Protein Degradation in *Lemna* with Particular Reference to Ripulose Bisphosphate Carboxylase

I. THE EFFECT OF LIGHT AND DARK

Received for publication August 27, 1986 and in revised form December 18, 1986

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ABSTRACT

Ribulose bisphosphate carboxylase from *Lemna minor* resembles the structure reported for the enzyme from other plants. When grown in the light, the enzyme appears to undergo little or no degradation, as measured by a double-isotope method. This situation is similar to that reported for wheat and barley, but is unlike that reported for maize, where the enzyme degrades at the same rate as total protein. Prolonged periods of darkness usually induce leaf senescence, characterized by the rapid degradation of chlorophyll and protein, with ribulose bisphosphate carboxylase undergoing preferential degradation. In *L. minor* there is selective protein degradation in the dark, but chlorophyll and ribulose bisphosphate carboxylase are stable when fronds are kept in the darkness for up to 8 days. It appears that *Lemna* is not programmed to senesce, or at least that darkness does not induce senescence in *Lemna*. Although there is no evidence for *in vivo* degradation or modification of ribulose bisphosphate carboxylase during prolonged periods of darkness, extracts from fronds which have been kept in the dark for periods in excess of 24 hours convert ribulose bisphosphate carboxylase to a more acidic form. The properties of the dark-induced system which acts on ribulose bisphosphate carboxylase, suggest that it may be a mixed function oxidase. This proposition that the selectivity of protein degradation is genetically determined, so that the rate at which a protein is degraded is determined by its charge or size, was tested for fronds grown in the light or maintained in the dark. There was no significant correlation between protein degradation and either charge or size, in light or dark.

A great deal of evidence has been marshaled to support the view that the key enzyme of photosynthesis, RuBPCase (EC 4.1.1.39), undergoes little or no degradation prior to leaf senescence. The strongest evidence that RuBPCase does not degrade while it is being synthesized has been presented by Huffaker and his colleagues (19, 29). The same group has shown that, during the dark-induced senescence of detached leaves of barley, the initial loss of protein is almost entirely due to the degradation of RuBPCase (28). This behavior has also been observed in the dark-induced senescence of attached wheat leaves (37, 38). The stability of RuBPCase prior to senescence, and its rapid degradation during senescence, has led to its classification as a leaf storage protein (19, 21).

The pattern of protein degradation in maize leaves does not appear to follow that established for barley and wheat leaves.

1 Abbreviations: RuBPCase, ribulose bisphosphate carboxylase; FPLC, fast protein liquid chromatography; PMSF, phenylmethylsulfonyl fluoride.
number of experiments, designed to test these correlations in plants, produced results broadly consistent with the general hypothesis (1, 10). However, more recent experiments, employing different methods, have suggested that, if the correlations exist, they are at best weak (8, 9). We have used FPLC and PAGE to improve the separation of proteins and report their use in testing the correlation between the physical properties of proteins and their rates of degradation when *Lemna* is maintained in light, and also in darkness.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions.** *Lemna minor* L was grown autotrophically under sterile conditions at 25°C, under constant light (35) or in total darkness. The growth medium contained: 5 mM KNO₃, 2.5 mM KCl, 2 mM KH₂PO₄, 1 mM MgSO₄·7H₂O, 364 µM CaCl₂·2H₂O, 19 µM EDTA-Na₂, 23 µM H₂BO₃, 5 µM MnCl₂·4H₂O, 160 mM CuSO₄·5H₂O, 382 mM ZnSO₄·7H₂O, 206 mM Na₂MoO₄·2H₂O, 52 mM CoCl₂·6H₂O, 3 mM KI, 1 mM piperazine-N₂-bis[2-ethanesulfonic acid] (PIPES), and pH adjusted to 6.8.

**Chemicals.** Sodium [³H] bicarbonate was obtained from Amersham International, U.K., and l-[3,4,5-³H]leucine and l-[³H]leucine from New England Nuclear, U.K. D-Ribulose-1,5-bisphosphoric acid and mol wt markers for SDS-gel electrophoresis were supplied by Sigma, U.K., and Pico-Flour 15 and PicoFlour 30 by United Technologies Packard, U.K. The FPLC system, Mono Q HRS/5 column, Superose 12 HR10/30 column, and the PD-10 prepacked Sephadex G-25M columns were supplied by Pharmacia, Uppsala, Sweden. Other biochemicals and general laboratory chemicals were obtained from Boehringer, U.K., Sigma, U.K., and from BDH, U.K.

