

## The Osmotic Behavior of Corn Mitochondria<sup>1</sup>

George H. Lorimer<sup>2</sup> and Raymond J. Miller

Department of Agronomy, University of Illinois, Urbana, Illinois 61801

Received January 20, 1969.

**Abstract.** The volume changes undergone by corn (*Zea mays* L.) mitochondria suspended in solutions of constant or varying osmolarity were studied. Within the range of osmotic pressure from 1.8 to 8.4 atmospheres, corn mitochondria behave as osmometers, if allowance is made for an osmotic "dead space" of about 6.9  $\mu\text{l}/\text{mg}$  protein. The final equilibrium volume of mitochondria swollen in solutions containing both ribose and sucrose were shown to depend upon the concentration of impermeable solute (sucrose) present and not upon the concentration of ribose present. Osmotic reversibility was found for mitochondria swollen in isotonic solutions of KCl or ribose. The passive swelling of corn mitochondria may be due to the osmotic flow of water coupled to the diffusion of a permeable solute.

The swelling-contraction characteristics of plant mitochondria have been well documented (6, 9, 11, 13, 16). Swelling (water influx) occurs when the mitochondria are transferred from sucrose to isomolar solutions of KCl; upon the addition of an oxidizable substrate, such as NADH or malate-pyruvate, or ATP they contract (water efflux). Plant and animal mitochondria appear to be different in that plant mitochondria will swell passively and contract actively whereas animal mitochondria swell actively (substrate dependent) and contract when the system becomes anaerobic. Recently there has been a suggestion that these seeming differences may be the result of preparation procedures (1, 5).

It is known that the inner compartment of animal mitochondria behaves as an osmometer (2, 12, 15). Sucrose is the osmoticant commonly used and can penetrate the outer but not the inner mitochondrial membrane (2, 12).

Corn shoot mitochondria are commonly isolated in 0.4 M sucrose. Experimental observations are usually conducted in electrolyte solutions. Since sucrose is a non-penetrating molecule but electrolytes slowly penetrate the membranes of plant mitochondria, the transfer from sucrose to electrolytes could result in osmotic shock. If the membrane is not semipermeable, diffusion of salts will occur. The importance of these osmotic phenomena in plant mitochondrial reactions is not known.

This study elucidates the effect of permeable and impermeable solutions on the passive swelling of corn shoot mitochondria.

### Materials and Methods

Mitochondria were isolated, with a procedure similar to that of Kenefick and Hanson (8), in the cold form from 100 g (fresh wt) of 3 and one-fourth day etiolated corn shoots (*Zea mays* L., WF9  $\times$  M14) by grinding in an ice cold mortar with 250 ml of 0.4 M sucrose, 0.02 M tris-HCl, pH 7.5, 0.005 M EDTA. The slurry was filtered through cheesecloth and the mitochondrial fraction collected by successive centrifugation at 1000g and 10,000g for 10 min. The mitochondria were then resuspended in a total of 50 ml of 0.4 M sucrose, centrifuged at 1000g for 10 min and the suspension centrifuged at 10,000g for 10 min. The pellet was resuspended with 0.4 M sucrose, 0.02 M tris-HCl, pH 7.5 (about 10 mg protein/ml) and the suspension stored on ice. All isolation procedures were conducted between 0 and 2°.

This procedure is somewhat different from other procedures for the isolation of corn mitochondria (8) in that the mitochondria were not centrifuged through a layer of 0.6 M sucrose. This was omitted, since it was important to know the final osmolarity of the solution in which the mitochondria were suspended. However, no differences in swelling behavior were found when mitochondria isolated this way were compared with mitochondria that had been centrifuged through a layer of 0.6 M sucrose, or from tissue that had been ground in a medium containing phosphate buffer.

Measurements of water content were made by centrifuging an aliquot of mitochondria in a lusteroid tube for 20 min at 10,000g. The supernatant was carefully removed and the bottom of the tube containing the pellet sliced off. The tube plus wet pellet was weighed, dried overnight at 105° and reweighed. No correction was made for extramitochondrial water or water between the outer and inner membranes.

