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Putrescine and Acid Stress[1]

INDUCTION OF ARGININE DECARBOXYLASE ACTIVITY AND PUTRESCINE ACCUMULATION BY LOW pH

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ABSTRACT

Incubation of peeled oat *Avena sativa* L. var Victory leaf segments on media of pH 5.0 or below leads to a rapid and massive increase in the titer of putrescine while incubation at pH values above 5.0 causes little or no change. The low pH effect is independent of the buffer system employed. Putrescine levels rise within 3 hours and reach their peak 8 to 9 hours after acidification. At this time, putrescine titer is eight times greater at pH 3.5 than at 6.0. None of the other polyamines shows a response to changes in external pH. The increase in putrescine is blocked by the addition of cycloheximide or DL-α-difluoromethylarginine, a specific inhibitor of the putrescine biosynthetic enzyme, arginine decarboxylase. In one experiment, arginine decarboxylase activity was 110% greater at pH 4.0 than at 6.0 after a 4-hour incubation, although the average increase over many experiments was 47%. The activity of the other possible putrescine biosynthetic enzyme, ornithine decarboxylase, falls throughout the incubation period and is virtually equal at pH 4.0 and 6.0.

Plants exposed to ionic stress frequently respond by increasing their endogenous level of Put.[2] While this phenomenon was first observed in plants exposed to K^+ deficiency (5), the titer of Put is now known to increase under conditions of Mg^{2+} deficiency, low external pH, high levels of NH_4^+, SO_2 fumigation, and osmotic shock (7, 17, 22). The enhancement in Put levels under conditions of ionic stress is often greater than 10-fold and is observed in such diverse families as Gramineae, Leguminoseae, Solanaceae, and Cucurbitaceae (22). It is also well known that Put and the PA derived from it can associate with and affect the behavior of proteins, nucleic acids, and membranes (2, 12, 16). Several authors have therefore proposed that the changes in the level of Put in response to ionic stress might be important in the regulation of the ionic environment within the cell (18, 21). In addition, Put, like many other PA, has been found to carry out several regulatory functions, including inhibition of senescence of excised leaves in the dark (13), induction of DNA synthesis and mitosis in oat protoplasts (14), and to be required for postpollination growth and development of tomato ovary and fruit (10).

Tracer studies have demonstrated that, in many systems, Put is produced from Agm, the decarboxylation product of arginine. Floating barley leaves on a medium containing radiolabeled arginine quickly leads to the appearance of label in Put (3). The rate of conversion can be enhanced by ionic stress, such as K^+ deficiency (3). A key rate-limiting enzyme in the conversion of arginine to Put is ADC, whose activity has been found to be increased by several different kinds of ionic stress (20, 23). Decarboxylation of ornithine also produces Put through catalysis by the enzyme ODC (1). It therefore becomes important to know which pathway leading to Put is enhanced by ionic stress. Fortunately, irreversible, enzyme-activated inhibitors for the enzymes ADC and ODC are now available. They are, respectively, DFMA and DFMO (11, 15). Addition of these inhibitors, along with the imposition of ionic stress, can determine which pathway is most important.

Most studies on the relationship between ionic stress and the physiology of Put have been performed with whole plants. We now report an *in vitro* system based on excised leaves of *Avena* for the study of the effect of acid stress on the synthesis of Put. Such a system permits experiments to be performed rapidly, reproducibly, and under precisely controlled conditions when compared with whole plants. With excised oat leaves, we have studied the response of Put to a broad range of external pH values, the short-term kinetics of the response, and the effect of inhibitors, including DFMA, DFMO, and cycloheximide. We have also determined the activities of the Put biosynthetic enzymes ADC and ODC in response to changes in external pH. The results demonstrate that the increase in the titer of Put in response to acid stress is rapid, massive, and reversible, and that this increase is accompanied by an increase in the level of ADC, but not ODC, activity.

MATERIALS AND METHODS

Plant Materials. *Avena sativa* L. (var Victory) and *Pisum sativum* L. (var Alaska) seeds were sown in water-saturated, coarse Vermiculite and grown in controlled growth rooms with a 16-h photoperiod at about 350 $\mu E/m^2 \cdot s$ and at 23°C, as described earlier (8).

