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Polyamines and Plant Stress: Activation of Putrescine Biosynthesis by Osmotic Shock

Abstract. *The putrescine content of oat leaf cells and protoplasts increases up to 60-fold within 6 hours of exposure to osmotic stress (0.4 to 0.6 molar sorbitol). Barley, corn, wheat, and wild oat leaves show a similar response. Increased arginine decarboxylase activity parallels the rise in putrescine, whereas ornithine decarboxylase remains unchanged.* DL-α*-Difluoromethylarginine, a specific irreversible inhibitor of arginine decarboxylase, prevents the stress-induced rise in increase in arginine decarboxylase activity and putrescine synthesis, indicating the preferential activation of this pathway.*

Although leaf mesophyll protoplasts of many species have been successfully cultivated and regenerated in vitro, cereal protoplasts have remained refractory (*1*). The addition of the polyamines putrescine, spermidine, and spermine to the isolation and culture medium stabilizes oat leaf protoplasts against lysis, increases their incorporation of [^{3}H]leucine and [^{3}H]uridine into macromolecules (*2*), induces limited mitosis (*3*), and retards leaf senescence (*4*). These observations prompted us to study the role of polyamines in the physiology of plants.

The pattern of polyamines in freshly isolated oat mesophyll protoplasts is strikingly different from that in mesophyll cells. The titer of putrescine, as confirmed by high-performance liquid chromatography and thin-layer chromatography (*5*), increases five- to tenfold in protoplasts, whereas spermidine and spermine remain constant or show a slight decrease. Since the osmoticum present in the protoplast isolation medium (*1*) is known to trigger an increase in ribonuclease and protease activities (*2*), changes characteristic of leaf senescence (*4*), we tested the possibility that the increase in putrescine titer is a direct result of the osmotic treatment.

The lower epidermis of primary leaves of 7- to 9-day-old oat cv. Victory seedlings was removed and the leaves floated under light in the presence and absence of 0.6*M* sorbitol, an osmoticum commonly used during protoplast isolation (*1, 3*). A significant increase in putrescine was evident after 2 hours of such incubation, and a level 60-fold higher than the initial value was reached by 6 hours (Fig. 1A). In contrast, putrescine concentrations were not significantly altered by incubation of leaf segments in phosphate buffer (1 m*M*, *p*H 5.8). It is unlikely that the osmoticum-induced increase in putrescine is due to its release from a bound form, since acid hydrolysis of the perchloric acid–soluble and insoluble fractions of leaf extracts causes little or no putrescine release after 16 hours at 110°C in 6*N* HCl (*6*). The other polyamines, spermidine and spermine, show a gradual decline during osmotic treatment, with a concomitant increase in 1,3-diaminopropane, the product of their oxidation by the polyamine oxidase present in cereal leaves (*7*). Osmotica such as mannitol, proline, betaine, and to a lesser extent sucrose, have a similar effect on the putrescine titer.

The response of putrescine to osmotic shock is not restricted to oat. Leaf segments of wild oat, barley, corn, and wheat floated on 0.4*M* sorbitol also showed significant increases in putrescine after 4 hours (Fig. 1B). The putrescine concentration at the start of the experiment was not significantly different from that of the control leaves incubated in buffer for 4 hours; however, there is a twofold increase in putrescine in corn leaf segments floated on buffer, possibly because of the damage induced by peeling. Wheat leaf segments, which cannot be peeled and do not show any obvious signs of wilting in the presence of osmoticum, show a 4.6-fold increase in putrescine titer (Fig. 1B). Thus, injury due to peeling is not essential for the increase in putrescine. Also, the threshold levels of osmoticum necessary for the response are probably much lower than the concentrations used in these experiments, since treatment with even 0.1*M* sorbitol increased the putrescine titer 2.5-fold after 4 hours. Increased putrescine levels have also been noted during deficiencies of potassium (*8, 9*) and magnesium (*9*), acid stress, (*6, 10*) and NH_4Cl feeding (*11*). Bacterial and mammalian cells, however, show an inverse correlation between polyamine content (putrescine, spermidine, and spermine) and the osmolality of the culture medium (*12*).

