EVIDENCE FOR A DIRECT INVOLVEMENT OF ELECTRON TRANSPORT IN THE HIGH-AFFINITY ION ACCUMULATION SYSTEM OF AGED BEET PARENCHYMA

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Summary

Effects of anaerobic conditions, uncouplers [2,4-dinitrophenol and carbonyl cyanide 3-chlorophenylhydrazone (CCCP)], inhibitors of oxidative phosphorylation (Dio-9 and oligomycin), and an ATP-trapping reagent (L-ethionine) on influx and net uptake of K^+, Na^+, and Cl^- from 0·5 mM KCl or NaCl solutions into aged beet parenchyma disks and on tissue ATP levels were determined.

Anaerobic conditions inhibited K^+ and Cl^- influx completely and Na^+ influx by 85% after 30 min. After 1 hr of anaerobiosis the ATP level in aged beet disks was not significantly lower than the initial aerobic level.

Dinitrophenol ($0 \cdot 25$–$1 \cdot 0 \times 10^{-3}$M) and CCCP ($1 \cdot 0 \times 10^{-5}$M) caused rapid net loss of K^+ from aged beet disks. Dinitrophenol completely inhibited K^+, Na^+, and Cl^- influxes within 30 min and caused depression of the ATP level to 4% of the control level after 2 hr. In contrast, L-ethionine (0·5–1·0 mM) caused severe depression of the tissue ATP level without inhibiting K^+, Na^+, or Cl^- influxes by more than 50% after 1 hr.

Dio-9 (10 μg/ml) had little effect on Na^+ or K^+ influx into aged beet disks. Oligomycin ($1 \cdot 3$–$2 \cdot 6$ μg/ml) caused a delayed inhibition of K^+ and Na^+ influxes and did not completely inhibit these fluxes over 2 hr.

The results provide evidence against the concept of a direct involvement of ATP as energy source for Na^+, K^+, and Cl^- transport by the high-affinity ion accumulation systems of aged beet parenchyma cells.

I. Introduction

Development of an enhanced capacity for ion absorption by beet storage-organ disks on aeration in water for an extended period ("aging") was first described by Asprey (1937).

Van Steveninck (1962, 1964) found that this enhancement results mainly from decreased K^+ efflux and increased Cl^- influx during the aging period.

Kinetic analyses of cation and anion uptake by a variety of plant tissues have revealed two apparently distinct mechanisms for salt accumulation: "system 1" operating effectively at external salt concentrations lower than 1 mM, and "system 2" operating at higher salt concentrations (see Epstein 1966).

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Recent studies by Osmond and Laties (1968) have demonstrated the existence of two such systems for accumulation of K^+ and Cl^- in aged beet parenchyma disks, system 1 operating in the range 0·01–0·5 mM KCl and system 2 operating at higher concentrations (1–50 mM KCl). It has been proposed that system 1 is located at the plasmalemma and is rate-limiting at low salt concentrations ($< 0{\cdot}5$ mM), while system 2 operates at the tonoplast at higher salt concentrations (1–50 mM), at which levels plasmalemma permeability to alkali cations and chloride may be increased (Torii and Laties 1966; Osmond and Laties 1968).

Isotope exchange studies by Pitman (1963) suggested that diffusion may account for a large part of the observed rates of K^+ uptake in beetroot tissue (cf. Laties, MacDonald, and Dainty 1964). However, electrophysiological studies by Poole (1966) have indicated that KCl uptake into aged beet disks from "low" salt concentrations (< 1 mM) is best described in terms of a linked, active transport of K^+ and Cl^-.

The energetics of these transport processes are incompletely defined. Early studies showed that alkali cation and chloride accumulation by aged beet disks from relatively high salt concentrations (in which system 2 would be rate-limiting) was associated with an increased respiration rate, the increase being defined as "salt respiration" (Robertson, Turner, and Wilkins 1947). This phenomenon was demonstrable in a variety of plant storage tissues (see Briggs, Hope, and Robertson 1961). It was proposed, on the basis of observed stoichiometries of ion accumulation and "salt respiration" and from the effects of uncouplers and other inhibitors on ion accumulation, that salt accumulation in the conditions of Epstein's and Laties' "system 2" was directly linked to oxygen-terminated electron transport (for reviews see Lundegardh 1955, 1960; Robertson 1960, 1967, 1968; Briggs, Hope, and Robertson 1961). Estimation of ATP levels in aged carrot xylem parenchyma disks in the presence of various inhibitors has indicated that alkali cation and chloride influxes from 40 mM salt solutions are linked to oxygen-terminated electron transport rather than to hydrolysis of ATP, and that a large proportion of salt respiration may be an indirect consequence of salt accumulation (Atkinson *et al.* 1966; Atkinson and Polya 1968). Little is known about the differential effects of inhibitors acting on transport systems at the plasmalemma and tonoplast. Arisz (1958) found selective effects of respiratory inhibitors on Cl^- uptake and on intracellular transport of Cl^- in leaves, and Lüttge and Laties (1967) found that K^+ and Cl^- transport systems at the plasmalemma of maize-root cells were more sensitive to uncouplers than those at the tonoplast. Cram (1969) has found that anaerobic conditions inhibit Cl^- influx at the plasmalemma of aged carrot slices, whereas this influx is unaffected by concentrations of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and oligomycin that affect respiration. CCCP and anaerobic conditions reduce active influx of Cl^- at the tonoplast, but it is suggested that energy is supplied to this "pump" by respiration without mediation of ATP. It was of interest to extend these studies on involvement of electron transport and ATP in accumulation of ions to the lower concentrations corresponding to system 1, using aged beet tissue, for which electrophysiological information is available. The results presented in this paper indicate that K^+, Na^+, and Cl^- influxes from dilute solutions into aged beet cells by this high-affinity system are not directly dependent on tissue ATP level, but rather are linked energetically to oxygen-terminated electron transport.

