The Role of Protein Synthesis in the Senescence of Leaves

I. THE FORMATION OF PROTEASE

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ABSTRACT

The senescence of oat leaves has been studied by following the loss of chlorophyll and protein and the increase of a-amino nitrogen, after detachment and darkening. Protein synthesis and the amounts of proteolytic enzymes in the leaves have been determined directly. The process of senescence is shown to be a sequential one in which protein synthesis, most probably the formation of a proteolytic enzyme with L-serine in its active center, is of prime importance. The evidence is as follows. Firstly, L-serine specifically enhances senescence, especially in presence of kinetin. Secondly, cycloheximide, which inhibits protein synthesis in other systems, delays senescence and prevents the serine enhancement. Although requiring higher concentrations, cycloheximide can be as effective as kinetin in inhibiting senescence. It is shown directly that cycloheximide prevents protein synthesis in oat leaves under the same conditions as when it prevents senescence. Thirdly, leaves have been shown to contain two proteinases, with pH optima at 3 and 7.5, whose activity increases during senescence, even though the total leaf protein is decreasing. The amounts of both these enzymes present after 3 days are clearly increased by serine, and are greatly decreased by cycloheximide or by kinetin. The role of kinetin in delaying senescence thus may rest on its ability to suppress protease formation.

Senescence has long been regarded as an essentially degradative process, and the rapid onset of proteolysis in isolated and darkened leaves has been documented since 1930 (3, 28, 29). Accordingly, when the action of cytokinins in preventing leaf yellowing was discovered, it was ascribed to the promotion either of amino acid accumulation and protein synthesis in general (e.g., 17 and 18) or of more specific types of DNA-dependent protein synthesis (19). Skoog and Armstrong in their recent review (23) conclude that the evidence "probably favors a more direct effect of cytokinins on synthesis than on uptake or prevention of degradation." However, recent studies in this laboratory (22) have indicated that the prevention of leaf senescence in oats by kinetin could be equally and perhaps better explained by its inhibiting the proteolysis which was demonstrated to be concomitant with senescence. In particular it was found, inter alia, that the amino acid L-serine enhances senescence, thus acting in the opposite direction to cytokinin (22, 26). In explanation it was suggested that the serine might become incorporated into the active center of one or more proteolytic enzymes participating in the senescence process.

The present investigation was therefore undertaken, firstly, to find out more in general about the progress of senescence in the oat leaf, and about the antagonism between L-serine and kinetin; secondly, and more specifically, to see whether there was evidence that proteolytic enzymes are, in fact, synthesized in the senescing leaf, and if their amount increases with time.

A preliminary account of some of the results has been presented earlier (12).

MATERIALS AND METHODS

General Procedure. Oats (cv. "Victory") obtained from the U. S. Department of Agriculture were husked, soaked, and planted in vermiculite. They were grown 50 cm below a bank of eight 20-w cool white fluorescent lights giving $6.4 \times 10^3$ watts/cm² at about 23 to 25 C for 7 days. The first leaves, which were then about 12 cm long, were cut off and the apical 3 cm placed on slides over moist filter paper in Petri dishes, a minor modification from the procedure previously used (25, 26), which in turn was based on work of Gunning and Barkley (6). Usually a single 10 μl drop was placed in the center of each leaf, as was previously done for the kinetin bioassay (25). This drop contained the test substance together with McIlwain buffer, pH 4.7, diluted 1:10, and 0.2% Tween 80. The isolated leaves were left for 72 hr (or occasionally for 96 hr) in darkness, at which time about 50% of the chlorophyll had disappeared. The chlorophyll and a-amino nitrogen of groups of five leaves were then extracted into boiling 80% alcohol for 30 min, the solution was made to 10 ml, its absorbance at 665 nm was read for the chlorophyll content, and an aliquot was taken for determination of a-amino nitrogen by the Moore and Stein method (16). The extracted leaves were then washed twice with 80% alcohol, the protein was solubilized with 1 N NaOH and determined by Miller's (14) modification of the Lowry et al. method (9). Initial values of chlorophyll, a-amino nitrogen, and protein were determined on a leaf sample at the beginning of each experiment, and the results below are expressed as a percentage of these initial values.

