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Osmotic Stress-Induced Polyamine Accumulation in Cereal Leaves[1]

I. PHYSIOLOGICAL PARAMETERS OF THE RESPONSE

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ABSTRACT

Putrescine and spermidine accumulate in cereal cells and protoplasts exposed to various osmotica (sorbitol, mannitol, proline, betaine, or sucrose). The response is fast (1–2 hour lag), massive (50- to 60-fold increase in putrescine), and is not due to release of putrescine from a bound form or to conversion from spermidine. It rather involves the activation of the biosynthetic pathway mediated by arginine decarboxylase (ADC; EC 4.1.1.19) (Flores and Galston 1982 Science 217: 1259). Polyamine accumulation and the rise in ADC activity in osmotically stressed tissue are prevented by ADC inhibitors (α-difluoromethylarginine, D-arginine, and L-canavanine) but are not affected by α-difluoromethylornithine and methylornithine, inhibitors of the alternative putrescine biosynthetic enzyme ornithine decarboxylase (EC 4.1.1.17). Putrescine accumulation by oat and corn leaves is maximal in solutions only slightly hyperosmotic (0.4 molar). The stress response, which declines with leaf age, is completely prevented by cycloheximide (10 to 50 micrograms per milliliter) when added during the first hour of exposure to osmoticum, and partially by transcription inhibitors (cordycepin, Actinomycin D, 5 to 20 micrograms per milliliter). Oat seedlings allowed to wilt by withholding water also show a rise in polyamine titer and ADC activity. This response is not readily reversible upon rewatering.

The diamine putrescine and the polyamines spermidine and spermine are now well established as ubiquitous components of higher plant cells. Evidence gathered in recent years in plant systems supports their role as regulators of cell proliferation and differentiation previously proposed for animal and bacterial cells (2). Thus, polyamines have been shown to promote macromolecular synthesis and stimulate nuclear division in cereal protoplasts (13), to be involved in the early proliferative stage of tomato fruit development (6), and in embryo formation from carrot cell suspensions (8).

Recent observations, however, suggest that unique additional functions may have evolved for polyamines in plant cells (9, 13). For example, polyamines are effective retardants of senescence in mono- and dicotyledonous plants (13). In etiolated pea seedlings, ADC[3] activity is regulated by photochrome (13), and in *Bryophyllum* the induction of Crassulacean acid metabolism results in a rhythmic oscillation in polyamine content in the leaves, paralleling that of organic acids (22). A more neglected aspect of polyamine physiology concerns the response to various stress conditions. In 1952, Richards and Coleman (26) found that putrescine was the main nitrogenous compound accumulating in potassium deficient barley plants. This was subsequently shown to be a universal response (27) correlated with an increase in ADC activity (28). Putrescine buildup was later shown in plants deficient in other mineral nutrients (3), fed with NH_4Cl (20) or subjected to acid (29) or salt (31) stress.

As part of our attempts at *in vitro* culture of cereal protoplasts, we observed a rapid and massive increase in the putrescine titer of mesophyll cells exposed to the osmotica used routinely for protoplast isolation. The osmoticum-induced putrescine rise was specifically prevented by α-difluoromethylarginine, an enzyme-activated irreversible inhibitor of ADC (11). Inhibitors of ODC, the alternative putrescine biosynthetic enzyme in higher plants and bacteria, did not prevent the stress response. In the following reports, we characterize the polyamine accumulation induced by osmotic treatment in detached peeled cereal leaves, and show that conventional water stress imposed by withholding water leads to similar results. The accompanying paper (12) relates the changes in polyamines to other alterations in soluble nitrogen pools known to occur upon water stress, and to some of the factors presumed to mediate such changes (ABA, ethylene).

MATERIALS AND METHODS

Plant Materials. The following plant species were used: (*Avena sativa*) cv Victory (The General Swedish Seed Co., Svalov, Sweden); wild oat (*Avena fatua*); barley (*Hordeum vulgare*) cvs Himalaya, Excelsior, Prior, and Proctor (Small grain Collection, U.S.D.A., Beltsville, MD); corn (*Zea mays*) cv Golden Cross Bantam; wheat (*Triticum aestivum*) cv Yamhill; and pea (*Pisum sativum*) cv Alaska (Asgrow Seed Co., Mechanicsburg, PA). The cereal seeds were germinated and grown in controlled environment growth rooms, in plastic pots containing washed coarse grade vermiculite, and subirrigated twice daily with a 1.2 g/l solution of Hyponex (7-6-19 by N:P:K:analysis; Hydroponics Chemical Co., Copley, OH). The mineral solution was supplemented every 2 weeks with 1.2 g/l Hyponex. Plants were grown under a 16-h photoperiod, 25°C (day and night), with 9:1 energy

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[3] Abbreviations: ADC, arginine decarboxylase; ODC, ornithine decarboxylase; CH, cycloheximide; DFMO, α-difluoromethylornithine; DFMA, α-difluoromethylarginine; PCA, perchloric acid; PEG, polyethylene glycol.