**Double-Isotope Labeling.** The relative rates of protein degradation were measured by a modification of the double-isotope technique of Arias et al. (2). The modification is similar to that described by Dice et al. (12) for pea-stem sections and can be used in either steady-state conditions or in circumstances where physiological changes are occurring (15). Data on protein degradation was obtained by labeling one batch of *Lemna* fronds with [³H]leucine, the other with [¹⁴C]leucine for the same period of time. The [³H]-labeled fronds were washed in unlabeled growth medium, then divided into four approximately equal samples and transferred to four flasks. The number of fronds in each flask was counted and the fronds were allowed to grow in unlabeled growth medium for varying periods of time. The [¹⁴C]-labeled fronds were harvested immediately after the labeling period and divided into four parts, in proportion to the number of [³H]-labeled fronds in each of the four flasks. The four samples were frozen in liquid N₂ and stored at −80°C until required. The samples of the [³H]-labeled fronds, after growth for varying periods of time, were mixed with the corresponding samples of [¹⁴C]-labeled fronds. Soluble protein was extracted, fractionated and counted for [¹⁴C] and [³H].

**Fractionation of Total Soluble Protein by Anion Exchange Chromatography.** A desalted extract was loaded into the Mono Q HRS/5 ion exchange column of the FPLC, previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The bound proteins were eluted with a gradient of NaCl (0–1 M). The flow rate was 2 ml/min and 1 ml fractions were collected.

**Fractionation of Total Soluble Protein by Gel Filtration.** A desalted extract was loaded into the Superose 12 HR10/30 gel filtration column of the FPLC, previously equilibrated with 100 mM Tris-HCl buffer (pH 7.5); the flow rate was 0.5 ml/min and 0.5 ml fractions were collected. The marker proteins used were: bovine thyroglobulin (mol wt = 669,000), horse spleen ferritin (mol wt = 440,000), rabbit muscle aldolase (mol wt = 158,000), horse heart malic dehydrogenase (mol wt = 70,000) and horse heart Cyt c (mol wt = 12,400). All of these proteins were detected by their λ at 280 nm.

**SDS-PAGE.** A discontinuous system (23) was used for SDS-PAGE. Electrophoresis was performed in a 10% or 12.5% (w/v) acrylamide slab gel, according to the method described by Blose and Feramisco (6). Samples were prepared for electrophoresis by boiling for 2 min in the presence of 1% SDS and 0.1 M DTT. The proteins were stained with Coomassie brilliant blue R. The marker proteins used were: bovine milk α-lactalbumin (mol wt = 14,200), soybean trypsin inhibitor (mol wt = 20,100), bovine pancreas trypsinogen (PMSF treated, mol wt = 24,000), bovine erythrocyte carbonic anhydrase (mol wt = 29,000), rabbit muscle glyceraldehyde-3-P dehydrogenase (subunit, mol wt = 36,000), egg albumin (mol wt = 45,000), bovine plasma albumin (mol wt = 66,000), rabbit muscle phosphorylase b (subunit, mol wt = 97,400), Escherichia coli β-galactosidase (subunit, mol wt = 116,000), and rabbit muscle myosin (subunit, mol wt = 205,000).

The radioactive protein bands were sliced and digested by the wet oxidation method (24). The effect of the presence of HClO₄/NaOH, acrylamide, and Coomassie brilliant blue R on the [¹⁴C] and [³H] counts was checked and found to be negligible.

**Assay of RuBPCase.** RuBPCase from *Lemna* fronds grown under light was assayed by a modification of the method described by Lorimer et al. (25). The enzyme was activated by incubation in 100 mM Tris-HCl buffer (pH 8.4), containing 10 mM MgCl₂, 10 mM NaHCO₃, and 5 mM DTT for 45 min at 35°C. The enzyme activity was measured in a 100 mM glycylglycine buffer (pH 8.0), containing 16 mM MgCl₂, 1.6 mM DTT, 16 mM NaHCO₃ (3.7 MBq/mmol), and 1.2 mM d-ribulose-1,5-bisphosphoric acid in 250 µl volume at 25°C. The reaction was stopped after 30 s by addition of 0.2 ml 2 N HCl. The contents of the vials were evaporated to dryness, dissolved in water, and counted.

