ION UPTAKE BY CARROT TISSUE AND MITOCHONDRIA

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Summary

It is shown that aged carrot disks which accumulate ions and show salt-stimulated respiration are suitable for the isolation of mitochondria.

The isolated mitochondria were enzymically similar to mitochondria prepared from fresh tissue. Both showed respiratory control.

The mitochondria of aged disks were capable of accumulating ions and the sensitivity of this process to inhibitors and uncouplers was similar to that of the tissue. Oligomycin did not inhibit substrate-supported ion accumulation by the mitochondria.

The results are discussed and their significance to the theories of ion uptake by plant storage tissues is considered.

I. Introduction

The accumulation of salts by washed slices of plant storage organs is accompanied by an increase in the respiratory rate. Both this "salt respiration" and "salt accumulation" are sensitive to inhibitors which act on the respiratory system. However, uncouplers of oxidative phosphorylation (2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone) whilst inhibiting salt accumulation increase the oxygen uptake of the tissue, making it impossible to observe any effect on salt respiration (Briggs, Hope, and Robertson 1961). These results suggest that the electron transfer and oxidative phosphorylation systems of mitochondria are involved in salt uptake. The involvement of these mitochondrial properties may be direct [i.e. charge separation being essential (Lundegårdh 1960; Robertson 1960)] or indirect [i.e. the formation of ATP being essential (Sutcliffe 1962)]. Recently Atkinson *et al.* (1966) reported on the ATP content of carrot disks, and argued that ion uptake was directly coupled to electron transport. However, Hodges (1966) has observed an inhibition by oligomycin of potassium and chloride uptake by various plant roots. Since it is known that oligomycin inhibits ATP formation but not ion uptake by isolated mitochondria this result strongly suggests an involvement of ATP. Both of these systems are insensitive to ouabain suggesting that Na^+- and K^+-activated adenosine triphosphatase (Charnock and Post 1963) is not involved.

It has been established that isolated plant mitochondria are the sites of aerobic respiration (Hackett 1959), and that they can accumulate ions (Lieberman and Baker 1965). However, there is no information available on the properties of mitochondria isolated from aged tissue slices. The present paper describes the isolation, enzymic activity, and ion uptake properties of mitochondria isolated from aged carrot disks which have been shown to be capable of accumulating ions.

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II. Materials and Methods

(*a*) *Preparation of Disks*

Disks (1 mm thick) were cut from cylinders (6 mm diameter) of excised xylem parenchyma of commercially obtained carrot (*Daucus carota* L.). The disks were well rinsed and aged in flasks of distilled water which was changed frequently and continually aerated with a stream of filtered air. The tissue was allowed to wash for about 96 hr after cutting to allow the respiratory drift to return to a steady ground state (Robertson and Turner 1945).

(*b*) *Preparation of Mitochondria*

Carrot tissue (300 g) was blended in a Waring Blendor (at maximum speed) for 20–25 sec in a medium (260 ml) of 0·4M sucrose containing 5 mM EDTA, 18 mM Tris, 1% bovine serum albumin (fraction V powder), and 0·05% L-cysteine (free base). The mixture was strained through muslin and centrifuged at 3500 *g* (maximum) for 15 min to remove debris and the supernatant was then centrifuged at 10,000 *g* (maximum) for 15 min to sediment the mitochondria. The mitochondrial pellet was washed by resuspending in 0·4M sucrose, with the aid of a Potter–Elvehjem homogenizer, and centrifuging at 10,000 *g* (maximum) for 15 min. The free-flowing carotene fragments on the surface of the pellet were decanted and the pellet washed again. The final pellet was resuspended in 6–8 ml of 0·4M sucrose. All apparatus was prechilled and all operations were performed in a cold-room or a refrigerated centrifuge (0–4°C).

(*c*) *Manometry*

Respiratory rates of carrot disks were measured by standard manometric techniques at 25°C with air as the gas phase (Umbreit, Burris, and Stauffer 1959). Disks were placed in distilled water with 4N KOH and filter paper in the centre well. Additions were added from side-arms. The manometers were shaken in a Braun rotary Warburg bath at 120 oscillations per minute with a 4-in. stroke.