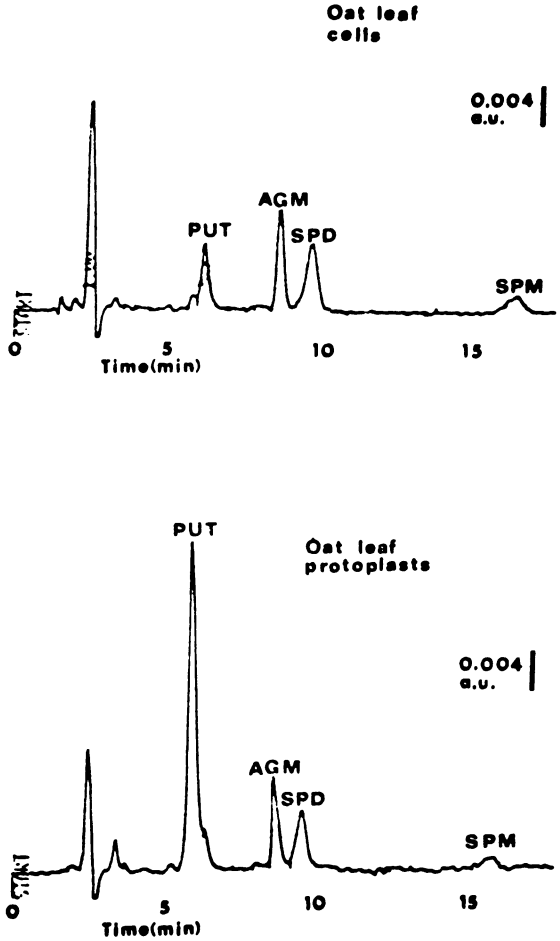

FIG. 1. Polyamine patterns in oat leaf cells and protoplasts. HPLC profiles of benzoylated extracts from oat cv. Victory mesophyll cells (A) or protoplasts (B), representing the same per cell equivalent.

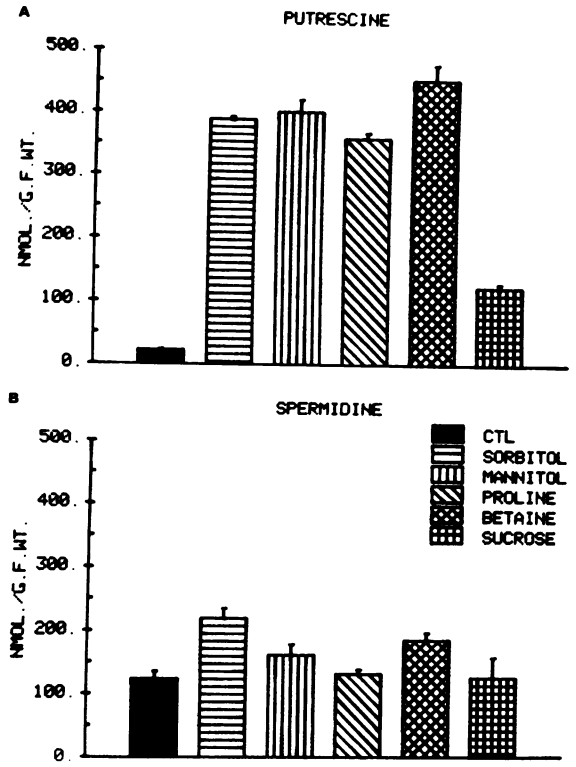

FIG. 2. Effect of different osmotica on polyamine accumulation. Oat leaf segments were incubated in 0.4 M osmoticum and extracted ("Materials and Methods") after 4 h under fluorescent light. Bars represent ± SEM.

mixture of fluorescent and incandescent light at an energy level of 17,600 ergs·cm^{-2}·s^{-1} at the plant level. Peas were grown in darkness at 26°C, 70% RH in fine grade vermiculite, and watered every 3 d after imbibition.

Isolation and Treatment of Excised Tissue. Unless otherwise indicated, the first leaf from 7- to 9-d-old seedlings were used. The lower epidermis was peeled, and 50 mm segments were floated, usually 12 per treatment (8 for corn leaf segments) over 10 ml of 1 mM phosphate buffer (pH 5.8) contained in a 100 × 15 mm Petri dish, in the absence or presence of osmotica and/or inhibitors. For osmotic shock experiments, 1M stock solutions of sorbitol, mannitol, proline, betaine, and sucrose (Sigma) in phosphate buffer were made fresh or kept at 4°C for up to 1 week, and diluted as required. PEG 6000 (average mol wt, Sigma) and PEG 3350 (Baker) solutions were always made fresh. Excised leaf segments were incubated for up to 10 h under fluorescent light (20 μE/m^2·s) at 24 to 26°C. At the end of the treatment period, triplicate samples consisting of four leaf segments each were rinsed twice with buffer and taken to polyamine analysis or enzyme assay. Samples were analyzed immediately, or stored in liquid N_2. These procedures yielded identical results. Protoplasts were isolated as described before (9).

Addition of Inhibitors. The irreversible polyamine biosynthesis inhibitors DFMO (MDL 71,782) and DFMA (MDL 71,797) were gifts from Dr. Peter P. McCann (Merrell-Dow Research Center, Cincinnati, OH). The competitive inhibitors α-methylornithine (Calbiochem), L-canavanine (Sigma), and D-arginine (Sigma) were obtained as hydrochlorides. Cordycepin (3'-deoxyadenosine) (Sigma, >98% purity) and cycloheximide (Sigma) were obtained crystalline. All inhibitors were stored in the desiccated form at −20°C, and solutions were made immediately before use.

Polyamine Analysis. Leaf samples were extracted in ice cold 5% PCA at 100 to 200 mg/ml for 30 min. The supernatant fraction after centrifugation at 12,000*g* for 30 min was used for TLC and HPLC analysis. Dansylation was performed as described previously (10). Dansylpolyamines were extracted into benzene, and the organic phase collected and stored in glass vials at −20°C. Dansylated extracts were stable for at least 1 month under these conditions. TLC was performed on LK6D high resolution silica gel plates (Whatman, Clifton, NJ). After development in chloroform:triethylamine (5:1 v/v) for about 1 h, dansylpolyamine bands were scraped, eluted in 2 ml ethylacetate, and quantified with an Aminco-Bowman spectrophotofluorimeter, with excitation at 350 nm and emission at 495 nm. Results obtained with the dansyl method were confirmed by HPLC analysis of benzoyl derivatives as described previously (10). Benzoylpolyamines were collected after extraction in diethyl ether, evaporated to dryness, and redissolved in 200 μl of methanol. Samples were run in an Altex Beckman model 322 liquid chromatograph through a 5-μM reversed phase ODS column. The solvent system consisted of ethanol:water run isocratically at 60 to 65% methanol at a flow rate of 1 ml/min. Unknowns were identified by using authentic benzoyl polyamines as external standards, and results were quantified with a 3390A Hewlett-Packard integrator.

