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Calcium Accumulation by Maize Mitochondria [1, 2]

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A number of laboratories have reported that animal mitochondria can actively accumulate various inorganic ions (8). Energy can be supplied by either substrate oxidation or ATP. The antibiotic, oligomycin, inhibits ATP-driven Ca uptake, but not substrate-driven uptake (6, 18). On the basis of these findings, plus the observation that ADP also inhibits substrate-driven ion accumulation, Brierley et al. (5) suggest that a high energy intermediate of oxidative phosphorylation is a common energy source for ATP formation and for ion transport.

Inasmuch as these studies may be relevant to the problem of the connection between respiratory energy and ion transport in plants, we have conducted similar studies on the accumulation of inorganic ions by plant mitochondria. Earlier attempts to demonstrate an energy dependent accumulation of ions by plant mitochondria had only limited success (17, 21). In general, our results are similar to those obtained with animal mitochondria, and support the view that a high energy intermediate of oxidative phosphorylation participates in Ca, Mg and phosphate uptake. However, a few differences do exist. The uptake of phosphate, Mg and Ca^{45} is dependent upon the presence of Ca. Substrate-driven Ca and phosphate uptake does not require exogenous Mg. In addition, we have been unable to find Ca plus Mg: phosphate uptake ratios suggestive of hydroxyapatite formation (5, 6, 18, 22). Preliminary communications on phases of this work have appeared elsewhere (11, 13, 25).

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Materials and Methods

Isolation of Mitochondria. Corn seeds (*Zea mays* L., WF9 × M14) were germinated in the dark at 28° on paper towels saturated with 10^{-4} M $CaCl_2$. After 3 and one-half days, the shoots were excised, chilled, and ground in an ice-cold mortar with 0.25 M sucrose, 0.05 M KH_2PO_4 and 0.005 M EDTA, adjusted to pH 7.5 with Tris (hydroxymethyl) aminomethane. The slurry was strained through cheesecloth. Mitochondria were isolated in a refrigerated centrifuge as the fraction sedimenting between 2000 × *g* for 5 minutes and 12,000 × *g* for 10 minutes. The mitochondria were twice washed, first in the grinding medium, next in 0.25 M sucrose and were finally suspended in 0.25 M sucrose. The mitochondria were quite active, giving $QO_2(N)$ values of about 1500 and P/O values of about 2.5 when oxidizing a mixture of pyruvate and succinate in the absence of inhibitors or uncouplers. Procedures for determining oxidative phosphorylation have been described (12).

Procedures for Measuring Ion Uptake. The experiments were carried out at 28° (except for experiments where temperature was varied) in centrifuge tubes in a shaking waterbath. Unless otherwise noted, the reaction period was 10 minutes. Total volume of mitochondria plus additives was 2.5 ml. Except for the sucrose concentration of 0.25 M, buffering with Tris to pH 7.5 and 4-8 × 10^4 cpm Ca^{45} per tube the reaction mixtures varied, and are given with the experimental data. When pyruvate + succinate served as substrate, 0.1 μmole coenzyme A, 0.4 μmole thiamine pyrophosphate and 0.6 μmole NAD per tube were used. About 0.1 mg mitochondrial N was used per tube, except for experiments where total Mg and P were determined; here about 0.7 mg N per tube was used.

At the end of the experimental period, 5 ml of ice-cold 0.5 M sucrose was layered beneath the reaction mixture, the tubes were chilled for a few minutes in crushed ice, and the mitochondria centrifuged through the sucrose in a centrifuge set at −4°. (Washing the mitochondria in sucrose by suspension and resedimentation produced variable results, apparently from leaching of ions from the mitochondria.) The pellet was suspended in water for assay.

Analytical Methods. Calcium uptake was measured with Ca^{45}. Aliquots of the aqueous suspension were plated on planchets, dried, and counted with a gas flow counter. For the determination of calcium, magnesium and phosphate (table VII and fig 7) the entire mitochondrial pellet was wet-ashed with H_2SO_4 and H_2O_2, the acid solution was diluted with water, boiled for 10 minutes to hydrolyze pyrophosphates, neutralized with KOH, and made to 10 ml with water. Aliquots were used for determination of inorganic phosphate by the method of Fiske and Subbarow (10); Mg by the procedure described by Brierley, et al. (5); nitrogen by nesslerization; Ca by determination of Ca^{45}. Oxygen uptake was followed with the vibrating platinum electrode (Gilson Oxygraph).

