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Development and Function of
Membrane Systems in Plant Tissue

MASTER

Annual Progress Report

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Abstract

Studies of phosphate absorption by corn mitochondria indicate that transport is mediated via a sulfhydryl-sensitive carrier, just as with animal mitochondria. Presumably the carrier is electroneutral with Pi^- exchanging for OH^- ions created by respiration. However, very little respiratory release accompanies this transport; most of the increase is due to turnover of the coupling system with internal phosphate. Depletion of internal phosphate in ATP formation lowers the rate of respiration.

Calcium ions react with mitochondria to release respiration and accelerate acetate transport in a sucrose medium.

The failure of many plant mitochondria to show uncoupler-stimulated ATPase lies with a low concentration of internal phosphate. If the internal concentration of phosphate is re-established by respiration the ATPase is reactivated. The phosphate gradient establishes a potential for ATP transport into the active sites.

Washed corn roots show a trebling of ion uptake rate in 4 hours. No known plant growth substances appear to be involved in this induction. The anti-auxin 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) is very inhibitory to the development of salt uptake capacity. 2,4,6-T also prevents the increase in linoleic acid in the microsomal membranes. Addition of linoleic acid to the washing medium stimulates the rate and extent of ion absorption rates.

I. Annual Research Report

15 July 1971 - 14 July 1972

A. Phosphate and acetate transport in corn mitochondria.

We continue to give emphasis to studies on the mechanism of active anion transport through mitochondrial membranes, especially of phosphate. Past work under this contract has shown Ca^{++} to strongly activate Pi transport. Our interpretation was that Pi transport was primary, with Ca activating the transport by reacting with a high energy intermediate, $\text{T}^{\sim}\text{X}$ or $\text{X}^{\sim}\text{P}$. Work with vertebrate mitochondria indicates the contrary; Ca^{++} may be actively transported by a high-affinity carrier protein without need for Pi (1). However, yeast (2) and blowfly mitochondria (3) seem to lack this carrier protein, and blowfly mitochondria (3) and brown fat mitochondria (4) resemble corn mitochondria in requiring phosphate for Ca^{++} uptake.

Last year we worked primarily on investigating the activation of Pi transport by polycations (protamine, pancreatic ribonuclease, and polylysine), and reported on the results in the annual report. This work has now been published (5, COO-790-45). It was found that polycations caused extensive phosphate swelling, simultaneously releasing acceptorless respiration. This loose coupling lowered ADP:O ratios and respiratory control ratios. Protamine appeared to compete with Ca^{++} for activation of Pi accumulation.

We suggested that the release of respiration was "loose coupling", with the energy diverted to ion uptake from ATP formation.

As noted in last year's report, there was one troublesome observation. If acetate were substituted for phosphate, protamine would accelerate the swelling but there was no respiratory release. If no protamine were used the rates of active phosphate and acetate swelling were lower, but phosphate still partially released respiration and acetate did not. This led us to suspect that it was not the act of transporting phosphate that increases respiration, but rather the presence of the accumulated phosphate in the matrix.

1. WILLIAM KENNETH BOBOL

This proves to be true. We have finished the initial work here and have submitted a paper describing it to Plant Physiology. Rather than repeat the details we attach the manuscript as part of this report (coded COO-790-46, reference 6).

The most important result from this investigation is that we now have good evidence that plant mitochondria transport phosphate into the matrix before it reacts with the high energy intermediates of the coupling system. As with animal mitochondria (7) organic mercurials such as mersalyl block P_i transport in corn mitochondria, and if the transport is blocked, so are reactions of P_i leading to ATP formation. Hence, reactions of P_i with the coupling mechanism are from the inside, not the outside as we previously assumed (8). This makes P_i transport a separate act from phosphorylation of ADP, and accounts for the failure of oligomycin to block P_i uptake.

In the paper referred to above (6) we conclude that respiration must furnish both a proton motive force (PMF, a proton gradient) and a high energy intermediate if the respiratory release with phosphate is to be accounted for. The energy demand for transport is small, but considerable energy is dissipated in recycling the coupling intermediates. Acetate, is transported but apparently does not react with the intermediates to any significant degree.

However, according to Mitchell's chemiosmotic hypothesis acetate transport by itself should release respiration. Brierley's (9) model for acetate swelling shows this.

reactant structure shown here.

transformation by direct reaction between reactants. Equation (c) model for

however, according to Hirschfeld's photochemical reaction scheme
 not sufficiently good not react with the intermediates to any significant degree.
 suggested to react with the combined intermediates. According to Hirschfeld's
 for, the overall reaction for reaction is small, but considerable energy is
 needed. Hirschfeld's (c) the reactionally between after products is to be discussed.
 under similar form a broken bonds bonds (H₂O) a broken diameter) and a H₂O.

In the Haber reaction to form (c) no complete free reaction
 according to the nature of oxygen to react at surface.
 this takes place between a substance for from photochemical reaction of H₂O and
 intermediates are from the surface, not the surface as to photochemical reaction (c).
 or it is related to H₂O formation. Hence, reactions of H₂ after the combined
 reaction to form intermediates, and if the reaction is possible, so the reaction
 is after surface intermediates (a) oxygen molecules as a catalyst block at
 before it reacts with the main energy intermediates of the combined reaction.
 does evidence that H₂ and intermediates reaction products into the reaction

The most important reaction from (c) is photochemical reaction is from the main reaction
 reference (c).

reaction is shown the mechanism as part of the reaction (coded COO-100-10)
 simplified a Haber reaction is to H₂O photochemical. Hence, this reaction the
 this reaction to be from, no more intermediates the reaction work here and here

Collapse of the proton gradient in acetate transport should permit additional electron transfer and greater oxygen consumption, but the respiratory response is small (Fig. 2B, COO-790-47). At the present Dr. Byron Bertagnolli is investigating this problem. The more important observations made with respect to active acetate transport and release of respiration are as follows:

1. In a buffered sucrose medium, increased acetate transport can be secured by addition of Ca^{++} (Figure 1A,1B) which also releases the respiration rate. Past experiments using only K acetate as the supporting medium never showed a release of respiration with Ca^{++} or augmentation of swelling. Earnshaw, while working in our laboratory, previously found a transient uptake of Ca^{++} during acetate pumping lasting about 30 seconds before efflux (he is now writing up his results).

