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The Role of Polyamines in Growth and Senescence of Plants Under Stress Conditions with Special Reference to Horticulturally Important Organs and Tissues

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Abbreviations

ACC: 1-aminocyclopropane-1-carboxylic acid
ADC: L-arginine decarboxylase
Agm: Agmatine
AVG: aminoethoxyvinylglycine
DAP: 1,3-diaminopropane
DFMA: difluoromethyl arginine
DFMO: difluoromethyl ornithine
IBA: indole-3-butyric acid
MGBG: methylglyoxal-bis(guanyl hydrazone)
ODC: L-ornithine decarboxylase
PA,PAs: polyamines (including di-, tri- and tetramines)
PAO: polyamine oxidase
Put: putrescine
SAMDC: S-adenosylmethionine decarboxylase
Sp,Spm: spermine
Spd: spermidine

1. Abstract

Polyamines and their biosynthetic enzymes, as well as polyamine precursors and intermediates, are ubiquitous in plant tissues. As a class of compounds, polyamines are naturally-occurring polycations which function in plant growth and development similar to their participation in various physiological events of bacteria and animals. The investigations which were conducted during the 3 years of the present research revealed some of the biochemical aspects of plant polyamines, and their participation in growth and in the response to stress conditions.

1. The intracellular concentration of several polyamines, the activity of arginine decarboxylase and ornithine decarboxylase, and precursor incorporation were found to be dependent upon the type of tissue and growth processes. In several cases, the increase in endogenous polyamines and in their biosynthesis enzymes is correlated with growth.
2. Polyamines are involved in several growth processes and in plant response to growth hormones (auxins, gibberellins, cytokinins) and exogenous stimuli (light, excision), and causal relationships have been established in some cases. Thus, auxin-induced root formation, root elongation, GA-induced elongation of light-grown internodes, and initiation of sprouting in potato tubers - were all accompanied by an increase in polyamine titer and their biosynthesis, and inhibitors of polyamine biosynthesis inhibited, in some cases, the growth process.
3. Polyamines retard several aspects of plant senescence and participate in plant response to certain stress conditions. Leaf senescence is retarded and PA have been shown to prevent chlorophyll loss, to inhibit protease and ribonuclease activity, and to inhibit ethylene biosynthesis. Solute leakage from cells and RNase activity of wounded tissues are also retarded by exogenous polyamines. Polyamine accumulation and metabolism is closely

linked to plant response to osmotic and salt stress: under these conditions, putrescine (and other PA) accumulate in specific tissues as a result of preferential activation of ADC and ODC and increased PA biosynthesis from their precursors arginine and ornithine. This may contribute to plant adaptation to salt stress.

We conclude that polyamines have an important intracellular role in plant growth and response to stress, possibly as 'messengers' of plant hormones and other growth stimuli. Further research should elucidate their molecular effects and their practical implications.

2. Body of the Report

2.1 General Introduction

Polyamines, as well as their biosynthetic enzymes and precursors, are ubiquitous in bacteria and in animals, and their stimulatory effect on growth has been established in many organisms. Moreover, a close correlation between the intracellular concentration of polyamines and the growth rate was found, and the increase in polyamine content and in the activity of their biosynthetic enzymes usually precedes, or is simultaneous with the increase in nucleic acid and protein levels. Although it is difficult to ascribe a single function and a single mechanism of action to polyamines, it is assumed that the biological role of polyamines stems from their polycationic nature. This is manifested at both the structural-organizational level and the biochemical level: (a) polyamines are able to stabilize cellular membranes and subcellular organelles, and, thus, they protect cells against lysis and preserve the normal uptake and structural functions of tissues and cells, and (b) due to their polycationic nature, polyamines bind strongly to both DNA and RNA, thereby stabilizing their secondary structure. As a result, polyamines have been shown to have a variety of stimulatory effects on RNA, DNA and protein biosynthesis as manifested by their controlling certain aspects of DNA-primed RNA polymerase, nucleases, stability of ribosomes and binding of amino acyl transfer RNA to ribosomes (16,48,174,176,193).

The chemistry, occurrence and certain biochemical aspects of plant polyamines have been studied quite extensively during the last 10-15 years by Smith and his colleagues, as well as by others (171,176). However, earlier studies of the biological function of polyamines in plant growth and development, except for their role in maintaining the cation/anion balance in cases of potassium deficiency (81,172), were limited to a series of work by Bagni et al (19,23,31). The renewed interest in plant polyamines resulted in several recent reports which indicate that

polyamines may have an important regulatory role in plant growth and development, similar to their functions in procaryotes and higher animals. The following aspects seem of special interest in this respect.

Growth effects: active growth and cell division of tumors of Scorzonera hispanica and Sesamum, as well as of habituated tissues of Nicotiana glauca, was correlated with higher levels of putrescine and spermidine (15,23,105,82). Differentiation of embryogenic cells of Daucus carota was accompanied by elevated content of polyamines and by higher rates of putrescine biosynthesis (126,127). A similar dramatic rise in ODC (ornithine decarboxylase) activity was observed in actively dividing carrot cells and in initial stages of tomato fruit development (75). It also appears that polyamine content and the activity of certain enzymes related to polyamine metabolism change significantly in germinating seeds (11,20,152,180,203).

Nucleic acids: polyamine-mediated activation of protein synthesis in isolated ribosomes, as well as strong binding to RNA species, have been shown in spinach, pea and Jerusalem artichoke (21,23). Spermidine interacts with chromatin-bound RNA polymerase of soybean hypocotyls which requires Mg^{++} or Mn^{++} (71). Polyamines stimulated the oxidation of NADPH by spinach flavoprotein-iron-sulphur complex, probably by affecting the binding between flavoprotein and iron-sulphur protein (135). Also, polyamines were extremely efficient in replacing the Mg^{++} requirement in the ribosome cell-free incorporation system of polyphenylalanine synthesis (45). The senescence-retarding effect of polyamines in oat protoplasts was found to be mediated, at least in part, by the inhibition of senescence-induced rise in ribonuclease activity, as verified by polyamine interaction with both the enzyme system and the substrate (65,94).

Senescence and tissue deterioration: recent investigations indicate that polyamines may play an important regulatory role in a variety of plant cells and

tissues which are subjected to stress conditions such as senescence, wounding, heat, salinity, etc. Thus exogenous application of arginine and lysine, as well as of cadaverine, spermidine and spermine, retarded the progressive senescence of oat leaf protoplasts, stabilized them, supported a higher incorporation of uridine and leucine into macromolecules, and augmented mitotic activity (9,65,67,94). These findings were later extended to detached leaves of several plant species and it was demonstrated that polyamines retard chlorophyll breakdown and prevent the rise of RNase and protease (9,44,96). In addition, it was found that polyamines may have a 'protective' action on seed germination at high temperature, and are correlated with plant growth under SO₂ pollution and saline conditions (14,138,150). Other recent observations indicate that polyamines may stabilize membrane functions during senescence (44,135,148).

While the involvement of polyamines in the regulation of plant growth and physiology was limited in the past to few studies (23,174), accumulating evidence during the last 5-6 years indicates that PA can be regarded as a new class of plant growth substances (5,6,7,66). Thus, in recent years PA have been shown to participate in various aspects of plant growth processes and in the response to specific stress conditions and exogenous stimuli (5,6).

The foregoing suggests that polyamines play an important role in the control of growth and senescence in plants in general, and particularly in plants subjected to stress conditions. Recent data which support this assumption, and which were achieved within the framework of the BARD grant, will be reviewed in the following.

2.2 General Materials and Methods

General analytical procedures which were employed throughout this study, as well as the various plant species and tissues, will be described in the following. Specific treatments and modifications of procedures will be mentioned later, where appropriate.

2.2.1. Layout of experiments.

Various types of experiments, using several methodologies, were employed:

1. Application of exogenous polyamines and their immediate precursors (e.g. putrescine, spermidine, spermine, arginine, ornithine).
2. Treatment of whole plants, or excised tissues, with structural analogues of PA and their immediate precursors, and with inhibitors of PA biosynthesis, such as L-canavanine, L-canaline, difluoromethyl ornithine (DFMO), difluoromethyl arginine (DFMA), and methylglyoxal bis (guanyl hydrazone) (MGBG).
3. Changes in endogenous PA levels in various plants and tissues in response to environmental changes or specific treatments.
4. Studies of PA biosynthesis, including the activity of several enzymes such as L-arginine decarboxylase (ADC), L-ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase, as well as the incorporation into polyamines of labeled L-arginine, L-ornithine, L-citrulline, putrescine, L-methionine and related compounds.
5. Studies of PA degradation and conjugation, and especially the activity of amine oxidases.
6. Investigation of the control of polyamine biosynthesis by phytochrome and gibberellin.
7. Studies of osmotic and pH stress on polyamine biosynthesis.
8. Involvement of polyamines in potato bud sprouting.

9. Studies of inhibition of protease activity by polyamines.
10. Investigation of the formation of 1.3-diaminopropane in leaves and its effects on senescence and ethylene biosynthesis.

2.2.2. Plant Material

Plant material for the experiments was collected from greenhouse-grown plants or from plants which were germinated and grown in a controlled phytotron or in a growth chamber. The following various types of plant material was used:

1. Intact or excised leaves from tobacco, dwarf bean, mung bean, radish, oat, barley, wheat and rice were used in studies of leaf senescence, following various treatments.
2. Buds and stem or root sections of mung bean, dwarf bean, pea, oat, potato, and other plants, were used to investigate the interrelationships and various growth responses (environmental conditions such as light, osmotic, ionic, and salt stress; as well as hormone application).
3. Discs removed from potato or beet root tubers were used for studying aging processes and wound response.
4. Adventitious root formation was investigated in mung bean hypocotyl cuttings.
5. Protoplasts were obtained from leaves of oat, Vigna and Trigonella by standard enzymatic techniques.
6. Oat leaves were obtained from uniform seedlings grown in controlled growth rooms. Their lower epidermis was removed by peeling to facilitate penetration of test substances.
7. Etiolated pea seedlings were grown in a thermostated, humid dark controlled growth room and exposed as needed to a standard red or far red source for phytochrome conversion experiments.
8. Dormant potatoes were bought in the open market and stored until spontaneous sprouting occurred.

2.2.3. Analytical procedures

Extraction and analysis of polyamines: plant material was collected, weighed, and extracted in cold 5% perchloric acid (PCA), using an all-glass homogenizer or a pestle and mortar. After centrifugation at 12,000-27,000 g for 20-30 min, the supernatant fraction was used for analysis of free polyamines. Two separate procedures were employed:

(1) dansylation and TLC - dansylation was carried out according to Seiler and Wiechmann (1974) and Flores and Galston (59) with some modifications.

To a 0.2-ml aliquot of the HClO_4 extract, sufficient Na_2CO_3 was added to bring it to a pH of approximately 7.0, followed by an overnight incubation in the dark (room temperature) with 0.4 ml dansyl-chloride (30 mg/ml, in acetone). Excess dansyl chloride was converted to dansyl proline by a 30-min incubation with 0.1 ml L-proline (100 mg/ml), after which dansylated PA were extracted in 0.5 ml toluene. Twenty to 150 μl aliquots of extracts were spotted on activated (1 h at 110°C) TLC plates pre-coated with 375 μm silica gel G-60 (Merck), and co-chromatographed with dansylated PA standards. Plates were developed in ethyl acetate:cyclohexane (2:3, v/v) or, occasionally, in benzene:triethylamine (5:1, v/v), and the spots were traced with long wave UV lamp. Dansylated derivatives were extracted in ethyl acetate and fluorescence measured in a Perkin-Elmer Fluorescence Spectrophotometer 104 (excitation at 365 nm, emission at >500 nm).

(2) Extracts were also analyzed for free polyamines by HPLC according to a benzylation procedure modified for higher plants. Separation, identification and quantification were done with a Beckman 320 HPLC model, equipped with a C-18 reverse-phase column (Altex, 5 μm) and Hewlett-Packard 3990A integrator. Benzoyl-polyamines were eluted with 64% methanol, at a flow rate of 1 ml/min, and detected at 254 nm. Results obtained by TLC and HPLC were reproducible and showed good correlation.

Extraction and assay of ADC, ODC and SAMDC:

Extraction and Assay of ADC and ODC. Sections were harvested, weighed, and ground in a prechilled mortar with a pestle (250-600 mg fresh weight/2 ml medium). Extraction medium consisted of 100 mM phosphate buffer (pH 7.2), 0.1 mM DTT, 1 mM pyridoxal-5'-phosphate and 20 mM Na-EDTA. The extracts were centrifuged at 12,000g for 20 min, and the supernatant, hereafter referred to as crude enzyme, was used immediately. The enzyme reaction was started by adding 100 μ l crude enzyme to a test tube containing 150 μ l of the extraction medium and 50 μ l arginine hydrochloride (Coomissariat a l'Energie Atomique Gif-sur-Yvette, France, 0.125 μ Ci, 20mCi/mmol) hydrochloride (Amersham, 0.125 μ Ci, 59 MCi/mmol) for ADC or ODC assay, respectively. The test tubes were then capped with rubber stoppers fitted with plastic center wells (Kontes Glass Co., NJ) containing 0.2 ml of Soluen-100 (Packard) on a Whatman No. 1 paper wick. The reaction was allowed to proceed for 60 min at 37°C in a shaking water bath. The reaction was terminated by injection of 0.2 ml 4% (v/v) HClO_4 into the reaction medium. After an additional 30-min shaking, the center wells were removed and placed in vials with 10 ml toluene-PPO-POPOP scintillation mixture. Counting efficiency was about 80%, and blank values were obtained by using boiled crude enzyme. The standard procedure was adopted following extensive preliminary experiments, using several compositions of the extraction and reaction media and modified assay procedures. Two to three separate extractions per treatment were used, each assayed in duplicate, and all experiments were repeated 2 to 4 times. ADC and ODC activities are presented as pmol $^{14}\text{CO}_2$ per g fresh weight or mg protein.

Extraction and Assay of SAMC.

For each extraction, approximately 5 g (9 discs) of tissue (potato tuber) were homogenized in chilled mortars with 1.5 ml of 100mM phosphate buffer at pH 7.6. The homogenates were centrifuged at 26,000g for 15 min at 4°C and the resulting clear supernatant fractions were assayed for enzyme activities.

SAMDC assays were done in 12 x 75 mm polystyrene culture tubes sealed with

polyethylene caps. A filter paper disc (6 mm diameter) impregnated with 50 μ l 2 N KOH was supported on a 22-gauge syringe-needle through the cap and used to trap the $^{14}\text{CO}_2$ liberated and activity was determined by measuring the release of $^{14}\text{CO}_2$ from the substrate SAM[^{14}C]carboxyl. The reaction mixture contained 100 μ l crude enzyme, 10 μ l 20 $\mu\text{Ci/ml}$ SAM[^{14}C]-carboxyl (60 mCi/mmol, New England Nuclear Co.) containing unlabeled SAM, to give a final concentration of 2.7 mM and 10 μ l 1 mM pyridoxal phosphate. The enzyme activity was linear with increasing concentrations of crude enzyme and with time of incubation up to 60 min. The temperature for optimal activity was between 35 to 40°C. Thus, the reaction mixtures were incubated for 45 min at 37°C and CO_2 liberated by injecting 0.2 ml 10% TCA into each reaction tube through the syringe needle. The tubes were stoppered and incubated for an additional 45 min in the water bath. The liberated $^{14}\text{CO}_2$ was determined by placing the paper disc into 2 ml Econofluor in mini-vials and counting in a scintillation counter. Enzyme activity was expressed as nmol of $^{14}\text{CO}_2$ liberated/g fresh weight.

Incorporation of labeled precursors: Plant material was labeled with L-[U- ^{14}C] ornithine hydrochloride (Amersham, 285 $\mu\text{Ci/mmol}$), or L-[U- ^{14}C] arginine hydrochloride (Amersham, 345 $\mu\text{Ci/mmol}$), or [U- ^{14}C] putrescine (Amersham). Plant material (leaves, or stem and root sections) was allowed to absorb the radiolabeled compound (usually at 0.5 $\mu\text{Ci}/0.2$ ml) after which additional volume of 1mM phosphate buffer, pH 5.8, was added to the vial. The plant material was removed from the uptake solution at the indicated time, and extracted in cold 5% PCA as described previously. Free polyamines were dansylated and separated on TLC plates (as described before), and the appropriate spots (co-chromatographed with known markers) were collected and radioactivity was measured in a liquid scintillation counter. Alternatively, PCA extract were dansylated and separated on Kodak pre-coated TLC plates for identification of radioactive spots after 10-20 day exposure onto film.

Protein Determination. The HClO_4 pellet was rinsed twice with 80% (v/v) acetone,

and solubilized in 1 N NaOH (45 min at 37°C). Protein was determined according to Lowry et al (116) with BSA standards.

Protease extraction. Fifteen leaf segments (4.5 cm each) were homogenized in a pre-chilled mortar and pestle with 2 ml cold 50 mM phosphate-citrate buffer, (pH 6.0). The homogenates were centrifuged at 12 000 x g for 15 min at 4°C. The resulting clear supernatant fractions were assayed for protease activity.

Protease assay. Protease activity was measured by using Axocoll (Calbiochem) as the substrate, in 1.0 ml a final volume.

Determination of ethylene production.

Air samples (1 to 5 ml) were withdrawn from the bottles with a syringe, and injected into a gas chromatograph apparatus (Perkin Elmer, Model F-11) equipped with an alumina column. Ethylene was identified and quantified by comparing the peaks with the retention time and peak heights of ethylene standards.

Determination of ACC content. Leaf tissue (0.3 g) was ground in liquid nitrogen, then extracted with 3 ml ethanol (80%, v/v) for 16 h at 4°C with constant shaking. The extract was centrifuged at 10,000g for 15 min, resuspended in 3 ml ethanol and recentrifuged as above. Combined supernatants were dried at 37°C in a N stream and residues were dissolved in a known volume of water. After centrifuging the extract at 2,000g for 10 min, we determined ACC content in the aqueous solution by the method of Lizada and Yang (111).

Determination of ACC synthase activity. Preparation and assay of ACC synthase were done as described previously. Leaves (approximately 1 g) were thoroughly washed with water and homogenized in 3 ml K-HEPES buffer (50 mM, pH 8.5, P-L Biochemicals Inc.) containing 0.5 µM pyridoxal phosphate and 4 mM dithiothreitol. The homogenate was centrifuged at 10,000g for 20 min. Proteins in the supernatant fraction were precipitated by slow addition of ammonium sulfate (90% saturation) under gentle stirring for 2 h at 4°C. After centrifugation (15,000g, 20 min), the pellet was

dissolved in 1 ml of dialysis solution, containing 2 mM K-HEPES (pH 8.5), 0.5 μ M pyridoxal phosphate and 0.1 mM dithiothreitol. Dialysis was carried out at 4°C overnight. ACC synthase activity was determined in a reaction mixture containing 0.4 ml enzyme

2.3 RESULTS

2.3.1. POLYAMINE BIOSYNTHETIC ENZYMES

Introduction

ADC (EC 4.1.1.19) and ODC (EC 4.1.1.17) are two key enzymes in the biosynthetic pathway of polyamines. Extensive studies (6,42,48,161) with mammalian cells established ODC as the key enzyme for synthesis of putrescine from L-ornithine, putrescine being metabolized later to spermidine and spermine. In plants however, ODC seemed to be of less importance and it has been claimed that putrescine is synthesized from L-arginine by ADC via the intermediate agmatine (170,176). This was supported by the relative high ADC activity found in plant tissues in response to several growth stimuli and physiological conditions. Thus, ADC activity was increased in embryonic cells of Daucus carota (126) in potassium-deficient barley (170), and in soybean seedlings grown on ammonium (109). Similarly, N⁶-benzyladenine enhanced ADC activity in cucumber Cotyledons (188), and ADC was found to be controlled by phytochrome and GA (53). Other observations indicate, however, that this is not always the case and that both ADC and ODC are active and involved in polyamine biosynthesis (166,182,213). In recent studies (75), ODC activity of developing tomato fruits was found to increase dramatically up to the 3rd day after pollination, while ADC activity was very low, and both arginine and ornithine were equally well decarboxylated in tobacco cell cultures (29).

In view of the contradictory results regarding the activity and importance of ADC and ODC in plants, a comparative study of these two polyamine-biosynthetic enzymes was undertaken.

Materials and Methods.

Mung bean (Vigna radiata L.) seeds were germinated and grown in vermiculite in a controlled phytotron (27°C-day and 22°C-night temperature, 16-h photoperiod, 78% RH). One cm subapical sections of epicotyls were harvested from 7-

to 8-day-old seedlings and were extracted for determination of enzyme activities. Occasionally, other plant parts were extracted as mentioned. Epicotyl sections of French bean (Phaseolus vulgaris L. var. Brittle Wax) and cotton (Gossypium hirsutum L. SJ-2), grown for 8 days under the same conditions, were also used in several experiments.

Enzymes were assayed as described previously (2.2.3).

Results and Discussion

General Properties. The linearity of ADC and ODC activity, with increasing concentrations of crude enzymes, is depicted in Figure 1. The greater increase in ADC activity of mung bean epicotyls resulted in an increasing ADC-to-ODC ratio, when expressed both on fresh weight basis and as specific activity. ADC and ODC activity proceeded at a linear rate for up to 30 min and 60 min, respectively (Fig. 2). All assays were, therefore, run for 30 to 60 min. Optimum pH for both enzymes was 7.2 (Fig. 3), ADC evincing a greater decline in activity at both lower and higher pH values. The K_m of crude ADC and ODC of mung beans was recently found to be $5.86 \times 10^{-4}M$ and $2.53 \times 10^{-4}M$, respectively (Fig. 4). This is close to the $1.4 \times 10^{-4}M$ K_m of partially purified ODC from tobacco cells.

Partial characterization of the enzymic system of mung bean was done by analyzing the effect of α -difluoromethyl-ornithine, an enzyme-activated irreversible inhibitor of ODC (Table 1). Treatment of excised roots with 0.1 mM and 1 mM α -difluoromethyl-ornithine resulted in 55 to 69% inhibition of ODC activity and in a considerable increase in ADC activity. In an attempt to further characterize plant ADC and ODC we investigated the possibility that inhibitory proteins may interfere with the enzyme assay in crude extracts. When extracts were subjected to acid precipitation at pH 4.6 (and in other studies down to pH 4.1), most of the ADC and ODC activity was retained in the supernatant, although a considerable amount of protein was precipitated. The presence of phenylmethylsulfonyl fluoride (PMSF) in

the extraction and assay media resulted in a 2-3 fold increase in the specific activity of both ADC and ODC (Table 2).

This effect was saturated at a concentration of 1.6×10^{-3} M PMSF. It is, thus, possible that plant ODC and ADC may have inhibitory factors similar to mammalian cell ODC inactivation factor (ODIF) (82).

Relative Activities in Various Tissues. The distribution of ADC and ODC activity in mung bean seedlings shows a decreasing gradient from the terminal bud to the roots (Table 3). The ADC and ODC activity of leaves were relatively high, and the lower protein content of the subapical epicotyl section was manifested in higher specific activity values. ADC-to-ODC activity ratio was especially high in epicotyl and roots. A comparison of ADC and ODC activity in intact seedlings and in sections incubated in buffer for 24 h (Table 4) indicates a considerable increase of enzyme activity as a result of excision and incubation. Both ADC and ODC activity increased equally in incubated leaf sections, while a greater increase in ODC activity was especially evident in epicotyl and root sections. The relative activity of the two polyamine biosynthetic enzymes was further investigated in three different plant species (Table 5). Cotton hypocotyls had the lowest ADC activity of the three species which were assayed, while no ODC activity could be detected in French bean hypocotyls. These differences in activity are reflected in the ADC-to-ODC ratio.

