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

II. THE EFFECT OF NUTRIENT STARVATION

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ABSTRACT

The concept of ribulose bisphosphate carboxylase as a storage protein is not supported in the case of *Lemna minor*, where the enzyme appears to be particularly stable under conditions of nitrogen starvation. Total nutrient starvation in light and in the dark induced the degradation of this enzyme, but not at an enhanced rate compared with other leaf proteins and, surprisingly, darkness inhibited the degradation of chlorophyll which occurs with total nutrient starvation in the light. The data suggest that *Lemna* is not programmed to senesce in response to nutrient starvation. Differences in the pattern of protein degradation, which occurred under the stress conditions employed, are not consistent with a simple model of protein degradation in which the degradative system is assumed to be located in the vacuole. The data is best explained by a dual system in which cytosolic proteins are degraded by a vacuolar/lysosomal system and chloroplast proteins are degraded within the chloroplast. Whatever the system of degradation, our data do not support the proposed correlation between the rate of protein degradation and either protein charge or size.

There is abundant evidence that protein degradation in plants is enhanced by a number of stresses (8, 25). In animal cells, inhibitors such as puromycin and cycloheximide inhibit stress-stimulated protein degradation, but there is little effect on the basal level of degradation, suggesting that dual protein catabolic pathways are involved (12, 14). In plants, cycloheximide inhibits both normal and stress-enhanced protein degradation, making it unnecessary to postulate dual pathways. We have therefore proposed a simple model for stress induced degradation of cytoplasmic proteins in which control is located in the tonoplast mediated by the action of hormones (9). The model is an extension of the Matile (23) hypothesis which ascribes a lysosomal role to the vacuole and for which strong experimental evidence has recently been provided by Boudet's group (4, 5).

The model predicts that the pattern of cytoplasmic protein degradation should be qualitatively independent of the nature of the stress. The model can be extended to the degradation of proteins in organelles, provided that the organelles are degraded within the vacuole. Indirect evidence supporting this is that the two major endoproteinases which constitute over 99% of the total endoproteolytic activity of barley leaves (24) are located exclusively in the vacuole (29). Direct evidence comes from the observation that in isolated protoplasts from senescing leaf cells, chloroplasts appeared to move into invaginations of the vacuole or to be taken up into the vacuole (33). Veenhuis *et al.* (31) have obtained evidence that peroxisomes of methanol grown *Hansenula polymorpha* are degraded within the autophagic vacuole following transfer to a methanol free growth medium. Evidence against the view that chloroplasts are degraded within the vacuole is the demonstration that RuBPCase[1] protein degrades faster than the chloroplast numbers decrease in senescing leaves of wheat (20) and barley (22). Furthermore, there is now good evidence that chloroplasts contain proteases that can degrade their own protein constituents (10, 27) and, more recently, ATP stimulated proteolytic activity has been demonstrated in chloroplasts (19, 21, 30).

The work described in this paper was undertaken to test the Matile (23) lysosomal hypothesis of protein degradation as modified by Cooke *et al.* (9) to account for protein degradation under stress. We have also paid special attention to the degradation of RuBPCase because in senescing leaves the degradation of this protein is particularly rapid (26, 32).

MATERIALS AND METHODS

Plant Material and Growth Conditions. *Lemna minor* L. grown autotrophically at 25°C under continuous light, in a complete sterile culture medium as previously described (15), was used as the source of plant material in all the experiments. Where appropriate, the growth conditions were modified to provide the following stress conditions: (a) total nutrient starvation, in which the *Lemna* fronds were grown in H_2O (deionized H_2O filtered through the Milli-Q reagent grade water system, Millipore, U.K.); (b) nitrogen starvation, in which the fronds were grown in complete medium lacking nitrogen; and (c) starvation in the dark, in which the fronds were grown in deionized and filtered H_2O in total darkness.

Chemicals. L-[3,4,5-^{3}H]Leucine and L-[1-^{14}C]leucine were obtained from New England Nuclear, U.K., and Pico-Fluor 15 and Pico-Fluor 30 from United Technologies Packard, U.K. The FPLC system, Mono Q HR5/5 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.* (1). The modification is similar to that described by Dice *et al.* (11) for pea-stem sections and can be used in either steady-state conditions or in circumstances where physiological changes are occurring (13). The protocols used in our double-labeling experiments were essentially the same as described previously (15). The control fronds were labeled with

[1] Abbreviations: RuBPCase: ribulose bisphosphate carboxylase; FPLC, fast protein liquid chromatography.

L-[1-^{14}C]leucine (2 GBq/mmol) and the stressed fronds were labeled with L-[3,4,5-^{3}H]leucine (5.44 TBq/mmol). Consequently, a high (^{14}C/^{3}H) ratio in a protein fraction is indicative of rapid degradation associated with that fraction.

