Active Swelling and Acetate Uptake in Corn Mitochondria*

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ABSTRACT: Partially swollen corn mitochondria in KCl contract on addition of substrate, whereas in potassium acetate they show an additional swelling linked to respiration. Electron micrographs and gravimetric measurements confirm the volume changes measured by optical density methods. The effects of active swelling in acetate and other anions on respiratory control, volume changes, salt accumulation, and oxidative phosphorylation were determined. Active swelling is due to an inward transport of K^+ and acetate accompanied by an osmotic equivalent of water. The transport competes with adenosine triphosphate formation and Ca^{2+} and P_i uptake and results in loss of respiratory control. The results support the hypothesis that acetate is a "reactive" anion, not a "passive" anion, and that anion transport is at the expense of a high-energy intermediate formed from respiration or adenosine triphosphate hydrolysis.

Other short-chain monocarboxylic acids will produce the same transport phenomenon unless the α-carbon is substituted with a hydroxyl or keto group, in which case the mitochondria contract. Contraction was shown to be associated with expulsion of K^+ and water.

Mitochondria from various sources have been shown to undergo energy-linked volume changes which are critically dependent upon the anionic composition of the external medium. The addition of substrate to partially swollen corn mitochondria leads to contraction in KCl but to rapid additional swelling in potassium acetate (Hanson and Miller, 1967). Similar observations have been made with beef heart mitochondria (Blair and Stollar, 1967). Substrate-dependent swelling in acetate has been studied in several laboratories and is associated with the uptake of Ca^{2+} or K^+ in the presence of transport-inducing agents (Moore and Pressman, 1964; Rasmussen *et al.*, 1964; Azzi and Azzone, 1965a; Azzone and Azzi, 1965; Chappell and Crofts, 1965, 1966; Chappell and Haarhoff, 1966; Harris *et al.*, 1966; Lynn and Brown, 1966; Rasmussen and Ogata, 1966; Harris *et al.*, 1967), as well as the concomitant accumulation of acetate (Rasmussen *et al.*, 1965; Ogata and Rasmussen, 1966). From these studies it has been concluded that the energized uptake of salt associated with swelling is due to the active transport of cation accompanied by passively permeating acetate. Permeation of acetate is thought to be as the undissociated molecule or in exchange for hydroxyl (Chappell and Crofts, 1966). Chappell and Haarhoff (1966) discuss possible "carriers" for mono- and dicarboxylic acids to account for certain inconsistencies in the permeation of the anions. Presumably these carriers accomplish a passive exchange of anion for hydroxyl. Van Dam and Slater (1967) believe there may be energy-linked nonspecific anion carriers, but suggest that these may really be cation carriers with anions drawn in with the cations.

The concept of passive anion permeation is now established to the point where the expression "permeant anion" is routinely used in the literature. Except for our laboratory there has been no challenge of the "active cation–passive anion" hypothesis, although Pullman and Schatz (1967) have pointed out the lack of unequivocal evidence.

In maize mitochondria the accumulation of Ca^{2+} and P_i is indicated to be accomplished by the uptake of P_i at the expense of X~P (Stoner *et al.*, 1964; Hodges and Hanson, 1965; Truelove and Hanson, 1966; Kenefick and Hanson, 1966). Ca^{2+} is thought to interact with X~P to produce an unstable complex which breaks down to deliver Ca^{2+} and P_i into the matrix. Hanson and Miller (1967) suggest that acetate reacts with the nonphosphorylated high-energy intermediate in a comparable fashion; that is, I~X + acetate → I + X~acetyl. Spontaneous breakdown of the unstable X~acetyl would release acetate inside the mitochondrion. Consistent with this hypothesis is the finding that fatty acid uncoupling in corn mitochondria is competitive with P_i in ATP formation, and that sufficient linoleate can be activated in the presence of oligomycin and Ca^{2+} to appreciably label the mitochondrial phospholipid (Baddeley and Hanson, 1967).

The investigation reported here was to determine the effect of acetate and other anions on respiratory control, volume changes, salt accumulation, and oxidative phosphorylation. Active swelling proves to be due to an inward transport of K^+ and acetate accompanied by an osmotic equivalent of water. Acetate transport competes with both ATP formation and phosphate uptake, and results in loss of respiratory control. The data support the hypothesis that acetate is a "reactive" anion,

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not a passive anion, and that anion transport is at the expense of a high-energy intermediate normally involved in oxidative phosphorylation.

Methods

Mitochondria were isolated by procedures previously described (Kenefick and Hanson, 1966) in a 0.4 M sucrose, 20 mM Tris-Cl buffer (pH 7.6), and 5 mM EDTA, but with the omission of ADP during washing. Isolation for the electron micrographs was with the Tris-phosphate buffer used by Kenefick and Hanson (1966). Optical density measurements were made on a Zeiss PMQ II spectrophotometer. Oxygen was measured with a Clark oxygen electrode fitted to a Gilson oxygraph. P_i was determined by the isobutyl alcohol–benzene extraction procedure as modified by Penniall (1966). Potassium was measured by flame emission on a Jarrell ash absorption–emission flame spectrometer with a Heath recorder. Acetate was measured as acetate-1-^{14}C (234,000 cpm/reaction mixture), phosphate as ^{32}P (118,000 cpm/reaction mixture), and Cl^- as ^{36}Cl (380,000 cpm/reaction mixture) on a Nuclear-Chicago or Packard liquid scintillation counter. Gravimetric determinations were done by measuring fresh and dry weights of mitochondrial pellets as previously described (Stoner and Hanson, 1966) with a Cahn electrobalance. Protein was determined by the Lowry (1951) method.