II. Methods

(a) *Preparation of Disks*

Disks from parenchyma of fresh beet roots (*Beta vulgaris* L.) were washed and aged for 5 days as described previously (Polya 1968).

(b) *Ion Uptake*

Aged beet disks were lightly blotted, weighed, and aerated with washed air in 0·5 mM KCl or NaCl. An initial ratio of approximately 10 ml of external solution per gram of slices was used in all experiments. Ion influxes were approximately linear functions of time for over 1 hr in these conditions; ion influx rates declined subsequently due to depletion of ions in the external solution. ^{22}Na, ^{36}Cl, and ^{42}K were used as tracers for Na^+, Cl^-, and K^+ respectively in order to determine ion influxes. Net ion flux and tracer uptake were determined by analysis of the external solution as described below at the times indicated in Section III. Na^+ and K^+ influxes were calculated from net ^{22}Na, ^{36}Cl, and ^{42}K uptake using initial specific activities. Specific activities did not change appreciably over the course of these experiments except when 2,4-dinitrophenol (DNP) caused increased efflux of Na^+, K^+, and Cl^-; in these cases influx is corrected for changes of specific activity. ^{22}Na and ^{36}Cl were added at levels of 10 μCi/l; ^{42}K (half-life 12·4 hr) was employed at higher levels (25–50 μCi/l) to permit quick and accurate counting.

^{22}Na, ^{36}Cl, and ^{42}K activities were determined at infinite thinness using a low-background gas-flow counter (model 4342, Nuclear Chicago, Des Plaines, Illinois); ^{42}K activities were corrected for decay. K^+ and Na^+ were determined using a flame photometer (EEL, Halstead, Essex). Cl^- was determined by titration against $Hg(NO_3)_2$ using diphenylcarbazone as an indicator (Schales and Schales 1941).

Net ion uptake and ion influx were determined after an initial 30-min period of free-space equilibration in all experiments. Inhibitors were added after this 30-min equilibration period at the times indicated in Section III. All experiments were carried out at 30°C except where indicated otherwise in Section III.

(c) *ATP Determination*

ATP levels in beet disks were determined by the luciferin–luciferase method as described previously (Atkinson *et al.* 1966; Atkinson and Polya 1968).

(d) *Chemicals*

Disodium adenosine triphosphate, firefly tails, L-ethionine, phlorizin, and oligomycin (85% oligomycin B, 15% oligomycin A) were obtained from Sigma Chemical Co., St. Louis. ^{22}Na and ^{36}Cl were obtained from the Radiochemical Centre, Amersham, and ^{42}K from the Australian Atomic Energy Commission, Lucas Heights, N.S.W. DNP was obtained from British Drug Houses, Poole, Dorset. Dio-9 and CCCP were kindly provided by Professors N. A. Walker and A. B. Hope respectively.

III. Results

(a) *Net Uptake and Influx of Potassium Ions*

Figure 1 shows that uncoupling agents had a rapid effect on net K^+ uptake by aged beet disks. DNP and CCCP, reagents which uncouple oxidative phosphorylation from mitochondrial electron transport (Loomis and Lipmann 1948; Heytler and Prichard 1962), caused severe inhibition of net K^+ uptake by aged beet disks within 30 min of application at levels of 10^{-4}M and 5×10^{-6}M respectively [Fig. 1(*a*)]. Higher levels of uncouplers, 10^{-3}M DNP and 10^{-5}M CCCP, caused an immediate large net loss of K^+ from the disks [Fig. 1(*b*)]. Imposition of anaerobic conditions immediately inhibited net K^+ uptake and caused net K^+ loss after 30 min [Fig. 1(*b*)]. In contrast,