Results

General Characteristics of Ca Uptake. The essential features of Ca accumulation as described for animal mitochondria are readily found with corn mitochondria. Table I gives a summary experiment showing succinate- and ATP-supported uptake, and the inhibition of both systems by 2,4-dinitrophenol (DNP). In these early experiments, ADP inhibited succinate-driven uptake (see later for exceptions). The combination of succinate and ATP produces more Ca uptake than either separately, in agreement with Vasington and Murphy (27) and Brierley et al. (6), but the effects are not always additive. The level of Ca uptake is lower than that reported with animal mitochondria because lower concentrations of Ca were used in these experiments. It was verified that massive accumulations of Ca could be obtained (as high as 12.8 μmoles Ca/mg N/10 min with 2.5×10^{-3} M Ca), but there was no obvious advantage in doing so, and it would not be possible to make parallel studies in which oxidative phosphorylation was measured because of the uncoupling action of Ca.

Table I. *Effect of Various Additives on Ca Uptake*

Reaction mixture contained 625 μmoles sucrose, 25 μmoles $MgCl_2$, 0.25 μmole $CaCl_2$ + Ca^{45}, 5 μmoles Pi, and where indicated 8 μmoles succinate, 6.5 μmoles ADP, 6.5 μmoles ATP and 1.25 μmoles DNP. See text for conditions common to all experiments.

Additives	Ca uptake mμmoles/mg N
Blank	71
Succinate	306
ADP	63
Succinate + ADP	106
Succinate + DNP	90
ATP	268
ATP + DNP	167
ATP + Succinate	519

Figures 1 and 2 show that both succinate- and ATP-supported Ca accumulation are temperature dependent, with an optimum similar to that for growth of the corn seedling (28°–30°). The optimum for animal mitochondria has been reported to be 36°−40° (3, 27).

Time course studies, such as those of figures 1 and 2, did not always show continuous uptake for 10 minutes. With the optimum pH of 7.5, uptake was occasionally very rapid for 5 minutes, followed by a gradual loss of the ion (fig 3). At pH 6.5 the rate of Ca uptake was lower, but continued at a decreasing rate for 20 minutes with no subsequent loss of the ion. Exploration of this phenomenon showed that the rate of succinate oxidation, as determined with the Gilson Oxygraph, often declined after 5 minutes at pH 7.5 (fig 4). At pH 6.5 this did not occur, although the rate of oxidation was about 30 % less than the initial rate at pH 7.5.

The depression of succinate oxidation with time at pH 7.5 is considered to be due to oxalacetate accumulation (1,30). We verified with the Warburg apparatus that the low rate of succinate oxidation could be greatly augmented by addition of pyruvate (29) or ATP (1, 26, 30). The oxidation rates of succinate or malate were nearly doubled by the addition of pyruvate, which alone was not oxidized at significant rates. Table II shows that these combinations of substrate

Table II. *Effect of Various Substrates on Ca Uptake*

Reaction mixture for experiment 1 contained 625 μmoles sucrose, 6.25 μmoles $MgCl_2$, 17 μmoles Pi, 0.25 μmole $CaCl_2$ plus Ca^{45}, and 40 μmoles of the various substrates except 5 μmoles of malate and succinate were used for sparker quantities. Cofactors were present in all treatments. Mitochondria were preincubated for 5 minutes with the substrates prior to addition of other ingredients. Final reaction time was 5 minutes. Reaction mixture for experiment 2 was the same except 12.5 μmoles $MgCl_2$, 25.5 μmoles Pi, and where indicated 6.5 μmoles ATP and 5 μmoles oxalacetate were used. Cofactors were omitted.

Experiment No.	Substrate	Ca uptake mμmoles/mg N
1	None	84
	Citrate	31
	Malate	134
	α-Ketoglutarate	186
	Succinate	296
	Pyruvate	98
	Pyruvate + Malate	468
	Pyruvate + Succinate	487
2	Oxalacetate	104
	Succinate	555
	Succinate + Oxalacetate	80
	Succinate + Oxalacetate + ATP	328
	Succinate + ATP	604

acids were also more effective in Ca accumulation, and that ATP would partially alleviate the oxalacetate inhibition of succinate-supported accumulation. The failure to obtain Ca uptake with citrate is attributed to the chelation of the Ca ion (27).

Effect of Mg and Phosphate on Ca Uptake. Phosphate proved essential for substrate-supported Ca uptake (table III). In other experiments it was found that arsenate, sulfate or chloride were ineffective. The addition of phosphate lowered the nonmetabolic binding of Ca by the mitochondria (no substrate treatment), probably by lowering the effective Ca concentration.

