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Effects of Osmotic Shock on Some Membrane-regulated Events of Oat Coleoptile Cells[1]

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ABSTRACT

Oat coleoptile sections (*Avena sativa* L. cv. "Garry") were osmotically shocked with 0.5 M mannitol followed by 1 mM Na-phosphate (pH 6.4) at 4 C. This treatment reduced uptake of α-aminoisobutyric acid, 3-*o*-methyl glucose, and leucine by 75 to 90% but inhibited $^{36}Cl^-$ uptake only 30%. Some recovery was observed 1 to 3 hours later. Respiration rates were unaffected by osmotic shock and protein synthesis was reduced 11%.

Osmotic shock also stimulated efflux of α-aminoisobutyric acid and K^+ and led to an increase in conductivity of the solution bathing shocked sections. The transmembrane electropotential of 75% of the shocked cells fell to −20 mv to −45 mv compared with the majority of unshocked cells at −80 mv to −120 mv.

We concluded that osmotic shock selectively modifies the plasma membrane. The inhibitions of uptake could be due to removal of specific components of the plasma membrane and/or to the lowered electropotential.

Efforts to understand the mechanism of membrane-controlled events in plants such as nutrient uptake and auxin action have proved difficult. This is because the use of whole tissue makes it hard to evaluate various secondary factors which may be complicating the study, and tissue homogenization leads to the destruction or reorganization of the membrane fraction of interest. Osmotic shock, however, a treatment of 0.5 M sucrose followed by water or 0.5 mM $MgCl_2$ at 4 C, is a nondestructive method which appears to modify events selectively at the plasma membrane of various microorganisms (11, 15). Uptake of some substances is markedly inhibited and protein is released, thus permitting an investigation of those proteins which seem to originate primarily from the periplasmic space (11). The proteins are probably important constituents of the uptake mechanism (17) and have also been linked to chemotaxis (10). In agreement with these results, Amar and Reinhold (2) found that α-aminoisobutyric acid uptake was inhibited and protein was released when bean leaf strips were osmotically shocked.

In this paper, we will characterize the effects of osmotic shock on *Avena* coleoptile cells. We will attempt to show that shock treatment results in a marked inhibition of amino acid uptake but not Cl^- uptake, and that this effect is related to a modification of the plasma membrane.

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MATERIALS AND METHODS

Seeds of *Avena sativa* L. cv. "Garry" were sown on vermiculite, watered with tap water, and the coleoptiles harvested after 90 to 96 hr in the dark at 23 C. About 50 to 75% of the cuticle and epidermis was then removed (estimated by scanning electron microscopy), two 5-mm sections were cut 3 mm from the tip, and the sections were washed for about 45 min in 1 mM Na-phosphate (pH 6.4). Ten sections were then apportioned to 20-ml beakers containing 2 ml of phosphate buffer. All treatments were replicated and experiments repeated at least twice.

Sections were osmotically shocked by replacing the phosphate buffer with 0.5 M mannitol for 10 min. This was then aspirated from the flaccid sections and ice-cold phosphate buffer added for 10 min more. Controls were aspirated at the same intervals but phosphate buffer at room temperature was added each time.

To measure uptake, sections were floated on phosphate buffer containing 1 mM $Ca(NO_3)_2$ and the desired radioactive compound (^{3}H-AIB,[2] 2.5 Ci/mmol, final concentration 0.4 μM, and 40 μM carrier AIB added; $^{36}Cl^-$, 7 mCi/g Cl, final concentration 0.5 mM; ^{3}H-*o*-methyl glucose, 1.5 Ci/mmol; final concentration 0.15 μM) for 15 min. The sections were then washed for 10 or 12 min in ice-cold unlabeled AIB and finally transferred to 4 ml Multisol (Interex Corp.) for extraction and counting.

Efflux of AIB was determined by floating 40 sections on phosphate buffer with 5 μM AIB (1 μM ^{3}H-AIB plus 4 μM carrier AIB) for 30 min; the solution was aspirated and fresh buffer containing 5 μM unlabeled AIB added every 20 min. Aliquots of the efflux medium were counted at each time interval and the sections were counted after 2 hr.