inhibitors that block the terminal step of oxidative phosphorylation without directly impairing electron transport, namely phlorizin (Keller and Lotspeich 1959), oligomycin (Lardy, Johnson, and McMurray 1958), and Dio-9 (Guillory 1964), failed to completely inhibit net K^+ uptake into aged beet disks. Figure 1(*a*) shows that 0·5 mM phlorizin failed to affect net K^+ uptake over 2 hr; oligomycin (2·5 μg/ml) failed to inhibit net K^+ uptake for 30 min and net K^+ uptake still continued at 32% of the control rate after 1·5 hr of treatment. Net K^+ uptake continued at 40% of the control rate over 2 hr in the presence of 25 μg/ml Dio-9.

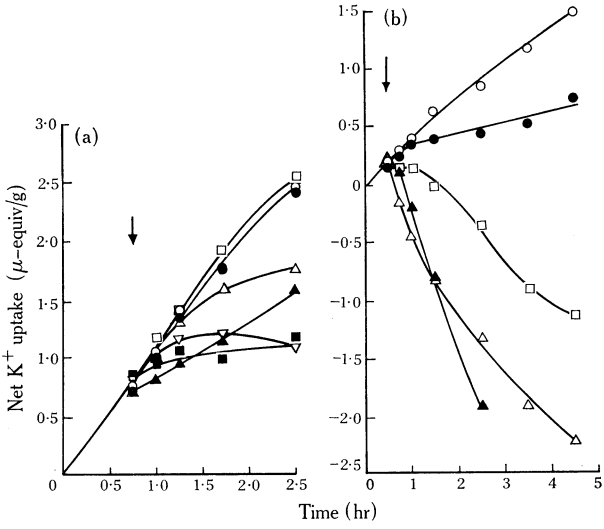

Fig. 1.—Net K^+ uptake by aged beet disks from 0·5 mM KCl at 25°C. Additions were made at the time denoted by the arrows to give the final reagent concentrations indicated. (*a*) ○, no addition; ●, 0·1% ethanol; □, 0·5 mM phlorizin–0·1% ethanol; ■, 5 μM CCCP–0·1% ethanol; △, 2·5 μg/ml oligomycin; ▲, 25 μg/ml Dio-9–0·1% ethanol; ▽, 10^{-4}M DNP. (*b*) ○, no addition; ●, 1 mM L-ethionine; △, 10 μM CCCP–0·2% ethanol; ▲, 1 mM DNP; □, uptake in anaerobic conditions (determined using oxygen-free nitrogen bubbled through external solution from time indicated by arrow).

Previous work has shown that L-ethionine can act as a highly effective ATP trap in aged carrot xylem parenchyma disks (Atkinson and Polya 1968). Figure 1(*b*) shows that 1 mM L-ethionine did not inhibit net K^+ uptake for 30 min, and net K^+ uptake continued at about 30% of the control rate between 0·5 and 4 hr after addition of inhibitor.

The sensitivity of K^+ accumulation by aged beet disks to uncoupling reagents such as DNP and CCCP and to anaerobic conditions, the much less severe effects of energy-transfer inhibitors such as oligomycin, phlorizin, and Dio-9, and the limited effect of ethionine all suggested that K^+ accumulation and retention in aged beet disks might be dependent on systems energized by electron transport rather than on a supply of ATP. To obtain more direct evidence it was necessary to study the effects of these inhibitors on ion influx and on the ATP level in the tissue.

Figure 2(*a*) shows that imposition of anaerobic conditions inhibited K^+ influx by 60% over 30 min, whilst the ATP level did not decrease significantly ($P > 0{\cdot}05$) over the same period [Fig. 2(*b*)]. During 30–60 min of anaerobic treatment K^+ influx was inhibited by 93%, whilst the ATP level reached a value significantly greater ($0{\cdot}05 > P > 0{\cdot}025$ in a two-sample *t*-test) than the control value [Figs. 2(*a*) and 2(*b*)].

In another experiment [Figs. 3(*a*) and 3(*b*)] anaerobic conditions inhibited K^+ influx by 50% within 30 min and this inhibition was associated with a 28% decline in ATP level over this period. After 30 min, K^+ influx was completely inhibited, but from 30 to 60 min the tissue ATP level recovered to a level not significantly lower than the initial ATP level ($P > 0{\cdot}50$).