In contrast with mammalian cells, in

Table 1. Polyamine titer and biosynthetic enzyme activity in oat leaf segments under osmotic stress. Peeled leaf segments (7 days old) were floated in buffer (control) or buffer plus 0.6*M* sorbitol in the presence or absence of polyamine inhibitors. After 4 hours of incubation at 25 to 26°C in darkness, triplicate samples were taken for polyamine analysis (*5*) and enzyme assay. Enzymes were extracted at 5°C in 0.1*M* phosphate buffer, *p*H 7.8. The clear supernatant obtained after centrifugation at 26,000*g* for 20 minutes was used. Assay conditions are described in (*14*). The results for the polyamine concentrations are expressed as means ± standard error. Numbers in parentheses express enzyme activities as percentages of the 4-hour control values. Protein was determined according to the Coomassie blue G-250 assay (*23*), with gamma globulin being used as the standard.

Treatment	Polyamine (nmole/g fresh weight)			Enzyme activity per hour (nmole of CO_2/mg protein)	
	Putrescine	Spermidine	Spermine	Arginine decarboxylase	Ornithine decarboxylase
Control (at 0 hour)	16 ± 1	161 ± 18	41 ± 4	2.27 (115)	4.24 (93)
Control (at 4 hours)	13 ± 2	152 ± 10	38 ± 3	1.98 (100)	4.55 (100)
Sorbitol	102 ± 5*	120 ± 13†	21 ± 2*	3.81 (192)*	4.65 (102)
Sorbitol plus $10^{-3}M$ DFMO	129 ± 7*	186 ± 10†	32 ± 3	5.31 (268)*	4.68 (103)
Sorbitol plus $10^{-4}M$ DFMA	20 ± 2†	123 ± 7†	36 ± 2	1.24 (63)†	4.64 (102)
Sorbitol plus $10^{-3}M$ DFMA	10 ± 1	128 ± 8	32 ± 2	0.41 (21)*	4.55 (100)

*Significantly different from control at 4 hours ($P < .01$). †Significantly different at $P < .05$.

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which putrescine is synthesized solely by decarboxylation of ornithine, putrescine can be formed in higher plants via agmatine through the action of arginine decarboxylase (E.C. 4.1.1.19). There is evidence for the widespread coexistence of these pathways (*4, 13*). Since both ornithine decarboxylase (ODC) (E.C. 4.1.1.17) and arginine decarboxylase (ADC) are present in oat leaves (*14, 15*), these enzyme activities were monitored in the osmotically stressed oat leaf segments. As shown in Table 1, ADC activity declined slightly during incubation in buffer but increased 92 percent in the presence of osmoticum; ODC activity remained unaffected. The changes in ADC activity were paralleled by a 7.8-fold increase in putrescine. The magnitude of these responses was similar to those previously observed under potassium deficiency in oat (*15*), upon acid feeding and hormone treatment in *Cucumis sativus* (*16*), and upon acid treatment in barley (*10*).

To further elucidate the mechanism of putrescine increase under osmotic stress, we tested the effect of several polyamine biosynthetic inhibitors. DL-α-Difluoromethylarginine (DFMA) is an enzyme-activated irreversible inhibitor of bacterial ADC that is effective in vivo and in vitro (*17*). It completely prevented the increase in putrescine while concomitantly inhibiting ADC activity in a concentration-dependent manner (Fig. 1 and Table 1). DFMA did not affect the spermidine and spermine titers. In contrast, DL-α-difluoromethylornithine (DFMO), an analogous inhibitor of mammalian ODC (*18*), had no effect on ODC activity in vivo or in vitro but potentiated the putrescine response to sorbitol and in addition increased spermidine levels. Although DFMO is ineffective against *Escherichia coli* and *Klebsiella pneumoniae* ODC (*19*), it augments ADC activity when added to exponentially growing cells. This regulatory phenomenon remains unexplained, but it is clear that DFMO relieves the block in spermidine formation from putrescine caused by osmotic shock (Table 1). No cross-inhibitions have been observed in assays in vitro, supporting the specificity of DFMA and DFMO. That ADC is indeed preferentially activated by osmotic shock is suggested also by these additional observations: (i) α-Methylornithine, a competitive inhibitor effective against ODC (*20*), has almost the same effect on stressed leaves as DFMO; that is, it increases the putrescine and spermidine titers above the sorbitol treatment. (ii) D-Arginine and L-canavanine, which inhibit oat ADC by 60 and 50 percent, respectively, when present at 1 m*M* in the in vitro assay (*15*), also prevent the increase in putrescine when added at the same concentration to osmotically shocked leaf segments.

