Plant Physiol. (1982) 69, 405–410
0032-0889/82/69/0405/06/$00.50/0

# Relation of Polyamine Synthesis and Titer to Aging and Senescence in Oat Leaves[1]

Received for publication May 14, 1981 and in revised form September 28, 1981

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## ABSTRACT

**Polyamine biosynthesis in senescing leaves of *Avena sativa* L. was measured by determining the activities of arginine decarboxylase (EC 4.1.1.19), ornithine decarboxylase (EC 4.1.1.17) and *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50). Polyamine content was also estimated by thin layer chromatography and high performance liquid chromatography. Arginine decarboxylase activity decreases progressively in aging attached first leaves and in senescing excised leaves in the dark. Conversely, it increases during light exposure of excised leaves, which retards senescence. Ornithine decarboxylase activity is high and constant in the attached leaf, irrespective of age; it decreases in excised leaves kept in the dark and in the light, irrespective of senescence. S-Adenosyl-L-methionine decarboxylase shows no correlation with age or senescence. Levels of putrescine, diaminopropane, agmatine, and spermidine are high in young leaves and decline with age. The best single indicator of senescence is usually spermidine, which decreases in excised leaves incubated in the dark, but increases in such leaves with time of light exposure. Spermidine generally has a reciprocal relationship with putrescine, indicating that spermidine synthase, which converts putrescine to spermidine, may exert important physiological control. These data support the view that polyamines play an important role in the regulation of plant development.**

PA,[2] such as Put, Cad, Spd, and Spm are synthesized in almost all biological systems, including higher plants (27). Their possible role in various growth and physiological processes in plants, microbial, and animal systems has been deduced from exogenous application of PA as well as from changes in their endogenous biosynthesis and content (4, 5, 11, 26). In general, PA levels are high in actively growing tissues (4, 11) and low in senescing tissues (1, 26). However, an elucidation of the possible role of PA in the regulation of plant development requires a careful analysis of PA content and synthesis during controlled development. We have undertaken such a study in senescing leaves of oats.

Putrescine, central to the synthesis of Spd and Spm (Fig. 1) arises from arginine via either of two alternate decarboxylation pathways. One involves an initial decarboxylation catalyzed by ADC, giving rise to agmatine, and loss of urea to form Put; and the other involves an initial loss of urea to form ornithine which then undergoes decarboxylation catalyzed by ODC. In bacteria and plants, Put synthesis can occur by either pathway (4, 27); in plants, the ADC pathway semes to be preferred in some cases (21, 27), while in animal tissues the ODC pathway predominates (4, 5). The Put formed by either pathway is converted to Spd and Spm by the consecutive addition of propylamino residues originating from decarboxylated SAM generated via SAMDC (4, 5, 27).

In plants, high levels of ADC activity have been reported in growing seedlings of *Lathyrus sativus* (22), during embryogenesis of carrot cell suspension cultures (19), and in tissues subjected to light and growth stimuli, high salinity, or potassium deprivation (6, 18, 27, 29). Increased ODC activity has been reported in actively dividing tobacco cells and in developing ovaries of tomato (12). In contrast, little is known about the levels of these enzymes in senescing plant tissues.

PA are potent senescence inhibitors when applied to many plant tissues (1, 2, 13, 16). Applications of exogenous Cad, Put, Spd, and Spm and their precursors L-arginine and L-lysine decrease the rate of senescence of oat leaf protoplasts, stabilize them against lysis, and increase their net synthesis of proteins and RNA (2, 10, 13). These treatments also induce some DNA synthesis and mitotic activity (14). Furthermore, PA treatments of excised leaf segments of a number of plant species retarded Chl breakdown and prevented the rise in RNase and protease activities that accompany senescence (16).

Since PA biosynthetic activity and titer are highest in young, growing cells, and since applied PA retard senescence, it would be expected that PA biogenesis and titer would decrease as senescence progresses. The present study was undertaken to investigate this possibility. We have accordingly measured activities of ADC, ODC, and SAMDC, as well as PA titer, in attached and detached oat leaves of various ages. The changes in these parameters during senescence have been related to Chl and protein breakdown, which are commonly used as indicators of senescence (31).

## MATERIALS AND METHODS

**Plant Material.** The first leaf of 4- to 21-day-old seedlings of *Avena sativa* L. (var. Victory) was used for measuring PA biosynthetic enzyme activities, endogenous levels of PA, Chl, and soluble protein contents. The seeds (Swedish Seed Company, Ltd., Svalöv, Sweden) were grown in subirrigated vermiculite in controlled growth rooms maintained at 25°C and with a 16-h photoperiod of about 12,000 lux.

