Plant Physiol. (1966) 41, 1601–1609

Contracted State as an Energy Source for Ca Binding and Ca + Inorganic Phosphate Accumulation by Corn Mitochondria[1]

D. G. Kenefick[2] and J. B. Hanson
Department of Agronomy, University of Illinois, Urbana, Illinois 61803

Received July 7, 1966.

Summary. An investigation has been made of the possibility of utilizing the potential energy of the contracted state of corn mitochondria to drive Ca + inorganic phosphate accumulation. Contraction was obtained with succinate or NADH oxidation. In the succinate experiments the mitochondria were contracted in buffered KCl layered over sucrose in centrifuge tubes and centrifuged down through distinct wash, reactive and isotope exchange layers. In the NADH experiments, ion accumulation was initiated upon exhaustion of the substrate. The results show that mitochondria in the contracted state will actively bind some ^{45}Ca, but no real accumulation occurs until inorganic phosphate is available. Substrate powered contraction in the presence of inorganic phosphate also provides a potential for accumulation upon subsequent reaction of the mitochondria with Ca. It is deducted that contraction is due to X~I formation, to which Ca will bind. Subsequent reaction with inorganic phosphate produces CaX~P, which is the transport moiety. When X~P is formed first, Ca also reacts to produce CaX~P. Hence it is immaterial which ion reacts first with the contracted state. Contraction is believed to result from the action of a mechanoenzyme, presumably I~. The stability of CaX~I must be low for the mitochondria swell very rapidly upon exhaustion of NADH or blocking of succinate oxidation by cyanide.

Previous papers from this laboratory have reported the characteristics of corn mitochondria in Ca + P_i accumulation and in swelling-contraction. Substrate-powered calcium accumulation is dependent upon phosphate, and phosphate accumulation is dependent upon calcium, or the related strontium ion which is about half as effective (10, 25). The mitochondria show no pronounced uncoupling responses to Ca; the small decline in P/O ratio with 1 mM Ca is due to diversion of phosphate from ATP formation into P_i accumulation (7). Swelling is spontaneous in buffered KCl, with rapid contraction not only with ATP and Mg but also with the simple addition of an oxidizable substrate (23). The ATP-powered contraction is oligomycin sensitive and releases P_i, accounting for the bulk of the ATPase activity (23). The substrate powered contraction is very closely linked to respiration and is inhibited about 40 % by 1 mM phosphate (23, 25). Calcium releases the phosphate inhibition of contraction, simultaneously increasing respiration and activating P_i accumulation (25). Dinitrophenol in low concentration (30 μM) uncouples the substrate-powered phosphate uptake but does not reduce respiration or the contraction process (25).

From these observations we have concluded that the contraction process is very closely linked to electron transport, and that the level of contraction reflects the level of non-phosphorylated high energy intermediate (23, 25). The P_i inhibition of substrate-powered contraction must lie in the loss of non-phosphorylated intermediate in formation of the phosphorylated form. Calcium relieves the phosphate inhibition by activating P_i accumulation from the phosphorylated form, recycling the intermediate for increased electron flow and contraction (25).

Current opinions on contraction in animal mitochondria appear to be centered about the osmotic consequences of active salt movement (3, 14, 20, 24). Mitochondria presumably swell because they accumulate salt and contract because they lose it. Pressman (18) and Harris et al. (8) think the amount of salt transferred to be inadequate. There seems to be little active support for Lehninger's hypothesis on mechanoproteins (12). However, our observations on corn mitochondria would be most readily explained by assuming that the high

[1] Supported in part by the United States Atomic Energy Commission (AT/11-1/790). The research represents a cooperative effort of the South Dakota State University and University of Illinois Experiment Stations.

[2] On leave from the Department of Agronomy, South Dakota State University, Brookings, South Dakota.

energy intermediate (I∼X) has as one element a mechanoprotein producing contraction. Swelling could then result from either of 2 processes: spontaneous hydrolysis of the intermediate (I∼X → I + X), or reversible phosphorylation of the intermediate (I∼X + P_i ⟷ I + X∼P). The addition of P_i to substrate-contracted mitochondria does in fact cause a rapid drop to a lower level of contraction (23, 25). Calcium reverses this drop producing a burst of contraction, respiration and P_i accumulation (25), indicating that the intermediates have been recycled [I + X∼P $\xrightarrow{Ca}$ (Ca + P_i) inside + I + X].

If this view is correct, the contracted state should represent a potential for doing the osmotic work of Ca + P_i accumulation. Demonstration of this potential requires techniques for producing contraction, removing the substrate, then exposing the mitochondria to Ca + P_i. Such experiments have been done and are reported here.

Materials and Methods

Mitochondria were isolated in the cold from 100 g of 3-day etiolated corn shoots (*Zea mays* L., WF9 × M14) by grinding in an ice cold mortar with 250 ml of 0.4 M sucrose, 5 mM EDTA and 50 mM KH_2PO_4 neutralized with Tris to pH 7.5. The homogenate was strained through cheese cloth and the mitochondrial fraction collected by centrifugation at forces between 1000 × *g* and 11,000 × *g* for 10 minutes. The pellet was resuspended in 50 ml of 0.4 M sucrose and centrifuged at 1500 × *g* for 10 minutes. The supernatant fluid was decanted into clean tubes and made up to 80 μM ADP, which was added to reduce endogenous substrate and phosphate levels. Ten ml of cold 0.6 M sucrose was layered under each suspension which was then centrifuged at 9400 × *g* for 12 minutes. The supernatant fraction was removed by aspiration and the pellet was resuspended and made up to 4 ml with 0.4 M sucrose (about 0.7 to 0.9 mg N/ml) and the suspension was then stored on ice. All isolation procedures were conducted between 0 and 2°.