2. Addition of mersalyl increases the rate of acetate swelling (cf. Figures 1A & 1B). This is the enhanced permeability effect of mercurials described by Brierley (10).

3. Addition of the uncoupler 2,4-dinitrophenol (DNP) gives an abrupt shrinkage as the energy supply is interrupted. However, after a new equilibrium is established, influx pumping of acetate is resumed. The efficiency of pumping is reduced in that the ratio of the initial swelling rate/respiration rate is reduced.

4. When uncoupling is secured with arsenate + ADP the rate and extent of acetate swelling is small (Figure 2). Addition of oligomycin, which blocks arsenate uncoupling, greatly increases the rate of acetate pumping at the same time that respiration declines. Arsenate uncoupling differs from DNP uncoupling in that the arsenylated analog of ATP (ADP-As) is thought to be formed and immediately hydrolyzed (11). Hence, Figure 2 really illustrates the competition between acetate uptake and ATP formation reported previously (8).

We are comparing the action of Ca^{++} on acetate uptake with that on

phosphate uptake. When Ca^{++} accompanies Pi into the mitochondria there is a reduction of osmotic pressure as the ions precipitate and go out of solution. This causes shrinkage. In addition, there appears to be considerable additional light-absorption due to the precipitates themselves. Hence, the transmission traces decrease instead of increasing as with acetate uptake.

Figure 3 shows this phenomenon. We had hoped to be able to load the mitochondria with Pi, block further transport with mersalyl, then add Ca^{++} and see if internal Pi reacting with the coupling system would still give uptake. As shown in Figure 3A we have had only had partial success in determining the answer. It proves necessary to use low concentrations of the mercurial otherwise membrane semipermeability is damaged. Low mersalyl does not completely stop Pi transport, and there is some uptake of Ca^{++} and Pi. K^+ and H^+ are lost, as is a trace of Mg^{++} . Balancing in terms of positive charge shows 280 equivalents taken up and 252 equivalents lost (assuming $\text{Pi}^{1.5-}$), which considering the possible leakage involved in recovering the mitochondria from the solution (they were collected at the indicated points and centrifuged down through an underlayer of 0.3M sucrose) can be considered approximately balanced. Hence, it appears that there is some uptake of Ca^{++} without Pi. Succinate or pyruvate could serve as accompanying anions.

Figure 3B shows normal Ca^{++} + Pi uptake without mersalyl. ~~The Ca/Pi uptake without mersalyl.~~ The Ca/Pi ratio is 1.68, very similar to previously determined ratios (12). In Figure 3A the ratio is 2.55, again indicating that some Ca^{++} can be accumulated without Pi. Possibly succinate or pyruvate entered as well. When mersalyl is present (Fig. 3A), Ca^{++} elicits a very brief burst of extra respiration, followed by a strong inhibition. The inhibition is very slight and gradual without mersalyl (Fig. 3B). Either the precipitation of the phosphate has lowered respiration, or the excess Ca^{++} is inhibitory to the electron transfer chain. Chance (13) has described the latter phenomenon.

We tend to believe the inhibition is due largely to loss of Pi, since the same phenomenon can be seen when Pi is depleted in ATP formation (see accompanying manuscript COO-790-46).

These recent studies all indicate that there is a limited mechanism for Ca^{++} transport in corn mitochondria which is not linked to Pi uptake. The uptake may or may not depend on turnover of endogenous Pi. It is very difficult to determine this last point directly, since the entering Ca^{++} tends to precipitate the Pi. However, we can now study the phenomenon using acetate as the counter ion, and will do so. It must be confessed, however, that the procedures and techniques we are now using tend to support a chemiosmotic transport hypothesis like that proposed by Brierley (9). In the coming year we hope to get evidence that will satisfy us if this is true.

B.

It was reported last year that we found it necessary to examine the properties of mitochondrial ATPase before proceeding with the nature of fatty acid transport. Mr. Dennis Jung found that corn mitochondria did have an uncoupler-stimulated ATPase, but that the level of activity could be stimulated in KCl vs sucrose solution (14,15) by sonication (15) and by respiration (16). This last phenomenon is hard to understand, and we have devoted a good deal of time to it. Since corn mitochondria do have some uncoupler-stimulated ATPase, we put much of our effort into investigating cauliflower mitochondria which have none (17).

Table 1 shows the effects of sonication, respiration, and the uncoupler DNP on the ATPase of corn and cauliflower mitochondria.

Table 1

ATPase activity of corn and cauliflower mitochondria.

Mitochondria were suspended in 200 mM sucrose, 20mM KCl, 2mM MgCl₂, 20mM TES (to pH 7.5 with Tris). Reaction was started with 3mM ATP plus 0.5mM NADH, 0.5mM potassium phosphate, and 0.1mM DNP as indicated. Reaction was run at 25°C for 20 minutes and Pi released determined. The extra ATPase was all oligomycin-sensitive. Where indicated the mitochondria were sonicated for 30 seconds before starting the reaction.

<u>Additives</u>	Cauliflower		Corn	
	<u>normal</u>	<u>nanomoles Pi/mg protein/minute sonicated</u>	<u>normal</u>	<u>sonicated</u>
none	11.6	178.0	44.1	144.6
DNP	11.6	183.4	115.0	145.0
NADH	11.6	178.0	42.8	132.1
NADH + DNP	62.4	187.0	140.2	145.5
NADH + Pi + DNP	149.6	185.2	158.3	141.3

The finding that phosphate was needed for maximum effect of respiration was unexpected. The Km for Pi in activating ATPase is about 40 μ M, about the same as for Pi transport (6) and for Pi transport-dependent oxidative phosphorylation (18). Phosphate is totally ineffective if the mitochondria are not respiring. Sonication relieves the need for respiration, Pi, and uncoupler. Evidently, the act of respiration is one which somehow potentiates the transport of ATP into the intact mitochondria. Respiration with Pi is necessary for the potentiation, and uncoupling is necessary for continued activity. How is this to be resolved?