**Conditions for Leaf Senescence.** The leaves were sterilized by dipping in 70% ethanol for 2 min followed by two washes with sterile-distilled $H_2O$, further immersion in 10% Clorox containing Tween 20 (1 drop/10 ml) for 5 min, and finally rinsing five times with sterile-distilled $H_2O$. All manipulations were performed aseptically in a laminar flow hood as detailed in an earlier paper (16).

**Extraction and Measurements.**

*PA Biosynthetic Enzymes.* The enzymes were extracted from leaves frozen in liquid $N_2$ and homogenized in chilled mortars. For ADC and SAMDC determinations, two 45-mm leaf segments

[1] Supported by National Science Foundation Grant DAR 7813294 to A. W. G.

[2] Abbreviations: PA, polyamine; Put, putrescine; Cad, cadaverine; Spd, spermidine; Spm, spermine; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; SAM, *S*-adenosyl-L-methionine; SAMDC, *S*-adenosyl-L-methionine decarboxylase; ACC, 1-aminocyclopropane-1-carboxylic acid.

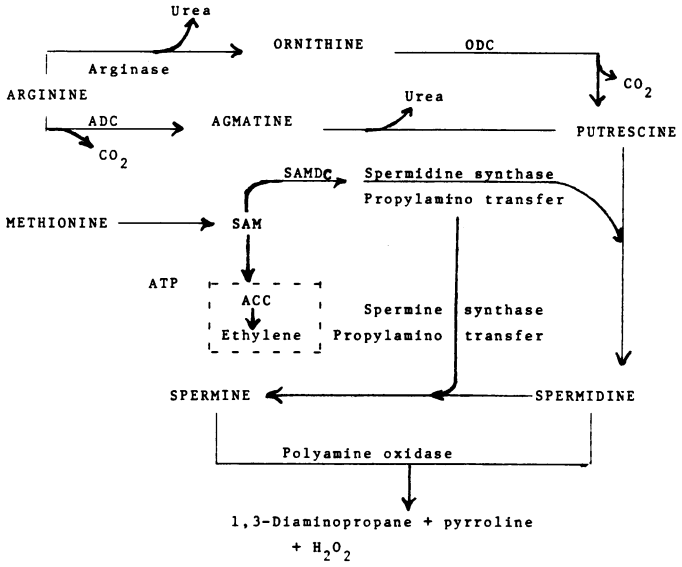


FIG. 1. Metabolic pathways for the major PA, showing linkage to ethylene biosynthesis.

from random sample were homogenized in 1 ml of 100 mM phosphate buffer at pH 7.6 and for ODC in 100 mM of Tris-HCl buffer at pH 8. The homogenates were centrifuged at 26,000*g* for 15 min at 4°C and the resulting clear supernatant fraction was used as the crude enzyme.

Enzyme assays were carried out in 12 × 75 mm polystyrene disposable culture tubes sealed with polyethylene caps. A filter paper disc 6 mm in diameter, impregnated with 50 μl 2 N KOH and transfixed with a 22-gauge syringe needle through the cap was used to trap the $^{14}CO_2$ liberated. Optimum assay conditions for each enzyme were determined and used. ADC activity was determined by measuring the release of $^{14}CO_2$ from the substrate, L-[U-$^{14}$C]arginine. The reaction mixture contained 100 μl crude enzyme and 10 μl 20 μCi/ml of L-[U-$^{14}$C]arginine (300 mCi/mmol, ICN) diluted with unlabeled L-arginine to give a final concentration of 9 mM. ODC activity was similarly measured by using DL-[1-$^{14}$C]ornithine as the substrate. Each assay consisted of 100 μl crude enzyme, 10 μl 20 μCi/ml of DL-[1-$^{14}$C]ornithine (54.9 mCi/mmol, New England Nuclear) diluted with cold L-ornithine to a final concentration of 66 mM, and 10 μl 5 mM pyridoxal phosphate. SAMDC activity was determined by measuring the release of $^{14}CO_2$ from SAM-[$^{14}$C]carboxyl. The reaction mixture contained 25 μl crude enzyme, 75 μl 100 mM phosphate buffer (pH 7.6), 10 μl 20 μCi/ml of SAM-[$^{14}$C]carboxyl (60 mCi/mmol, New England Nuclear) diluted with cold SAM, to give a final concentration of 2.7 mM and 10 μl 1 mM pyridoxal phosphate. In control assays, this concentration of pyridoxal phosphate did not give nonenzymic decarboxylation of SAM. Furthermore, boiling the enzyme prior to assay did not liberate $CO_2$. Based on these controls, the $CO_2$ liberated was assumed due to decarboxylation of SAM by SAMDC in the crude extracts. Enzyme activity, in all cases, was directly dependent upon crude extract volume. These procedures represent modifications of conventional methods (8, 19, 24).

