Metabolism of Oat Leaves during Senescence

VIII. THE ROLE OF L-SERINE IN MODIFYING SENESCENCE

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ABSTRACT

The mechanism whereby l-serine specifically promotes the dark senescence of detached oat (Avena) leaves has been examined. The fact that this promotion is strong in darkness but very weak in white light has been explained, at least in part, by the finding that added serine is partly converted to reducing sugars in light. Labeled serine gives rise to 14C-sugars and 14CO2. In the absence of CO2, serine does cause chlorophyll loss in light and undergoes a decreased conversion to sugar.

As to the large promotion of protease activity which accompanies senescence in the dark, reported earlier, careful purification of the proteases shows that the l-[14C]serine is not incorporated into these enzymes, although it is incorporated into the total protein. Cycloheximide decreases the overall synthesis both of protease and of total protein, but again [14C]serine does not impact radioactivity to the purified acid proteases. Even when serine is simply added to the protease assay the proteolysis is significantly increased. It is concluded that serine promotes the protease activity by synergizing with the enzyme, or by activating an apoenzyme.

One of the early findings about leaf senescence in Avena was that senescence in darkness is accelerated by the amino acid l-serine (12). Two other amino acids, related to serine in its synthetic pathway by the glycolytic system, had similar but much smaller effects (6). Although senescence is characterized by rapid proteolysis, nevertheless, in order for senescence to occur, the synthesis of some protein(s) appears to be necessary. For this reason, it was suggested that the role of serine might be to become incorporated into the active site of a protease. One of the two major proteinasises of the oat leaf does appear to have serine at its active factor (2), but this does not, of course, prove the serine to be the limiting factor. Because it is rare for protein synthesis in tissues to be controlled by the availability of one particular amino acid, a further study of the fate of serine in senescing oat leaves has been undertaken. The results show that the behavior of serine in the light differs from that in the dark; in the light it does not promote Chl loss but is partly converted to reducing sugars. In the dark, where it does promote the loss of both Chl and protein, i.e. true senescence, no evidence could be found for its direct incorporation into the proteases of the leaf, but rather the data indicate a kind of synergism between this amino acid and the protease that has an acid pH optimum, resulting in an increased rate of proteolysis. The results also confirm the earlier conclusion (11) that the action of light on senescence, although not dependent on photosynthesis, is nevertheless modulated by CO2. Horton et al. (4) and Yang et al. (18), from quite different experiments, have reached the parallel conclusion that the action of light on ethylene production is also mediated by CO2.

MATERIALS AND METHODS

As in preceding communications, the plant materials were the first leaves of 7-d-old oat (Avena sativa cv Victory) seedlings grown in vermiculite under about 30 µE m-2 s-1 daylight fluorescent lights at 23 ± 2°C.

To follow senescence, eight 3-cm subapical segments were floated on 10 ml of the test solution in a Petri dish under white light of the same intensity as above, or in total darkness. After (usually) 3 d in darkness or 6 d in light, the leaves were extracted for 15 min in boiling 80% ethanol and the Chl, α-amino nitrogen, and reducing sugars determined in the extract in the usual way (12).

For metabolism experiments, six or seven subapical segments (about 120 mg fresh weight) were floated on water for 15 min, then incubated at 25°C in 2 ml of 25 mM unlabeled l-serine containing 0.1 ml l-[U-14C]serine (New England Nuclear Co.) with or without 3 µg/g kinetin. Incubation was in a 25-ml three-necked flask, one neck being stoppered (used to introduce the leaves), one fitted with a rubber serum cap to take the CO2 samples, and the third serving for circulation of air (or CO2-free air). For the minus-CO2 experiments of Tables II and III, the CO2 was absorbed in a vial of 2 ml ethanolamine attached to the serum-capped neck from which 0.1-ml samples could be taken. The CO2-free water control had the same amount of ethanolamine so that the difference was due only to the serine. The ethanolamine was of course omitted from the plus-CO2 experiments. The air stream was at 200 ml/min; when CO2-free air was used, the air bubbled through two flasks of 20% KOH before entering the three-necked flask. For CO2 sampling, the air was of course closed off. During experiments in light, two 100-w incandescent bulbs were placed 50 cm from the flask, otherwise the flask was tightly wrapped in heavy foil. Oxygen consumption (Table IV) was measured with the Clark O2 electrode as in reference 11.