(*d*) *Conductivity*

Salt accumulation by carrot disks was measured with a Pye conductance bridge.

(*e*) *Oxygen Electrode*

Oxygen electrode studies were made with a Clark oxygen electrode (Yellow Springs Instrument Co., Cleveland, Ohio) connected to a Varian G-14 recorder (Varian Associates, Palo Alto, California). The reaction medium was 0·25M sucrose containing 10 mM Tris–HCl buffer (pH 7·2), 5 mM $MgCl_2$, 10 mM potassium phosphate buffer (pH 7·2), and 5 μM cytochrome *c*, 0·5 mM EDTA, 8 mM Tris–succinate, and 150–250 μg mitochondrial nitrogen. The reaction was carried out in a sealed Perspex vessel (3·2 ml) and stirred with a small magnetic stirrer (Rank Bros., Bottisham, Cambridge). Constant temperature (25°C) was maintained by circulating water around the vessel.

(*f*) *Ion-uptake Studies*

Flasks containing 0·25M sucrose, 12 mM Tris–HCl buffer (pH 7·2), 15 mM $MgCl_2$, 3·5 mM potassium phosphate buffer (pH 7·2), 5 μM cytochrome *c*, 8 mM Tris–succinate, and mitochondria were shaken in a water-bath (25°C). Samples were taken at different time intervals and the mitochondria separated from the medium either by centrifuging through 3 ml of 1.0M sucrose or by filtering (Millard, Wiskich, and Robertson 1965) through Millipore filters (1·2 μ mean pore size, Millipore Filter Corp., Bedford, Massachusetts). Although the mean pore size was larger than the expected mitochondrial size, the filters were still completely effective in retaining the mitochondria (see Millipore publication ADM-30).

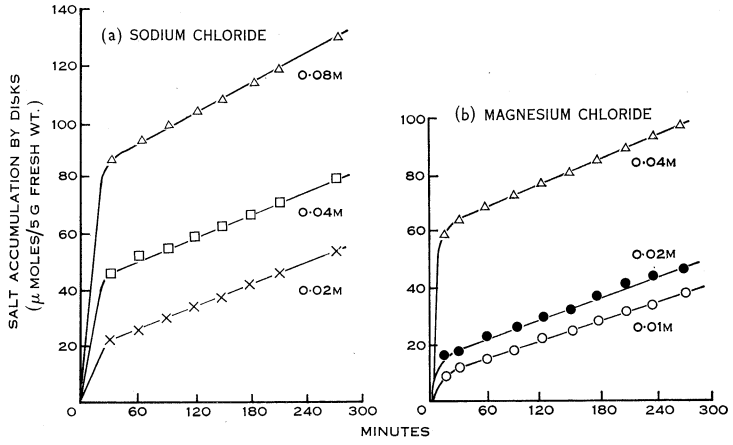

Fig. 1.—Accumulation of sodium chloride (*a*) and of magnesium chloride (*b*) by aged carrot disks. The different concentrations used are indicated.

(*g*) *Estimation of ATP Formation*

The same reaction medium as used in (*f*) above was used with the following additions: 1 mM ADP, 12 mM glucose, and excess hexokinase. The glucose-6-phosphate so formed was assayed spectrophotometrically as described by Wiskich, Morton, and Robertson (1960).

(*h*) *Ion Analyses*

The mitochondria, after separation from the reaction medium, were extracted with 5·5% perchloric acid. Mitochondrial and carotene fragments were removed by centrifuging at 25,000 *g* (maximum) for 10 min. This was essential as the carotene interfered with the phosphate analysis. Phosphate was determined by the method of Marsh (1959) and magnesium by the method of Vogel (1961).

(*i*) *Mitochondrial Nitrogen*

Samples were digested in concentrated sulphuric acid using mercury as catalyst. Distillations and titrations were carried out essentially as described by McKenzie and Wallace (1954).

