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Permeability Properties of the Inner Membrane of Mung Bean Mitochondria and Changes during Energization[1]

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ABSTRACT

The permeability properties of the inner membrane of mung bean mitochondria were studied by osmotic swelling techniques. Rapid mitochondrial swelling was observed in isotonic ammonium phosphate, which indicated that an active phosphate/hydroxyl antiporter was present. The phosphate carrier was specifically inhibited by sulfhydryl reagents. Mitochondria did not swell in isotonic ammonium salts of malate, succinate, or fumarate, either in the presence or absence of 10 millimolar phosphate. Additionally, no swelling was observed in ammonium citrate upon addition of malate plus phosphate. Consequently, no evidence was obtained with the osmotic swelling technique for a coupled exchange of phosphate for dicarboxylic acids across the membrane.

On the basis of valinomycin-induced swelling in potassium salts, suggestions were obtained that chloride, sulfate, and phosphate anions permeated more rapidly than acetate anions. Sulfhydryl reagents increased the rate of valinomycin-induced swelling in potassium phosphate, but had no effect on swelling in potassium acetate.

Tributyltin induced a low rate of mitochondrial swelling in KCl in the absence of substrates, which indicated the presence of a low activity (Na^+) K^+/H^+ antiporter in the membrane. In the presence of NADH, rapid swelling, followed by a contraction, was observed upon addition of tributyltin. Swelling was insensitive to uncouplers, whereas contraction was uncoupler-sensitive. O_2 uptake (state 4) was greatest (3- to 4-fold stimulation) during the contraction phase, which indicated that the observed contraction was coupled to the pH gradient formed during electron transport. The results suggested that energization increased the activity of the (Na^+) K^+/H^+ antiporter in the inner mitochondrial membrane.

The inner mitochondrial membrane is thought to present a permeability barrier to free movement of solutes into and out of the mitochondrial matrix. Selective movement of solutes across the inner membrane involves specific transport systems that facilitate the exchange-diffusion transport of certain metabolites (21). Transport systems and solute permeation can be studied spectrophotometrically (4, 18). As mitochondria swell (because of net solute uptake), light scattering is decreased thereby causing an apparent absorbance decrease.

The species of an anion undergoing net translocation can be determined by the osmotic swelling technique. Two systems have proven to be very useful (12). One system involves the use of the ionophore, valinomycin. Induction of swelling by valinomycin in the K^+ salt of the anion indicates electrogenic anion uptake. Valinomycin is an antibiotic that specifically increases membrane permeability to K^+ (16) and thereby causes swelling when the mitochondrial membrane is permeable to the anion. In the second system, spontaneous swelling of mitochondria in an isotonic ammonium salt has been interpreted to indicate that the anion is transported in an electroneutral fashion (*i.e.* in exchange for OH^- ions, which is equivalent to uptake of the corresponding acid). The ammonia diffuses into the matrix where it hydrolyzes to $NH_4^+ + OH^-$. The OH^- ions are then available to drive uptake of the anion (4, 12). Valinomycin will not induce swelling in the K salt of an anion that is transported electroneutrally.

In this report, the osmotic swelling technique was used to study transport across the inner membrane of mung bean (*Phaseolus aureus* Roxb.) mitochondria. Mitochondria isolated from mung bean hypocotyls are used frequently in studies of electron transport and oxidative phosphorylation; however, little is known about the semipermeability properties of the inner membrane. The objectives of the present study were (*a*) to characterize the inner membrane of mung bean mitochondria relative to the permeability of cations and anions; and (*b*) to determine if energization affects permeability to monovalent cations.

MATERIALS AND METHODS

Isolation of Mitochondria. Mitochondria were isolated from 4-day-old, dark-grown mung bean hypocotyls as described by Ikuma and Bonner (10) and modified by Moreland and Boots (14).