Isolation and Culture of Excised Tissue. The first leaf from 8- to 11-d-old oat seedlings was used in most experiments. The lower epidermis was peeled off and a 4-cm median leaf segment cut out. Pea tissue was obtained by cutting 6-mm leaf discs from the second trifoliate leaf with a cork bore.

The culture medium consisted of a macronutrient and a buffering component. The composition of the macronutrients was kept constant at the following concentrations: KNO_3, 3.0 mM; NH_4NO_3, 3.0 mM; $CaCl_2$, 2.0 mM; $MgSO_4$, 0.5 mM; and $NH_4H_2PO_4$, 0.5 mM. In media buffered at pH 8.0, it was necessary to reduce the concentration of $NH_4H_2PO_4$ to 0.1 mM. All nutrient media were autoclaved before use.

The composition of the buffering component in most experiments consisted of succinic acid at 5.0 mM and variable amounts of Tris-base (1.0–9.0 mM) as required to adjust the pH to the desired value. In media buffered at pH 8.0, the concentration of

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[2] Abbreviations: Put, putrescine; PA, polyamine(s); Agm: agmatine; ADC: arginine decarboxylase; ODC: ornithine decarboxylase; DFMA, D,L-α-difluoromethylarginine; DFMO, D,L-α-difluoromethylornithine; Spm, spermine; Spd, spermidine; Cad, cadaverine.

Tris-base was maintained at 5.0 mM and succinic acid added as required to adjust the pH. In one set of experiments, several different buffer systems were tested. Details of these experiments are listed in Table I.

Excised material was incubated for up to 24 h under 'daylight' fluorescent light (20 $\mu E/m^2 \cdot s$) at 22 to 25°C. Each oat sample consisted of three leaf segments and each pea sample consisted of eight leaf discs. All samples were incubated in 15- × 60-mm Petri dishes containing 15 ml of nutrient medium. Most determinations were performed in triplicate.

Addition of Inhibitors. In one set of experiments, cycloheximide (Sigma) was added to pH 4.0 buffered medium at 5.0 μg/ml. In another set of experiments, the specific inhibitors of ADC and ODC, DFMA and DFMO (Merrell Research), respectively, were added to pH 4.0 buffered medium at 0.5 mM.

Determination of PA Levels. Levels of the PA were determined by a method modified from Flores and Galston (6). Briefly, samples were homogenized in 5% (v/v) PCA at 0.1 g/ml and the extract was dansylated by adding 0.4 ml of dansyl-Cl (1-dimethylamino-5-napthalenesulfonylchloride; Sigma) in acetone (5 mg/ml) and 0.2 ml of saturated Na_2CO_3 to 0.2 ml of the PCA-soluble extract. Samples were mixed and incubated overnight. Dansyl-PA were extracted into 0.5 ml benzene, and 20 μl of the benzene extract was spotted onto silica gel thin-layer plates (Whatman, LK6D). TLC plates were developed in chloroform:triethylamine (5:1). Standards of the PA, Spm, Spd, Put, and Cad, were run each time PA levels were determined. After development, bands were scraped into 2.0 ml of ethyl acetate and the fluorescence at 500 nm was determined in an Aminco spectrophotofluorimeter with an activating wavelength of 350 nm.

Determination of ADC and ODC Activities. Samples for the determination of ADC activity were homogenized in 0.1 M sodium-phosphate buffer (pH 7.6) at 0.1 g fresh weight/ml in an ice bath. Samples for the determination of ODC activity were homogenized in 0.1 M Tris-HCl buffer (pH 8.0) in a similar manner. After centrifugation at 20,000*g* for 10 min in a Sorvall RC-5B centrifuge, the supernatant (crude extract) was used immediately to assay enzyme activity. The reaction vessel consisted of a 12- × 75-mm polystyrene tube sealed with a polyethylene cap. This cap supported a 22-gauge needle onto which a 6-mm filter paper disc, impregnated with 40 μl of 2 N KOH, was affixed. The reaction mixture consisted of 160 μl of crude extract, 20 μl of 10 mM pyridoxal-P, and 20 μl of radiolabeled compound. For assay of ADC activity, the labeled compound was L-[U-^{14}C]arginine (300 mCi/mmol, Schwarz-Mann) at 2.8 × 10^7 cpm/ml diluted with unlabeled arginine added to give a final concentration of 10 mM. For assay of ODC activity, the labeled compound consisted of DL-[1-^{14}C]ornithine (54.9 mCi/mmol, New England Nuclear Co.) at 1.7 × 10^7 cpm/ml with unlabeled ornithine added to give a final concentration of 50 mM. Reaction mixtures were incubated for 60 min with gentle shaking at 37°C, at which time the reaction was terminated and the labeled CO_2 released by the injection of 0.2 ml of 10% (w/v) TCA directly through the needle. Trapping of the labeled CO_2 onto the filter disc continued for 45 min. Discs were then removed and allowed to air dry before being immersed in 2.0 ml Biofluor (New England Nuclear Co.). The radioactivity liberated was determined by counting for 10 min in a Beckman LS 7000 scintillation counter. Enzyme activity is expressed as pkat/mg protein (1 katal = 1 mol/s). The conditions given above were found to be optimal for the assay of ADC and ODC in oat leaves.