Enzyme Assays. ADC and ODC were assayed in crude oat leaf extracts (9, 11). Leaves were extracted either fresh or after storage in liquid N_2, in 100 mM phosphate buffer (pH 7.6) for ADC, and 100 mM Tris-HCl buffer (pH 8.0) for ODC, at a ratio of 100 mg/ml buffer. The clear supernatant fraction after centrifugation at 27,000*g* for 20 min was used as the crude enzyme.

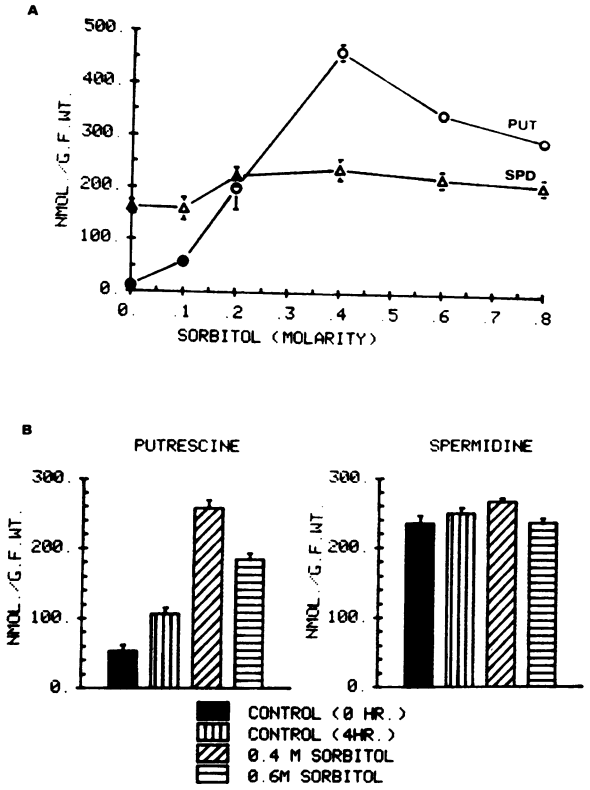

FIG. 3. Effect of osmoticum concentration on polyamine accumulation. A, Response of oat leaf segments. Polyamine titer determined after 4 h incubation in osmoticum or control buffer, under fluorescent light. B, Response of corn leaf segments. Experimental conditions as above. Bars represent ± SEM.

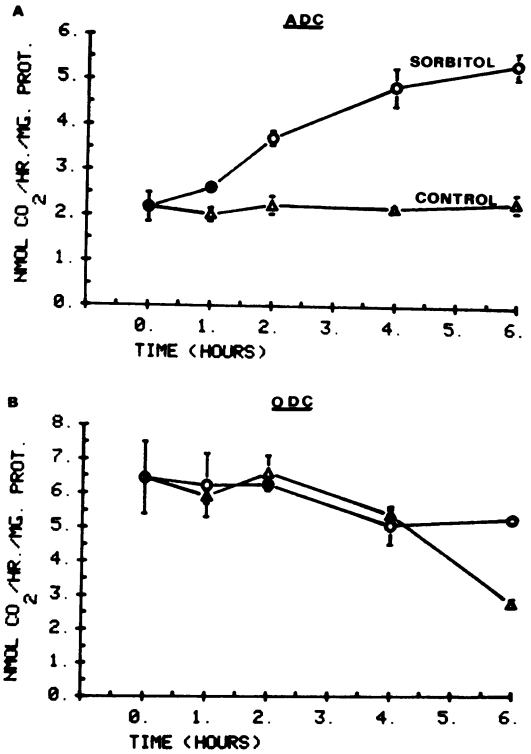

FIG. 4. Effect of osmotic stress on arginine and ornithine decarboxylase activity. A, Arginine decarboxylase. Leaf segments were floated over buffer (Δ) or buffer + 0.4 M sorbitol (O) under light. Samples were taken at the indicated times and assayed for enzyme activity ("Materials and Methods"). Bars represent ± SEM. B, Ornithine decarboxylase. Experimental conditions as above.

Table I. *Distribution of Free and Bound Putrescine in Oat Leaves*

Samples were extracted in 5% PCA. The 27,000*g* supernatant fraction was analyzed for free putrescine. Analysis for total putrescine was performed after hydrolysis of the PCA-soluble or -insoluble fractions in 6 N HCl at 100°C for 18 h. Numbers represent means ± SEM, corrected for recovery by use of 1,6-diaminohexane as an internal standard.

	Putrescine		
	PCA-soluble		PCA-insoluble
	Free	Total	
	nmol/g fresh wt		
Control			
0 h	26 ± 3	24 ± 2	ND[a]
4 h	22 ± 2	25 ± 3	ND
0.4 M Sorbitol			
2 h	85 ± 7	91 ± 5	T[b]
4 h	395 ± 18	408 ± 23	T

[a] ND, below detection limit (5 nmol/g·fresh wt).
[b] T, trace (10–15 nmol/g·fresh wt).

All assays were performed on fresh extracts. Enzyme assays were carried out in 12 × 75 mm disposable polystyrene tubes (Sarstedt) sealed with polyethylene caps. A 6-mm diameter filter paper disc (Schleicher & Schuell) impregnated with 50 μl of 2 N KOH and transfixed with 1.5-inch-long, 22-gauge needle was used to trap the $^{14}CO_2$ liberated during the reaction. The reaction mixture contained 100 μl of crude enzyme and 10 μl of L-[U-^{14}C]arginine (20 μCi/ml, 300 mCi/mmol, 1CN) diluted with cold arginine to give a final concentration of 9 mM. Assays were run at 30°C for 30 min in a water bath with continuous shaking, after which time 0.2 ml of 10% TCA was added to release the CO_2 formed by decarboxylation. After a further 30-min incubation, the filter paper was dried, placed in scintillation minivials with 3 ml Biofluor (New England Nuclear), and radioactivity counted in a Beckman LS7000 scintillation counter. Enzyme activity was expressed as nmol $^{14}CO_2$ released/h·mg protein. Protein was determined according to Bradford (5), using bovine gamma globulin (Sigma) as a standard (9).