In studies of K^+ and acetate uptake, treatments were started by the addition of mitochondria (2.0–2.5 mg of protein) to the reaction mixtures as noted in the legends. After incubation for 3 min the mitochondria were centrifuged at 27,000*g* for 5 min. Numerous attempts to centrifuge the mitochondria through different wash layers of sucrose ranging in concentration from 0.2 to 0.6 M resulted in removal of all but background levels of K^+ and acetate. In earlier work it had been found that collection and washing on Millipore filters produced the same leaching. It was necessary to accept the high "blank" level of K^+ and acetate in the pellet and measure changes from this. After centrifugation the supernatant was aspirated and the tubes were thoroughly wiped with cotton tip applicators. The pellet was resuspended in 5.0 or 6.0 ml of 10% trichloroacetic acid, vigorously stirred, and the extracted mitochondria were centrifuged down at 27,000*g* for 5 min. Aliquots of the supernatant were used for specific ion determinations. The amount of K^+ and acetate remaining in the extracted mitochondrial pellet was less than 0.5% of the total measured. Controls containing the complete reaction mixture but lacking mitochondria were used to correct for residual K^+ and acetate adhering to the tube. This correction ranged between 5 and 8% of the total K^+ and acetate measured.

Electron Microscopy. Mitochondria were incubated in the reaction mixture for the time intervals noted in the legends. After incubation 5.2 ml of 0.2 M sucrose was layed beneath the mixture; the mitochondria were centrifuged down at 15,000*g* for 5 min. The pellet was suspended in 2 ml of solution containing 0.2 M sucrose, 0.1 M sodium cacodylate (pH 7.2), and 2.5% glutaraldehyde and allowed to stand overnight. Then they were washed three times in 3.5 ml of 0.2 M sucrose and 0.1 M sodium cacodylate (pH 7.2). The pellet of the third wash was suspended in 0.5 ml of 1% OsO_4 in 0.1 M sodium cacodylate (pH 7.2) and collected by centrifugation. The pellet was resuspended in 1 ml of 1% OsO_4 in 0.1 M sodium cacodylate (pH 7.2) and allowed to stand 1 hr. The fixed pellet fragments were put through four changes of ethanol from 50 to 100% with a 5-min dehydration in each followed by a second dehydration in the last step for 1 hr in 100% ethanol.

Embedding was carried out essentially according to Luft (1961). The fragments were prerinsed with propylene oxide and allowed to stand in 2 ml of fresh propylene oxide for 15 min. Fresh propylene oxide (2 ml) was added with an equal volume of Epon 812 resin accelerator mixture. After standing 2 hr the fragments were placed in fresh resin accelerator and left standing overnight. After a further treatment with resin accelerator for 2 hr the fragments were transferred to individual capsules (Beem) containing resin accelerator. The capsules were heated at 33° for 18 hr followed by 46° for 18 hr. The resin in the capsules was stained in 1% uranyl acetate for 15 min and Reynold's lead citrate for 1–5 min.

Tissues were sectioned using a Porter Blum MT-1 microtome and a diamond knife. The sections were poststained for 1–5 min in 2% uranyl acetate followed by 1–5 min in Reynold's lead citrate. Sections were studied and photographed with a Philips EM-200 microscope.

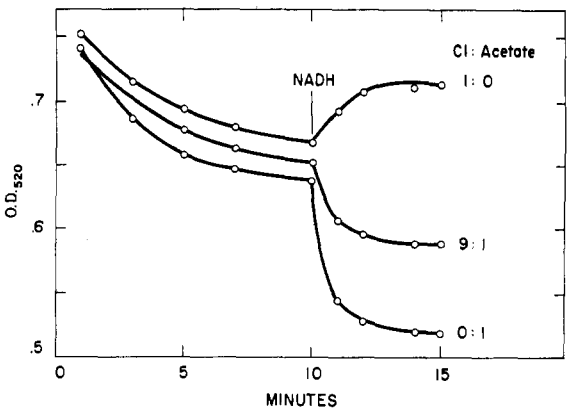

FIGURE 1: Active swelling and contraction of passively swollen mitochondria in chloride and acetate. The reaction mixture of 2.6 ml contained: 20 mM Tricine buffer (pH 7.6), 1 mg/ml of bovine serum albumin, 0.68 mg of mitochondrial protein, and 100 mM K^+ salt of Cl or acetate as indicated. Respiration was initiated by addition of 1 μmole of NADH.

Results

Volume Changes and Salt Uptake in Acetate and Chloride. Figure 1 shows the typical volume response of corn mitochondria in solutions of potassium acetate or KCl. Passive swelling measured between 1 and 10 min after the addition of mitochondria resulted in the same optical density change in both salt solutions. In 22 experiments the average optical density decrease was 0.103 ± 0.006 in acetate and 0.109 ± 0.007 in chloride. Hence, at pH 7.6 the passive permeation of the anions is equivalent.

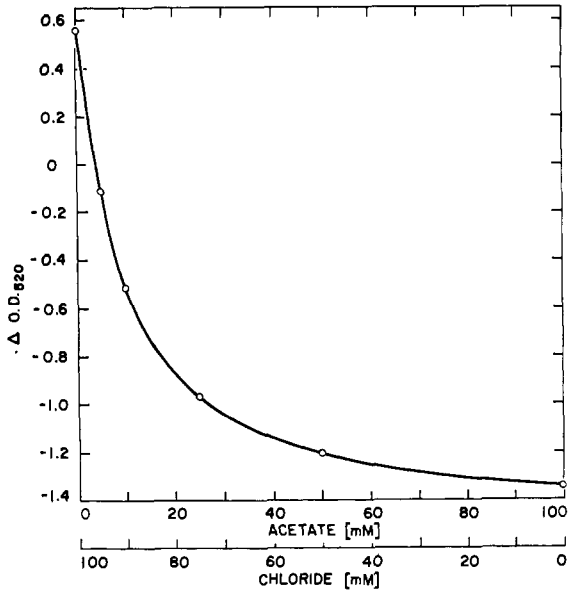

FIGURE 2: Absorbancy changes of mitochondria in solutions containing different proportions of KCl and potassium acetate. Mitochondria were passively swollen 10 min followed by addition of 1 μmole of NADH to each reaction mix and the change in optical density was recorded during the next 4 min. Other conditions are as in Figure 1.