Determination of Protein Levels. The concentration of protein in samples used for enzyme assays was determined by the method of Bradford (4) using BSA as a standard.

RESULTS

Effect of Different Buffer Systems. To demonstrate that the results we observed were due to actual changes in the external pH and not a result of the particular buffer system employed, several different buffers were tested at a low (usually 4.0) and a high (6.0) pH to observe their effect on Put levels. One of the buffers employed, Mes, was used at a pH value of 4.8. Although this is below the published pH range for Mes, we found it effective in maintaining the pH. The advantage of using Mes is that, unlike the other buffers tested, it is neither taken up nor metabolized (9). Excised and peeled oat leaf segments were floated on nutrient media buffered with the various buffer systems for 8 h. After this time, tissue was harvested and the concentration of Put determined. In each case (Table I), Put titer was increased at low pH. The magnitude of the increase was generally 5- to 6-fold.

Response to a Broad Range in External pH. To determine the response of Put titer to external pH, we floated excised leaf tissue on nutrient media buffered at pH values between 3.5 and 8.0 for a period of 8 h. The results were remarkably similar in both peas and oats (Fig. 1). In each case, the concentration of Put changed little in the range of pH 8.0 to 6.0. Levels were slightly higher at pH 5.0 than 6.0. Below pH 5.0, however, Put levels increased dramatically. The level of Put was 8 times greater at pH 3.5 than at pH 6.0 in oats. In peas, the increase was 5-fold. Significantly,

Table I. *Effect of Different Buffer Systems on the Titer of Put in Excised Oat Leaf Segments as a Function of External pH*

[Put] is given as mean ± SD.

Buffer System	Acid	Base	pH at Start	pH at 8 h	Put
	mM				*nmol/g fresh wt*
1. Succinic acid/Tris	5.0	2.5	4.0	4.2	347 ± 25
	5.0	9.0	6.0	5.9	70 ± 15
2. Succinic acid/Tris	9.5	5.0	4.0	4.1	280 ± 11
	2.6	5.0	6.0	5.9	51 ± 8
3. Succinic acid/NaOH	5.0	2.0	4.0	4.0	318 ± 33
	5.0	8.5	6.0	5.7	51 ± 8
4. Mes/Tris	10.0	0.5	4.8	4.9	109 ± 14
	10.0	5.0	6.0	5.8	46 ± 6
5. KH phthalate/Tris	5.0	0.0	4.0	4.2	283[a]
	5.0	4.5	6.0	5.8	69

[a] This buffer system was tested in duplicate only.