(*j*) *Chemicals*

Adenosine phosphates were obtained from P–L Biochemicals, Wisconsin. Enzymes, horse-heart cytochrome *c*, antimycin A, nicotinamide adenine dinucleotide phosphate (NADP), and 2-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO) were obtained from Sigma Chemical Co., St. Louis. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was a gift from E. I. Dupont & Co., Delaware, and oligomycin a gift from Dr. Elizabeth McCoy, University of Wisconsin.

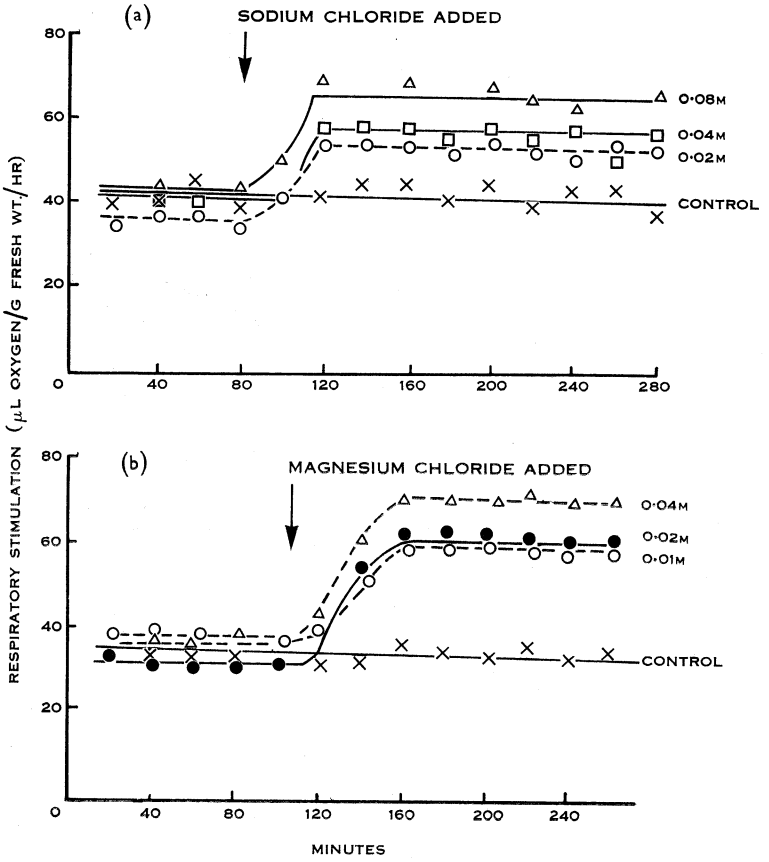

Fig. 2.—Respiratory stimulation of aged carrot disks on addition of (*a*) sodium chloride and (*b*) magnesium chloride at the concentrations indicated.

III. Results

(*a*) *Salt Accumulation by Aged Carrot Disks*

The washed carrot disks were capable of accumulating salts, as shown in Figure 1 for both monovalent and divalent chloride salts. The total uptake of salt (Fig. 1) must be regarded only as an approximation because of the assumptions made in calculating concentration changes from changes in conductivity. These are that the salt is removed as both ions with no preference for any ion species, i.e. net uptake is not confused by an ion-exchange phenomenon. The results shown in

Figures 1 and 2 are typical of those presented by Robertson and Turner (1945), and Robertson and Wilkins (1948). Figure 2 shows that the oxygen uptake of these carrot disks was stimulated by the addition of salt. The effects of various chloride salts summarized in Table 1 show that the carrot disks responded to added salts in a similar manner to other plant storage tissue used by previous workers.