We found that activation of the ATPase by respiration in the presence of Pi continued for some minutes after respiration had ceased. Indeed, during respiration there was a "negative" ATPase -- that is, oxidative phosphorylation occurred -- unless DNP were present. From these observations we devised the

experiment in Figure 4, which shows a respiratory trace, a swelling trace, and the relative DNP-stimulated ATPase of mitochondria taken from the reaction vessel at the indicated intervals. Sequential additions of ADP and DNP were made to the aliquots, one following the other within 10 seconds. Subsequent ATPase reaction was carried out for 20 minutes.

When the mitochondria are still respiring it makes no difference which order the DNP and ATP are added to the aliquot. Within a minute, of course, the NADH is gone, but over the following 19 minutes there is a very active DNP-stimulated ATPase.

The respiring mitochondria are actively accumulating Pi as shown by the swelling trace. After respiration ceases there is a period of 2 - 3 minutes of shrinkage as the Pi diffuses back out. During this period there is a post-oxidative activation of ATPase, provided the DNP is not added first. DNP quickly collapses the Pi gradient across the membrane, giving rapid Pi leakage, as shown in previous papers (5,6). Hence, at least one function of respiration is to build up the internal Pi concentration. The $\Delta[\text{Pi}]$ then drives ATP uptake. Without a pool of internal Pi to diffuse out there is no potential for bringing ATP in.

This phase of the investigation is about ready for publication. In the coming year we will study more closely the uncoupling effect, and see if we can secure it with transportable fatty acids including acetate.

C. Induction of solute transport in corn roots by washing.

Mr. Ramesh Pant and Dr. Richard Smith have been working on phases of this project. Pant has been investigating the effects of washing corn root tissue in various growth regulators on the development of ion accumulation rates, ribonuclease activity and acid phosphatase activity.

We have found no promotive effects of any growth regulator tested (several auxins and antiauxins, cytokinins, gibberellins, abscisic acid or ethylene) on the increased rates of ion accumulation which accompany washing. Auxins,

antiauxins and kinetin are quite inhibitory, but only at concentrations of 0.1mM and above. The other regulators have no effect. The antiauxin, 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) is most inhibitory, as previously reported (19).

Ribonuclease activity increases with washing approximately in parallel with increased ion absorption. In this case the auxin 2,4-D is promotive (50%) as is GA (25%), and these growth regulators have additive effects. Kinetin is slightly inhibitory, while ABA and Ethrel (which produces ethylene) have no effect.

Washing root tissue for ²⁴ hours in 0.1mM CaCl₂ gives no increase in RNA and protein content unless 2,4-D is included, in which case increases in RNA of up to 55% and in protein up to 24% have been found. One could thus ascribe the 60% inhibition of development of ion uptake by 2,4-D to diversion of metabolites and energy to other synthetic processes in the roots. However, this does not account for the action of the antiauxin 2,4,6-T which has no effect on RNA and protein synthesis under the same conditions that it completely inhibits the increase in ion uptake.

Washing of potato tuber tissue is reported to increase synthesis of unsaturated fatty acids (20). We find small change in the fatty acid composition of the microsome fraction from corn roots after 4 hours of washing, a change dramatically affected by 0.1mM 2,4,6-T. Microsomal membranes were analyzed because it was here that we found the increase in ATPase (21).

% of total fatty acids

<u>Fatty Acid</u>	<u>Fresh</u>	<u>Washed</u>	<u>Washed + 2,4,6-T</u>
Myristic 14:0	10.0	10.3	18.3
Unitentified 16-18(?)	1.6	2.3	3.0
Palmitic 16:0	34.2	31.4	27.7
Stearic 18:0	trace	trace	trace
Oleic 18:1	trace	trace	trace
Linoleic 18:2	54.2	56.0	51.0

Hence, it appears that we have located an inhibitor, 2,4,6-T, which will completely suppress the development of ion uptake during washing, and appears to do so by interference with some phase of lipid metabolism. We need now to do work on the extracted phospholipids as well, using tracers to study turnover.

We have also looked to see if linoleic acid, the dominant unsaturated acid which increases with washing would increase ion absorption if incorporated into the washing medium. It does so, having maximum effectiveness at 10^{-6} M.

<u>*Washing</u>	<u>^{32}P absorption (% of control)</u>
none (fresh)	100
4 hours	236,268
4 hours + 10^{-6} M linoleic acid	330,333

*Washing of 2 cm corn root sections was in 0.1mM CaCl_2 + 10mM K phthalate buffer, pH 5.5. at 25°C with aeration. Linoleic acid was added in ethanol, and equivalent ethanol was added to the control. After washing the tissue was rinsed and assayed for ^{32}P uptake (19).

Dr. Richard Smith, a visiting professor from the University of Florida has been working on a rather venturesome project. Our earlier work (21) showed that a microsomal ATPase increased in parallel with ^{32}P absorption rates during washing of corn root sections. However, there is a large disparity in the relative amounts of increase -- 30% for ATPase and 300% for ^{32}P absorption. The correlation may be only fortuitous.

There is only one way to establish that the microsomal ATPase is a transport ATPase. One must show that ions can be transported in or out of the microsomal vesicles at the expense of ATP.

This has been a most exasperating project. Much of the preliminary work has produced nothing. Recently we have been able to load the microsomal vesicles with KCl during the homogenizing process. When these are placed in

a buffered sucrose medium with MgCl_2 the action of ATP is first to accelerate the movement of K^+ from the vesicles to the solution, followed after 30 minutes by absorption of this extra K^+ . However, we are not sure of what is going on here and the amount of K^+ moved is small. We wonder if it is possible that the microsomes have only limited capacity to swell and shrink (unlike the case with the folded inner membrane of the mitochondrion). In this case, the net K^+ movements may not be the best parameter to follow. We intend to do cross-loading experiments with either K^+ inside and Na^+ outside or vice versa. Perhaps this way we can amplify the K^+ transport. A great deal of empirical experimentation on solutions, isolation conditions, assay conditions, etc., remains to be done. It is discouraging work, but if these membranes transport ions at the expense of ATP there must be a way of showing it.