For all three enzymes, the reactions, which progressed linearly for 60 min, were incubated for 45 min at 37°C, and stopped by injecting 0.2 ml 10% TCA into each reaction tube through the syringe needle. The tubes were stoppered and incubated in the water bath for an additional 45 min. The liberated $^{14}CO_2$ 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 nM of $^{14}CO_2$ liberated/mg protein or /g fresh weight.

*Polyamines.* Ten leaf segments weighing about 400 mg were homogenized with 2 ml 5% $HCLO_4$ in a prechilled mortar. The homogenates were placed on ice for about 1 h and then centrifuged at 26,000*g* for 15 min. The resulting supernatant fractions were dansylated according to a method modified from Seiler and Wiechmann (25): 0.2 ml of the supernatant fraction was added to a mixture of 0.4 ml 5 mg/ml of dansylchloride (Sigma) in acetone and 0.2 ml of a saturated solution of $Na_2CO_3$ and allowed to react overnight in the dark at room temperature. To the mixture was added 0.1 ml proline (100 mg/ml $H_2O$); it was then vortexed for 10 s and kept in the dark for 30 min. The dansylated products were then extracted with 0.5 ml benzene and separated on silica gel thin layer (TLC) plates (Whatman, LK6D). These plates, equipped with a preadsorbent which packs the components of the mixture at the origin, permit sharp separations.

For separation of the PA, 50 μl benzene extract was spotted on the TLC plate and developed in solvents containing chloroform: triethylamine (25:2, v/v) or cyclohexane:ethyl acetate (5:4, v/v). PA standards were similarly dansylated and chromatographed. The dansylated PA separated well, permitting scraping of the fluorescent spots, which were eluted with 2 ml ethyl acetate. The products were quantified with the use of an Aminco-Bowman spectrophotofluorimeter, in which the emission at 500 nm was recorded after excitation with UV (350 nm).

To specify further the changes in endogenous levels of PA during senescence, treatments which showed meaningful changes by TLC were confirmed by HPLC. Thus, $HClO_4$ extracts from attached leaves of 4- and 21-day-old seedlings and excised leaf segments incubated for 48 h in light or dark were benzoylated according to the method of Redmond and Tseng (23) and used for HPLC separation. Known PA standards were similarly handled.

**HPLC Analysis.** Benzoylated samples were separated in a Beckman (Altex) Model 322 liquid chromatograph equipped with an C18 reverse phase column and a 254 nm absorbance detector. Conversion of the chromatographic peaks to amounts of each of the PA was done by an Altex C-RIA integrator. Details of the chromatographic system and analytical procedure have been described by Flores and Galston (9).

With both TLC and HPLC, standard curves of PA (Sigma), run together with tissue extracts in each experiment, were reproducible within 5%. The limit of detectability was at the low level of 25 to 50 pmol. PA concentrations were expressed as μg/g fresh weight. The presence of each PA in leaf extracts was confirmed by adding known PA standards to the unknown mixtures and noting specific rise in a single peak.

*Chlorophyll Content.* Chlorophyll extracted with hot 80% ethanol was measured spectrophotometrically at 665 nm and expressed as percentage of the initial value.

*Protein Content.* Protein content of the crude enzyme extracts was measured according to the method of Lowry *et al.* (17), using BSA as a standard.

The data presented were from single experiments which are representative of 2 to 4 experiments or their mean. Each experiment was performed in duplicate and all enzyme assays in triplicate.

## RESULTS

**Enzyme Activities and Senescence in Attached Leaves.** Enzyme activities, measured in the first leaf from 4-, 7-, 14-, and 21-day-old oat seedlings and compared with protein loss, were used as a measure of senescence. Figure 2 shows that the specific activity of ADC is highest in leaves from 4-day-old seedlings and decreases with age of seedlings. This decrease in enzyme activity corresponds with an increase in protein breakdown. In contrast, SAMDC activity increased and ODC activity did not change significantly with increasing age of leaves. The results thus suggest that changes in ADC, but not in ODC or SAMDC, may be involved in the