Although not absolutely essential, phosphate

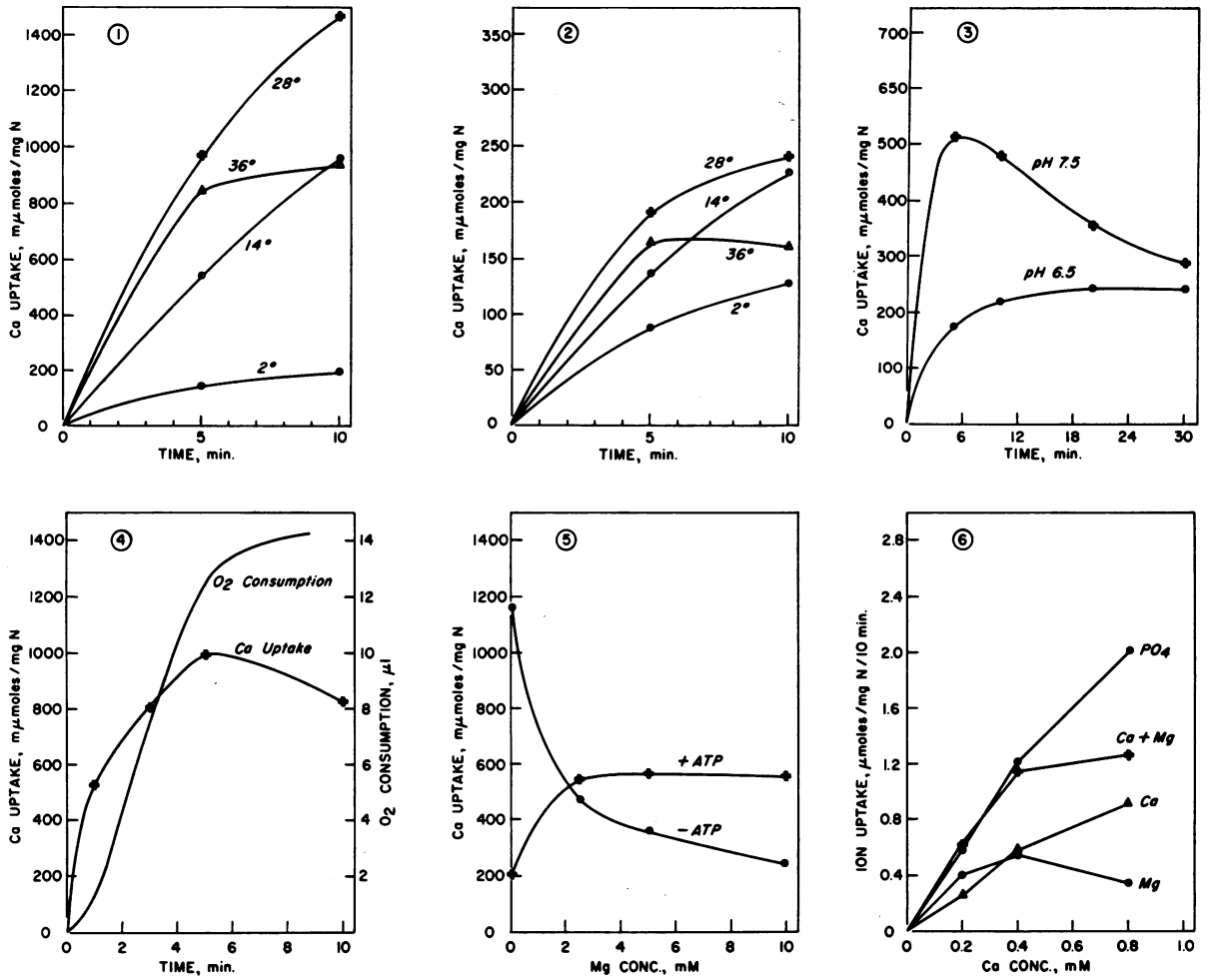

FIG. 1. Effect of temperature on substrate supported Ca uptake. Reaction mixture contained 625 μmoles sucrose, 6.25 μmoles $MgCl_2$, 17 μmoles Pi, 0.25 μmole $CaCl_2$ plus Ca^{45}, 40 μmoles pyruvate, 5 μmoles succinate and cofactors. See text for conditions common to all experiments.

FIG. 2. Effect of temperature on ATP supported Ca uptake. Reaction mixture contained 625 μmoles sucrose, 25 μmoles $MgCl_2$, 17 μmoles Pi, 0.25 μmole $CaCl_2$ plus Ca^{45} and 5 μmoles ATP.

FIG. 3. Effect of reaction pH on succinate supported Ca uptake. Reaction mixture contained 625 μmoles sucrose, 6.25 μmoles $MgCl_2$, 17 μmoles Pi, 0.25 μmole $CaCl_2$ plus Ca^{45}, and 20 μmoles succinate. Final pH's were adjusted to 6.5 and 7.5 with Tris.

FIG. 4. Relationship between Ca uptake and oxygen consumption with succinate as substrate at pH 7.5. Reaction mixture contained 625 μmoles sucrose, 6.25 μmoles $MgCl_2$, 25.5 μmoles Pi, 0.25 μmole $CaCl_2$ plus Ca^{45}, and 40 μmoles succinate. Reaction in Oxygraph vessel initiated by adding 0.1 ml mitochondria to above reactants in a volume of 2 ml at 25°. Oxygen consumption is replotted from the trace obtained with the Gilson Oxygraph.