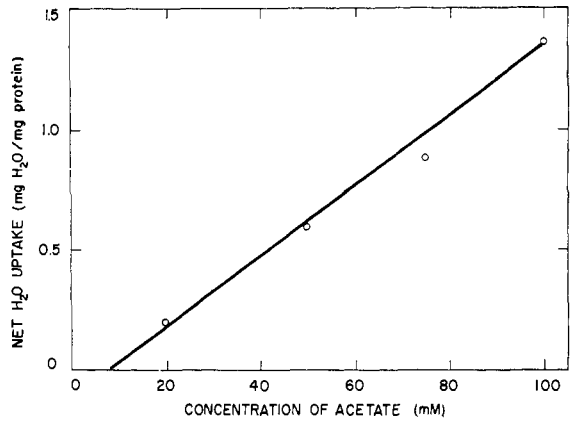

FIGURE 3: Gravimetric determinations of the net uptake of water with different proportions of KCl and potassium acetate. Each reaction mixture of 2.6 ml contained: 20 mM Tricine buffer (pH 7.6), 2.6 mg of bovine serum albumin, 2 mM $MgCl_2$, 40 μM CoA, 230 μM NAD, 170 μM thiamine pyrophosphate, 10 mM succinate plus 10 mM pyruvate as substrate where added, and 100 mM potassium salt composed of different ratios of acetate and Cl. The reactions were started by the addition of 0.5 ml of mitochondria suspension containing 3.5–4.5 mg of protein. The net uptake of H_2O is the difference between the water content in the presence and absence of a substrate. Each point is the average of at least two experiments.

On the addition of substrate the mitochondria contract in chloride, whereas they actively swell still further in acetate. Active swelling is like contraction (Stoner and Hanson, 1966) in that malate, succinate, NADH, or ATP plus Mg^{2+} will serve as an energy source. Unlike contraction, which involves binding of divalent ions, active swelling in acetate does not require Ca^{2+} or Mg^{2+} (Hanson and Miller, 1967).

In a medium containing a mixture of the two anions, acetate dominated over chloride so that a solution with a chloride:acetate ratio of 9:1 appreciable swelling was still evident (Figure 1). Figure 2 shows the extent of active swelling as a function of the proportion of acetate in 100 mM potassium salt. Only when the concentration of acetate was reduced to 4–5 mM was swelling completely inhibited. Falcone and Hadler (1968) found 7 mM acetate to be the critical level in gramicidin-induced transport. As can be seen in Figure 3, an increase in the proportion of acetate in the medium resulted in a linear increase in the amount of water taken up. However, measurements of water content are laborious and depend on small differences in weight. For experiments where qualitative volume changes were adequate the light-absorbancy technique was used.

The uptake of K^+ and acetate from media varying in acetate:chloride is shown in Figure 4. Within error, the K^+ and acetate were accumulated in a 1:1 ratio. By labeling the chloride present in a solution of 50 mM potassium acetate plus 50 mM KCl with ^{36}Cl it was determined that no active accumulation of the Cl^- occurred. Hence, no simple permeation of solution is involved in active swelling; acetate is actively transported and chloride is not. With respect to swelling it is reasonable to assume as others have (Chappell and Crofts, 1965; Tedeschi and Hegarty, 1965; Azzi and Azzone, 1966a; Azzi *et al.*, 1966; Ogata and Rasmussen, 1966) that the active uptake of salt is accompanied by an osmotic equivalent of water (*cf.* Figures 3 and 4).

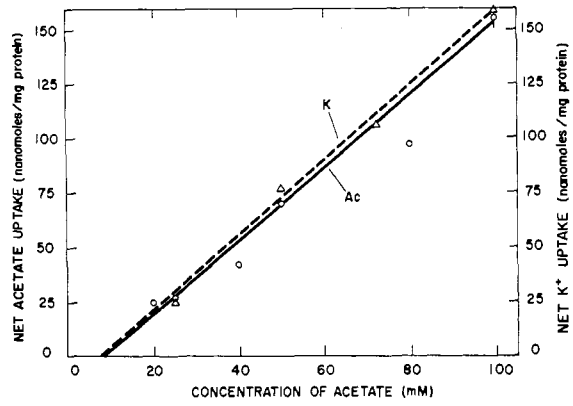

FIGURE 4: Net uptake of K^+ and acetate with different proportions of KCl and potassium acetate. The reaction mixture is the same as given in Figure 3. The net uptake of ions is the difference between the amounts bound in the presence and absence of substrate. See Methods section for details of the procedure.

There is one difficulty with this simple osmotic explanation of active swelling: osmotic swelling requires semipermeable membranes readily permeated by water and only slowly permeated by solute. Actively accumulated salt can thus produce a rapid water flux. As pointed out under Methods, the accumulated salt in swollen corn mitochondria is readily washed out by sucrose solutions. Lynn and Brown (1965) have reported from

TABLE I: Swelling and ATP Formation in Solutions of KCl and Potassium Acetate.[a]

Conditions	Swelling (optical density × 10^3/min)	Respiration (nmoles of O_2/min)	ATP (μmoles/10 min)
100 mM potassium acetate	−24		
+Substrate	−125	26	
+Substrate + ADP + P	−37	68	2.53
160 mM KCl	−27		
+Substrate	−3	46	
+Substrate + ADP + P	−24	102	2.72

[a] Each reaction mixture of 2.6 ml contained: 20 mM Tricine buffer (pH 7.4), 1 mg/ml of bovine serum albumin, 40 μM CoA, 230 μM NAD, 170 μM thiamine pyrophosphate, 50 mM glucose, 0.6 mg of hexokinase, 1 mM $MgCl_2$, 100 mM KCl, or potassium acetate as indicated. The reaction was started by the addition of 0.82 mg of mitochondrial protein. Where added the concentration of substrate was 10 mM succinate plus 10 mM pyruvate, ADP was 77 μM, and P_i was 4 mM. The change in optical density as well as respiration rates were recorded between 0.5 and 1.5 min after addition of mitochondria to the reaction mixture.

studies with rat liver mitochondria that hypertonic solutions of sucrose cause immediate contraction and release of salts in acetate-swollen mitochondria. One would not expect this result in a simple osmotic system; only the water should have been removed rapidly. The centrifugation technique for washing and collecting mitochondria can also result in drastic changes in the K^+ content of mitochondria (Harris *et al.*, 1967). The expectation would be that excess pressure would remove water but not solute. As an osmotic system the mitochondrion has complexities not yet properly described.