The assay for ODC was run as above. The assay mix contained 100 μl crude enzyme, 10 μl of 20 μCi/ml DL-[1-^{14}C]ornithine (54.9 mCi/mmol, NEN), diluted with unlabeled ornithine to a final concentration of 66 mM, and 10 μl 5 mM pyridoxal phosphate. Although the above conditions were optimal, the pH peak was between 7.6 and 8.0 for both enzymes. For some experiments (Table V) a compromise buffer, 100 mM phosphate at pH 7.8 was, therefore, used. Under these conditions, ODC activity was somewhat lower than in Tris buffer, but the reaction was linear with time and amount of enzyme, and the trends observed were the same as for other experiments. Specific activities for ADC and ODC are expressed as nmol $^{14}CO_2$/h·mg protein. Since ODC is active only on L-ornithine, the ratio of D to L-ornithine (50:50) in the labeled sample obtained from NEN was always taken into account in the calculations.

ADC and ODC activities in etiolated pea buds were measured as above in the 27,000*g* supernatant of 100 mM phosphate buffer (pH 7.0) extracts (9). To study the effect of inhibitors *in vitro* (Fig. 6), the crude extracts were preincubated with 0.01 to 1.0 mM DFMA or DFMO for up to 30 min, then excess-labeled substrate was added and the assay run as usual. Residual activity was compared to that of controls preincubated in buffer.

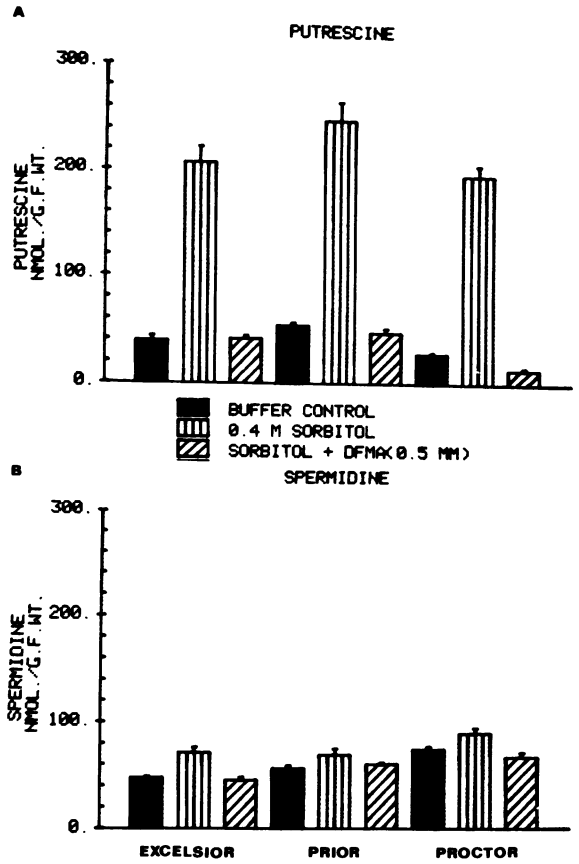

FIG. 5. Response of different barley cultivars to osmotic stress. Peeled leaf segments from the first leaf of 8- to 10-d-old barley seedlings were incubated under light in the various treatments, and samples taken after 4 h. Bars represent ± SEM.

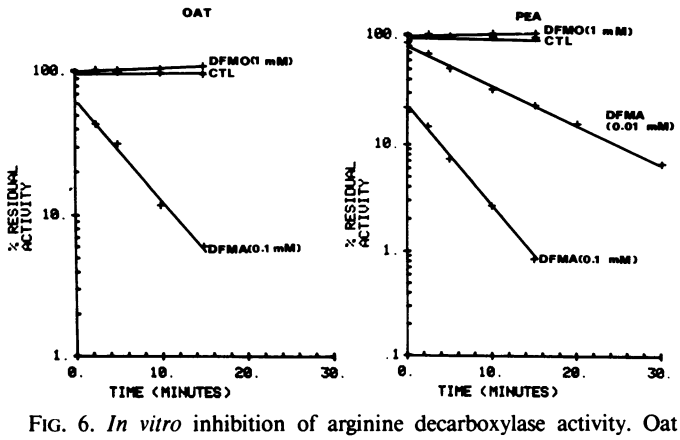

FIG. 6. *In vitro* inhibition of arginine decarboxylase activity. Oat leaves and etiolated pea buds were extracted and assayed as described in "Materials and Methods". The supernatant fraction was preincubated in 0.01 to 1.0 mM inhibitor for the indicated times before assay.

RESULTS AND DISCUSSION

The polyamine patterns of oat mesophyll protoplasts and of the mesophyll cells from which they are derived are shown in Figure 1. While agmatine, spermidine, and spermine remain unchanged on a per cell basis, the putrescine content is over 10 times higher in protoplasts. This change occurs in the 2- to 3-h period of digestion of peeled oat leaves with cellulolytic enzymes (0.5% Cellulysin Calbiochem in 0.4 M sorbitol at 30°C in darkness). The increase in putrescine is not a direct result of the toxic,

Table II. *Effect of Competitive Polyamine Biosynthetic Inhibitors on the Response to Osmotic Stress*

Oat leaf segments were incubated in the corresponding treatment under light.

Treatment	Putrescine	Spermidine	Spermine
	nmol/g fresh wt		
4-h Control	21 ± 2	124 ± 3	43 ± 6
0.4 M Sorbitol	427 ± 24[a]	141 ± 8	41 ± 3
+ 1 mM α-methylornithine	487 ± 17[a]	185 ± 11	45 ± 4
+ 1 mM D-arginine	19 ± 3	133 ± 8	39 ± 4
+ 1 mM L-canavanine	23 ± 3	129 ± 9	37 ± 5

[a] Significantly different from 4-h control at $P < 0.01$.

low mol wt components present in crude Cellulysin, since the same results are obtained with Cellulysin that has been desalted through Sephadex G-50. It is also not dependent on the source of cellulase. Putrescine titer is high in protoplasts isolated with Onozuka cellulase, which has widely differing ratios of proteolytic:cellulolytic:pectinolytic activity (14).