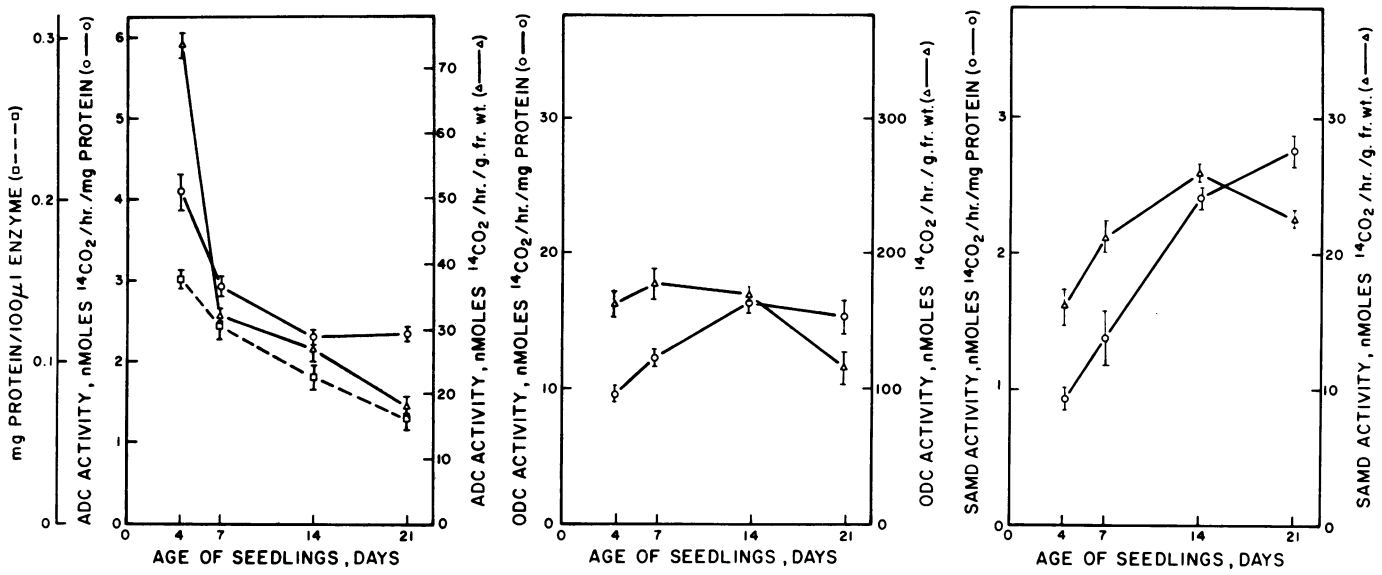


FIG. 2. Activities of ADC, ODC, and SAMDC and soluble protein content in attached first leaf from 4- to 21-day-old oat seedlings.

senescence of attached leaves.

**Enzyme Activities and Senescence in Detached Leaves.** Enzyme activities were also measured in detached first leaves of 2-week-old oat seedlings, incubated either in the dark or light for various periods of time. The results show that senescence, as measured by the progressive loss of Chl and soluble proteins, is accelerated with time of dark incubation and retarded with light incubation. These results are in agreement with earlier observations (16, 31). Furthermore, the specific activities of both ADC and ODC decreased dramatically with time of dark incubation (Fig. 3), but ADC activity, again paralleling degree of senescence, increased sharply with time of light exposure (Fig. 4). ODC activity decreased even more sharply during first 48 h in the light while SAMDC activity, when expressed as nmol $^{14}CO_2$/g fresh weight decreased only slightly with time of incubation in both dark and light (Figs. 3 and 4). The correlation of loss of Chl and soluble proteins with enzyme activity supports the overall view that ADC, and to some extent ODC, may be involved in senescence of excised leaves. In the light, where ADC activity increases and ODC activity decreases, putrescine synthesis appears to occur mainly via ADC. Furthermore, the differential specific activities of ADC in the dark and in the light show that its activity can be regulated. In etiolated Alaska pea seedlings, we have found that ADC activity can be controlled by phytochrome (6) while in light-grown dwarf Progress pea seedlings it can be controlled by $GA_3$ (7). In both systems, ADC activity parallels growth. In addition, ADC activity in cotyledons of cucumber has been reported to be increased by benzyladenine treatment and decreased by abscisic acid treatment (29).

In contrast to ADC and ODC, much less is known about SAMDC in plants. Although increases in SAMDC activity have been observed in germinating corn seeds (30), decreases in SAMDC activity with senescence and aging have not been established in either plants or animals. While these results indicate that SAMDC is unlikely to be directly involved in senescence, there may be competition between SAMDC and ACC synthase for the SAM pool which is a common precursor for PA (antisenescence) and ethylene (senescence-inducing) biosynthesis (3).