Some measurements were made on the water and K^+ extrusion during contraction in chloride. In five experiments the net K^+ loss compared to a control lacking substrate was 44 ± 8 nmoles of K^+/mg of protein. Water loss in two experiments averaged 0.51 mg/mg of protein. These results support the idea that contraction results from an efflux of K^+ salts followed by an osmotic equivalent of water (Rasmussen *et al.*, 1964; Chappell and Crofts, 1965; Tedeschi and Hegarty, 1965; Azzi and Azzone, 1966b, 1967). Changes in chloride or endogenous anions were not determined, but an efflux of chloride is known to occur (Azzi and Azzone, 1967). The important point here is that in the absence of a reactive anion, energy-linked K^+ transport is outward; only with a reactive anion such as acetate is there an influx of K^+.

Volume Changes and Salt Accumulation in Relation to Oxidative Phosphorylation. Corn mitochondria in KCl exhibited respiratory control with ADP while those in potassium acetate showed a complete lack (Figure 5). However, in an acetate medium the presence of an ATP-generating system added initially resulted in phosphorylation (Table I). Indeed the P/O ratio was higher in acetate due to the lower respiration rate. However, in these cases where ADP and P_i plus a hexokinase trap were added initially to mitochondria in acetate, the respiratory rate was rapid and sensitive to oligomycin (Figure 6, first trace). However, if ADP and P_i were not initially present the respiratory rate was slower, and the separate addition of P_i, ADP, and oligomycin had no effect on the rate (Figure 6, second trace). Thus in an acetate medium, coupling of respiration to ATP formation depends upon having P_i and ADP present initially. Addition of ADP and P_i after the mitochondria have embarked on active salt uptake and swelling is without effect: the mitochondria are completely uncoupled. This is not true with chloride. Sequential addition of P_i and ADP stimulates respiration, and the stimulation is oligomycin sensitive (Figure 6). Mitochondria in chloride possess respiratory control with ADP and P_i (Figures 5 and 6) and the capacity to contract (Figure 1) but these are competitive events. If the energy is used in ATP

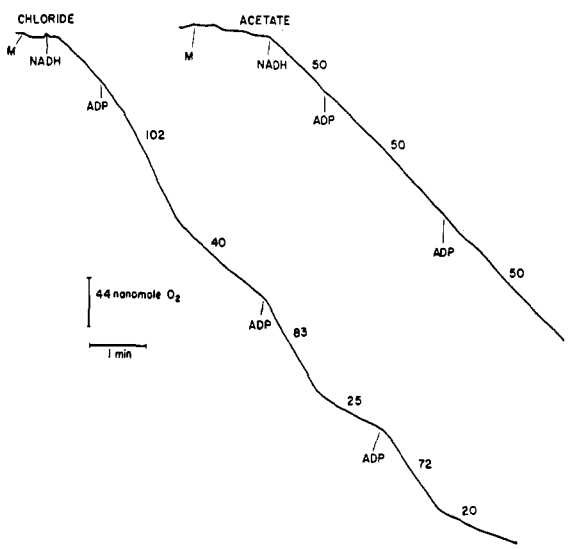

FIGURE 5: Respiratory control in solutions of KCl and potassium acetate. Medium contained in 2.6 ml: 20 mM Tricine (pH 7.6), 1 mg of bovine serum albumin/ml, 1 mM $MgCl_2$, 2 mM P_i, 100 mM of the salt indicated, and 0.81 mg of mitochondrial protein. Where indicated the following additions were made: 2 μmoles of NADH, and 0.2 μmole of ADP. Temperature 25°. Numbers refer to rates in nanomoles of O_2 per minute.

TABLE II: Inhibition of Acetate Uptake by ADP and P.[a]

Treatment	K^{+} [b]	Acetate[b]	ΔK^{+} [b]	ΔAcetate[b]
−Substrate	521 ± 12	537 ± 21		
+Substrate	681 ± 10	692 ± 25	160	155
+Substrate, ADP, P	640 ± 21	569 ± 33	119	32
+Substrate, ADP, P, oligomycin	722 ± 6	700 ± 15	201	163

[a] The incubation mixture was identical with the potassium acetate solution in Table I. Reactions were started by adding 0.3 ml (2.0–2.5 mg of protein) of mitochondria to 2.3 ml of solution. The final concentration of the additions as noted in the table were: 10 mM succinate plus 16 mM pyruvate (substrate), 77 μM ADP, 4 mM P_i, and 1 μg of oligomycin/ml. The results are an average of five experiments. See Materials and Methods for details of the procedure.
[b] Values are given as nanomoles per milligram of protein.

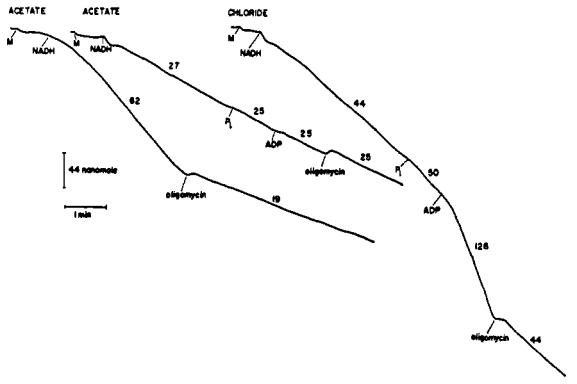

FIGURE 6: Conditions for uncoupling in potassium acetate. Reaction mixtures of 2.6 ml contained: 20 mM Tricine buffer (pH 7.6), 1 mg/ml of bovine serum albumin, 1 mM $MgCl_2$, 100 mM potassium salt as indicated, 50 mM glucose, 0.6 mg of hexokinase (Sigma type III), and 0.88 mg of mitochondrial protein. The reaction mixture in the first acetate tracing also had 4 mM P_i and 77 μM ADP present initially. Where indicated the following additions were made: 2 μmoles of NADH, 5.2 μmoles of P_i, 0.2 μmole of ADP, and 1 μg of oligomycin/ml (Sigma). Temperature was 25°. Numbers refer to rates in nanomoles of O_2 per minute.