Analyses of variance were carried out on each experiment except the time course (Fig. 1), where 95% confidence limits were calculated. All values cited are the means of three or four replicates, each comprising five leaves.

Extraction of Protease. Two hundred and fifty 5-cm apical sections of leaves (approximately 7 gm fresh weight) grown as above were finely cut with scissors into 25 ml of ice-cold solution containing 0.50 mM tris, pH 8, 3.5% NaCl and 5 mM dithioerythritol. When smaller samples were used, the ratio of

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leaves to solution (10:1) was kept constant. The leaves were then macerated for 1 min at full speed with a VirTis 23 homogenizer, the homogenate was strained through 1 mm mesh nylon net, and the resulting solution was centrifuged at 40,000g for 30 min. No protease activity was detected in the 40,000g pellet. The supernatant solution was therefore applied to a Sephadex G-50 (coarse) column which was equilibrated and eluted with 10 mM phosphate buffer at pH 7. The protein fraction was collected in an aliquot twice the volume of the supernatant solution applied, and its protease activity was assayed. The temperature was kept below 4° C during the above operations.

Assay of Protease. Of the various substrates tried (hemoglobin, bovine serum albumin, gelatin, and casein), hemoglobin was found to be the best. Gelatin had too large a blank, casein was too insoluble at acid pH, and BSA was poorly attacked. Protease activity was determined as the release of α-amino nitrogen (measured by the method of Moore and Stein [16]), because this method was far more sensitive than the measurement of aromatic amino acids with the Folin reagent. Two peaks of activity were found at pH 3 (the larger) and pH 7.5, with a minimum at pH 5. Interestingly, the acid pH optimum shifted to 4 when BSA was the substrate.

The assay mixture comprised: buffer: 1 ml of one-fifth strength McIlvain pH 3 or 25 mM tris-HCl, pH 7.5; substrate: 0.25 ml of 4% Bacto Hemoglobin in water; enzyme solution: 0.5 ml. After 1.5 hr of incubation at 40 C (the release of α-amino nitrogen at pH 3 and pH 7.5 was essentially linear for 1.5 hr), the reaction was stopped with 0.25 ml of 40% trichloroacetic acid and the solutions let stand for at least 0.5 hr in a refrigerator. Tubes were then centrifuged at full speed in a Sorvall XL centrifuge for 2 min, and 0.1 ml of the supernatant solution was analyzed for α-amino nitrogen. Protease activity was expressed as nmoles of α-amino nitrogen released per hour by 0.5 ml enzyme solution, as calculated from a standard graph using L-leucine as the reference standard. To obtain specific activities, these figures were divided by the micrograms of protein present in 0.5 ml of enzyme solution. Blank determinations were carried out similarly, except that the enzyme solution was added immediately before the trichloroacetic acid at 1.5 hr.

Uptake and Incorporation of 14C-Leucine. 14C-Leucine was applied to leaves in 5 μl droplets of buffer to which were added an additional 5 μl of buffer containing the experimental substances. At the end of the experiment, the leaves were washed for 20 min in running tap water, and the chlorophyll, soluble nitrogen, and protein were extracted and determined as described above. The radioactivity in the soluble nitrogen and protein fractions was determined by pipetting 0.5 ml of each fraction (per replicate of five 3-cm apical leaf segments) into planchetts, evaporating to dryness and counting in a Nuclear Chicago Model 1042 gas flow counter.

Kinetin, L-serine, cycloheximide, and phenazine methosulfate were obtained from Sigma Chemical Company, DL-homoserine was obtained from Calbiochem, and diisopropylfluorophosphate was obtained from K and K laboratories.

Presentation of Data. In Figures 1 through 6 the data are presented as percentage of the initial values in the leaves. To facilitate comparison with other work the following are the initial values for the five 3-cm apical leaf segments which serve as a unit in these experiments: protein content: 2.61 mg (BSA equivalent); amino nitrogen content: 1.55 nmol amino N; chlorophyll content: the extracts in 10 ml 80% (v/v) alcohol averaged \( A_{440 \text{ nm}} = 0.90 \pm 0.05 \).