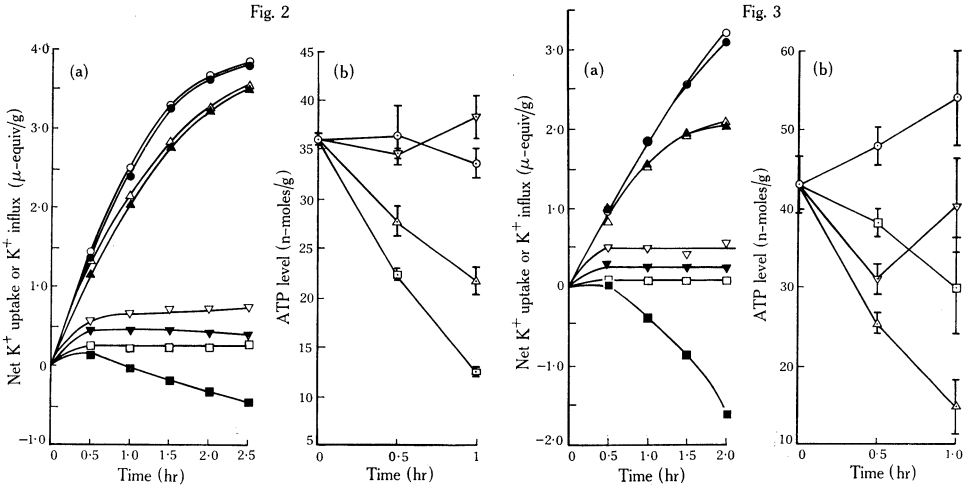

Fig. 2.—Net K^+ uptake (*a*, closed symbols), K^+ influx (*a*, open symbols), and ATP levels (*b*) of aged beet disks aerated at 30°C in 0·5 mM KCl (○, ●, ⊙), 0·5 mM KCl–0·5 mM L-ethionine (△, ▲, ◬), 0·5 mM KCl–$2{\cdot}5\times10^{-4}$M DNP (□, ■, ⊡), and 0·5 mM KCl made anaerobic by passage of oxygen-free nitrogen (▽, ▼, ▽). In (*b*) the vertical bars represent standard deviations from triplicate estimations. Disks from the same batch were used in all experiments.

Fig. 3.—Net K^+ uptake (*a*, closed symbols), K^+ influx (*a*, open symbols), and ATP levels (*b*) of aged beet disks aerated at 30°C in 0·5 mM KCl (○, ●, ⊙), 0·5 mM KCl–1·0 mM L-ethionine (△, ▲, ◬), 0·5 mM KCl–5×10^{-4}M DNP (□, ■, ⊡), and 0·5 mM KCl made anaerobic by continuous passage of oxygen-free nitrogen (▽, ▼, ▽). Vertical bars are as in Figure 2. Beet disks used in (*b*) were not from the same batch as those used in (*a*), but were otherwise identically prepared and treated.

$2{\cdot}5\times10^{-4}$M DNP completely inhibited K^+ influx and caused an increased K^+ efflux after 30 min of administration [Fig. 2(*a*)], the tissue ATP level falling by 40% over this period. After 60 min of treatment, the ATP level had fallen to 37% of the control value. 5×10^{-4}M DNP inhibited K^+ influx by 90% within 30 min; K^+ influx was completely inhibited and K^+ efflux greatly increased after 30 min [Fig. 3(*a*)]. The ATP level in beet tissue treated with 5×10^{-4}M DNP fell by 21 and 45% after 30 and 60 min respectively [Fig. 3(*b*)].

To test the hypothesis that the rapid inhibition of K^+ influx by $2 \cdot 5$–5×10^{-4}M DNP could be attributed to the concomitant drop in tissue ATP level and consequent inhibition of an ATP-dependent transport process, or of alterations in membrane integrity due to inhibition of protein synthesis (see Polya 1968) which requires ATP, the effects of $0 \cdot 5$–$1 \cdot 0$ mM L-ethionine on K^+ influx and tissue ATP level were examined. Figure 2(*a*) shows that K^+ influx continued at 82% of the control rate after 60 min of treatment with $0 \cdot 5$ mM L-ethionine, despite a 35% depression of tissue ATP [Fig. 2(*b*)] to a level not significantly different ($P > 0 \cdot 50$) from the ATP level obtaining in tissue treated with $2 \cdot 5 \times 10^{-4}$M DNP for 30 min.

Similarly Figures 3(*a*) and 3(*b*) show that, despite depression of tissue ATP level by 72% after 60 min of treatment, 1 mM L-ethionine failed to completely inhibit K^+ influx, which continued at 62% of the control rate after 60 min of treatment. In contrast, 5×10^{-4}M DNP completely inhibited K^+ influx after 30 min despite smaller depressions in tissue ATP level than those obtaining with administration of 1 mM L-ethionine.

(*b*) *Sodium and Chloride Ion Influx and Net Uptake*

The results presented in Section III(*a*) indicate a dependence of K^+ influx on some DNP-sensitive system linked to oxygen-terminated electron transport. It was of interest to see if a similar basis for the energetics of Na^+ and Cl^- influxes could be determined.