The reaction was stopped by adding boiling 80% ethanol, the leaves washed with water and extracted further with hot 80% ethanol, rewashed, and the combined extracts taken to dryness in vacuo. From the residue the pigments were extracted with ether. The water-soluble residue was then placed on a 6-×1-cm column of Dowex 50-X8 (H+), from which the amino acids were subsequently eluted with 50 ml of N NH4OH. The effluent from the column was cleared with a few drops of benzene and placed on a similar column of Dowex 1-X 10 (formate). The neutral

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with cocktail '30 a 70 B' of the Research Products International Co., Elk Grove Village, IL.

The first-named column was regenerated with 2 N HCl and the second with a mixture of 60 ml 1 M sodium formate and 30 ml 0.1 N HCOOH.

For the protease experiments, 5.0 g fresh weight of the sub-apical segments, 3 cm long, were floated on the test solutions, then homogenized in 40 ml ice-cold isolation medium and centrifuged 30 min at 20,000 g. The supernatant was treated with (NH₄)₂SO₄; the major part of the proteases precipitated when the (NH₄)₂SO₄ concentration was increased from 35 to 75% (cf. Ref. 2). The precipitate was centrifuged and the pellet was washed twice in isolation medium (pH 6.8 buffer + 10 mM mercaptoethanol) at 0°C, resuspended in isolation medium, and dialyzed for 2 × 2 h. For the experiments where the unpurified extract was used, protease activity was then determined directly against hemoglobin, which has been shown to be readily hydrolyzed by the leaf protease (2). For the experiments in Figures 3 and 4, the solution was placed on a column of Sephadex G-100-120, 2 × 50 cm long, run with 100 mM phosphate buffer (pH 5.6), and the successive 10-ml fractions assayed for protease as before. This experiment was repeated three times, using four levels of [¹⁴C]serine. The data of Figures 3 and 4 refer to the highest levels of the serine used.

For the determinations of Figure 5, the segments were floated on four test solutions: (a) 1.75 ml water; (b) 1.25 ml water + 0.5 ml 200 mM l-serine; (c) 0.75 ml water + 1.00 ml 200 mM CHI⁴; (d) 0.75 ml water + 1.0 ml 200 mM CHI added after 24 h. The fractions from the Sephadex column were assayed, and those with activity placed on an affinity column. From this, the activity was eluted first at pH 5.7, then at 3.1, as described previously.

For the experiment of Table VI, the protease was prepared as usual from oat leaf segments which had senesced for 3 d in the dark, by (NH₄)₂SO₄ precipitation, re-solution, and dialysis. The co-factors to be tested were added to the buffered medium and the activity measured for 1 h at 50°C with hemoglobin as substrate.

Protein determinations were by the Lowry method (5). For the [¹⁴C] assay, 500 µl of each fraction was mixed with 4.5 ml counting cocktail 30 a 70 B and counted on a Beckman LS-230 scintillation counter.

### RESULTS

**Metabolism of Serine during Senescence.** Because senescence in the dark is rapid, the effect of serine in promoting it can be seen more clearly by antagonizing the action of kinetin, which by itself always delays senescence. However, a reexamination of the leaf metabolism in such experiments has shown that the decrease in Chl is accompanied by a marked increase in reducing sugar (Fig. 1). At 30 mM serine, in presence of kinetin, the reducing sugar increases by 50 µg per segment, or for 10 segments about 2.8 µmol. Although this might reflect some overall change in metabolism, it might also mean that the serine is being directly converted to carbohydrates. Of the 300 µmol of serine in the Petri dish, about 31% or 93 µmol was taken up in the 72 h. The respiration rate indicates that about 5 µmol were respired, leaving 88 µmol to be converted. If 2 mol serine form 1 mol hexose, the yield would be only 6%, but its significance would be that it changes a substance that promotes senescence into one that delays it (see Table IV of Ref. 13). In light, as will be seen, the yield is nearer to 30%.