It should be mentioned that although most studies indicate that the effect of plant hormones on growth and development is accompanied by an increase in ADC and/or ODC activity (as will be mentioned later), some exceptions do occur. Thus, treatment of mung bean seedlings of different age with gibberellic acid (GA_3) resulted in a marked inhibition of ADC activity, especially in older plants, while ODC activity was very little affected (Fig. 5). Levels of putrescine and spermidine, however, were considerably increased in these experiments due to the GA_3 treatment (Fig. 6). It is, thus, likely that ADC activity was inhibited by accumulation of its product,

putrescine, or that putrescine conjugation to ADC modifies its activity, similar to the suggested transglutaminase-mediated incorporation of putrescine to ODC in mammalian cells (160).

It has been claimed that L-arginine decarboxylation is the main pathway for putrescine biosynthesis in plants (170,176). However, the simultaneous activity of ADC and ODC in mung bean plants and the similarity in their enzymic properties (Figs. 1-3) indicate that no exclusive pathway for putrescine formation should be considered a priori. Rather, the synthesis of putrescine and related polyamines may involve both ADC and ODC as well as other enzymes (Fig. 4). Because both ADC and ODC can be the rate-limiting enzymes in putrescine formation, factors which control their relative activity are of special importance for polyamine biosynthesis and effects in plants. Thus, ADC and ODC activity can be regulated by: (a) concentration and availability of arginine and ornithine; (b) interconversion of arginine and ornithine and the degree of their metabolism other than to putrescine; (c) activation, half-life, and compartmentation of ADC and ODC; (d) de novo enzyme synthesis in response to specific hormonal and physiological stimuli. The tissue-specific activation of ADC or ODC (e.g. a markedly higher ADC-to-ODC ratio in roots. Table 3), the differential stimulation of decarboxylation activity in response to excision and incubation, (e.g. ODC increasing more than ADC. Table 4), and the considerable difference in ADC and ODC activity in various plant species (Table 5) all may be related to the above-mentioned factors. The possible modulation of ADC and ODC is partially evidenced by the observation that blockage of the ODC route results in the activation of the alternative ADC route (Table 1). This is in accordance with earlier observations on multiple pathways for putrescine biosynthesis in Escherichia coli. ODC inhibition by α -difluoromethyl-ornithine did not affect ADC activity of tobacco cell cultures. In addition, arginine and ornithine are equally well incorporated into putrescine, spermidine and spermine (R. Friedman, A.

Altman, U. Bachrach).

Different ADC-to-ODC activity ratios and different patterns of activity are found in various tissues of a given plant, and in various plant species. Recently it was found that the different patterns of ADC and ODC activity in the peel and pulp of developing Citrus fruit follow the growth pattern and other physiological activity of these two tissues (Nathan, Altman and Monselise, unpublished data). The range of activities found by us in various plants and tissues is 0.3-750 nmoles CO_2 /mg protein for ADC and 0.03-250 nmoles CO_2 /mg protein for ODC. These values are in the range of ADC and ODC activity found in bacteria and ODC activity found in mammalian tissues, and in some cases considerably higher.

The above-mentioned studies, as well as additional data which will be reported later, thus, suggest that both ADC and ODC are active in plant tissues and that their relative contribution to putrescine (and polyamine) biosynthesis is dependent upon the type of tissue and the growth process. The presence of two enzymes with a similar function, resulting in two alternative pathways of putrescine biosynthesis, may have an important bearing on the control of polyamine levels and effects in plants. This will be discussed later.

Figure 1. ADC and ODC activities of mung bean epicotyls as a function of crude enzyme concentration.

Figure 2. ADC and ODC activities of mung bean epicotyls as a function of reaction time.

Figure 3. Effect of pH on ADC and ODC activities of mung bean hypocotyls.

Figure 4. Arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) activity with increasing substrate concentrations (L-arginine and L-ornithine, respectively). Assayed according to Altman *et al.* (4).

Figure 5. Effect of gibberellic acid (GA) treatment on ADC and ODC activity in the sub-apical region of mung bean seedlings of different age. Seedlings, germinated on different dates, were sprayed with water (control) or 10^{-4} M GA₃ 2 days prior to harvesting. Seedlings were 6, 8, 10 or 12 days old when harvested. The 10 mm sub-apical region of the epicotyl was extracted and enzyme activities were determined according to Altman *et al.* (Adapted from Galston, Altman and Levin, unpublished data).

Figure 6. Effect of gibberellic acid (GA) treatment on polyamine levels in the sub-apical region of mung bean seedlings of different age. Plants were treated as described in the legend for Figure 5. Free polyamines were extracted and assayed according to Friedman *et al.*

Table 1. Effect of α -Difluoromethyl-Ornithine on ADC and ODC
Activity of Excised Mung Bean Roots

Lateral roots from 8-day-old plants were excised and incubated for 24 h (25°C, dark) in buffer solution or with α -difluoromethyl ornithine.

	ADC	ODC	ADC	ODC	ADC/ ODC
	pmol CO ₂ -mg protein ⁻¹ h ⁻¹		%		ratio
Control	981	527	100	100	1.86
α -difluoromethyl ornithine, 1 mM	1,556	239	159	45	6.51
0.1 mM	1,200	165	122	31	7.27

Table 2. Partial purification and 'activation' of L-arginine and L-ornithine decarboxylases of mung bean^a

	pmole CO ₂ -mg protein ⁻¹ h ⁻¹	% of crude control	
	ADC	ODC	ADC ODC
Crude, control	221	66	100 100
Crude + PMSF	688	251	310 380
Acid prec., supernatant	278	75	125 114
Acid prec., supernatant + PMSF	657	186	297 281
Acid prec., pellet	27	6	12 9
Acid prec., pellet + PMSF	68	15	13 23

^aADC and ODC were extracted from leaves of 11-day old mung bean plants as described previously, either without or with phenylmethylsulfonyl fluoride (PMSF) at final concentration of 3×10^{-3} M. The 12000 x g supernatant was subjected to acid precipitation at pH 4.6 (phosphate buffer), and the resulting supernatant and pellet were assayed separately.

Table 3. Distribution of ADC and ODC Activity in Mung Bean Seedlings. Eight-day-old seedlings were separated as follows: terminal bud, dissected above the petioles of the first pair of leaves; leaves, blades of the first pair of leaves; epicotyl, 1-cm section of the subapical epicotyl; hypocotyl, 1-cm section of hypocotyl just below the cotyledonary node; roots, 1-cm terminal section of lateral roots.

	ADC	ODC	ADC	ODC	ADC/ ODC
	pmol CO ₂ xg fresh wt ⁻¹ h ⁻¹		pmol CO ₂ xmg protein ⁻¹ h ⁻¹		ratio
Terminal bud	1,252	408	1,475	483	3.05
Leaves	1,404	503	1,465	524	2.80
Epicotyl	400	96	2,484	582	4.27
Hypocotyl	47	16	554	179	3.09
Roots	92	15	354	62	4.71

Table 4. ADC and ODC Activity in Intact and Dissected Organs of Mung Bean Seedlings. Intact plants were assayed as in Table 3. Leaf discs and 1-cm sections of subapical epicotyl and lateral roots were incubated for 24 h (25°C dark) in a buffer solution.

	Intact		Sections		ADC sections	ODC sections
Organ	ADC	ODC	ADC	ODC	ADC intact	ODC intact
	pmol CO ₂ mg protein ⁻¹ h ⁻¹				ratio	
Leaf	429	135	600	207	1.40	1.53
Epicotyl	1,703	302	2,972	858	1.75	2.84
Root	542	70	1,020	200	1.88	2.86

Table 5. ADC and ODC Activity in Three Plant Species. Activity was measured in crude extracts of subapical 1-cm epicotyl sections of 7-day-old seedlings.

	ADC	ODC	ADC	ODC	ADC/ ODC
	pmol CO ₂ g fresh wt ⁻¹ h ⁻¹		pmol CO ₂ mg protein ⁻¹ h ⁻¹		ratio
Mung bean	2,709	1,294	7,292	3,381	2.3
French bean	1,002	0	1,681	0	
Cotton	290	172	259	152	1.7

2.3.2. POLYAMINE AND WOUNDED STORAGE TISSUES.

Introduction

Wounded plant tissues evince several dramatic changes which culminate either in rapid deterioration (senescence) and death, or else, if appropriate conditions are supplied, in renewed cell division. These changes may include a marked rise in respiratory activity (87), activation of nucleic acid and protein synthesis (88), increased activity of RNase, proteases, peroxidases, and several other enzymes (37,88,145), rapid ethylene evolution (212), and loss of membrane function, including structural changes and solute leakage (28,147,201).

Polyamines stabilize bacterial protoplasts against lysis (118,191), possibly by binding to cytoplasmic membranes (70). Recent data suggest that polyamines may function in a similar way in plant tissues, regulating some aspects of plant growth and development (5,66). Thus, oat leaf protoplasts were stabilized through PA-mediated inhibition of senescence (9,65), and PA have been shown to stabilize leaf membrane function during senescence (148), and to decrease membrane leakage of damaged beet root discs (134,135). While the role of PA in these processes has not been fully elucidated, the polycationic nature of PA lends support to the hypothesis that they function by binding to negatively-charged sites of nucleic acids and of cell membranes (16,48,92). This binding may stabilize nucleic acids and cell membranes against senescence-induced enzymatic degradation and solute leakage, respectively.

The present study was aimed at elucidating the following questions: (a) Do exogenously-supplied PA inhibit stress-induced RNase activity and solute leakage of storage tissues, similar to their effect in senescing leaves and protoplasts? (b) Is the effect limited to a general rise in RNase, or is it involved in the specific wound-induced RNase? (c) Can PA stabilize damaged membranes against excessive solute efflux? Two parameters were employed: RNase activity of potato tuber discs and

betacyanin leakage from beet root discs.

Materials and Methods

Potato tubers (Solanum tuberosum L.) and beet roots (Beta vulgaris L.) were purchased locally and stored at 3-6°C until used. Discs 10 mm in diameter and 2 mm thick were prepared aseptically from surface-sterilized tubers (CaOCl₂, 9%w/v, for 15 min.). Freshly prepared potato discs were pretreated for 0.5 h (following 5 min. vacuum infiltration) in solutions of polyamines and related compounds (HCl salts), all dissolved in 1 mM KH₂PO₄-Na₂HPO₄ buffer, pH 5.8. Two to five discs were then incubated at 25°C in darkness on a moist filter paper in petri dishes for periods of up to 24 h. Freshly prepared beet root discs were washed for 0.5 h in running tap water and then for 0.5 h in several changes of deionized water. Beet root discs were either pretreated with polyamine solutions as mentioned previously, or incubated in solutions which contained polyamines and other additives, all dissolved in 1mM phosphate buffer, pH 5.8 as mentioned. Two to four discs were incubated in 5 ml appropriate solutions in vials, at 25-40°C in darkness, for 1-24 h.

Ribonuclease activity of potato discs was assayed with purified yeast RNA as described previously (Altman 1982). RNase activity is expressed as $\Delta A_{240\text{nm}}/\text{h} \times \text{g}$ fresh weight of discs. Betacyanin leakage was followed by measuring the absorbance of the ambient solution at 540 nm, and data are expressed per 2 or 4 discs.

Results and Discussion

RNase activity in potato tuber discs

Several polyamines and metabolically-related compounds inhibited the dramatic rise in ribonuclease activity of potato discs following wounding (Table 6). Structure-related differences are, however, evident: the triamine, spermidine, and the tetra-amine, spermine were most effective, followed by L-arginine. Both L-ornithine, and especially the di-amine, cadaverine, were less effective. RNase inhibition in wounded discs is concentration-dependent, as found with discs

pretreated with various concentrations of spermidine (Table 7). Only moderate inhibition was noticed at concentrations of up to 1mM, and it increased progressively at 10 mM, when the wound-induced rise in RNase activity was inhibited. Higher and lower spermidine concentrations were not tested. In order to elucidate further the nature of the PA effect on the increase in ribonuclease activity, two degrees of wounding were examined (Table 8). RNase activity of standard discs was 3 times higher than that of spermidine and spermine-treated discs, supporting previous data. Excessive wounding of discs ('chopped') resulted in a continued increase in RNase activity, and this additional increase was almost completely inhibited by PA.

Betacyanine leakage from beet root discs

Since increased RNase activity is just one of the senescence-related phenomena of wounded storage tissues, the effect of PA on an additional parameter was investigated (Table 9). Betacyanin leakage from beet root discs increased upon incubation at 40°C or after RNase treatment at 25°C for 24 h; spermidine pretreatment of discs considerably inhibited membrane leakage under both conditions. Similar results were found with spermidine and spermine at 5 mM. In order to eliminate long-term effects of overall senescence and deterioration, which may interfere with initial effects of excision, betacyanin leakage was also investigated in discs incubated no longer than 3 h (Table 10). Spermine inhibited to about 50% leakage from discs incubated at 25°C. Leakage was considerably enhanced by incubation at 37°C or by freezing and thawing of discs (both treatments implying membrane damage), and spermine effectively inhibited this excessive betacyanin leakage. The data indicate that the spermine effect was saturated at a concentration of 1 mM. The protective effect of PA was further elucidated by following betacyanin leakage under conditions which may induce membrane damage (Fig. 7). The various treatments resulted in various degrees of enhanced betacyanin leakage, and the presence of 1 mM spermine in the incubation solution effectively inhibited the leakage. This is

especially evident with frozen discs at all incubation periods, and with protease-treated discs at 5 and 22 h.

These results are supported by two additional independent observations: (1) the survival of partially-imbibed mung bean seeds which were subjected to cryogenic storage in liquid N_2 was considerably increased by spermine, but not by the standard cryoprotectant dimethyl sulfoxide (DMSO) (Table 11), and (2) spermine effectively blocked the rise in peroxidase activity in discs of *Duranta* leaves (Table 12). The latter effect was similar in both green and white discs (excised from different areas of the variegated leaves), indicating that the polyamine effect on senescence is not necessarily linked with chlorophyll loss in senescing leaves.

Increased RNase activity (Tables 6,7 and 8) and betacyanin leakage (Tables 9 and 10, Fig. 7) reflect the response of storage tissues to wounding and other adverse conditions. This response may be attributed both to short-term, direct, wound effects and to long-term, indirect, senescence effects resulting from metabolite deprivation and general deterioration (28,88). In either case, nucleic acid degradation and loss of membrane functions can be visualized as primary causing events. Thus, mechanical damage to leaves and tubers of *Solanum tuberosum* causes a rapid dramatic increase in RNase activity, which continues to increase for the first 15-24 h following the damage (145). The increased RNase activity in response to mechanical damage and wounding is probably related to enzyme activation rather than to de novo synthesis (88). Similarly, excision of beet root and potato storage tissue resulted first in extensive membrane degradation (83) which is reflected in the activity of several lipases (64,72). Increased synthesis and rearrangement of membrane components occur at later stages only, prior to the increase in mitotic activity (28). Changes in solute efflux, in response to various treatments, thus, represent destabilization-stabilization properties of membranes.

How do polyamines inhibit the rise in RNase activity and betacyanin leakage, as

found in the present study? The efficiency of tri- and tetraamines, as compared with other compounds (Table 6), and the concentration-dependent effect (Table 7), support previous observations and hypotheses that binding of PA to negatively-charged sites of polynucleotides and to cell membranes stabilizes them (16,70,118,191). Thus, inhibition of RNase activity by PA can be attributed to 2 separate mechanisms:

(1) direct binding to the substrate, the RNA, or (2) membrane stabilization following wound-induced damage (e.g., release of RNase from lysosomal compartments?). The fact that potato discs, but not the yeast RNA, were pretreated with PA and the observed correlation with wound-induced RNase (Table 8), favors the latter. The increased leakage of betacyanin in response to several destabilizing treatments and its retardation by PA (Tables 9 and 10, Fig. 7, 135), suggest that PA may act directly on wound-induced membrane changes which are enhanced at high temperature and by freezing and thawing. While the components of intact membranes are relatively stable to enzymatic degradation, they can become substrates for proteases and other hydrolases as soon as they dissociate from the complex (88). This may explain the observed effects upon treatment of wounded discs with protease and RNase (Table 9, Fig. 7).

The foregoing, as well as previous data (3,5,9,135,148) suggest that PA may interact with primary senescence-induced destabilization of plant cell membranes. Binding of these biologically-active polycations to phospholipid components of the membrane is one possible mechanism.

Figure 7. The protective effect of spermine on induced betacyanin leakage from beet root discs. Freshly prepared discs were incubated in buffer (control) or in buffer solutions containing 500 μ g/ml bovine pancreas RNase or 500 μ g/ml *Streptomyces* protease; discs were frozen, thawed quickly and incubated. All incubations were carried out at 25°C, with or without 1 mM spermine.

Table 6. Inhibition of wound-induced RNase of potato tuber discs by polyamines and related metabolites. Discs were incubated for 24 h following a pretreatment with the listed compounds, all at 10 mM. RNase activity of freshly-cut discs: $0.045\Delta A_{260\text{nm}}/\text{g fresh wt} \times \text{h}$. Values are significantly different if followed by different letters ($P = 0.05$). Inhibition in % was calculated from 24 h control activity.

Compound	RNase activity, 24 h after wounding	
	$\Delta A_{260\text{nm}}/\text{g fresh wt} \times \text{h}$	% inhibition
Control (buffer)	0.224 a	0
L-ornithine	0.105 c	53
L-arginine	0.082 cd	63
Cadaverine	0.151 b	33
Spermidine	0.058 d	74
Spermine	0.056 d	75

Table 7. The effect of various concentrations of spermidine on wound-induced RNase of potato discs. Discs were incubated for 24 h following a pretreatment with spermidine. RNase activity of freshly cut discs: $0.065\Delta A_{260\text{nm}}/\text{g fresh wt} \times \text{h}$. Inhibition in % was calculated from 24 h control activity.

Spermidine (mM)	RNase activity, 24 h after wounding	
	$\Delta A_{260\text{nm}}/\text{g fresh wt} \times \text{h}$	% inhibition
0	0.187 a	0
0.1	0.183 a	2
0.5	0.141 b	25
1.0	0.132 b	29
5.0	0.099 c	47
10.0	0.062 d	67

Table 8. Inhibition of wound-induced RNase in 'intact' and 'chopped' potato discs by polyamines. Discs were pretreated with polyamines and incubated for 24 h either with no additional wounding ('intact') or following excessive additional wounding ('chopped'). Data are expressed also as % of initial activity ($0.077 \Delta A_{260\text{nm}}/\text{g fresh wt x h}$).

Pretreatment	RNase activity, 24 h after wounding			
	Discs		Chopped discs	
	$\Delta A_{260\text{nm}}/\text{g fresh wt x h}$	%	$\Delta A_{260\text{nm}}/\text{g fresh wt x h}$	%
Control (buffer)	0.360 b	468	0.574 a	746
Spermidine, 10 mM	0.105 c	136	0.140 c	182
Spermine, 10 mM	0.110 c	143	0.130 c	169

Table 9. Inhibition of betacyanin leakage from beet root discs by spermidine. Discs were aged for 24 h at 25°C or 40°C in buffer or in 400 µg/ml RNase solution, after being pretreated for 1 h with 10 mM spermidine.

Aging conditions	$A_{540}/2 \text{ discs x 24 h}$	
	Control	Spermidine
Buffer, 25°C	0.075±0.015	0.080±0.018
Buffer, 40°C	0.180±0.024	0.110±0.029
RNase, 25 °C	0.208±0.030	0.120±0.025

Table 10. The protective effect of spermine on initial leakage of betacyanin from beet root discs. Freshly prepared discs were incubated at 25°C or 27°C without or with spermine; discs were frozen, thawed quickly and then incubated at 25°C.

$A_{540}/4$ discs x 1 or 3 h						
Incubation solution	25°C		37°C		frozen/ thawed	
	1 h	3 h	1 h	3 h	1 h	3 h
Control (buffer)	0.071	0.115	0.381	0.534	0.132	0.157
Spermine, 1 mM	0.036	0.053	0.181	0.411	0.055	0.046
Spermine, 10 mM	0.030	0.054	0.120	0.311	0.085	0.067

Table 11. Polyamine Effect on Survival of Cryopreserved Mung-Bean Seeds. Seeds were pretreated for 4 1/2 h at 25°C prior to immersion in liquid N₂. After thawing, seeds were germinated at 25°C for 24 h.

Pretreatment	% germination
Water (control)	43 ± 4
DMSO, 5%	41 ± 5
Spermine, 10 mM	52 ± 3
Spermine, 1 mM	63 ± 4
Spermine, 0.1mM	63 ± 4

Table 12. The Effect of Spermine on Peroxidase Activity of Duranta repens Leaf Discs.

Discs were prepared from green and white sections of variegated leaves, and aged for 24 h in the dark, on various solutions.

	Peroxidase Activity, O.D. 425 nm/disc	
	Green	White
Fresh discs	0.171	0.045
Aging, phosphate buffer	0.590	0.460
Aging, spermine, 10 ⁻⁴ M	0.540	0.450
Aging, spermine, 10 ⁻³	0.115	0.036

2.3.3. POLYAMINE EFFECTS AND METABOLISM IN SENESCING RADISH AND BEAN LEAVES.

Introduction

Among their other physiological effects, PA are involved in the control of several stress-related phenomena, such as senescence, wounding, heat and salinity, both in plant organs and in isolated tissues and cells. Thus, exogenous application of PA and related precursors retarded the progressive senescence of oat leaf protoplasts, stabilized them against lysis and supported a higher incorporation of uridine and leucine (9,65). These findings were later extended to detached leaves of several plant species, and it was demonstrated that polyamines retard chlorophyll loss and prevent the rise of RNase and proteases (5,44,96). In addition, PA may have a 'protective' action on seed germination at high temperature and are correlated with plant growth under SO₂ pollution and saline conditions (138,150). While their role in the above mentioned processes has not been yet fully elucidated, PA may stabilize both nucleic acids and membrane functions during senescence (94,135,148). The wide distribution of PA as naturally-occurring polycations lends support to this hypothesis.

The present study was undertaken to determine the effect of exogenous polyamines on several parameters of leaf senescence, and to elucidate some metabolic changes in endogenous polyamines. Detached radish leaves and chloroplast suspensions, as well as intact and detached bean leaves, were used.