Nitrogen was determined on digested mitochondrial samples by nesslerization. Light scattering was measured in a Zeiss PMQ II spectrophotometer, and O_2 uptake with a Clark electrode in conjunction with a Heath recorder. In the ^{45}Ca and ^{32}P analysis mitochondria were centrifuged from the KCl layer through wash or reaction layers and collected at the bottom of the tube. The supernatant layers were carefully removed by aspiration. Extraction of ^{45}Ca was accomplished in 10 % trichloroacetic acid. Inorganic ^{32}P was extracted by an isobutanol procedure (26). The precipitate in either procedure was removed by centrifugation. Aliquots were counted in a Nuclear Chicago liquid scintillation spectrometer. (Specific experimental procedures are recorded in the legends.)

Results

Centrifuge Experiments. The objective in these experiments was to form the contracted state by reacting the mitochondria with succinate, then to centrifuge the contracted mitochondria down through discrete sucrose layers for washing, reacting with ions, exchanging off superficially bound isotope, and finally collecting the pellet for analysis. Figures 1 and 2 present the experimental set up and give typical results.

It was first determined that reaction with substrate in the presence of P_i produced conditions which would subsequently lead to ^{45}Ca uptake (fig 1). Presumably a high energy phosphorylated intermediate (X∼P) was formed during substrate oxidation in the presence of P_i. Sufficient intermediate must have survived removal from the substrate and subsequent washing to react with ^{45}Ca and produce uptake of the ion.

Next, the mitochondria were added to the KCl medium with or without succinate, but without P_i, and the mixture was layered on top of the sucrose (fig 2). Under this condition in the presence of substrate the mitochondria contract vigorously (23, 25), which as far as we know is the only physical

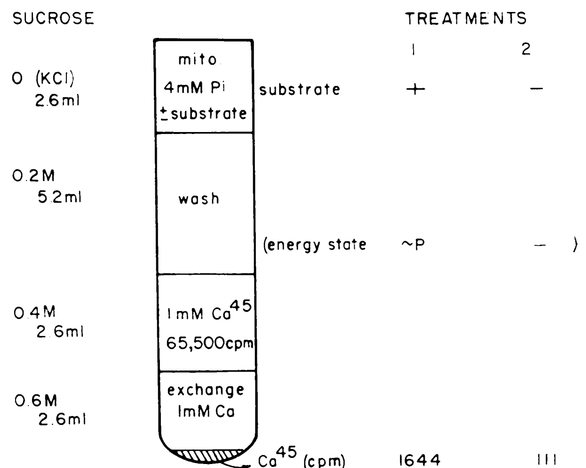


FIG. 1. ^{45}Ca uptake by mitochondria subsequent to reaction with substrate acids and P_i. Sucrose layers were prepared in 15 ml centrifuge tubes with additives as indicated. Mitochondria (0.1 ml; 76 μg N) were added to 2.5 ml of 0.1 M KCl, 0.02 M Tris (pH 7.6), 1 mg/ml BSA, 4 mM KH_2PO_4, 230 μM NAD, 170 μM TPP, ± substrate (10 mM succinate + 10 mM pyruvate) and the mixture layered on top of the sucrose. The tubes were placed in the #969 swinging bucket rotor of the International BD-2 centrifuge set at 15°. Mitochondria were then subjected to a stepwise increase of centrifugal force of 4500 × *g* for 2 min, 8700 × *g* for 2 min and 40,000 × *g* for 6 min (calculated from maximum radius). The pellet was extracted and analyzed for ^{45}Ca.

manifestation that an energy potential has been conserved. Any conserved energy for ion transport would need to be in a non-phosphorylated high energy state associated with the contraction. As shown in figure 2, the only appreciable uptake of ^{45}Ca occurred in the presence of P_i. Neither acetate, arsenate nor citrate would substitute for P_i (table I). Evidently the non-phosphorylated intermediate associated with contraction (X∼I) must first react with P_i producing the phosphorylated intermediate (X∼P) before ^{45}Ca is accumulated.

However, it will be noted in figure 2 that reaction with substrate caused a small increase in ^{45}Ca content of the mitochondria (tube 1 vs tube 3). Although small the increase was quite consistent and could not be ignored. It was the first evidence our laboratory had ever been able to obtain of active Ca binding in the absence of P_i as described for animal mitochondria (1, 5, 20, 22). With continuously available substrate, and proportionately much larger Ca accumulation, any such binding had probably been masked. In addition, the method of isolation used here produces mitochondria which are more active in contraction and have better respiratory control, suggesting that higher and more stable levels of the binding intermediate were produced.