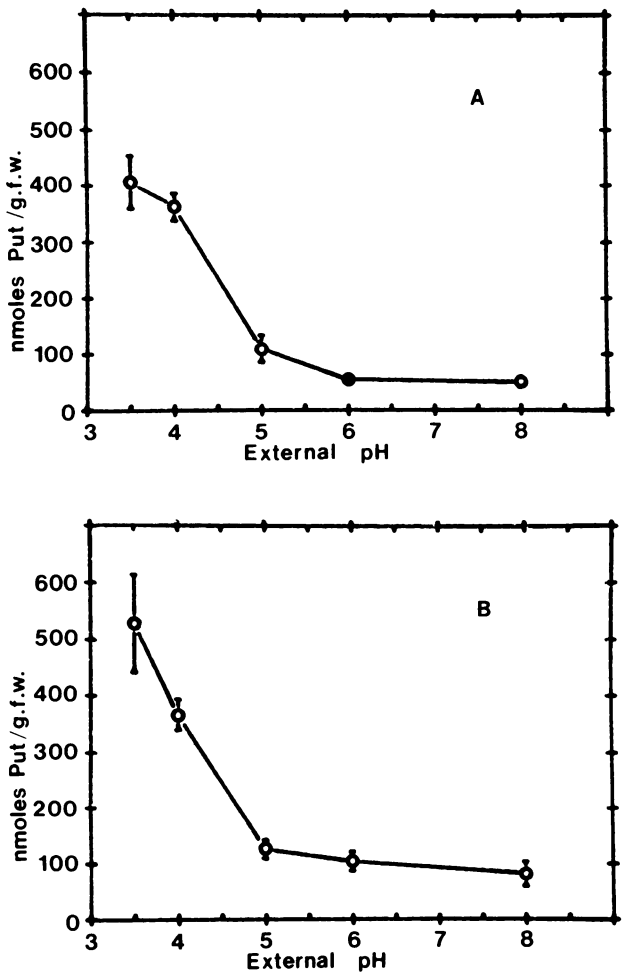

FIG. 1. Response of endogenous Put concentration to a range of external pH in excised oat leaf segments (A) and pea leaf discs (B) incubated for 8 h. Error bars represent ± 1 SD. Error bars overlap the data point for pH 6 in (A).

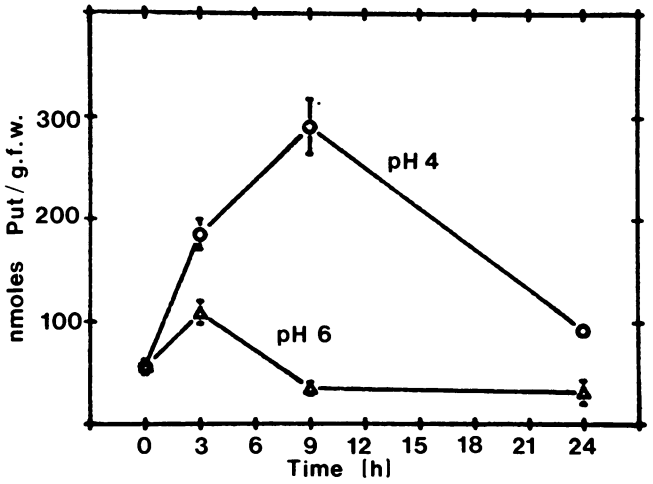

FIG. 2. Kinetics of Put accumulation in excised oat leaf segment incubated at pH 4.0 (○) or pH 6.0 (△) for up to 24 h. Error bars represent ± 1 SD.

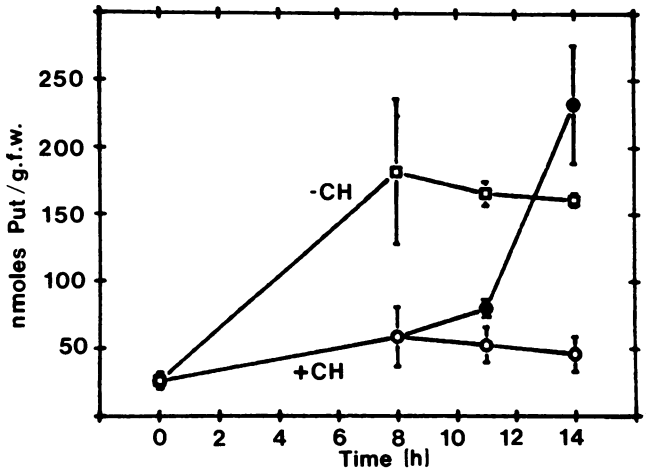

FIG. 3. Effect of cycloheximide (CH) on the accumulation of Put in oat leaf segments incubated at pH 4.0. Control (□); cycloheximide (○); incubated for 8 h with cycloheximide then moved to a cycloheximide-free medium (●). Error bars represent ± 1 SD.

none of the other PA, Cad (present in peas only), Spd, or Spm, showed any response to changes in external pH (data not shown). The similarity in the response of Put to low pH in both peas and oats, two widely divergent species, suggests that an increase in Put in response to acid stress might be a general phenomenon of plant leaf tissue.