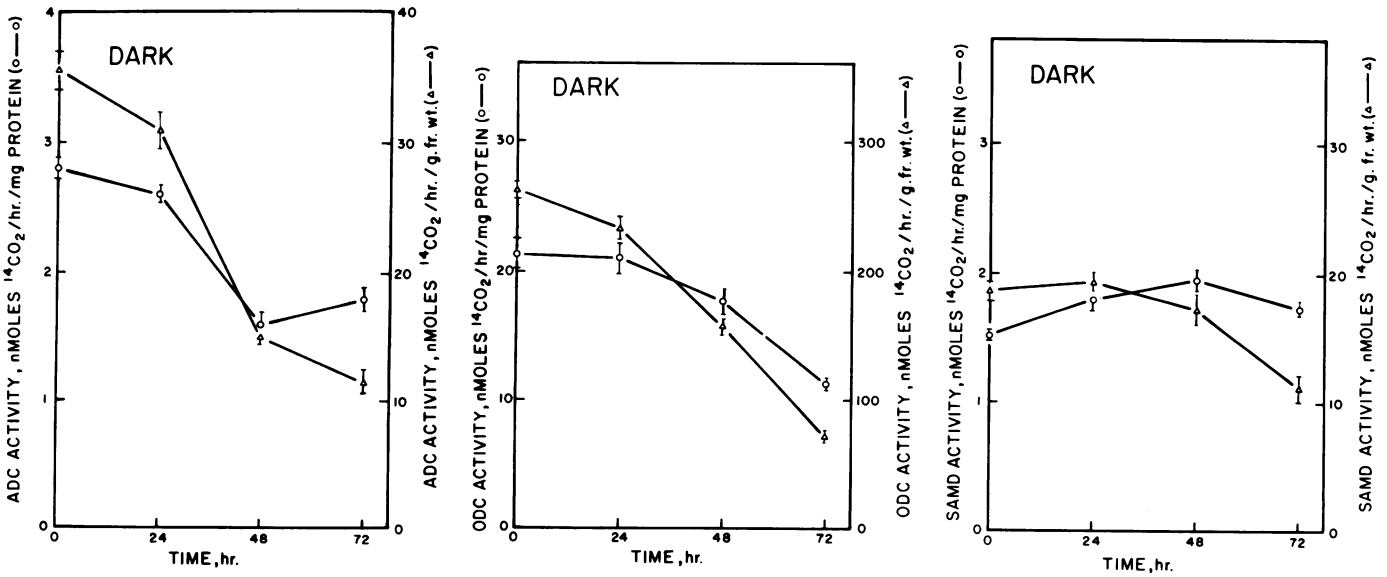


FIG. 3. Kinetics of ADC, ODC, and SAMDC activities in detached first leaf from 14-day-old oat seedlings incubated in the dark.

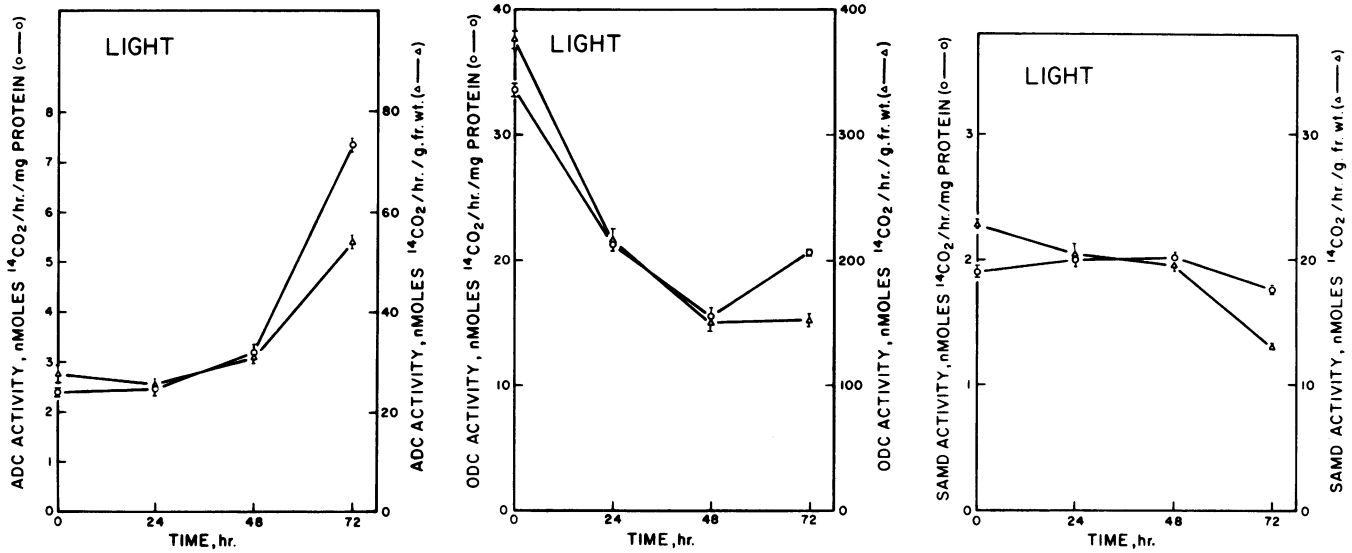


FIG. 4. Kinetics of ADC, ODC, and SAMDC activities in detached first leaf from 14-day-old oat seedlings incubated in the light.