Further investigation of the active Ca binding phenomenon showed that if the ^{45}Ca were introduced into the top layer with the substrate the amount of binding was considerably enhanced. Experiments illustrating different aspects of the energized binding are given in table II. The following points can be made:

A) Addition of the ^{45}Ca directly in the succinate layer produces 3 to 4 times as much ^{45}Ca in the pellet as does application in the third layer (expts 1, 2). Part of this may well be due to reaction time. The ^{45}Ca is with the mitochondria and succinate for 3 to 5 minutes while the mixture is prepared, layered, and the centrifuge set in operation. This is sufficient time for the mitochondria to achieve complete contraction and thus maximum binding potential. However, if the ^{45}Ca is not in the top layer some of the potential for binding may be dissipated as the mitochondria move away from the contracting substrate and down through the wash layer. Loss of potential coupled with rapid movement through the third layer could lower Ca binding.

B) Addition of ^{45}Ca with succinate is almost as effective as addition of P_i with succinate (expts 1, 2, 3). Certainly the amount of transport potential is of the same order of magnitude. It should be recognized that these experiments were intended to provide a single cycle of transport operation. That is, the transport mechanism was energized without Ca and P_i or with these ions singly, not with both ions together where there is constant recycling and massive transport (eg., 10, table IV). In a single cycle of operation the transport mechanism might be expected to bind roughly equivalent Ca or P_i since corn mitochondria show Ca/P_i accumulation ratios of about 1 (10, 25).

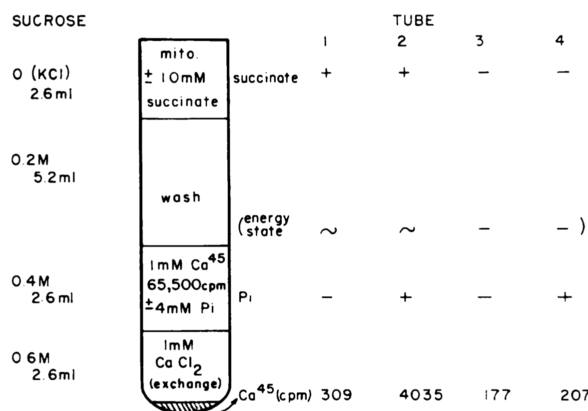


FIG. 2. Utilization of energy associated with the contracted state to produce ^{45}Ca accumulation. Conditions of experiment like those of figure 1, except that substrate was 10 mM succinate only and the KH_2PO_4 was moved to the third layer as indicated. Contraction of mitochondria as Δ OD_{520} in the top KCl layer was separately determined to be from 0.668 at the end of the swelling period to 0.838 in 5 minutes, the approximate length of time required to prepare and add the top layers, precool, and start centrifuging.

Table I. *Necessity for P_i to Secure ^{45}Ca Accumulation*

Experiments were performed in the centrifuge as illustrated in figure 2. 72,000 cpm of ^{45}Ca were added.

Tube	Layer 1 (top)	2	3	4	Pellet cpm
1	succinate	wash	^{45}Ca	Ca exch	363
2	succinate	wash	^{45}Ca + 1 mM P_i	Ca exch	1388
3	succinate	wash	^{45}Ca + 1 mM Acetate	Ca exch	333
4	succinate	wash	^{45}Ca + 1mM AsO_4	Ca exch	450
1	succinate	wash	^{45}Ca	Ca exch	291
2	succinate	wash	^{45}Ca + 4 mM P_i	Ca exch	2124
3	succinate	wash	^{45}Ca + 4 mM AsO_4	Ca exch	306
4	succinate	wash	^{45}Ca + 4 mM citrate	Ca exch	249

Table II. *Active ^{45}Ca Binding in the Contracted State Produced by Succinate, and Subsequent Transport with P_i*
Experiments were performed in centrifuge tubes as in figures 1 and 2, with 1 mM Ca and 4 mM P_i and 10 mM succinate as indicated. In expt 4 the P_i was 1 mM labeled with 356,000 cpm ^{32}P. Expt 1, 2, and 3 had 65,500 cpm of ^{45}Ca; expt 5 had 53,700. In expt 5 the Ca exchange layer was increased to 5.2 ml, reducing layer 2 to 2.6 ml.

Expt	Tube	Layer 1 (top)	Layer 2	Layer 3	Layer 4	Pellet
1	1	succinate	wash	^{45}Ca	Ca exch	503
	2	succinate + P_i	wash	^{45}Ca	Ca exch	2364
	3	succinate	wash	^{45}Ca	Ca exch	582
	4	succinate + ^{45}Ca	wash	wash	Ca exch	1701
2	1	succinate	wash	^{45}Ca	Ca exch	258
	2	succinate + P_i	wash	^{45}Ca	Ca exch	1905
	3	succinate	wash	^{45}Ca + P_i	Ca exch	1539
	4	succinate + ^{45}Ca	Ca exch	wash	wash	1176
3	1	P_i	wash	^{45}Ca	Ca exch	204
	2	succinate + P_i	wash	^{45}Ca	Ca exch	3057
	3	^{45}Ca	Ca exch	wash	P_i	288
	4	succinate + ^{45}Ca	Ca exch	wash	P_i	2604
4	1	Ca	wash	$^{32}P_i$	wash	96
	2	succinate + Ca	wash	$^{32}P_i$	wash	2925
5	1	succinate + ^{45}Ca	wash	P_i	Ca exch	2931
	2	^{45}Ca	wash	P_i	Ca exch	335
	3	succinate + ^{45}Ca	wash	wash	Ca exch	1190
	4	^{45}Ca	wash	wash	Ca exch	355

C) The binding reaction of Ca in the presence of succinate conserves energy which can be subsequently used in $^{32}P_i$ transport (expt 4).