Materials and Methods

Radish seeds (Raphanus sativus L. var. radicula cv. 'Giant Butter') were germinated in vermiculite, at 26±1°C and under a 16-h photoperiod of 'Tadiran' cool white fluorescent light at 22 W m⁻² (400-700 nm) for 8-9 days. The pair of primary leaves, which was fully expanded at this stage, were excised and floated on 1 mM KH₂PO₄-Na₂HPO₄ buffer, pH 5.8, or on solutions with 10 mM polyamines or related compounds, in petri dishes. Occasionally, other concentrations were used. All

compounds were used as HCl salts, and were dissolved in the same phosphate buffer. Leaves were aged for various periods of time, up to 96 h, at $25 \pm 1^\circ\text{C}$ in darkness (or, as mentioned, in the same light as above). Five pairs of leaves were incubated in one petri dish, 3 replicates for each treatment, and all experiments were repeated 2-4 times. Data were analyzed using Duncan's multiple range test. Cell-free chloroplast suspensions were prepared by homogenizing 300 surface-sterilized leaves (CaOCl_2 , 4.5% w/v, for 5 min) in 60 ml of 0.05 M Tris-HCl-0.4 M sucrose medium at pH 8.0, using an all-glass homogenizer in the cold. The homogenate was filtered through 4 layers of cheese-cloth, and 2 ml aliquots of the chloroplast suspension were 'aged' in vials under aseptic conditions. Polyamines were added to the incubation medium, and for the preparation of disrupted chloroplasts, SDS was added at a final concentration of 0.25% (w/v).

Bean seeds (Phaseolus vulgaris L. var. Brittle Wax) were germinated and grown as described previously. These plants were used in two experimental systems:

1. Accelerated senescence of detached leaves: the first pair of leaves from 15 day old plants were excised and placed in a dark humid chamber (25°C) for various periods of time, as indicated.
2. Sequential senescence of intact leaves: plants were grown for 30 days, and sequential leaves along the plant axis were removed for analysis (four pairs of leaves, from the bottom-oldest to the top-youngest).

Chlorophyll was extracted in cold 80% (v/v) acetone and, after filtration, the clear supernatant was measured spectrophotometrically at 652 nm according to Bruinsma (41). Enzyme activity, polyamine levels and the incorporation of precursors were determined as described previously.

Results and Discussion

Preliminary experiments indicated that rapid senescence of excised leaves of several plant species, as monitored by chlorophyll loss in the dark, could be

considerably retarded by floating the leaves on spermidine solutions rather than on buffer alone. This was found in detached leaves of radish, French bean and wheat, and in leaf discs from tobacco and Parthenocissus. Since the most dramatic effects were observed in radish, the activity of various polyamines in delaying senescence was, thereafter, followed in excised primary leaves of radish.

A comparison of the effect of several naturally occurring polyamines and related metabolites revealed marked differences in their ability to prevent chlorophyll loss (Table 13). The diamines putrescine and cadaverine, as well as the triamine spermidine and the tetra-amine spermine, were all highly active in chlorophyll retention. Both arginine, and especially ornithine, which are metabolized to putrescine, were less active. This effect was evident when chlorophyll content was expressed on both fresh weight basis and per 10 leaves (the effect on fresh weight change will be shown later). Polyamine concentrations of 1-5 mM were also effective, but results were more consistent at 10 mM. Calcium ions, at 1-10 mM, could not replace polyamines in preventing chlorophyll loss.

The 'anti-senescence' effect of polyamines was further elucidated by following the kinetics of chlorophyll loss for up to 72 h of aging in the dark (Figs. 8 and 9). Only slight changes in chlorophyll content (per leaf) were found during the initial 24 h, followed by a marked decrease thereafter. Both cadaverine (Fig. 8) and spermine (Fig. 9) completely inhibited chlorophyll degradation and maintained its initial level for at least 72 h of aging (and up to 96 h in other experiments). The increase in chlorophyll content, when expressed on fresh weight basis is due to the slight inhibitory effect of cadaverine and spermine on change in fresh weight.

The separate effects of polyamines on fresh weight increase and on chlorophyll retention was further followed under conditions of accelerated leaf aging (light; Table 14). A marked increase in the fresh weight of leaves was found when they were incubated with buffer in the light, while this increase was inhibited by

cadaverine. The fresh weight of dark-incubated leaves increased only slightly. The nature of the cadaverine effect on fresh weight reduction was not investigated. The above mentioned effects are reflected in the chlorophyll content when expressed on fresh weight basis and, therefore, actual changes in chlorophyll are better judged when expressed per unit leaf. Thus, the rapid loss of chlorophyll in the dark was to a great extent prevented by cadaverine, keeping the chlorophyll levels similar to those found in light-incubated leaves. In the light, cadaverine even slightly accelerated chlorophyll loss when compared with aged controls.

As mentioned earlier (Figs. 8 and 9), large chlorophyll losses were evident only after 48 h of aging. Thus, it was of interest to ascertain whether polyamines may affect some earlier events of leaf senescence (Table 15). Considerable loss of protein and a marked rise in ribonuclease activity was, indeed, found during the initial 24 h of aging, when chlorophyll was only slightly degraded. Spermidine was highly effective in preventing protein degradation, and inhibited RNase activity of leaves almost completely.

The nature of the polyamine 'anti-senescence' effect was further investigated by studying chlorophyll and protein changes in cell-free systems (Table 16), rather than in intact senescing leaves (previous experiments). Only a slight loss of chlorophyll was noted in suspensions of intact chloroplasts, both in the dark and in the light, while 50% loss in protein content was found under the same conditions. In contrast, chlorophyll losses amounted to 50% in disrupted chloroplasts incubated in the dark, while complete bleaching occurred in the light. In neither case did cadaverine prevent chlorophyll or protein loss, indicating its functions in the context of the senescing leaf but not in a cell-free system. Similar results were found with spermidine.

Differences in activity of various PA have been ascribed to the number of free amine groups, which can affect their cationic properties (5,94,96,191). In the

present study (Table 13) diamines and polyamines were similarly active, indicating that either putrescine is rapidly metabolised to spermidine and spermine, or that 2 free amine groups are sufficient to saturate the system. The activity of L-arginine (and of L-ornithine) may be explained by their metabolic conversion to putrescine, via arginine decarboxylase or ornithine decarboxylase, respectively (176). Thus, the effectivity of L-arginine, as compared with that of L-ornithine, may reflect a preference of the arginine decarboxylase pathway (7).

Leaf senescence involves a sequence of degradative events which result in chlorophyll loss, nucleic acid and protein breakdown, loss of photochemical activities, and disintegration of chloroplast ultrastructure (194,195,210). While the nature of triggering events and the array of resulting changes may depend on the plant material (e.g. intact vs detached leaf senescence) and on aging conditions (e.g. light vs dark senescence), it is usually believed that chlorophyll loss is the end result of previous senescence events. This is supported by the present data, indicating a marked effect of PA on RNase activity and protein degradation, both of which precede chlorophyll loss (Table 14, Figs. 8 and 9). The findings that PA prevented the marked chlorophyll loss in darkness but did not compensate for chlorophyll loss under light conditions (Table 14) further support the possibility that PA arrest some primary degradative changes which occur in dark-aged leaves, rather than directly affecting chlorophyll. Indeed, PA have been found to promote, rather than inhibit, chlorophyll loss in light-incubated leaves (96,164). Finally, the data of Table 16 indicates that the whole 'machinery' of events within the senescing leaf is needed for PA to exert their effect on retardation of chlorophyll loss.

It is, thus, concluded that PA prevent chlorophyll loss by affecting earlier senescence-linked events which occur in dark-incubated leaves.

While exogenous application of compounds is an important and necessary step in

understanding the leaf senescence syndrome, endogenous metabolic changes in PA are, clearly, more significant. Earlier studies established that PA levels of several plant tissues are correlated with their age and stage of development (180), and that kinetin and benzyladenine significantly increase putrescine level and biosynthesis in Cucumis sativus cotyledons (188). However, PA levels and metabolism with respect to senescence have been studied only little. Altman and Bachrach (5) first indicated that senescence is correlated with lower levels of putrescine and other PA, both in intact leaves which senesce sequentially and in detached leaves. Moreover, indole-3-butyric acid and 6-benzyladenine increased putrescine levels in aged mung bean hypocotyls and potato tuber discs, respectively. Kaur-Sawhney et al. (98) have recently demonstrated that the activity of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), the putrescine biosynthetic enzymes, decrease progressively in senescing excised oat leaves in the dark. Also, levels of putrescine, spermidine, and related compounds, were found to be high in young leaves, and declined with age (see also later in this report).

Thus, in order to further elucidate the role of polyamines in leaf senescence, we compared PA levels, the activity of biosynthetic enzymes and the incorporation of label from arginine and ornithine into PA. These parameters were investigated in two systems: accelerated senescence of detached bean leaves in a humid chamber in the dark, and sequential senescence of intact leaves along the plant axis. Polyamine levels and their biosynthesis (enzyme activity and precursor incorporation) were all correlated with leaf age and senescence, as outlined in the following.

A progressive decrease in chlorophyll and protein content of both detached (Figs. 10,11) and intact (Fig. 12) bean leaves during their senescence was found. The decrease of protein slightly preceeded that of chlorophyll.

The endogenous spermidine content was shown to be the highest, followed by putrescine, with spermine having the lowest level. While spermine content showed

little variation in either system, considerable changes in spermidine and putrescine were found. A bi-phasic curve of polyamine changes was found under accelerated senescence conditions (a decrease, followed by an increase, and then a further decrease). In contrast, the sequential senescence was accompanied by a progressive decrease until the third leaf, followed by an increase in the fourth leaf (Figs. 13,14).

In the accelerated senescence system, the activity of the enzymes arginine decarboxylase and ornithine decarboxylase evinced a similar bi-phasic curve. In the sequential senescence system, an increase in enzyme activity was found from the youngest leaf to the third (which was followed by a decrease in the oldest leaf) (Figs. 15,16). Changes in enzyme activity usually preceded those of PA levels.

The incorporation of U-¹⁴C- arginine and ornithine into polyamines was studied in detached senescing leaves. The efficiency of ornithine incorporation into various polyamines was greater than that of arginine. The incorporation of both precursors followed the typical bi-phasic curve described above. Thus, the incorporation of label from arginine, and especially from ornithine, changed only slightly during the initial 2 days of leaf senescence, but increased considerably towards the later stages, followed by another decrease (Fig. 17).

In view of these findings, the effect of applied polyamines on chlorophyll and protein content and on the activity of PA biosynthetic enzymes of detached leaves was also studied. Pretreatment of leaves with spermidine (prior to their excision), resulted in a considerable inhibition of protein and chlorophyll loss after eight days of senescence (Figs. 18,19). Similarly, spermidine treatment resulted in a considerable increase of arginine decarboxylase, and especially of ornithine decarboxylase, after eight days of senescence (Fig. 20). In contrast, application of polyamines to already senescing leaves did not have the same effect, indicating that leaves should be treated with polyamines before the onset of senescence.

The above-mentioned data clearly indicate that both the polyamine content and their biosynthesis enzyme activity and precursor incorporation) are closely linked to senescence in leaves, thus they may have a role in controlling this process. It is suggested that senescence-induced changes in the leaf are sensed by the PA biosynthetic 'machinery' at an early stage, resulting in renewed putrescine and spermidine biosynthesis - probably from free amino acids which accumulate upon proteolysis. Further elucidation of the role of polyamines in leaf senescence calls also for a study of their catabolism (oxidation and conjugation).

Figure 8. Kinetics of chlorophyll loss in radish leaves: the effect of cadaverine. Leaves were floated on phosphate buffer (control) or on 10 mM cadaverine.

Figure 9. Kinetics of chlorophyll loss in radish leaves: the effect of spermine. Leaves were floated on phosphate buffer (control) or on 10 mM spermine.

Figure 10. Protein loss during senescence of detached bean leaves.

Figure 11. Chlorophyll and fresh weight loss during senescence of detached bean leaves.

Figure 12. Protein and chlorophyll levels in sequentially developing and senescing bean leaves (leaf 1: apical, youngest leaf, to 4: basal, oldest leaf).

Figure 13. Endogenous polyamine levels in senescing detached bean leaves. A: on protein basis, B: on fresh weight basis.

Figure 14. Endogenous polyamine levels in sequentially-developing and senescing bean leaves. A: on protein basis, B: on fresh weight basis.

Figure 15. Arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) activity of senescing detached bean leaves. A: on protein basis, B: per leaf disc.

Figure 16. Arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) activity in sequentially-developing and senescing bean leaves. A: on protein basis, B: on fresh weight basis.

Figure 17. Incorporation of $[U]^{14}\text{C}$ arginine and $[U]^{14}\text{C}$ ornithine into various polyamines in senescing detached bean leaves. A: dpm in various polyamines on protein basis, B: same, as % of total dpm in the acid extract of tissues.

Figure 18. The effect of polyamine pretreatment of bean leaves on their protein content during the senescence period.

Figure 19. The effect of polyamine treatment of bean leaves on their chlorophyll content during the senescence periods.

Figure 20. The effect of polyamine pretreatment of bean leaves on the activity of arginine decarboxylase (A) and ornithine decarboxylase (B) during leaf senescence.

Table 13. The effect of polyamines and related metabolites on chlorophyll retention in detached radish leaves. Detached leaves were floated in the dark on phosphate buffer (control) or on 10 mM test solutions, and chlorophyll was measured after 72 h aging. Values are significantly different if followed by different letters ($P = 0.05$), Multiple Range Test.

Experiment	Chlorophyll/g fresh wt		Chlorophyll/10 leaves	
	mg	%	mg	%
Control, 0 time	20.5 a	100	6.2 a	100
Control, 72 h	4.9 d	24	3.2 c	52
L-ornithine	7.8 d	38	4.8 b	77
L-arginine	12.9 c	62	5.2 b	84
Spermidine	16.1 b	79	6.4 a	103
Spermine	17.3 ab	84	6.5 a	105
Putrescine	17.4 ab	85	6.5 a	105
Cadaverine	19.0 a	93	6.2 a	100

Table 14. The effect of cadaverine on chlorophyll loss from radish leaves aged under dark or light conditions. Detached leaves were floated for 48 h on phosphate buffer (control) or 10 mM cadaverine. Values within each column are significantly different if followed by different letters ($P = 0.05$). Data are also expressed a percent of initial values.

Experiment	Fresh wt		Chlorophyll/ g fresh wt		Chlorophyll/ 10 leaves	
	mg/10 leaves	%	mg	%	mg	%
0-time	340	100	16.8	100	5.6	100
Dark, control	361 b	106	5.5 b	33	2.0 b	35
Dark, cadaverine	266 b	78	12.9 a	77	3.4 a	61
Light, control	540 a	159	6.9 b	41	3.7 a	66
Light, cadaverine	250 b	74	12.2 a	72	3.0 a	54

Table 15. The effect of spermidine on chlorophyll and protein content and on ribonuclease activity of aged radish leaves. Detached leaves were floated for 24 h in the dark on phosphate buffer (control) or on 10 mM spermidine. Data are expressed per 10 leaves and as % of fresh leaves. Values within each column are significantly different if followed by different letters (P = 0.05).

Experiment	Chlorophyll/ 10 leaves		Protein/ 10 leaves		RNase/ 10 leaves	
	mg	%	mg	%	$\Delta A_{269\text{nm}}/\text{h}$	%
0-time	5.9 a	100	0.45 a	100	0.128 b	100
24 h aging, control	4.9 a	84	0.24 b	53	0.194 a	152
24 h aging, spermidine	5.2 a	89	0.50 a	111	0.022 c	17

Table 16. Chlorophyll and protein degradation in vitro, in 'intact' and 'disrupted' isolated chloroplasts. Two ml samples of cell-free chloroplasts were incubated for 48 h in the dark or in the light with or without 10 mM cadaverine, in Tris-sucrose medium ('intact') or in Tris-sucrose + 0.25% (w/v) SDS medium ('disrupted'). Data are expressed as % of the following initial values: chlorophyll: 3.66 mg/2 ml; protein: 0.084 mg/2 ml.

Experiment	Intact (Tris-sucrose)		Disrupted (Tris-sucrose + SDS)	
	Chloro- phyll	Protein	Chloro- phyll	Protein
Dark, control	89	50	51	39
Dark, cadaverine	90	38	59	36
Light, control	84	45	7	41
Light, cadaverine	81	57	10	41

2.3.4. INTERRELATIONSHIPS OF POLYAMINES AND ADVENTITIOUS ROOT FORMATION.

Introduction

Cuttings excised from plants either senesce and deteriorate, or resume active cell division and regenerate adventitious roots. It is well established that root formation involves intensive mitotic activity and metabolic changes, and is dependent on an array of endogenous physiological factors (79,80). Whereas auxins seem to be a universal 'inducer' of adventitious roots (2), other factors are also involved and these factors may become limiting under specific conditions. Thus, inhibitors (79), rooting 'cofactors' (79,80), auxin antagonists (26), and nutrients (80,137) have been shown to modulate root formation. In addition, it is still unclear which hormone (auxin) messengers are involved, and what are the primary events in auxin-induced root formation.

Recent studies, including our own, indicate that PA may play a significant role in plant growth and senescence (5,6), similar to their activity in mammalian tissues and procaryotes (16,48,192). It has been shown that active growth of germinating seeds, of habituated and crown gall tissues, of pollen tubes, of potato buds and tomato fruits (15,75,99,182,203), as well as embryo and organ differentiation (5,126,127), are correlated with significant changes in PA content and metabolism. In addition, PA have been shown to respond to environmental and hormonal stimuli (54,99), and to play an important role in protoplast, tissue, and organ senescence (3,9,96).

It, therefore, seems that cuttings may serve as an important experimental system to elucidate the involvement of PA in their senescence and in auxin-induced root formation. In addition to the effect of exogenous PA, endogenous changes in PA, and possible inhibitors of PA biosynthesis were also investigated. Both L-canavanine and L-canaline, the structural analogs of L-arginine and L-ornithine, respectively (156,157), may affect putrescine formation from these two amino acids. Similarly,

MGBG can block spermidine and spermine biosynthesis by inhibiting S-adenosylmethionine decarboxylase (151). L-Canavanine has been shown previously to inhibit IAA-dependent elongation of Avena coleoptiles (36), and the growth of Phaseolus roots (205) and soybean cell suspensions (52).

Materials and Methods

Plant Material and Rooting Experiments. Mung bean (Vigna radiata [L.] Wilczek) seedlings were grown in vermiculite in a controlled phytotron (27°C day and 22°C night temperature, 78% RH, 16 h photoperiod). Seven- to 9-d-old seedlings were excised 2.5 cm below the cotyledonary node, the cotyledons were removed, and the resulting cuttings (consisting of the hypocotyl and the intact epicotyl, with a pair of primary leaves) were used in rooting experiments.

Freshly prepared hypocotyl cuttings were introduced into glass vials containing 15 ml of test solution which covered the entire hypocotyl. This solution was renewed after 24 h, and then at 48-h intervals. Control solution consisted of 1 mM phosphate buffer at pH 5.8, to which various compounds were added, as mentioned. IBA was used at $5 \times 10^{-5}M$, unless otherwise mentioned. PA, L-arginine, L-ornithine, L-canavanine, L-canaline, and MGBG were used at the mentioned concentrations, all in buffer. Unless otherwise stated, all treatments were typically performed during the initial 24-h period, after which the cuttings were transferred to buffer solutions. Cuttings were maintained in a controlled growth chamber ($24 \pm 1^\circ C$, 16 h photoperiod of cool white fluorescent light at 3600 lux) for 5 to 6 days, after which the adventitious roots (longer than 0.5 mm) were counted.

Polyamine content, ADC and ODC activities, and the incorporation of precursors were determined as described previously.

Results and Discussion.

Root Formation. Initially it was found desirable to look at the effect of exogenous PA on natural and on IBA-induced root formation. In neither case did

putrescine or spermine (and spermidine, data not presented) promote root formation (Table 17). Putrescine at $10^{-4}M$ and more so spermine, inhibited root formation, whereas higher concentrations ($>5 \times 10^{-4}M$) were found to be toxic.

Since PA are synthesized in plants from L-arginine and L-ornithine, the effect of these precursors and their analogs on root formation was studied. Whereas L-arginine did not affect natural root formation and partially inhibited IBA-induced root formation (Table 18), its analog, L-canavanine, totally prevented root formation in control cuttings and significantly inhibited IBA-induced root formation. Arginine completely reversed the inhibition and this effect was concentration dependent. The above mentioned results were more evident with $10^{-5}M$ IBA, as compared with its higher concentration. Similar results were found with L-ornithine and its analog, L-canaline. Root formation and inhibition is also illustrated (Fig. 21). In an attempt to clarify the nature of the L-canavanine inhibition, the timing effect of IBA and L-canavanine application was investigated (Table 19). Root formation was completely arrested by $10^{-4}M$ L-canavanine, regardless of its time of application with respect to IBA application; thus, a later IBA treatment did not overcome the initial canavanine effect, neither did an IBA pretreatment prevent the canavanine inhibition. Surprisingly, the low canavanine concentration treatment resulted in a significant promotion of IBA-induced root formation when applied after IBA but inhibited it when administered prior to IBA. MGBG, another possible inhibitor of PA biosynthesis, significantly inhibited root formation (Table 20). The MGBG effect was found to be specific: inhibition of root initiation at the base of the cutting, resulting in a second wave of root formation above the damaged area of the hypocotyl (Fig. 21).

Polyamine Content. IBA treatment of cuttings resulted in remarkable increase in endogenous polyamine content, more than 2-fold of the control (Table 21). This increase was especially evident in the hypocotyl and epicotyl, and a

considerable increase in the putrescine/spermidine ratio was found in all organs. A study of PA changes over a 48-h period (Table 22) clearly indicates a continuous decrease in putrescine and spermidine levels in untreated cuttings which produce only few roots and presumably senesce, but this decrease in putrescine is prevented by IBA treatment. These changes are reflected in the putrescine to spermidine ratio.

Polyamine levels were further followed as a function of IBA concentrations. Whereas continuous increase in root formation was found at IBA concentrations higher than $5 \times 10^{-6}M$, putrescine level of hypocotyls peaked at $5 \times 10^{-6}M$ IBA where only slight promotion of rooting was observed (Fig. 22). Putrescine and spermidine levels dropped dramatically at $5 \times 10^{-5}M$ IBA, followed by a second increase. It should be noted, however, that the highest IBA concentration was supraoptimal, resulting in abnormal root formation and tissue hypertrophy. Similar changes in putrescine content were found in the epicotyl section of the cutting (data not reported). On the other hand, continuous increase in putrescine content was observed in leaves of cuttings treated with increasing IBA concentrations (Fig. 23).

The effect of auxin on the incorporation of arginine, ornithine, and putrescine, was studied in either fresh or IBA pretreated cuttings. Figure 24 demonstrates the incorporation of ^{14}C -arginine into various dansyl products. It is clear that arginine is quickly incorporated into PA as well as to other dansyl products (Fig. 24, Table 23).

IBA treatment results in increased incorporation into putrescine and decreased incorporation into spermidine and product b (unidentified as yet). Similar results were found with ^{14}C -ornithine incorporation (Table 23), IBA treatment resulting in higher putrescine/spermidine ratios. In IBA-treated cuttings, the incorporation of the ^{14}C -putrescine into spermidine and spermine decreases, and putrescine/spermidine ratios are fairly high.

The auxin-induced changes of precursor incorporation into PA were observed in the

hypocotyl sections only, where roots are formed and not in the epicotyl or the leaves. It is, thus, assumed that changes in PA metabolism are associated with the effect of auxin on root initiation.

The distribution of labelled PA derived from arginine and ornithine was examined during 72 hours. The formation of various PA from both arginine (Fig. 25) and ornithine (not shown) has a very similar pattern. The effect of auxin treatment on PA metabolism can be better judged when the results are expressed as putrescine/spermidine ratio (i.e. the ratio of label incorporated into putrescine to that into spermidine). Such a comparison indicates (Fig. 26) that IBA treatment is accompanied by consistent increase of this ratio, from both precursors.