TABLE 1

SALT ACCUMULATION AND RESPIRATION OF AGED CARROT DISKS INDUCED BY VARIOUS CHLORIDES

Measurements were made as described in Section II. Salt concentration 0·4M in each case

Salt	Salt Accumulation Rate (μmoles/g fresh wt./hr)	Respiratory Rates (μl O_2/g fresh wt./hr)		
		Salt Respiration	Ground Respiration	Total Respiration
KCl	1·8	20	40	60
NaCl	1·7	20	40	60
$MnCl_2$	1·7	26	42	68
$MgCl_2$	1·6	36	36	72
$CaCl_2$	1·1	21	48	69

(*b*) *Behaviour of Isolated Mitochondria*

In agreement with Dalgarno and Birt (1962) the preparations were always contaminated with particulate carotene. Nevertheless, their behaviour resembled that of other isolated plant mitochondria. A typical oxygen electrode trace obtained with mitochondria isolated from aged disks is shown in Figure 3. Respiratory control by the level of ADP as defined by Chance and Baltscheffsky (1958) is shown by the repeated stimulation of oxidation rate on addition of ADP (their state 3) and depression of oxidation rate on exhaustion of ADP (their state 4). Respiratory control ratios are calculated from the state 3/state 4 ratio (see Wiskich and Bonner 1963).

It was found that both cysteine and bovine serum albumin were essential components of the homogenization media if mitochondria showing respiratory control were to be isolated. However, aged carrot disks required less bovine serum albumin (0·3%) in the medium than fresh carrot tissue (1·0%). Dalgarno and Birt (1963) have previously indicated beneficial effects of bovine serum albumin in the preparation of carrot mitochondria. They have correlated this effect with the binding of free fatty acids released during the preparation of the mitochondria, and the inhibitory effects of added fatty acids. Presumably, then, a large fraction of the free fatty acids becomes unavailable during the washing period. Data obtained within the same experiment from oxygen electrode traces, using succinate as substrate, show that the coupling properties of mitochondria isolated from fresh and aged carrot disks

(expressed in terms of respiratory control ratios) were 1·5 and 1·9 respectively. The corresponding ADP/O ratios (i.e. g-moles ATP esterified/g-atoms oxygen consumed) were 1·1 and 1·6.

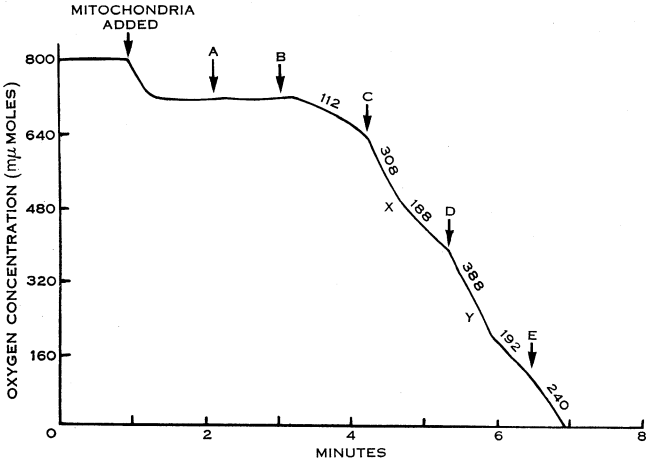

Fig. 3.—Oxygen electrode trace of succinate oxidation by mitochondria prepared from aged (160 hr) carrot disks. Additions (final concentrations) at times indicated by arrows were as follows: *A*, 5 μM cytochrome *c*; *B*, 15 mM Tris–succinate; *C*, *D*, 0·2 mM ADP; *E*, 0·15 mM ADP. At *X* and *Y*, ADP/O ratios were 1·4 and 1·6 and the corresponding respiratory control ratios were 1·6 and 2·2 respectively. Rates are expressed as mμmoles O_2/min.

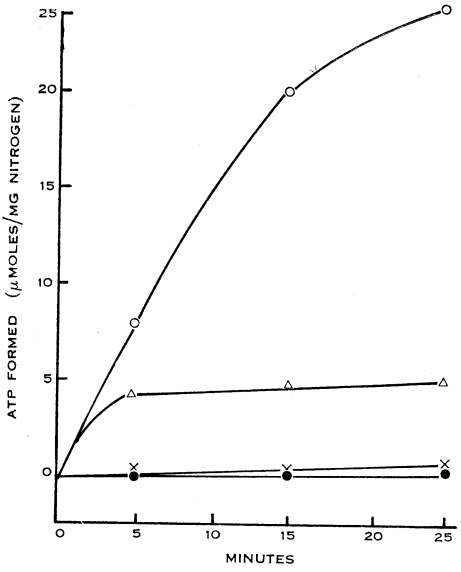

Fig. 4.—Effect of oligomycin on ATP formation by aged carrot mitochondria. The medium was the same as that used for ion-uptake studies.