Respiration rates were determined with an O_2 electrode. Ten 5-mm sections were placed in the chamber containing a stirred solution of 1 mM Na-phosphate with 1 mM $Ca(NO_3)_2$ (pH 6.4) at 25 C.

Estimates of protein synthesis were made by floating sections on ^{14}C-leucine (0.24 Ci/mmol, final concentration 1 μM) for 1 hr. The sections were then washed in 10 mM leucine and groups of 10 ground in 1 ml of 0.1 N NaOH with a motor-driven glass homogenizer for 90 sec; the apparatus was washed with 1 ml 0.1 N NaOH and 100-μl aliquots of the homogenate placed on each of two filter paper discs. After drying, one of the discs was counted immediately as a measure of leucine uptake. The other was washed for 1 hr in 10% (w/w) trichloroacetic acid; 15 min in 5% trichloroacetic acid at 90 C, 15 min in 95% ethanol-anhydrous ether (1:1), 15 min in 95% ethanol:ether (1:3), and finally in ether for 15 min. This disc was then dried and counted as a measure of leucine incorporation.

A conductivity meter (Radiometer Copenhagen, type CDM

[2] Abbreviations: AIB: α-aminoisobutyric acid; O-MG: 3-*O*-methylglucose.

2e with a CDC 114 electrode) was used to examine ion leakage. Forty coleoptile sections were used for each treatment and following osmotic shock, they were floated on 2 ml of distilled H_2O. After 1 hr, the conductivity of an aliquot of this solution was measured. The K^+ concentration of the efflux medium was determined with a Perkin-Elmer model 306 atomic absorption spectrometer at 384 nm.

Cell electropotentials were measured by inserting a glass microcapillary electrode filled with 3 M KCl (tip diameter less than 1 μm; resistance less than 10 megohm) into individual cells near the cut surface of the section. The potential between this electrode and an Ag/AgCl reference electrode in the bathing solution was determined with an electrometer (WP Instruments, model 725) and strip chart recorder (Esterline Angus, Minis-ervo). During potential measurements, the tissue was perfused in a chamber with 1X mineral solution as described by Etherton (9).

RESULTS

The effects of various types of shock on uptake of AIB into coleoptile cells are presented in Table I. A cold treatment, given either alone or before plasmolysis, is without effect. Mannitol treatments for 10 min inhibit uptake by 50% over control, but when rehydration occurs at 1 C, the inhibition is more pronounced. Osmotic shock as used in this paper, therefore, will refer to a 10-min treatment of 0.5 M mannitol followed by 10 min of cold Na-phosphate (pH 6.4).

The uptake activity remaining after osmotic shock is inhibited further by NaN_3 and like activity of unshocked sections, is stimulated by incubation in pH 4 (Table II). Uptake of the nonmetabolized sugar, 3-*o*-methyl glucose (o-MG), was inhibited to the same extent as AIB (data not shown), but the uptake of $^{36}Cl^-$ (Table II) was inhibited much less by shock. The remaining activity is stimulated by low pH and inhibited by NaN_3.

A partial recovery of AIB uptake from an inhibition of 70% of control to 27% of control can be seen 90 min after shock (Table III). Superimposed on this recovery was a 3-fold increase in uptake capacity by controls. The uptake stimulation is not due to removal of the epidermis, since uptake into sections with the epidermis intact increased by the same amount (data not shown). The control increase as well as recovery by shocked sections was inhibited by NaN_3.

In order to characterize the recovery of uptake capability after shock without the increase also seen in controls, sections were first incubated for 6 hr in Na-phosphate buffer. Preliminary experiments showed no further increases in uptake capacity by unshocked sections after that time. The preincubated sections were inhibited by shock to the same degree, or even more than, fresh sections (91%) (Table IV). A recovery to about 50% of control occurred after 180 min. Recovery approaching less than 30% of controls was never seen.

For a similar experiment using Cl^-, the small inhibition of uptake by osmotic shock (34%) was reduced to 18% when measured 60 min after shock; here, too, complete recovery was never seen. There was also a much smaller increase in Cl^- uptake capability by unshocked sections over time compared with AIB uptake (Table III).