Untreated mung bean cuttings produce only few roots and occasionally senescence, whereas IBA-treated hypocotyl form many roots and show vigorous growth (Table 18, Figs. 21 and 22). Fully developed cuttings with roots (5 d) evince a remarkable increase in putrescine and spermidine in response to IBA treatment (Table 21), and a corresponding increase in putrescine to spermidine ratio, indicative of active cell division and growth (16). Moreover, early changes in PA content and biosynthesis in hypocotyls are detected before any growth of adventitious roots occurs (Tables 22 and 23, Figs. 25 and 26); thus, the considerable initial drop in PA level (and especially of putrescine) of senescing cuttings is prevented by an IBA treatment, which also results in higher putrescine to spermidine ratio already during the first 48 h. Such early changes in proteins and nucleic acids have been previously reported for root formation (12), and it is possible that these changes are related to PA content and metabolism, similar to other biological systems.

The lack of response to applied PA (Table 17) suggests that either PA are not involved in root formation, or that applied PA do not reach the site of action, or that endogenous levels of PA are optimal for root production. However, the inhibition of IBA-induced root formation by L-canavanine and L-canaline, and its

reversal by the corresponding amino acids (Tables 18 and 19), indicate that PA may be necessary for root formation. It is well established that putrescine is synthesized in plants from arginine and ornithine (177) and that the antimetabolite canavanine may retard growth by competing with arginine and effectively inhibit enzymes of arginine metabolism (156). Moreover, the first report of canavanine effect in plants indicated that it inhibited IAA-dependent elongation of Avena coleoptiles, and a specific role for arginine was suggested (36). Recent reports (52,205) clearly show that canavanine inhibits growth of several other plant tissues. It is noteworthy that arginine has been previously found to be active as an auxin-cofactor in root inhibition on Hibiscus cuttings (199). Indeed, our data show that the synthesis of putrescine and spermidine from arginine is much higher than from ornithine (Table 23). These results suggest that arginine may be a preferred precursor for PA during root formation. In addition, the major changes in PA content and biosynthesis occur in the hypocotyl where roots are formed and less so in leaves, and the highest putrescine to spermidine ratio occurs at IBA concentration, which is only slightly inductive for root formation, whereas massive root growth may lead to relatively greater utilization of putrescine. MGBG, an inhibitor of spermidine and spermine biosynthesis, has previously shown to inhibit S-adenosylmethionine decarboxylase activity of etiolated Lathyrus sativus seedlings (187) and in the present study it effectively retarded root formation (Table 20).

The foregoing suggests that polyamines are involved in auxin-induced root formation, although their specific role has yet to be elucidated in order to establish a causal relationship.

Figure 21. Root formation in mung bean hypocotyl cuttings. 1, Control; 2, IBA, $5 \times 10^{-5}M$; 3, IBA, $5 \times 10^{-5}M$ + MGBG, $10^{-4}M$; 4, IBA, $5 \times 10^{-5}M$ + L-canavanine, $10^{-4}M$.

Figure 22. Rooting and polyamine content of hypocotyls of mung bean cuttings treated with different IBA concentrations. Plant material was extracted for PA determination at end of a 96-h rooting period.

Figure 23. Polyamine content of leaves of mung bean cuttings tested with different IBA concentrations. Plant material was extracted at end of a 96-h rooting period.

Figure 24. The incorporation of ^{14}C -arginine into various polyamines and other dansylated products in mung bean cuttings. The incorporation was continued for 5 or 24 h in control cuttings, or for 24 in IBA-treated cuttings.

Figure 25. Distribution of label from ^{14}C -arginine in various polyamines, in control and IBA-treated mung bean cuttings, during a 72 h period.

Figure 26. The ratio of label incorporation into putrescine, to that into spermidine, from either ^{14}C -arginine or ^{14}C -ornithine. Calculated from data of Figure 25 and additional data.

Table 17. Effect of Exogenous Polyamines on Root Formation.

Cuttings were treated with polyamines during the initial 24-h period. Values within a column are significantly different when followed by a different letter (P = 0.05, multiple range test).

No. of Roots/Cutting		
Treatment	Control	IBA ($5 \times 10^{-5}M$)
Control	6.7 d	38.7 a
Putrescine		
$10^{-5}M$	6.7 d	26.5 b
$10^{-4}M$	6.1 d	25.7 b
Spermine		
$10^{-5}M$	6.3 d	24.7 b
$10^{-4}M$	7.9 d	18.8 c

Table 18. Effect of Polyamine Precursors and Their Analogs on Root Formation in Mung Bean Cuttings.

Cuttings were treated with the various compounds during the initial 24-h period, and then transferred to buffer ($\pm$ SD of the mean).

No. of Roots/Cutting			
Treatment	Control	$10^{-5}M$	IBA $5 \times 10^{-5}M$
Control	6.7 $\pm$ 2.7	15.7 $\pm$ 7.0	22.1 $\pm$ 6.4
Arginine			
$10^{-5}M$	7.9 $\pm$ 2.7	9.3 $\pm$ 9.6	18.5 $\pm$ 6.0
$10^{-4}M$	8.6 $\pm$ 3.1	11.2 $\pm$ 5.3	13.4 $\pm$ 5.7
Canavanine			
$10^{-4}M$	0	4.6 $\pm$ 5.3	14.1 $\pm$ 4.1
$10^{-4}M$ +arginine $10^{-5}M$	0	7.1 $\pm$ 5.2	13.3 $\pm$ 7.6
$10^{-4}M$ +arginine $10^{-4}M$	0	12.8 $\pm$ 9.8	27.5 $\pm$ 10.9
Ornithine			
$10^{-5}M$	7.3 $\pm$ 3.7		
$10^{-4}M$	7.9 $\pm$ 3.5		
Canaline			
$10^{-4}M$	0	4.6 $\pm$ 5.0	8.4 $\pm$ 6.5
$10^{-4}M$ +ornithine $10^{-5}M$	0	9.5 $\pm$ 6.3	18.1 $\pm$ 8.2
$10^{-4}M$ +ornithine $10^{-4}M$	0	11.2 $\pm$ 6.1	16.5 $\pm$ 6.6

Table 19. Timing of the Canavanine Effect on Root Formation in Mung Bean Cuttings.

Experiment 1: Cuttings were pretreated with L-canavanine combined with IBA for 24 h (A), or pretreated with IBA for 24 h, followed by treatment with L-canavanine for additional 72 h (B). Experiment 2: Cuttings were pretreated with L-canavanine alone for 24 h, followed by treatment with IBA for additional 72 h. Multiple range test, $P = 0.05$, the two experiments analyzed separately.

No. of Roots/Cuttings		
Treatment	Control	IBA ($5 \times 10^{-5}M$)
Experiment 1		
Control	5.6 c	15.6 b
(A) 0-24 h		
Canavanine, 10^{-5}	7.5 c	21.8 b
Canavanine, 10^{-4}	0 d	0.4 d
(B) 24-96 h		
Canavanine, 10^{-5}	7.9 c	32.7 a
Canavanine, 10^{-4}	0.4 d	0 d
Experiment 2		
Control	6.3 b	25.5 a
Canavanine, 10^{-5}	7.3 b	3.8 b
Canavanine, 10^{-4}	0 c	0 c

Table 20. Effect of MGBG on Root Formation in Mung Bean Cuttings

Cuttings were treated with MGBG during the initial 24-h period (multiple range test, $P = 0.05$).

No. of Roots/Cutting		
	Control	IBA ($5 \times 10^{-5}M$)
Control	8.4 $\pm$ 2.0 c	27.1 $\pm$ 8.4 a
MGBG, $10^{-5}M$	9.1 $\pm$ 2.9	18.7 $\pm$ 5.5 b
MGBG, $10^{-4}M$	3.3 $\pm$ 4.3 d	15.3 $\pm$ 6.3 b

Table 21. Effect of IBA on Polyamine Content in the Hypocotyl of mung Bean Cuttings.

Cuttings were separated to the corresponding organs after 96-h rooting period. Number of roots per cutting: control, 7; IBA, 24. IBA applied at 10^{-5} M.

	Putrescine		Spermidine		Putrescine to Spermidine	
	Control	IBA	Control	IBA	Control	IBA
	nmol/mg protein				ratio	
Hypocotyl	95.1	203.5	22.5	37.9	4.23	5.37
Epicotyl	105.1	214.0	36.7	57.0	2.86	3.75
Leaves	34.2	50.0	9.1	9.5	3.76	5.26

Table 22. Effect of IBA on PA Content in the Hypocotyl of Mung Bean Cuttings.

Cuttings were pretreated with 10^{-5} M IBA for 24 h and then incubated for additional 24 h in buffer. The entire hypocotyl was used for PA determination.

Time	Putrescine		Spermidine		Putrescine		Spermidine		Putrescine to Spermidine	
	Control	IBA	Control	IBA	Control	IBA	Control	IBA	Control	IBA
h	nmol PA/ g fresh wt				nmol PA/ mg protein				ratio	
0	41.9		19.1		46.4		21.1		2.19	
24	28.4	37.1	15.8	18.1	33.1	44.2	18.3	21.4	1.80	2.05
48	25.7	49.3	12.9	13.4	25.4	42.8	12.4	11.5	1.99	3.68

Table 23. Incorporation of ^{14}C -Arginine, ^{14}C -Ornithine and ^{14}C -Putrescine into polyamines.

Cuttings were pretreated for $24 \text{ h} \pm 5 \times 10^{-5} \text{ M}$ IBA, followed by a 24 h incorporation period.

	dpm/mg protein ⁻¹				% of total activity in dansyl products			
	<u>put</u>	<u>spd</u>	<u>sp</u>	<u>put/spd</u>	<u>put</u>	<u>spd</u>	<u>sp</u>	<u>put/spd</u>
^{14}C -Arginine								
control	1726	2388	239	0.75	16.2	23.8	2.3	0.62
IBA	2386	1440	0	1.66	20.1	12.1	0.0	1.66
^{14}C -Ornithine								
control	450	2534	1404	0.18	1.3	7.8	5.0	0.10
IBA	907	827	257	1.10	12.1	11.0	3.5	0.78
^{14}C -Putrescine								
control	1300	4600	850	0.28	8.3	26.5	4.9	0.31
IBA	1320	1820	430	0.73	13.1	18.2	4.3	0.71

2.3.5. POLYAMINE METABOLISM WITH RESPECT TO SALT STRESS.

Introduction

The accumulation of putrescine in leaves of K^+ -deficient plants was first reported by Richard and Coleman (155), and subsequent studies of Smith and co-workers, and others, have established the specific role of PA in maintaining a cation-anion balance in plant tissues (171). The high accumulation of putrescine was established in many plants in response to both K^+ and Mg^{++} deficiency, and the activity of ADC was also found to increase under these conditions. It was suggested that putrescine is formed in leaves to replace K^+ , the major inorganic cation, reflecting a homeostatic mechanism for controlling cellular pH in higher plants. Saline conditions also caused, in some cases, an increase in putrescine levels, and it is likely that differential uptake of ions, resulting in changes in cell pH and ion balance, is the cause of putrescine accumulation. Specific information on PA in salt-stressed plants is scarce, but in one case salinity did not cause a change in free polyamines (150), while in another study spraying of broad bean plants with putrescine resulted in improved growth under saline conditions (14). A series of works by Stroganov (186) indicated, already long ago, that salinity-induced changes in protein metabolism is accompanied by accumulation of free amino acids as well as putrescine and cadaverine. In view of these early reports, and our former data as to polyamine contribution to tissue stabilization under stress conditions, the interrelationships of PA and salinity stress were studied. We examined plant response to high NaCl concentrations in the growth medium in an attempt to clarify the following questions:

1. Are the polyamine biosynthetic enzymes, arginine and ornithine decarboxylases, involved in the response of plant seedlings to salt stress?
2. Do intact or excised plant tissues respond to NaCl stress in the same way, with respect to PA-biosynthesis?

3. Is the plant response to salt stress a result of osmotic or ion-specific changes?
4. Do different plants react similarly to salinity? In what way do glycophytes and halophytes differ with respect to salt-induced changes in polyamine metabolism?

Materials and Methods

Plant Material

Mung bean (Vigna radiata L.) seeds were germinated and grown in vermiculite in a controlled phytotron (27°C-day and 22°C-night temperature, 16h photoperiod, 78% RH).

Young seedlings, 23 weeks old, of Atriplex halimus L., Pulicaria undulata (L.) Kostel., Salsola Rosmarinus (Ehr.) Solms-Laub and Mesembryanthemum Forskahlei Hochst. were collected in February from saline soil in the northern regions of the Dead Sea valley. The seedlings were then grown in trays with the original soil (saline soil, 2mMhos conductivity), in a controlled phytotron (22°C day and 15°C night temperature, 8h short day and 78% RH).

Salt Stress Treatment

1. Vermiculite: trays with 4 day old seedlings were soaked for one hour in saline water (5 atm NaCl). The plants were afterwards treated in the same way for three days with NaCl solutions of increasing concentrations (7, 5, 15 and 22.5 atm).
2. NaCl solutions: 7 day old seedlings were transferred from the vermiculite medium into aerated solutions containing NaCl (5 or 7.5 atm) for 20 to 48 hours. In other experiments, mung bean seeds were germinated and grown in saline soil (2mMhos conductivity) taken from the Dead Sea valley.

Halophytes were subjected to 'stress' by either transferring seedlings from saline soil to aerated tap water, or growing the seedlings in prewashed soil (0.3 mMhos conductivity).

Results and Discussion

1. Activation of arginine and ornithine decarboxylases.

Exposure of mung bean plants to high concentrations of NaCl results in

growth inhibition, especially of roots, in decreased leaf turgidity, and in partial necrosis. This salt stress was accompanied by a decrease in ADC and ODC activity in the leaves. However, the activity of ODC in the stem, and especially in the roots, was markedly increased under the same conditions (Tables 24,25). The data indicate, also, that the changes in ADC and ODC activity were directly correlated with NaCl concentration, and that the response of plants to saline solutions was more pronounced than when grown in vermiculite (although the exposure in vermiculite was longer and the osmotic potential was higher). Since the observed effects might be of osmotic nature, it was important to examine whether the above-mentioned changes can be brought about by equi-osmotic solutions, other than by NaCl. Table 26 presents data of ADC and ODC activities as affected by NaCl or KCl stress. Similar results were obtained when plants were treated with NaCl and KCl in equi-osmotic concentrations, suggesting that the observed metabolic changes are due to osmotic stress rather than to specific ion toxicity. In addition, these data indicate that the salt-induced rise in ODC activity operates both in intact plants and in excised roots. The response of mung bean roots to NaCl stress is rapid, as indicated by the data of ADC and ODC kinetics over a 24 h period (Fig. 27). The activity of ODC increased already 2 h after exposure to a salt stress, and continued to increase later (previous data), while ADC activity decreased during the first 6 h (both in the control and with NaCl) and slightly increased only later. Since ion concentration is known to affect many enzymatic activities, it was questioned whether the observed effects of NaCl are due to 'in vitro' activation or inhibition of the enzymes. Data of relevant experiments, with different concentrations of NaCl in the assay medium (Table 27), indicate that this is not the case. In fact, increasing concentrations of NaCl inhibited ADC and ODC in vitro activity, supporting the conclusion that the observed rise in ODC activity is a result of plant response to salt stress. Finally, changes in the activity of arginine and ornithine decarboxylases were observed in

several other plant species: sorghum and tomato plants evinced a very high activity of both enzymes in roots under NaCl stress, while in vetch seedlings an increase in ornithine decarboxylase (300%) and a slight decrease in arginine decarboxylase activity was observed (Table 28).

2. Polyamine content and biosynthesis

Mung bean seedlings growing in high NaCl media evince a high putrescine content in the shoot, while the content in the root is decreased. The major increase in putrescine occurs in the epicotyl (400%), with a moderate increase in the leaves and hypocotyl (180% and 140%, respectively). Putrescine/spermidine ratios in response to salt stress, are higher in shoot organs as well as in the roots (Table 29).

The changes in polyamine content are correlated with the osmolarity of the medium: higher concentrations of NaCl result in higher putrescine/spermidine ratios (data not presented). As previously described, salt stress activates the polyamine biosynthetic enzymes, ADC and ODC, mainly in the root system. In view of these results, it was of interest to ascertain whether the polyamines are synthesized more intensively when plants are exposed to salt stress. Table 30 shows the effect of NaCl on arginine and ornithine incorporation into putrescine, spermidine and spermine. The data clearly indicate that the incorporation of label from both arginine and ornithine into putrescine and spermidine is enhanced in the roots as a result of salt stress, but inhibited in leaves. The incorporation of arginine and ornithine is accompanied by lower values of putrescine/spermidine incorporation ratio in salt stress leaves. Similar lower values are detected in the roots when ornithine serves as the precursor for putrescine biosynthesis. It should be noted that the salt-induced 'activation' of PA biosynthesis is even higher when data are expressed on fresh weight basis, since protein content of NaCl-treated plants was significantly higher than control plants, in all cases (e.g. Table 29).

3. Response of halophytes and glycophytes.

Leaves of several halophytes, grown in their natural habitat, contain the three major polyamines, although putrescine and spermidine levels are considerably lower than in mung bean leaves (Table 31). Since our previous results indicated that the response of mung bean (and other glycophytes) to stress is related to considerable changes in PA biosynthesis, the behavior of halophytes, in this connection, is of great interest. First, high activity of ODC, and especially of ADC, was observed in leaves of Pulicaria, while no activity could be detected in leaves of two other halophytes, Atriplex and Mesembryanthemum (Table 31). However, high activity of ADC and ODC was found in the roots of all the above mentioned halophytes (Table 32) as well as in leaves of halophytes which were transferred from their natural habitat to different growth conditions. The term 'stress' has different meanings for halophytes than for glycophytes: thus, mung bean seedlings have stunted growth of shoot and roots, rolled and necrotic leaves when cultivated in saline media, while halophytes evince very similar 'stress' response when transferred from saline soil or salt solutions to leached soil or to tap water, respectively. This similar response to a change in the natural growth habitat is clearly reflected in the activity of ADC and ODC (Table 32). Thus, mung bean roots evince a typical activation of ODC in saline soil, while ADC and ODC activity of roots of the 2 halophytes (Pulicaria and Atriplex) is considerably promoted when plants are 'stressed' by their transfer from saline soil to leached soil. It can be argued that ADC and ODC activity, as affected by salt stress, may be due to direct activation by sodium and chloride ions. Results of an in vitro experiment, with increasing NaCl concentrations in the reaction mixture, indicate (Fig. 28) that this is not the case, and that in vitro enzyme activity of mung bean roots is inhibited by high NaCl concentrations, while their activity in Pulicaria roots is very little affected, even at high concentrations. This supports our assumption that the observed in vivo

effect results from a physiological response to a change in the osmotic potential of the root environment.

Our data indicate that mung bean plants respond to salt stress by an increased activity of polyamine biosynthetic enzymes, especially ODC, and increased polyamine biosynthesis in the roots. These findings affirm the hypothesis, suggested first by Stroganov (186) and later by Smith (170,171), that polyamines accumulate under stress conditions as a result of activation of their biosynthetic enzymes.

In our studies we found that polyamines accumulate in shoot organs and decrease in the roots, where active synthesis takes place. It is therefore suggested that putrescine, which is synthesized in the root system, is transported to the shoot where it accumulates.

The survival, growth and adaptation to salinity, of salt-sensitive and salt-tolerant plant species, is accompanied by some changes in plant metabolism. In response to stress, both halophytes and non-halophytes develop an adaptation mechanism which prevents the growth reduction and injury caused by increased membrane permeability, ion toxicity and water deficiency. High concentrations of organic solutes such as glycinebetaine, proline, arginine or sucrose, which accumulate in NaCl stressed plants, may have an important role in the 'protection' against protein changes, inactivation of enzymes and osmotic imbalance in the cells. The metabolism of proline, arginine and glycine in stressed plants may be closely related to the metabolism of polyamine via their precursors ornithine and arginine. Although a direct connection between PA biosynthesis and proline accumulation in stressed plants has not been shown yet, it is suggested that such biosynthetic interrelationships do exist:

- a. Salt stress leads to enhanced ornithine and arginine synthesis as well as to proline synthesis from their common precursor glutamine.
- b. Activation of ADC and ODC, as established in this work, leads to the accumulation

of polyamines, mainly putrescine.

- c. Polyamines may be involved in the adaptation of plants to salinity by either a direct regulation of protein synthesis, or by the regulation of ion balance and pH in the cells.

In addition, PA can be involved in stabilization of plant cell membranes against stress, as shown before for protoplast stabilization, leaf senescence and wounding. It is suggested, therefore, that the interaction of polyamines with primary stress-induced destabilization of cell membranes can be contributed to plant adaptation to salinity.

Figure 27. Kinetics of ADC and ODC activity of mung bean roots in control and NaCl solutions. Excised lateral roots were incubated for periods up to 24 h in water or in NaCl solution (7.5 atmospheres).

Figure 28. The in vitro effect of NaCl concentrations on ADC and ODC activity of mung bean and *Pulicaria* roots. Root crude extracts were assayed in the presence of increasing NaCl concentrations in the reaction mixture.

Table 24. The effect of salt-stress on ADC and ODC activity of mung bean plants. Plants were grown for 96 h in vermiculite with final NaCl levels of 22.5 atmospheres.

	pmoles CO ₂ / mg prot.		% of control	
	ADC	ODC	ADC	ODC
Leaf, control	1176	386	100	100
NaCl	567	166	48	43
Root, control	388	30	100	100
NaCl	158	129	41	430

Table 25. Effects of NaCl concentrations on ADC and ODC activity of intact mung bean plants.

Plants were grown for 48 h in NaCl solutions of 5 or 7.5 atmospheres.

	pmoles CO ₂ / mg prot.		% of control	
	ADC	ODC	ADC	ODC
Leaf, control	320	273	100	100
NaCl, 5 atm	388	170	47	62
NaCl, 7.5 atm	dead			
Stem, control	2400	796	100	100
NaCl, 5 atm	2972	991	124	125
NaCl, 7.5 atm	4863	1534	203	193
Root, control	445	57	100	100
NaCl, 5 atm	347	164	78	288
NaCl, 7.5 atm	483	477	109	837

Table 26. The effect of equi-osmotic concentrations of NaCl and KCl on ADC and ODC activity of excised roots, or on roots from intact mung bean plants.

Plants with their intact roots, or excised roots, were incubated for 20 h in NaCl or KCl solutions at 7.5 atmospheres.

	pmol CO ₂ / g fresh wt.		% of control	
	ADC	ODC	ADC	ODC
Excised roots				
control	2813	466	100	100
NaCl	1565	960	56	206
KCl	1352	713	48	153
Intact plant roots				
control	681	99	100	100
NaCl	556	269	82	272
KCl	456	192	67	194

Table 27. The in vitro effect of NaCl on ADC and ODC activity of mung bean roots.

The crude extract of mung bean roots was assayed in the presence of increasing NaCl concentrations in the reaction mixture.

osmotic values of NaCl (Atm)	nmoles CO ₂ /mg protein	
	ADC	ODC
0	11.16	2.86
1.5	11.86	2.33
2.5	8.39	1.91
5.0	7.56	1.82
7.5	6.63	1.12

Table 28. The effect of salt stress on ADC and ODC activity of several plant species.