D) If the ^{45}Ca bound in the presence of succinate is reacted with P_i in the third layer, a good deal more ^{45}Ca appears in the pellet after exchange (expt 5). It would appear that the ^{45}Ca is bound to some energized component which is accessible to exchange with the unlabeled Ca used to reduce superficially bound ion. If the actively bound ^{45}Ca first reacts with P_i, however, transport to the matrix must occur, preventing ready exchange.

NADH Contraction and Resulting Transport. There are 2 basic difficulties with the foregoing experiments. The first is that one cannot simultaneously follow swelling and contraction and ion transport. The principle difficulty however is that a bit of succinate might be accumulated during reaction in the top layer, and survive washing to be oxidized in the third layer, directly producing ion accumulation. An attempt was made to assess this possibility by reacting 0.2 ml of mitochondria in the KCl medium ± succinate, as in figure 2, then centrifuging them down through 0.4 M sucrose. The mitochondria were immediately suspended in 0.2 ml of 0.4 M sucrose at room temperature and added to the KCl medium in the oxygen electrode chamber. No endogenous respiration could be found for the mitochondria not exposed to succinate, but there was a trace of respiration from those which were exposed to succinate. The maximum respiration found was 9 mμmoles O_2, the oxygen being consumed in 2 minutes at 28°. This respiration rate is so low that it would hardly be adequate in itself to provide the Ca accumulation noted in the foregoing experiments (eg., tube 2, fig 2), but it could contribute.

Cyanide was used in an attempt to block respiration once the mitochondria had attained full contraction (table III). Immediately after the addition of cyanide, P_i was introduced to 1 treatment. As reported in the legend of table III, parallel measurements of light scattering show swelling to be very rapid on introduction of cyanide, but when P_i is also added the rate slows markedly and there is additional ^{45}Ca uptake. However, parallel determinations with the oxygen electrode showed 1 mM cyanide to only inhibit respiration by 80 to 90 %. Hence there is still the possibility that this residual respiration might be coupled to ^{45}Ca + P_i uptake, and thus not all of the uptake would be due to utilization of the potential of the contracted state.

To overcome these problems, experiments were done with NADH, which gives rapid contraction but is quickly exhausted. Figure 3 shows a typical result in which contraction, respiration and ion accumulation were followed in 3 parallel treatments. Rapid respiration was associated with contraction and with maintenance of the contracted state. When the NADH was exhausted the mitochondria swelled rapidly, and when collected showed no evidence of ^{45}Ca binding. If P_i were added just at the point of NADH exhaustion, swelling was retarded and some ^{45}Ca was accumulated. There

Table III. *^{45}Ca Accumulation from the Contracted State After Blocking Respiration with Cyanide*

Mitochondria were placed in 2.6 ml of 0.1 M KCl, 0.02 M Tricine (pH 7.6), 1 mg/ml bovine serum albumin (BSA) 1 mM ^{45}Ca (103,000 cpm total), and allowed to swell at room temperature (about 25°) for 10 minutes (Δ OD_{520} = −0.149 from 0.778 in parallel measurement). Contraction, where indicated, was initiated with 10 mM succinate + 10 mM pyruvate with 40 μM CoA, 230 μM NAD and 170 μM TPP (Δ OD = +0.078, leveling out at 3 min). At 15 minutes 1 mM KCN was added to all tubes, followed immediately by 4 mM P_i as indicated. Respiration as determined separately with the oxygen electrode was inhibited 89 % by KCN addition and was paralleled by immediate swelling of the mitochondria. (Δ OD = −0.133 in 4 min without P_i and −0.036 with P_i). 10 ml of 0.6 M sucrose was layered beneath the reaction mixture, and at 19 minutes the mitochondria were centrifuged down and analyzed for ^{45}Ca.

Swelling 0–10 min	Contraction 10–15 min	+KCN ± P_i 15–19 min	^{45}Ca in pellet cpm
+	+	+ P_i	4225
+	−	+ P_i	1010
+	+	− P_i	1925
+	−	− P_i	1120

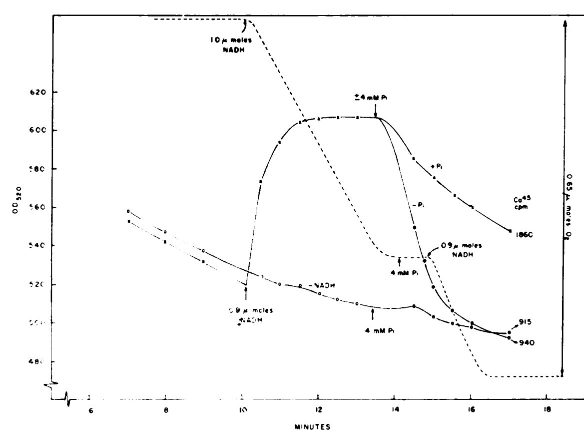


Fig. 3. The relationship between swelling-contraction (solid line), rate of oxygen uptake (broken line) and ^{45}Ca uptake (right margin) by mitochondria under conditions of NADH depletion observed in the presence and absence of 4 mM P_i. All measurements were made in parallel from a common stock of 27.5 ml of 0.10 M KCl, 0.02 M Tricine pH 7.6, 1 mg BSA/ml, 1 mM $CaCl_2$ + ^{45}Ca (61,000 cpm/2.6 ml) to which 1.1 ml mitochondria (86 μg N per final 2.6 ml aliquot) were added to initiate the 10 minute swelling period. Immediately 3 each 2.6 ml aliquots were added to cuvettes, 6 each 2.6 ml aliquots were added to centrifuge tubes for duplicate ^{45}Ca uptake and 1 2.6 ml aliquot to the oxygen electrode cell. At 10 minutes 0.9 μmoles NADH was added to each of the cuvettes and centrifuge tubes and 1.0 μmole to the oxygraph cell and contents were mixed. When oxygen uptake stopped, 4 mM P_i (pH 7.6) was added to appropriate samples and ^{45}Ca uptake was determined in the centrifuge tubes as lescribed in the legend of table III.