<sup>1</sup> Supported in part by a grant from the Office of Saline Water, Department of Interior (14-01-0001-1088).

<sup>2</sup> Present address: Department of Biochemistry, Michigan State University.

A continuous record of the volume changes was obtained by recording the percentage transmittance (%T) at 520 m $\mu$  of the mitochondrial suspension which was placed in a water jacketed cuvette designed for the purpose. All experiments were performed at 25° in solutions buffered at pH 7.5 with 0.02 M tris-HCl, except where otherwise indicated. Protein was determined by the procedure of Lowry *et al.* (10), using bovine serum albumin standards.

The total intra- and extra-mitochondrial sucrose accessible space was estimated using C-14 polyglucose (New England Nuclear Corporation) as a space marker (2). After centrifugation, the mitochondrial pellet was dissolved with 0.5 ml of 1 M formic acid and the radioactivity measured on aliquots of the formic acid solution.

*Theoretical Treatment of the Volume Changes.* Tedeschi and Harris (15) demonstrated that the volume of rat liver mitochondria could be predicted by

$$V_i = \frac{Q}{P} + b \quad (I)$$

where  $V_i$  is the total volume of the mitochondria,  $P$ , the osmotic pressure of the external medium, and  $b$ , the osmotic "dead space", (the volume of the mitochondria at infinite osmotic pressure of impermeable solute). In an ideal osmotic system, the proportionality constant,  $Q$ , is equal to  $mRT$ , where  $m$  corresponds to the moles of osmotically active material within the mitochondria,  $R$  to the gas constant and  $T$  to temperature.

Since the outer membrane of the mitochondrion appears to be permeable to sucrose (2,12), equation I will not necessarily predict the volume of the osmotically active compartment, that enclosed by the inner membrane. In order to predict the volume of this compartment, it is necessary to take into account the volume of intra- and extra-mitochondrial water. Equation II includes both these factors,

$$V_{s,i} = V_i - (V_{es} + V_{isn}) = \frac{Q}{P} + b \quad (II)$$

where  $V_{s,i}$  corresponds to the sucrose inaccessible space,  $V_{es}$ , to the extramitochondrial space and  $V_{isn}$ , to the intramitochondrial sucrose accessible space.

Assuming  $b$  to be constant, a plot of  $V_i$  against  $1/P$  will be linear only if  $(V_{es} + V_{isn})$  is constant or varies linearly with  $V_i$ .

Consider a sphere bounded by a membrane that is essentially freely permeable to water but impermeable to the solutes within the sphere. When it is suspended in a solution of infinitely large volume (relative to the volume of the sphere) containing a mixture of permeable and impermeable solutes, the permeable solutes will slowly diffuse into the sphere along the concentration gradient. As the osmotic pressure of the internal solution increases, there will be a rapid osmotic adjustment as the freely permeating water (15) enters under the influence of the osmotic pressure gradient. Expansion of the sphere results from an osmotic flow of water coupled

to the diffusion of the permeable solute down the concentration gradient.

It is convenient here to describe 2 such systems in order to clarify the conceptual basis of the overall system. Since the membrane is essentially freely permeable to water, the system can be considered to be at osmotic equilibrium at all times. Thus,

$$P_i = P_o \quad (III)$$

where the subscripts  $i$  and  $o$  refer to the internal and external solutions respectively.

In the case where the sphere is transferred from a solution of impermeable solute to an isotonic solution containing both permeable and impermeable solutes, at time zero,

$$P_i = P_o + P'_o \quad (IV)$$

where  $P$  and  $P'$  refer to the osmotic pressures of the impermeable and permeable solutes respectively. As the permeable solute diffuses into the sphere, the osmotic pressure of the internal solution increases and thus water enters causing an increase in volume. At equilibrium, the concentration of permeable solute is the same on both sides of the membrane. Thus,

$$P_i + P'_i = P_o + P'_o \quad (V)$$

A consequence of equation V is that the swelling is not infinite, as it would be if no impermeable solute was present, but is finite with the limit determined by  $P_o$ .