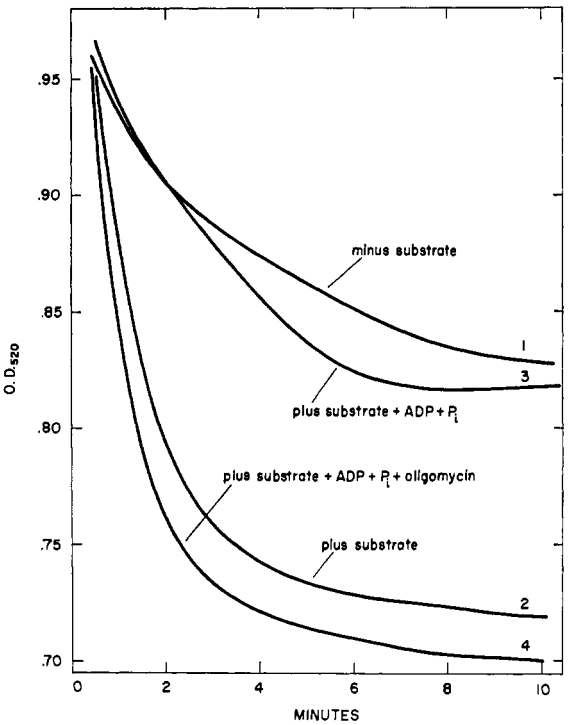

FIGURE 7: Reversal of active swelling by ADP and P_i. Basic reaction mixture was identical with the potassium acetate solution in Table I. The reactions were started by the addition of 0.1 ml of mitochondria (0.95 mg of protein) to 2.5 ml of solution. Curve 1, minus substrate: curve 2, plus 10 mM succinate and 10 mM pyruvate; curve 3, same as 2 plus 77 μM ADP and 4 mM P_i; curve 4, same as 3 plus 1 μg of oligomycin/ml.

formation, less is available for maintaining contraction (Table I). Note that in the experiment of Table I the mitochondria were not allowed to passively swell. The action of respiration in this case is to prevent passive swelling (Stoner and Hanson, 1966).

Initial inclusion of the ATP generating system prevented the active swelling in acetate (Table I, Figure 7). Addition of oligomycin to prevent ATP formation shifted the mitochondria back to active transport and swelling; the transport is accomplished at the expense of oligomycin-insensitive respiration. Plant mitochondria thus behave like chloroplasts in that volume changes and ATP formation are competitive acts (Dilly and Vernon, 1964; Dilly, 1966). It has been shown in rat liver mitochondria that ADP prevents both small and large amplitude P_i-induced swelling (Azzone and Azzi, 1965). However, oligomycin was reported to inhibit swelling as well (Azzi and Azzone, 1965b).

Table II provides data on the reduction in transport of K^{+} and acetate during ATP formation. Only acetate transport shows a strong inverse relationship. There are K^{+} influxes associated with the presence of ADP and/or P_i that we cannot account for at present, but possibly are associated with the uptake of these anions.

Once the mitochondria were swollen in acetate, the addition of ADP and P_i, or Ca^{2+} and P_i, gave only a slight reversal of swelling (Figure 8). Again, active acetate transport or the associated swelling irreversibly damages the coupling mechanism. In similar studies the respiratory inhibitors antimycin and cyanide as well

TABLE III: The Effect of Ca^{2+}, Mg^{2+}, and P_i, on the Uptake of Potassium Acetate.[a]

Treatment	ΔK^+ [c]	ΔAcetate[c]	ΔP_i [c]
−Substrate[b]	(564)	(554)	(132)
+Substrate	177	142	7
+Substrate, 1 mM $CaCl_2$	25	32	694
+Substrate, 1 mM $MgCl_2$	142	150	41

[a] The basic reaction mixture contained the same constituents as given in Table I with the exception that 4 mM P_i was present and the hexokinase trap was not added. Protein was 1.8 mg/reaction mixture. The results are an average of two experiments. [b] Values in parentheses are total ion content of the pellet. Changes are relative to this base. [c] Values are given as nanomoles per milligram of protein.

TABLE IV: Proportion of Contracted, Partially Swollen, and Swollen Mitochondria in KCl and Potassium Acetate.[a]

Treatment	Mitochondrial Morphology (%)[b]		
	Contracted	Partially Swollen	Swollen
100 mM KCl − substrate	37	19	43
100 mM KCl + substrate	72	22	6
100 mM Potassium acetate − substrate	36	7	59
100 mM Potassium acetate + substrate	5	27	68

[a] Mitochondria were permitted to swell for 10 min in 100 mM KCl or Potassium acetate, 20 mM Tricine (pH 7.6), 1 mg of bovine serum albumin/ml, and 1 mM $CaCl_2$. NADH was added as indicated and the active swelling or contraction reaction continued for 5 min. Swollen and contracted mitochondria were recovered and processed for electron microscopy. Between 130 and 270 mitochondria were scored for each treatment. [b] See text for a description of these types.

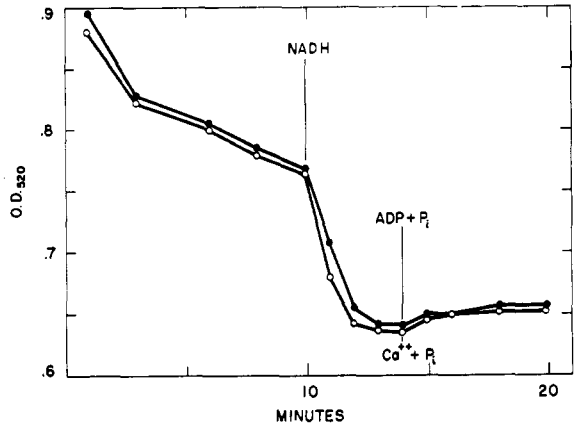

FIGURE 8: Effect of adding ADP plus P_i or Ca^{2+} plus P_i on actively swollen mitochondria. The medium of 2.6 ml contained: 20 μM Tricine buffer (pH 7.6), 1 mg of bovine serum albumin/ml, and 100 mM potassium acetate. Active swelling was initiated by the addition of 2.0 μmoles of NADH. The final concentration of added components was 1 mM $CaCl_2$, 2 mM P_i, and 77 μM ADP.

as ATP failed to induce shrinkage of acetate swollen mitochondria. However, respiratory inhibition (cyanide) or uncoupling (DNP) of acetate transport was readily secured if the reagents were added initially.