To determine whether sugar molecules are directly formed from serine, L-[¹⁴C]serine was applied. The leaves were infiltrated with the labeled serine for 30 min, then one sample was fractionated and two other samples left for 48 h—one in light

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**Abbreviation:** CHI, cycloheximide.

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**Table I. Partition of Radioactivity from L-[¹⁴C]Serine in Oat Leaves**

Leaves were infiltrated with 25 µM L-serine. Data as per cent of total uptake.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>After 30 min</th>
<th>After 48 h in Darkness</th>
<th>After 48 h in White light</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO₂</td>
<td>0.6</td>
<td>2.9</td>
<td>13.0</td>
</tr>
<tr>
<td>Amino acids</td>
<td>62.7</td>
<td>78.9</td>
<td>9.2</td>
</tr>
<tr>
<td>Organic acids</td>
<td>3.6</td>
<td>2.5</td>
<td>7.0</td>
</tr>
<tr>
<td>Sugars</td>
<td>1.2</td>
<td>7.7</td>
<td>30.0</td>
</tr>
<tr>
<td>Insolubles</td>
<td>13.8</td>
<td>6.4</td>
<td>15.0</td>
</tr>
</tbody>
</table>

**Table II. Data Showing that L-Serine, without Effect in Light in Air, Causes Even Greater Chl Loss in Light Minus CO₂ than in Darkness**

Data after 2 d.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chl</th>
<th>Reducing Sugars</th>
<th>Nonreducing Sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A₆₆₀ µg/segment</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table III. Effect of Photorespiratory Products on Chl and Reducing Sugar Content in CO₂-Free Air**

<table>
<thead>
<tr>
<th>Conc. mm</th>
<th>Chl A₆₆₀ µg/segment</th>
<th>Reducing Sugars</th>
<th>Nonreducing Sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial value</td>
<td>0.276</td>
<td>27.2</td>
<td></td>
</tr>
<tr>
<td>After 2 d in white light</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water control</td>
<td>0.189</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>0.130</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>0.112</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>L-Glycine</td>
<td>0.120</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>L-Glycine</td>
<td>0.110</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>L-Serine</td>
<td>0.105</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>

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[Figure 1. Chl and reducing sugars of oat leaf segments as function of L-serine concentration, after 3 d in darkness.]

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[Table I. Partition of Radioactivity from L-[¹⁴C]Serine in Oat Leaves]

[Table II. Data Showing that L-Serine, without Effect in Light in Air, Causes Even Greater Chl Loss in Light Minus CO₂ than in Darkness]

[Table III. Effect of Photorespiratory Products on Chl and Reducing Sugar Content in CO₂-Free Air]
When photosynthesis is inhibited by removal of CO$_2$ from the air, serine still continues to increase the level of reducing sugars (Table II). Of the 99.4 µg increase in reducing sugars due to serine in air, 40.7 apparently was provided by hydrolyzing nonreducing sugar; the remaining 58.7 µg must have come partly from photosynthesis and partly from serine. In the absence of CO$_2$, the 31.4 µg increase in reducing sugar evidently all came from serine. In the dark, serine yields 6.4 µg reducing sugar and 6.4 µg nonreducing sugar. (As shown earlier [14], this nonreducing sugar is not sucrose, but a fructosan that is hydrolyzable by invertase.)

Although in normal air serine causes little or no Chl loss in the light, in CO$_2$-free air it causes even greater Chl loss than in the dark. Thus, when photosynthetic assimilation of CO$_2$ is prevented, the action of serine on Chl content in the light is the same as in the dark.