● Control.
× Control plus glucose and hexokinase.
○ Control plus glucose, hexokinase, and ADP.
△ Control plus glucose, hexokinase, ADP, and oligomycin (1 μg/ml).

To compare absolute data obtained with mitochondria isolated from different types of plants it is necessary to establish a suitable reference unit for the amount or number of mitochondria. Total nitrogen is usually used but this will be unsatisfactory

if, as in these experiments, there is contamination of the mitochondrial preparation by nitrogen-containing material.

The mitochondria prepared as in Section II(*b*) behaved normally in that oxidation of tricarboxylic acid substrates was completely inhibited by 1·5 μg/ml of antimycin A, 60 μM HOQNO, and 0·1 mM KCN. Furthermore, oxygen electrode studies showed that coupled oxidation was sensitive to the antibiotic oligomycin, and to the uncouplers 2,4-dinitrophenol and CCCP. Figure 4 shows that ATP formation was inhibited by oligomycin (Lardy, Johnson, and McMurray 1958) and that added ADP was essential for ATP formation. The ATP formed in the presence of oligomycin could be accounted for by considering adenylate kinase activity.

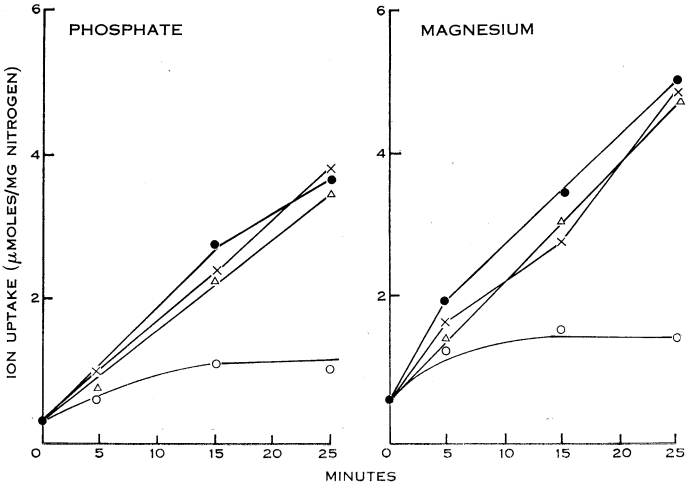

Fig. 5.—Effect of phosphorylating conditions on ion uptake. × Control. ● Control plus glucose and hexokinase. ○ Control plus glucose, hexokinase, and ADP. △ Control plus glucose, hexokinase, ADP, and oligomycin (1 μg/ml).

(*c*) *Ion Uptake by Isolated Mitochondria*

Accumulation of magnesium and phosphate by isolated mitochondria from aged carrot disks was studied under the conditions described by Millard, Wiskich, and Robertson (1964). Figure 5 shows that accumulation of both ions was inhibited by ATP formation. Oligomycin, which inhibits ATP formation, restored accumulation to the control level but the other additions (glucose and hexokinase) were without effect. However, as noted by Millard, Wiskish, and Robertson (1965) this was true only if the added hexokinase was not suspended in ammonium sulphate. Table 2 shows that hexokinase suspended in ammonium sulphate inhibited magnesium and phosphate accumulation and that recovery of ion uptake in the presence of oligomycin approached only this inhibited level.

The effects of inhibitors of the electron transport chain and of uncouplers of oxidative phosphorylation are shown in Table 3. It is evident that ion accumulation was dependent on continuous electron flow (substrate oxidation) and on an undamaged

oxidative phosphorylation system. However, it was observed (Fig. 5) that oligomycin did not inhibit ion uptake.