II. Publications since last report

COO-790-43. Leonard, R. T. and J. B. Hanson. Induction and development of increased ion absorption in corn root tissue. *Plant Physiol.* 49: 430-435, 1972.

COO-790-44. Leonard, R. T. and J. B. Hanson. Increased membrane-bound adenosine triphosphatase activity accompanying development of enhanced salt uptake in washed corn root tissue. *Plant Physiol.* 49: 436-440, 1972.

COO-790-45. Hanson, J. B. Ion transport induced by polycations and its relationship to loose coupling of mitochondria. *Plant Physiol.* 49: 707-715, 1972.

COO-790-46. Hanson, J. B., B. L. Bertagnolli, and W. D. Shepherd. Phosphate-induced stimulation of acceptorless respiration in corn mitochondria. *Plant Physiol.* (accepted for publication).

III. Personnel on the project

Dr. J. B. Hanson, Principal Investigator. Approximately 40% of time

spent on the project during current funding year. Activity for the balance of the year will be the same.

Dr. Richard Smith, Visiting Professor. 100% time for 6 months.

Dr. Byron L. Bertagnolli, Research Associate. 100% time.

Mr. Dennis Jung, Graduate Student. 100% time (University of Illinois fellowship).

Mr. Ramesh Pant, Graduate Student. 100% time (AID fellowship).

Mr. Willy Lin, Technician. 40 hours week, summer session.

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20. Willemot, C. and P. K. Stumpf. Can. J. Bot. 45: 579, 1967.
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1A

1B

Figure 2

Fig. 3A

	nanomoles / μ g protein		Δ equivalents
	(1)	(2)	
K^+	197	62	-135 (+)
Ca^{++}	35	175	+280 (+)
Mg^{++}	13	11	-4 (+)
P_i	17	72	+83 (-)
H^+ extruded		30	-30 (+)
			280 (+) gained
			252 (+) lost

Fig. 3B

nanomoles / μ g protein

(3)	(4)
158	147
43	1000
14	12
20	588
-	

FIGURE 4

DNP STIMULATION OF CAULIFLOWER ATPase IN THE PRESENCE OF NADH AND PHOSPHATE

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COO-790-46

Phosphate-induced stimulation of acceptorless
respiration in corn mitochondria.¹

¹Supported by U.S. Atomic Energy Commission (AT 11-1-790).

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ABSTRACT

By use of the organic mercurial mersalyl to block phosphate transport, it has been shown that only a small fraction of the respiratory increase of corn mitochondria to additions of inorganic phosphate is due to energy expended in P_i accumulation. Most of the respiratory release occurs from accelerated turnover of the coupling mechanism with internal phosphate in an oligomycin-sensitive reaction. Addition of ADP to mersalyl-blocked mitochondria depletes internal P_i in ATP formation and respiration declines.

Arsenate produces the same responses as phosphate but is more effective in respiratory release. It appears that the arsenylated analog of ATP is sufficiently stable to exit from the mitochondria, giving shrinkage.

Inhibition of the ADP-ATP antiporter with atractyloside shows that the increased respiration with internal phosphate is not due to turnover of ADP acceptor through exogenous ATPase.

Use of valinomycin to facilitate movement of K^+ greatly accelerates the rate of phosphate transport, but there is no consistent correlation between respiration and transport. In the absence of phosphate, valinomycin dramatically releases respiration with only trivial swelling.

The data indicate that loose coupling is due only fractionally to energy expenditure in ion transport. An explanation consistent with the observations can be derived by assuming that both a high energy intermediate ($I \sim X$) and a proton motive force (PMF) - or its electrochemical equivalent - arise at coupling sites. Dissipation of these energy conserving acts in acceptorless or state 4 respiration requires turnover of $I \sim X$ or $X \sim P$ by hydrolytic attack of OH^- from the matrix phase and H^+ or K^+ from the external phase.

In a recent paper (11) we suggested that high state 4 respiration rates in corn mitochondria are linked to energy expenditure in phosphate transport. State 4 respiration was considerably augmented by polycations which served to increase phosphate transport, producing swelling. It seems to be widely accepted that energy-linked ion transport in mitochondria is a process capable of releasing coupled respiration (3,20,21), although it is sometimes pointed out that the stoichiometry is disturbing (3), and that permeant anions are required (20).

Some further experimentation in which acetate was substituted for phosphate gave a puzzling result. Although acetate definitely gave active swelling there was only a trivial release of respiration with or without polycations. The swelling is known to result from acetate transport, with any of a wide spectrum of cations serving as counter ion (24). Hence, there is something exceptional about phosphate transport which makes it effective in accelerating respiration.

Perhaps it is not the act of phosphate transport which releases respiration, but rather the presence of accumulated phosphate in the matrix. A technique for resolving this question is available in the use of organic mercurials to block phosphate transport (8,9,24). One should be able to add phosphate to respiring mitochondria and start the respiratory increase and swelling. Now mersalyl can be added to block further transport. If transport is responsible for the respiratory increase, respiration should decline. If the presence of the accumulated phosphate is responsible, respiration should not decline.

In this paper we report on this and related experiments. The results clearly show that high state 4 respiration rates in corn mitochondria are dependent on internal phosphate with only a small respiratory increment due to active transport. Internal phosphate appears to accelerate respiration by reaction with the coupled phosphorylation system.

METHODS

Mitochondria

Corn mitochondria (Zea mays L., WF9(Tms) x M14) were isolated and assayed as previously described (11). Final suspension of the mitochondria was in the same medium as used for the basic reaction mixture: 200 mM sucrose, 10 mM TES buffer, 1 mM $MgSO_4$, 1 mg/ml bovine serum albumin, adjusted to pH 7.6 with KOH. Mitochondria concentrations were adjusted to approximately 10 mg protein/ml, with protein determinations corrected for the BSA of the suspending medium. Reaction vessels contained 4.4 ml of the basic medium plus 0.1 ml of mitochondria suspension (about 1 mg protein), with

additives as indicated in figures and tables. Temperature was 28 or 29 C. Recordings of O_2 consumption and percent transmission were as previously described (11).