is no question here about identifying substrate carry over, for if the P_i was added before the NADH was gone there was a burst of additional contraction which is related to the higher oxidation rates in the presence of Ca and P_i (fig 4).

In following up this latter observation we examined NADH oxidation as affected by Ca and P_i (insert, fig 4). Calcium strongly activates NADH oxidation. Hackett (6) has reported on this phenomenon in plant mitochondria. It is a specific divalent cation effect over and above the general ionic strength effect. Hackett also reported an optical density increase that occurred on the addition of divalent ions to a mitochondria suspension. He suggested that the optical density changes denoted a change in physical structure facilitating the access of NADH to reactive sites of the respiratory chain. Hackett's explanation is in accord with our results, as the increased oxidation of NADH upon addition of Ca is not associated with Ca accumulation (fig 3, 4 and table IV). Only when P_i is added

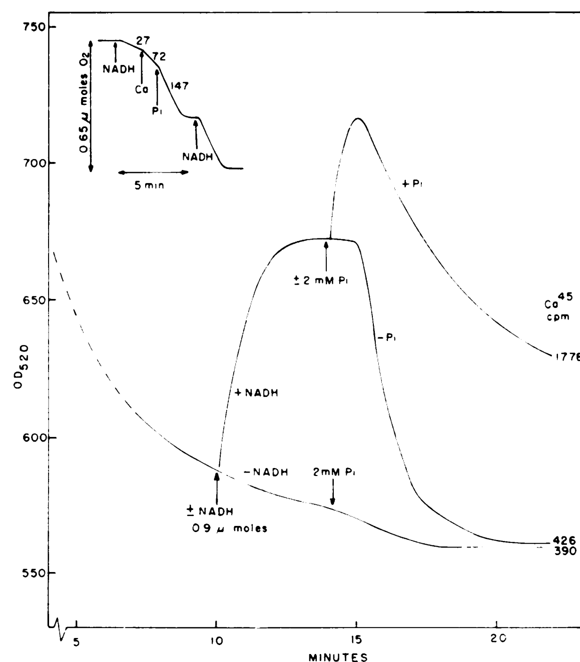


Fig. 4. The effect of P_i addition on contraction, oxygen consumption (insert) and ^{45}Ca uptake when added prior to NADH depletion. Data collected in the manner described in figure 3. Mitochondria (87 μg N/0.1 ml) were added to 2.5 ml of reaction mixture containing 0.5 mM $CaCl_2$ + ^{45}Ca.

Table IV. *^{45}Ca Binding and Accumulation Powered by NADH Oxidation*

Centrifuge tubes contained 2.5 ml of 0.1 M KCl, 0.02 M Tris (pH 7.6), 1 mg/ml BSA, 1 mM $CaCl_2$ + ^{45}Ca (58,500 cpm total) with 0.9 μmole NADH and 4 mM P_i as indicated. Reaction started by addition of 0.1 ml mitochondria (0.093 mgN) and run for 4 minutes at room temperature. Tubes were quickly chilled and 10 ml of ice cold 0.6 M sucrose was layered beneath the reaction mixture. The mitochondria were centrifuged through the sucrose in a total of 5 minutes where force attained during this period was 28,000 × *g*, collected and analyzed for ^{45}Ca. Respiration of 0.1 ml of mitochondria was determined with the oxygen electrode in the same buffered KCl + BSA mixture with 1 mM Ca, 0.9 μmole NADH and 4 mM P_i as indicated. NADH oxidation rate before addition of Ca or P_i was 37 mμmoles per minute.

Treatment	^{45}Ca uptake cpm/pellet	Respiration rate mμmoles/min
^{45}Ca	515	0
NADH + ^{45}Ca	981	90
NADH + ^{45}Ca + P_i	19820	138

Table V. *Removal of Bound ^{45}Ca by Exchange*

Experimental procedure was the same as described in table IV, except differential ^{45}Ca exchange was accomplished by the presence or absence of 1 mM $CaCl_2$ in the 0.6 M sucrose layer. Total counts of ^{45}Ca added per tube was 103,600 cpm.

Additives to KCl medium	Centrifugation through sucrose ± $CaCl_2$ cpm/pellet			
	No exchange	Exchange	Loss	% Loss
^{45}Ca	1055	770	285	27
^{45}Ca + NADH	2160	1570	590	27
^{45}Ca + P_i	935	585	350	37
^{45}Ca + P_i + NADH	71,265	68,630	2635	4

is Ca accumulation found, although again there is some extra binding of ^{45}Ca linked to substrate oxidation in the absence of P_i (table IV). Proportionately, this extra calcium is just as subject to exchange as is that held in the absence of substrate (table V). In these experiments the mitochondria were recovered prior to substrate exhaustion and swelling, and thus did not lose the bound ^{45}Ca (cf. figs 3, 4).