Extraction of Total Soluble Protein. The extraction of *Lemna* total soluble protein was performed as reported previously (15) by mixing the suitable pair of ^{14}C- and ^{3}H-labeled fronds, except that for the homogenization of fronds stressed in H_2O in the dark, the extraction medium used was 100 mM-Tris-HCl buffer (pH 7.5), containing 1 mM phenylmethylsulfonyl fluoride, 2 mM KCN, and 2 mM EDTA, to protect against the action of unspecific oxidases (15).

Fractionation of Total Soluble Protein, SDS-PAGE, and Measurement of Radioactivity in Samples. Total soluble proteins from *Lemna* were fractionated in the FPLC Mono Q ion exchange column, in the FPLC Superose 12 gel filtration column or by SDS-PAGE, as previously reported (15). The peaks corresponding to RuBPCase and nucleic acids separated by FPLC and also the subunits of RuBPCase separated by SDS-PAGE were identified as in the previous paper (15). It should be noted that there is some variability in the point at which the RuBPCase is eluted from the Mono Q column. Polyacrylamide gel electrophoresis and the measurement of radioactivity in samples were performed as described by Ferreira and Davies (15).

Chl Determination. Chl was extracted in 80% (v/v) acetone and determined according to Arnon (2).

RESULTS

Chl Degradation. The effect of various growth and stress conditions on the amount of Chl present in *Lemna* fronds was measured over several days (Fig. 1). Chl *a* and *b* were also determined separately, but since the ratio was constant the data are not reported here.

Effect of Nitrogen Starvation on Protein Degradation. Two batches of *Lemna* fronds were grown in complete medium (400 ml) containing either 1.47 MBq of L-[3,4,5-^{3}H]leucine or 0.47 MBq of L-[1-^{14}C]leucine for 25.5 h. During that time, 73% of the L-[3,4,5-^{3}H]leucine and 81% of the L-[1-^{14}C]leucine were taken up by the fronds, as judged by measuring the loss of radioactivity from the solutions. The ^{3}H-labeled fronds were transferred to three flasks containing growth medium lacking nitrogen. At zero time, after 5 d and after 10 d, the fronds were removed and combined with fronds that had been labeled with L-[1-^{14}C]leucine, as described in the previous paper (15). Protein was extracted and fractionated by FPLC.

Degradation of Protein in Relation to Charge. Protein from each of the samples obtained over a 10 d period was fractionated on the Mono Q column and the (^{14}C/^{3}H) ratio determined for each fraction. The results, presented in Figure 2, show that as judged by changes in the (^{14}C/^{3}H) ratio there was little or no degradation in the protein fractions corresponding to RuBPCase. Furthermore, there was no correlation between the rate of protein degradation of other soluble proteins and their charge.

Degradation of Protein in Relation to Molecular Weight. Pro-

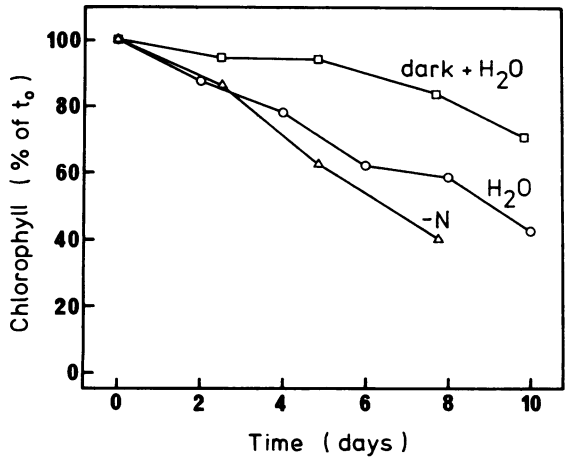

FIG. 1. Changes in Chl in *L. minor* stressed in water (total nutrient starvation), in complete medium lacking nitrogen (nitrogen starvation), and in water in the dark. *Lemna* fronds were stressed in water (○), in complete medium lacking nitrogen (△), and in water in the dark (□) for various periods of time. At intervals, Chl was determined as described in "Materials and Methods" (100% corresponds to 664 μg Chl/g fresh wt).