In addition to triggering increases in ribonuclease and protease activities (13), the osmoticum present in the protoplast isolation medium has been shown to cause changes in the membrane potential and to inhibit photosynthesis in mesophyll protoplasts and chloroplasts of spinach (16). We thus tested the possibility that the increased putrescine content of protoplasts is a direct result of osmotic stress. The experimental setup was identical to that used for protoplast isolation, except for the absence of cellulase in the incubation medium ("Materials and Methods"). No change was evident in putrescine content when leaves were floated in buffer. However, after a 2-h incubation in the presence of osmoticum, a significant increase in putrescine was apparent, and by 6 h the titer was 50- to 60-fold higher than the initial value (11). The diamine content reaches a plateau after 8 to 10 h of exposure to osmotic shock (H. E. Flores, unpublished data). Spermidine and spermine titer showed a gradual decline during the initial 4 h of osmotic treatment. Such changes were variable, but always correlated with an increase in diaminopropane, the direct oxidation product of spermidine and spermine by the polyamine-specific oxidase of widespread occurrence in cereal leaves (9, 13). Due to the presence of this highly active enzyme, polyamine oxidation during leaf sampling and extraction may have obscured any increases taking place early during the osmotic treatment. Nonetheless, an eventual rise in spermidine was evident after 6 to 10 h (data not shown). When care was taken to ensure a rapid extraction of the cells in PCA, a rise in spermidine was consistently observed after 4 h of osmotic stress (Figs. 2, 3; Tables II, III).

Sorbitol and mannitol are widely used osmotica for protoplast isolation and osmotic stress studies. They may not, however, be completely excluded from the cells. While isolated chloroplasts and protoplasts may function as near-perfect osmometers under a wide range of osmotic potentials provided by sorbitol in the medium (9), mannitol does penetrate cells, albeit slowly (32). We observed (Fig. 2) that osmotica with widely different assimilation routes, such as mannitol, sorbitol, proline, betaine, and sucrose, all induce putrescine rise (and in the case of sorbitol, mannitol and betaine, spermidine increase); therefore, while we are aware of the possible entry into the cells of these 'nonpenetrating' substances, our results support the conclusion that the observed responses in the detached cereal leaf system are due mainly to withdrawal of water from the cells.

Several cereals in addition to oat show putrescine accumulation when exposed to osmotic shock (Figs. 3, 5). For wild oat (*Avena fatua*), barley, and corn leaf segments floated on 0.4 M sorbitol, the putrescine increase is striking after 4 h, but there are no significant changes if the leaves are incubated in buffer

Table III. *Prevention by Cycloheximide of the Response to Osmotic Stress*

Peeled oat leaf segments were floated over control buffer or buffer + sorbitol for a total of 4 h under light. At the indicated times, 25 μg/ml CH (Sigma) was added to the sorbitol-treated segments. Samples were taken at 4 h for polyamine analysis or enzyme assay.

Treatment	Time of CH addition	Putrescine	Spermidine	Spermine	ADC	ODC
	h		*nmol/g·fresh wt*		*nmol CO_2/h·mg protein*	
0-h Control		19 ± 3	158 ± 10	28 ± 3	2.09	8.14
4-h Control		19 ± 4	149 ± 8	25 ± 2	2.27	8.14
0.4 M Sorbitol		466 ± 21[a]	210 ± 16	35 ± 4	4.20[a]	7.60
Sorbitol						
+ CH 10 μg/ml	0	85 ± 13[b]	97 ± 8[b]	30 ± 5	2.54	7.86
+ CH 25 μg/ml	0	55 ± 5[b]	125 ± 8	28 ± 3	1.98	7.91
+ CH 50 μg/ml	0	63 ± 5[b]	91 ± 4[b]	37 ± 3	2.05	7.75
+ CH 50 μg/ml	0	103 ± 9[a]	100 ± 7[b]	41 ± 5	2.72	7.97
+ 1 mM L-Arginine						
+ CH 25 μg/ml	1	341 ± 5[a]	210 ± 13[b]	32 ± 4	3.38[a]	6.35[b]
+ CH 25 μg/ml	2	502 ± 25[a]	257 ± 8[a]	29 ± 5	3.53[a]	6.00[b]
+ CH 25 μg/ml	3	561 ± 20[a]	311 ± 15[a]	30 ± 3	4.11[a]	4.79[a]

[a] Significantly different from the 4-h control at $P < 0.01$.
[b] Significantly different from the 4-h control at $P < 0.05$.

Table IV. *Polyamine Titer after Recovery from Osmotic Stress*

Peeled oat leaf segments were either incubated in control buffer (1 mM phosphate, pH 5.8) or buffer + sorbitol. Samples were taken for polyamine analysis at the indicated time or after transfer to control buffer and further incubation. All incubations carried out under light.

Length of Treatment		Putrescine	Spermidine	Spermine
0.4 M Sorbitol	Buffer			
h		*nmol/g·fresh wt*		
0	0	21 ± 2	112 ± 8	34 ± 3
0	8	12 ± 4	92 ± 4	16 ± 3
1		78 ± 5	119 ± 5	40 ± 4
1	3	65 ± 8	124 ± 2	38 ± 5
2		184 ± 5	120 ± 4	33 ± 2
2	2	153 ± 4	136 ± 5	59 ± 11
4		405 ± 5	143 ± 5	46 ± 4
4	4	271 ± 4	99 ± 3	51 ± 6
8		807 ± 58	162 ± 11	39 ± 3

(data not shown). In such cases, the lower epidermis can be removed without much damage to the underlying mesophyll (14). In contrast, peeling causes significant damage to corn leaves, and this is reflected as a 2-fold increase in putrescine upon incubation on control buffer (Fig. 3). The presumptive wound response can be reduced if the peeled corn leaf segments are rinsed in buffer before incubation in sorbitol, but it is clear (Fig. 3) that the osmoticum itself induces putrescine accumulation in corn leaves. In wheat leaves, which cannot be peeled, the presence of osmoticum causes a 4- to 6-fold increase in putrescine (11), supporting the view that injury due to peeling is not essential for the increase in putrescine.