In the case where the sphere is transferred from a solution of impermeable solute to a hypertonic solution containing the same concentration of impermeable solute but additional permeable solute, at time zero,

$$P_i < P_o + P'_o \quad (VI)$$

However, since water is essentially freely permeable, the sphere contracts rapidly until equation IV holds. Thereafter, the sphere expands as the permeable solute enters, in a similar manner to the case described above. At equilibrium, the sphere will assume its original volume. This particular case has been described by Johnson and Wilson (7) for sea urchin eggs.

This study is principally concerned with the events occurring after the minimum volume has been reached. In the isotonic case, the minimum volume corresponds to the initial volume.

## Results

*Centrifugation Time and Volume.* Half ml aliquots of mitochondrial stock solution were suspended in 4 ml of buffered 0.4 M sucrose or 0.05 M sucrose. The mitochondrial suspensions were centrifuged at 10,000g for increasing intervals of time. After 10 min of centrifugation the sedimentation of the mitochondria suspended in 0.05 M sucrose was complete. In 0.4 M sucrose 20 min centrifugation was necessary to obtain a constant pellet volume. The differences in sedimentation behavior probably reflect the differences in the viscosity of the suspensions.

In all other volume determinations, the mitochondria were centrifuged at 10,000*g* for 20 min.

**Volume and Transmittance Changes.** One ml aliquots of the mitochondria stock solution were suspended in 8.0 ml of varying concentrations of sucrose (0.4 M–0.05 M) and the resultant pellet volume determined from 7.0 ml of the diluted suspension. In a parallel experiment, 1.0 ml of the diluted solution was further diluted 1:8 with solutions of appropriate sucrose concentration. Aliquots (2.5 ml) of this were used to determine the  $\Delta\%T$ , relative to the  $\%T$  in 0.4 M sucrose.

The results of 3 such experiments are shown in Fig. 1. There is a linear relationship between the volume of the mitochondria and the  $\Delta\%T$ , relative to the  $\%T$  in 0.4 M sucrose.

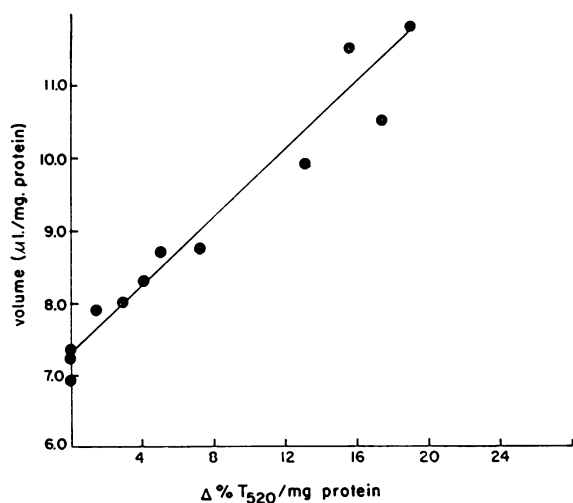


FIG. 1. The relationship between the volume of the mitochondria and the change in  $\%T$  (percent transmittance), relative to the  $\%T$  in 0.4 M sucrose. (Composite results of 3 experiments)

Thus, a continuous record of the  $\%T$  closely reflects changes in the volume of the mitochondria.

**Behavior of Mitochondria in Solutions of Varying Osmolarity.** Mitochondria were suspended in solutions of various osmolarities of sucrose (8.4–1.8 atm) and their volumes determined.

The plot of reciprocal osmotic pressure against volume (Fig. 2) reveals mitochondria do behave approximately as osmometers within this range. Extrapolation to infinite osmotic pressure yields a value for the osmotic "dead space" of about 6.9  $\mu\text{l}/\text{mg}$  protein. At sucrose concentrations both above and below these limits (8.4–1.8 atm) the mitochondria did not behave as osmometers. These deviations from osmotic behavior may have been due to lysis at low osmotic pressures ( $< 1$  atm) and due to collapse of the outer membrane under influence of centrifugal force at the higher osmotic pressure