To see if the effect of osmotic shock on uptake was an indirect one through some aspect of metabolism, respiration was measured. Control rates of O_2 uptake (6.1 $\pm$ 1.7 μmol/g fresh wt·hr) were inhibited over 50% by a 15-min pretreatment with 5 mM azide to 2.9 $\pm$ 1.4 μmol/g fresh wt·hr but osmotic shock had no detectable effect on respiration rates. Measurements made 2 to 6 hr after shock were also not different from control (data not shown).

Another important component of cellular metabolism, protein synthesis, was also examined. The data in Table V show that, like the uptake of AIB and o-MG, leucine uptake is inhibited by 75%, but the incorporation to uptake ratio indicates that osmotic shock inhibits leucine incorporation into protein by only 11%.

In order to determine the effect of osmotic shock on efflux of AIB, sections were floated on ^{3}H-AIB for 30 min. The radioactive tissue was then shocked as before and aliquots of shock media and subsequent wash solutions were counted. The data show (Fig. 1) that during the plasmolysis step (0–10 min), similar amounts of AIB were released; the rehydration step, however (10–20 min), produced a significant increase in AIB

Table I. Effects of 0.5M Mannitol and/or Cold Treatment on Subsequent Uptake of ^{3}H-AIB

Treatment		Uptake	Inhibition over Control
0 to 10 min	10 to 20 min		
		cpm/section/15 min	
Buffer (25C)	Buffer (25C)	392	%
Mannitol (25C)	Buffer (1C)	99	...
Buffer (25C)	Buffer (1C)	400	0
Buffer (1C)	Mannitol (25C)	206	48
Buffer (25C)	Mannitol (25C)	228	42

Table II. Effect of Acid and Azide on Uptake of ^{3}H-AIB and $^{36}Cl^-$ after Osmotic Shock

Following a 45 min preincubation period, the sections were transferred to ^{3}H-AIB or $^{36}Cl^-$ at pH 4.0 or pH 6.5 with or without NaN_3.

Compound	Treatment	pH 6.5		pH 4.0
		-NaN_3	+1mMNaN_3	
		cpm/section/15 min		
^{3}H-AIB	No Shock	230	29	800
^{3}H-AIB	Shock	30	23	48
$^{36}Cl^-$	No Shock	472	129	2040
$^{36}Cl^-$	Shock	296	74	1107

Table III. Recovery of ^{3}H-AIB Uptake after Osmotically Shocking Coleoptile Sections

Sections were floated on 1mM Na-phosphate buffer pH 6.4 with 1mM $Ca(NO_3)_2$ during the recovery period. NaN_3 at 1mM was added during the uptake period only.

Treatment	Time after Shock			
	10 min		90 min	
	-NaN_3	+NaN_3	-NaN_3	+NaN_3
	cpm/section/15 min			
No Shock	185	43	507	63
Shock	56	40	371	50

Table IV. Recovery of AIB Uptake after Osmotically Shocking Sections Preincubated for 6 hr

Sections were preincubated for 6 hr on 1mM Na-phosphate buffer (pH 6.4) then transferred to fresh buffer containing 1mM $Ca(NO_3)_2$ for the various time periods after shock.

Treatment	Time after Shock		
	10 min	90 min	180 min
	cpm/section/15 min		
No Shock	1563	1156	2105
Shock	146	274	551

Table V. Effect of Osmotic Shock on Uptake of ^{14}C-leucine and its Incorporation into Protein

Following osmotic shock and a 30 min uptake period of ^{14}C-leucine in 1mM Na-phosphate buffer (pH 6.4) and 1mM $Ca(NO_3)_2$, the sections were homogenized. One aliquot was taken for uptake determination and another was purified (see Methods) for determination of incorporation.

Treatment	Uptake	Incorporation	Incorporation/ Uptake
	cpm/section/hr		ratio
No Shock	250	219	0.85
Shock	60	46	0.76