The same behavior is shown by two of the photosynthetic products related to serine, namely glycolic acid and glycine. These have little effect on Chl retention in ordinary air, but Table III shows that in CO$_2$-free air they both promote loss of Chl. This shows also that unlike the keto-acids (15) they do not substitute for CO$_2$.

These data, together with those presented previously [11], show that the effect of light on senescence, although dependent on photosynthesis, is nevertheless modulated by CO$_2$.

The influence of serine on senescence in the dark is, as would be expected, accompanied by a corresponding rise in respiration. Table IV shows that the normal ‘climacteric’ rise, typical of senescence, is increased by serine. Kinetin, on the other hand, prevents the rise for at least the first 5 d (13) and at the concentration used is unable greatly to counteract the effect of the serine.

Since 1 g fresh weight comprises about 60 segments, it is possible to correlate the respiratory rates in Table IV with the reducing sugar contents in Table II. Even at the low respiration rates in the first column, and using both reducing and nonreducing sugar, it is evident that the carbohydrates alone could not support all of the observed respiration. This agrees with the low RQ (around 0.7) that we reported in 1974 to occur during dark senescence [13].

**Interrelation of Serine with Protease.** While the above results provide a partial explanation of the ineffectiveness of serine in light—namely, its partial conversion to carbohydrates—they still do not explain its promotion of senescence in darkness. The increase in proteolytic activity previously recorded [6, 12] was therefore studied in detail. Figure 2 shows how serine influences the time course of enzyme development at the two pH values. Measured at pH 4.1, serine increases protease activity on day 3 (the peak) by nearly 3 times; at pH 6.6, the increase is 2 times and the peak is apparently delayed 1 d as well by the serine. The rapid drop in protease activity on day 4 was earlier [6] ascribed to autolysis of the protease, or the hydrolysis of one protease by the other.

To determine whether the newly formed protease actually contains the added serine, leaves were allowed to take up L-[U-14C]serine for 48 h in the dark, and then extracted as described in “Materials and Methods.” After several preliminary trials, two complete series were conducted, one group senescing in the labeled serine alone, the other in presence of unlabeled serine. The following day, ninhydrin was added to the 50 mm as well. From both groups, the proteins in the extract were precipitated with (NH$_4$)$_2$SO$_4$, and the pellet redissolved, dialyzed, and separated on G-100–120 Sephadex columns.

Figure 3, left side, shows that the protease activity appears to coincide with a peak in radioactivity, but the right side shows that when further purified on the affinity column the protease is not released until after acidification, while the radioactivity has
It was thought that perhaps the sensitivity of the assay was being lowered by the large amount of protein that is being synthesized. Since CHI was earlier shown not only to delay senescence but also to inhibit protein synthesis by 80 to 90% in these leaves (6), a similar experiment was carried out in which the leaf segments senesced in the dark in presence of 100 μg/g CHI (Fig. 5). Comparison of the figure with Figures 3 and 4 makes clear that CHI given for the first 24 h does greatly decrease the incorporation of radioactivity into protein. Table V not only supports that but shows that both total protein and protease activity are decreased by the CHI. When CHI was given only during the second 24 h, the decreases were smaller. However, the peak of protease activity, representing 40 to 60 μmol protein hydrolyzed/mg protein·h, again does not correspond to any peak in radioactivity.

It must be concluded that there is no evidence for the incorporation of serine into the leaf proteases. How then can it promote the proteolysis? One possibility is that serine might act as a synergist. The effect of simply adding serine to the protease assay was therefore studied. Leaves were allowed to senesce for 3 d in the dark, and were extracted, and the (NH₄)₂SO₄ precipitate, after redissolution and dialysis, was allowed to act for 1 h at 50°C on hemoglobin and the free α-amino nitrogen measured. Figure 6 shows that the rate of release of α-amino nitrogen by the acid protease is indeed greatly increased by 1 mM l-serine. To determine the specificity of the reaction, several other addenda were tested, including salts, three reagents that modify senescence, glycine because it had been found to show a small serine-like effect in promoting senescence (6), and proline as a result of an early preliminary survey. Table VI shows that serine is evidently the most potent synergist of those tested. The smaller effects of glycine and proline may or may not be significant. The effects of the other addenda are insignificant or negative. Whether the whole effect of serine can be explained by this type of synergism will require further study.