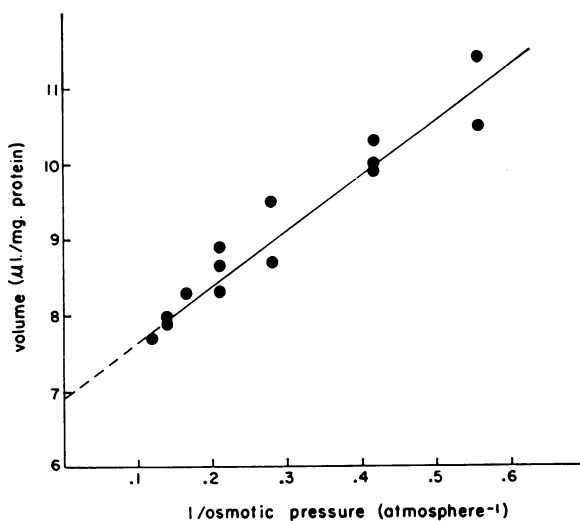


FIG. 2. The relationship between the volume of the mitochondria and osmotic pressure over the range 1.8 to 8.4 atm.

( $> 8.5$  atm). It is also possible that some of the deviations may have been caused by an elastic component in the membrane. Bentzel and Solomon (2) believe this effect is small.

Application of equation VII (table I), derived from equation I, reveals that the mitochondria behave as osmometers,

$$\frac{V_1 - b}{V_2 - b} = \frac{P_2}{P_1} \quad (\text{VII})$$

where  $V_1$  and  $V_2$  refer to the volumes of the mitochondria in solutions of osmotic pressure  $P_1$  and  $P_2$  respectively. A consequence of this finding is that the sum of the terms  $V_{es}$  and  $V_{isa}$  in equation II must be constant, and can therefore be lumped as part of the osmotic "dead space"  $b$ , as is done in equation I.

Table I. *The Osmotic Behavior of Corn Mitochondria*  
 $b^1$  (osmotic "dead space") = 6.9  $\mu\text{l}/\text{mg}$  protein.

$V_1$	$V_2$	$P_1$	$P_2$	$V_1 - b^1$	$P_2$
				$V_2 - b$	$P_1$
$\mu\text{l}$	$\mu\text{l}$	atm	atm	ratio	ratio
8.4	9.9	5	2.5	0.5	0.5
8.8	10.6	4	2.0	0.5	0.5

In order to confirm this deduction, the total extra- and intra-mitochondrial sucrose accessible space in the pellet was determined, using C-14 polyglucose as a space marker. The difference in the total polyglucose accessible space of the mitochondrial pellets isolated from solutions of 0.4 M and 0.05 M sucrose did not exceed 0.8  $\mu\text{l}/\text{mg}$  protein. Experiments with C-14 sucrose revealed the same

value for the ratio of the total sucrose accessible volume in the pellet to the total pellet volume as did those performed with C-14 polyglucose. This indicates that polyglucose is able to permeate the same volume as sucrose. The value for the ratio referred to above was found to be about 0.6, a figure that agrees with the lower values of this ratio determined by Malamed and Recknagel (12) for rat liver mitochondria. Bentzel and Solomon (2) obtained a ratio of 0.7 in 0.2 M sucrose.

The reason why the sum of the terms  $V_{es}$  and  $V_{isa}$  appears to be constant is not immediately apparent. If the mitochondria are centrifugally packed in the manner of rigid spheres, then some 26% of the total pellet water is external to the mitochondria (2,4). A 2-fold increase in mitochondrial volume will result in a 2-fold increase in the extra-mitochondrial water; i.e.,  $V_{es}$  doubles in value. Electron micrographs of swollen mitochondria indicate that  $V_{isa}$  becomes smaller as the mitochondria swell. Fortuitously, perhaps, the changes in the 2 terms  $V_{es}$  and  $V_{isa}$  offset one another such that the sum remains constant.

Half ml aliquots of mitochondria were suspended in solutions of KCl and ribose, containing 0.05 M sucrose and sufficient KCl or ribose to give a known osmotic pressure. Standards containing various osmolarities of sucrose were also prepared. The solutions were allowed to equilibrate for 30 min before volume determination. The results (Fig. 3) indicate that ribose is not an osmoticant, since the equilibrium volume was equal to that in 0.05 M sucrose, no matter what concentration of ribose was used. This is as predicted in equation V for a permeable solute.