FIG. 5. Effect of Mg and ATP on Ca uptake. Reaction mixture contained 625 μmoles sucrose, 4.5 μmoles Pi, and 0.25 μmole $CaCl_2$ plus Ca^{45}.

FIG. 6. Effect of Ca concentrations on Ca, Mg and phosphate uptake. Reaction mixture contained 625 μmoles sucrose, 10 μmoles $MgCl_2$, 17 μmoles Pi, 40 μmoles pyruvate, 5 μmoles succinate, cofactors, and Ca plus Ca^{45} as indicated.

Table III. *Ca Uptake as a Function of Phosphate Concentration*

Reaction mixture contained 625 μmoles sucrose, 0.5 μmole $CaCl_2$ plus Ca^{45}, and cofactors. Substrate, where indicated, was 40 μmoles pyruvate and 5 μmoles succinate. Reaction time was 6 minutes.

Phosphate (μmoles/tube)	Ca uptake (mμmoles/mg N)		
	+ substrate	— substrate	net
0	840	843	−3
8.5	2390	758	1632
17.0	2720	620	2100
34.0	2710	542	2168

Table IV. *Effect of Phosphate and ADP on ATP Supported Ca Uptake*

Reaction mixture contained 625 μmoles sucrose, 25 μmoles $MgCl_2$, 0.25 μmole $CaCl_2$ plus Ca^{45}, and where indicated 5 μmoles ADP, ATP, and 17 μmoles Pi.

Additives	Ca uptake mμmoles/mg N
Blank	79
ATP	140
ATP + Pi	497
ATP + Pi + ADP	167

greatly enhanced ATP-driven Ca accumulation (table IV). The addition of ADP reversed the phosphate effect.

Added Mg was of no value in substrate-supported Ca accumulation, and actually lowered both nonmetabolic binding and metabolic uptake (table V). The Mg probably "diluted" the Ca, competing with Ca for exchange and uptake sites. It is possible, of course, that the endogenous Mg (about 1 μmole/mg mitochondrial N) would be adequate for a Mg-requiring reaction. However, the mitochondria were isolated and washed with EDTA, which should have lowered the in vivo concentration, and one would expect additional Mg to promote activity if Mg were actually needed.

Table V. *Effect of Mg on Substrate Supported Ca Uptake*

Reaction mixture contained 625 μmoles sucrose, 17 μmoles phosphate, 1 μmole $CaCl_2$ + Ca^{45} cofactors, and where indicated 40 μmoles pyruvate + 5 μmoles succinate and 6.25 μmoles $MgCl_2$. Incubation was for 5 minutes. The endogenous Mg content of the mitochondria was 945 mμmoles/mg N (0.154 μmole/tube).

Substrate	$MgCl_2$	Ca uptake (mμmoles)
—	—	797
+	—	3420
—	+	654
+	+	3040

A very definite requirement for additional Mg in the ATP-supported Ca uptake system is illustrated in figure 5. The total picture is complicated, however, by a competition between Mg and Ca for binding sites, and by what appears to be a lowering of effective Ca concentration by ATP. In the absence of energy source (ATP), the addition of Mg decreases the level of nonmetabolic Ca binding. When ATP, but not Mg, is added there is a sharp reduction in bound Ca, probably due to the formation of a Ca-ATP complex (7, 27). With the addition of Mg there is an increase in Ca uptake, suggestive of metabolic uptake. To verify this we determined the effect of temperature on ATP-stimulated Ca uptake in the presence and absence of Mg (table VI). A significant temperature

Table VI. *Effect of Temperature and Mg on ATP Supported Ca Uptake*

Reaction mixture contained 625 μmoles sucrose, 5 μmoles Pi, 0.25 μmole $CaCl_2$ + Ca^{45}, 6.5 μmoles ATP, and where indicated 12.5 μmoles $MgCl_2$.

Temperature	Ca uptake (mμmoles/mg N)	
	— Mg	+ Mg
2° (ice)	146	230
28°	157	504

dependence is only found with Mg. The small increase in Ca uptake at ice temperatures with Mg could be due to either the low rate of metabolic uptake (fig 2), or to some alleviation of the Ca binding to ATP.

The Essentiality of Ca for Ca^{45}, Mg and Phosphate Uptake. Efforts to find Mg plus phosphate accumulation similar to that reported for animal mitochondria (3, 4, 5, 23) were completely unsuccessful. Only in the presence of Ca can Mg and phosphate be accumulated (table VII). Without Ca some phosphate is lost when the mitochondria are incubated with substrate, although the amounts are rather variable.

The most striking observation was that the mito-

Table VII. *Effect of Ca on Substrate Supported Mg and Phosphate Uptake*

Reaction mixture contained 625 μmoles sucrose, 10 μmoles $MgCl_2$, 17 μmoles Pi, cofactors and where indicated 0.5 μmole $CaCl_2$, 40 μmoles pyruvate and 5 μmoles succinate as substrate.