TABLE 2

EFFECTS OF PHOSPHORYLATION AND AMMONIUM SULPHATE ON ION UPTAKE BY CARROT MITOCHONDRIA

Samples were analysed after incubation for 25 min as described in Section II. The hexokinase was suspended in ammonium sulphate

System	Magnesium Uptake (μmoles/mg mitochondrial nitrogen)	Phosphate Uptake (μmoles/mg mitochondrial nitrogen)
Control	6·00	5·50
Control plus glucose and hexokinase	4·22	3·04
Control plus glucose, hexokinase, and ADP	2·71	2·52
Control plus glucose, hexokinase, ADP, and oligomycin (1 μg/ml)	4·42	3·56

The uptake of monovalent cations could not be studied in the same way as divalent cations, presumably because the efflux of these ions was too rapid to allow

TABLE 3

EFFECT OF INHIBITORS AND UNCOUPLERS ON ION ACCUMULATION BY MITOCHONDRIA FROM AGED TISSUE

The mitochondria were incubated for 15 min (as described in Section II), separated from the medium, and analysed for magnesium and phosphate

Expt.	Additive	Magnesium Uptake (μmoles/mg mitochondrial nitrogen)	Phosphate Uptake (μmoles/mg mitochondrial nitrogen)
1	None	2·20	0·72
	Succinate (10 mM)	4·94	1·35
	Dinitrophenol (0·1 mM)*	1·41	0·50
	CCCP (0·8 μM)*	1·45	0·62
	HOQNO (60 μM)*	1·00	0·50
	Antimycin A (1 μg/ml)*	1·20	0·70
2	None	2·10	0·25
	Succinate (10 mM)	4·33	1·74
	KCN (0·1 mM)*	2·60	0·63

* Succinate (10 mM) present.

for any large amounts of net accumulation. Nevertheless, the inhibition by ammonium sulphate (Table 2) was thought to be due to a competition between NH_4^+ and Mg^{2+}

(Millard, Wiskich, and Robertson 1965). Analyses for ammonia did not reveal any accumulation but the alkaline conditions within the mitochondria (Brierley *et al.* 1963) would tend to dissociate the ammonium ion, yielding free ammonia which could diffuse out again. Similarly, potassium was found to reduce magnesium and phosphate uptake (Fig. 6). Although potassium analyses were not done, studies with ^{22}Na suggested that the net accumulation could be detected only in the first few minutes of the experiment. Further experiments on monovalent cation uptake are being conducted.

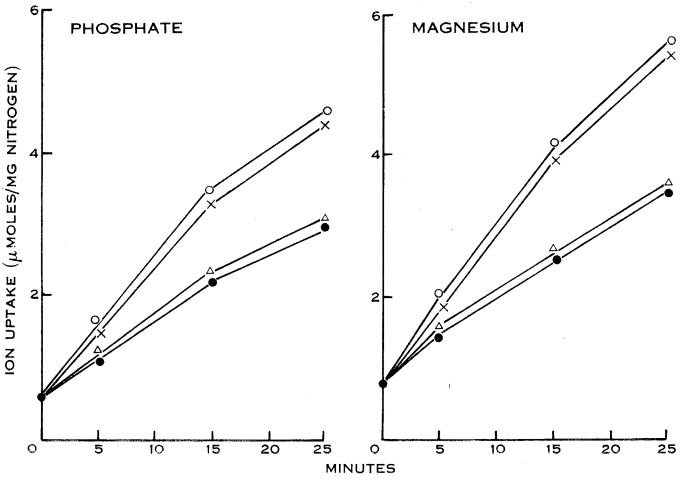

Fig. 6.—Effects of potassium chloride and Tris.HCl (both 10 mM) on magnesium and phosphate uptake. ○ Control. × Control plus Tris.HCl. △ Control plus potassium chloride. ● Control plus Tris.HCl and potassium chloride.

IV. Discussion

Mitochondrial fractions have been prepared from both fresh and aged disks of carrot xylem parenchyma. The preparations were always contaminated with carotene and possibly other substances. This contamination proved impossible to prevent. Nevertheless, the preparations behaved, enzymically, like most other plant preparations (Hackett 1959; Lieberman and Baker 1965), and showed respiratory control. The beneficial effect of bovine serum albumin in preparing active mitochondria, especially from fresh carrot tissue, has been confirmed. This requirement was noted and discussed by Dalgarno and Birt (1962, 1963).