The KCl plot indicates that KCl is a poorer osmoticant than sucrose; i.e., some of the mitochondria (approximately 50%) behave effectively as if

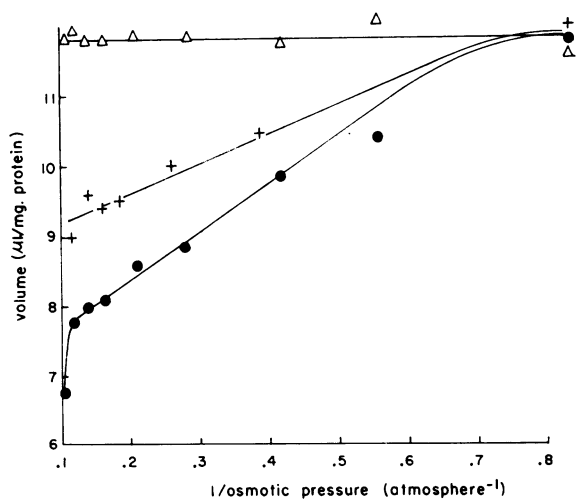


FIG. 3. The osmotic behavior of corn mitochondria in sucrose ●, KCl + and ribose Δ. The solutions of KCl and ribose contained 0.05 M sucrose plus sufficient KCl or ribose to give a known osmotic pressure.

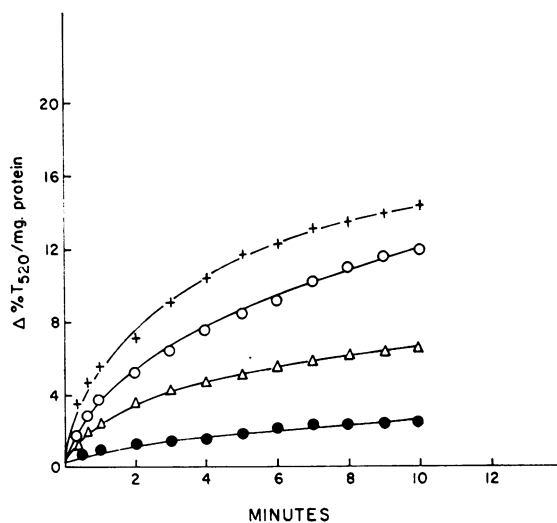


FIG. 4. The change in transmittance (an index of volume) with time. 0.4 M ribose +, 0.4 M xylose ○, 0.4 M glucose Δ and 0.4 M maltose ●.

the KCl were an osmoticant. Electron micrographs (13) reveal that about 40% of the mitochondria remain the same size in KCl as those in 0.4 M sucrose. It is this portion of the mitochondria that behaves effectively as if KCl were an osmoticant.

*The Behavior of Mitochondria in Solutions of Constant Osmolarity.* Mitochondria were transferred from a solution of 0.4 M sucrose to isotonic solutions of various sugars. Fig. 4 illustrates the volume changes (reported as  $\Delta\%T/mg$  protein) undergone by the mitochondria. In this particular experiment, the reaction was not allowed to run to equilibrium.

Fig. 4 shows that the rate of change of volume increases as the molecular size decreases, a phenomenon to be expected for nonelectrolytes if their rate of uptake is in approximate proportion to the olive-oil water partition coefficient as found for rat liver mitochondria (2). These findings agree with similar permeability data obtained from other types of membranes (3).

*Osmotic Reversal of Ribose Induced Swelling.* If, indeed, the swelling of mitochondria is osmotic in nature, then it should be reversed by increasing the concentration of impermeable solute. Such an osmotic reversibility has been demonstrated (9, 11, 14, 16).

Half ml aliquots of mitochondrial solution were pretreated for 30 min by suspension in solutions of 0.4 M sucrose, 0.05 M sucrose or 0.35 M ribose in 0.05 M sucrose. The concentration of the impermeable solute, sucrose, was then increased by the addition of 0.6 M sucrose in 0.35 M ribose to the tube containing 0.35 M ribose in 0.05 M sucrose. The final concentration of sucrose was then 0.4 M. Volume measurements were made. The results (table II) demonstrate that the swelling in ribose is osmotically reversible, as equation V would predict.