Ca	Substrate	Mitochondrial content (mμmoles/mg N)			
		Mg	Δ Mg	PO_4	Δ PO_4
—	—	1340	−1	2880	−130
—	+	1339		2750	
+	—	1368	160	2765	635
+	+	1528		3400	

Table VIII. *Effect of Ca Concentration on Substrate Supported Ca^{45} Uptake*

Reaction mixture contained 625 μmoles sucrose, 13 μmoles $MgCl_2$, 17 μmoles Pi, Ca^{45}, cofactors and where indicated 40 μmoles pyruvate and 5 μmoles succinate.

Ca Conc (mM)	Additive	Ca^{45} uptake cpm/mg N
Carrier	None	36,390
free Ca^{45}	Pyruvate + Succinate	36,715
+ 0.05	"	96,472
+ 0.10	"	105,575
+ 0.50	"	127,760
+ 1.00	"	119,722
+ 2.00	"	77,267

chondria seem to require Ca to effectively accumulate Ca^{45} (table VIII). Carrier-free Ca^{45} is not actively accumulated. The Ca^{45}, however, is rapidly taken up if carrier Ca is added.

Effect of Inhibitors on Ca Uptake. As mentioned previously, the uncoupler 2,4-dinitrophenol inhibits both substrate- and ATP-driven Ca accumulation (table I). Chloramphenicol was also found to inhibit both substrate- and ATP-supported Ca uptake (table IX). However as reported elsewhere (25), the sites of action of these 2 inhibitors appear to be quite different.

Arsenate in equimolar concentrations with phosphate inhibited substrate-supported Ca uptake by 15 to 20 %.

As with animal mitochondria (4, 6, 18) oligomycin blocks ATP-driven Ca uptake but does not inhibit

Table IX. *Effect of Chloramphenicol on Substrate- and ATP-Supported Ca Uptake*

Reaction mixture for experiment 1 contained 625 μmoles sucrose, 6.25 μmoles $MgCl_2$, 17 μmoles Pi, 0.25 μmole $CaCl_2$ plus Ca^{45}, cofactors and substrate (40 μmoles pyruvate plus 5 μmoles succinate). Reaction mixture for experiment 2 was the same except 12.5 μmoles ATP were substituted for the pyruvate and succinate. Incubation time was 5 minutes. Chloramphenicol was added in ethanol with equivalent amounts of ethanol in all tubes.

Energy source	Chloramphenicol mg/tube	Ca uptake mμmoles/mg N
Substrate	None	1270
"	1	1210
"	2	713
"	4	284
None	None	104
ATP	None	426
"	2	181
"	4	87

Table X. *Effect of Oligomycin on Substrate- and ATP-Supported Ca Uptake*

Reaction mixture contained 625 μmoles sucrose, 12.5 μmoles $MgCl_2$, 0.25 μmole $CaCl_2$ + Ca^{45}, 17 μmoles Pi, cofactors, and where indicated 40 μmoles pyruvate, 5 μmoles succinate, and 6.5 μmoles ATP. Oligomycin was dissolved in ethanol and all other treatments had an equivalent amount of ethanol.

Treatment	Ca uptake mμmoles/mg N
Blank	183
Pyruvate + Succinate	1195
Pyruvate + Succinate + 2 μg/ml Oligomycin	1004
+ ATP	1129
+ ATP + 2 μg/ml Oligomycin	269

the substrate-driven system (table X). Oligomycin (2 μg/ml) practically eliminates oxidative phosphorylation of corn mitochondria (C. D. Stoner, unpublished data), and presumably functions by blocking one of the terminal steps of the phosphorylation sequence (3, 15). For this reason, oligomycin should be effective in reversing the ADP inhibition of Ca uptake, as shown previously for phosphate uptake (5). That is, oligomycin by inhibiting ATP formation at a terminal step, should allow greater diversion of energy to ion accumulation. Such actually occurs, as shown in table XI.

We did not always find ADP to inhibit Ca uptake with pyruvate + succinate as substrate. Examples are given in table XII where there were actually small promotions with 2.5 mM Mg and a large promotion with 10 mM. This latter result is similar to that reported by Vasington and Murphy (26). However, the inclusion of a hexokinase trap, which would constantly regenerate the ADP, always produced an in-

Table XI. *Effect of ADP and Oligomycin on Substrate Supported Ca Uptake*

Reaction mixture contained 625 μmoles sucrose, 200 μmoles glucose, 6.25 μmoles $MgCl_2$, 17 μmoles Pi, 0.25 μmole $CaCl_2$ plus Ca^{45}, cofactors, and where indicated, substrate (40 μmoles pyruvate plus 5 μmoles succinate), 12.5 μmoles ADP, 5 μg oligomycin and 20 K.M. units hexokinase. Oligomycin was suspended in ethanol and all other treatments had an equivalent amount of ethanol. Reaction time was 5 minutes.