Excised roots of the various plants were incubated for 20 h in NaCl solutions at 5 atmospheres.

		pmoles CO ₂ / mg protein		% of control	
		ADC	ODC	ADC	ODC
Mung bean	control	2300	401	100	100
	NaCl	1829	572	79	143
Vetch	control	623	165	100	100
	NaCl	441	480	71	290
Sorghum	control	1526	2641	100	100
	NaCl	2668	3888	175	147
Corn	control	3633	2429	100	100
	NaCl	3652	2462	100	101
Tomato	control	1484	619	100	100
	NaCl	3311	1124	223	182

Table 29. The effect of salt stress on polyamine content of mung bean plants.

Mung Bean plants, grown in vermiculite, were irrigated on the 4th, 5th and 6th day from germination with saline water containing 10.5, 21 and 31.5 g/NaCl, respectively. The 7-day-old seedlings were separated and polyamine content was determined in the various organs.

	n moles/g fresh weight				put/spd ratio		Protein content mg/g fresh wt.	
	putrescine	spermidine	putrescine	spermidine	putrescine	spermidine	putrescine	spermidine
	Cont	NaCl	Cont	NaCl	Cont	NaCl	Cont	NaCl
Leaves	46.2	79.9	72.8	66.8	0.63	1.20	18.6	26.5
Epicotyl	33.5	133.4	55.5	75.2	0.60	1.76	6.4	11.2
Hypocotyl	47.1	66.3	54.1	77.6	0.88	0.87	6.0	8.9
Roots	40.3	33.0	91.5	39.2	0.45	0.86	6.6	8.0

Table 30. Incorporation of (U-¹⁴C) arginine and (U-¹⁴C) ornithine into polyamines of mung bean plants as affected by salt stress.

Eight-day-old mung bean plants were grown for 24 h in tap water without or with NaCl (10.5 g/l). Leaf discs and 1.5 cm lateral root sections were then excised and incubated for 24 h in similar solutions which contained also 0.5 μ Ci (U-¹⁴C) arginine or (U-¹⁴C) ornithine.

	dpm/ μ g protein						put/spd incorporation ratio	
	putrescine		spermidine		spermine		Cont	NaCl
	Cont	NaCl	Cont	NaCl	Cont	NaCl		
¹⁴ C-Arginine								
Leaves	0.48	0.20	0.12	0.20	0.04	0.16	4.02	1.03
Roots	6.72	9.39	3.75	4.80	1.00	0.97	1.79	1.95
¹⁴ C-Ornithine								
Leaves	0.30	0.18	0.07	0.08	0.05	0.04	4.31	2.25
Roots	3.20	5.07	1.32	5.94	1.00	1.04	2.42	0.85

Table 31. Polyamine content and ADC and ODC activity of leaves of mung bean (*Vigna*) and several halophytes.

Eight-day-old *Vigna* seedlings (grown in vermiculite in the phytotron) and ca. 3 week-old seedlings of the halophytes (grown in natural habitat) were used for the determinations.

	PA, nmole/mg protein			put/spd ratio	nmole CO ₂ / mg protein	
	Putrescine	Spermidine	Spermine		ADC	ODC
<i>Vigna radiata</i>	15.03	17.26	6.80	0.87	1.95	0.88
<i>Atriplex halimus</i>	4.24	5.82	4.52	0.72	0	0
<i>Pulicaria undulata</i>	2.28	4.42	1.98	0.51	4.67	1.65
<i>Mesembryanthemum</i>	3.73	7.47	2.82	0.50	0	0

Table 32. ADC and ODC activity of roots of mung bean plant and two halophytes grown in saline or in washed soil.

Young plants were grown for 18 days in saline soil (conductivity of 2.0 mmhos) or in soil which was washed to leach all salts (conductivity of 0.3 mmhos). Enzyme activity was assayed in samples of 1.5 cm section of lateral roots.

	nmoles/g fresh wt/h		nmoles/mg protein/h		ADC/ODC ratio
	ADC	ODC	ADC	ODC	
Mung bean, washed	3.55	0.65	2.00	0.37	5.5
saline	4.01	1.33	2.42	0.80	3.0
Pulicaria undulata, washed	62.50	12.99	19.12	3.97	4.8
saline	31.55	5.77	21.92	4.00	5.5
Atriplex halimus, washed	6.51	4.73	7.90	5.77	1.4
saline	3.80	2.86	4.26	3.21	1.3

2.3.6. CONTROL OF POLYAMINE BIOSYNTHESIS BY PHYTOCHROME AND GIBBERELLIN.

Introduction

Since phytochrome and polyamines act by regulating similar aspects of cell growth, a question arises as to their possible functional relationship. Thus far, there has been no report connecting phytochrome status with the activity of biosynthetic enzymes of polyamines. Here, we present evidence for a two way, organ-specific phytochrome regulation of ADC activity in buds and epicotyls of etiolated pea seedlings. Since the Pfr-induced changes in ADC activity parallel the opposite Pfr-induced growth changes in these organs, the possibility of a causal connection between polyamine level and growth is strengthened.

Furthermore, since GA_3 is known to reverse the effects of red light in etiolated peas and to stimulate stem growth, especially in dwarf cultivars we also investigated the influence of GA on the control of polyamine biosynthesis.

Materials and Methods

For phytochrome studies seeds of Pisum sativum L. var. Alaska were imbibed for 10 h in tap water in the dark and sown in prewashed vermiculite. After 6 days in the dark at 27 C and about 70% RH, seedlings were selected for uniformity of third internode length (30-50 mm) and sharply recurved apical hooks.

Light Sources and Irradiation. The R source (600-900 nm) consisted of four 15-w red fluorescent tubes (Sylvania) wrapped with two layers of Dupont red cellophane. Energy at the seedling level was $2.0 \text{ kiloergs cm}^{-2}\text{s}^{-1}$. The FR source (710-760 nm) consisted of five 300-w internal reflector incandescent flood lamps filtered through 16 cm water and a Westlake FR Plexiglas FRF 700 filter, emitting $19 \text{ kiloergs cm}^{-2}\text{s}^{-1}$ energy at the seedling level. Both R and FR irradiations lasted for 5 min. After light treatment, the seedlings were kept in the dark for different time periods until harvest and assay. In the experiments involving photoreversibility, R and FR irradiation was followed immediately by FR or R, respectively.

Enzyme Extraction. For preparation of the crude enzyme extract, eight buds or eight subapical 5-mm epicotyl sections from the third internode were harvested under a photomorphogenically inactive green safelight and ground in chilled mortar with 1 ml 100 mM phosphate (pH 7.0). The homogenates were centrifuged at 30,900g for 15 min at 4°C; the supernatant fraction was used for enzyme assay and protein estimation. For GA studies seeds of a dwarf variety of Progress pea (Pisum sativum; Asgrow, Inc.) were imbibed overnight in tap water and sown in bottom-perforated plastic containers in coarse vermiculite. They were subirrigated twice daily with a solution of 1.2 g/l Hyponex (Hydroponics Chemicals Co., Copley, OH) in a temperature-controlled room at 24°C, with a 16-h photoperiod of about 2,000 ft-c of mixed daylight fluorescent-incandescent light (about 9:1 energy ratio). Nine days after sowing, experimental plants were selected for uniformity and received spray treatment.

Application of GA₃ and AMO-1618. Nine-day-old plants were sprayed with GA₃ (Sigma; 5, 20, or 34.6 µg/ml) or AMO-1618 (Calbiochem; 100 or 200 µg/ml). All solutions were prepared in 0.03 M phosphate buffer (pH 6.4) containing 0.05% pluronic L101 (Wyandotte Chemical Corp.) to aid penetration. Buffer solution with pluronic L101 was used as a control. Two days after spraying, the plants were harvested for enzyme assays and determination of polyamine levels.

Enzyme Extraction. Seven complete excised fourth internodes from light-grown Progress pea seedlings were homogenized in prechilled mortars, with 1 ml 100 mM phosphate buffer (pH 7.0) for ADC, or 100 mM Tris-HCl buffer (pH 7.1) for ODC activity. The homogenates were centrifuged at 27,000g for 15 min at 4°C, and the supernatant fractions were used for enzyme assays and protein estimations.

Enzyme Assays. ADC activity was determined by measuring the release of ¹⁴CO₂ from (U-¹⁴C)-L-arginine (New England Nuclear; 300 mCi/mmol) as described earlier (53). For ODC assay, a similar method was used with DL-(1-¹⁴C)-ornithine (New England Nuclear; 54.9 mCi/mmol) as substrate. For the pH optima and cofactor

requirement experiments, the components of the reaction mixture were as described in the text. Protein in the crude enzyme was determined according to Lowry et al.(116), using BSA as a standard.

Extraction and Measurement of Polyamines. Seven complete excised fourth or fifth internodes were extracted with 1 ml 5% perchloric acid. The homogenates were centrifuged at 27,000g for 15 min at 4°C. Aliquots of the supernatant fraction (0.2 ml) were added to a mixture of 0.4 ml of dansyl-Cl (Sigma; 5 mg/ml acetone) and 0.2 ml of a saturated solution of Na_2CO_3 . After incubation overnight, the dansylated products were extracted with 0.5 ml benzene. Fifty μl benzene extract were then spotted on a silica gel thin layer plate (Whatman; LK6D), and thin layer chromatograms were developed with chloroform:triethylamine (25:2, v/v). The fluorescent spots, located in a dark chamber with ultraviolet light, were scraped off and eluted with ethyl-acetate (Baker), and the dansylated polyamines were measured in an Aminco-Bowman spectrophotofluorimeter with an activating wavelength of 350 nm and a fluorescent wavelength of 495 nm. Standard curves permitted calculation of concentration equivalents.

Replication of Experiments. The data presented are from single experiments which are representative of 2 to 4 experiments or their means, each performed in duplicate or triplicate. For experiments using 7-day-old dark-grown Alaska pea seedlings, procedures of plant-enzyme assay, red-light treatment, and application of GA are the same as those described earlier (53); in this case, however, the spraying of GA was usually performed 30 min before red irradiation.

Results and Discussion

Control by phytochrome. Six day old etiolated pea plants were given a 5 min exposure to red light and the terminal buds (mainly leaf tissue) and subapical epicotyl (stem) tissue harvested and compared with dark controls. In buds, where the specific activity of ADC rose gradually in the controls over the eight hour test

period, red light caused a highly significant doubling of ADC specific activity within two hours, and a continued rise until the end of the experiment (Figure 29). This effect was mediated by phytochrome, as shown by mutual red/far-red reversibility (Figure 30). In epicotyls, on the other hand, where the dark controls show a fairly steep rise in ADC activity over the eight hour test period, red light completely prevents this rise (Figure 31). This effect, too, is completely red/far-red reversible (Figure 32). Cycloheximide (5 µg/ml) strongly inhibits the increase of ADC activity in both rapidly-growing organs (darkened epicotyls and red-irradiated buds) but is without much effect on the already inhibited red-irradiated epicotyls and darkened buds. It thus appears that phytochrome controls the de novo synthesis of ADC in both organs, but in opposite ways. ADC is the only enzyme yet known whose activity is controlled in this organ-specific, opposite manner. This correlation lends support by our subsequent demonstration (69) that polyamine titer does change in etiolated pea seedlings following red light treatment. In buds, red light increased the titers of agmatine, putrescine and spermidine (Figure 33), while in epicotyls red light decreased the titers of each of these polyamines. In both organs, agmatine, the direct product of arginine decarboxylation, was the polyamine most affected.

Is the rise or fall of ADC activity merely a secondary consequence of the rise or fall of growth rate? We used a peculiarity of the etiolated pea system to show that changes in ADC are in fact independent of growth. If the terminal bud is excised together with a long piece of epicotyl consisting of several internodes, then red light promotes the growth of the terminal bud, although to a lesser extent than in the intact plant. If the bud is excised with only a short piece of attached epicotyl, red light cannot promote bud growth. Yet in both systems, red light promotes ADC activity in the buds. This shows that red light has its effect on ADC activity irrespective of its linkage to growth.

Control by gibberellin.

Effect of GA₃ and AMO-1618 on ADC and ODC Activities. It is well known that GA₃ elicits dramatic stem elongation when applied to light-grown dwarf peas (38). In general, AMO-1618 works in the opposite direction, but its effects cannot be interpreted in terms of GA alone, inasmuch as it also inhibits the formation of steriods. It is thus, gratifying that similar opposite effects of GA₃ and AMO-1618 on ADC activity were found in fourth internodes of light-grown Progress pea seedlings. Figure 34 shows that a single spray treatment with 5 µg/ml GA₃ enhanced ADC activity more than 3-fold as compared to the control, while 34.6 µg/ml GA₃, presumably slightly supraoptimal, caused a 250% increment in ADC activity. Conversely, 100 µg/ml AMO-1618 produced a slight inhibition of ADC activity.

It is both surprising and significant that GA₃ treatment significantly decreased ODC activity in fourth internodes (Figure 35). AMO-1618 also inhibited ODC activity even more strongly than it inhibited ADC activity. Thus, ADC, but not ODC, responds to GA₃ treatment in a direction that makes sense for polyamine levels and growth effects (see below).

Effects of GA₃ and AMO-1618 on Polyamine Levels in Internodes and Their Correlation with Growth. Table 33 shows that GA induces about 1.5 and 3.0 times as much elongation in fourth and fifth internodes, respectively, as it does in controls. By contrast, AMO-1618 inhibited elongation by about 10%.

Levels of Put, Spd, and Spm, determined by TLC in fourth and fifth internodes after length measurements had been made, revealed a close correspondence between internode growth and polyamine content. As shown in Table 34, Put and Spd increase about 1.5-fold after GA treatment and decline about 15% in fourth internodes after AMO-1618 application. In fifth internodes, GA treatment caused much stronger increase of Put and Spd levels that paralleled the increased internode elongation (Table 33). Similarly, AMO-1618 produced parallel decreasing effects on Put and Spd

levels and on elongation. Spm seemed not to correlate well with growth; for this polyamine, the differences induced by GA_3 and AMO-1618 are slight, both in fourth and fifth internodes (Table 34).

GA Effect on the Red-Light-Induced Inhibition of ADC Activity in Etiolated Alaska Pea Epicotyls. We have shown previously (53) that transformation of phytochrome from Pr to Pfr decreases the growth and ADC activity in epicotyls of etiolated Alaska pea seedlings in a parallel fashion. Table 35 shows that the inhibition of ADC activity by red light can be completely reversed by GA_3 and that GA_3 has no effect on ADC activity in dark-grown plants. Thus, in both green and etiolated pea seedlings, gibberellin acts opposite to Pfr phytochrome.

A clear and marked stem elongation in response to applied GA_3 has made the intact light-grown dwarf pea plant a convenient system for the investigation of the possible relation between GA action and polyamine metabolism. To date, there is scant information on possible correlations between GA and polyamines, despite the fact that the similar effects of both classes of compounds are well established (23). Our results show that, of all the PAs, the level of Put is most markedly raised by GA treatment. Inasmuch as this diamine arises from arginine via either ADC or ODC, the activities of these enzymes were studied in reference to the effects of applied GA and AMO-1618. The promotion of ADC activity by GA in intact Progress pea internodes resembles that in excised cucumber cotyledons (188). Unlike in many animal systems (10, 16, 207) and some plant systems (75) ODC activity is not related to growth rate in our pea system. Furthermore, the inhibition of ODC activity by both GA and AMO-1618 indicates the existence of a complex regulatory mechanism for PA biosynthesis in this plant.

In addition, our kinetic study shows that, in the fourth internodes of light-grown Progress pea seedlings, the stimulation of ADC activity by GA_3 appears 6 h after its application, and two peaks of the stimulation have been observed at 9 h and

at about 27 h. On the other hand, the promotion of the length growth of 4-h internodes by GA_3 does not occur until 12 h. A distinct promotion appears at 24 h and continues to increase with time up to 36 h. The results suggest that an increment of ADC activity preexists before the stimulation of length growth, possibly implicating a cause-and-effect relationship between GA-induced promotion in polyamine biosynthesis and growth.

The correspondence of GA effects on ADC activity and stem elongation in the light-grown Progress pea system is comparable to the phytochrome effect on ADC activity and epicotyl elongation in the etiolated Alaska pea system (53). Actually, the dwarfism of light-grown Progress peas can be considered a result of photo-inhibition of stem elongation, inasmuch as, in the dark, Progress pea behaves like the normal Alaska pea. Thus, we have to confront an old and unsolved question, i.e., the meaning of the reversal by GA of photoinhibition of stem elongation. Lockhart (112), noting the reversal of red-light inhibition of Alaska pea stem growth by applied GA, postulated that light acts through inhibition of gibberellin synthesis (113). However, the data of other authors could not confirm this hypothesis (86, 100, 133). Our results (Figure 34 and Table 35) make it possible that both the reversal of photoinhibition of elongation by GA in the etiolated Alaska pea system and the promotion of elongation by GA in light-growth Progress pea system depend on the same mechanism, i.e., one mediated via polyamines and their biosynthesis.

It is noteworthy that PA level per unit fresh weight or dry weight is fairly constant, but PA per organ or starting tissue weight changes dramatically. This suggests a regulatory role for PA levels in growing tissue. A similar analysis was made by Suresh et al. (188), using paired cotyledons as a basis for comparison. The control by GA of polyamine biosynthesis and the correlation between PA increase and level of induced growth compels further examination of the possible role of polyamines in controlling the growth of plant cells.

Figure 29. (left) The kinetics of change of ADC specific activity in buds of etiolated pea seedlings. Bars indicate standard error of the mean.

Figure 30. (right) FR reversibility of R-induced promotion of ADC activity in buds of etiolated pea seedlings. Measurements made 6 h after light treatment. Bars indicate standard error of the mean. D, dark; R, red; FR, far-red.

Figure 31. (left) The kinetics of change in ADC specific activity in epicotyls of etiolated pea seedlings.

Figure 32. (right) FR reversibility of the R-induced inhibition of ADC activity in epicotyls of etiolated pea seedlings.

Figure 33. Polyamine titer, determined by HPLC, in buds (left) and internodes (right) of 6-day-old etiolated seedlings: dark control and red light treated. Spd, spermidine; Spm, spermine; Put, putrescine; Agm, agmatine.

Figure 34. Effect of GA and AMO-1618 on ADC activity in fourth internodes of light-grown Progress pea seedlings. Measurements from a single representative experiment were made 2 days after GA and AMO-1618 treatments. The SE are in the range of 4.46 to 16.20.

Figure 35. Effect of GA and AMO-1618 on ODC activity in fourth internodes of light-grown Progress pea seedlings. Measurements from a single representative experiment were made 2 days after GA treatment. The SE are in the range of 1.83 to 16.94.

Table 33. Effect of GA and AMO-1618 on the Growth in Length of Internodes of Light-Grown Progress Pea Seedlings.

Measurements were made 2 days after GA and AMO-1618 treatments.

Treatment	Growth in Length			
	Fourth internodes		Fifth internodes	
	mm	%	mm	%
Control	9.78±0.24	100	6.68±0.51	100
GA (20 µg/ml)	16.03±0.59	165	21.50±0.73	322
AMO-1618 (200 µg/ml)	9.18±0.42	94	5.62±0.50	84

Table 34. Effect of GA and AMO-1618 on Polyamine Content of Internodes of Light-Grown Progress Pea Seedlings.
Measurements were made 2 days after GA and AMO-1618 treatments (in triplicate).

Treatment	Putrescine				Spermidine				Spermine			
	Internode 4	Internode 5	Internode 4	Internode 5	Internode 4	Internode 5	Internode 4	Internode 5				
	nmol/ internode	%	nmol/ internode	%	nmol/ internode	%	nmol/ internode	%				
Control	13.60±0.50	100	5.39±0.18	100	54.89±3.74	100	21.74±0.32	100	18.94±1.07	100	13.68±1.06	100
GA (20 µg/ml)	21.66±1.50	159	15.33±2.87	284	92.54±2.39	169	39.37±5.19	183	23.15±0.81	122	17.99±1.69	132
AMO-1618 (200 µg/ml)	12.08±0.00	89	4.75±1.31	88	46.46±2.65	85	19.14±0.71	88	17.30±0.61	91	12.97±2.12	95

Table 35. Effect of GA and Red Light on ADC activity in Etiolated Alaska Pea Seedlings.

Measurements were from a single representative experiment made 6 hrs after red irradiation.

Treatment	ADC activity	Relative Activity
	cpm $\times 10^{-3}$ /mg protein	%
Dark	55.49 $\pm$ 6.62	100
Red	36.66 $\pm$ 5.14	66
Dark + GA (5 μ g/ml)	53.73 $\pm$ 1.50	97
Red + GA (5 μ g/ml)	57.32 $\pm$ 1.80	103

2.3.7. CONTROL OF POLYAMINE BIOSYNTHESIS BY OSMOTIC AND pH STRESS.

Introduction

Polyamine metabolism is remarkably sensitive in higher plants to various stress conditions (senescence, wounding, heat, salinity, etc.). Studies were therefore undertaken to examine the effect of osmotic and pH stress on polyamine biosynthesis.

Materials and Methods

Victory oat seeds were sown as described in General Material and Methods. The primary leaves of 7-9 day old leaves were harvested, the lower epidermis removed by stripping with a fine forceps, and the leaves floated in the light in the presence or absence of osmoticum or particular pH buffers. Polyamine titer and the biosynthetic enzyme activities were determined as described in General Material and Methods.

Results and Discussion

A significant increase in putrescine was evident after 2 hours of floating of peeled leaves on 0.6M sorbitol, and a level 60-fold higher than the initial value was reached by 6 hours (Figure 36A). In contrast, putrescine concentrations were not significantly altered by incubation of leaf segments in phosphate buffer (1 mM, pH 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 6N HCl (214). 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 (175). 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 0.4M sorbitol also showed significant increases in putrescine after 4 hours (Figure 36B). 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 (Figure 36B). 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 0.1M sorbitol increased the putrescine titer 2.5-fold after 4 hours. Increased putrescine levels have also been noted during deficiencies of potassium (155,25) and magnesium (25), acid stress, (214,179) and NH_4Cl feeding (108). Bacterial and mammalian cells, however, show an inverse correlation between polyamine content (putrescine, spermidine, and spermine) and the osmolality of the culture medium (130).

In contrast with mammalian cells, in which putrescine is synthesized solely by decarboxylation of ornithine, putrescine can be formed in higher plants via agmatine through the action of arginine decarboxylase. There is evidence for the widespread coexistence of these pathways. Since both ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) are present in oat leaves (98,170,177), these enzyme activities were monitored in the osmotically stressed oat leaf segments. As shown in Table 36, 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 (170), upon acid feeding and hormone treatment in Cucumis sativus (188), and

upon acid treatment in barley (179).

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 (91). It completely prevented the increase in putrescine while concomitantly inhibiting ADC activity in a concentration-dependent manner (Figure 36 and Table 36). DFMA did not affect the spermidine and spermine titers. In contrast, DL- α -difluoromethylornithine (DFMO), an analogous inhibitor of mammalian ODC (122), 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 (90), 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 36). 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, 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 mM in the in vitro assay (177), 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 (214) occurs within a few hours. The apparent time lag for the putrescine increase (Figure 36), 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 $\mu\text{g/ml}$) 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 Figure 36 with Table 36), it is possible that a previously described phytochrome control of ADC activity is involved (53).