Although most of the previously reported responses of putrescine to stress take place during several days, the increase in putrescine in cells exposed to osmotic shock or high external acidity (*6*) occurs within a few hours. The apparent time lag for the putrescine increase (Fig. 1), together with the effects of polyamine biosynthesis inhibitors, led us to suppose that new synthesis of ADC was involved in the response. Cycloheximide (10 to 50 μg/ml) prevents the increase in putrescine if added during the first hour of osmotic shock but is ineffective when given at a later time, thus confirming the supposition. Since the response of putrescine to osmotic stress is greatly enhanced by light (compare Fig. 1 with Table 1), it is possible that a previously described phytochrome control of ADC activity is involved (*21*).

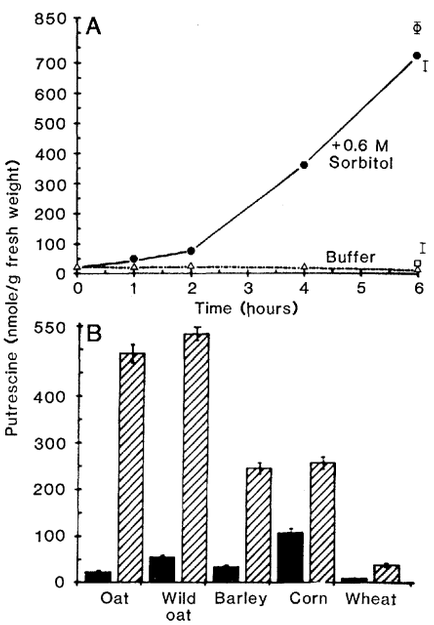

Fig. 1. (A) Changes in putrescine titer in peeled oat leaf segments floated on buffer (△), buffer plus 0.6*M* sorbitol (●), and sorbitol in the presence of 10^{-3} *M* DFMO (○) or 10^{-3} *M* DFMA (□) (see text). Leaf segments were incubated at 25°C under fluorescent light (about 12 kilolux). At each time point, triplicate samples were extracted at 5°C in 5 percent perchloric acid (PCA) for 30 minutes, at a ratio of 100 mg of leaf per milliliter of PCA. After centrifugation at 26,000*g* for 20 minutes, the supernatant fraction was benzoylated or dansylated and analyzed as in (*5*). Error bars are shown to the right of the curves. (B) Putrescine titer in cereal leaves under osmotic stress. Peeled leaf segments from 7-day-old seedlings of oat cv. Victory, *Avena fatua* (wild oat), barley cv. Himalaya, and 9-day-old hybrid corn Golden Cross Bantam were floated over buffer (solid bars) or buffer plus 0.4*M* sorbitol (hatched bars) as in (A). Wheat cv. Yamhill leaf segments were floated unpeeled. After 4 hours of incubation under light, triplicate samples were extracted and dansylated, and the polyamines were analyzed by thin-layer chromatography (*5*).

If the restriction in spermidine and spermine formation from putrescine (possibly involving polyamine synthases as well as ADC) persists during the culture of cereal protoplasts, it could lead to a depletion of these polyamines. Since their titers show close correlations with cell division (*22*), the manipulation of endogenous spermidine and spermine by the use of inhibitors such as DFMO could lead to renewed mitosis. This would increase the effectiveness of polyamine addition to the medium, which has thus far been of limited value (*2, 3*) for cereal protoplast culture.

Note added in proof: We have now found that the putrescine content also rises in oat seedlings allowed to wilt by withholding water. With this treatment, spermidine and free ammonia titers also rise. The increased polyamine levels do not fall rapidly after rewatering of the plants and subsequent recovery from stress.

HECTOR E. FLORES
ARTHUR W. GALSTON
Department of Biology,
Yale University,
New Haven, Connecticut 06511

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Glycolipids in Mammalian Epidermis: Structure and Function in the Water Barrier

Abstract. *In the epidermis of terrestrial vertebrates, lipid lamellae between the horny cells are thought to form a barrier to water loss. The lipids are extruded from living cells after assembly in lamellar granules. This assembly might be promoted by recently identified 1-(3'-*O*-acyl)-β-D-glucosyl-*N*-(ω-hydroxyacyl)sphingosines, which have 30- and 32-carbon hydroxy acids as amides and linoleic acid esterified to glucose. Such a role for these molecules could explain the effects of essential fatty acid deficiency, in which the lamellar granules fail to assemble and the barrier to water diffusion is lost.*

The thin horny layer of terrestrial vertebrate skin forms an efficient barrier to water loss (*1*), which is greatly impaired by extraction with polar solvents (*2*). In mammals, birds, and reptiles, the intercellular spaces in the horny layer contain multiple membranous sheets (*3*). These appear to be derived from lamellar granules discharged from the uppermost living epidermal cells after the granules have accumulated during cell progression from the germinative basal layer toward the horny layer (*4*). In animals deprived of linoleic acid, the skin becomes scaly and more permeable to water (*5*), the lamellar granules appear empty, and the horny layer is deficient in intercellular membranes (*6*). A mechanism for this effect of essential fatty acid deficiency has not been explained.