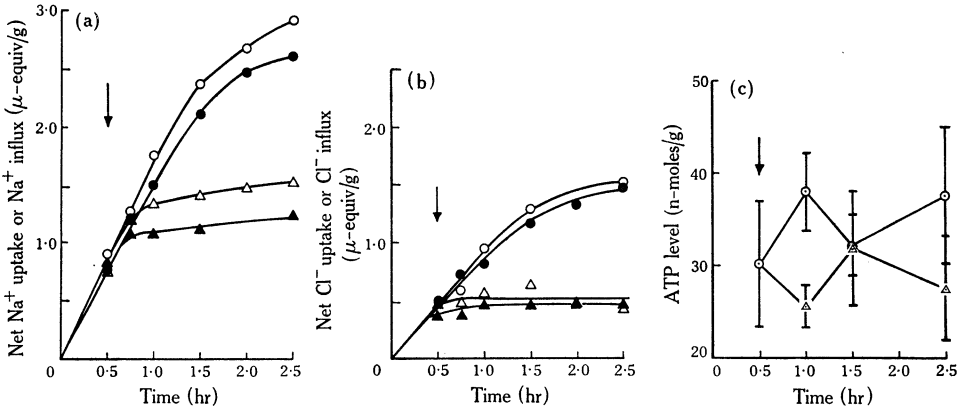

Fig. 4.—Effect of anaerobic conditions on net Na^+ uptake (*a*, closed symbols), Na^+ influx (*a*, open symbols), net Cl^- uptake (*b*, closed symbols), Cl^- influx (*b*, open symbols), and ATP levels (*c*) of aged beet disks incubated in $0 \cdot 5$ mM NaCl at 30°C. Disks were aerated continuously (○, ●, ○) or conditions made anaerobic (△, ▲, △) by continuous passage of oxygen-free nitrogen from the time indicated by the arrows. Disks from the same batch were used in all experiments. Vertical bars in (*c*) represent standard deviations from triplicate estimations.

Figures 4(*a*) and 4(*b*) show that imposition of anaerobic conditions inhibited Na^+ influx into aged beet disks from $0 \cdot 5$ mM NaCl by 90% after 30 min; Cl^- influx was completely inhibited after 30 min. Anaerobic conditions caused a temporary depression after 30 min in the ATP level of aged beet disks incubated in $0 \cdot 5$ mM NaCl,

but after 60 min the ATP level in anaerobic disks was not significantly different ($P > 0{\cdot}50$) from that obtaining in the aerobic control disks. After 2 hr of anaerobic conditions, the ATP level in anaerobic disks was not significantly lower ($P > 0{\cdot}20$) than the initial level in aerobic disks [Fig. 4(*c*)].

Figures 5(*a*) and 5(*b*) show that 5×10^{-4}M DNP completely inhibited both Na^+ and Cl^- influx into aged beet disks within 15 min. Interpolation in Figure 5(*c*) indicates that the tissue ATP level was depressed by 30% over this period. After 2 hr of treatment with 5×10^{-4}M DNP the ATP level fell to about 4% of the control level, and