ATP Measurements

Two ml samples were withdrawn from the reaction vessel in the spectrophotometer before and 1 minute after adding 300 umoles ADP. In determination of adenylate kinase blanks the same procedure was followed but initial additions were made of 20 uM rotenone, 3 uM antimycin A, and 20 ug oligomycin, and addition of NADH was omitted. The samples were added to 3 ml of 20 mM glycine buffer (pH 7.7) in a boiling water bath. After 15 minutes the tubes were cooled in an ice bath and the denatured mitochondria centrifuged down at 5000 g for 10 minutes. Aliquots of the extract were assayed for ATP by a luciferase assay (15) using an Aminco Chem-Glow Photometer and firefly lantern extract from Sigma.

Phosphate uptake

We have had a great deal of trouble measuring net Pi uptake by respiring mitochondria due to leakage of phosphate during recovery of the mitochondria. Experimentation has confirmed Fonyo's report (8) that mercurials are effective in blocking passive exit of phosphate. By adding mersalyl after Pi is accumulated and by centrifuging through silicone oil it is possible to get reproducible values for Pi content of mitochondria. There does appear, however, to be leaching of endogenous Pi from the mitochondria into the reaction medium prior to addition of exogenous Pi and mersalyl, since endogenous Pi could not be detected after recovery of the mitochondria

from the reaction medium.

Phosphate uptake was determined in parallel with O_2 consumption and %T measurements using 10 ml erlenmeyer flasks shaken in a bath at 28°C. Reactions were started with 1 ml of mitochondria (about 10 mg protein), 0.3 ml of basic medium, and 0.2 ml of NADH solution (9.4 umoles). Variable amounts of potassium phosphate were added, and after 1 minute further transport was blocked with 0.25 mM mersalyl. The mitochondria were centrifuged down through 50 μ l of Dow-Corning silicone oil, #560, in 0.3 ml aliquots using the Beckman 152 microfuge at 140 volts, and the pellets were collected by excising the tip of the tube. Phosphate was extracted by vortexing the pellets in ice cold 5% trichloroacetic acid, and the extract was assayed for Pi by a modification of the isobutanol-benzene extraction of the molybdate complex (16).

RESULTS

Phosphate accelerated respiration

The basic problem under consideration here is illustrated in fig 1. Addition of substrate - and we here use NADH to eliminate organic acid influx pumping - starts an acceptorless respiration. Associated with this respiration there is some swelling as shown by the increase in percent transmission. Presumably this component of respiration is at least partly linked to solute transport, but as yet there is no resolution of what solute is entering. It might be the TES buffer (a zwitterion), NADH, the $MgSO_4$, or even sucrose.

Focus here is on the release of additional respiration following

addition of phosphate. In 12 determinations like fig 1 the mean respiration rate increased from 82 to 136 nanomoles O_2 /min mg protein (average respiratory control ratios were 3.44 and ADP:O ratios were 1.78). The respiratory rate can be considered a "state 4" rate since it is like those obtained on exhaustion of added ADP, and since endogenous ADP is present giving ATP formation (shown later in Table II). Oligomycin brings the respiration back towards the rate without P_i (in the 12 experiments, the average rate after oligomycin addition was 92 nanomoles O_2 /min mg protein).

Since P_i addition also causes active swelling, we suggested that active transport was responsible for the respiratory increase (11).

However, as shown in fig 2, this is not likely to be true. Acetate is also pumped in giving swelling, but there is only trivial release of respiration. Low respiration rates during potassium acetate pumping in corn mitochondria have been noticed before (24). We have also checked acetate swelling in the presence of protamine, and again there is no significant increase in respiration rate although additional swelling is obtained.

Mersalyl, an effective inhibitor of the phosphate transporter (24), almost completely suppresses both the increase in respiration and swelling on addition of phosphate (fig 2C). The standard explanation here is that inhibiting phosphate transport inhibits utilization of energy for transport. Failure to obtain state 3 respiration on addition of ADP is due to lack of P_i transport to the matrix for ATP formation. The sharp decrease in transmittance with ADP probably represents loss of endogenous P_i (and a

small amount of accumulated Pi) in ATP formation, which is also reflected in a 17% decline in respiration rate (see below). However, there is in addition some configurational change which occurs in oligomycin-blocked mitochondria on adding ADP (figs 1 and 5) which must also contribute to the decrease in transmittance.

Figure 3A and B differs from fig 2C in that the mersalyl is added after Pi has been accumulated and the higher "state 4" respiration is attained. Mersalyl has but little effect on state 4 respiration (9,23). This result was repeatedly confirmed, and it provides strong evidence that the respiratory increase on addition of Pi is due to the presence of internal Pi, not the act of transporting it. Additional evidence is provided by the decline in respiration after addition of ADP. Entrance of ADP and exit of ATP are not blocked by mersalyl, so ATP should be formed at the expense of the internal, immobilized Pi (24). The ATP so formed then exchanges for external ADP, accompanied by K^+ to equalize the difference in charge. As a net result there is loss of Pi and K^+ from the matrix giving the observed shrinkage (fig 3). But with the loss of internal Pi there is also a decline in respiration, again indicating that acceptorless respiration is governed at least in part by internal Pi concentration.

In fig 3A the mitochondria were freshly isolated; in fig 3B the same mitochondria had been held in ice for several hours. They differ in 2 respects: acceptorless respiration was lower in fresh mitochondria and there was a burst of respiration on addition of ADP, just as reported for liver mitochondria (9,23). With the aged mitochondria there was no

respiratory burst. However, aging with its additional "loose coupling" does not noticeably alter the response to additions of Pi, mersalyl or ADP. The coupling mechanisms for Pi transport and ATP formation are still quite active.