RuBPCase from fronds kept in darkness was prepared for assay in a different way, to protect against the action of unspecific oxidases, that otherwise would have inactivated the enzyme during the preparation of the extract. *Lemna* fronds were extracted (2.5 ml/g fresh weight) in 100 mM Tris-HCl buffer (pH 8.1), containing 10 mM MgCl₂, 10 mM NaHCO₃, 10 mM DTT, 2 mM KCN, 2 mM EDTA, 1 mM PMSF, centrifuged for 15 min, at 10,000g, 1°C in a Micro-Haematocrit centrifuge (Hawksley, England), and left for about 1 h in ice to ensure the activation of the enzyme. The enzyme activity was then measured as described above.

**Measurement of Radioactivity in Samples.** Fractions from the anion exchange column were dissolved in Pico-Flour 30 scintillant, whereas fractions from the gel filtration column were dissolved in Pico-Flour 15. The reason for this choice is that, when Pico-Flour 30 is used, the counting of [³H] and [¹⁴C] is not affected by the presence of NaCl, whereas when Pico-Flour 15 was used, [³H] and [¹⁴C] counts were reduced by the presence of NaCl, with the effect on [³H] counts being greater than on [¹⁴C] counts. The digested protein bands from SDS-PAGE were dissolved in Pico-Flour.
Fluor 30–once again, Pico-Fluor 30 proved to be almost insensitive to the presence of HClO₄/H₂O₂, acrylamide, and Coomassie brilliant blue R, whereas with Pico-Fluor 15, the efficiency of ³H and ¹⁴C counting was greatly reduced by the presence of those compounds. All samples were counted for ¹⁴C and ³H in a quench calibrated PW4700 liquid scintillation counter (Philips, Holland), using a dual label program with automatic quench compensation.

To check the results obtained when measuring the ¹⁴C and ³H contents of double-labeled proteins by direct liquid scintillation counting, an experiment was set to compare this method with the direct oxidation of the samples, using a biological materials oxidizer (R. J. Harvey Instrument Corp., Hilldale, NJ). Three ¹⁴C and ³H-labeled protein solutions with different (¹⁴C/³H) ratios were counted by both methods. The three samples measured after oxidation gave ratios of 0.463, 1.034, and 3.406. The same samples measured by direct liquid scintillation counting gave ratios of 0.494, 1.025, and 3.405.

**Chl Determination.** Chl was extracted in 80% (v/v) acetone and determined according to Arnon (3).

**RESULTS**

**Identification of RuBPCase.** Total soluble protein from 1.2 g of *Lemna fronds* was loaded into the FPLC Mono Q ion exchange column and eluted with a gradient of NaCl. Fractions (1 ml) were collected and assayed for RuBPCase activity. Figure 1A shows that the main protein peak (as judged by A at 280 nm and catalytic activity) corresponds to RuBPCase. The peak emerging at fraction 41 is identified as nucleic acid on the basis of its absorption spectrum. In some experiments a small amount of RuBPCase activity could be detected before the main peak was eluted—presumably due to limited proteolysis of RuBPCase.

A sample of the main protein peak (identified as RuBPCase in Fig. 1A) was incubated with SDS (1% w/v) and DTT (25 mM) for 30 min at 37°C, then boiled for 2 min and, after cooling, was loaded into the FPLC Superose 12 gel filtration column, which had been previously equilibrated with Tris-HCl buffer (100 mM, pH 7.5) containing SDS (1% w/v). Elution of the column showed (Fig. 1B) a low mol wt peak corresponding to DTT and two protein peaks corresponding to the large and small subunits of RuBPCase. To determine their mol wt, samples of each of the protein peaks isolated by chromatography on the Superose 12 column, were run in a 12.5% (w/v) polyacrylamide SDS-gel. A sample of the main protein peak from the Mono Q ion exchange column (identified as RuBPCase, Fig. 1A) was also subjected to PAGE. The results shown in Figure 2, together with data from other experiments involving lower mol wt markers (see "Material and Methods") give the mol wt of the large subunit as 52 kD and that of the small subunit as 14 to 15 kD.

It was found that when 10% (w/v) acrylamide SDS-gels were used (results not shown), the small subunit of *Lemna RuBPCase* electrophoresed with the front. Similar behavior has also been observed with the enzyme from *Thiobacillusintermedius*, tobacco and rye (7). When 12.5% (w/v) acrylamide SDS-gels were used we were able to resolve the small subunit of RuBPase into a single electrophoretic species (Fig. 2).

The RuBPCase isolated by ion exchange FPLC contains a number of impurities which can be seen in Figure 2 (lane 3). The main contaminant has a molecular mass of about 42 kD.
and was still present as a contaminant after the large subunit had been purified by gel filtration (Fig. 2, lane 4). The small subunit of RuBPCase, purified by gel filtration, also contained an impurity which coincides with the large subunit and may represent the trailing edge of the large subunit peak, emerging from the gel filtration column.

