major factor that distinguishes Sr^{2+} transport in plant mitochondria from transport in rat liver mitochondria is the lack of Sr^{2+} accumulation in the absence of P_i. Associated with this is the absence of a transient stimulation of respiration and H^+ ejection. Some of the same observations have been reported for Ca^{2+} loading with corn and mung bean mitochondria[20,31]. These distinctions indicate mitochondria from plants and vertebrate animal sources vary in critical properties of divalent cation transport and lend credence to the possibility that the mechanisms of transport differ depending on the mitochondrial source.

Since salt transport appears to be closely associated with the coupling mechanism in both plant and animal mitochondria, it may be relevant to point out that a basic difference in the coupling process of mitochondria from these two sources is the looser coupling generally seen in plant mitochondria as measured both by

respiratory control and ADP/O values (see Table III). The lower coupling in plant mitochondria may be due either to the technical difficulties associated with isolating organelles and maintaining their integrity from plant cells surrounded by rigid cell walls, or to an inherent looser coupling between oxidation and energy-transducing processes in plant mitochondria. Since extensive work has been concerned with improving the former, with little success relative to animal preparations, it appears necessary to accept the looser coupling condition in studying plant mitochondria.

With rat liver mitochondria it is generally concluded that the uptake of divalent cations and anions requires the presence of a permeant anion such as P_i or acetate in the reaction medium. During active cation uptake the passive accumulation of these anions is thought to maintain a charge balance across the inner mitochondrial membrane[2,5]. However, with bean mitochondria anions other than P_i, including acetate and arsenate, will not substitute for P_i in Sr^{2+} uptake (Table IV), yet these ions induce swelling both in the presence and absence of added substrate (Fig. 4.) Thus, the permeability of the membrane, to the anions present during Sr^{2+} transport, does not correlate with the ability to support active Sr^{2+} uptake.

The high specificity of P_i in supporting divalent cation transport shown here is consistent with an active transport carrier involving P_i. Hanson and Miller[29] have concluded that Ca^{2+} uptake in mitochondria is intimately dependent on the presence of P_i and that P_i is actively transported. They have suggested that a phorphorylated intermediate $X \sim P$ may be involved in the transport process[10,30]. Consistent with the hypothesis is the K_m of 0.12 mM for P_i uptake with Sr^{2+} which is in agreement with a K_m of 0.2 mM for P_i during oxidative phosphorylation in bean mitochondria[32].

It is evident that a specificity also exists for divalent cation uptake although the specificity shows a broader range than with P_i. Recently Sherry[33] has evaluated the underlying mechanism of selectivity for relative binding capacity of divalent cations in Group II of the alkaline earth metals. It was suggested that divalent cation selectivity resides in the difference between the free energy of cation site interaction and the cation free energy of hydration. From calculations of the free energy of exchange for closely spaced sites seven select series were predicted from a possible 24 permutations. Diamond and Wright[34] have summarized studies of diverse biological phenomena in which a substantial correlation between selectivity and one of the seven predicted sequences was observed. In the studies reported here Sequence I which is $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$, or Sequence II where the order of Sr^{2+} and Ca^{2+} is reversed conform with the order of relative effectiveness in inhibiting divalent cation uptake (Table V). These two sequences would be favored at weak binding sites, where the free energy of hydration dominates exchange and where the site spacing is relatively large[33].

In addition to the selectivity for divalent cations and P_i in the transport system studies here, it is apparent that the system requires a mechanism to regulate salt uptake so that a constant ratio of metal (M^{2+}) to P_i is maintained. These properties would all be met in a transport site which favored formation of a $M^{2+}:P_i$ complex with a fixed stoichiometry for the two ions. When the proper ratio of the $M^{2+}:P_i$ complex is formed, the system is poised to deliver salt into the mitochondrial internal matrix. A similar system has been recently suggested for calcification in bovine tendon matrix where a $Ca^{2+}:P_i$ complex is thought to form as an inter-

mediate stage in a sequence of reactions leading to Ca^{2+} and P_i deposition[36]. For each specific divalent cation that is transported, a ratio of $M^{2+}:P_i$ would be favored which presumably would depend on the free energy exchange values as well as steric consideration for the $M^{2+}:P_i$ binding site interaction.

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