**Effect of Temperature on Enzyme Activities and Senescence.** To examine further the role of PA biosynthesis in senescence, enzyme activities were measured and correlated with Chl and protein loss in excised leaves incubated in the dark at various temperatures, ranging from 5 to 35°C. Figure 5 shows that between 5 and 25°C, the higher the temperature the greater is the reduction in Chl and protein contents of the leaves and thus, the more rapid the senescence. At 35°C, there is damage to the leaf, and the trend is interrupted. Activities of ADC and ODC in these leaves show corresponding decreases with increase in temperature and time of incubation (Fig. 6). The decrease in enzyme activities was lowest in leaves incubated at the lowest temperature (5°C) and highest in leaves incubated at the highest temperature (35°C). The activities of SAMDC, however, increase during the initial 24 h at 25 and 35°C and then decrease with time of incubation. Dialysis of the crude extracts did not alter the enzyme activities, suggesting that the effects noted are not due to changes in the concentration of small molecules or PA themselves. These observations further support the hypothesis that PA biosynthesis is correlated with and possibly involved in senescence.

**Polyamine Titer in Attached Leaves.** As the activities of PA biosynthetic enzymes decrease during senescence, the levels of endogenous PA also decline. The results in Table I show that Put, Spd, and diaminopropane (a breakdown product of Spd and Spm) are present in senescing attached oat leaves, while agmatine and Cad were not detected with TLC. A striking feature of the PA changes is the sharp decline in Put, diaminopropane and Spd with age of seedlings. The sharpest decline was in Spd, with a less sharp decline in Put and diaminopropane. Spm was detected only in trace amounts. The results reported in Table I were obtained by TLC, using chloroform and triethylamine as the solvents; separations in ethylacetate-cyclohexane mixtures gave similar results. Decline of PA titer with age was also confirmed by HPLC. Clearly, the senescence of attached oat leaves is accompanied by decreased PA levels, reflecting decreased activity of ADC and possibly also of ODC.

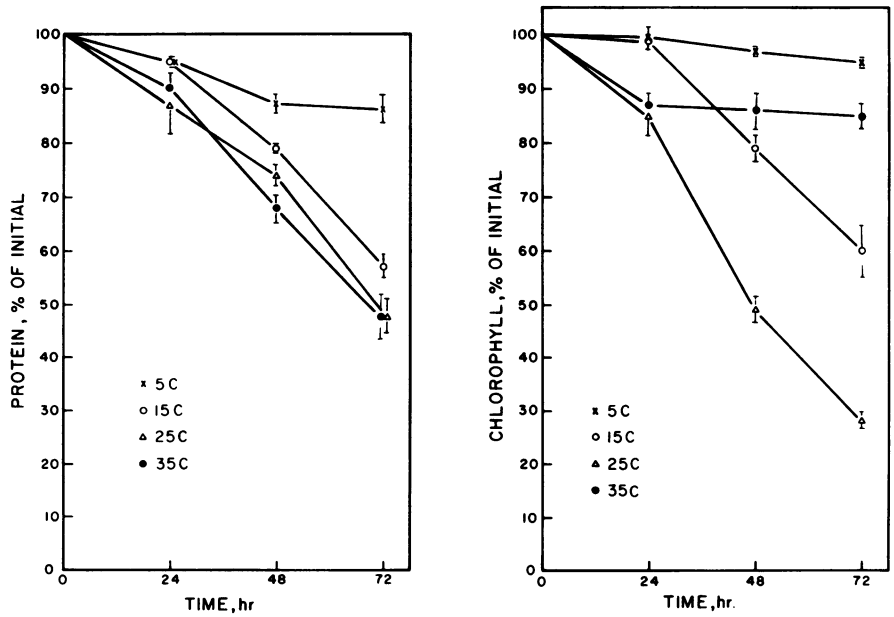


FIG. 5. Kinetics of decrease in Chl and soluble protein contents in detached first leaf from 14-day-old oat seedlings incubated in the dark at temperatures from 5 to 35°C.