On the basis of the L₄S₈ structure of higher plant RuBPCase, the molecular mass is estimated to be 532 kD. In separate experiments, the molecular mass of the native RuBPCase was estimated by chromatography on a Superose 12 column as about 500 kD (Fig. 4).

Degradation of the Soluble Proteins of Lemna Grown in Continuous Light. Two batches of Lemna fronds were grown in complete medium (750 ml) containing either 1.48 MBq of L-[3,4,5-³H]leucine (5.44 TBq/mmol) or 0.56 MBq of L-[1-¹⁴C]leucine (2 GBq/mmol) for 25.5 h. During that time 78% of the L-[3,4,5-³H]leucine and 94% of the L-[1-¹⁴C]leucine were taken up by the fronds, as judged by measuring the loss of radioactivity from the solutions. After the labeling period, the ³H-labeled fronds were washed in unlabeled medium and transferred, as approximately equal samples, to four flasks containing complete medium. The number of fronds in each flask was counted and the fronds were allowed to grow for 0, 3, 6, or 9 d before being harvested and combined with the corresponding samples of ¹⁴C-labeled fronds as described in the Methods section. Protein was extracted and fractionated by FPLC using the Mono Q ion exchange column and also with the Superose 12 gel filtration column.

The design of this experiment is such that a comparison of the (¹⁴C/³H) ratios of the isolated protein fractions provides a measure of the relative rates of in vivo degradation—a high (¹⁴C/³H) ratio is indicative of a relatively rapid rate of protein breakdown.

Degradation of Lemna Proteins in Relation to their Charge. Protein samples, for each time period, were fractionated by FPLC on the Mono Q column and ³H and ¹⁴C determined in each fraction (Fig. 3). The basic proteins are not retained by the column and the more acidic proteins are eluted as the concentration of NaCl is increased. The data presented in Figure 3A constitutes a control, giving the noise level in the experiment. With increasing periods of time, a trough in the (¹⁴C/³H) ratio appears and increases (Fig. 3, A–D). It should be noted that this trough, corresponding to the position of RuBPCase, is produced by increases in the (¹⁴C/³H) ratio of those proteins which are either more acidic or more basic than RuBPCase. The (¹⁴C/³H) ratio corresponding to the peak of RuBPCase does not change throughout the experiment, suggesting that there is no degradation of RuBPCase. On the other hand, the (¹⁴C/³H) ratio increases for other proteins, particularly for one or more proteins emerging from the Mono Q column at the beginning of the NaCl gradient (fraction 12). However, there is no clear correlation between protein charge and the (¹⁴C/³H) ratio which is an index of protein degradation.

Degradation of Lemna Proteins in Relation to Their Molecular Weight. Protein samples for each of the 4 times, were fractionated by FPLC on the Superose 12 column and ³H and ¹⁴C determined in each fraction (Fig. 4). The “noise level” in this experiment is established by the control (Fig. 4A). With increasing time, the increase in the (¹⁴C/³H) ratio observed for many of the fractions indicates protein degradation, although this degradation does not appear to be correlated with the size of the proteins. The trough in (¹⁴C/³H) ratio which appears to increase with time (Fig. 4, A–D), corresponds to a mol wt of about 500 kD which is tentatively identified as RuBPCase.

Turnover of the RuBPCase Subunits. Samples from the RuBPCase peak separated on the Mono Q column for each time period, were taken and run in SDS-PAGE (12.5% w/v acrylamide). The protein bands corresponding to the subunits of Ru

![Figure 3](https://plantphysiol.org/figure/3.png)

**Fig. 3.** Relative degradation rates of *Lemna* total soluble proteins, fractionated by anion exchange chromatography, after 0, 3, 6, and 9 d under continuous light. Double-labeled soluble proteins from *Lemna* were isolated, loaded into the FPLC Mono Q column equilibrated with 20 mM Tris-HCl buffer (pH 7.5) and eluted with a gradient of NaCl. 1 ml fractions were collected. A high ¹⁴C/³H ratio indicates a high rate of degradation. A, B, C, and D. Degradation of soluble proteins after 0, 3, 6, and 9 d, respectively. E, ³H and ¹⁴C dpm per fraction, corresponding to degradation after 9 d, and shape of the NaCl gradient used. Note break in ordinate. O, ¹⁴C/³H ratio; Δ, ³H dpm; □, ¹⁴C dpm. Arrows: 1, RuBPCase; 2, nucleic acids, as judged by the A₂₆₀/A₂₃₀ ratio, binding properties to the anion exchanger and level of incorporation of label. Kendall's rank correlation coefficients were: -0.09 for d 3, -0.18 for d 6, and -0.25 for d 9. The small shifts in the RuBPCase peaks are due to slight variability between FPLC runs, which particularly affect the salt concentrations at which proteins emerge.
PROTEIN TURNOVER IN LIGHT AND DARK