Previous work with corn mitochondria indicated that active P_i uptake occurs at the expense of X∼P, with Ca^{2+} serving as the activating and accompanying cation (Hodges and Hanson, 1965; Hanson and Miller, 1967). Experiments were done to determine if acetate uptake would be suppressed by induction of phosphate uptake with Ca^{2+}. There was a pronounced reduction in both K^+ and acetate uptake (Table III). As previously reported, Mg^{2+} is relatively inefficient in activating phosphate uptake (Hodges and Hanson, 1965; Kenefick and Hanson, 1967) and did not reduce acetate transport. No attempt was made to calculate acetate–phosphate stoichiometry since the acetate uptake produced complete uncoupling, while phosphate uptake activated by Ca^{2+} does not (Hanson and Miller, 1967).

Electron Microscopy. Electron micrographs were examined for morphological changes in the mitochondria due to passive swelling in chloride or acetate, active swelling in acetate (Figure 9A), and contraction in chloride (Figure 9B). Mitochondria from each treatment were classified into one of three basic structural types: contracted, partly swollen, or swollen on the basis of the following characteristics. (1) Contracted mitochondria (C in Figure 9) are characterized by a small volume of very dense, homogeneous matrix and very prominent cristae (intermembrane spaces) formed by the tortuous collapse of the inner membrane. Diameters were 0.6–0.9 μ. (2) In partially swollen mitochondria (P in Figure 9) the matrix is less dense, occupies a larger volume, and is granular in character. The number and size of the cristae is reduced. The mitochondrial size is

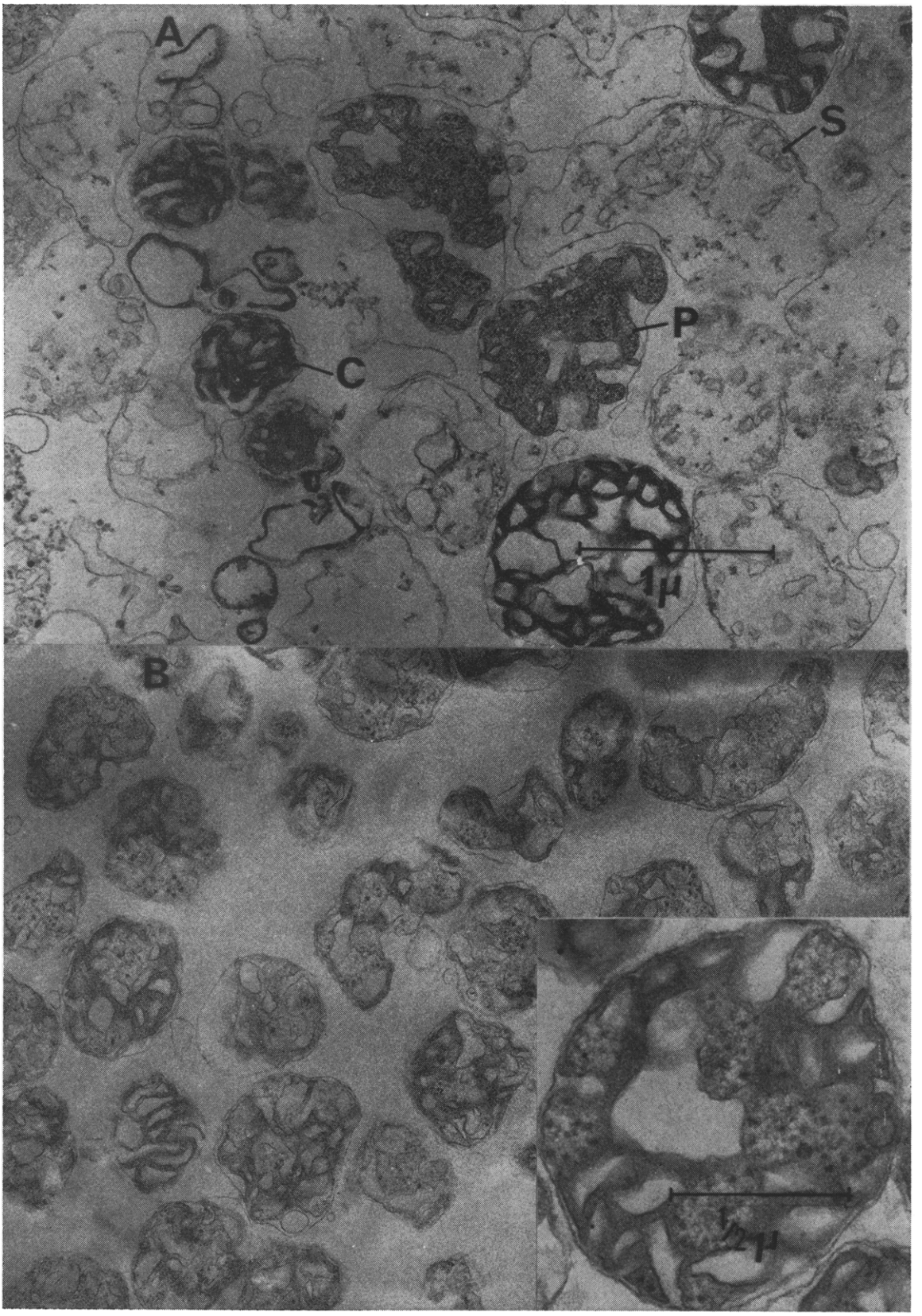

FIGURE 9: Electron micrographs of mitochondria actively swollen in potassium acetate and actively contracted in KCl. The mitochondria were incubated 10 min in 2.6 ml of media containing: 20 mM Tricine buffer (pH 7.6), 2.6 mg of bovine serum albumin, 1 mM $CaCl_2$, and 100 mM potassium acetate (A) or 100 mM KCl (B). After 10 min of passive swelling 1 μmole of NADH was added and the mitochondria were collected after 5 min by centrifugation through 10 ml of 0.2 mM sucrose at 31,000*g* for 5 min. The pellet was resuspended in 2.6 ml of 0.4 M sucrose and prepared for electron microscopy (see Methods).