Kinetics of the Response to External pH. The time course of the changes in Put titer in response to external pH was determined by floating excised oat leaf segments on nutrient media buffered at pH 4.0 or 6.0 for up to 24 h (Fig. 2). At various times, tissue was harvested and the concentration of Put measured. After a small increase at 3 h, Put levels remained low and constant at pH 6.0. In segments incubated at pH 4.0, however, Put levels rose rapidly until 9 h. At this time, the level of Put at pH 4.0 was 6 times the original concentration and 8 times greater than the level of Put in segments at pH 6.0 at 9 h. After 9 h, the Put titer in segments at pH 4.0 fell, though even at 24 h the level of Put at pH 4.0 was 3 times that at pH 6.0. Throughout the experiment, the fresh weight and protein and Chl levels remained equivalent in segments at pH 4.0 and 6.0. Thus, results reported on a fresh weight basis are equally valid when expressed on protein basis.

Effect of Inhibitors. Addition of cycloheximide to pH 4.0 buffered nutrient medium completely inhibited the rise in Put in excised oat leaves for at least 14 h (Fig. 3). Moving segments exposed to a medium with cycloheximide to one lacking the inhibitor resulted in an accumulation in Put titer within 6 h. The accumulation was preceded by a lag of at least 3 h, during which Put levels rose only slightly. These results indicate that protein synthesis might be required for the induction of Put synthesis observed under acid stress.

DFMA and DFMO, specific inhibitors of the Put biosynthetic enzymes, ADC and ODC, respectively, were also added to test solutions. They provide excellent probes for the involvement of specific enzymes in the accumulation of Put in response to low external pH. Addition of these compounds separately to pH 4.0 buffered nutrient media resulted in an inhibition of the increase in Put by DFMA only (Table II). This is strong evidence for a role of ADC in the low pH induced accumulation of Put. Addition of DFMO was without effect (Table II). These negative results with DFMO alone cannot by themselves rule out a role for ODC in the pH response. However, since ODC activity falls in segments incubated at pH 4.0, even as Put levels rise (see below), it seems likely that ODC is of little or no significance in controlling Put levels in pH-stressed leaves.

Activity of Biosynthetic Enzymes in Response to External pH. The observations that the level of Put increases rapidly in response to low external pH, and that this response can be abolished by the addition of DFMA, suggest that there should be an increase in ADC activity in oat leaf tissue incubated at low pH. Figure 4

Table II. *Effect of Inhibitors of Put Biosynthesis on the Accumulation of Put in Oat Leaf Segments Incubated for 8 Hours at pH 4.0*

[Put] is given as mean SE.

Treatment	Put Concentration	% of Control
	nmol/g fresh wt	
Control	371 ± 17	100
DFMO	347 ± 38	94
DFMA	82 ± 19	22

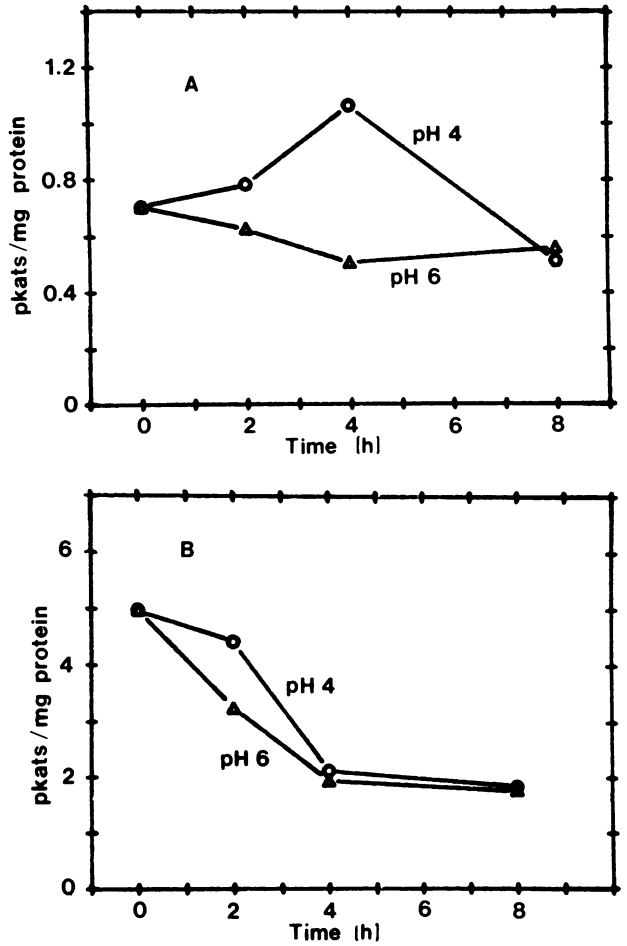

FIG. 4. Time course of ADC (A) and ODC (B) activities in oat leaf segments incubated at pH 4.0 (○) or at pH 6.0 (△) for up to 8 h. Each data point represents the mean of two independent samples.