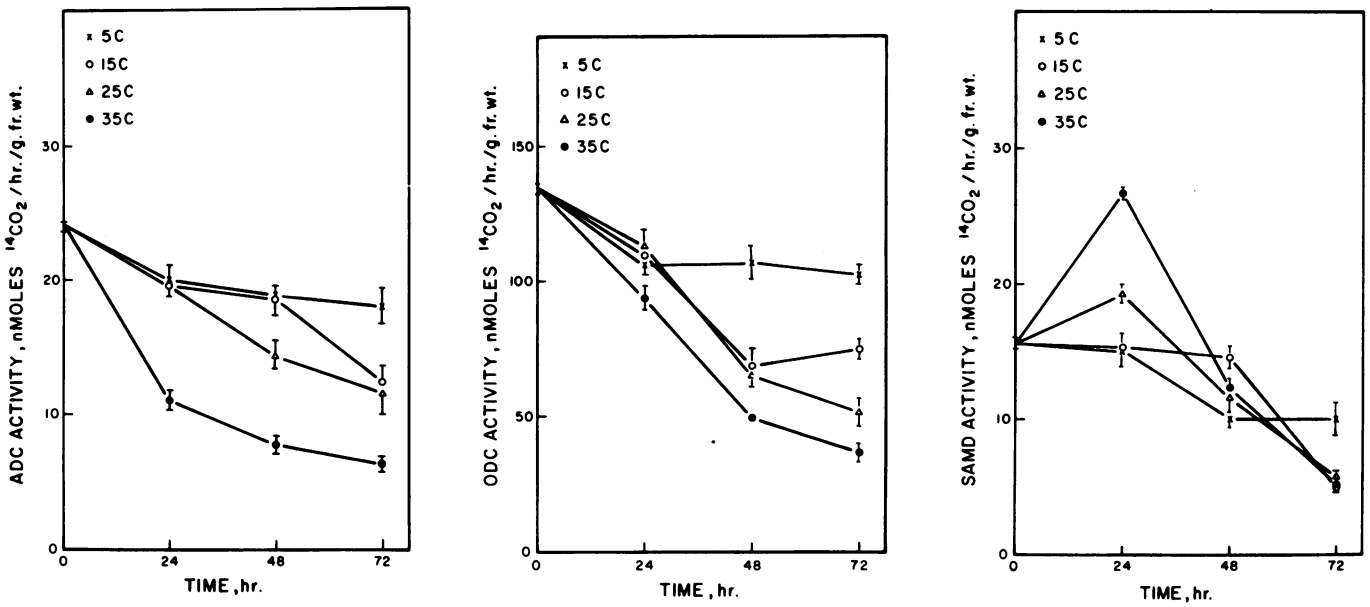


FIG. 6. Kinetics of ADC, ODC, and SAMDC activities in detached first leaf from 14-day-old oat seedlings incubated in the dark at temperatures from 5 to 35°C.

Table I. *Levels of Polyamines in the First Leaf of 4- to 21-Day-Old Oat Seedlings*

Amounts were calculated from TLC separations. Solvent system was chloroform:triethylamine (25:2,v/v).

| Age of Seedlings | PA Content | | | Relative PA Content | | |
|---|---|---|---|---|---|---|
| | Diaminopropane | Put | Spd | Diaminopropane | Put | Spd |
| *d* | *μg/g fresh wt ± SE* | | | % | | |
| 4 | 16.4 ± 2.1 | 4.6 ± 0.6 | 23.8 ± 2.8 | 100 | 100 | 100 |
| 7 | 9.5 ± 0.1 | 2.4 ± 0.2 | 9.3 ± 1.2 | 58 | 53 | 39 |
| 14 | 4.3 ± 0.4 | 2.6 ± 0.2 | 6.8 ± 0.7 | 26 | 55 | 29 |
| 21 | 4.4 ± 0.4 | 2.8 ± 0.4 | 5.3 ± 0.4 | 27 | 60 | 22 |

Table II. *Levels of Polyamines in the First Leaf of 2-Week Old Oat Seedlings, Excised and Incubated in the Dark or Light at 25°C*

Amounts were calculated from TLC separations.

| Time of Incubation | PA Content | | | | | | Relative PA Content | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Diaminopropane | | Put | | Spd | | Diaminopropane | | Put | | Spd | |
| | Dark | Light | Dark | Light | Dark | Light | Dark | Light | Dark | Light | Dark | Light |
| *h* | *μg/g fresh wt ± SE* | | | | | | % | | | | | |
| 0 | 11.8 ± 0.6 | 11.8 ± 0.6 | 4.4 ± 0.3 | 4.4 ± 0.3 | 11.3 ± 0.5 | 11.3 ± 0.5 | 100 | 100 | 100 | 100 | 100 | 100 |
| 24 | 12.3 ± 1.0 | 20.8 ± 1.6 | 8.2 ± 0.4 | 6.1 ± 0.4 | 12.2 ± 1.0 | 13.9 ± 0.9 | 104 | 175 | 189 | 140 | 108 | 123 |
| 48 | 4.4 ± 0.3 | 3.5 ± 0.5 | 12.8 ± 0.8 | 7.1 ± 0.6 | 15.2 ± 1.3 | 29.7 ± 2.0 | 37 | 30 | 293 | 164 | 134 | 263 |
| 72 | 4.9 ± 0.5 | 7.4 ± 0.6 | 10.6 ± 1.0 | 6.8 ± 0.5 | 5.2 ± 0.5 | 24.2 ± 1.1 | 41 | 63 | 242 | 155 | 44 | 215 |