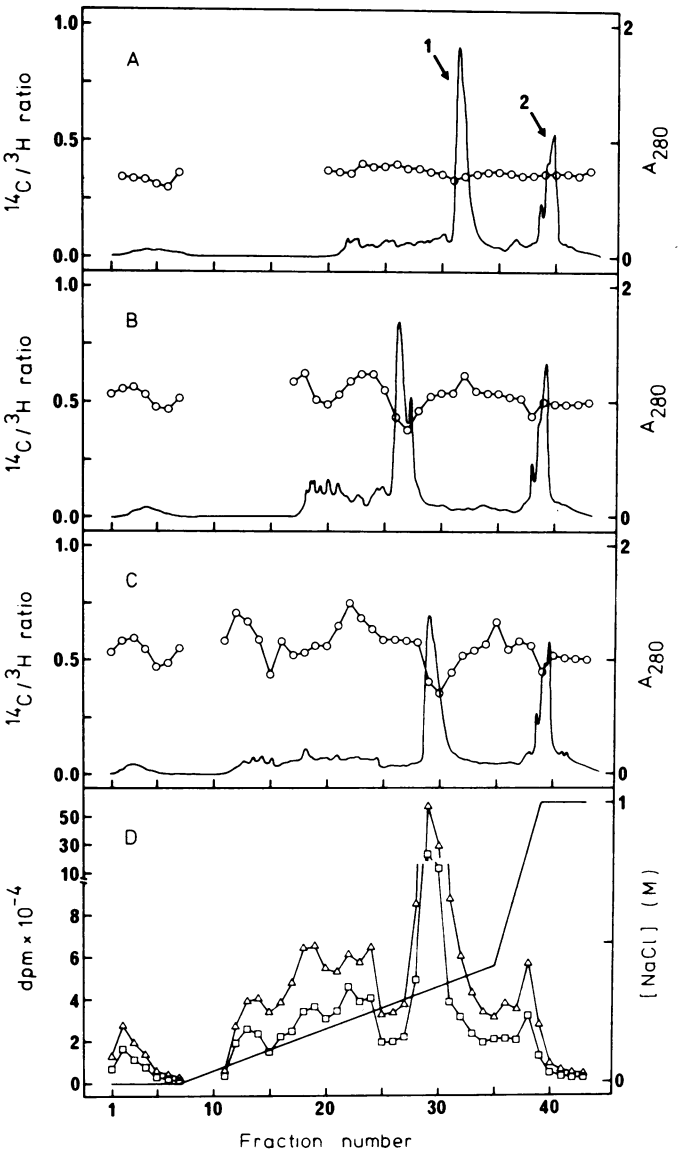

FIG. 2. Relative degradation rates of *Lemna* total soluble proteins fractionated by anion exchange chromatography, after 0, 5, and 10 d in complete medium lacking nitrogen (nitrogen starvation). Double labeled soluble proteins from *Lemna* were isolated, loaded into the FPLC Mono Q column equilibrated with 20 mM-Tris/HCl buffer (pH 7.5), and eluted with a gradient of NaCl; 1-ml fractions were collected. A high (^{14}C/^{3}H) ratio indicates a high rate of degradation. A, B, and C, Degradation of soluble proteins after 0, 5, and 10 d in complete medium lacking nitrogen, respectively; D, ^{3}H and ^{14}C dpm per fraction, corresponding to degradation after 10 d (C), and shape of the NaCl gradient used. Note break in ordinate. ○, (^{14}C/^{3}H) ratio; △, ^{3}H dpm; □, ^{14}C dpm. Arrows: 1, RuBPCase; 2, nucleic acids.

tein from each of the three samples was separated on the Superose column and the relationship between protein degradation and mol wt is shown in Figure 3. There appears to be no correlation between general protein degradation and mol wt and the protein fractions corresponding to RuBPCase appear to undergo little or no degradation.

Effect of Nutrient Starvation on Protein Degradation. Two batches of *Lemna* fronds were grown in complete medium (400 ml) containing either 1.48 MBq of L-[3,4,5-^{3}H]leucine or 0.56 MBq of L-[1-^{14}C]leucine for 25.5 h. During that time, 81% of the L-[3,4,5-^{3}H]leucine and 97% of the L-[1-^{14}C]leucine were taken up by the fronds, as judged by measuring the loss of radioactivity from the solutions. The ^{3}H-labeled fronds were transferred to four flasks containing water. The samples were collected after 0, 3, 6, and 9 d and combined with ^{14}C-labeled fronds, as described previously (15). Protein was extracted and fractionated by FPLC.