### Table 1. Effect of Anaerobiosis on Senescence

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>Percentage of Initial Values after 4 Days in Darkness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>83.8 161 66.7</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>86.5 127 60.8</td>
</tr>
<tr>
<td>Air</td>
<td>23.8 520 31.4</td>
</tr>
<tr>
<td>LSD at ( P = 0.05 )</td>
<td>5.2 57 8.4</td>
</tr>
</tbody>
</table>

### RESULTS

**Time Course of the Senescent Process.** It was noticed at the outset that after 24 hr in the dark the color of the leaves appeared unchanged. The times of onset of chlorophyll breakdown and protein hydrolysis were therefore determined by taking a succession of samples on which chlorophyll, free amino nitrogen, and protein were all measured. Figure 1 shows that the first noticeable effect is an increase in amino nitrogen detectable at 6 hr, followed by a decrease in protein which is evident at 24 hr, and a decrease in chlorophyll which does not begin much before 36 hr after starting up. Two other time courses carried out under similar conditions gave basically the same results; in each case an increase in α-amino nitrogen was visible at about the 6th hr, whereas no decrease in chlorophyll was detectable before about the 36th hr. One possible interpretation of this result is that proteolysis commences in the cytoplasm and involves the plastids only later (cf. 7).

**Response of Senescence to Varied Treatments.** That senescence is an active process is shown very simply by placing the isolated and darkened leaves in anaerobic conditions. Such treatment might well be expected to cause protein breakdown, but instead, both in 100% \( N_2 \) and in 100% \( CO_2 \), yellowing was strongly inhibited (Table 1). In both cases the protein was preserved, and correspondingly the increase in α-amino nitrogen was prevented. The leaves in \( N_2 \) appeared normal and healthy after 3 days, but those in \( CO_2 \) were wilted and had guttated. These differences were not further explored, but the qualitative significance of the effect of anaerobiosis is clear. Wood and

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1 Abbreviations: BSA: bovine serum albumin; CYC: cycloheximide.
Cruikshank (28) also found exposure to \( N_2 \) to inhibit senescence in oats and in two grasses. In general, drastic chemical treatments, which might be thought to be damaging to the leaves, were found, on the contrary, to prevent senescence to varying degrees. Acids and bases at 0.1 \( \text{N} \) and above caused marked chlorophyll retention, even when (with HCl) the immediate area around the droplet was yellow-brown, evidently due to phoephyllin formation. Even 1 \( \text{M} \) solutions of \( \text{NH}_4\text{NO}_3 \), urea, or potassium acetate inhibited yellowing. The effect of acetate was not related to pH, and acetic acid was about equally effective. Triacetin and tributyryl, applied undiluted, were clearly effective, but olive oil had no effect. Ethanol at 50% or higher clearly inhibits yellowing. These results are not inconsistent with the data published earlier (25), since that dealt only with substances active in very low concentrations.

**Interaction between Serine and Kinetin.** A feature of the action of L-serine is that it has a greatly enhanced effect in promoting senescence when kinetin is present. Figure 2 shows the effects on chlorophyll retention of serine alone and in presence of four concentrations of kinetin. Figure 3 shows the comparable effects on protein preservation and on the accumulation of free amino acids. It is clear that the dual actions of kinetin in preserving chlorophyll and preventing proteolysis are strongly offset by serine; at 0.1 \( \text{M} \), serine can completely overcome the effect of 10 \( \mu\text{g/m}\)l kinetin.

Since L-serine also promotes yellowing when the agent preserving the chlorophyll is IAA or adenine at high concentration (22), it is clear that it is not a direct antagonist of the cytokinins.

**Effect of Cycloheximide on Senescence.** The rapid initiation of proteolysis when the leaves are detached and darkened must mean either that a protease is liberated from some bound or inactive form, as may occur in tobacco leaves (3), or else that protease commences to be synthesized. In experiments with detergents, no evidence for the first alternative was found, though it was not disproved. However, experiments with agents which inhibit protein synthesis have given strong evidence in favor of the second alternative. The most powerful such agent is cycloheximide (also called actidione).