The dead epidermal horny layer of mammals, birds, and reptiles contains ganglioside sulfates, ceramides, cholesterol, cholesteryl sulfate, and free fatty acids, but no phospholipids (*7, 8*). The living epidermal cells also contain these lipids, as well as phospholipids and several series of glucosylceramides (*8*). In mammalian epidermis, the major and least polar glucosylceramide was reported to have fatty acids esterified in the 3-position of glucose, and the amide-linked fatty acid was said to contain 35 carbon atoms and have two hydroxyl groups and two double bonds somewhere near the middle of the molecule (*9*). The esterified acids contained a high proportion of linoleic acid, ranging from 34 percent in neonatal mice to 56 percent in human skin and 77 percent in pig epidermis.

We have investigated the acylglucosylceramides from pig epidermis and found (*10*) amide-linked ω-hydroxy acids having mainly saturated and monounsaturated chains of 30 and 32 carbon atoms, respectively. Linoleic acid comprised 74 percent of the esterified acids. The dimensions of such molecules (Fig. 1) would allow the hydroxy acid chains to extend across both hydrocarbon palisades of a fluid bimolecular lipid membrane, with the hydroxyl group anchored in one polar surface and the glucose portion in the other surface. Furthermore, the fatty acids esterified to the glucose should be capable of extending into the hydrocarbon region of a second membrane, holding it in close apposition. This could constitute the mechanism by which multiple layers of intracellular membrane are assembled for inclusion in lamellar granules (Fig. 2). A deficiency of linoleic acid might result in synthesis of insufficient or inappropriate acylglucosylceramides, resulting in the observed nonassembly of lamellar granules during essential fatty acid deficiency (*6*).

Assembly of the lamellar granules appears to take place in the endoplasmic reticulum and Golgi regions (*11*). From the known compartmentalization of lipid synthesis (*12*), it can be inferred that the acylglucosylceramides are also produced in these regions. Thus, while the synthesis of 16-carbon fatty acids is accomplished by soluble enzymes in the cytoplasm, further extensions in chain length are mediated by enzymes bound in the endoplasmic reticulum. Such chain extension could continue, so that the growing chains progressed across the entire lipid region of the endoplasmic membrane until, at a length of 30 carbon atoms, the methyl group of the fatty chain would emerge into the endoplasmic space, there to be hydroxylated by a microsomal oxidase (*13*). Once thus anchored in the membrane, incorporation of the hydroxy acid into glucosylceramide would most likely occur in situ. Subsequent attachment of linoleic acid to the glucose portion could provide attraction for adjacent folds of membrane, resulting in the observed stacking of disk-shaped sections of membrane in cisternae (*11*).

Lipids with the molecular dimensions of the acylglucosylceramides should not be required for maintaining the extracellular sheets of lipid membranes once they are formed, since similar membrane structures exist in myelin and in liposomes prepared in vitro from myelin lipids or isolated phospholipids (*14*), all of which lack such molecules. We suggest that the function of the linoleic acid–containing structures is limited to aggregation of the disks of membrane that are stacked in the lamellar granules. These remain coherent, even though they are no longer enclosed in a bounding membrane, for some time after extrusion from the granular cells. Reorganization of the disks into intercellular sheets could depend on the observed disappearance of the acylglucosylceramides. Adequate polarity for the maintenance of the intercellular lamellae would then be provided by the ganglioside sulfates, which together with ceramides, cholesterol, cholesteryl sulfate, and free fatty acids,

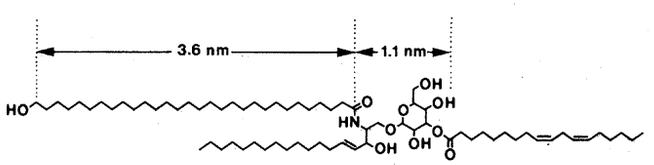

Fig. 1. Structure of the major acylglucosylceramide from pig epidermis, showing the dimensions of the molecule.

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