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 (74), 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 (9,95) for cereal protoplast culture.

We have also found that the putrescine content 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.

Low pH has also been reported to cause elevation of putrescine titer in cereal leaves (179). Accordingly we floated peeled oat leaves and excised pea leaf discs for 8 hours in the light on buffered mineral nutrient solutions containing KNO_3 , 3.0 mM; NH_4NO_3 , 3.0 mM; CaCl_2 , 2.0 mM; MgSO_4 , 0.5 mM and $\text{NH}_4\text{H}_2\text{PO}_4$ 0.5mM. Succinic acid (5.0 mM) coupled with variable concentrations of Tris-base provided a range of pH's from 8.0 to 3.5. Additional buffer systems were checked, and gave similar results, showing that the effects obtained were due to pH and not to choice of buffer.

In both oat and pea leaves, putrescine titer was low and constant at pH's greater than 6; putrescine rose slightly between pH 6 and 5, and steeply between pH 5 and 3.5

(Figure 37). In oat, the effect of lowered pH was seen within 3 hours and peaked at about 9 hours (Figure 38), and was sensitive to cycloheximide and DFMA but not to DFMO. It appears that the pH effect, like the osmotic effect, is due to activation of arginine decarboxylase.

Does the stress-induced rise in putrescine titer have any adaptive value for the stressed cell? It could, of course, itself serve as a buffer to resist changes in cellular pH, or to serve as a facilitator of the transport of H^+ or other cations across the plasma membrane. Putrescine is also known to inhibit the activity of ribonuclease (65) and acid protease (97), two enzyme systems associated with injury and senescence in plant cells (96). Putrescine can also protect membranes of fragile protoplasts against spontaneous or injury-induced lysis (9).

Table 36. Polyamine titer and biosynthetic activity in oat leaf segments under osmotic stress.

Peeled leaf segments (7 days old) were floated in buffer (control) or buffer plus 0.6M 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 and enzyme assay. The results for the polyamine concentrations are expressed as means $\pm$ 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, with gamma globulin being used as the standard.

Treatment	Polyamine (nmole/g fresh weight)			Enzyme activity per hour (nmole of CO ₂ /mg protein)	
	Putres- cine	Sper- midine	Sperm- ine	Arginine decarbox- ylase	Ornithine decarbox- ylase
Control (at 0 hour)	16 $\pm$ 1	161 $\pm$ 18	41 $\pm$ 4	2.27 (115)	4.24 (93)
Control (at 4 hours)	13 $\pm$ 2	152 $\pm$ 10	38 $\pm$ 3	1.98 (100)	4.55 (100)
Sorbitol	102 $\pm$ 5*	120 $\pm$ 13+	21 $\pm$ 2	3.81 (192)*	4.65 (102)
Sorbitol plus 10 ⁻³ M DFMO	129 $\pm$ 7*	186 $\pm$ 10+	32 $\pm$ 3	5.31 (268)*	4.68 (103)
Sorbitol plus 10 ⁻⁴ M DFMA	20 $\pm$ 2+	123 $\pm$ 7+	36 $\pm$ 2	1.24 (63)+	4.64 (102)
Sorbitol plus 10 ⁻³ M DFMA	10 $\pm$ 1	128 $\pm$ 8	32 $\pm$ 2	0.41 (21)*	4.55 (100)

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

+Significantly different at P < .05.

Figure 36. (A) Changes in putrescine titer in peeled oat leaf segments floated on buffer (Δ), buffer plus 0.6M sorbitol ($\circ$), and sorbitol in the presence of 10^{-3} M DFMO ($\circ$) or 10^{-3} M DFMA ($\square$) (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 26,000g 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 farua* (wild oat), barley cv. Himalaya, and 9-day-old hybrid corn Golden Cross Bantam were floated over buffer (solid bars) or buffer plus 0.4M sorbitol (hatched bars) as in (A). Wheat cv. Yamhill leaf segments were floated unpeeled. After 4 hours incubation under light, triplicate samples were extracted and dansylated, and the polyamines were analyzed by thin layer chromatography (5).

Figure 37. Putrescine titer in excised and peeled oat leaf segments and pea leaf discs after 8 h incubation on nutrient solutions buffered to various external pH values. (Error bars represent ± 1 S.D.).

Figure 38. Time course of changes in putrescine levels in excised and peeled oat leaf segments on solutions of two different external pH values, pH 4 (—) and pH 6 (—A—). Error bars represent ± 1 S.D.).

2.3.8. INVOLVMENT OF POLYAMINES IN POTATO BUD SPROUTING.

Introduction

Both the activity of PA biosynthetic enzymes and PA titer have been reported to increase dramatically during rapid growth in many plant systems, such as germinating seeds of Zea, Pisum, Triticum, and Tragopogon (203), developing seedlings of Phaseolus (20) and Lathyrus (152), ovaries of tomato, and rapidly dividing tobacco cells in suspension culture (75), during crown gall-tumor development (23) and embryogenesis of carrot suspension cells (126,127). Although little is known about the activities of PA biosynthetic enzymes in dormant tissues such as tubers and senescencing plant parts, contents of PA in these systems are extremely low (5,168). We have observed that activities of PA biosynthetic enzymes and PA titer decrease during aging and senescence of oat leaves (98). Furthermore, exogenous application of the PA or their precursor amino acids retards senescence of leaves in several monocotyledonous and dicotyledonous plants (96) and of protoplasts from oat leaves (9,65,93,95). PA appear to inhibit senescence by preventing Chl, protein, and RNA breakdown in leaves (96) and by increasing macromolecular synthesis and mitotic activity in protoplasts (95). The antisenescence properties of PA and their correlation with cell proliferation and differentiation lend support to the contention that they act as growth factors (16,23,66). Despite these observations, the relative roles of individual PA biosynthetic enzymes in dormant and actively dividing plant tissues is not well understood. We have, therefore, examined the activities of ADC, ODC, and SAMDC and the endogenous levels of Put, Spd, and Spm in dormant and actively growing tissues of potato tubers.

Materials and Methods

Idaho Russet baking potatoes (Solanum tuberosum L.) (U.S. No. 1) were purchased from the local supermarket. The tubers were firm, with no sign of sprouting, and were therefore regarded as dormant. Medium-size tubers were selected

and allowed to sprout by storing them in the dark at room temperature for about 2 months. Samples of tissue were taken from dormant, initial, and advanced (profuse) sprouted tubers. Three areas from the same tuber showing different sprouting activity were selected for sampling (Figure 39): (A) bud tissue in the apical region (hereafter referred to as apical buds) which developed sprouts; (B) bud tissue from the lateral region (hereafter referred to as lateral buds) which remained dormant throughout the storage period; and (C) nonbud tissue which does not develop sprouts. Tissue sections (7 mm in diameter, 3 mm in length) containing the skin and outer cortex were removed. During the periods when sprouting occurred, the sprouts were excised with the apical bud tissue and assayed together. Six to eight tubers were used for each experiment and slices of each area were randomized. Suitable amounts were selected for determining ADC, ODC, and SAMDC activities and PA titer. Enzyme activities and polyamine levels were determined as described previously.

Results and Discussion

Enzyme Activities in Dormant and Sprouting Potato Tubers. When firm dormant potato tubers are kept in the dark at room temperature, sprouting starts to occur after about 30 days and is profuse after about 60 days (Figure 39). These stages are described for convenience as dormant, initial sprouting, and profuse sprouting (Figure 39). Under our storage conditions, the apical buds sprouted while most of the lateral buds remained dormant. Measurements of enzyme activities at dormancy and progressive stages of sprouting (Figure 40) show that ODC, SAMDC, and ADC activities are approximately the same in the apical and lateral buds and nonbud tissues of the dormant tuber. However, with initiation of sprouting after 30 days of storage, ODC and SAMDC activities in the apical buds increase and continue to increase with progressive sprouting. ODC activity at advanced sprouting after 60 days was more than four times and SAMDC more than two times the respective activities at the dormant stage. ADC activity, on the other hand, showed a slight increase with

initiation of sprouting and then declined to the value at the dormant stage. Dialysis of the enzyme extracts did not alter the enzyme activities, suggesting that the higher ODC and SAMDC activities at sprouting are not due to a low molecular weight activating molecule. Likewise, the low ADC activity is not due to a low molecular weight inhibitor. The above observations were confirmed by mixing (1:1, v/v) extracts from buds of dormant tubers with sprouted apical buds which showed approximately half the initial values. The activities of ODC and SAMDC in the lateral buds and nonbud tissues remained essentially the same, whereas ADC activity decreased with progressive sprouting (Figure 40).

The maximum increase in enzyme activities clearly occurred at the initiation of sprouting and was limited to the apical buds; ODC activity increased more than 300% from dormant to initial sprouting and about 200% from initial sprouting to profuse sprouting (Figure 41). SAMDC showed a similar trend. These findings suggest that the high activities of the PA biosynthetic enzymes, ODC and SAMDC, are related to active cell division initiated by the breaking of dormancy. Similar correlation of ODC and SAMDC activities with cell division have been shown in other plant systems, such as developing ovaries of tomato, actively dividing tobacco cells in suspension cultures (75) and germinating corn seeds (190). Correlation of ODC and SAMDC activities with cell proliferation has also been reported in animal tissues (16,48), while low enzyme activity with declining cell activity has been observed in resting and aging animals, in nongrowing tissues (16,48) and in senescing leaves (98). The observed 5-fold increase in ODC activity at the profuse sprouting stage suggests that ODC is the primary enzyme involved in polyamine biosynthesis during sprouting of the potato tuber. In plants, polyamine biosynthesis via ADC has thus far been considered the more probable pathway, partly because more work has been done with ADC rather than ODC (16,177). A recent investigation (75), however, shows much higher activities of ODC than ADC in developing ovaries of tomato and in the exponential

growth phase of tobacco cells in suspension cultures. In animal systems also, ODC is considered the key enzyme in PA biosynthesis (16,48). Our observations with the potato tuber, together with those of Heimer et al. (75) suggest that either ODC or ADC can act as the rate-limiting enzyme for PA biosynthesis in different actively growing plant tissues.

Polyamine Titer. Measurements of PA levels show that Put, Spd, and Spm are present in tissues from dormant and sprouting potato tubers (Table 37). Cadaverine was not detected either by HPLC or TLC. In dormant tubers, PA titer in apical buds, lateral buds, and nonbud tissues is essentially the same (Table 37). However, at break of dormancy accompanying initiation of sprouting, PA in the apical buds increased sharply, and like the enzyme activities, continued to increase with progressive sprouting. Spm increased more than three times, Spd about two times, and Put about 1.5 times the initial values at dormancy. At profuse sprouting, the increase in Spm titer was the highest. Levels of Spm in the apical buds increased by more than 10 times, while Spd and Put increased by about three and two times, respectively, when compared with the initial levels (Figure 42). Furthermore, the HPLC profiles of PA show that peaks of the individual PA are well resolved and that PA titer increases with progressive sprouting. Spd and Spm are the first to rise significantly, followed by Agm and Put. A similar trend was observed when samples were analyzed by TLC (data not presented).

The profuse sprouting in the apical buds appears to suppress growth of lateral buds. Consequently, in the lateral buds the endogenous levels of most of the PA, like the biosynthetic enzymes, remain essentially the same during sprouting as in the dormant stage. Similarly, PA titer in the inactive nonbud tissue remains unchanged. In both types of tissues however, Spm titer increased with sprouting, but the levels are much lower than those in the apical buds (Table 37).

The high activities of ODC and SAMDC and the corresponding increases in PA levels

during initial sprouting are consistent with the view that increased synthesis of PA is one of the primary events controlling the inception of growth in previously dormant tissue. This is further supported by the correlation of low activities of ODC and SAMDC and low titer of PA with no sprouting in lateral buds inhibited by apical dominance. Correlation of high endogenous levels of PA with break of dormancy by hormones or by slicing has also been reported in tubers of Jerusalem artichoke (23,168).

To investigate further the role of ADC in PA synthesis during sprouting in potato tuber, we determined levels of Agm (best resolved by HPLC) which is an intermediate in putrescine synthesis from arginine mediated via ADC. Although there was no appreciable rise in ADC activity during sprouting, Agm levels increased sharply in the apical buds. In contrast, both ADC activity and Agm levels remained essentially unchanged in lateral buds and nonbud tissues. In apical buds, the Agm titer increased by about five times in the advanced stage of sprouting as compared with the dormant stage (Table 37). The high levels of Agm during sprouting might depress ADC activity by end-product inhibition, or the conversion of Agm to Put might constitute the rate-limiting step in this tissue. Although the lack of correlation between Agm and ADC activity during sprouting is puzzling, the results indicate that the observed increased level of putrescine does not directly involve ADC. These results further support the contention that ODC is the rate-limiting enzyme in Put synthesis in potato tubers.

The high localized PA titer in advanced sprouting and the low titer in dormant tubers suggest that PA, like the well-known hormones ABA, gibberellins, and cytokinins, can act as growth factors. PA probably differ from hormones, however, in being relatively immobile intercellularly. ABA levels generally increase with dormancy, whereas gibberellins and cytokinins increase with break of dormancy. Such changes in endogenous levels of these hormones have been well established in seeds,

and vegetative and flower buds, which like the potato tuber, undergo a period of dormancy. In the potato tuber also, dormancy is associated with low endogenous levels and sprouting with high levels of growth promoting hormones (76,200). The increased PA synthesis during potato tuber sprouting observed here suggests that PA are also involved in the break of dormancy, perhaps as second messengers. The action of exogenous PA in preventing senescence of excised leaves (5,96), protoplasts (9), and chloroplast thylakoids (148) support their proposed physiological role.

Figure 40. Activities of ODC, SAMDC, and ADC in apical and lateral buds and nonbud tissue of dormant and sprouting potato tubers at different stages of sprouting: 0 day, dormant; 30 days, initial sprouting; 60 days, profuse sprouting.

Figure 41. Comparison of ADC, SAMDC, and ODC activities in apical buds of dormant and sprouting potato tubers.

Figure 42. PA content in apical buds of dormant and sprouting potato tubers.

Table 37. Polyamine Content in Progressive Stages of Sprouting Potato Tubers. PA extracts in PCA were benzoylated, separated and quantified by HPLC.

PA Content in Tuber Tissue				
PA	Type Tissue	Dormant	Initial sprouting	Profuse sprouting
$\mu\text{g/g fresh wt}^*$				
Agm	Apical buds	40.4 $\pm$ 2.1	65.3 $\pm$ 3.1	181.9 $\pm$ 8.7
	Lateral buds	40.4 $\pm$ 2.1	50.6 $\pm$ 1.7	55.6 $\pm$ 2.0
	Nonbud tissue	20.8 $\pm$ 2.0	32.8 $\pm$ 1.7	37.8 $\pm$ 6.6
Put	Apical buds	4.5 $\pm$ 0.7	6.4 $\pm$ 0.1	9.3 $\pm$ 0.3
	Lateral buds	4.5 $\pm$ 0.7	4.4 $\pm$ 0.3	4.5 $\pm$ 0.1
	Nonbud tissue	6.0 $\pm$ 0	4.5 $\pm$ 0.2	4.7 $\pm$ 0.4
Spd	Apical buds	7.7 $\pm$ 1.7	14.5 $\pm$ 1.6	20.7 $\pm$ 2.3
	Lateral buds	7.7 $\pm$ 1.7	9.6 $\pm$ 0	9.9 $\pm$ 0.1
	Nonbud tissue	9.0 $\pm$ 0.4	8.5 $\pm$ 0.6	7.3 $\pm$ 0.1
Spm	Apical buds	1.0 $\pm$ 0.2	3.4 $\pm$ 0.5	10.2 $\pm$ 1.0
	Lateral buds	1.0 $\pm$ 0.2	3.4 $\pm$ 0.5	10.2 $\pm$ 1.0
	Nonbud tissue	0.6 $\pm$ 0.1	1.0 $\pm$ 0.3	2.3 $\pm$ 0.1

* $\pm$ SE; n=6 to 12.

2.3.9. INHIBITION OF PROTEASE ACTIVITY BY POLYAMINES

Introduction

One of the early events in leaf senescence is the well documented rise in protease activity (194). However, little is known about the regulation of proteolysis itself during leaf senescence. Considerable evidence has accumulated which shows that involvement of polyamines in cellular activity may be mediated via interaction with macromolecules (74). Since exogenous application of polyamines retards senescence and the rise in protease activity is one of the early events in senescence, we reasoned that these compounds may in fact decrease senescence through inhibition of protease activity. We therefore undertook to investigate the effects of various naturally-occurring polyamines on protease activity in connection with their action in controlling senescence of excised oat leaves.

We now report that application of exogenous polyamines to leaf segments retards senescence by decreasing protease activity and chlorophyll loss. Furthermore, we provide evidence that the decrease in protease activity occurs by binding of the polyamines to the enzyme.

Materials and Methods

Plant material

Oat seeds (*Avena sativa*, cv. Victory) obtained from Swedish Seed Co. (Svalov) were grown in controlled rooms (96). The first leaf of two-week-old seedling was used.

Conditions of treatments

For in vivo effects of polyamines excised leaves were sterilized as in (98) and the lower epidermis removed by stripping with fine forceps. Four such median (4.5 cm) leaf segments were floated, stripped side down in Petri dishes on 5 ml of either 1 mM phosphate buffer (pH 5.8), or on buffered solution containing 1 mM of various polyamines (HCl salt, Sigma). Polyamines at 1 mM were optimum for reducing

chlorophyll breakdown (96). We also found that spermidine treatment for 48 h is most effective in preventing the rise of protease activity and chlorophyll breakdown with no visible toxic effects (not shown). Therefore, 1 mM spermidine was used for most of these experiments. Spermine was most effective in 1 h treatments, but it produced toxic effects when used for 48 h. After incubation for 48 h in the dark, the leaves were thoroughly washed to remove excess polyamines and used for measurements of chlorophyll content and protease activity. All manipulations were performed under aseptic conditions in a laminar flow hood. For in vitro effects of polyamines on protease activity, various concentrations of polyamines were added to the crude exyame extract at time of assay.

Enzyme extraction and assay.

Extraction of proteases and assay were done as described under General Material and Methods.

Protein content

Soluble protein content in the enzyme extracts was measured as described earlier.

Analysis of results

Initial values of protease activity and chlorophyll content were measured on leaf samples at the start of each experiment. The results are expressed as units of activity or as percentage of the initial values. The data presented are from single experiments which are representative of 2-6 experiments of each treatment.

Results and Discussion

In vivo inhibition of protease activity

When excised oat leaves are incubated in the dark, their protease activity increases dramatically (Table 38). Increased activity was observed in two commonly reported proteases having pH optima at 4.2 and 6.6 with Azocoll as substrate. The acid protease increased >6 times the initial value during 48 h dark incubation, while the neutral protease activity increased only 2.5 times. This rise in both protease

activities was inhibited when leaves were floated on polyamine solutions. Spermine was most effective, followed by spermidine, putrescine and cadaverine. In all cases, the inhibition was greater for acid than for neutral proteases, and remained unaltered when the enzyme was either passed through Sephadex G-50 or exhaustively dialyzed against buffer in the cold and subsequently treated with the polyamine. These results indicate that protease activity is directly inhibited by polyamines and not mediated by a low M_r factor.

Determinations of chlorophyll content, a measure of leaf senescence, showed that during dark incubation for 48 h, the chlorophyll content in control leaves decreased by 73% when compared with the initial value (Table 38). Exogenous application of the polyamines prevented this decrease. As in the reduction of protease activity, the tetramine spermine and triamine spermidine were more effective than the diamines in preventing chlorophyll breakdown.

In vitro inhibition of protease activity

Various concentrations of polyamines were added to the enzyme extract during assay of enzyme activity. The results show that both acid and neutral proteases are inhibited (Fig. 43). As before, the polyamines were more effective than the diamines. At lower concentrations of spermine and spermidine, the inhibition was more pronounced for acid than for neutral proteases. In contrast, cadaverine and putrescine produced only small decreases in both types of protease. With all polyamines, the inhibition of protease increased with increasing concentrations.

Interaction of polyamines with enzyme or substrate.

To determine whether polyamines inhibit protease activity by interacting with enzyme or with substrate, spermidine (5 mM) was preincubated with enzyme or substrate preparations for 30 min at room temperature. The enzyme-polyamine mixture was then passed through Sephadex G-50 column or exhaustively dialysed, while the substrate - polyamine mixture was thoroughly washed prior to assay. The results in Table 39

shown that pretreatment of the enzyme with spermidine prior to addition of substrate inhibited both the acid and neutral protease activities, while pre-treatment of the substrate with spermidine did not. This suggests that polyamines inhibit protease activity by binding to the enzyme.

Effect of calcium on polyamine inhibition of protease activity and chlorophyll loss

At physiological pH, the polyamines function as polyvalent cations and are known to bind strongly to the acid groups of nucleic acid and proteins (163,206). Thus, it is possible that polyamines may prevent senescence of leaves by virtue of their polyvalent cationic properties. If this were true, then treatments with other cations, such as Ca^{+} , would be expected to reduce the effects of polyamines on inhibition of protease activity and chlorophyll breakdown. Exogenous application of CaCl_2 (1-10 mM) along with spermidine (1 mM) antagonized the spermidine-mediated inhibition of rise in acid protease activity and chlorophyll degradation. Treatments with Ca^{+} alone reduced protease activity only slightly and did not prevent chlorophyll loss. In vitro application of Ca^{+} produced no inhibition of protease activity.

The observed increases in protease activity followed by chlorophyll breakdown in excised oat leaves incubated in the dark, and their partial suppression by senescence-inhibiting polyamines indicate that protease, among other hydrolases, may be involved in leaf senescence. Analysis of extracts of oat leaves have revealed that they contain at least 2 proteases, most active at pH 4.2 and pH 6.6, which digest Azocoll, supporting (194,120). The greater inhibition by polyamines of the rise in acid protease activity concurs with effects produced by kinetin and cycloheximide (194,120). Thus the acid protease appears to be the functional protease during early senescence.

Although the mechanism by which polyamines inhibit protease activity is not clear

at present, the differential extent of inhibition by the diamines and polyamines can probably be attributed to the number of free amino groups, which affect their cationic properties (16,48). Thus, spermine, a tetra-amine, is the most effective inhibitor, followed by the triamine spermidine, which in turn is more effective than the diamines putrescine and cadaverine. Similar results have been obtained on inhibition by polamines of RNase activity in plants (5,65,94) and animal cells (92) and are further confirmed by the polyamine-mediated inhibition of chlorophyll loss in dark-incubated leaf segments of several leaves (96).

The inhibition of protease activity is not dependent on interaction with a low- M_r substance, be that of the animal lysosome. Polyamines may therefore also act by restricting the release of proteases from the central vacuole.