The effect of osmoticum concentration on polyamine increase is shown in Figure 3. As little as 0.1 M sorbitol can cause a significant rise in putrescine in oat cells. The response peaks at 0.4 M sorbitol, and declines at higher levels of osmoticum. Similarly, in corn (Fig. 3B), putrescine and spermidine rise is maximal at 0.4 M sorbitol. It is known that the osmotically triggered increase of protease and RNAase activity is more pronounced during extreme plasmolysis (9), parallel to a decline in protein and nucleic acid synthesis (13). This may be why at high osmoticum levels the polyamine rise is 'suboptimal', since the response appears to require protein synthesis (Table III). Since osmoticum levels below the threshold for incipient plasmolysis (0.36 to 0.4 M sorbitol) induce polyamine rise, this suggests that the turgor rather than the osmotic component of the water potential is involved. Without implying a necessary connection with them, other processes are known to be extremely sensitive to variations in turgor pressure. Auxin-induced cell elongation in *Avena* coleoptiles is drastically inhibited by 0.15 to 0.5 M mannitol, while cell wall extensibility is inhibited at higher mannitol concentrations. Both processes, however, show a direct dependence on turgor pressure. In *Nitella* internode cells, small reductions in turgor pressure, effected through changes in PEG or mannitol in the external medium, cause immediate deceleration of growth (15). This must be considered when assessing the significance of the polyamine changes, since the effect of reduced turgor pressure on membranes and ABA metabolism or compartmentation are strongly implied as the processes that respond directly to water stress (15).

It may be argued that the osmoticum-induced increase in polyamines is due to their release from a bound form. In fact, polyamines conjugated to hydroxycinnamic acid moieties via amide linkages are of widespread occurrence, in particular among dicotyledonous plants (30). In some plants, as is the case with tobacco leaves, flowers, and cell suspensions, they may account for over 90% of the total amine pool (H. E. Flores, unpublished data). That this does not seem to occur in our cereal leaf system is shown in Table I. Acid hydrolysis of the PCA-soluble and insoluble fractions of leaf extracts caused little or no putrescine or spermidine (results not shown) release. Putrescine might also be produced from spermidine by the action of a polyamine oxidase such as that present in *Pseudomonas* or in mouse liver (23). However, the polyamine oxidases known so far in cereals, attack the side of the secondary nitrogen that is distal, rather than proximal, to the aminopropyl moiety of spermidine, and therefore, the products of oxidation are 4-aminobutyraldehyde and 1,3-diaminopropane, rather than putrescine and 3-aminoproprionaldehyde in the former case. Furthermore, even if a bacterial type of polyamine oxidase existed in oat cells, the total (free plus bound) levels of spermidine (150–200 nmol/g fresh weight) could account for only a small fraction of the putrescine formed during osmotic stress. We must thus conclude that putrescine biosynthesis is actually induced by osmotic treatment. Experiments involving polyamine biosynthesis inhibitors (below)

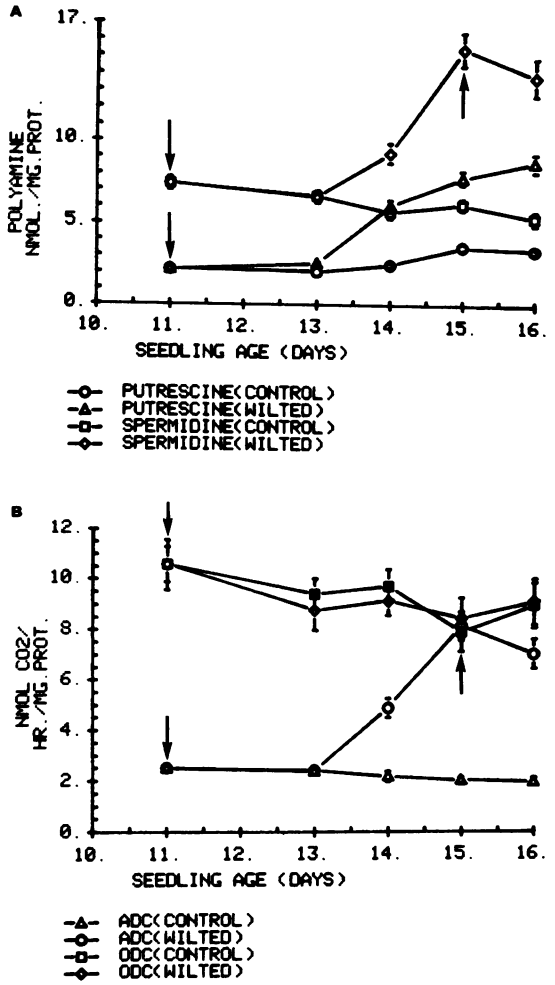

FIG. 7. Effect of wilting on polyamine titer, ADC, and ODC activity. Water was withheld from 11-d-old oat seedlings (↓) until day 15, at which time (↑) plants were rewatered and allowed to recover overnight. At the indicated times, samples were taken from the midportion of the first leaf for polyamine analysis and enzyme assay ("Materials and Methods"). Bars represent ± SEM.

Table V. *Response to Osmotic Stress in Relation to Leaf Age*

The first leaf of cv Victory oat seedlings was peeled at the indicated age and floated over control buffer (1 mM phosphate, pH 5.8) or buffer + sorbitol for 4 h under light. Numbers represent mean ± SEM.