Treatment	Ca uptake mμmoles/mg N
Blank	158
Blank + Oligomycin	141
Substrate	790
Substrate + Oligomycin	628
Substrate + ADP	268
Substrate + ADP + Oligomycin	400
Substrate + ADP + Hexokinase	176
Substrate + ADP + Hexokinase + Oligomycin	256

Table XII. *Effect of ADP on Substrate Supported Ca Uptake*

Reaction mixtures for experiments 1 and 2 contained 625 μmoles sucrose, 6.25 μmoles $MgCl_2$, 17 μmoles Pi, 0.25 μmole $CaCl_2$ plus Ca^{45}, cofactors, and where indicated 40 μmoles pyruvate, 5 μmoles succinate, 5 μmoles ADP, 100 μmoles glucose and 20 K.M. units of hexokinase. Conditions were the same for experiment 3 except 25 μmoles Mg were used.

Experiment No.	Additive	Ca uptake mμmoles/mg N
1	None	140
	Pyruvate + Succinate	512
	Pyruvate + Succinate + ADP	592
	Pyruvate + Succinate + ADP + Hexokinase Trap	340
2	Pyruvate + Succinate	468
	Pyruvate + Succinate + Hexokinase Trap	468
	Pyruvate + Succinate + ADP	492
	Pyruvate + Succinate + ADP + Hexokinase Trap	289
3	None	129
	Pyruvate + Succinate	620
	Pyruvate + Succinate + ADP	844
	ADP	166

hibition of Ca uptake (table XII). We therefore concluded that the promotive effects of ADP alone were due to ATP formed, and might be related to the ATP plus substrate promotion found earlier (tables I and II). No attempt was made to determine the relationship between high Mg concentration and the ADP promotion.

The Stoichiometry of Ca, Mg and Phosphate Accumulation. Figure 6 shows the changes in ion content found by acid digestion of the mitochondria after 10 minutes incubation with varying levels of Ca. The minus Ca treatment was used to establish control levels of ion since there is no active accumulation in the absence of Ca (tables VII and VIII), but the nonmetabolic binding of ions is compensated for. Some experiments were performed with cold perchloric acid or trichloracetic acid extraction of the mitochondria, but the extraction of inorganic phosphate proved highly variable. This variability may have resulted from esterification of phosphate into various organic fractions. It was believed that the important statistic was the total phosphate accumulated, regardless of disposition after entry, and change in total ion content was thus determined.

At the lower levels of Ca, the divalent ion: phosphate ratio is about unity. In 7 such experiments the ion uptake in the presence of 0.2 mM Ca was 245±14 mμmoles Ca, 309 ± 47 mμmoles Mg, and 647 ± 43 mμmoles phosphate per mg N. As the concentration of Ca is raised to 0.8 mM the ratio declines (fig 6) as a result of the drop in Mg uptake. Calcium and Mg seem to compete for uptake, with Ca showing the greater affinity for the accumulation mechanism.

Discussion

The initial task of demonstrating substrate and ATP-powered Ca accumulation by plant mitochondria was largely a matter of following descriptions in the literature. Corn mitochondria clearly possess the same basic system as animal mitochondria for using high-energy intermediates in Ca, Mg and phosphate accumulation. If one recalls, however, that plants are the primary accumulators of the very diffuse nutritive ions in the soil environment, certain adaptive specializations of membrane transport might be expected.

The Ca requirement for Ca^{45}, Mg and phosphate accumulation (tables VII and VIII) is an example of this. Animal mitochondria can accumulate Mg and Pi in the absence of added Ca (3, 5) although Lehninger et al. (18) note that Ca is more effective than Mg in supporting P uptake. Corn mitochondria require Ca, and perhaps it would be surprising if they did not. Calcium is the dominant cation on the exchange complex of most soils, and calcium is widely known to have some role in membrane integrity and regulation of ion accumulation. Calcium deficient plants show disintegration of membrane structure (19) and it has been shown that root tissues depleted of Ca by EDTA are ineffective in phosphate accumulation (9). The fundamental question raised by the experiments reported here is whether Ca has a structural role in the membrane which prevents phosphate from leaking out as fast as it is accumulated, whether it is a cofactor for a phosphate-transporting enzyme, or if it simply immobilizes phosphate which has been accumulated. We do not have as yet sufficient evidence to distinguish between these alternatives.

Of equal interest is the failure of the corn mitochondria to accumulate divalent cations and phosphate in ratios typical of inorganic salt formation (5, 6, 18, 22). This may be due in part to the technical procedures followed; i.e., use of low Ca concentrations, and analysis for total rather than acid-extractable inorganic phosphate. Under the condition of our experiments, however, the stoichiometry of uptake is about 1 phosphate molecule accompanied by 1 divalent

ion (fig 7). (We have not yet studied the stoichiometry of uptake at high concentrations of Ca, nor have we investigated the uptake of other cations in the medium, such as Tris.) Because no evidence can be found for a Mg requirement in substrate-powered accumulation, perhaps only the Ca should be considered. Here the ratio becomes approximately 2 phosphate molecules accompanied by 1 Ca. Too little is known of the transport mechanism to attempt to attribute any biochemical significance to either ratio.