Since polyamines are known to act as cations at physiological pH (16,48) their action may involve electrovalent linkages with negatively charged groups of the enzyme or substrate. Such a linkage has been observed in several animal and bacterial systems (92), and in the inhibition of RNase activity in oat leaf protoplasts (94). Our experiments support the view that spermidine inhibits protease activity by interacting with the enzyme and not the substrate, since decreased activity was observed only when spermidine was preincubated with the enzyme extract prior to the assay. The binding of spermidine to the crude enzyme from oat leaves is insensitive to EDTA, which suggests that binding is not the transglutaminase type as reported in animal cells (206). Furthermore, since Ca^{+} prevents the spermidine effect only when added in vivo, the protease inhibition may be mediated by an initial ionic attachment mechanism, in which Ca^{+} and spermidine compete for the same ionic sites on membranes. A similar mechanism has been suggested previously in experiments on chlorophyll retention (96) and ethylene inhibition in oat leaves (63) and apple fruit tissue (13).

Many effects of polyamines may result from repression of de novo protease

synthesis as well as by direct inhibition of protease activity. The rise in protease activity during dark senescence of leaves is inhibited by cycloheximide (120), suggesting its de novo synthesis. Consequently, the repression of protease activity by polyamines in vivo may be due to a specific inhibition of hydrolase synthesis. The direct inhibition of protease activity as manifested by the in vitro experiments probably results from neutralization of the negative sites on the enzyme by the positively charged polyamines thereby rendering it less accessible to the substrate. A similar mechanism has been proposed for RNases from oat leaf protoplasts (94), and bacterial and animal cells (92). Polyamines are also known to stabilize plant membranes (135,148) and acid hydrolases from wheat (208) and from a number of plants (34) may be localized in the central vacuole, analogous to that of animal ⁵⁰lysosome.

Overall, results suggest a regulatory interaction between polyamines and the de novo synthesis and activity of proteases. The inhibition of protease activity in vitro by polyamines appears to be specific for oat leaves since similar in vitro experiments with papain (papaya latex, Sigma), bromelin (pineapple stem, Sigma) and trypsin (bovine pancreas, Sigma) showed no inhibition by spermidine over pH 4.2-8. Although the polyamines have been shown to interact with several enzymes concerned with the synthesis and breakdown of macromolecules, this is the first report on inhibition of protease activity by polyamines.

Thus, polyamines appear to prevent chlorophyll loss in excised oat leaves incubated in darkness by preventing an early senescence-linked rise in protease activity. This is presumably accomplished through electrovalent attachment of protonated amino groups to anionic sites on proteins. A similar action in plants also prevents a rise in and inhibition of RNase activity (5,65,94), stabilizes membrane to lysis (9), and leakage (134). Decrease in polyamine biosynthesis with increased senescence in attached and excised oat leaves (98) further establishes the role of polyamines in leaf senescence.

Fig. 43 In vitro inhibition of protease activity in oat leaf extracts. Various concentrations of the polyamines were added to the enzyme extract at time of assay.

Table 38. In vivo inhibition of protease activity and chlorophyll loss in oat leaves by 1 mM polyamines and diamines.

Treatment	Protease activity (units. $\frac{\text{mg protein}^{-1} \cdot \text{h}^{-1} \pm \text{SE}}$)		Chlorophyll content (% loss)
	Acid	Neutral	
Control			
initial	0.8 $\pm$ 0.04	1.7 $\pm$ 0.08	0
final	5.2 $\pm$ 0.11 (0)	4.0 $\pm$ 0.19 (0)	73
Cadaverine	3.4 $\pm$ 0.22 (39)	4.3 $\pm$ 0.06 (+7)	33
Putrescine	3.0 $\pm$ 0.06 (42)	2.8 $\pm$ 0.04 (30)	42
Spermidine	1.2 $\pm$ 0.02 (77)	1.0 $\pm$ 0.03 (62)	10
Spermine ^a	0.6 $\pm$ 0.01 (81)	0.8 $\pm$ 0.06 (64)	5

^aPeeled leaves floated on spermine for 1 h; leaves then washed and floated on buffer for the remaining 47 h.

Protease activity and chlorophyll loss were measured in leaves after 48 h incubation in the dark. Figures in parentheses represents % inhibition relative to final control.

Table 39. Interaction of spermidine with enzyme or substrate

	Protease activity (%inhibition)	
	Acid	Neutral
Enzyme	0	0
+ 1 mM spermidine	57	48
pre-treated with 5 mM spermidine for 30 min at 24°C and passed through Sephadex G-50 column ^a	53	45
Substrate (Azocoll)		
pretreated with 5 mM spermidine and washed 4X prior to assay	0	0

^aFinal concentration of spermidine during assay was 1 mM

2.3.10 THE FORMATION OF 1,3-DIAMINOPROPANE IN LEAVES AND ITS EFFECTS.

Introduction

Dap, an oxidation product of the naturally - occurring PAs, Spd and Spm, occurs in many cereal plants (174). The oxidation is catalyzed by PA-oxidase, a cell wall-localized enzyme in oat leaves. Our recent observations (98) showed that endogenous levels of Dap, like those of other PAs, decrease in attached oat leaves with increasing age of seedlings and in excised leaves with increasing time of dark incubation. This suggested that Dap, like other PAs, may be involved in the control of senescence. Although other PAs have been implicated in processes controlling cell growth (16,23,48,66) and senescence (5,9,44,95,96) virtually nothing has been reported about the biological activity of Dap in plant cells.

We now report that Dap accumulates following exogenous application of Spd or Dap to excised dark incubated oat leaves. Exogenous Dap, like Spd, inhibits senescence, possibly by preventing the increases in protease activity and ethylene production that precede Chl breakdown. Inhibition of senescence is possibly effected through an initial interaction between Dap and membranes.

Materials and Methods

The first leaf of 14-day old seedlings of oat (Avena sativa L., var Victory) was used for most experiments. The seeds (Swedish Seed Company, Ltd., Svalov, Sweden) were grown in Vermiculite in controlled growth rooms maintained at 24±1°C and with a 16-h photoperiod of about 12,000 lux.

Incubation of leaf segments. For measurements of protease activity, leaves were sterilized as described earlier. The lower epidermis was peeled off and 45 mm-long leaf segments were rinsed in distilled water and floated in Petri dishes, stripped side down, on 5 ml of 1 mM phosphate buffer (pH 5.7). All manipulations were performed aseptically in a laminar flow hood. For measurements of ethylene production, leaf segments were similarly handled and floated on solutions in bottles

sealed with rubber serum caps. For estimating senescence by measurements of residual Chl content, the following materials were used: Leaf segments from oats, barley (Hordeum, vulgare cv. Himalaya), corn (Zea mays cv. Golden cross bantam) and wheat (Triticum aestivum cv. Yamhill) and leaf discs (10 mm) from mature leaves of 1 to 2 month old plants of bean (Phaseolus vulgaris cv. Taylor's Horticultural), radish (Early Scarlet Globe) and broccoli (Waltham). Leaves of these materials were floated on buffer in Petri dishes, with Dap, Spd (HCl salt, Sigma) and other effectors included as indicated in the appropriate tables. Incubation was carried out at 25 C in the dark or in the light in growth rooms as described above.

Determination of protease activity. Protease activity was measured in oat leaves as described in general Materials and Methods.

Determination of PA content. PAs were extracted from leaf segments (98) with 5% perchloric acid, dansylated and separated on silica gel thin layer plates and quantified as described earlier.

Determination of Chl content. Chl extracted with hot 80% ethanol was measured spectrophotometrically at 665 nm and expressed as percent of the initial value.

Determination of protein content. The crude protease extract was used to measure soluble protein content according to the method of Lowry et al (116), using bovine serum albumin as a substrate. The data presented were from single experiments which are representative of 2-4 experiments. Each experiment was performed in duplicate.

Results and Discussion

Accumulation of Dap. When excised oat leaves were incubated on Spd - containing medium in the dark, there was a large accumulation of Dap, which increased with increasing concentration of Spd and time of treatment (Table 40). Dap titer increased 44 fold with 0.5 mM Spd and 66 fold with 1.0 mM Spd during 48 h treatments, while a 3 h exposure to 1.0 mM Spd increased the titer by only 9 fold. However, if the leaves were floated first on medium containing the PA oxidase inhibitor,

hydroxyethyl hydrazine, and then on Spd, the accumulation of Dap in leaves was greatly reduced. Apparently, the accumulation of Dap occurs through a rapid uptake of Spd and its oxidation by PA oxidase, which is abundant in oat leaves (174). Similar accumulation of Dap after exogenous Spd application was reported in the cyanobacterium Anacystis nidulans (153). The results in Table 38 further show that a high accumulation of Dap also occurs with exogenous application of Dap; this increases with increasing Dap concentration, being 35 fold in 0.5 mM Dap and as high as 120 fold in 1 mM treatments for 48 h. The accumulation must involve rapid and direct uptake of Dap since a 3 h treatment with 1 mM Dap caused a 26 fold increase and treatments with hydroxyethyl hydrazine did not alter the endogenous titer.

Effect of Dap on Protease Activity. Excision and dark-incubation of cereal leaves cause a sharp rise in protease activity (96,194). The activity (Table 41) includes at least two proteases, showing pH optima at 4.2 and 6.6 with Azocoll as a substrate, supporting earlier reports (194). The exogenous application of Dap (in vivo) inhibited the rise in both acid and neutral proteases, the inhibition increasing with increasing Dap concentration. The increase in acid protease was higher than the neutral protease in control leaf segments during the 48 h senescence period, and the inhibition of acid protease by Dap (0.5 mM) was also more than the neutral protease (Table 41). While these results support the contention that acid protease predominates during early stages of leaf senescence in cereal leaves, this is not the case in other plants such as tobacco and nasturtium (194).

Similar inhibitory effects on in vivo protease activity were observed with Spd and Spm (97) and with cytokinins (194). The Dap - mediated inhibition of proteases is antagonized by Ca^{2+} ; for example, addition of 1 mM CaCl_2 together with 1 mM Dap reduced the inhibition of acid protease by about half. These results suggest that protonated Dap (Dap^{2+}) and Ca^{2+} may compete for the same electronegative sites on membranes.

To determine whether Dap might retard senescence by directly inhibiting protease activity, 1 mM Dap was added in vitro to the enzyme extract during the assay of enzyme activity. Such in vitro addition of Dap was not as effective in decreasing protease activity as in vivo addition (data not shown). Rapid proteolysis as one of the early events in cereal leaf senescence is well documented (143,194). It is also known that cycloheximide at concentrations that inhibit protein synthesis prevents the rise of protease activity and the accompanying Chl loss. Consequently, the increase in protease activity has been suggested as due to its de novo synthesis (194). Thus Dap, which inhibits more than 90% of the rise in acid protease when applied exogenously, may retard senescence more by inhibiting the synthesis than the action of proteases. These results gain support from our work on inhibition of ACC⁴ synthase, the enzyme involved in ethylene biosynthesis (63) and from a recent report on the inhibition of protein synthesis by Dap in animal cells (197). It has also been suggested (35) that compartmentation of proteases may occur in lysosome-like organelles. Since PAs are known to stabilize membranes (16,48,134) Dap may act by limiting the release of proteases from these organelles.

Effect of Dap on Chl Content. Excised oat leaf segments floated on medium containing Dap and incubated in the dark showed a striking decrease in Chl loss with increase in Dap concentrations (Table 42). Even a short exposure to 1 mM Dap decreased Chl breakdown. In contrast, in the light, where Chl breakdown is generally lower than in the dark, Dap treatments promoted its breakdown (bleaching). These differential effects of Dap on Chl breakdown in the dark and in the light confirm our earlier results with other PAs (96). Further experiments are needed to determine the effects of Dap on protease activity and ethylene production in light which should help in understanding the differential effects of Dap on senescence in the dark and in the light.

The inhibition of Chl breakdown in the dark by Dap was also examined in other

cereals. The results in Fig. 44 show that, as in oat leaves (Table 42), increasing Dap concentrations cause progressive decreases in Chl loss in wheat, corn and barley leaf segments. These findings, together with data showing decreases in Dap titer in senescing oat leaves (98), suggest that Dap may play a role in controlling senescence in cereal leaves.

To examine whether Dap is similarly involved in the control of leaf senescence in dicotyledonous plants, we examined its effect on Chl breakdown in dark-incubated leaf discs from broccoli and radish, which also senesce rapidly. In these plants, Dap-treated leaf discs show a marked accumulation of Dap, but Dap was only slightly effective in preventing Chl breakdown (data not shown). Furthermore, control leaf discs of broccoli and radish do not contain Dap, probably indicating the absence of PA oxidase. Thus, Dap which occurs naturally in cereals, may control senescence there, but its absence and failure to act when applied exogenously to radish and broccoli probably indicates that it does not control senescence in dicots.

Antagonism by Ca^{2+} of Dap-mediated Inhibition of Chl Loss. The data in Table 43 show that the inhibition of Chl loss by Dap in leaves incubated in the dark and its increase in leaves exposed to light are greatly reduced by the simultaneous addition of CaCl_2 to the incubation medium. These results support previous findings on the decrease by Ca^{2+} of the Dap-mediated inhibition of protease activity (Table 42). Likewise, in the light, where Dap promotes Chl loss, the presence of Ca^{2+} partially inhibits the Dap effect. However, when the leaves were first floated on CaCl_2 for 3 h and then on Dap for the remaining 45 h in darkness, no antagonism of Dap retardation of Chl loss was observed. Similar results were obtained when leaves were first floated on Dap and then on CaCl_2 . In both light and dark, Ca^{2+} by itself had no effect on Chl loss when compared with controls. As in our earlier observations (96), these results indicate a competition by Ca^{2+} and protonated PAs for attachment to membranes and entry into the cell. At physiological pH, the polyamines carry a

net positive charge and hence function as polyvalent cations. Cationic PAs bind to negative sites on membranes, ribosomes and nucleic acids (1,16,48). Since Dap is a diamine and hence positively charged, it may bind similarly. The antagonism with Ca^{2+} supports this view and indicates an initial ionic attachment of Dap to membranes. Further confirmation is obtained from our work on the antagonism by Ca^{2+} of the Dap- and Spd-mediated inhibition of ethylene production in oat leaf segments (63) aging apple tissue (13) and orange peels. The results thus suggest that the Dap-mediated retardation of senescence occurs by an initial binding of Dap to a membrane site which it shares with Ca^{2+} , and that once both are inside the cell, Ca^{2+} no longer interferes with Dap action.

Dap-mediated Inhibition of Chl Loss as Affected by Mg^{2+} . The Dap-mediated increased Chl breakdown in leaf segments exposed to light may result from its action in displacing Mg^{2+} from the pyrrole rings of the Chl molecule, similar to effects of EDTA and other chelators which remove Mg^{2+} (104). Addition of 1 mM or 10mM MgCl_2 , however, did not decrease the Dap-mediated Chl breakdown in the light (data not shown). The bleaching effect caused by Dap and by other PAs (96) may also involve a direct inhibition of a process responsible for Chl synthesis, since Dap is known to inhibit enzymes such as proteases and ACC synthase (as demonstrated in the present study), and ornithine decarboxylase (149).

Ethylene and ACC production by unpeeled and peeled oat leaves.

When excised oat leaves are incubated on moist filter paper in the dark, they produce relatively little ethylene or ACC (data not shown). Peeling of the lower epidermis prior to incubation on buffer induced substantial increase in formation of ethylene and ACC (Table 44). In the presence of AVG, a competitive inhibitor of ACC synthesis from SAM (33,215), ethylene and ACC production were inhibited by 97 to 96%, respectively. Ethylene production from exogenously applied ACC which is the immediate ethylene precursor was not affected by AVG (data not shown). This suggests

that wound ethylene production induced by peeling is controlled by ACC synthesis and follows the proposed pathway: methionine — SAM — ACC — C_2H_4 . Mechanical wounding of bean leaves (103) or pericarp tissue of tomato induces ethylene production in a similar way.

Effect of DAP and Spd on ethylene production and chlorophyll loss.

Kinetic studies show that ethylene is produced continuously during the dark incubation, starting after a lag period of approximately 60 min following peeling. Data in Fig. 45A shows that ethylene production after 24h is about 15-folds and after 48h about 25-fold higher than the initial value. This time-course of ethylene production is different from that found when ethylene production is stimulated by a toxic substance, such as Cd_{2+} . In the latter case, ethylene production rate peaks after several hours of treatment and declines rapidly thereafter.

Figure 45A also shows that exogenous application of Dap or Spd strikingly inhibited ethylene production, and that the Pa-mediated inhibition was antagonized by simultaneously - applied Ca^{2+} . Application of Ca^{2+} alone had no effect on ethylene production when compared with controls. The basic pattern of ethylene production during the incubation period was not changed in the presence of Dap or Spd. Inhibition of ethylene production by PAs has also been reported for senescing petals of *Tradescantia* (189), leaf tissue of beans, and apple protoplasts (13).

Figure 45B shows that Chl content decreased only little during the first 24h of dark-incubation, but rapidly between 24 and 48h, as was reported earlier (68,96). Dap and Spd inhibit CHl loss and, as with ethylene production, inhibition of CHl loss is antagonized by Ca^{2+} . This underlines the probable involvement of membrane attachment in the Dap- or Spd- Ca^{2+} interaction (57).

Effect of Dap and Spd on ACC synthesis and ACC conversion to ethylene.

ACC synthesis from SAM is catalyzed by ACC synthase (215). The *in vivo* activity or synthesis of this key enzyme of ethylene biosynthesis is promoted by IAA (216),

mechanical wounding (34) or chemical treatment. ACC synthase activity in untreated leaves was not detectable, but peeling followed by dark incubation induced considerable activity (Table 44). The ACC synthase activity extractable after a 24h incubation in the presence of Dap or Spd was reduced by 41% and 46%, respectively, relative to controls, and Ca^{2+} did not reduce this inhibition significantly. The observed Spd-mediated inhibition of ACC synthase agrees with an earlier report of inhibited incorporation of 3,4[^{14}C]methionine into ACC, but not into SAM, in Spd-treated orange peel discs (57).

ACC synthase seems to be a pyridoxal phosphate-linked enzyme (216). PAs, especially spermine, Spd and Dap are known to react with pyridoxal phosphate by forming a Schiff's base (101). Further experiments are needed to determine whether this reaction causes an irreversible inhibition of ACC synthase, and would therefore account for the observed reduction of enzyme activity after treatment with Dap or Spd. Alternatively, since Dap can inhibit protein synthesis, possibly by binding with polysomes, in animal cells (197) the buildup of Dap following administration of exogenous Spd might inhibit the formation of ACC synthase.

Despite the fact that ACC synthase activity was decreased by Dap or Spd, ACC content was increased (Table 44) by these compounds, because they also inhibit ACC conversion to ethylene. Ca^{2+} antagonized Dap- or Spd-mediated inhibition of ACC conversion.

To examine more directly the effect of these PAs on ACC conversion, peeled leaves were preincubated on Dap or Spd solutions and then transferred to solutions containing ACC. AVG was included during the preincubation and incubation periods to inhibit endogenous production of ACC. AVG does not inhibit ACC conversion to ethylene. The results in Table 45 demonstrate the strong inhibition of ACC-dependent ethylene production by Dap and Spd, and the antagonism by Ca^{2+} , thus confirming the results presented in Table 44. These results also agree with those of Apelbaum

et al. (13) who suggested that polyamines inhibit a membrane associated ACC conversion system. The Ca^{2+} antagonism again indicates that an initial ionic attachment mechanism is involved. In additional experiments, we found that the Spd-mediated inhibition of ACC conversion to ethylene was only 35% when the preincubation was carried out in the light, compared to the 90% inhibition after preincubation in the dark.

Relationship between ethylene production and chlorophyll loss.

Ethylene production increases during leaf senescence (68), but, it is not clear to what extent ethylene plays a causal role in senescence. According to Gepstein and Thimann (68), senescence of oat leaves in light, but not in darkness, depends on ethylene production. Our results with peeled leaves support their conclusion (data not shown). Ethylene induced by peeling and by exogenously applied ACC, or ethylene itself, only slightly enhanced CHl loss but AVG, which blocks ethylene biosynthesis, did not reduce CHl loss effectively. However, Dap and Spd- inhibited CHl loss substantially, but this loss can not be due to the inhibition of ethylene biosynthesis, since exogenous ethylene did not reverse the PA effect. Thus, despite the fact that Dap and Spd inhibit ethylene biosynthesis and leaf senescence, the former is not the cause of the latter. Rather it appears that they are linked in the sense that PAs affect the structure of membranes, and that ACC-dependent ethylene synthesis and senescence, being membrane localized processes, are accordingly independently affected. Alternatively, PA may antagonize ethylene action as is known for cytokinins.

The timing of CHl loss and ethylene production during senescence as observed in this study agrees with previous observations (68). Senescence in plant tissue is generally accompanied by changes in the structure of membranes, due to lipid peroxidation (56) or a change in the phase behavior of membrane lipids (106). A link between these two processes is likely to exist, but is not yet established. Chl

destruction, as observed during senescence, can, at least in part, result from lipid peroxidation in the chloroplast membrane. Interestingly, the ACC converting system in cells of higher plants has been suggested to be associated with membranes, possibly the lipid fraction (121). Thus, lipid peroxidation and oxidation of ACC may occur at similar sites in the cell. Furthermore, both processes require oxygen uptake and possibly the formation of hydroxyl free radicals. As shown in Table 46, Chl loss and ethylene production were inhibited in the presence of Co_2^+ , which acts as a scavenger of free radicals, or in a nitrogen atmosphere. In isolated chloroplasts, ethylene formation from methionine in the presence of pyridoxal phosphate can be inhibited by catalase and superoxide dismutase (103). The activities of these enzymes determine the abundance of oxygen-derived free radicals, and are known to decrease during senescence (56). The observed inhibitory effects of Dap and Spd on Chl loss and ACC conversion, however, is not likely to be due to a direct inhibition of peroxidation reactions. Protection of membranes from peroxidative damage should preserve the capacity to convert ACC to ethylene (107).

The Ca^{2+} antagonism suggests that protonated PAs compete with Ca^{2+} for electronegative sites on the membrane. The surface charge of biological membranes can act as a regulator of membrane-bound enzymes (84). Because of the electrostatic nature of the interaction with PAs, a structural change in the membrane is possible (209). Pa-induced alterations of the lipid phase transition temperature has been reported for microsomal membranes in animal cells (84), while Ca^{2+} is known to abolish phase transitions for lipid bilayers in the range 0 to 70°C (85). This could explain the Ca^{2+} antagonism observed in the experiments reported here. Subtle structural changes in the membranes, which could be viewed as substrates for lipid degrading enzymes, could account for the preservation of structural stability during senescence. Such stabilization has been observed in thylakoid membranes of barley chloroplasts (148), storage tissue of beet roots (3,135) and oat protoplasts (9).

Also, Dap⁻ and Spd-mediated inhibition of membrane breakdown due to lipid peroxidation may be related to a decrease in proteolytic activity in the presence of these PAs. The ACC converting system may also lose its affinity for its substrate, ACC, or for a possible cofactor as a result of the Dap⁻ or Spd-mediated changes. The translocation of ACC might also be inhibited; a regulation of ethylene biosynthesis at the site of ACC translocation has been proposed (117).

In examining the effects of several membrane-deteriorating treatments, we find that Chl loss and wound-induced ethylene production are diminished equally Table 47. Osmotic shock treatment is least inhibitory, while freezing followed by thawing or incubation on 2,4-dinitrophenol inhibit the two processes most effectively.