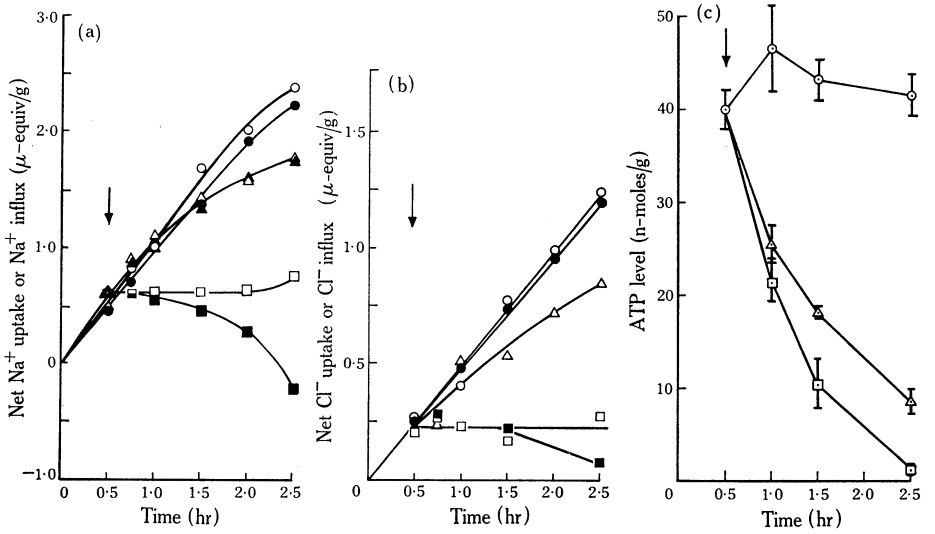

Fig. 5.—Net Na^+ uptake (*a*, closed symbols), Na^+ influx (*a*, open symbols), net Cl^- uptake (*b*, closed symbols), Cl^- influx (*b*, open symbols), and ATP levels (*c*) of aged beet disks aerated at 30°C in 0·5 mM NaCl (○, ●, ⊙), 0·5 mM NaCl–1 mM L-ethionine (△, ▲, ◬), and 0·5 mM NaCl–5×10^{-4}M DNP (□, ■, ⊡). Inhibitors were added to give these final concentrations at the time indicated by the arrows. Vertical bars represent standard deviations from triplicate estimations. The disks used in these experiments were from the same batch.

both Cl^- and Na^+ effluxes increased over this period. In contrast, 1 mM L-ethionine depressed the tissue ATP level by 60% over 60 min [Fig. 5(*c*)] but Na^+ and Cl^- influxes continued at 50 and 60% of the control rates respectively after 60 min of treatment [Figs. 5(*a*) and 5(*b*)]. 1 mM L-ethionine depressed the tissue ATP level to 20% of the control level after 2 hr of treatment, but Na^+ and Cl^- influxes were not inhibited by more than 60% over this period [Figs. 5(*a*), 5(*b*), and 5(*c*)].

(*c*) *Effects of Mitochondrial Phosphorylation Inhibitors on Cation Fluxes and ATP Level in Aged Beet Disks*

The results presented in Sections III(*a*) and III(*b*) provide no evidence for a direct involvement of ATP in the system 1 K^+, Na^+, and Cl^- influxes of aged beet root parenchyma cells. The effects on cation influxes of compounds that inhibit the terminal step of oxidative phosphorylation without directly inhibiting electron transport were therefore studied.

Table 1 shows that Dio-9 at 10 μg/ml, a level much higher than that used to inhibit mitochondrial oxidative phosphorylation *in vitro* (Guillory 1964), failed to inhibit Na^+ influx and net Na^+ uptake by more than 5% after 3·5 hr of treatment.

TABLE 1

EFFECT OF DIO-9 ON SODIUM INFLUX IN AGED BEET DISKS

Aged beet disks were aerated at 30°C in 0·5 mM NaCl–0·1% ethanol (control) or 0·5 mM NaCl–0·1% ethanol–10 μg/ml Dio-9. Na^+ influx and net Na^+ uptake (both expressed as μ-equiv/g of slices) were determined as described in Section II

Time (hr)	Na^+ Influx (control)	Na^+ Influx (with Dio-9)	Net Na^+ Uptake (control)	Net Na^+ Uptake (with Dio-9)
0·25	0·39	0·26	0·36	0·33
0·5	0·76	0·66	0·74	0·67
1·0	1·30	1·16	1·21	1·19
1·5	1·74	1·57	1·67	1·56
2·0	2·00	1·90	2·07	1·87
3·5	2·41	2·22	2·42	2·22

TABLE 2

EFFECT OF DIO-9 ON ATP LEVELS IN AGED BEET DISKS

In experiment 1, aged beet disks were aerated at 30°C in 0·5 mM NaCl–0·1% ethanol (control) or 0·5 mM NaCl–0·1% ethanol–10 μg/ml Dio-9. Standard deviations were determined from triplicate estimations; significant differences between ATP levels were determined by two-sample *t*-tests. The disks used were from the same batch prepared for use in the experiments shown in Table 1; in both experiments the disks were aerated in 0·5 mM NaCl for 60 min before addition of ethanol or Dio-9 in ethanol solution. In experiment 2, aged beet disks were initially aerated for 30 min in 0·5 mM KCl at 30°C; disks were then treated with 0·5 mM KCl–0·1% ethanol (control), 0·5 mM KCl–0·1% ethanol–10 μg/ml Dio-9 (A), or 0·5 mM KCl–0·1% ethanol–35 μg/ml Dio-9 (B). Standard deviations are given from triplicate estimations. ATP levels are expressed as n-moles/g of slices in all cases

Experiment 1				Experiment 2	
Time (hr)	ATP Level (Control)	ATP Level (with Dio-9)	Significance of Difference*	Treatment	ATP Level
0	33·2±1·5			Zero-time sample	48·4±2·3
0·5	29·9±2·1	35·6±4·9	$0{\cdot}4 > P > 0{\cdot}2$	Control for 1 hr	40·4±1·6
1·0	38·3±4·1	29·8±5·6	$0{\cdot}1 > P > 0{\cdot}05$	A for 1 hr	35·6±3·9
2·0	33·1±3·0	42·2±3·0	$0{\cdot}025 > P > 0{\cdot}01$	B for 0·5 hr	30·8±2·2
				B for 1 hr	28·2±4·6

* From two-sample *t*-test.