O_2 Uptake and Mitochondrial Swelling. O_2 utilization was measured polarographically with a Clark electrode in a 2-ml thermostatted cell at 25 C. Mitochondrial swelling was measured simultaneously as a decrease in *A* at 520 nm (4). Reaction mixtures contained 0.3 M mannitol or salts, as indicated in the figure legends, and 10 mM Hepes (pH 7.1), unless noted otherwise. Each 2-ml reaction mixture contained approximately 0.4 mg mitochondrial protein, as determined by the method of Lowry *et al.* (11). Mung bean mitochondria do not contain significant endogenous substrates; hence, it was not necessary to add respiratory inhibitors such as cyanide in the passive swelling experiments. To determine inhibition of swelling by sulfhydryl reagents, mitochondria (0.4 mg protein) were preincubated at 25 C for 3 min with mersalyl (0.16 μmol/mg protein), pCMBS[2] (0.16 μmol/mg protein), or NEM (2.5 μmol/mg protein), before addition to the indicated reaction mixture. Valinomycin and FCCP were prepared in acetone.

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[2] Abbreviations: Ac: acetate; FCCP: carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NEM: *N*-ethylmaleimide; pCMBS: *p*-chloromercuriphenyl sulfonic acid.

RESULTS AND DISCUSSION

Metabolite Transport Systems. Metabolite transport systems have been demonstrated in isolated cauliflower (15), beet root (15), and potato tuber (19) mitochondria by means of the ammonium salt swelling technique, originally applied to rat liver mitochondria (4). Mitochondria from the aforementioned sources swell rapidly in isotonic ammonium phosphate, which indicated the presence of an active Pi^-/OH^- antiporter (4). Ammonia is considered to diffuse into the matrix where it hydrolyzes to form $NH_4^+ + OH^-$. The OH^- ions then exchange for external Pi on the Pi/OH^- antiporter (4). The net result is uptake of $Pi + NH_4^+$ which increases the internal osmolarity and, hence, the mitochondria swell osmotically. As shown in Figure 1 (trace A), mung bean mitochondria also swell rapidly in ammonium phosphate. As shown below (Fig. 3A), pretreatment of mitochondria with mersalyl, pCMBS, or NEM inhibited the rate of swelling in ammonium phosphate. Other investigations have reported that sulfhydryl reagents inhibited swelling of potato tuber (19) and bean (7) mitochondria in ammonium phosphate but not ammonium acetate. Hence, mung bean mitochondria possess a Pi/OH^- antiporter with properties similar to the Pi carrier from other plant sources.

Mitochondria from cauliflower (15), beet root (15), and potato tuber (19) swell in ammonium malate, but only after addition of catalytic amounts of Pi. Swelling has been interpreted to result from recycling of Pi on the Pi/OH^- and Pi/dicarboxylic acid transporters. No swelling of mung bean mitochondria was observed in ammonium malate, succinate, or fumarate, either in the presence or absence of 10 mM Pi (Fig. 1, trace C). Similar results were obtained when the pH of the reaction mixture was varied between pH 6.5 and 8.0 (data not shown). Similarly, no swelling was observed in ammonium citrate upon addition of malate + Pi (Fig. 1, trace B), which was reported to cause rapid swelling of beet root (15), cauliflower (15) and potato tuber (19) mitochondria. Cauliflower mitochondria, isolated and assayed under the conditions described for mung bean mitochondria, swell dramatically in ammonium malate plus Pi and in ammonium citrate in the presence of catalytic amounts of malate and Pi (data not shown). The observed swelling of cauliflower mitochondria was consistent with previous findings (15) and indicated that the lack of swelling of mung bean mitochondria (Fig. 1, traces B and C) probably could not be attributed to our techniques. Additionally, energized swelling of mung bean mitochondria was not observed in a medium containing NADH, malate, and Pi, that was reported by Day and Hanson (6) to cause swelling of corn mitochondria in response to malate accumulation. Hence, no evidence was obtained using the osmotic swelling technique for the presence of a Pi/dicarboxylate antiporter in mung bean mitochondria.