shows the results of one representative experiment in which this expectation was verified. After 4 h incubation, ADC activity at pH 4.0 was approximately 50% greater than its initial value and more than double the activity in tissue at pH 6.0. The 4 h increase of ADC activity at pH 4.0 over that at pH 6.0, averaged over several experiments was 47%. The time course of the increase in ADC activity at low pH supports the hypothesis that ADC is the relevant biosynthetic enzyme in the acid-induced increase in Put titer. ADC activity begins to rise after only 1 h incubation at pH 4.0, while Put titer responds to low pH only after 2 h (data not shown). Moreover, the period during which Put accumulation is fastest coincides with the maximum in ADC activity.

While ADC activity increases dramatically within 4 h, there is no increase in ODC activity at low pH (Fig. 4). In fact, ODC activity falls from the start in excised tissue incubated at either pH 4.0 or 6.0 and is virtually equal at pH 4.0 and at pH 6.0 after 4 h.

DISCUSSION

The development of an *in vitro* system for the study of the effects of ionic stress on the titer of Put in oat leaves has enabled us to perform experiments which otherwise would have been very difficult. The environmental conditions can be more accurately controlled with excised and peeled leaves than in whole plants and the effect is observed within hours rather than days. A differential response to external pH can, in fact, be observed within only 3 h. The addition of inhibitors is straightforward and has demonstrated the importance of protein synthesis and the involvement of ADC in the response to pH. The use of an *in vitro* system also allows for the easy addition of radiolabeled precursor compounds. Our preliminary results show that labeled arginine fed directly to excised oat leaves is converted into Put at a faster rate at pH 4.0 than at pH 6.0. Suresh *et al.* (26) studied the effect of HCl at 5 to 10 mM on Put content and ADC activity in excised *Cucumis* cotyledons. Their results are similar to ours, although the magnitude of the increase in Put that they report (2-fold) is far less than the increase we observe in excised oat leaf segments. Moreover, they made no attempt to observe the effect of Put biosynthetic inhibitors or to compare ADC with ODC activity. In a recent study, Flores and Galston (7) determined the response of Put titer and ADC activity in excised and peeled oat leaves in response to osmotic shock, effected by 0.4 M mannitol. As in the case of pH stress, Put levels and ADC activity increase dramatically within hours and the increase is sensitive to DFMA.

Most of the stress conditions which induce Put biosynthesis, including acid stress, have in common the fact that they affect the ionic balance in the cell. Thus, it is often suggested that Put provides necessary cationic or basic equivalents under conditions in which such ionic species are deficient. This, however, cannot provide an explanation for all stress-induced increases in Put, *e.g.* osmotic stress. Other small molecules are known to increase in response to imposed stress, most notably proline (19) and methylated quaternary ammonium compounds, such as glycine betaine (25). One possible function for proline and glycine betaine in salt-stressed plants is that they provide compatable osmotica in the cytoplasm, maintaining a sufficiently low internal osmotic potential for retention of water within the cell (24, 25). Put, proline, and glycine betaine, all synthesized in plants under stress, therefore appear to have similar homeostatic roles, each helping to maintain an intracellular environment conducive to growth. Several important differences exist between these compounds, however. The concentrations of proline and glycine betaine are often several times that of Put, even during periods of appropriate stress. Moreover, Put is well known for its specific interactions with proteins, nucleic acids, and membranes. Thus, if Put is important in resistance to ionic stress, it might differ from the other compounds mentioned above by affecting microenvironments around macromolecules and cellular structures, rather than producing global changes throughout the cytoplasm. Experiments to demonstrate that Put is, in fact, associated with resistance to ionic stress are currently under way in our laboratory. These include correlation of Put induction with the degree of resistance to ionic stress and the effect of specific inhibitors of Put biosynthesis on resistance to ionic stress.

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