Effect of Dap on ethylene production and PA titer. Since increased ethylene production is characteristic of senescing tissues and ethylene and PA biosynthesis have a common precursor in S-adenosylmethionine, we investigated the effect of Dap on their role in senescence. The results (Table 48) show that exogenously applied Dap is a potent inhibitor of ethylene production. This marked decrease in ethylene production should result in an increase in PA titer through a diversion in S-adenosylmethionine metabolism. However, the results (Table 48) show that exogenous Dap decreased Put and Spd levels, although it increased Dap titer (Table 40). Dap is known to inhibit ornithine decarboxylase activity in several animal systems (16,149). This enzyme is rate limiting in some systems for the synthesis of Put, a precursor for Spd. Hence, the observed low titer of Put and Spd could result from the inhibition of ornithine decarboxylase (or possibly arginine decarboxylase) by the accumulated Dap in leaves floated on medium containing Dap. Furthermore, when leaf segments were floated on medium containing 0.2 mM AVG, a specific inhibitor of ethylene biosynthesis from methionine, ethylene production was almost completely inhibited, but there was only a slight effect on the endogenous levels of PAs, and no effect on prevention of Chl loss (data not shown). Thus, ethylene biosynthesis

probably is not involved in Chl loss in darkness. In contrast, PA synthesis has been shown to increase in senescing orange peels treated with AVG.

These data suggest that Chl loss and ethylene biosynthesis in vivo depend on specific physical properties of membranes, and that PA-mediated inhibition of ACC conversion reflects a loss of membrane functions due to changes in membrane structure. It will be interesting to investigate a possible relationship between lipid peroxidation or a change in phase behavior of membrane lipids and PA-mediated inhibition of senescence and ACC-dependent ethylene production. Suitable experiments are now in progress.

In summary, Dap and Spd inhibit ethylene biosynthesis at the step from SAM to ACC by inhibiting ACC synthase activity and, more effectively, at the conversion of ACC to ethylene. The Ca^{2+} antagonism suggests an initial ionic attachment at membranes and the involvement of membranes in the in vivo conversion of ACC to ethylene. Inhibition of ACC conversion, Chl loss and, as previously reported, ribonuclease (65) and protease activity may reflect the alteration of specific membrane functions as a result of structural changes mediated by Dap and Spd.

LEGEND TO FIGURE

Fig. 44. Effect of Dap on Chl Content in Excised Cereal Leaves Incubated in the Dark. Leaves were excised, peeled and floated on 1 mM phosphate buffer or on buffer containing various concentrations of Dap and incubated for 48 h in the dark.

Fig. 45. Time courses of ethylene production (A) and Chl loss (B) during senescence of peeled oat leaves in darkness and effect of Ca^{2+} on the inhibition of Spd and Dap. Spd, Dap and Ca^{2+} concentrations were 1 mM. Values for ethylene production reflect the amount of ethylene accumulation at the indicated time, based on a fresh wt basis. Initial value of Chl was 13.8A units/g fresh wt.

Table 40. Accumulation of Dap in Excised Oat Leaves Incubated in the Dark for 48 h on Medium Containing Spd or Dap

Pa Conc	Dap Content
mM	µg/g fresh wt
Control	3.9
Spd, 0.5	175.7
Spd, 1.0	266.6
Spd, 1.0 ^a	36.0
Spd + HEH ^b	15.2
Dap, 0.5	137.9
Dap, 1.0	482.7
Dap, 1.0 ^a	110.5
Dap + HEH ^b	105.0

^aPeeled leaves floated on Spd or Dap for 3 h, washed thoroughly and floated on buffer for the remaining 47 h.

^bPeeled leaves floated on 2 mM HEH (hydroxyethylhydrazine) for 2 h followed by HEH + 1 mM Spd or Dap for 3 h, washed thoroughly and floated on buffer for the remaining 43 h.

Table 41. Inhibition of Protease Activity by Exogenous Application of Dap to Excised Oat Leaves. Peeled leaves floated on solutions containing Dap for 48 h in the dark. Initial values for acid and neutral proteases were 0.7 ± 0.07 and 1.3 ± 0.07 units/mg protein/h, respectively.

mM	Protease Activity			
	Acid		Neutral	
	Units/mg protein/h $\pm$ SE	% inhibition of increase	Units/mg protein/h $\pm$ SE	% inhibition of increase
0 (Control)	3.5 $\pm$ 0.12	0	3.1 $\pm$ 0.13	0
0.5	1.6 $\pm$ 0.03	68	2.3 $\pm$ 0.05	44
1.0	0.9 $\pm$ 0.05	93	1.3 $\pm$ 0.13	100
1.0 mM Dap +	2.17 $\pm$ 0.18	48	—	—
1.0 mM CaCl ₂	2.17 $\pm$ 0.18	48		

Table 42. Effect of Dap on Chl Content in Excised Oat Leaves Incubated in the Dark (2 days) or Light (5 days). Initial value for Chl was 2.1 ± 0.06 .

Dap Conc	Chl Content			
	Dark		Light	
	Absorbance	% Loss*	Absorbance	% Loss*
mM	665 nm $\pm$ SE		665 nm $\pm$ SE	
0 (Control)	0.5 ± 0.02	77	1.2 ± 0.08	40
0.25	0.6 ± 0.03	73	1.0 ± 0.04	50
0.5	1.3 ± 0.06	41	0.4 ± 0.02	80
1.0	1.9 ± 0.01	13	0.6 ± 0.02	70

*Relative to initial value

Table 43. Prevention by Calcium of Dap-mediated Inhibition of Chl Loss

Treatments	Chl Loss (%)	
	Dark (2 days)	Light (5 days)
Control	77	40
CaCl ₂ (1 mM)	73	44
Dap (1 mM)	13	76
CaCl ₂ + Dap	68	58
CaCl ₂ (3h) ^a + Dap	5	—
Dap (3h) ^b + CaCl ₂	27	—

^aPeeled leaves floated on CaCl₂ for 3 h, washed thoroughly and floated on Dap for the remaining 45 h.

^bPeeled leaves floated on Dap for 3 h, washed thoroughly and floated on CaCl₂ for the remaining 45 h.

Table 44. Effect of Dap (1mM) and Spd (1mM) on ACC synthase activity, ACC content and ethylene production.

Measurements were carried out after a 24-h dark-incubation. Values are expressed on a fresh wt basis. The Ca^{2+} concentration used was 1 mM. Values in a column followed by the same letter are not significantly different at the 5% level of probability (Duncan's Multiple Range, $n=4$).

	ACC synthase		ACC content		C_2H_4	
	(nmol/g/h)		(nmol/g)		(nmol/g/24h)	
Initial	ND ^a		0.5			
Control	0.56	A	12.6	B	14.0	A
Dap	0.33	B	16.4	A	1.0	D
Dap + Ca^{2+}	0.39	B	9.5	C	9.0	B
Spd	0.30	B	16.0	A	1.5	D
Spd + Ca^{2+}	0.37	B	6.7	C	5.7	C
Ca^{2+}	NA ^b		BA		15.0	

a: NB, Not detectable

b: NA, Not assayed

Table 45. Effect of Dap (1mM) and Spd (1mM) on ACC-dependent ethylene production. Peeled leaves were floated for 3 h on solutions containing Spd or Dap and then transferred to solutions containing ACC (0.1 mM). AVG (0.2 mM) was included during the preincubation and incubation periods. Ethylene production rate was calculated from the difference between ethylene produced after 1 and after 3 h incubation on ACC and is expressed on a fresh wt basis. Ca^{2+} concentration used was 1 mM.

	Ethylene production	% Control
	(nmol/g/h)	
Control	16.8	100
Dap	1.2	7
Dap + Ca^{2+}	15.7	93
Spd	1.5	10
Spd + Ca^{2+}	13.7	81
Ca^{2+}	17.9	107

Table 46. Effect of Co^{2+} (2mM) and N_2 on Chl loss and ethylene production. Measurements were carried out after a 48-h dark-incubation. Co^{2+} was added as CoCl_2 . For the N_2 treatment, bottles were flushed with N_2 for 15 min and then sealed. Ethylene production for the control was 25.0 nmol/g fresh wt/48 h (=100%). Initial value for Chl was 13.8 A units/g fresh wt.

	Chl loss (% of initial value)	Ethylene production (% of control)
Control	68	100
Co^{2+} D	8	11
N_2	9	6

Table 47. Effect of various stress treatments on Chl loss and ethylene production. Measurement was carried out after a 48-h dark incubation. The 'heat' treatment included a 3-h incubation at 50 C. The 'osmotic shock' treatment consisted of incubation for 10 min on 0.5 M mannitol at 25 C followed by 10 min on buffer at 3 C. The 'Freezing-Thawing' treatment included freezing of the leaves at -20 C followed by thawing at 25 C. Concentrations of 2,4-dinitrophenol and CdCl_2 were 0.1 mM. Ethylene production rate for the control was 25 nmol/g fresh wt/48 h (=100%). Chl loss for control was 9.4 A units/g fresh wt./48 h (=100%).

	Chl loss (% of initial value)	Ethylene production (% of control)
Control	100	100
Osmotic shock	77	54
CdCl_2	32	36
Heat ²	29	25
2,4-Dinitrophenol	20	2
Freezing-Thawing	20	2

Table 48. Effect of Dap on Ethylene Production and PA titer in Excised Oat Leaves Incubated for 27 h in the Dark.

<u>Treatment</u>	<u>Ethylene Production</u>		<u>PA Titer</u>			
			Put	Spd	Put	Spd
	n mol/g fresh wt/24h	% inhibition	µg/g fresh wt±SE		% Decrease	
Control	14.0	0	11.0±1.0	2.9±0.87	0	0
Dap, 0.5 mM	2.2	84	5.2±1.1	2.2±0.35	53	25
Dap, 1.0 mM	1.0	93	3.6±0.5	2.0±0.1	67	31

2.4 GENERAL DISCUSSION AND CONCLUSIONS

While the involvement of polyamines (PA) in the regulation of plant growth and physiology was limited in the past to few studies (23,171,176), accumulating evidence during the last 5-6 years indicates that PA can be regarded as a new class of plant growth substances.

The above mentioned conclusions have been inferred from various types of experiments, using several methodologies:

1. Application of exogenous polyamines and their immediate precursors.
2. Treatment of whole plants or excised tissues with structural analogues of PA and their immediate precursors, and with inhibitors of PA biosynthesis.
3. Changes in endogenous PA levels in various plants and tissues.
4. Studies of PA biosynthesis, including the activity of several enzymes such as L-arginine decarboxylase (ADC), L-ornithine decarboxylase (ODC), spermidine and spermine synthases, S-adenosylmethionine decarboxylase, as well as the incorporation of labeled L-arginine, L-ornithine, L-citrulline, putrescine, L-methionine and related compounds.
5. Studies of PA degradation and conjugation, and especially the activity of amine oxidase.

Sharp and rapid changes in PA content of plant cells have now been documented for two kinds of situations: 1. Where application of plant growth hormones gibberellin (54), auxin (61,62) or cytokinin (88) cause a promotion of growth through increase in cell division activity or cell elongation, the activity of PA producing enzymes ADC or ODC are increased in specific plants. Following this, an increase is seen in the titers of Put, Spd and sometimes Spm. Similarly, where P_{fr} phytochrome promotes growth, red light promotes both ADC activity and PA accumulation, and where P_{fr} phytochrome inhibits growth, ADC activity and PA titers are decreased. 2. Where stress is applied to particular plant tissues not undergoing cell division, the

activity of ADC or ODC is sharply increased, resulting in elevated Put levels.

It is now clear that polyamines fulfil at least several criteria to be regarded as growth regulators (6). In order to elucidate their role in the control of plant growth and physiology, several relevant questions ought to be asked: (a) what are the dose-response relationships with applied PA and their immediate precursors? (b) in what way are PA related to known hormonal effects? (c) how do the levels of putrescine, spermidine, spermine and related compounds change in various plant species, in different tissues, and in response to various growth and development processes?, and (d) what are the metabolic steps involved in PA biosynthesis and degradation? We think that the bulk of the evidence indicates an intracellular role for the PAs; they may perhaps be envisioned as 'second messengers' whose titer rises and falls in response to external and internal stimuli like light, hormones and stress.

Gradual or abrupt loss of normal membrane functions may be the common mechanism leading to the various stress-related phenomena. The specific site - e.g. the plasmalemma, the tonoplast, thylakoid membranes, lysosomal membranes - probably determines the type of response, i.e., protoplast lysis and cell leakage, cellular pH and ion imbalance, chloroplast deterioration, and hydrolase activation, respectively. Since PA have been shown to inhibit, or at least to delay all these processes, it can be concluded that they stabilize normal membrane functions. In addition, biosynthesis and activation of nucleic acids and proteins are also impaired under stress conditions, and this may be another important feature of cell 'senescence'. Hence, binding of PA to acidic sites of phospholipids and nucleic acids may both stabilize membrane functions and regulate nucleic acid activities. This is supported also by recent suggestions that spermidine promotes the negative supercoiling of intracellular DNA which is necessary for DNA replication (129). Thus, PA offer a polycationic versatility, not found with inorganic cations, through metabolic

control. This may be brought about by oxidation of PA, their acetylation, conjugation, etc.

Polyamines are the newest class of compounds to be considered recently as possible plant growth regulators. A crucial question is whether they merely reflect general changes in plant metabolism, which accompany growth, or are they involved in the regulation of these changes. The evidence, so far, points to the possibility that polyamines fulfill at least several criteria to be regarded as growth regulators.

Conclusions

1. Polyamines and their biosynthetic enzymes, as well as polyamine precursors and intermediates, are ubiquitous in plant tissue. The titer of endogenous PA and the activity of ADC and ODC, and of other PA biosynthetic and degrading enzymes, is dependent upon the type of tissue and growth process.
2. Polyamines are involved in several growth processes and in plant response to growth hormones and exogenous stimuli, and in some cases causal relationships have been established. This includes auxin-induced root formation, root elongation, GA-induced elongation of light-grown internodes, and initiation of sprouting in potato tubers.
3. Polyamines retard several aspects of plant senescence and participate in plant response to certain stress conditions. Leaf senescence is retarded and PA have been shown to prevent chlorophyll loss, to inhibit protease and ribonuclease activity, and to inhibit ethylene biosynthesis. Solute leakage from cells and RNase activity of wounded tissues are also retarded by exogenous polyamines. Polyamine accumulation and metabolism is closely linked to plant response to osmotic and salt stress: under these conditions, putrescine (and other PA) accumulate in specific tissues as a result of preferential activation of ADC and ODC, and increased PA biosynthesis from their precursors arginine and ornithine.

This may contribute to plant adaptation to salt and osmotic stress.

4. It is concluded that polyamines have an important intracellular role in plant growth and response to stress.

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3. EVALUATION OF THE RESEARCH ACHIEVEMENTS WITH RESPECT TO THE ORIGINAL RESEARCH PROPOSAL, AND PLANS FOR THE FUTURE.

Following, are the objectives that were mentioned in the original research proposal:

1. To study the effects of applied polyamines on growth and physiological changes in intact plants and in horticulturally important detached organs subjected to normal and stress conditions.
2. To investigate the quantitative and qualitative changes of endogenous polyamines in response to environmental stress conditions (wounding and excision, salt, water and temperature stresses).
3. To elucidate the interrelationships of application of hormones which are known to delay various aspects of senescence, especially cytokinins, and endogenous changes in polyamines.
4. To study the biosynthesis and catabolism of polyamines in response to the above mentioned factors.
5. To apply the findings of the above mentioned aspects as practical treatments in field studies.

We also mentioned, in the proposal that 'Although the senescence of intact plants and of detached organs has been studied extensively, our knowledge of this important problem, which has theoretical and practical implications, is far from being complete.' We then concluded that 'new lines of research as to the nature of senescence in horticulturally-important organs and to the feasibility of alternative practical treatments, which may open new vistas, are most needed.'

The literature survey and our preliminary data on polyamines in plants led us to propose the following working hypothesis, which constituted the backbone of our research proposal to BARD: 'polyamines play a central role in the control of growth and senescence of plants in general, and in plants subjected to stress conditions in

particular, and may find a use as 'antisenescence' agents'.

In retrospect, after 3 years of intensive experimental work, we feel that the working hypothesis has been materialized, that polyamines are intimately involved in plant growth and response to stress, and that most of the original objectives have been pursued and elucidated. This is supported by the experimental data, as summarized in the conclusions (chapter 2.4).

Needless to say, not all the questions have been fully answered during the 3 years of the research, and much is still left to be investigated. It seems to us that special attention should be given to further elucidation of objectives 3 and 5, i.e. the applied aspects of polyamines and plant growth, and to the molecular mechanisms of polyamines effects, i.e. the more basic aspects. Thus, the work projected for the future includes: (1) in vivo and in vitro effects and interrelationships of polyamines with leaf proteins and chloroplast membranes, (2) the contribution of polyamines and their precursors and metabolites in ion balance and membrane turnover under salinity stress, (3) selection of cell lines resistant to PA inhibitors and with altered PA metabolism, (4) PA metabolism in competent and incompetent protoplasts with respect to their division and regeneration, (5) localization of ADC and ODC in tissues at the electron microscopic level, and (6) purification of ADC and preparation of antibodies to ADC to elucidate the molecular mechanism of its activation.

When our research proposal was first submitted to BARD, little was known on polyamines in plant physiology. As a result of our research, as well as research which was initiated recently in few other laboratories, polyamines are now regarded as 'legitimate' endogenous plant growth regulators, in the broad context of the term. Thus, this new line of research indeed opened some new vistas.

4. DESCRIPTION AND RESULTS OF COOPERATION.

The cooperation between the two research group during the first year of the research was more than successful. Prof. Galston spent a full 3 months in our laboratory at Rehovot, and during this time both in-depth discussions as well as a good amount of laboratory experiments were carried out. The joint experiments were aimed at studying the role of polyamine biosynthetic enzymes (arginine and ornithine decarboxylases) in hormone-dependent growth processes.

Following the in-depth cooperation between the 2 research groups during the first year, we continued this successful cooperation during the second year. We exchanged data and conclusions of research areas, as well as relevant abstracts and preprints. A. Altman met A.W. Galston and his colleagues during his 2 week visit to the U.S.A. and Yale University. Thorough discussions and analysis of results were held, and we participated in the Gordon Research Conference on Polyamines and presented original data which were supported by the BARD grant.

The third year of the research was devoted to the conclusion of our joint efforts within the framework of the BARD program. Exchange of information, joint evaluation of results and intensive discussions were held between the participating investigators on two occasions towards the end of the research program (July 1982): (1) The Bat-Sheva Seminar on Polyamines in Growth and Differentiation Processes, in Israel, and (2) The International Conference on Plant Growth Substances, in Great Britain. Special sessions, devoted to plant polyamines, were held in these two international meetings, and both A. Altman and A.W. Galston presented invited review papers on the work which was supported by BARD.

Final discussions of the achievements, as well as plans for future research were held recently at Yale University when A. Altman arrived to the U.S. for his sabbatical leave. Thus, we were able to plan and execute the joint final report to BARD.

This close cooperation, which was aided by the fact that the BARD grant was distributed among all the three investigators, resulted in several major achievements. As described in the present final report, as well as in 16 published papers and 5 manuscripts, the participation of polyamines in various plant growth processes and in plant response to stress is now well established and better understood.

5. THESES

1. D. Amir: Changes in polyamines during leaf senescence of Phaseolus vulgaris.
M.Sc. Thesis.
2. H. Flores: Studies on the physiology and biochemistry of polyamiens in higher plants. Ph.D. Thesis.
3. R. Friedman: Polaymine effects and metabolism in plant tissue with respect to growth and stress. Ph.D. Thesis.

6. LIST OF PUBLISHED ARTICLES

1. Altman, A. 1982. Retardation of radish leaf senescence by polyamines. *Physiol. Plant* 54:189-193.
2. Altman, A. 1982. Polyamines and wounded storage tissues: inhibition of RNase activity and solute leakage. *Physiol. Plant*. 54:194-198.
3. Altman, A. and U. Bachrach. 1981. Involvement of polyamines in plant growth and senescence.. In: C.M. Caldarera et al. eds. *Advances in Polyamine Research*, vol. 3. Raven Press, 365-375.
4. Altman, A., R. Friedman, D. Amir and N. Levin. 1982. Polyamine effects and metabolism in plants under stress conditions. In *Plant Growth Substances*, 1982, Academic Press (Proc. 11th Inter. Conf. Plant Growth Substances).
5. Altman, A., R. Friedman and U. Bachrach. 1980. Polyamine metabolism in relation to growth of mung bean seedlings. 13th FEBS meeting Proc. p. 274. Abstract.
6. Altman, A., R. Friedman and N. Levin. 1982. Arginine and ornithine decarboxylases, the polyamine biosynthetic enzymes of mung bean seedlings. *Plant Physiol.* 69:876-879.
7. Altman, A., R. Friedman and N. Levin. 1983. Alternative metabolic pathways of polyamine biosynthesis in relation to plant growth. *Advances in Polyamine Research*, Vol. 4. Raven Press.
8. Dai, Y., R-K Sawhney and A.W. Galston. 1982. Promotion of gibberellic acid polyamine biosynthesis in internodes of light-grown dwarf peas. *Plant Physiol.* 69:103-105.
9. Flores, H.F. and A.W. Galston. 1982. Polyamines and Plant Stress: Activation of putrescine biosynthesis by osmotic shock. *Science* 217:1259-1261.
10. Friedman, R., A. Altman and U. Bachrach. 1982. Polyamines and root formation in mung bean hypocotyl cuttings. 1. Effect of exogenous compounds and changes in endogenous polyamines. *Plant Physiology* 70:844-848.

11. Fuhrer, Y., R.K-Sawhney, L. Shih and A.W. Galston. Effects of exogenous 1,3-diaminopropane and spermidine on senescence of oat leaves. II. Inhibition of ethylene biosynthesis and possible mode of action. Plant Physiology vol, pp?
12. Galston, A.W., Y. Dai, H. Flores and N. Young. 1983. The control of arginine decarboxylase activity in higher plants. Advances in Polyamine Research, Vol. 4. Raven Press.
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15. Kaur-Sawhney, R., L. Shih, T. Cegielska and A.W. Galston. 1982. Inhibition of protease activity by polyamines. Relevance for control of leaf senescence. FEBS Letters 145:345-349.
16. Shih, L., R.K-Sawhney, J. Fuhrer, S. Samanta and A.W. Galston. Effects of exogenous 1,3-diaminopropane and spermidine on senescence of oat leaves. I. Inhibition of protease activity, ethylene production and chlorophyll loss as related to polyamine content. Plant Physiology, in press.

Manuscripts

1. Friedman, R., A. Altman and U. Bachrach. 1981. Polyamines and root formation in mung bean hypocotyl cuttings. 2. Incorporation of polyamine precursors and activity of biosynthetic enzymes.
2. Friedman, R., A. Altman and U. Eachrach. Polyamines and plant response to salinity. 1. Activation and inhibition of arginine and ornithine decarboxylases of mung bean.

3. Friedman, R., A. Altman and U. Bachrach. Polyamines and plant response to salinity. 2. Polyamine content and biosynthesis in mung bean.
4. Friedman, R., A. Altman and U. Bachrach. Polyamines and plant response to salinity. 3. The response of glycophytes and halophytes.
5. Amir, D. and A. Altman. Changes in polyamine content and metabolism in senescing leaves of Phaseolus vulgaris.

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