**DISCUSSION**

The conversion of serine to glucose in leaves has been known to be light-dependent since 1965–1966 (8, 10). There are two reasons for this. First, when serine is deaminated and dehydrogenated by serine deaminase, it yields pyruvate, which can then be converted to phosphoglycerate; this compound then enters the normal reductive photosynthetic cycle. The reaction requires ATP and is therefore greatly accelerated by light. But the second, more specific reason, is that at least four of the enzymes of the reductive photosynthetic cycle are activated by light (see the review of Buchanan [1]). Rates in the dark are only a small fraction of those in the light, so that even with a moderate supply of ATP from mitochondria the conversion of serine to glucose would be extremely slow.

Oxidation of serine to CO₂, however, can occur readily in either dark or light. Since the pyruvate formed (as above) yields acetyl-CoA, it can enter directly into the oxidative Krebs cycle. Two recent studies exemplify these reactions in leaves. With rey
leaves that have formed L-[^14]C serine from ^14CO2 in light, their transfer to darkness causes the radioactivity of serine inter alia to decrease rapidly, showing that the serine and other labeled amino acids are rapidly metabolized (9). Vallisneria leaves that were fed[^14]C serine in the light converted it more or less completely to sucrose and malate (3). Participation of a reversible reaction at some stage is shown by the fact that the oxidation of serine to CO2 is inhibited by high CO2 concentrations such as 1200 mmol/L (17). Serine of course can undergo other conversions too. In tomato roots, [^14]C serine is converted to labeled phosphatidyl-ethanolamine and phosphatidyl-choline (16).

The partial conversion of serine to reducing sugars in the absence of CO2, shown in Tables I and III, is in contradiction to the data of Waidyanathan et al. (15) on wheat leaves. In those experiments, serine was not appreciably metabolized in CO2-free air, 91% of it remaining unchanged. It may be that the carbohydrate or organic acids present in the oat leaves provided enough CO2 for some serine metabolism; it is notable that in the wheat leaves of those authors pyruvate or ^3-ketoglutarate would substitute for CO2 to allow serine metabolism. The conversion of serine to reducing sugars here is only one-third as much as in the presence of CO2.

The major problem resulting from the research reported here is how to explain the apparent synergistic effect of L-serine on proteolysis. The effects are exerted, though to differing extents, on both the major protease enzymes of the oat leaf. It is at least highly suggestive that the larger effect is on the acid protease, which appears to have serine at its active site (2). Hence, a possible interpretation would be that additional serine molecules become loosely attached to the protein, either increasing the activity of the enzyme, or perhaps interfering with the action of some associated inhibitors. Since these proteases were not maximally purified, the presence of inhibitors is not excluded; if indeed they are present this would make the phenomenon studied of considerable physiological importance. It must also be noted that [^14]C serine is indeed clearly incorporated into total protein, as was shown earlier (12), and this protein may well include enzymes that activate proteases, as in the well-known conversion of an apoenzyme into an active enzyme by splitting off a small part of the polypeptide molecule. A perhaps comparable, though biochemically quite different, system is the activation of protein enzymes by phosphorylation. In either case, the serine would not be incorporated into the protease itself, and the amount of serine entering into such modifying enzymes would be far less than that needed for incorporation into the total protease; thus, it could easily be below our detection limits.

These proposed mechanisms are of course highly speculative, but they are put forward to show that the observed protease activation without detectable incorporation of the serine can be explained in more than one way. Support for any one of them will depend on the preparation of a large enough quantity of the pure proteases for a detailed study of the enzyme, the mechanisms that control or modify their activity, and their relations to non-protein molecules in the ambient solution.

LITERATURE CITED