BPCase were sliced, digested and counted. The ($^{14}$C/$^3$H) ratios for each of the RuBPCase subunits appear to decrease with time (Table I). This result suggests that RuBPCase does not degrade when *Lemna* fronds are kept in continuous light. The decrease in the ($^{14}$C/$^3$H) ratio can be explained by the incorporation into RuBPCase of $^3$H-labeled amino acids released by the degradation of other proteins. This recycling of amino acids with reincorporation into RuBPCase is further illustrated in Figure 5 for the subunits and the native enzyme.

Degradation of the Soluble Proteins of *Lemna* Kept in Darkness. Before starting the double-labeling experiments, the effect of darkness on the level of Chl and the activity of RuBPCase was determined over 6 d. The results presented in Figure 6 show that there was surprisingly little loss of Chl and after an initial fall there was no further decrease in RuBPCase activity. We are not sure whether or not the initial fall in RuBPCase activity was real or artifactual due to the necessity to limit the conditions employed to activate RuBPCase and so avoid the action of the oxidases induced by prolonged darkness.

Degradation of *Lemna* Proteins in Relation to Their Charge. Two batches of *Lemna* fronds were grown in complete medium (750 ml) containing either 1.44 MBq of L-[3,4,5-3H]leucine (5.44 TBq/mmol) or 0.56 MBq of L-[1-14C]leucine (2 GBq/mmol) for 27 h. During that time 80% of the L-[3,4,5-3H]leucine and 92% of the L-[1-14C]leucine were taken up by the fronds. After the labeling period, the fronds were washed and divided into four parts as described in the previous section. The $^3$H-labeled fronds were kept in total darkness for 0, 4, 6, and 8 d before being harvested, and combined with the appropriate number of $^{14}$C-labeled fronds. Protein was extracted and fractionated by FPLC using the Mono Q column. As in the previous light experiment, a high ($^{14}$C/$^3$H) ratio is indicative of a relatively rapid rate of protein degradation.

The results of this experiment are shown in Figure 7. The pattern of protein degradation in darkness is significantly different from that observed in the light. For example, protein or proteins which emerge from the Mono Q column at the beginning of the NaCl gradient are degraded more rapidly in light than in darkness, whereas the opposite behavior is found in the protein(s) which emerge from the column just before the RuBPCase peak. However, there was no clear correlation between protein charge and degradation in the dark, as previously noted for continuous light. A trough in the ($^{14}$C/$^3$H) ratio corresponding to the RuBPCase peak suggests that this protein is degraded less rapidly than some of the proteins which are more acidic or more

**Table I. Relative Degradation Rates of Both Subunits of Light Grown *Lemna* RuBPCase, Separated by SDS-PAGE**

<table>
<thead>
<tr>
<th>Time</th>
<th>RuBPCase small subunit</th>
<th>RuBPCase large subunit</th>
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<tbody>
<tr>
<td>d</td>
<td>0.41</td>
<td>0.42</td>
</tr>
<tr>
<td>0</td>
<td>0.33</td>
<td>0.41</td>
</tr>
<tr>
<td>3</td>
<td>0.30</td>
<td>0.33</td>
</tr>
<tr>
<td>6</td>
<td>0.32</td>
<td>0.33</td>
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Samples of double-labeled RuBPCase, partially purified by ion exchange chromatography (Fig. 3), were boiled for 2 min in the presence of SDS and DTT and subjected to electrophoresis in a 12.5% (w/v) acrylamide SDS-gel. The proteins were stained with Coomassie brilliant blue R and the bands corresponding to the large and small subunits of RuBPCase were sliced and digested as described in "Materials and Methods." The ($^{14}$C/$^3$H) ratio correspondent to each band was calculated by liquid scintillation counting. A high ($^{14}$C/$^3$H) ratio indicates a high rate of degradation.