It must be emphasized that the Ca-activated respiration occurs only when NADH is substrate. As previously reported (7) addition of Ca to an acceptorless system oxidizing an organic acid substrate yields only a very small and transitory respitory response unless P_i is also present.

Discussion

The experiments demonstrate 3 additional characteristics of the Ca + P_i transport mechanism of corn mitochondria:

1) There is a binding of Ca to the activated transport mechanism, and the bound Ca is exposed to exchange for external Ca.

2) In the final realization of Ca + P_i transport it makes no difference in which order the ions are presented to the activated mechanism.

3) Activation of the transport mechanism is somehow associated with contraction.

In short, the contracted state formed in association with substrate oxidation provides a potential for Ca binding in the absence of P_i and Ca + P_i accumulation in the presence of P_i. In the centrifuge experiments some contribution to binding or accumulation may have been made by accumulated succinate. Even a low rate of respiration during centrifugation would tend to maintain the contraction and binding potential achieved in the top layer (cf. 5). With the NADH experiments there is no question about residual respiration since none could be detected, but there was still ^{45}Ca accumulation if P_i was added. However, without P_i there was rapid swelling and any actively bound ^{45}Ca was released.

Due to the ready dissipation of the contracted state in saline media (ie: rapid spontaneous swelling) it is difficult to make a quantitative assessment of how much potential is available. One can estimate from tables IV and V, where no ^{45}Ca-Ca exchange was carried out, that 20 to 25 mμmoles ^{45}Ca were bound in association with the contracted state (treatments with ^{45}Ca + NADH less those with ^{45}Ca alone, related to total ^{45}Ca available). From this it can be calculated that the net accumulation of 1650 mμmoles of ^{45}Ca in 4 minutes (table V, treatment with ^{45}Ca + P_i + NADH with exchangable ^{45}Ca removed) represents an average turnover of 15.5 per minute of the extra ^{45}Ca bound in the presence of NADH, assuming this actively bound ^{45}Ca to be the source of that accumulated in the

presence of P_i. Since the time course of ^{45}Ca accumulation shows a rapid initial rate which declines over a few minutes (10, 25), the initial turnover must be even greater. In addition there is a process dissipating the contracted state as shown by the rapid swelling on exhaustion of substrate (fig 3, 4) or the addition of cyanide (table III). Even though the P_i needed for accumulation retards this dissipation it still exists, and hence the turnover of the Ca-binding intermediate must be still larger by some additional factor which we cannot estimate. At the present the best one can do is state that qualitatively part of the potential associated with contraction can be utilized in ^{45}Ca binding or Ca + P_i accumulation, and let the quantitative aspects await experimental refinements.

In terms of the standard type I hypothesis of oxidative phosphorylation, the contracted state must correspond with formation of a non-phosphorylated high energy intermediate. Formation of the intermediate seems to be generally accepted as meaning formation of a covalent bond with a high standard free energy of hydrolysis. The exception to this lies in the chemiosmotic hypothesis of Mitchell (15, 16, 17). It is not clear to us how one applies Mitchell's hypothesis to formation of a contracted state with the potential to transport Ca, particularly when there is an evident requirement for phosphorylation to secure actual transport. Contraction might well result from dehydration of a membrane constitutent following polar H^+ and OH^- ejection, but subsequent use of the potential through a phosphorylated intermediate would still suggest that a chemical bond equivalent in energy to an acid anhydride must have been formed. As a matter of convenience, the results will be discussed from the viewpoint of the older carrier-covalent bond hypothesis.

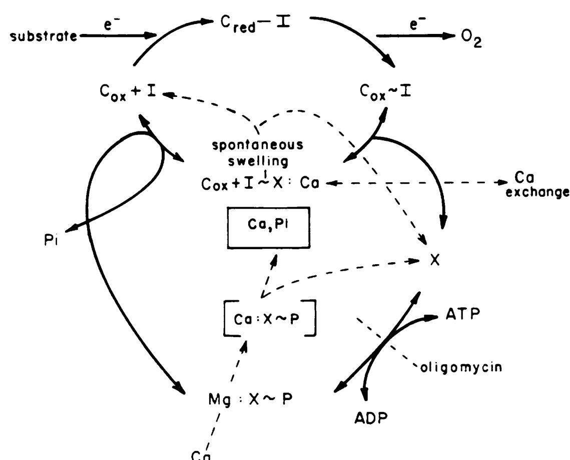


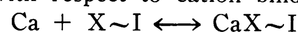
FIG. 5. A schematic representation of the oxidative phosphorylation pathway as it appears to exist in corn mitochondria (solid lines) and the reactions leading to spontaneous swelling, Ca exchange and Ca + P_i accumulation (dotted lines). See text for details.

As previously pointed out (25), there are actually 2 non-phosphorylated bonds which are candidates for the potential chemical energy stored during contraction. This is shown in figure 5, which is a schematic representation of the situation which appears to exist in corn mitochondria. The conventions used are those of Chance and Williams (2) except that the initial high energy bond is considered to be with the oxidized form of the carrier (13, 19). Cyanide, which should produce reduced conditions, inhibits substrate powered contraction in corn mitochondria (23, table III). The unknown I would then correspond to a carrier-linked enzyme.