Leaf Age/Treatment	Putrescine	ADC	ODC
	nmol/g·fresh wt	*nmol/CO_2/h·mg protein*	
1 week			
Control	45 ± 2	1.77	5.03
0.4 M Sorbitol	389 ± 13[a]	4.87[a]	5.34
2 weeks			
Control	62 ± 7	1.30	5.46
0.4 M Sorbitol	205 ± 11[a]	2.49[a]	5.49
3 weeks			
Control	78 ± 5	1.65	5.27
0.4 M Sorbitol	116 ± 4[b]	2.16[b]	5.65

[a] Significantly different from control at $P < 0.01$.
[b] Significantly different from control at $P < 0.05$.

and precursor labeling (12) support this conclusion.

Acid stress causes a significant accumulation of putrescine in barley seedlings (29). More recently, it was shown in the peeled oat leaf system (33) that putrescine accumulates when the pH of the external solution is lower than 5.5. We monitored the pH of the control and sorbitol solutions throughout the 4-h span of an osmotic shock experiment, and found no significant differences (data not shown). If the osmotic stress-induced polyamine increase is mediated through a decrease in the pH of the external solution, it would have to be localized, *e.g.* in the cell wall. However, several differences between the acid and osmotic stress responses make this possibility unlikely. First, putrescine rise under osmotic stress is detectable after 1 to 2 h (11), *versus* 3 to 5 h for the acid response, and the total rise is at least 2-fold higher; spermidine and ammonia also increase, while no such changes occur in response to acid stress. Second, the increases in polyamines and ADC under osmotic stress are not readily reversible (Fig. 7; Table IV), while the ADC increase during acidification is transient and of smaller magnitude (50% *versus* 2- to 3-fold for osmotic stress). Putrescine titer falls rapidly upon withdrawal of the acid condition and eventually even under continuous acid treatment. Third, the osmotic stress effect occurs both in light- and dark-incubated segments (12), while acid-induced putrescine formation occurs only under light (33). Thus, in addition to sharing with acid stress all the features discussed below (increase in ADC activity, prevention by ADC inhibitors, and dependence or protein synthesis), osmotic stress induces a more permanent shift in polyamine metabolism, as well as a whole syndrome involving other facets of nitrogen metabolism.

As judged by the Evans Blue exclusion test for viability, oat leaf segments recover well after stress withdrawal (H. E. Flores, unpublished data). Turgidity was regained completely when sorbitol was rinsed away, and no necrotic areas were observed. This is of interest since osmotic stress induces putrescine rise of the same order of magnitude as that found in K^+-deficient cereals, which show extensive cell death. Experiments involving putrescine feeding have been taken to imply that the accumulation of diamines is the direct cause of the necrosis observed in K^+-deficient (7) or salt-stressed plants (31). In our system, the cells do recover whether the stress involves hours or days, so it is tempting to consider that the eventual synthesis of spermidine from putrescine is the key protective factor for the stressed cell. It is not necessary to postulate then, as has been done in the case of K^+ deficiency and salt stress, that putrescine *per se* is deleterious to the plant. Putrescine accumulation may reflect a potentially protective response that does not proceed to completion. It should be possible to test whether various stress situations impair the steps in polyamine biosynthesis involving *S*-adenosylmethionine decarboxylase and/or aminopropyltransferase (spermidine synthase).

It is now accepted that in most, if not all, higher plants, putrescine may be synthesized by two alternative pathways: directly via ornithine decarboxylation, or through arginine decarboxylation via agmatine and *N*-carbamylputrescine (1), although the relative contribution of each pathway during growth and development is not yet clear. As shown in Figure 4A, ADC activity does not change significantly during incubation of oat leaf segments in the control buffer, but a rise is apparent after 1 h of osmotic shock, reaching a plateau at 2 to 3 times the starting value after 4 to 6 h. This increase in ADC is correlated with and precedes the accumulation of putrescine and subsequent rise in spermidine (9, 11). ODC activity does not change appreciably under stress and, in fact, declines in the control treatment (Fig. 4B).

The correlation between ADC increase and putrescine accumulation is of similar magnitude to that observed under K^+ deficiency (28), upon acid feeding (29, 33), and during NH_4^+ nutrition (20), but is not, by itself, proof that only ADC mediates the stress-induced rise in polyamines. The availability of enzyme-activated specific inhibitors of polyamine synthesis allowed us to establish a causal link (11). In contrast to the classical irreversible

inhibitors, compounds such as DFMO and DFMA are chemically inert substrate analogs, that when acted upon by the enzyme generate an electrophilic form inside the active site; this reactive intermediate forms a covalent link with a nucleophilic residue in the active site, leading to irreversible inactivation of the enzyme (21). We showed previously (11) that DFMA, the enzyme-activated irreversible inhibitor of ADC (18), completely prevents the rise in putrescine and ADC activity in osmotically shocked oat leaf segments, without any effect on ODC activity. DFMO, the corresponding ODC inhibitor (21), had no effect on ODC activity and did not prevent putrescine rise. In three different barley cultivars we observed the prevention of the stress response by DFMA (Fig. 5), suggesting that the ADC pathway also operates in this species. As in the case of oat leaves, DFMO is ineffective (data not shown). The effects obtained *in vivo* have been confirmed in the *in vitro* assay for ADC (Fig. 6). ADC activity is effectively inhibited upon preincubation with DFMA in oat and pea extracts. The effect of DFMO does not differ significantly from that of preincubation in buffer.