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**Fig. 4.** Relative degradation rates of *Lemna* total soluble proteins, fractionated by gel filtration, after 0, 3, 6, and 9 d under continuous light. Double-labeled soluble proteins from *Lemna* were isolated and loaded into the FPLC Superose 12 column equilibrated with 100 mM Tris-HCl buffer (pH 7.5); 0.5 ml fractions were collected. A high ($^{14}$C/$^3$H) ratio indicates a high rate of degradation. A, B, C, D, Degradation of soluble proteins after 0, 3, 6, and 9 days, respectively. E, $^3$H and $^{14}$C dpm per fraction, corresponding to degradation after 9 d, D, O, $^{14}$C/$^3$H ratio; $^3$H dpm; $^3$C dpm. Arrows: 1, nucleic acids, as judged by the absorption spectrum, mol wt and level of incorporation of label. 2, RuBPCase as judged by mol wt about 500,000 and catalytic activity, but certainly contaminated with other proteins of similar mol wt. Mol wt markers: a, thyroglobulin, mol wt = 669,000; b, ferritin, mol wt = 440,000; c, aldolase, mol wt = 158,000; d, malic dehydrogenase, mol wt = 70,000; e, Cyt c, mol wt = 12,400. Kendall's rank correlation coefficients were: 0.21 for d 3, 0.25 for d 6, and 0.26 for d 9.
grown under continuous light A, O, Relative rate of degradation of RuBPCase, partially purified by ion exchange chromatography (Fig. 3); O, relative rate of degradation of the soluble protein fraction (no. 12) fractionated by ion exchange chromatography, which seemed to be the one with the fastest degradation. B, O, Relative rate of degradation of RuBPCase, partially purified by gel filtration (Fig. 4). C, Δ and □, relative rates of degradation of RuBPCase large and small subunits respectively, partially purified by ion exchange chromatography and then separated by SDS-PAGE. A high \((^{14}\text{C}/^{3}\text{H})\) ratio indicates a high rate of degradation. For each case, the straight line was obtained by linear regression, using the least squares method. Kendall's rank correlation coefficients were: 0.02 for d 4, 0.31 for d 6, and 0.14 for d 8.

FIG. 5. Relative rate of degradation of RuBPCase from \(L.\ minor\) grown under continuous light. A, O, Relative rate of degradation of RuBPCase, partially purified by ion exchange chromatography (Fig. 3); O, relative rate of degradation of the soluble protein fraction (no. 12) fractionated by ion exchange chromatography, which seemed to be the one with the fastest degradation. B, O, Relative rate of degradation of RuBPCase, partially purified by gel filtration (Fig. 4). C, Δ and □, relative rates of degradation of RuBPCase large and small subunits respectively, partially purified by ion exchange chromatography and then separated by SDS-PAGE. A high \((^{14}\text{C}/^{3}\text{H})\) ratio indicates a high rate of degradation. For each case, the straight line was obtained by linear regression, using the least squares method. Kendall's rank correlation coefficients were: 0.02 for d 4, 0.31 for d 6, and 0.14 for d 8.

Fig. 6. Changes in Chl and RuBPCase activity in \(L.\ minor\) kept in continuous darkness. \(Lemna\) fronds were kept in total darkness for various periods of time. At intervals, Chl and RuBPCase activity were determined as described in “Materials and Methods.” O, Chl; 100% corresponds to 694 \(\mu\text{g}\) Chl-g\(^{-1}\text{fresh wt.}\). Δ, RuBPCase activity; 100% corresponds to 1.27 \(\mu\text{mol} \text{CO}_2\text{fixed-min}^{-1}\text{-g}^{-1}\text{fresh wt.}\).

Fig. 7. Relative degradation rates of \(Lemna\) total soluble proteins, fractionated by anion exchange chromatography, after 0, 4, 6, and 8 d in the dark. Double-labeled soluble protein corresponding to a mixture of light \((^{14}\text{C}) + \text{dark (}^{3}\text{H})\) grown fronds were isolated, loaded into the FPLC Mono Q column equilibrated with 20 mM Tris-HCl buffer (pH 7.5) and eluted with 40 mM NaCl. 1 ml fractions were collected. A high \((^{14}\text{C}/^{3}\text{H})\) ratio indicates a high rate of degradation. A, B, C, and D, Degradation of soluble proteins after 0, 4, 6, and 8 d in darkness, respectively. E, \(^{3}\text{H}\) and \(^{14}\text{C}\) dpm per fraction, corresponding to degradation after 8 d in the dark. D, and shape of the NaCl gradient used. Note break in ordinate. O, \((^{14}\text{C}/^{3}\text{H})\) ratio; Δ, \(^{3}\text{H}\) dpm; □, \(^{14}\text{C}\) dpm. Arrows: 1, RuBPCase; 2 nucleic acids.
**DISCUSSION**