The 2 non-phosphorylated bonds are $C_{ox}\sim I$ and $I\sim X$. Both of these occur in what might be called the "I-cycle", the cycle involving oxidation and reduction of the electron carriers. The lower, or "X-cycle", is concerned with phosphorylation and transport reactions. The cycles mesh through $I\sim X$. Contraction is somehow closely linked to respiration, and thus to operation of the I-cycle. As discussed elsewhere (25) uncouplers do not affect contraction except to increase it when they increase respiration and depress it when they depress respiration. Perhaps then contraction is associated with formation or utilization of $C_{ox}\sim I$ as indicated in the schemes of Crofts and Chappell (4) and Lardy et al. (11), respectively. However, there should be rapid equilibration between the 2 states, and for lack of other evidence it would be better to simply equate the level of $I\sim$ with the extent of contraction. It follows then that rate of contraction should parallel the rate of attaining steady state oxidation in the electron donating carriers at coupling sites (eg., cytochrome *b*) following the initial burst of reduction on addition of substrate.

Spontaneous hydrolysis of $I\sim X$ would lead to rapid swelling. Spontaneous swelling is not found in sucrose except under special conditions of aging and chelation (23), so there must be a role for inorganic salts in promoting spontaneous hydrolysis. Our laboratory has tried repeatedly to define this role in terms of active salt transport with resultant osmotic swelling, but we have failed to date (J. Carter, unpublished thesis research). However, a reaction of cations with $I\sim X$ to give spontaneous hydrolysis and swelling seems to be a reality under some circumstances (eg: the rapid swelling after NADH exhaustion in the presence of Ca, figs 3, 4), but no reliable evidence for associated transport has ever been found unless P_i is included. This cation activated secondary swelling is under current study.

The evidence reported here (table II) strongly suggests that the basic formulation of Chance (1) is correct with respect to cation binding: that is,

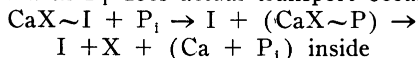


$$Ca + X\sim I \longleftrightarrow CaX\sim I$$

Hydrogen or other cations bound to X would be displaced. Calcium so bound appears to still be available for ready exchange with external Ca, and thus cannot be considered transported. The Ca must be held in a dynamic steady state, much as

described by Drahota et al. (5). Once respiration ceases (as in fig 3) CaX~I cannot be maintained and the contracted state dissipates with a half time of about 1 minute, releasing bound Ca. In the centrifuge experiments the mitochondria must have been moved rapidly enough that some CaX~I survived (dissipation in the pellet would not alter the ^{45}Ca analysis).

The above comments refer to the active binding (see fig 5 for schematic representation). Only on reaction with P_i does actual transport occur:



$$\text{CaX}\sim\text{I} + P_i \rightarrow \text{I} + (\text{CaX}\sim\text{P}) \rightarrow \text{I} + \text{X} + (\text{Ca} + P_i) \text{ inside}$$

The recycling of I and X leads to the higher respiration rate which occurs with Ca + P_i (fig 4, table IV, 7).

As suggested before (25) perhaps corn mitochondria fail to transport Ca with other anions (table I, 10, 25) because they cannot form the equivalent X~acyl or because X~AsO_4 is stable only in the hydrophobic membrane phase.

Figure 5 is drawn with the assumption that X~binds cations, and since the endogenous Mg of corn mitochondria is quite high (10), a further assumption is made that MgX~I and MgX~P are the normal intermediates. (fig 5 does not show MgX~I since CaX~I was needed to illustrate the binding of Ca). As with other mitochondria, there is a Mg requirement for energy-linked processes driven by ATP, such as contraction (23), ATPase (7, 23) and Ca + P_i uptake (10). It is further visualized that Ca mediates an aborted reaction by displacing Mg to yield an unstable CaX~P which degrades in a polar fashion to give transport (25). The Ca can be bound while the intermediate is in the X~I form, some of which survives to produce transport of Ca + P_i after reaction with P_i (table II). Thus it is possible for the contracted state to yield transport without regard for whether Ca or P_i reacts first.

Nothing in the work our laboratory has done to date on the swelling-contraction phenomenon indicates that contraction consumes bond energy; that is, I~being utilized in producing contraction. Rather, contraction seems to represent, or be associated with, the conservation of energy derived from substrate oxidation. The need for a continuous input of energy is to offset the continued wastage of energy in spontaneous hydrolysis of I~X. For this reason we assume that contraction is a function of the relative amount of intermediate in the non-phosphorylated high energy state (23). If this is true, then a mechanoenzyme such as discussed by Lehninger (12) could be implicated. This is not to deny that there will be an osmotic consequence of ion transport, but only that the basic contraction mechanism operates independently of osmotic volume adjustments. Coupled electron transport might involve a mechanical response in a coupling mechanism which is associated with a potential for Ca binding and Ca + P_i transport. To be consistent, I~ should then represent a mechanoenzyme in the contracted state. The only apparent alternative is to draw on the charge separation hypothesis (15, 16, 17, 21), assuming that creation of additional transmembrane electro-chemical potential or dehydration of a membrane element produces the contraction. Hind and Jagendorf (9) have suggested that a transmembrane pH gradient created by light is responsible for associated changes in light scattering and phosphorylation potential in chloroplasts. Plant mitochondria might be expected to show related phenomena. At the moment we have no evidence for evaluating these possibilities. As mentioned above, dehydration by polar loss of H^+ and OH^- could yield an anhydride bond equivalent to the non-phosphorylated intermediate of the type I scheme, and thus not be basically different. That is, the scheme we use (fig 5) is concerned with events contingent upon formation of a high energy non-phosphorylated bond (I~X), with nothing known as to how the bond is formed. We do believe, though, that formation of the bond could involve a mechanical response in the membrane or matrix responsible for the noted increase in light scattering, water expulsion, and conformational changes in the cristae. Furthermore, cations would be implicated in the mechanical response, at least to the extent of governing its stability.