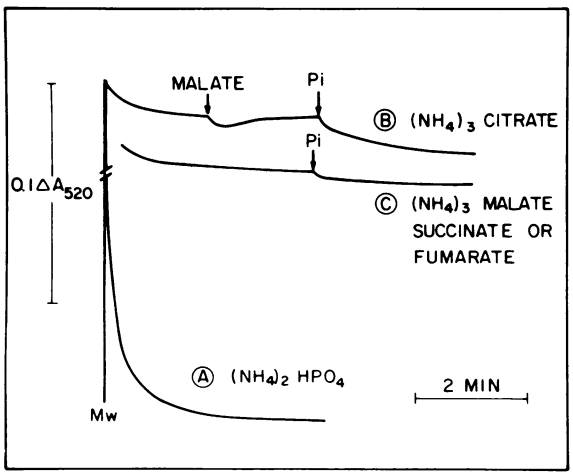

FIG. 1. Osmotic volume changes of mung bean mitochondria (Mw) injected into: (A) 0.1 M $(NH_4)_2HPO_4$; (B) 0.1 M $(NH_4)_3$ citrate; and (C) 0.10 M ammonium salts of malate, succinate, or fumarate. Malate (10 mM) and 10 mM Pi were added as indicated. All reaction mixtures were adjusted to pH 7.1 and additionally contained 10 mM Hepes-NaOH (pH 7.1). The final *A* of traces B and C were equal and were approximately 0.15 units greater than the final *A* of trace A.

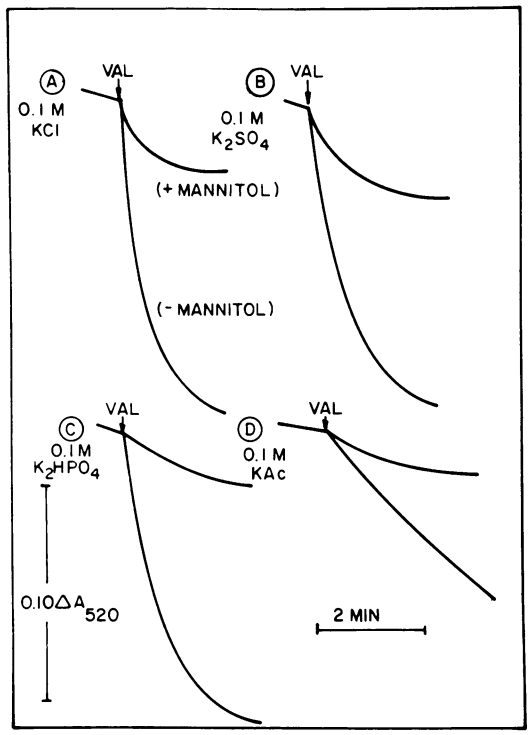

FIG. 2. Valinomycin-induced swelling of mung bean mitochondria suspended in: (A) 0.15 M KCl; (B) 0.1 M K_2SO_4; (C) 0.1 M K_2HPO_4; and (D) 0.1 M KAc. As indicated, swelling was initiated by addition of 0.1 μM valinomycin (VAL). The upper trace in each figure contained 0.2 M mannitol in addition to the indicated salt.

Wiskich (20) and Day and Hanson (6) have suggested that operation of the Pi/dicarboxylate antiporter is required for maximum rates of substrate oxidation. The postulate is supported by the stimulation of malate oxidation in the absence of ADP by exogenous Pi (6, 20). However, stimulation of receptorless malate oxidation by Pi does not necessarily indicate a Pi/dicarboxylate exchange. With mung bean mitochondria, exogenous Pi (10 mM) stimulated the oxidation of malate and NADH (1- to 2-fold) in an oligomycin-sensitive reaction (data not shown). Hanson *et al.* (8) previously demonstrated oligomycin sensitivity of the phosphate stimulation of NADH oxidation and concluded that internal Pi accelerated turnover of the coupling mechanism. Interaction of internal Pi with the coupling mechanism may also explain the results obtained with mung bean mitochondria.