**Methodology.** The experiments described in this paper yield values for protein degradation in terms of the ratio $^{14}$C in protein sample immediately after labeling/$^{3}$H remaining in protein sample after labeling and chasing in cold medium for time $t$. The experiments have been designed so that a high value for the ratio $^{14}$C/$^{3}$H indicates rapid protein degradation. The general theoretical basis for transforming this ratio into a rate constant of degradation ($K_D$) or a half-life ($t_{0.5}$) was outlined in the introduction. To accommodate the particular protocol used in this work, $K_D$ can be calculated from the equation derived by Wilde et al. (36)

$$K_D = \ln \left( ^{14}C/^{3}H \right)_{\text{test}} - \ln \left( ^{14}C/^{3}H \right)_{\text{control}}$$

where $\left( ^{14}C/^{3}H \right)_{\text{test}}$ is the isotopic ratio after a chase period of time $t$, and $\left( ^{14}C/^{3}H \right)_{\text{control}}$ is the isotopic ratio at the end of the labeling period.

We have presented values for protein degradation as the direct ratio $^{14}$C/$^{3}$H rather than as $K_D$ or $t_{0.5}$, because these logarithmic transforms are familiar terms, with an implied but spurious precision. Thus the assumption of a linear relationship between $^{14}$C/$^{3}$H and $t_{0.5}$ and $K_D$ is not valid if amino acid recycling occurs (39), and we have shown (11) that 50% of the leucine released during protein degradation undergoes recycling. Furthermore, growth which occurs during the chase period further distorts the relationship. Thus if a protein is synthesized but not degraded, then the radioactivity ($^{3}$H) in that protein will increase during the chase, as $^{3}$H-amino acids released from other proteins are incorporated into that protein. Thus $\left( ^{14}C/^{3}H \right)_{\text{test}}$ will be less than $\left( ^{14}C/^{3}H \right)_{\text{control}}$, and $K_D$ will be negative.

**Protein Turnover in Continuous Light.** The double-labeling experiments show that in the chase period, the values of $\left( ^{14}C/^{3}H \right)$ vary from protein to protein, indicating that proteins turnover at different rates, generating a characteristic pattern of protein degradation. A protein fraction eluting at the beginning of the salt gradient (Fig. 3) appears to turnover particularly rapidly. Light stimulates the degradation of a 32 kD thylakoid protein which is the apoprotein of the secondary electron transport carrier on the reducing side of PSII (18, 22). Unfortunately the techniques used in this work do not provide any identification of proteins beyond their charge and size.

The dominant feature of the pattern of protein degradation is the trough associated with RuBPCase. In general a trough will be generated by adjacent protein fractions degrading faster than RuBPCase so that the $\left( ^{14}C/^{3}H \right)$ values of the proteins on either side of RuBPCase are raised above $\left( ^{14}C/^{3}H \right)_{\text{control}}$. However, the experiments show that $\left( ^{14}C/^{3}H \right)_{\text{test}}$ for RuBPCase is less than $\left( ^{14}C/^{3}H \right)_{\text{control}}$. This implies that RuBPCase is not degraded and furthermore $^{3}$H-labeled amino acids released from other proteins undergoing degradation are incorporated into RuBPCase during the chase period.

The structure of RuBPCase isolated from *Lemna* appears essentially the same as that reported for the enzyme isolated from other leaves (27). The lack of degradation of RuBPCase noted for *Lemna* has previously been reported in other C-3 species (19), whereas in maize which happens to be a C-4 plant, RuBPCase appears to turnover in the light (31). The possibility that RuBPCase turnover is fundamentally different in C-3 and C-4 plants deserves further experimentation. However, it should be noted that there is evidence that in field grown wheat Ru-
BPCase undergoes a diurnal cycle of protein turnover (26), although the possibility that this is due to fluctuations in water stress cannot be ruled out.

The lack of turnover of RuBPCase means that the half-life of soluble proteins, other than RuBPCase, in *Lemma* must be about 3 d, compared to the average half-life of about 7 d reported for the total soluble proteins of *Lemma* growing under identical conditions (20).