Figure 4 presents data compiled from a series of experiments to determine the effect of Pi concentration on acceptorless respiration and swelling. Respiration rates rose rapidly with Pi additions up to about 1 mM. A second but lower rate of Pi-dependent respiratory increase occurred between 4 and 8 mM Pi; this might be related to the extensive swelling occurring here. The K_m for Pi in ATP formation is transport-dependent and is about 0.25 mM with undamaged membranes (2), which falls in the range of the initial respiratory increase and swelling (fig 4) and suggests that the second phase may be non-physiological. As a compromise which would secure good Pi uptake without apparent damage as judged by the ability of ADP to reverse swelling (fig 3) we settled on 5 mM Pi additions. It will be noted in fig 4 that subsequent addition of mersalyl only slightly depressed respiration at any concentration, and that DNP uncoupled respiration was independent of Pi concentration. However, mersalyl does have some stimulating effect on NADH oxidation (fig 2C, 11), and thus the difference between the $\pm$ mersalyl respiration curves might be larger if a correction were made here.

Determinations of Pi uptake are given in Table I. Table II shows the results from determining ATP before and after adding ADP as in fig 3. It was necessary in these experiments to determine ATP arising from

adenylate kinase (an intermembrane enzyme, ref 7) and subtract this as a blank control. The amount of ATP thus formed is reasonably like the amount of Pi accumulated (c.f. Table I), except with 10 mM Pi additions. As mentioned above there may be some swelling damage at high Pi concentrations.

Oligomycin suppresses the enhanced respiration obtained with internal Pi, and subsequent addition of ADP fails to produce the pronounced shrinkage attendant on loss of Pi as ADP (fig 5; c.f. figs 1,3). (There is, however, the small, sharp decrease in transmittance mentioned above.) Perhaps oligomycin interferes with the reaction of internal Pi with the primary high energy intermediate, much as proposed by Ernster et al. (6), and in this fashion prevents Pi turnover and inhibits respiration. An alternative view is that of Lardy et al. (14) that oligomycin prevents phosphoryl transfer to ADP.

In mersalyl-blocked mitochondria with high Pi content there is sufficient endogenous adenylate to form about 4 nanomoles ATP/mg protein (Table II). Racker (22) thinks that loose coupled respiration arises from hydrolysis of endogenous ATP, recycling ADP. If this ADP recycles through an ATPase external to the inner membrane, the recycling can be blocked with atractyloside, the inhibitor of the ADP-ATP exchange transport (13).

Figure 6A shows that atractyloside blocks the normal state 3 respiration and shrinkage initiated by ADP addition (c.f. fig 1). There are two oddities: atractyloside increases the state 4 respiration, a phenomenon explained for liver mitochondria as due to damage caused by phosphate (1);

subsequent addition of ADP actually decreases respiration. Blocking phosphate transport with mersalyl does not change these responses (fig 6B; c.f. fig 3). It is probably worth noting that the respiratory increase with atractyloside is associated with a sharp drop in transmission, even when Pi transport is inhibited with mersalyl (fig 6B). Furthermore, atractyloside eliminates the sharp decrease in %T on addition of ADP (fig 6A) which is found even in oligomycin-blocked mitochondria (fig 5). These responses to atractyloside are not readily explained simply on the basis of blocking the ADP-ATP exchange transporter. However, the results do show that if phosphate-induced respiration is due to turnover of ADP it cannot be through ATPases external to the inner membrane.

Arsenate Accelerated Respiration:

If turnover of the phosphorylating mechanism is responsible for the high state 4 respiration, it should be possible to augment the rate by substituting arsenate for phosphate. Arsenate is believed to form an unstable intermediate, possibly at the level of ADP - As formation (6).

In a preliminary experiment it was confirmed that mersalyl blocks arsenate transport (24). Figures 7A and B compare arsenate with phosphate in an experiment like that of fig 3. The experiment with Pi includes an addition of cysteine to demonstrate the reversible nature of mersalyl inhibition of Pi transport (24). Qualitatively, the responses to arsenate and Pi are the same. Quantitatively, arsenate was more effective in releasing respiration, but subsequent addition of ADP produced less rapid shrinkage and proportionately greater respiratory decline.

However, why should there have been any shrinkage and respiratory decline at all? It appears that the arsenylated analog of ATP is more stable than is commonly assumed (6), allowing for a slow but significant loss of arsenate from the matrix.

It should be noted that if the extra respiration on addition of Pi or As were linked solely to transport, arsenate should have produced greater swelling. It did not.

Kinetics of Pi transport

It is evident from the foregoing experiments that the integrated process of ATP formation can be separated into 2 parts: (1) phosphate transport followed by (2) ADP transport, ATP formation, and ATP exit. The second process is quite rapid (fig 3). By fitting tangents to the shrinkage curves on addition of ADP it can be estimated from the ~~total~~ ^{net} ATP formed (Table II) that the initial rate of ATP formation is 7 to 8 nmoles ATP/sec·mg protein. This corresponds to a rate of 8-10 nmoles/sec·mg protein for ATP formation in normal oxidative phosphorylation with NADH as substrate (eg: the rate for the second addition of ADP in fig 1 is 8.5 nmoles/sec·mg protein). However, initial Pi transport is much slower (fig 3) being from 1.5 to 2.0 nmoles/sec·mg protein. Hence, Pi transport is the rate limiting factor in ATP formation. But this limitation only shows when the Pi transport process is separated out. When both Pi and ADP are present, as in state 3 (fig 1), Pi transport must be keeping pace with ATP formation.

In ~~the~~ net uptake the phosphate anion must be accompanied by a cation to maintain electrical neutrality, largely K^+ in these experiments (11).

However, in coordinated ATP formation little or no uptake of K^+ is required: $Pi^- + ADP^{3-}$ in; ATP^{4-} out (13). Hence, the low rate of Pi uptake as in fig 3 might result from an opposing electropotential due to resistance to K^+ penetration. If this resistance were lowered with a K^+ ionophore such as valinomycin it should be possible to show a respiratory release with salt pumping as has been done with animal mitochondria (3,20,21).