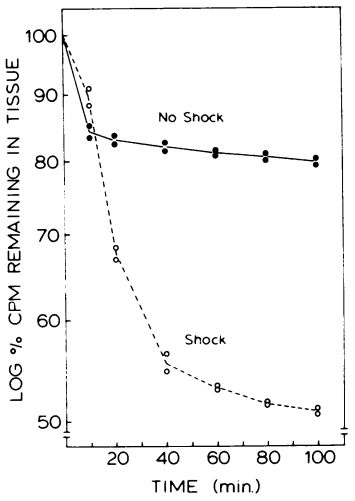

FIG. 1. Effect of osmotic shock on AIB efflux from coleoptile sections. After a 30-min loading period on ^{3}H-AIB, the sections to be shocked were transferred to 0.5 M mannitol for 10 min. An aliquot of this solution was then counted (10-min points) and buffer at 4 C added. Ten min later, an aliquot was removed for counting (20-min points) and fresh buffer at 25 C added every 20 min for 80 min more. Control solutions were changed at the same periods, but buffer at 25 C was added every time. Each point represents the radioactivity from 40 sections.

release compared with control and an accelerated rate of efflux continued for at least 80 min more during subsequent changes of phosphate buffer.

Ion leakage is also accelerated after osmotic shock (Table VI). A small increase in conductivity is detected during rehydration in cold water (shock fluid) and the solution from shocked sections had twice the conductivity as that from unshocked sections during the next 60 min. The K^+ concentration of the efflux solution reflected the conductivity data. Distilled H_2O was used after plasmolysis for these experiments, but the substitution of 1 mM Na-phosphate (pH 6.4) did not change the conductivity results.

The net effect of osmotic shock on ion flux was estimated by measuring membrane potentials. Coleoptile sections were preincubated for 3 hr on 1 mM Na-phosphate buffer, then shocked and perfused with buffer during implantation of the microelectrode (Table VII). Potential values for shocked cells showed considerable variation, so the potentials were divided into three arbitrary categories: from −20 mv to −45 mv (the range of potentials which result 10 min after addition of 1 mM NaN_3, as shown by Anderson *et al.* [4] and our unpublished results); from −80 to −120 mv (values which are representative of the majority of unshocked cells); from −50 to −75 mv (representing an intermediate degree of depolarization).

In all four experiments (Table VII), approximately 70% of the shocked cells were depolarized to −20 to −45 mv; the remainder of the cells were largely in the intermediate range, and 6% appeared normal. There was no detectable repolarization during the 3 hr after shock, no changes in potential were noted as the microelectrode moved from the cut surface to about eight cells deep, and there were no differences between cells near the outer or inner epidermis. Often it was necessary to place the microelectrode deeper into the tissue of shocked sections in order to record potentials above −20 mv. In each experiment, the same electrodes were used on both shocked and unshocked sections. This procedure insured that the low readings obtained with osmotically shocked tissue were not due only to improperly placed or coarse electrode tips.

Table VI. Electrolyte Leakage Following Osmotic Shock

After the sections were plasmolyzed for 10 min in 0.5M mannitol, they were transferred to ice cold distilled H_2O for 10 min. An aliquot of this solution (shock fluid) was used for conductivity measurements. Fresh distilled H_2O at 25 C was then added for 1 hr more. Aliquots of this solution were taken for measurement of conductivity and of K^+ concentration by atomic absorption spectrometry.

Treatment	Conductivity		K^+
	Shock Fluid	1 hr Post-Shock	1 hr Post-Shock
	μmhos		μeq/l
No Shock	20.3	22.0	90
Shock	24.3	49.4	275

Table VII. Changes in Cell Transmembrane Electropotentials after Osmotic Shock

Potentials were recorded 30 min to 3 hr after shock. For each treatment, two to four different sections were probed on a given day and the results of 4 similar experiments from 4 different days are pooled.

Treatment	Membrane Potential		
	−20mV to −45mV	−50mV to −75mV	−80mV to −120mV
	cell number		
No Shock	1	11	31
Shock	70	24	6

DISCUSSION

The results seem to support the proposition that osmotic shock affects events regulated by the plasma membrane. This conclusion is based on the evidence that shock inhibits the uptake of AIB (2, Table I), o-MG, and leucine (Table V) but has little to no effect on respiration or protein synthesis (Table V). The respiration data also imply that energy production (though not measured directly) is not seriously affected by shock. If energy levels were reduced, Cl^- uptake and protein synthesis would be inhibited as markedly as amino acid uptake.

It should be noted that when protein synthesis was measured, amino acid pool size was not estimated. The possibility exists that osmotic shock leads to large decreases in endogenous pools which would then result in less dilution of isotope and the same apparent incorporation of ^{14}C-leucine as in the controls. Britten and McClure (8) have shown that some amino acid pools are eliminated after shocking *Escherichia coli*, but their shock treatment (a transfer from isotonic to hypotonic medium) was different than that used in our study, and the precursor pools for protein synthesis were not affected in the bacterium.