Table II. *The Osmotic Reversibility of Mitochondria Swollen in Ribose*

Pretreatment	Treatment	Rel. vol.
0.4 M sucrose	0.4 M sucrose	1.0
0.05 M sucrose	0.4 M sucrose	0.9
0.05 M sucrose	0.05 M sucrose	1.5
0.35 M ribose +	0.35 M ribose +	1.5
0.05 M sucrose	0.05 M sucrose	
0.35 M ribose +	0.35 M ribose +	0.9
0.05 M sucrose	0.4 M sucrose	

*The Osmotic Reversal of KCl Induced Swelling.*

This experiment is identical to that described for the reversal of ribose induced swelling except that 0.224 M KCl was used in place of 0.35 M ribose. The results (table III) demonstrate that the swelling in KCl is osmotically reversible by the addition of impermeable solute, as equation V would predict.

Table III. *The Osmotic Reversibility of Mitochondria Swollen in KCl*

Pretreatment	Treatment	Rel. vol.
0.4 M sucrose	0.4 M sucrose	1.0
0.05 M sucrose	0.05 M sucrose	1.5
0.224 M KCl +	0.224 M KCl +	1.3
0.05 M sucrose	0.05 M sucrose	
0.224 M KCl +	0.224 M KCl +	0.8
0.05 M sucrose	0.4 M sucrose	

## Discussion

Of the procedures commonly used in mitochondria studies none is more critical than the relationship between the volume of the mitochondria and the change in % transmittance at 520  $m\mu$ . Fig. 1 indicates that a linear relationship exists for volume changes induced by altering the concentration of sucrose. Earnshaw (personal communication) has found a similar relationship for volume changes of bean hypocotyl mitochondria, induced by suspension of the mitochondria in solutions of KCl. The data in Fig. 1 was not corrected for changes in refractive index with changes in sucrose concentration. Tedeschi and Harris (15) show that the relationship between transmittance and refractive index is linear. Correcting Fig. 1 for refractive index would only change the slope, not the linear relationship.

The measurement of the mitochondrial pellet volume includes extra-mitochondrial water. Thus values for the mitochondrial volumes are over estimated. This is reflected in the value for the osmotic "dead space" of about 6.9  $\mu\text{l}/\text{mg}$  protein. This corresponds to over 90 % of the pellet volume in 0.3 M sucrose (Fig. 3). If the mitochondria are centrifugally packed in the manner of rigid spheres,

then some 26 % of the "dead space" is extra-mitochondrial water.

The behavior of corn mitochondria as osmometers within the range of about 1.8 to 8.4 atm osmotic pressure (Fig. 2), confirms for plant mitochondria (11, 16) similar observations with animal mitochondria (2, 12). It is not known if the deviations outside this range of osmotic pressures are real phenomena or experimental artifacts or both.

For an 8-fold change in the concentration of sucrose (0.4 M-0.05 M), the mitochondria undergo a volume change of about 1.5 to 2-fold. The large variation in the volume changes is probably a reflection of the fact that the isolation procedure includes the manual grinding of the tissue, a step that is not easy to standardize. That the mitochondria do not also undergo an 8-fold change in volume, is due to the magnitude of the osmotic "dead space" (table I).

The principal purpose of this study was to elucidate the mechanism of passive swelling of mitochondria. The results indicate that the volume changes occurring in ribose represent a diffusion controlled osmotic flow of water. Two facts point to this conclusion. Firstly, the equilibrium volume of the mitochondria suspended in ribose depends not on the quantity of ribose that has entered the mitochondria but upon the concentration of impermeable solute present (Fig. 3). This result is predicted from equation V, which is based upon the equilibration of the permeable solute across the membrane. Secondly, the swelling in ribose is osmotically reversible by increasing the concentration of impermeable solute in the external medium.