Figure 4 supplements the table published elsewhere (26) in showing how CYC, applied in the usual 10-\( \mu\text{l} \) droplet, with or without kinetin, can inhibit the senescence process. There are marked effects at all concentrations, and at the highest level CYC is as effective as 5 \( \mu\text{g/m}\)l of kinetin. Some slight loss of protein occurs in 3 days, but no loss of chlorophyll. This supports the deduction from Figure 1 that proteolysis probably does not begin in the chloroplasts.

Puromycin had a moderate but less complete effect on...
chlorophyll loss. Among inhibitors of RNA synthesis, 6-methylpurine gave about 50% inhibition of chlorophyll loss and protein breakdown at 500 μg/ml in the droplet.

Figure 5 shows how the effect of CYC in presence of kinetin can be partially offset by L-serine. The three-way interaction between CYC or kinetin on the one hand and serine on the other is clear, though of course it does not of itself prove that all three are acting on the same unit process. Evidence for that will be given below. A peculiar feature of these curves is that at a CYC concentration of 30 μg/ml the action of kinetin is partly antagonized. This shows most strongly at the higher concentrations of kinetin. It is lessened by the presence of serine. It is shown also by 6-methylpurine. The action of CYC alone (curves marked "control") shows no infection at 30 μg/ml.

In order to see at what point the senescence process can be stopped, the droplets were applied at different times after leaf detachment. Figure 6 shows that both compounds still exert some effect even when applied 48 hr after commencing the experiment. This suggests that if some protein or proteins which are synthesized in the leaves after detachment are necessary for the degradation of chlorophyll and protein, this synthesis is continuous during the experimental period. Considered with the results in Figure 1, it is logical to deduce that the protein synthesized is a proteolytic enzyme which is "turned over" during the course of senescence. The close similarity in the timing for CYC and kinetin suggests that kinetin also may prevent either the formation or the activity of proteolytic enzymes (Table II).

Some idea of the rate at which CYC acts is given by experiments in which the droplets were taken off before the end of the 3-day period. Figure 7 shows that CYC at 1 mg/ml was just as effective when it was on the leaf only for 12 hr as for the following 72. The figure shows clearly that at the lower CYC concentrations the preservation of chlorophyll increases with time of contact, but at 1 mg/ml the preservation is as complete at 12 hr as at any other time. It may be noted again that this concentration of CYC is actually more effective than kinetin at 10 μg/ml, as shown also on the left side of Figure 5.

**Action of CYC on the Incorporation of Leucine into Protein.** Thus CYC, which is known to inhibit protein synthesis, retards the senescence process, which involves protein breakdown. This paradox certainly supports the idea that senescence is initiated by a synthesis of some protein. However, it was...
Fig. 6. Effect of time of application of kinetin (10 ppm) and CYC (1000 ppm) on senescence. The leaves were all detached at time zero and kept in the dark. The 72-hr points mean that nothing was applied.

Table II. Similarity of Action of CYC and Kinetin on Senescence

<table>
<thead>
<tr>
<th>Substance in 10 μl Droplet</th>
<th>Percentage of Initial Values after 3 Days in Darkness:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chlorophyll</td>
</tr>
<tr>
<td>Water alone</td>
<td>46.4</td>
</tr>
<tr>
<td>Kinetin, 10 μg/ml</td>
<td>92.9</td>
</tr>
<tr>
<td>CYC, 1 mg/ml</td>
<td>99.0</td>
</tr>
</tbody>
</table>

necessary to establish that CYC does in fact inhibit protein synthesis under the conditions of the present experiments, i.e., in detached oat leaves kept for 3 days in the dark. To establish this, the effect of CYC on the incorporation of 14C-leucine into protein was studied. The 10-μl droplets applied to the isolated 3-cm leaf sections were made to contain 14C-leucine plus CYC at several concentrations. In a parallel series, kinetin at 7.5 μg/ml was present in addition. After 12, 24, or 72 hr in darkness the droplets were washed off (20 min in running tap water), and the soluble nitrogen and protein was extracted as described in "Materials and Methods."