TABLE V: The Effect of a Series of Cations on Passive and Active Swelling in Acetate.[a]

Salt	Initial Optical Density[b]	Passive Swelling[c] (ΔOD $\times$ 10^3)	Active Swelling[d] (ΔOD $\times$ 10^3)
Potassium acetate	0.740	−112	−70
Sodium acetate	0.729	−120	−77
Lithium acetate	0.640	−72	−50
Ammonium acetate	0.518	+3	+4
Magnesium acetate	0.800	+2	−103
Tetraethylammonium acetate	0.759	−131	−56
Tetramethylammonium acetate	0.805	−153	−80
Tris-acetate	0.695	−122	−50

[a] The reaction medium of 2.6 ml contained: 20 mM Tricine buffer (pH 7.5), 1 mg of bovine serum albumin/ml, and 100 mM acetate salt listed. [b] The optical density 1 min after addition of mitochondria. [c] The optical density change between 1 and 10 min after adding mitochondria. [d] The optical density change between 10 and 15 min following addition of 1 μmole of NADH to passively swollen mitochondria.

TABLE VI: Volume Changes and Respiratory Control in Potassium Salts of Various Acids.

Anions[a]	pK_a, 25°	ΔOD $\times$ 10^3, 2 min	Respiration (μmoles of O_2/min) State 4	State 3	Respiratory Control
Chloride		+48	41	127	3.1
Pyruvate	2.50	+32	46	137	3.0
Lactate	3.86	+31	54	125	2.3
L-Alanine	2.35	−51	77	110	1.4
Glycine	2.35	−62	68	115	1.7
Formate	3.76	−76	78	78	1.0
Acetate	4.73	−81	70	70	1.0
Propionate	4.86	−81	78	78	1.0
Arsenate[b]	6.77	−75	95	95	1.0
Phosphate[b]	6.70	−10			

[a] The experimental system contained: 20 mM Tricine buffer (pH 7.6), 2.6 mg of bovine serum albumin, 100 mM potassium salt of the anion as listed, 4 mM $MgCl_2$, 4 mM P_i, and 0.81 mg of mitochondria protein/ml in 2.6 ml. [b] pK_2.

slightly larger than the contracted form with diameters of 0.7–1.0 μ. (3) In swollen mitochondria (S in Figure 9) the matrix is too dilute to be recognized except as thin patches of granular or amorphorous material. In some instances no outer membrane can be recognized. The diameter of the swollen forms was 0.9–1.4 μ.

As is evident from the electron micrographs and the summary in Table IV of the proportion of different morphological types, swelling is not uniform throughout the population. Some mitochondria swell readily, others are resistant and remain contracted. Not every mitochondrion in acetate is responding to the addition of substrate with salt and water transport, but there is a sizeable increase in the numbers that do. Thus optical density recordings of volume changes (Figure 1) record only a complex statistical mean on the proportion and extent of mitochondrial swelling and contraction. Contraction of swollen mitochondria in KCl (insert in Figure 9) failed to reestablish the uniformly dense matrix of the mitochondria which have never swollen. The matrix is now sorted into two components, the original amorphous phase plus a less dense nucleoplasm containing what appear to be ribosomes, and which does not contract. The amorphous matrix contains large, dense bodies which are presumably calcium phosphate precipitates (Ca^{2+} plus P_i were added here to obtain these deposits). Where these large particles are low in contrast, they seem to be surrounded by a membrane (insert Figure 9).

Effect of Other Cations and Anions on Volume Changes. The effect of various cations on active swelling in acetate is shown in Table V. Very little passive swelling is observed in Mg^{2+} but the addition of substrate induced a rapid and extensive active swelling. The acetate salts of inorganic cations Na^+ and K^+ show similar swelling curves for both passive and active swelling. In Li^+ the initial optical density was lower and the passive and active swelling was reduced. Lynn and Brown

(1965) have reported inhibition of passive swelling by Mg^{2+} acetate in liver mitochondria. However, they found that Na^+ and NH_4^+ salts of acetate caused faster rates of passive swelling than did K^+. Ammonium acetate provides essentially no osmotic support to corn mitochondria, which is consistent with the results obtained by Chappell and Crofts (1966) with rat liver mitochondria and Crofts *et al.* (1967) with chloroplasts. The monovalent organic cations investigated produce responses very much like the inorganic.

Hanson and Miller (1967) suggested that the important property of acetate in active swelling is the reactivity of the carboxyl groups. Therefore a number of monocarboxylic acids were examined for their capacity to support active swelling. The results are summarized in Table VI. Support of active swelling is not a property of all these carboxylic acids, nor is it related in any obvious way to the pK of the carboxyl group (see also Chappell and Haarhoff, 1966). Although the concentration of nondissociated molecules may be involved, it does not appear to be as important as substitution on the α-carbon with respect to regulating active swelling and producing uncoupling. This is particularly noticeable in the three carbon series, propionate, alanine, lactate, and pyruvate. Active swelling is rapid in propionate and respiratory control is completely lost. Substitution with an α-amino group still gives some active swelling, but a little respiratory control can now be found. However, with α-hydroxyl or α-keto substitution, active swelling is lost and the mitochondria contract and give respiratory control. Lactate and pyruvate must thus be classed with chloride as unreactive anions.