The question still remains as to the mechanism of malate transport. No evidence was obtained by the osmotic swelling technique for an active Pi/dicarboxylate antiporter. Possibly, the nature of the transport systems in the mitochondrial inner membrane reflects the function of the tissue from which the organelles are isolated. The significance of the differences between beet root, cauliflower, potato tuber, and corn mitochondria as reported in the literature for dicarboxylate transport and the results reported herein for mung bean mitochondria remains to be established.

Ionophore-induced Swelling. Valinomycin induced rapid swelling of mung bean mitochondria suspended in isotonic KCl, K_2SO_4, and K_2HPO_4 (Fig. 2, traces A, B, and C), but only slow swelling in KAc (Fig. 2, trace D). Because swelling requires net uptake of both cation and anion, the results indicated that the inner membrane of mung bean mitochondria was permeable to Cl^-, SO_4^{-2}, and Pi anions. The limited swelling in KAc indicated a low, but

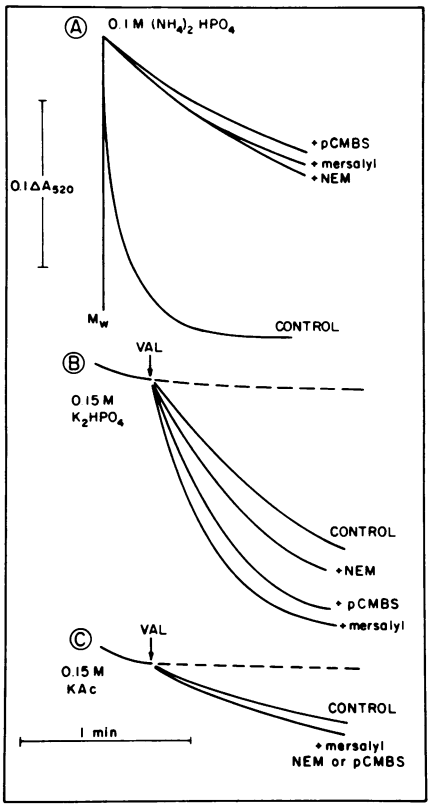

FIG. 3. Effect of sulfhydryl reagents on the swelling of mung bean mitochondria in: (A) 0.1 M $(NH_4)_2HPO_4$; (B) 0.1 M K_2HPO_4; and (C) 0.15 M KAc. Additions of 0.1 μM valinomycin (VAL) were as indicated. Dashed lines in B and C indicate the *A* changes observed when acetone was added in place of valinomycin. Pretreatment of mitochondria with mersalyl, NEM, or pCMBS was as described under "Materials and Methods."

significant, membrane permeability to Ac^- ions.

Mannitol (0.2 M) largely prevented valinomycin-induced mitochondrial swelling in the salts tested (Fig. 2, A–D). Addition of sorbitol to mitochondria swollen in a mannitol-free medium caused substantial shrinkage (data not shown). The results suggested that the observed swelling was of a pseudoenergized type (1, 2). The nonpermeant solute mannitol inhibited this type of swelling by providing an external osmotic gradient that opposed, or reversed, swelling.

Results reported in the literature concerning the permeability of plant mitochondria to electrogenic Cl^- uptake are contradictory. Turnip (13), and in this report, mung bean, mitochondria have been judged to be permeable to Cl^-, whereas bean (7) mitochondria have been reported to be impermeable. De Santis *et al.* (7), with bean mitochondria, established only that Cl^- did not enter the matrix in an electroneutral fashion, *i.e.* the possible presence of an electrogenic flux was not considered.