Figures 8 and 9 demonstrate that the 14C content of the protein is indeed drastically decreased by CYC. Figure 8 shows the percentage incorporation of the leucine into protein, and Figure 9 shows the specific radioactivity of the protein formed. At 3 days the optimum concentration of 300 μg/g decreases the specific radioactivity by nearly a factor of 10, i.e., from 3500 to 400 cpm/mg. Even 12 hr after application the decrease is large. The presence of kinetin, while slightly increasing the specific radioactivity at all three time periods, does not prevent CYC from exerting its inhibiting effect, and the "plus-kinetin" curves essentially parallel those without kinetin. At 12 hr, indeed, kinetin has no effect at all on the percentage incorporated (Fig. 8). The peculiar, but doubtless real effect that after 72 hr the concentration of 1 μg/ml exerts a somewhat lesser inhibition than the lower concentrations is not easy to explain, but suggests a secondary effect, perhaps an interference with proteolysis. Two other experiments gave closely similar results.

The total intake of radioactivity was affected little by the treatment with CYC. In the early hours the intake was actually somewhat promoted by CYC, but at 72 hr the differences were not significant (Table III). The incorporation of 14C into protein, on the other hand, was cut to 14% of the control value. It must be concluded that CYC causes a very strong and specific inhibition of protein synthesis when applied to the detached leaves under exactly the same conditions and over the same concentration range as those where it prevents the breakdown of chlorophyll and of protein.

It should be added that in all these experiments with CYC the leaves remained in green and turgid condition throughout. Since the droplets applied were small and the material does not by any means all enter during the experiment, no toxicity was evident even at the relatively high concentration of 1 mg/g. In contrast, when the leaves were placed with their bases im-
mersed in CYC 100 µg/ml, or when they were floated for only 2 hr on 100 µg/ml CYC and then removed, they did become flaccid after 48 hours.

**Role of Protease in Senescence.** It was deduced above that de novo synthesis of proteolytic enzymes may be the primary biochemical change in senescence. To test this hypothesis directly, senescing leaves, maintained in darkness, were extracted with aqueous medium and the extract applied to a Sephadex column as described in "Materials and Methods." All the protease activity was found in the extract. As described in "Materials and Methods," there are at least two proteolytic enzymes present, having optima at pH 3 and pH 7.5. These pH optima and the minimum at pH 5 for activity are almost identical to those reported by Robinson (20), who assayed proteolytic activity in bean root tips, using hemoglobin as a substrate. The only difference is that his second peak was at pH 7, not 7.5.

**Table III. Effect of CYC on the Conversion of 14C-L-leucine to Protein**

A 10-µl droplet of solution containing 14C-L-leucine plus the stated concentration of CYC was applied to the center of the 3-cm apical parts of five leaves, kept in the dark at 23-25°C for 1 or 3 days. Leaves were then washed and extracted.

<table>
<thead>
<tr>
<th>Concentration of CYC (µg/ml)</th>
<th>Total Activity in Five Leaves (cpm)</th>
<th>Activity in Protein Fraction (cpm)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At 24 hr</td>
<td>At 72 hr</td>
<td>At 24 hr</td>
<td>At 72 hr</td>
</tr>
<tr>
<td>0</td>
<td>17,000</td>
<td>65,300</td>
<td>2,570</td>
<td>5,820</td>
</tr>
<tr>
<td>30</td>
<td>18,600</td>
<td>74,200</td>
<td>730</td>
<td>2,140</td>
</tr>
<tr>
<td>100</td>
<td>19,700</td>
<td>59,000</td>
<td>430</td>
<td>870</td>
</tr>
<tr>
<td>300</td>
<td>26,000</td>
<td>51,900</td>
<td>580</td>
<td>830</td>
</tr>
<tr>
<td>1,000</td>
<td>30,800</td>
<td>61,700</td>
<td>640</td>
<td>1,430</td>
</tr>
<tr>
<td>Maximum inhibition of incorporation</td>
<td>83%</td>
<td>86%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 10 shows how the activity of the two proteolytic enzymes changes with time after detachment. The total activity at pH 3 is greater than that at pH 7.5 and rises more sharply, reaching a maximum at 4 days, and then falling steeply to the 6th day. In contrast, the total activity at pH 7.5 rises steadily and does not decrease during the experimental period. The specific activity at pH 3 follows the same trend as the total activity, but the specific activity of the pH 7.5 enzyme markedly diverges from the total activity after the 2nd day, showing that this enzyme is more stable than the other. The decrease in specific activity at pH 3 indicates that the enzyme is subject to attack in the general proteolysis which is going on at this time (cf. Fig. 1). The pH 7.5 enzyme apparently substantially contributed to the total proteolysis only in the final stages of senescence. It is important to note that the total activity of these enzymes is rising (up to 4 days), while the level of total protein in the leaves and extract is falling.