**Polyamine Titer in Excised Leaves.** The patterns of PA change in excised darkened leaves (except Put) are similar to those in attached leaves (Tables I and II). In addition, the levels of Put and Spd in the leaves incubated in the dark are strikingly different from those in the light (Table II). For example, in the dark, the Put concentration increases dramatically with incubation time, showing a peak at 48 h of about 300% of initial value; by contrast, Spd at first increases slightly, then decreases sharply, reaching 44% of the initial value after 72 h. In the light, the trends are reversed; Put level increases slowly and spermidine level increases sharply.

The strikingly high levels of Put and Spd in leaf segments incubated for 48 h in the dark or light, respectively, were confirmed by HPLC, the results being identical to those obtained by TLC. Determinations of agmatine, the direct decarboxylation product of arginine, by HPLC showed that it is higher in light-exposed leaves than in leaves incubated in the dark (data not presented).

## DISCUSSION

The declining level of PA with senescence in the dark together with reversal of the pattern in the light suggests that PA may play an important role in controlling senescence. This notion contrasts with the general belief that cytokinins are the major senescence inhibitors in plants. Although unequivocal evidence is not available in either case, both classes of substances occur naturally in plants, are found in highest concentrations in meristematic tissues,

and prevent the development of the senescence syndrome when applied exogenously. Senescence has also been correlated with a decline in gibberellin or auxins, or to a build up of ABA (31). The evidence obtained thus far is, however, not convincing that any one of these substances is solely responsible for senescence control. Indeed, all of them may affect the production or action of ethylene, which may be the true senescence controller.

Although changes in levels of cytokinins have not been established in senescing leaves, our preliminary experiments show that exogenous application of kinetin not only prevents Chl breakdown but also prevents the block in Spd synthesis, resulting in increased levels of Spd in leaves incubated in the dark. Furthermore, cytokinin applications to mung beans, potatoes, and other plant systems have been shown to increase PA levels, even prior to observed effects on senescence (1). These data further strengthen the role of PA in controlling senescence.

As the activities of ADC and ODC decrease during dark incubation, levels of Put would also be expected to decrease. However, the results show that Put levels increase, while Spd, a further transformation product of Put, decreases with senescence. This buildup of Put and low levels of Spd could be due to a block in the SAMDC or Spd synthase step. The latter enzyme catalyzes the transfer of propylamine groups from decarboxylated SAM to Put (Fig. 1). Increases in Put titer may result from stress caused by excision and starvation resulting from dark incubation. Increases in Put levels have been reported in ion-deficient, mainly potassium-deficient, plants (27, 28), $SO_2$-fumigated plants (20) and in plants grown under saline conditions (18). Smith (28) has demonstrated that in potassium-deficient plants, the high Put levels are correlated with an increase in ADC activity. In contrast, our results with oat leaves show that ADC activity decreases with dark incubation, thus confirming that the accumulated Put results from a block in the Spd synthesis step. In the light, where ADC activity increases with a corresponding increase in Spd level, there appears to be no such block.

In addition to Put and Spd, diaminopropane, a breakdown product of Spd formed through the action of PA oxidase in cereal leaves (Fig. 1), decreased in detached leaves senescing in either darkness or light (Table II). A similar decrease was observed in senescing attached leaves (Table I). The decrease in diaminopropane is in agreement with our earlier report (15) in which PA oxidase activity was found to decline during senescence.

These results thus show that ADC is closely linked with PA titer and senescence in both attached and excised oat leaves. Because ADC is regulated by light, and parallels leaf senescence behavior, it is more likely to be involved in senescence. The results also suggest that the decrease in activities of the PA biosynthetic enzymes and titer are critical events in a complex induction system, and not simply results of leaf senescence.

*Acknowledgments*—We are grateful to Professor Seymour S. Cohen and Bob Balint, Department of Pharmacological Sciences, State University of New York at Stony Brook, Long Island, New York for their advice on the TLC separations of PA.

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