The permeability properties of mung bean mitochondria with respect to electrogenic phosphate uptake appear qualitatively different from turnip (13), bean (7), and rat liver (12) mitochondria, which have been reported to be impermeable to Pi because valinomycin did not induce swelling in isotonic K-phosphate.

An alternate explanation for valinomycin-induced swelling in K_2HPO_4 is that the ionophore facilitates operation of the K^+/H^+ antiporter of the inner membrane, as suggested by Hensley and Hanson (9). In that case, swelling would occur by a combination of K^+/H^+ antiport and Pi/OH^- antiport, with the net result being uptake of K^+ + Pi. If operation of the Pi/OH^- antiporter is required for swelling in K_2HPO_4 + valinomycin, then swelling should be sensitive to sulfhydryl reagents that inhibit the antiporter (21). Mersalyl, pCMBS, and NEM inhibited the rate of mitochondrial swelling in ammonium phosphate greater than 90% (Fig. 3A) but had no effect on swelling in ammonium acetate, which occurs entirely by diffusion (data not shown). Specific inhibition of the Pi/OH^- antiporter by the sulfhydryl reagents was therefore suggested, which is consistent with results obtained with mitochondria isolated from other sources (7, 15). In contrast, swelling induced by valinomycin in K_2HPO_4 was stimulated by the sulfhydryl reagents (Fig. 3B). The stimulation did not involve valinomycin action, because swelling in KAc + valinomycin was not affected (Fig. 3C). Rather, an increase in electrogenic Pi uptake was suggested. The lack of inhibition of valinomycin-induced swelling in K_2HPO_4 by sulfhydryl reagents indicated that the Pi/OH^- antiporter was not involved.

Energized Swelling in Chloride-containing Solutions. Trialkyltins catalyze an exchange of Cl^- for OH^- ions across membranes (17). As expected, 1 μM tributyltin induced rapid mitochondrial swelling in isotonic NH_4Cl, slight swelling in KCl, and no swelling in mannitol (Fig. 4). Rapid swelling in NH_4Cl would be expected, because hydrolysis of ammonia in the matrix would produce OH^- ions for tributyltin-catalyzed exchange with external Cl^- (17). Presumably, the additional swelling in KCl caused by tributyltin reflected the presence of a low activity K^+/H^+ antiporter in the mitochondrial membrane.

Addition of tributyltin to mitochondria oxidizing exogenous NADH in isotonic KCl caused a rapid swelling that was followed by a contraction (Fig. 5). The induced swelling corresponded to a 1.5-fold increase in the state 4 rate of NADH oxidation. During the contraction phase, O_2 uptake was maximal (3.7-fold increase). After net contraction had ceased, the rate of O_2 uptake decreased to an intermediate rate. Similar results were obtained when mitochondria were suspended in isotonic NaCl (data not shown). The strong stimulation of O_2 uptake during the contraction phase suggested that contraction was driven by electron transport. The slight stimulation of O_2 uptake during the swelling phase may be attributed to dissipation of the pH gradient by tributyltin-catalyzed uptake of external Cl^- in exchange for internal OH^- ions. However, this would be limited by the normally low membrane permeability to cations.

The rapid mitochondrial swelling caused by the addition of tributyltin during receptorless NADH oxidation in isotonic KCl was largely prevented by FCCP when the uncoupler was added before the substrate (Fig. 6, trace A *versus* trace B). Addition of FCCP during the swelling phase allowed swelling to proceed, but completely prevented the contraction (Fig. 6, trace B *versus* trace

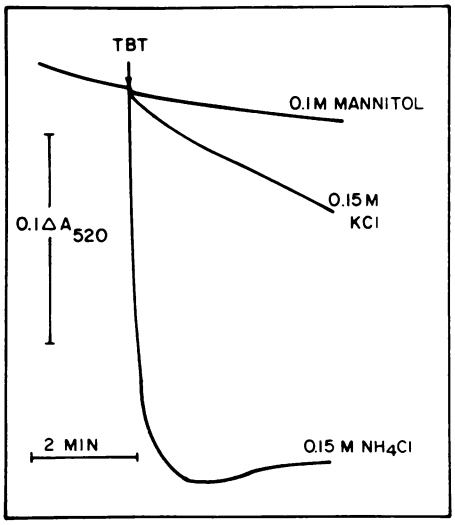

FIG. 4. Osmotic volume changes induced by 1 μM tributyltin chloride (TBT) to mung bean mitochondria suspended in 0.15 M ammonium and potassium chloride and 0.3 M mannitol.