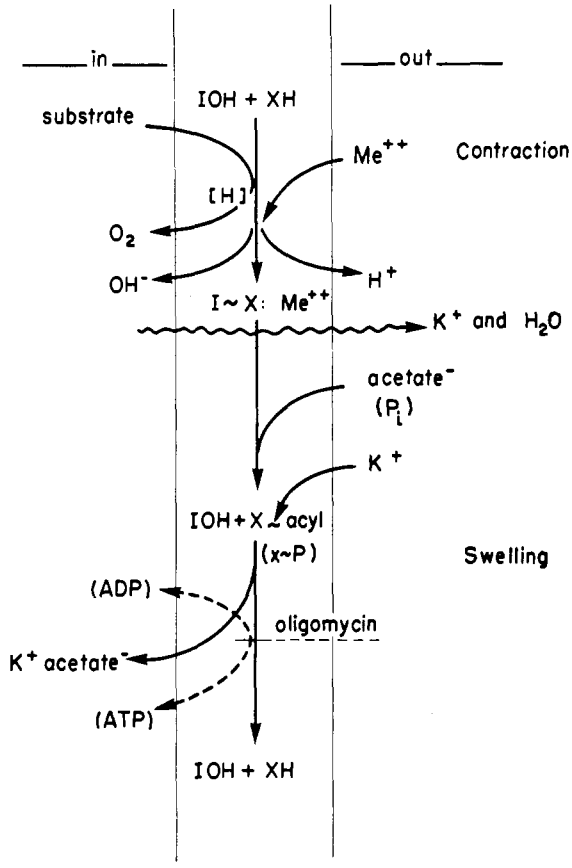

FIGURE 10: Diagrammatic scheme of ion transport in mitochondria. Upper part of the diagram refers to the contraction observed with nonreactive anions, and illustrates the active expulsion of H^+ and K^+ accompanying the formation of I~X. Efflux of anions is passive and not shown. Lower part of the diagram shows the active uptake of reactive anions such as acetate. Formation of X ~ acyl and X~P are competitive events.

Discussion

It is evident that the nature of active salt transport in corn mitochondria is governed by the anion. With chloride, there is an expulsion of K^+ (and H^+, unpublished work in progress) and probably chloride (Azzi and Azzone, 1967). With acetate, there is a rapid influx of salt. In all cases an osmotic equivalent of water fluxes with the salt, and despite the anomalies mentioned in the text it is reasonable to assume that the swelling and contraction are osmotic acts dependent upon salt transfer.

Thus the problem of salt transport is critically dependent on the anion present. As outlined earlier it is commonly thought that acetate freely permeates and chloride does not. If passive swelling can be used as a criterion to judge permeability here, this concept must be rejected. Passive swelling rates are not significantly different for chloride and acetate (Figure 1) and thus the anions must permeate at comparable rates. It is only on introduction of an energy supply *via* respiration or ATP hydrolysis that effect of anions on volume changes is revealed. Acetate is rapidly transported inward, and as reported here (Tables I–III, and Figure 7) acetate transport competes for energy with ATP formation and calcium-activated phosphate transport. Our laboratory has proposed that the transport of phosphate (Stoner *et al.*, 1964; Hodges and Hanson, 1965; Kenefick and Hanson, 1966), acetate (Hanson and Miller, 1967), and linoleate (Baddeley and Hanson, 1967) is at the expense of the hypothetical high-energy intermediate, X~P or X~acyl. The role of the cation is in reactive discharge of the acid group, with the cation accompanying the released anion.

The alternative would be that X~I is utilized in cation transport which would also be competitive with ATP formation, producing uncoupling or lack of respiratory control. With active cation transport the role of the anion would be limited to passively accompanying the cation. If it could not there would be a definite limit to inward cation transport due to a sharply rising electrochemical potential across the membrane. However, in corn mitochondria chloride passively permeates as rapidly as does acetate. There is no reason to believe that chloride would lag behind acetate if the rate of K^+ influx was stepped up by active K^+ transport. Although the rapid permeation of nondissociated acids is widely recognized, this property cannot be uniformly applied to active salt influx (Table VI; Chappell and Haarhoff, 1966). In order to solve this problem Chappell and Haarhoff (1966) suggest an anion carrier system with certain general specificities. We agree that anion carriers

which can recognize α substitution (and undoubtedly other structural modifications) must exist. For the present, however, these anion carriers can be grouped into "X." Indeed, inward transport of anions from "X" appears to be a characteristic of the inner mitchondrial membrane even in such a process as phosphorylation of ADP at the expense of X~P (Heldt, 1966).

It has previously been hypothesized that contraction of maize mitochondria involves the establishment and steady-state maintenance of a high-energy intermediate (Stoner *et al.*, 1964; Stoner and Hanson, 1966; Truelove and Hanson, 1966; Kenefick and Hanson, 1966) and the evidence has been summarized (Hanson and Hodges, 1965). The hypothesis is supported here by data which show diversion of energy for contraction into ATP formation (Table I). The energy conserved in the chloride system maintains contraction; if the conserved energy is used to form ATP, swelling sets in. It has also been demonstrated that Ca^{2+} and P_i can be transported after respiration ceases by energy conserved in the contracted state (Kenefick and Hanson, 1966). If acetate uptake is due to its reactivity with a high-energy intermediate, then chloride permits conservation of energy by virtue of its nonreactivity in the mitochondrial system. This inertness of chloride in the transport system permits observations on the physical parameters associated with the primary act of energy conservation at coupling sites. Important among these is the observation that K^+ (and H^+, unpublished data) is driven outward, not inward. If a carrier system for cation and/or proton transport does exist, it has polarity such that positive charges appear externally. The efflux of chloride or other anions will be passive along the potential gradient. The result is that salt is actively expelled in chloride, and this expulsion is not linked to utilization of I~X as with inward transport. The energy expended in electron transport at coupling sites appears in two phenomena; salt expulsion and I~X formation. If a cation pump exists it is active in association with energy conservation during respiration and has an outward polarity.

Figure 10 summarizes diagrammatically these observations. No attempt is made to characterize the nature of the coupling event, but Mitchell's concept of I~X formation by polar H^+ and OH^- ejection is adopted as a means of explaining H^+ appearance in the medium (Mitchell, 1966). A result of this is that I~X is "poised" with an enhanced membrane potential. Collapse of one will lead to collapse of the other. There is nothing to indicate the mechanism of K^+ efflux and it is simply indicated as occurring. The same is true for the extra divalent cation binding; it occurs in association with energy conservation and thus is conveniently indicated as $I\sim X{:}Me^{2+}$, but the actual site of binding is unknown. Introduction of a reactive anion such as acetate leads to salt influx at the expense of I~X through an X~acyl intermediate. If the accompanying cation has a role it lies with the stability of the X~acyl bond. X is here a general symbol for phosphoryl, arsenyl, and acyl carriers, and these are so structured to have an obligate inward polarity. One of these X carriers must be implicated in phosphorylation of ADP, and if it is not the same as the acyl carrier, the two must compete for activation at coupling sites.

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