It has been established that biochemically intact mitochondria can be isolated from aged carrot disks, and that these mitochondria do not differ significantly from those isolated from fresh carrot. Thus there is no evidence to suggest that mitochondrial properties differ in these fresh and aged disks. This is important because it is known that metabolic changes occur during the initial stages of aging of carrot disks (Ap Rees and Beevers 1960) and of potato slices (Laties 1963). Furthermore,

aged carrot disks show a cyanide-insensitive basal respiration, and a cyanide-sensitive salt respiration (Robertson, Turner, and Wilkins 1947). The oxidation of mitochondria isolated from both fresh and aged tissue was completely cyanide-sensitive (cf. Wiskich and Bonner 1963).

If mitochondria are involved in salt accumulation by storage tissues, as suggested by Robertson (1960), it is of prime importance to establish that mitochondria isolated from such tissues are not only biochemically intact but also capable of ion accumulation. The results presented here show that mitochondria isolated from aged carrot disks are capable of accumulating salt. The characteristics of this ion uptake were similar to those reported for mitochondria isolated from other plant tissues (Millard, Wiskich, and Robertson 1964, 1965; Hodges and Hanson 1965). The effects of respiratory inhibitors and uncouplers on ion uptake by isolated mitochondria paralleled their effects on the intact tissue. Accumulation of monovalent ions by isolated mitochondria has not been demonstrated directly. However, the inhibition of magnesium and phosphate accumulation by monovalent ions (Fig. 6) suggests an interaction between these ion species.

General Considerations

It has been suggested that in salt accumulation by intact tissue, electron transport (and not ATP) is of importance (Robertson 1960), i.e. the ion movement act may be an alternative to ATP formation. Since information on ion uptake by intact tissue and by mitochondria isolated from the same tissue is now available, a more definite appraisal of this hypothesis is possible. However, it must be emphasized that the properties of isolated mitochondria, even under the most critical of circumstances, give no more than an indication of the potential properties *in vivo*. Extrapolation of data of *in vitro* studies with isolated mitochondria to the intact tissue is not always reliable.

The single relevant point about ion uptake with isolated mitochondria, which supports the hypothesis that electron transport and not ATP is essential for ion movement, is the insensitivity to oligomycin. In fact, ATP formation competes with ion uptake (Fig. 5). However, ATP in the absence of added substrate will support mitochondrial ion uptake (Hodges and Hanson 1965). Both sources of energy (substrate and ATP) generate, and both processes (ion uptake and ATP formation) compete for, the same energized state. The competition favours ATP formation rather than ion uptake, so it becomes difficult to explain, in terms of the electron flow hypothesis, the decrease in ATP level (and presumably the increase in ADP level) on addition of salt to aged carrot slices (Atkinson *et al.* 1966). A direct consequence is that the distinction between salt and basal respiration may not be as definite as has been supposed. The report of Hodges (1966) that oligomycin inhibits ion uptake may be considered to support the idea that ATP is directly involved in ion movements. However, this result is complicated by the failure of oligomycin to inhibit respiration under the same conditions. If, as Hodges suggests, the ATP utilization processes and the oligomycin-sensitive sites are at the cell surface a connection between ion uptake and respiration should be evident.

Thus, there is no doubt that mitochondria can accumulate ions and in fact may be the carriers or sites of carriers in the metabolic uptake of ions. The evidence presented by Mertz (1961) that mitochondria, isolated from tissue incubated in a solution containing ^{45}Ca, contain substantial amounts of exchangeable ^{45}Ca supports this suggestion. The connection between ion uptake and respiration in plants remains obscure but when evidence from experiments with isolated mitochondria (as reported here and elsewhere) and with isolated chloroplasts (Jagendorf and Uribe 1966) is considered, and recent developments on the theory of oxidative phosphorylation (Mitchell and Moyle 1965) and the apparent absence of a system similar to the Na^+- and K^+-activated adenosine triphosphatase are taken into account, it appears that ATP *per se* need not be implicated.

V. Acknowledgments

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