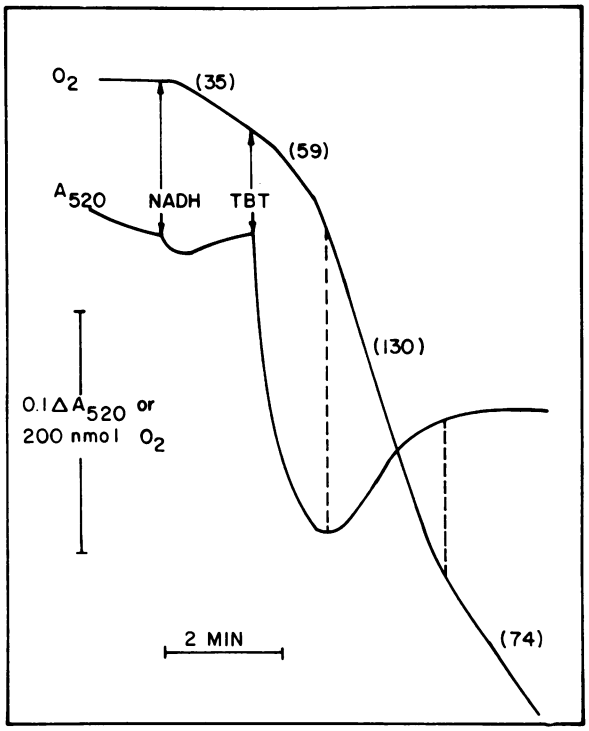

FIG. 5. Simultaneous measurements of O_2 uptake and change in A at 520 nm of mung bean mitochondria. The reaction mixture contained 0.15 M KCl and 10 mM Hepes (pH 7.1). NADH (1 mM) and 1 μM tributyltin (TBT) were added as indicated. Rates of O_2 uptake, expressed as nmol O_2/mg protein·min, are shown parenthetically.

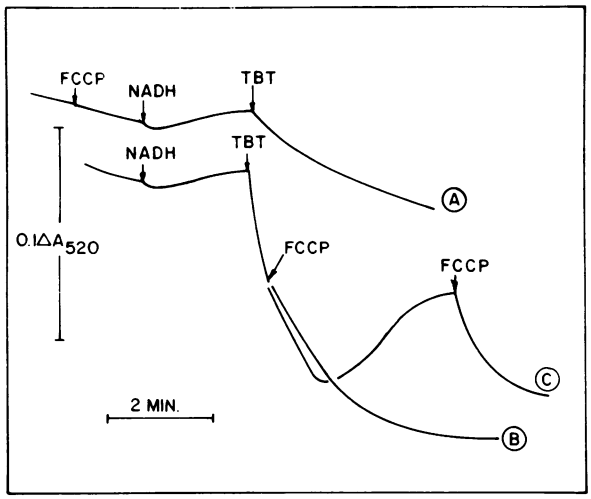

FIG. 6. Effect of FCCP on osmotic volume changes of mung bean mitochondria. The reaction mixture contained 0.15 M KCl and 10 mM Hepes (pH 7.1). NADH (1 mM), tributyltin (1 μM; TBT), and FCCP (1 μM) were added as indicated.