The results for the KCl induced swelling and contraction are essentially similar to those for ribose. This leads to the conclusion that the swelling in KCl is, likewise, a diffusion controlled osmotic flow of water. However, the system in KCl is clearly not as simple as that in ribose. Undoubtedly there will be some Donnan effects. Furthermore, Fig. 3 suggests that some 50 % of the mitochondria behave as if KCl were an osmoticant. The value of 50 % agrees with similar values obtained by scoring electron micrographs of mitochondria swollen in KCl (13). Either 50 % of the mitochondria are impermeable to KCl or possess a mechanism for the "pumping out" of one or both of the ionic species. It is known that plant mitochondria swollen in KCl will contract upon the addition of an oxidizable substrate (13).

Preliminary experiments not reported here indicate that ribose swollen mitochondria will not contract upon the addition of an oxidizable substrate. If the metabolically induced contraction in KCl represents an osmotic efflux of water, as the results here would suggest, then it must be caused by the efflux of one or both of the ionic species present. If then some 50 % of the mitochondria retained sufficient endogenous substrate during isolation, they would

possess a mechanism for "pumping out" one or both of the ionic species present. They would thus remain in the contracted state. Support for this view comes from the observation by Stoner and Hanson (13) that the uncoupling agent, carbonyl cyanide M-chlorophenyl hydrazone (CCP) increases the rate of swelling. CCP inhibits the metabolically induced contraction.

### Literature Cited

1. AZZI, A. AND G. F. AZZONE. 1967. Ion transport in liver mitochondria. II. Metabolism linked ion extrusion. *Biochim. Biophys. Acta* 135: 444-53.
2. BENTZEL, C. J. AND A. K. SOLOMON. 1967. Osmotic properties of mitochondria. *J. Gen. Physiol.* 50: 1547-63.
3. COLLANDER, R. AND H. BARLUND. 1933. Permeabilitätsstudien an *Chara ceratophylla*. II. Die Permeabilität für Nichtelektrolyte. *Acta Botan. Fennica*, 11: 1-114.
4. CONWAY, E. J. AND M. DOWNEY. 1950. An outer metabolic region of the yeast cell. *Biochem. J.* 47: 347-55.
5. DUMFORD, S. W. 1968. The effect of divalent inorganic cations on the energy driven reactions of corn mitochondria. Ph.D. Thesis, University of Illinois, Urbana, Illinois 61801.
6. EARNSHAW, M. J. AND B. TRUELOVE. 1968. Swelling and contraction of *Phaseolus* hypocotyl mitochondria. *Plant Physiol.* 43: 121-29.
7. JOHNSON, J. A. AND T. A. WILSON. 1967. Osmotic volume changes induced by a permeable solute. *J. Theoret. Biol.* 17: 304-11.
8. KENEFICK, D. G. AND J. B. HANSON. 1966. Contracted state as an energy source for Ca binding and Ca + inorganic phosphate accumulation by corn mitochondria. *Plant Physiol.* 41: 1601-09.
9. LONGO, C. AND O. ARRIGONI. 1964. Functional properties of isolated plant mitochondria. *Exptl. Cell Res.* 35: 572-79.
10. LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-76.
11. LYONS, J. M., T. A. WHEATON, AND H. K. PRATT. 1964. Relationship between the physical nature of mitochondrial membranes and chilling sensitivity in plants. *Plant Physiol.* 39: 262-68.
12. MALAMED, S. AND R. O. RECKNAGEL. 1951. The osmotic behavior of the sucrose inaccessible space of mitochondrial pellets from liver. *J. Biol. Chem.* 234: 3027-30.
13. STONER, C. D. AND J. B. HANSON. 1966. Swelling and contraction of corn mitochondria. *Plant Physiol.* 40: 255-66.
14. TEDESCHI, H. 1961. Osmotic reversal of mitochondrial swelling. *Biochim. Biophys. Acta* 46: 159-69.
15. TEDESCHI, H. AND D. L. HARRIS. 1955. The osmotic behavior and permeability to non-electrolytes of mitochondria. *Arch. Biochem. Biophys.* 58: 52-67.
16. YOSHIDA, K. AND S. SATO. 1968. Swelling and contraction of isolated plant mitochondria. I. Passive swelling in sugar and electrolyte solutions. *J. Faculty Sci. Univ. Tokyo, III*, 10: 49-62.