We can only speculate about how the plasma membrane is modified by shock treatment. Perhaps osmotic shock leads primarily to the removal or inactivation of certain sites responsible for uptake. Evidence for this is seen in Table II after the uptake occurring in 1 mM azide is subtracted (it is assumed that counts associated with tissues treated with this inhibitor resulted only from diffusion and/or trapped isotope which would also occur in untreated tissues); the per cent stimulation of AIB uptake by acid is similar in both control and shock treatments. This effect of low pH suggests that the activity remaining after shock is normal and is not due to a partial inhibition of all uptake sites. The mechanism of the stimulation of amino acid transport by low pH will be dealt with in another report (Etherton and Rubinstein, in preparation).

Evidence that osmotic shock leads to the removal of periplasmic protein was reported by Anraku (6), Wilson and Holden (22), Wiley (20), Kalckar (13), Berger and Heppel (7), Aksamit and Koshland (1), and Willis and Furlong (21); in these cases, a protein ligand was isolated from the shock fluid which would bind with the substance whose uptake was inhibited. A partial or complete restoration of uptake activity by adding back components of the shock fluid has been reported in bacterial systems by Anraku (5), Wilson and Holden (22), Hazelbauer and Adler

(10), and Kalckar (13). Amar and Reinhold (2) were also successful in restoring AIB uptake with addition of shock fluid to shocked leaf strips; they detected no AIB binding, however. Using *Avena*, we have been able to measure only very small amounts of protein released from shocked sections, and in preliminary experiments there was no promotion of AIB uptake using shock fluid (1 ml from 20 sections concentrated 10 × by ultrafiltration). One possibility for this negative result is that protein released from the membrane is being trapped by the cell wall of the 5-mm coleoptile sections; the bean leaves used by Amar and Reinhold (2) were cut to 1 mm in width, resulting in a shorter diffusion path than intact coleoptile sections. It is also possible that protein from bean leaves originates from within cells close to the cut surface and the stimulation of uptake by shock fluid protein may be due to a reduced pH occurring in the unbuffered solutions which were used.

Another explanation for the apparent inhibition of AIB uptake is that osmotic shock leads to massive changes in the membrane such that the uptake of AIB is countered by its rapid efflux (Fig. 1). If membrane permeability were markedly changed, one might expect an increase in uptake as the AIB diffused rapidly into the cell. Such large changes in permeability should also result in large alterations of Cl^- uptake.

Evidence is being accumulated which suggests that the cell membrane potential and proton gradient can control AIB uptake (Etherton and Rubinstein, in preparation). The possibility exists that ion leakage and depolarization of the cell membrane potential which result from osmotic shock (Tables VI and VII) are causally related to the inhibition of AIB uptake. We must interpret these results with caution, however. Even 3 hr after osmotic shock, deformation of the tissue is occasionally seen when the microelectrode is inserted, and this might lead to rupture of the cell membrane (artifacts of this nature have been discussed by Anderson and Higinbotham [3]). If the lowered potentials after shock reflect the true state of the coleoptile cells, the inhibition of AIB uptake may not be due directly to the loss or inactivation of a specific carrier complex, but only to the lowered potential.

Two other interesting findings appeared during these investigations. First, AIB uptake but not Cl^- uptake into unshocked coleoptiles increased markedly over the 3 to 6 hr after cutting. This increase was prevented by azide so it was probably not due to a nonspecific, passive increase in membrane permeability. Similar changes in uptake capacity during washing have been reported for pea epicotyls (18) and corn roots (14) as well as some other tissues. Paralleling the larger uptake capacity in these tissues is a hyperpolarization of the membrane potential (15, 19).

The other finding of interest was an apparent lack of a relationship between cell electropotential and Cl^- uptake after shock treatment. Of the cells which could be measured in shocked sections, 70% were depolarized, but Cl^- uptake was inhibited just 30%. As discussed above, the measurements of electropotential for shocked cells must be scrutinized, but if the values were real, it would suggest that Cl^- uptake was not controlled solely by cell membrane potential.

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