There are additional observations which are puzzling. One of these is the promotion of ATP-powered Ca accumulation by phosphate and the ADP reversal of this promotion (table IV). Another is the action of DNP in depressing ATP-powered Ca uptake. To explain these results we resort to the scheme given in figure 7. The scheme is adapted from hypotheses and comments from several laboratories [e.g., see the volume edited by Chance (8)], but most directly from the comments of Boyer (2).

Calcium and phosphate uptake are assumed to be at the expense of the phosphorylated high-energy intermediate, $X \overline{\ } I \sim P$. Slater et al. (24) consider that the nonphosphorylated intermediate provides energy for ion accumulation and other energy-linked processes, with phosphorylation of the intermediate providing a separate pathway to ATP formation. If this is true there must be an alternative phosphorylated pathway from $X \overline{\sim} I$ to Ca plus P accumulation in corn mitochondria, for Ca uptake is dependent upon phosphate (table III); we have not been able to find Ca uptake where arsenate, sulfate or chloride were substituted. The participation of phosphorylated substances in ion transport by a variety of animal systems has been reported (14). For the present we will assume a phosphorylated intermediate is involved and for purposes of simplicity a single phosphorylated intermediate is shown in figure 7.

The action of ADP in inhibiting Ca uptake (and ADP is always inhibitory in the presence of a hexokinase trap, table XI and XII) is indicated to be through competition for the phosphorylated intermediate (5). The action of oligomycin on Ca uptake by corn mitochondria (tables X and XI) is the same as that reported for animal mitochondria, and figure 7 is drawn in agreement with Brierley's basic scheme (3). According to figure 7, then, energy from substrate oxidation can be utilized either to form ATP or to power salt uptake.

When ATP is the energy source for Ca uptake, the reversible formation of phosphorylated intermediate will lead in turn to the reversible formation of nonphosphorylated intermediate plus a stoichiometric yield of inorganic phosphate. Now if additional phosphate is added to the system (table IV) mass action will tend to maintain a higher level of phosphorylated intermediate, and thus higher rates of Ca uptake. A further addition of ADP (table IV), however, would for the same reason tend to reduce

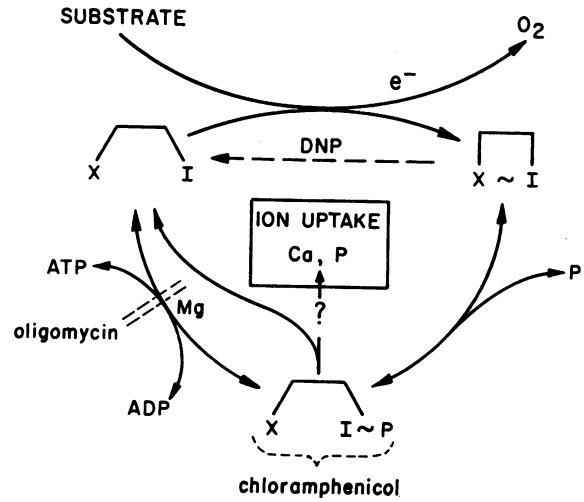

FIG. 7. A scheme depicting how a high energy, phosphorylated intermediate may participate in Ca, Mg and Pi uptake and the apparent sites of action of various inhibitors.

the level of phosphorylated intermediate, lowering Ca uptake.

When both ATP and substrate are provided there is an enhanced Ca uptake (table I). To explain this result one must assume that there is some endogenous "leakage," when only 1 source of energy is provided, of 1 or both high energy intermediates, and that supplying energy from 2 sources tends to maintain a higher level of $X \overline{\ } I \sim P$.

The scheme also allows for the action of DNP in depressing ATP-powered Ca uptake. The nonphosphorylated intermediate is assumed to be hydrolyzed (24, 28), thus setting up an irreversible degradation of ATP to ADP, Pi and the noncoupled intermediate ($X \overline{\ } I$); in short, a DNP-stimulated ATPase. Corn mitochondria have such an ATPase (25). The reduction in phosphorylated intermediate will be reflected in lower Ca uptake (table I). This interpretation would similarly explain the DNP inhibition of substrate driven Ca uptake (table I).

Chloramphenicol blocks both substrate and ATP powered Ca uptake by apparently blocking the action of the phosphorylated high energy intermediate (25).