If the proteases are of functional significance during the senescence process, then kinetin should prevent the increase, and, furthermore, serine should increase the activity in the presence of kinetin. On the other hand, CYC should prevent the increase. That is, the effects of these agents on protease activity should be opposite to their effects on the chlorophyll and protein contents of leaves. Table IV shows the interaction between kinetin and serine with respect to protease activity after 3 days. Kinetin markedly decreases the activity at both pH values, total activity at pH 3 being halved and the specific activity reduced almost to one-third. Serine has little effect (cf. Fig. 3) alone but increases the activity some 50% in the presence of kinetin. Thus, the effects of these substances on protease activity are, in fact, opposite to their effects on the chlorophyll and protein levels of leaves. The amounts of protein in the enzyme extract vary as expected, i.e., in the opposite sense to the changes in the proteases.

CYC, at the concentrations effective on senescence, powerfully decreases the development of protease activity (Fig. 11). Since CYC was shown in the preceding section to inhibit protein synthesis in senescing leaves, Figure 11 doubtless repre-
Table IV. Effects of Kinetin and Serine on Protease Activity
Leaves were treated in darkness with 10-μl droplets of reagent for 72 hr after detachment, then extracted. Protease was prepared and assayed as in "Materials and Methods.'

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein in Extract (mg/ml)</th>
<th>Protease Activity pH 7.5</th>
<th>Protease Activity pH 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
<td>per mg protein</td>
<td>total</td>
</tr>
<tr>
<td>Initial value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.878</td>
<td>537</td>
<td>0.61</td>
</tr>
<tr>
<td>Kinetin 10 μg/ml</td>
<td>0.459</td>
<td>1386</td>
<td>2.90</td>
</tr>
<tr>
<td>Serine 0.2 M</td>
<td>0.707</td>
<td>764</td>
<td>1.09</td>
</tr>
<tr>
<td>Kinetin plus serine</td>
<td>0.456</td>
<td>1497</td>
<td>3.28</td>
</tr>
</tbody>
</table>

Fig. 11. Influence of CYC (10-μl droplet) on the development of total and specific activity of proteases during 3 days in darkness. Solid lines: total activity (left-hand scale); dashed lines: specific activity, i.e. per μg protein (right-hand scale).

sents the inhibition of enzyme synthesis. Control experiments showed that kinetin concentrations from 1 ng/g to 1 μg/g, and CYC concentrations from 0.1 to 100 μg/g, have no detectable effect on the activity of the isolated enzymes. From these results it appears, therefore, that CYC acts similarly to kinetin in delaying senescence by preventing the synthesis of proteolytic enzymes. The mechanism of the prevention of synthesis may well be quite different in the two cases, however.

DISCUSSION

This research started with the aim of finding an explanation for our earlier observations (22) that L-serine enhances senescence of oat leaves and that, while a few other amino acids have a similar but much weaker effect, its D-isomer and other related compounds are inactive. The logical deduction (though made only tentatively) that L-serine becomes incorporated into a proteolytic enzyme which operates to cause senescence, has been rather well supported by the present experiments. The deduction involves a corollary, namely that when cytokins prevent senescence or its accompanying chlorophyll breakdown they do so not so much by promoting protein synthesis, as is often thought (e.g., 17), but rather by preventing proteolysis.