Acknowledgment

We are very appreciative of the excellent technical assistance of Mr. Joseph C. Nicholas.

Literature Cited

1. Chance, B. 1965. The energy linked reaction of calcium with mitochondria. J. Biol. Chem. 240: 2729–48.
2. Chance, B. and G. R. Williams. 1956. The respiratory chain and oxidative phosphorylation. Advan. Enzymol. 17: 65–134.
3. Chappell, J. B. and A. R. Crofts. 1965. Gramicidin and ion transport in isolated liver mitochondria. Biochem. J. 95: 393–402.
4. Crofts, A. R. and J. B. Chappell. 1965. Calcium ion accumulation and volume changes of isolated liver mitochondria. Reversal of calcium ion induced swellings. Biochem. J. 95: 387–92.
5. Drahota, Z., E. Carafoli, C. S. Rossi, R. L. Gamble, and A. L. Lehninger. 1965. The steady state maintenance of accumulated Ca^{++} in rat liver mitochondria. J. Biol. Chem. 240: 2712–20.
6. Hackett, D. P. 1961. Effect of salts on DPNH oxidase activity and structure of sweet potato mitochondria. Plant Physiol. 36: 445–52.
7. Hanson, J. B., S. S. Malhotra, and C. D. Stoner. 1965. Action of calcium on corn mitochondria. Plant Physiol. 40: 1033–40.
8. Harris, E. J., R. Cockrell, and B. C. Pressman. 1966. Induced and spontaneous movements of potassium ions into mitochondria. Biochem. J. 99: 200–13.

9. HIND, G. AND A. T. JAGENDORF. 1965. Light scattering changes associated with the production of a possible intermediate in photophosphorylation. J. Biol. Chem. 240: 3195–201.
10. HODGES, T. K. AND J. B. HANSON. 1965. Calcium accumulation in maize mitochondria. Plant Physiol. 40: 101–08.
11. LARDY, H. A., J. L. CONNELLY, AND D. JOHNSON. 1964. Antibiotics as tools for metabolic studies. II. Inhibition of phosphoryl transfer in mitochondria by oligomycin and aurovertin. Biochemistry 3: 1961–68.
12. LEHNINGER, A. L. 1962. Water uptake and extrusion by mitochondria in relation to oxidative phosphorylation. Physiol. Rev. 42: 467–517.
13. LEHNINGER, A. L. AND C. L. WADKINS. 1962. Oxidative phosphorylation. Ann. Rev. Biochem. 31: 47–78.
14. LYNN, W. S. AND R. H. BROWN. 1965. Role of anions in mitochondrial swelling and contraction. Biochim. Biophys. Acta 110: 445–58.
15. MITCHELL, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature 191: 144–48.
16. MITCHELL, P. 1962. Molecule, group and electron translocation through natural membranes. Biochem. Soc. Symp. 22: 142–69.
17. MITCHELL, P. AND J. MOYLE. 1965. Stoichometry of proton translocation through the respiratory chain and adenosine triphosphatase systems of rat liver mitochondria. Nature 208: 147–51.
18. PRESSMAN, B. C. 1965. Induced active transport of ions in mitochondria. Proc. Natl. Acad. Sci. 53: 1076–83.
19. RACKER, E. 1965. Mechanisms in Bioenergetics. Academic Press, New York.
20. RASSMUSSEN, H., B. CHANCE, AND E. OGATA. 1965. A mechanism for the reactions of calcium with mitochondria. Proc. Natl. Acad. Sci. 53: 1069–76.
21. ROBERTSON, R. N. 1960. Ion transport and respiration. Biol. Rev. 35: 231–64.
22. ROSSI, C. S. AND A. L. LEHNINGER. 1964. Stoichiometry of respiratory stimulation, accumulation of Ca^{++} and phosphate, and oxidative phosphorylation in rat liver mitochondria. J. Biol. Chem. 239: 3971–80.
23. STONER, C. D. AND J. B. HANSON. 1966. Swelling and contraction of corn mitochondria. Plant Physiol. 41: 255–66.
24. TEDESCHI, H. AND H. J. HEGARTY. 1965. Osmotic reversal of Ca^{++} induced mitochondrial swelling. Biochem. Biophys. Res. Commun. 19: 558–64.
25. TRUELOVE, B. AND J. B. HANSON. 1966. Calcium-activated phosphate uptake in contracting corn mitochondria. Plant Physiol. 41: 1004–13.
26. WIELE-MALHERBEE, H. AND R. H. GREEN. 1951. The catalytic effect of molybdate on the hydrolysis of organic phosphorus bonds. Biochem. J. 49: 286–92.