Table 2 shows that Dio-9 at 10 μg/ml failed to significantly lower the ATP level of aged beet tissue over 1 hr, and after 2 hr of treatment the ATP level in treated disks was significantly greater ($P < 0{\cdot}025$) than that in the controls [cf. Fig. 2(*b*)]. Similarly, Dio-9 at 10 μg/ml had only a slight effect on K^+ influx over 2 hr [Fig. 6(*b*)]. Higher levels of Dio-9 (17·5–70 μg/ml) administered to aged beet disks at 30°C

caused rapid loss of pigment, Na^+, and K^+. Most of the fall in ATP level, measured in the presence of Dio-9 at 35 μg/ml (Table 2), occurred within 0·5 hr; Dio-9 at such high levels may be acting as an uncoupler or as an unspecific detergent to solubilize membrane components.

Figure 6(*a*) shows that oligomycin (1·3–2·6 μg/ml) did not completely inhibit Na^+ influx over 2 hr. Na^+ influxes in the presence of 1·3 and 2·6 μg/ml oligomycin were 40 and 33% respectively of influx in the ethanol control after 1·5 hr of treatment. There was a definite lag of about 30 min before any major inhibition of Na^+ influx became apparent. Similarly, oligomycin (2·6 μg/ml) inhibited K^+ influx by 42% after 30 min; K^+ influx continued at 24% of the control rate after 1·5 hr of treatment [Fig. 6(*b*)].

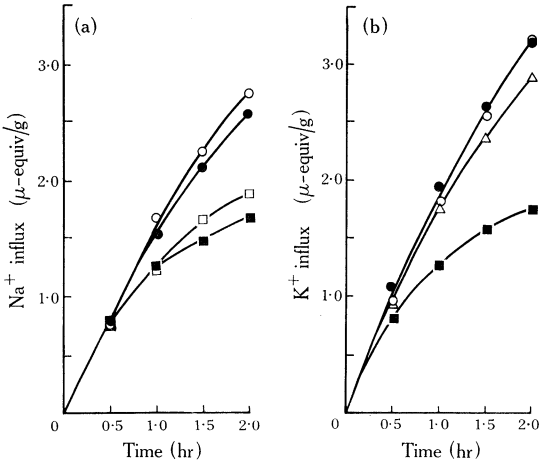

Fig. 6.—Na^+ (*a*) and K^+ influx (*b*) into aged beet disks aerated at 30°C. (*a*) ○, 0·5 mM NaCl; ●, 0·5 mM NaCl–0·1% ethanol; □, 0·5 mM NaCl–0·1% ethanol–1·3 μg/ml oligomycin; ■, 0·5 mM NaCl–0·1% ethanol–2·6 μg/ml oligomycin. (*b*) ○, 0·5 mM KCl; ●, 0·5 mM KCl–0·1% ethanol; △, 0·5 mM KCl–0·1% ethanol–10 μg/ml Dio-9; ■, 0·5 mM KCl–0·1% ethanol–2·6 μg/ml oligomycin.

IV. Discussion

From the results presented here it is evident that aged beet disks have special advantages for studies of the relationship of ion uptake to electron transport and ATP metabolism. In previous investigations to find if salt accumulation by aged storage tissue was "driven" directly by ATP hydrolysis (Atkinson *et al.* 1966; Atkinson and Polya 1968), one of the main pieces of evidence for a more direct involvement of oxygen-terminated electron transport than of ATP hydrolysis was the rapid inhibition of KCl accumulation when aged carrot disks in 40 mM KCl were made anaerobic. Although this inhibition preceded extensive depletion of the tissue ATP there was a fall in ATP that was promoted by external KCl (Atkinson *et al.* 1966) and that was associated with a rise in ADP (Atkinson and Polya 1968). This made interpretation of the earlier results difficult, though the salt effect on ATP levels is consistent with the demonstration that this tissue contains salt-stimulated adenosine triphosphatase of a different kind from that involved in Na^+–K^+ transport systems of animal membranes (Atkinson and Polya 1967). The failure of carrot disks to maintain a high level of phosphorylation of the adenine nucleotide system in anaerobic conditions may be related to the reported absence in carrot tissue of the stimulation of phosphofructokinase by AMP (Dennis and Coultate 1966). This stimulation is considered to be a major factor in activation of the glycolytic system