A number of observations in the literature bear on this problem. Firstly, the fact that proteolysis is the dominant process in the metabolism of starving leaves was realized at least as long ago as 1944 (28). The classical experiments of Yemm on isolated barley leaves (29), since they did not include quantitative determination of the chlorophyll, had left the exact relation between yellowing and proteolysis open, while the work of Michael with Tropaeolum leaves (13) had shown that the chlorophyll-protein ratio remained constant during senescence, so that proteolysis in the cytoplasm and in the chloroplast were thought to be simultaneous. In more recent work with cucumber cotyledons, which yellow more slowly, proteolysis was seen to begin early and indeed, as in our Figure 1, to precede the breakdown of chlorophyll (7). Further, Anderson and Rowan (1) found in 1965 not only that the senescence of tobacco leaves is accompanied by loss of protein, but that there is an actual increase in the activity of a proteinase (extratable at pH 5).

Secondly, in regard to the action of cytokinin, Mizrahi et al. (15) concluded that the enhancement of protein content in kinetin-treated Tropaeolum leaves could not be accounted for by increased synthesis but that kinetin must be acting to depress protein degradation. Tavares and Kende (24) came to a similar conclusion in regard to the action of 6-benzylaminopurine in retarding senescence of corn leaves. In harvested tobacco leaves, whose yellowing is again quite slow, Anderson and Rowan (2) found that the "only significant effect" of kinetin treatment was the inhibition of the rise in e-amino nitrogen. More to the point for our own studies, these workers found the peptidase to be decreased by kinetin. Thus there is a growing trend of evidence in this direction.

Our evidence that some protein synthesis must precede senescence does not appear to have been as directly foreshadowed in the literature, but Dr. Sahai Srivastava has informed us that in barley leaves held in the dark under his conditions the yellowing is significantly decreased not only by cytokinins but also by chloramphenicol, puromycin, and tetracycline. These antibiotics are known to inhibit, directly or indirectly, the synthesis of proteins. The striking effect of CYC in preventing senescence of oat leaves (Table II and Fig. 4) is certainly paralleled by its action under the same conditions in preventing the incorporation of leucine into protein. These effects are not due to toxicity, although it is true, as seen in Table I, that active metabolism is necessary for normal senescence to occur. As was mentioned above, we have found that true toxicity, with curling and loss of turgor, can indeed be produced by floating these leaves on CYC solution. With the droplet placed on the upper surface of the leaf, however, there was no sign of damage. Indeed, at the highest concentration, 12 hr contact was enough to produce the maximum effect on chlorophyll retention (Fig. 7). McHale and Dove (11) similarly found that kinetin or 6-benzylaminopurine at 50 μg/g were toxic when the leaves were floated on the solution, but not when the solution, absorbed on balls of cotton wool, was applied to the upper leaf surface. They concluded that the latter was the "best way" of application.

There is some uncertainty about the strict specificity of the action of CYC on protein synthesis in leaves, since it also stimulates respiration (10), affects ion uptake by beet discs (though not by leaves) (5), and interferes with steps in the synthesis of RNA (21). The latter, however, is interrelated with protein synthesis, and we have shown that 6-methyl-purine, which also affects RNA synthesis, prevents senescence in oat
leaves (26), though not so powerfully as CYC. Udvardy et al. (27) have reported that several enzymes, including phosphonoesterase, phosphodiesterase, and RNase, all increase in oat leaves kept in light, and the increase in these enzyme activities is prevented by CYC. In any event, the data of Figures 8 and 9 leave no doubt but that protein synthesis is powerfully inhibited.

Some of the effects of amino acids on the senescence process remain to be investigated further. These include the actions of cysteine and alanine, the antagonism between arginine and serine (22), and the curious interactions between low concentrations of CYC, serine, and kinetin (Fig. 5). While a detailed study of these effects will be presented in a later paper, it should be made clear now that the interactions between cytokinins and amino acids are important. For since the cytokinins are naturally occurring growth substances, it follows that at least in some circumstances their action must be modified or antagonized by free amino acids such as serine, cysteine, and glycine (for all three act to promote senescence in varying degrees). A more noteworthy deduction is that senescence has something of an autocatalytic character, for as peptolysis progresses, the concentration of such free amino acids will increase and thus proteolysis and yellowing will be still further promoted.

LITERATURE CITED