C). When FCCP was added after the contraction phase, swelling was reinitiated (Fig. 6, trace C). The results suggested that swelling required, but was not coupled to, the pH gradient generated by electron transport. The sensitivity of the contraction phase to FCCP indicated that this phase was driven by the pH gradient.

Models for the observed swelling and contraction are presented in Figure 7. Both models involve net movement of K^+ and Cl^- ions, and an electron transport-activated (Na^+) K^+/H^+ antiporter in the inner mitochondrial membrane. It is postulated that the pH gradient generated by electron transport is responsible for activation of the (Na^+) K^+H^+ antiporter. During swelling (Fig. 7A), the endogenous (Na^+) K^+/H^+ antiporter is coupled to tributyltin-catalyzed exchange of Cl^- and OH^- ions, thereby maintaining electroneutrality. A net uptake of KCl occurs and osmotic flow of water results in swelling. The pH gradient generated by electron transport is not dissipated, which is consistent with: (*a*) inhibition of swelling by mannitol (data not shown); (*b*) the relatively low stimulation of acceptorless respiration observed during swelling (Fig. 5); and (*c*) insensitivity to FCCP (Fig. 6). Mitochondrial contraction may be explained by the model proposed by Chavez *et al.* (5) and Brierley and Jurkowitz (3) for beef heart mitochondria (Fig. 7B). The pH gradient generated by electron transport drives the efflux of internal K^+ in exchange for external protons. Activity of the (Na^+) K^+/H^+ antiporter thereby reduces the magnitude of the pH gradient and allows for increased respiration. As a result, the membrane potential (inside negative) would increase, but would be dissipated by efflux of the permeant Cl^- anion (Fig. 2A). Contraction may be controlled by the concentration gradient of K^+ and Cl^- ions across the inner membrane. Initially, contraction would be prevented by the relatively high external concentration of KCl. However, after swelling is initiated, the internal concentration of salt increases to the point that further uptake is prevented. Energized contraction would then lead to net extrusion of K^+ and Cl^-. The steady-state (no A change) reached after net contraction had ceased appeared to be dynamic rather than static (*i.e.* swelling and contraction occurred at equal rates), because addition of FCCP (Fig. 6, trace C) or anaerobiosis (data not shown) caused additional swelling.

Maximal activity of the endogenous (Na^+) K^+/H^+ antiporter apparently required energization of the inner membrane. It is conceivable that energization caused a membrane conformational change that increased cation accessibility to the antiporter, perhaps by removal of a lipid barrier. Hensley and Hanson (9) have suggested that corn mitochondria contain a K^+/H^+ antiporter because of valinomycin-induced stimulation of respiration and active contraction of Pi-loaded mitochondria. Our results with mung bean mitochondria are generally consistent with those for

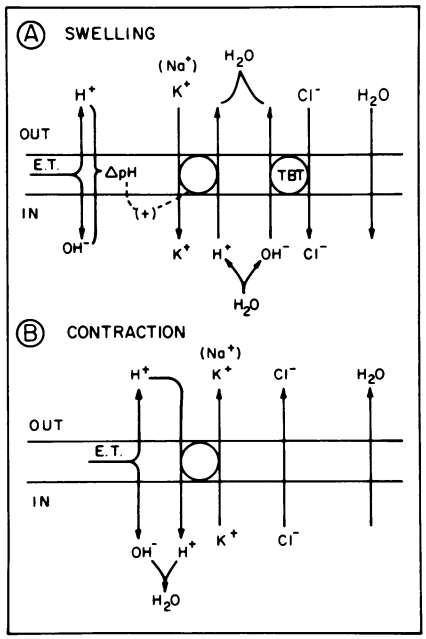

FIG. 7. Proposed models for energy-dependent (A) swelling and (B) contraction of mung bean mitochondria. (+): activation; (TBT): tributyltin-catalyzed transport; E.T.: electron transport.

corn mitochondria (9). However, we have also shown that activity of the K^+/H^+ antiporter increased dramatically upon energization of the mitochondria.

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