The results obtained with DFMA have been confirmed with competitive inhibitors (Table II). D-arginine and L-canavanine, which inhibit oat ADC by 60 and 50%, respectively (28), when present at 1 mM in the incubation medium completely prevent the stress response by oat leaf segments. In contrast, α-methylornithine, which does inhibit oat ODC *in vitro* (R. Kaur-Sawhney, personal communication), has the same effect *in vivo* as DFMO, that is, it cannot prevent the putrescine and ADC rise, but rather potentiates them (Table II). Taken together, our results strongly suggest that stress-induced putrescine is made entirely through the ADC-mediated pathway. We must caution, however, that the use of irreversible inhibitors in any plant system may not be fully warranted. In bacteria, DFMO is very effective *in vitro* against *Pseudomonas* ODC, but fails to inhibit the enzyme from *Escherichia coli* and *Klebsiella pneumoniae* (17). We have observed a similar situation in higher plants. Oat, pea, potato, and amaranth ODC are resistant to DFMO (H. E. Flores, unpublished data), while tomato ODC is inhibited (6). DFMA is without effect on ADC from potato tubers and amaranth leaves, while being extremely effective on the enzyme from oat, pea (Fig. 6), and carrot (8). Thus, an essential requisite in the use of these inhibitors as probes for polyamine function in plant systems, is the demonstration of their effectiveness *in vitro*.

The rise in putrescine and ADC in cells exposed to osmotic shock or high external acidity is seen after a time lag of 1 to 2 h, consistent with a requirement for protein synthesis. Table III shows the effect of transcription inhibitors on the response to osmotic stress. CH at 10 to 50 μg/ml completely prevents the rise in putrescine and spermidine. The effect of transcription inhibitors is not as clear. Actinomycin D at 10 to 20 μg/ml and cordycepin at 5 to 10 μg/ml prevent the rise in putrescine by 28 and 11%, respectively (data not shown); if transcription of the mRNA for ADC mediates the stress response, the results may reflect incomplete penetration of the inhibitors. The kinetics of prevention of the stress response by CH is shown in Table III. If added at the start of the osmotic treatment, CH prevents the putrescine and ADC rise effectively, but at progressively later times there is an escape from the prevention by CH which is completed by 1 to 2 h. This is consistent with the supposition that a protein synthesis dependent event occurring during the 1 h of exposure to osmotic stress is eventually translated into a rise in ADC and putrescine accumulation. For the full rise in putrescine to be expressed, the tissue needs to be stressed for several hours (Table IV). If leaf segments are floated on sorbitol for 1 h, transferred to buffer, and putrescine titer determined after a total of 4 h, the increase is significant, but much smaller than if the tissue had been stressed continuously for the 4-h period.

Are the short term changes observed in osmotically stressed leaf segments comparable to a water stress condition in whole plants? The results shown in Figure 7 support this view. If 11-d-old oat seedlings are allowed to wilt by withholding the water supply, polyamine titer remains unchanged for 2 d before rising significantly at the time when water stress symptoms are obvious (wilting, decrease in water content per leaf segment, protein loss). In parallel, ADC activity goes up almost 4-fold, while ODC is not affected (Fig. 7B). These changes were followed in the first leaf, which at this stage is more susceptible to wilting than the emergent second leaf. If plants are allowed to recover overnight after rewatering, putrescine titer continues rising somewhat, while spermidine and ADC decline slightly. This incomplete reversibility upon stress removal is also apparent in the *in vitro* leaf segment system (Table IV).

CONCLUSION

We have shown that when cereal leaves experience osmotic stress there is an activation of the ADC-mediated pathway for putrescine synthesis, leading to a fast, massive, and not readily reversible increase in polyamine titer. This phenomenon shares several features with stress-induced changes in polyamine physiology previously shown to occur within several days (K^+ deficiency, acid and salt stress, NH_4^+ nutrition) (20, 26, 29, 31). As in the case of pH stress (33) the *in vitro* leaf system allows for the induction and control of a rapid response; it is clear from our results that protein, and possibly RNA synthesis, are required for maximal polyamine accumulation. The striking similarities in the response to the above stresses strongly suggest that there is an underlying common mechanism. The obvious possibilities of there being internal pH changes, or alterations in K^+ fluxes in our system need to be addressed. The use of specific ionophores should prove valuable in this respect. The use of specific biosynthesis inhibitors also becomes apparent as a powerful tool in assessing the physiological significance of these changes. However, several gaps in our basic understanding of polyamine physiology in higher plants need to be filled if any of the above tools is to be used effectively. A most pressing problem is the almost complete lack of information on the localization of polyamines in the plant cell. Because of the rapid redistribution of these charged molecules occurring during cell extraction and fractionation, this has remained an unsolved area in polyamine research. The availability of rapid methods of cell fractionation from plant protoplasts may provide some answers. We have isolated chloroplasts and vacuoles from oat protoplasts by procedures that minimize redistribution (4, 24). Spermidine is present in chloroplasts, and at lower levels in vacuoles. The cell wall digest contains only diaminopropane, which agrees with the known localization of polyamine oxidase in the cell wall (9, 13), while putrescine and spermine are absent from all fractions except intact protoplasts. In view of the high putrescine content of oat protoplasts, its absence from the above fractions indicates that the methods used are clean and specific, but so far they can only eliminate several potential sites of the stress induced polyamine rise. Developed chloroplasts seem not to be necessary for stress-induced putrescine formation, since isogenic albino and green leaf tissue of the *albostrians* barley mutation both show elevated putrescine levels after osmotic shock (D. Church and A. W. Galston, unpublished data). Specific histochemical methods have been developed recently for spermidine in animal cells (19), and may be adapted for diamine localization in plant cells. The availability of labeled DFMA and DFMO should also prove useful in localizing the biosynthetic enzymes, as such an approach has already been succesful with animal cells (25).

Since the adaptive significance of stress-induced changes in polyamine metabolism is not apparent, we are left to speculate on this point. The metabolic consequences of water deficit are frequently compared with those of senescence. We have already

mentioned the rise in protease and RNAase activity that accompanies osmotic stress and leaf senescence in cereal protoplasts. Putrescine synthesis in stressed leaves may simply represent another biochemical consequence of senescence. However, it is known that polyamine titer and ADC activity decrease with increased ages of oat leaves (13). Putrescine and ADC activity increase maximally in young leaves subjected to osmotic stress (Table V); as the leaves get older, the magnitude of the response declines significantly. If stress-induced putrescine is of survival value, it would be advantageous to the plant to show a greater response in metabolically more active organs. The results presented above are in accord with this concept.

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