**Protein Turnover in Continuous Dark.** The (14C/13C) ratio for RuBPCase is almost constant over 9 d of darkness, whereas some of the proteins—particularly those eluting from the Mono-Q column, just before RuBPCase are, as judged by the (14C/13C) ratio, degraded much more rapidly. This stability of RuBPCase in *Lemma* which is manifest as a trough in the pattern of degradation (Fig. 7), stands in marked contrast to the rapid degradation of RuBPCase which is usually associated with dark-induced senescence (28). Chl, whose degradation is usually associated with dark-induced senescence, is also stable in *Lemma* for at least 9 d of darkness. Furthermore, the marked increase in activity of glutamate dehydrogenase which is associated with dark-induced senescence in other leaves (33) does not occur in *Lemma* (data not presented). We therefore conclude, that *Lemma* is not programmed to senesce, or at least that darkness does not induce senescence in *Lemma*.

One other feature concerning the stability of RuBPCase in darkness should be noted. When light grown *Lemma* fronds are extracted and the protein fractionated by ion exchange FPLC, RuBPCase appears as a single distinct peak. However, when fronds which have been maintained in darkness for 72 h are extracted and fractionated by FPLC (Fig. 9) the characteristic peak of RuBPCase is absent and a number of smaller peaks of protein emerge from the Mono-Q column at higher salt concentrations, suggesting that they are oxidized derivatives of RuBPCase. This shift in the position of the RuBPCase peak is inhibited by extracting the *Lemma* fronds in the presence of KCN (2 mM) and EDTA (2 mM). It thus appears likely that darkness induces a mixed function oxidase, which in extracts of *Lemma* can oxidize and inactivate RuBPCase. However, in *vivo*, the dark-induced oxidase does not attack RuBPCase—presumably due to compartmentation. The presence of the oxidase in extracts of dark grown fronds complicates the assay of RuBPCase, which requires preincubation of extracts with 10 mM MgCl2, 10 mM NaHCO3, and 5 mM DTT for 45 min at 35°C. Since these conditions allow the dark-induced oxidase to oxidize RuBPCase, we have compromised by incubating the extracts of dark grown *Lemma* for 1 h in ice, and by including 2 mM KCN, 2 mM EDTA and 1 mM PMSF in the incubation mixture. The apparent fall and rise of RuBPCase activity over the 9 d period of darkness is, we believe, due to the induction of a mixed function oxidase during the first 48 h of darkness and its subsequent decline in activity during prolonged darkness (Fig. 6).

**Correlation of Protein Degradation with the Physical Properties of Proteins.** The pattern of protein degradation in light and dark shows considerable heterogeneity in the rates at which individual proteins degrade. A number of studies (reviewed by Goldberg and St. John [17]) have suggested that the half-lives of proteins are determined by the physical properties of the proteins rather than by the specificity of the proteolytic system. Most of the evidence supporting this general hypothesis has been obtained as correlates between *in vivo* rates of degradation, determined by the double-isotope method, and the physical properties of the proteins such as charge or size. The criterion on which a correlation has been claimed has usually been visual inspection of the data. It is difficult to define an adequate statistical treatment. A theoretical basis for the proposed linearity of the correlation between degradation and size can easily be advanced, but it is difficult to provide a basis for linearity in the correlation with charge. To deal with these uncertainties and with the theoretical and technical difficulties associated with the double-isotope method, we have analyzed our data by means of Kendall’s rank correlation coefficient. The value of this coefficient (given in the legends to Figs. 3, 4, and 5) is in each case very low, suggesting the absence of a correlation between protein degradation and either charge or size. A similar lack of correlation has been reported for *Lemma* proteins, when the proteins were separated on the basis of their age rather than their charge or size (8).

This lack of correlation contrasts with reports indicating a reasonably good correlation (10, 13, 14). One possible explanation of the discrepancy lies in the accuracy of determining the (14C/13C) ratio. Thus in the experiments with pea epicotyls reported by Dice et al. (12), the incorporation of 14C and 13C into protein was two to three orders of magnitude lower than reported in the present experiments. Artifacts cannot always be excluded, for example, when we examined the relationship between charge and rate of degradation using Pico-Fluor 15, we observed a good correlation between the (14C/13C) ratio and the charge of the protein. Further investigations have established the artificial nature of this result which was due to the effect of the NaCl in the column eluate on the efficiency of counting 3H when Pico-Fluor 15 was the scintillant.

We do not take the lack of correlation between a single property of proteins and the rate of degradation as arguing against the proposition that protein degradation is genetically determined. Indeed if a number of factors, such as charge, size and the recently proposed N-end rule (4) contribute to the overall rate of protein degradation, a strong correlation between degradation and a single property is unlikely.

**LITERATURE CITED**


34. Thomas L, JL Stoddart 1975 Separation of chlorophyll degradation from other senescence processes in leaves of a mutant genotype of meadow fescue (Festuca pratensis). Plant Physiol 56: 438-441