of animal cells and yeast in response to a transient fall in ATP level and associated rise in AMP (cf. Krebs 1964; Atkinson 1966). In contrast to aged carrot disks, aged beet disks show little if any fall in ATP levels under anaerobic conditions; after 1–2·5 hr under oxygen-free nitrogen the levels did not differ significantly from initial levels or from those in controls in air [Figs. 2(*b*), 3(*b*), and 4(*c*)]. It is thus likely that beet disks have homeostatic mechanisms that compensate for loss of mitochondrial phosphorylation in anaerobic conditions. Since there is no net fall in ATP level, and since it is in the mitochondria that any decrease in ATP : ADP ratios in anaerobic conditions is likely to be most marked, it is probable that ATP levels at the plasmalemma (the proposed site of the high-affinity ion-transport system; cf. Osmond and Laties 1968) are at least as high in anaerobic beet disks as in controls. The striking inhibition of influx of Na^+, K^+, and Cl^- by anaerobic conditions [Figs. 2(*a*), 3(*a*), 4(*a*), and 4(*b*)] from external solutions with concentrations corresponding to the range of the high-affinity system 1 therefore implicates oxygen-dependent electron transport in a more direct way than through mitochondrial generation of ATP.

Supporting evidence for the existence of ion-transport systems linked to electron transport and not immediately dependent on ATP supply from the plant cytoplasm has come from studies of the depletion of ATP in aged carrot disks without an associated inhibition of ion uptake (Atkinson and Polya 1968). In the work described here, ethionine was again a useful reagent for artificial regulation of ATP levels in plant cells. Extensive falls in ATP levels [Figs. 2(*b*), 3(*b*), and 5(*c*)] were associated with only small decreases of influx of Na^+, K^+, and Cl^- in 30 min [Figs. 2(*a*), 3(*a*), 5(*a*), and 5(*b*)]; even after 1 hr, influx was more than half that in controls. With the demonstration (Polya 1968) that inhibition of protein synthesis in beet disks causes a marked inhibition of ion uptake, it is possible that the delayed effects of ethionine may reflect the requirement of protein-synthesizing systems for a supply of ATP.

Dio-9 only inhibited ion uptake extensively and caused a significant fall in ATP at levels high enough to cause cell destruction and leakage of pigment [Tables 1 and 2 and Fig. 6(*b*); cf. Section III]. The delayed effect of oligomycin on ion uptake into beet disks (Fig. 6) is at present unexplained. In previous work with 40 mM KCl, uptake by beet disks was inhibited more rapidly than uptake into carrot disks by oligomycin (Atkinson *et al.* 1966; Atkinson and Polya 1968). Known inhibitory effects of oligomycin on membrane adenosine triphosphatases (Van Groningen and Slater 1963) as well as on mitochondrial oxidative phosphorylation (Lardy, Johnson, and McMurray 1958) make it difficult to define the site of action of this reagent, and the effects reported here cannot be taken as evidence for an obligatory ATP involvement in uptake of Na^+, K^+, and Cl^- by beet disks.

The inhibition of ion influx by DNP was as rapid as that resulting from anaerobic conditions [Figs. 2(*a*), 3(*a*), 5(*a*), and 5(*b*)] and, although this uncoupler eventually causes almost complete loss of ATP [Fig. 5(*c*)], the inhibition of ion uptake precedes extensive ATP depletion [Figs. 2(*b*), 3(*b*), and 5(*c*)]. In one case [Fig. 3(*b*)] more extensive depletion of ATP by ethionine was associated with a much smaller inhibition of ion uptake. Although the site of inhibition by DNP cannot yet be defined, it may be identical with the system inhibited by anaerobic conditions. The extensive depletion of tissue ATP in the presence of DNP may be due in part to activation of mitochondrial adenosine triphosphatase by this compound (Selwyn 1967).

From studies of fluxes and potentials in aged beet disks, Poole (1966) suggested that salt accumulation required coupled active transport of both K^+ and Cl^-. Similar linked active transport of alkali cations and chloride has been shown in the algae *Nitella translucens* (Smith 1967) and *Hydrodictyon africanum* (Raven 1968). The algal transport system is dependent on light and may provide a useful analogy with the beet-disk system. In both cases there is active transport across the plasmalemma, and in both cases the simplest models implicate the electron transport systems of chloroplasts or mitochondria respectively as the source of energy for the active transport.

The problems associated with models involving mitochondrial pumps to explain ion transport across the plasmalemma (Osmond and Laties 1968) have been discussed previously (Atkinson and Polya 1968), but the effects of inhibitors described in this paper are consistent with a direct involvement of mitochondria, which also link ion uptake directly to electron transport (Lehninger, Carafoli, and Rossi 1967). A major problem arises from the absence of demonstrations of energy-linked alkali cation or chloride influx into isolated plant mitochondria, and the possible requirement of permeability modifiers for ion transport by plant mitochondria. A further possibility remains that membrane systems other than the mitochondrial system may be involved in the coupling of active ion fluxes across the plasmalemma to oxygen-terminated electron transport (cf. Conover 1967).

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