Valinomycin does initiate a large increase in respiration and there is abrupt phosphate swelling which shortly approaches steady state (fig 8). Blocking the Pi transporter with mersalyl has little effect on respiration, but after a few seconds there is a sudden induction of shrinkage, presumably due to a backflow of potassium phosphate through other than Pi transporter sites; there is no such shrinkage in liver mitochondria (24). Addition of ADP increases the shrinkage. Thus some ATP formation seems to occur, but there is evidence for permeability changes in the spontaneous efflux of Pi on addition of mersalyl, and in the failure to obtain complete shrinkage (c.f. fig 3).

Figure 8B shows the result of adding valinomycin before Pi . There is an impressive release of respiration (K^+ is present due to adjustment of pH with KOH). However, there is only trivial swelling until Pi is added. In liver mitochondria, release of respiration by valinomycin in the absence of Pi or acetate is linked to substrate uptake, for example succinate (22). Using NADH as substrate in corn mitochondria shows that valinomycin can act much like an uncoupler; i.e. facilitating membrane penetration by K^+ releases respiration by a means not linked to salt transport.

Discussion

These experiments reveal only a small increment of additional respiration associated with salt pumping (acetate, Fig. 2B; difference in respiration before and after adding mersalyl, Fig. 4). It appears that accumulated phosphate is largely responsible for increased acceptorless respiration. When further Pi transport is blocked by mersalyl, the internal phosphate (or arsenate) can be depleted in ATP formation (Table II) and the respiration falls (Figs. 3 and 7). The additional respiration associated with accumulated Pi is sensitive to oligomycin (Fig. 5).

Although organic mercurials might be suspected of interfering with a number of sulfhydryl enzymes, mersalyl seems not to produce deleterious side effects on phosphorylation. Sulfhydryl protecting reagents readily reverse the inhibition of Pi transport and thereby reestablish state 3 respiration (9,24; Fig. 7A). By use of mersalyl the process of oxidative phosphorylation can be divided into two component processes: Pi transport to the matrix by the $\text{Pi}^- - \text{OH}^-$ antiporter (4,5), and phosphorylation of ADP at the F_1 coupling sites (22). An essential part of the phosphorylation is the operation of the $\text{ADP}^{3-} - \text{ATP}^{4-}$ antiporter (13). When this is blocked with atractyloside ATP is not formed, and the shrinkage associated with ATP formation is not seen (cf. Fig. 1 and 3 with 6).

It is now central to chemical (19), chemiosmotic (17,18) and conformational (26) hypotheses that a respiration-linked proton motive force (PMF), ^{a proton gradient} or its electrochemical equivalent, drives ion transport. Collapse of this membrane potential in ion transport releases respiration. Most of these opinions on salt transport regulating respiration rates come from

experiments on animal mitochondria using ionophores (3,20,21). Corn mitochondria also show a burst of swelling and respiration with addition of valinomycin, but again mersalyl has little effect on the respiration rate (Fig. 8A) and actually initiates loss of accumulated salt. In the absence of phosphate, and hence of appreciable swelling, valinomycin still releases respiration (Fig. 8B). There is thus no consistent correlation between valinomycin induced respiratory increases and salt transport. Valinomycin accelerated swelling in potassium phosphate (or acetate or chloride, unpublished data) is simply one manifestation of lowered resistance to potassium transport. Another is a respiratory release somewhat reminiscent of uncoupling agents, which may or may not be associated with net salt uptake.

Since collapse of proton motive force in ion transport can only account for a small portion of the respiratory release with P_i , the high state 4 respiration observed must involve turnover of the phosphorylating mechanism as well. Sensitivity of the release to oligomycin supports this concept. Hence, respiration must generate both a proton motive force for transport and "squiggle" ($P-X$) for phosphorylation. Maximum release of respiration requires that both be dissipated.

Figure 9 is our attempt to give a coherent explanation of these observations. The scheme seems internally consistent and stoichiometric, but there is no way at present to establish if it is correct.

Figure 9A depicts P_i and adenylate transport associated with oxidative phosphorylation as they are now understood (13,24). The principal ambiguity lies with the primary coupling event. From the chemiosmotic viewpoint (17,18) the creation of PMF through respiratory loops is primary,

and P-X is derived secondarily at the expense of the electrochemical potential. From the chemical viewpoint, P-X is formed by direct coupling to electron transfer carriers, and the proton gradient arises at the expense of P-X (4,10,19). Our laboratory has suggested a modified chemical hypothesis (12,25) wherein the dehydration necessary to formation of the primary covalent bond (i.e. P-X , and acid anhydride or thioester) is accomplished by the polar ejection of H^+ and OH^- ions. Figure 9 begs the question as to which mechanism is involved, or whether there are conformational changes, but emphasizes that both a proton gradient and a chemical intermediate arise from respiration. The proton gradient drives P_i uptake through a $\text{P}_i^- - \text{OH}^-$ antiporter (4,5), and P-X provides the primary high energy bond needed for phosphorylation. Our past assumption that P_i reacts with P-X from the outside (25) is obviously erroneous and must be dropped.

As discussed in Results, no net accumulation of phosphate salt needs to occur during oxidative phosphorylation since simultaneous operation of the $\text{ADP}^{3-} - \text{ATP}^{4-}$ antiporter collapses the potential.

The sites of action for mersalyl (24) atractyloside (13) and oligomycin (6) in Fig. 9A are those commonly accepted.

In terms of this hypothesis, how does one account for acceptorless respiration, uncoupling, and the respiratory increment due to internal P_i ? In each case both PMF and squiggle must be dissipated if a respiratory cycle at coupling sites is to be completed.

Addition of NADH (or other substrate) initiates a low rate of acceptorless respiration. Figure 9B is intended to suggest that part of this respiration may lie with simple reversal of the coupling process,

limited by the rate of H^+ or other cation penetration. Mitchell (17,18) asserts that proton and cation permeability is normally low, but in corn mitochondria the permeability could well be finite, in particular where some aging has occurred (cf. Fig. 3A and B). Accessibility of $I\sim X$ to OH^- from the inside is here proposed to be not rate limiting. Hence the action of proton shuttling uncouplers such as DNP would not be to directly collapse the PMF as proposed by Mitchell (17,18), but rather to indirectly collapse it by accelerating hydrolysis of $I\sim X$.