Relationship to Ion Transport in Cells. Whether ion accumulation by isolated mitochondria is a phenomenon similar to ion transport across other membranes is uncertain. However, an attractive feature of the scheme outlined in figure 7 is that ion transport is not directly dependent upon substrate oxidation; a comparable DNP sensitive system operating in other cell membranes could be powered by ATP coming from mitochondria. In current work we find an ATP-stimulated binding of Ca by a microsome fraction which is indifferent to the presence of oxidizable substrate. However, the microsome fraction is much less effective than the mitochondria (20–50 % increase in Ca binding due to ATP vs. 200–400 % with mito-

chondria), and the result might be attributed to contaminating mitochondrial fragments which for some reason will not oxidize pyruvate + succinate. Peachey (20) has shown that divalent cations accumulate in granules inside the mitochondria under in vivo as well as in vitro conditions. Jackson et al. (16) have also linked phosphate accumulation by roots to oxidative phosphorylation by mitochondria. More work is needed before a definite conclusion can be made concerning the similarity of ion transport by mitochondria and ion absorption by entire roots.

Summary

The accumulation of Ca, Mg and phosphate by etiolated corn shoot mitochondria can be supported by substrate oxidation or by adenosine triphosphate. This accumulation is temperature dependent and is sensitive to pH. Substrate-supported uptake is depressed by the presence of adenosine diphosphate.

Substrate-supported Ca uptake requires phosphate but not exogenous Mg. Calcium is necessary for carrier-free Ca^{45}, Mg or phosphate uptake. The ratio of divalent cation: phosphate accumulated is approximately 1 at low Ca concentrations.

The adenosine triphosphate-driven Ca uptake requires exogenous Mg and is stimulated by inorganic phosphate; the stimulation is reversed by the addition of adenosine diphosphate.

Oligomycin inhibits adenosine triphosphate-supported Ca uptake but has no effect on substrate-supported Ca uptake. Dinitrophenol and chloramphenicol inhibit with either energy source.

The data suggest that a phosphorylated high-energy intermediate of oxidative phosphorylation participates in the accumulation of divalent ions and phosphate by corn mitochondria.

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Photomorphogenic Responses of Dodder Seedlings [1]

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In 2 previous reports the photocontrol of hook opening and twining of dodder seedlings was attributed to action of phytochrome (8, 18). An additional photoreaction has since been found to affect control over these responses. This paper furnishes a description of both controlling photoreactions, and the relationship between them.

Dodder seedlings respond to a single cycle of exposures to light. When the seedlings are grown in darkness, they form a hook much like the hypocotyl hook of a bean plant. A single saturating red irradiance causes the hook to open but the rate of opening is greatly accelerated under continuous white light. Twining depends on a prolonged exposure to blue or far-red radiant energy. Its display is favored by a final brief irradiance with far-red. The seedlings undergo circumnutational movements without a terminal exposure to far-red, but they do not incline sharply and twine.

Materials and Methods

All of the experiments were carried out with seedlings of *Cuscuta indecora* Chois. Fresh mature seeds were used. They were scarified for 1 hour with concentrated sulfuric acid. A measured number of seeds was planted in glass bowls (10 cm in diameter and 3.8 cm deep) on 2 layers of moist Whatman No. 3 filter paper. Five ml of water was added per bowl. After planting, each bowl was covered with the top of a petri dish. The seedlings were grown in darkness for 72 hours before the light treatments were given. All steps were carried out at 25°. Preliminary preparations and inspections were made in dim green light. Complete descriptions of the routines and methods for each kind of experiment are given in the next section.

Standard Light Sources. Standard cool-white fluorescent or incandescent lamps were used to provide white light. Heat produced by the incandescent lamps was removed with a water filter. Far-red was obtained by filtering the radiation from three 150-w internal-reflector incandescent lamps through 5 cm of tap water and either a sheet of black plastic (FRF 700 filter, Westlake Plastics Company, West Lenni Road, Lenni Mills, Pennsylvania) or 2 layers each of red and dark blue cellophane. Red light was obtained by filtering radiation from cool-white fluorescent lamps through 2 layers of red cellophane. The intensity of red and far-red radiation was adjusted to approximately 450 μw cm^{-2} over the wavelength bands of 600 to 700 and 700 to 770 mμ, respectively.

Three other lamps were used which, in addition to the standard red and far-red sources, provided sources that emit increasing amounts of far-red to red radiant energy. The 5 sources are listed below in order of increasing far-red. The ratio of radiant energy in the 700 to 770 mμ band, divided by the energy in the 600 to 700 mμ band, appears in parentheses: standard red source (0.147), standard incandescent lamp (0.94), incandescent lamp with ruby-red bulb (2.0), incandescent lamp with dark-red bulb [GE-BCJ (5.3)], and the far-red source [plastic filter (64.0)].

Monochromatic Light Sources. An interference-filter monochromator, such as described by Withrow (17), and the Beltsville spectrograph were used to determine action spectra. The interference filters used are listed in table I. Copper sulfate solution (100 g/liter) was used in the aqueous filter at all wavelengths below 550 mμ. Water was used for all longer wavelengths. Colored glass cut-off filters

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