Figure 9B is drawn to conform with a previous suggestion (12) that one of the acid groups may be partially neutralized (e.g.: X^-K^+ instead of XII), with K^+ or other cation substituting for H^+ . Alternatively, there may be H^+-K^+ exchange. In either case the reaction of valinomycin in releasing respiration in the absence of a permeant anion (Fig. 8B) might lie in facilitating K^+ plus OH^- hydrolysis of $I\sim X$. It is possible, of course, that the KOH hydrolysis occurs from the inner phase following valinomycin-facilitated penetration of K^+ .

Figure 9C deals with the central problem of how internal phosphate contributes to a high state 4 respiration rate. Since addition of ADP to mersalyl-blocked, P_i -loaded mitochondria produces ATP (Table II), and since oligomycin inhibits the extra respiration due to internal P_i (Fig. 1,5) it appears that P_i must be turning over through " $X\sim P$ ". The question is whether turnover of endogenous ADP through endogenous ATPase is responsible, or whether $X\sim P$ hydrolysis occurs. Probably both are involved, but we favor the latter process as the dominant one for these reasons:

- 1 - There is a continued rise in respiration with increasing P_i

(Fig. 4). With a limited amount of internal adenylate, saturation might be expected at low levels of P_i .

2 - The atractyloside experiments (Fig. 6) show that the ATPase would have to be in the matrix. The F_1 -ATPase can hardly be yielding net formation and net degradation of ATP at the same time. However, there might be appropriate phosphatases; the matter needs special study.

3 - Substitution of arsenate for phosphate gives a very rapid turnover of "X~As", but the shrinkage on addition of ADP does not indicate that ADP-As stability is a major factor.

4 - Depletion of P_i (or As) in ATP formation lowers respiration. There should be adequate internal ATP for recycling ADP + P_i acceptor in this situation.

We conclude that the high acceptorless or state 4 respiration rates typical of plant mitochondria cannot be ascribed solely to ion transport, but also involves uncoupling due to finite H^+ or K^+ penetration, and to turnover of the phosphorylating mechanism with internal P_i .

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Table I

Pi uptake by respiring mitochondria

Endogenous Pi content was too low to be reliably detected colorimetrically; hence, values reported as Pi content represent increase in Pi one minute after adding exogenous Pi to respiring mitochondria.

<u>Pi added</u>	<u>Pi content</u>	
	<u>Exp 1</u>	<u>Exp 2</u>
	nmoles Pi/mg protein	
2 mM	5.2	3.4
5 mM	32.2	31.2
10 mM	83.4	91.0

Table II

ATP formation at the expense of internal Pi.

Experiments performed as in fig 3 with samples taken just before and one minute after adding ADP. Adenylate kinase also determined over 1 minute. See Methods for details.

<u>Pi added</u>	<u>ATP content</u>			
	<u>endogenous</u>	<u>NADH + Pi + Mersalyl</u>	<u>+ ADP</u>	<u>loss</u> <u>adenylic kinase</u>
			(nmoles ATP/mg protein)	
5 mM*	0.4	3.7	138.7	34.7
2 mM**		---	67.8	10.8
5 mM	0.9	4.9	92.5	35.5
10 mM		---	112.5	55.5

*Mean values from 4 experiments. Adenylate kinase formed 104.0 nmoles/min·mg protein.

**Single experiment. Adenylate kinase = 57.0 nmoles/min·mg protein.

FIGURE LEGENDS

Figure 1. Acceptorless, state 3 and state 4 respiration of corn mitochondria. Medium was 4.5 ml of 0.2 M sucrose, 10 mM TES (pH 7.6), 1 mM MgSO_4 , 1 mg/ml bovine serum albumin. Additions: NADH = 2.6 μmoles , Pi = 5 mM (K salt, pH 7.6) ADP = 330 nmoles, oligomycin = 5 μg , DNP = 0.2 mM. Transmission was measured at 520 nm. See Methods for details.

Figure 2. Respiratory and swelling - shrinkage responses to additions of phosphate and acetate. Medium as in fig 1 but with addition of 10 mM KCl; Ac = 10 mM K acetate, Mers = 25 μM mersalyl. Other additions as in fig 1.

Figure 3. Respiratory and swelling - shrinkage responses of corn mitochondria to additions of phosphate, mersalyl and ADP. Medium as in fig 1, additions as in figs 1 and 2. A. Freshly isolated mitochondria. B. Same mitochondria after about 3 hours on ice.

Figure 4. Respiratory and swelling responses in experiments like 2A, but with variable additions of inorganic phosphate. Swelling was determined 1 min after Pi addition. Number of experiments averaged are given in parentheses.

Figure 5. Inhibition of Pi -released respiration by oligomycin. Conditions as in fig 3 with addition of 5 μg oligomycin as indicated.

Figure 6. Effect of atractyloside on respiration and ADP-induced shrinkage. Condition as in fig 3, with addition of 50 μM atractyloside as indicated.

Figure 7. Comparison of phosphate and arsenate in releasing respiration.

Conditions as in fig 3 with additions of 5 mM potassium arsenate and 1 mM cysteine as indicated. Note that cysteine removes the mersalyl inhibition of Pi transport, permitting state 3 respiration.

Figure 8. Release of respiration by valinomycin in the presence and absence of phosphate. Conditions as in fig 3, with additions of 1.4 ug valinomycin as indicated.

Figure 9. Schematic representation of acceptorless respiration in corn mitochondria. See text for discussion. A. Normal oxidative phosphorylation under state 3 conditions. B. Acceptorless and uncoupled respiration. C. Acceleration of respiration by accumulated Pi (or arsenate).

Fig 1

Fig 2

Fig 5

Fig. 6

Fig 7

A

Chemiosmotic

Chemical

Modified chemical

B

C