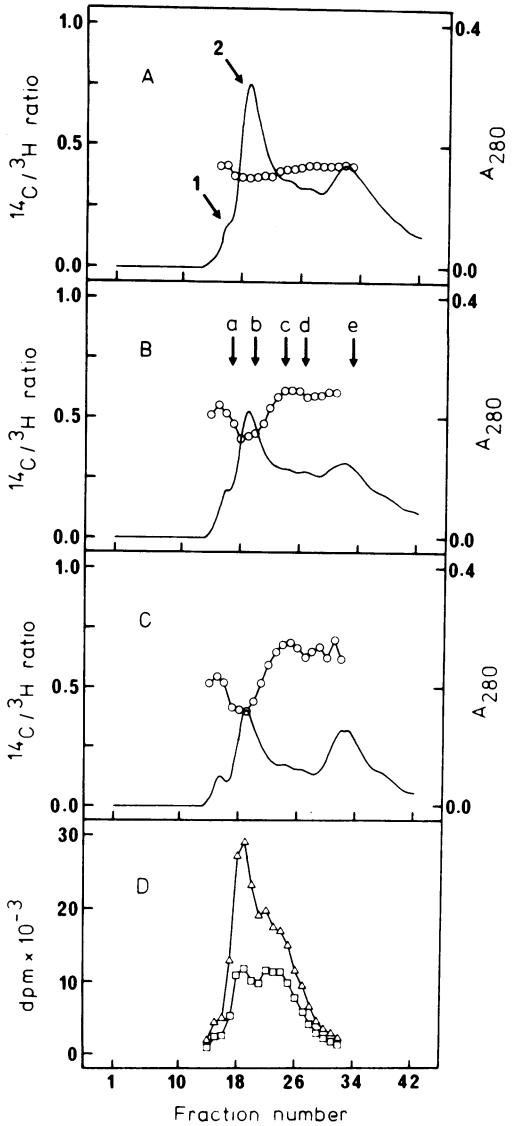

FIG. 3. Relative degradation rates of *Lemna* total soluble proteins fractionated by gel filtration, after 0, 5, and 10 d in complete medium lacking nitrogen (nitrogen starvation). Double labeled soluble proteins from *Lemna* were isolated and loaded into the FPLC Superose 12 column equilibrated with 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, and C, Degradation of soluble proteins after 0, 5, and 10 d in complete medium lacking nitrogen, respectively. D, ^{3}H and ^{14}C dpm per fraction, corresponding to degradation after 10 d (C). O, (^{14}C/^{3}H) ratio; Δ, ^{3}H dpm; □, ^{14}C dpm. Arrows: 1, nucleic acids; 2, RuBPCase. Mol wt markers as in Figure 5.

Degradation of Protein in Relation to Charge. Protein from each of the four samples obtained over a 9 d period was fractionated on the Mono Q column and the (^{14}C/^{3}H) ratio determined for each fraction. Figure 4 shows that under these conditions there is clear evidence that RuBPCase undergoes degradation. However, there appears to be no linear correlation between protein degradation and charge. It should be noted that basic proteins which do not bind to the Mono Q column appear to undergo little or no degradation under conditions of complete starvation of nutrients.

Degradation of Protein in Relation to Molecular weight. Protein samples corresponding to 0, 3, and 9 d were fractionated on the Superose column and the relationship between degradation and size is shown in Figure 5. The data confirm the conclusion from Figure 4, that RuBPCase undergoes degradation under conditions of total nutrient starvation. However, there is no linear correlation between the rate of protein degradation and mol wt—proteins with a mol wt about 100 kD appear to degrade more rapidly than proteins which are either larger or smaller.

Effect of Darkness and Nutrient Starvation on Protein Degradation. Two batches of *Lemna* fronds were grown in complete medium (400 ml) containing either 1.47 MBq of L-[3,4,5-^{3}H] leucine or 0.47 MBq of L-[1-^{14}C]leucine for 27 h. During that time, 73% of the L-[3,4,5-^{3}H]leucine and 97% of the L-[1-^{14}C] leucine were taken up by the fronds, as judged by measuring the loss of radioactivity from the solutions. The ^{3}H-labeled fronds were transferred to three flasks containing water and placed in total darkness. After 0, 5, and 10 d, the fronds were combined with ^{14}C-labeled fronds as previously described (15). Protein was extracted and fractionated by FPLC.

Degradation of Protein in Relation to Charge. Protein from each of the samples was fractionated on the Mono Q column and the (^{14}C/^{3}H) ratio determined for each fraction. The results shown in Figure 6 should be compared with Figure 4, which depicts the effect of nutrient starvation in light on protein degradation. The comparison shows that RuBPCase is degraded under starvation conditions either in light or darkness, but in neither case is there any close correlation between protein degradation and charge. However, it should be noted (a) that the basic proteins which hardly degrade at all in the light, degrade more rapidly in the dark and (b) the protein fraction which elutes from the Mono Q column at the beginning of the NaCl gradient is not the protein fraction with the highest degradation rate as in all other cases we have examined.

Degradation of Protein in Relation to Size. Protein from each of the samples was fractionated on the Superose column and the (^{14}C/^{3}H) ratio determined for each fraction. The results shown in Figure 7 should be compared with Figure 5, which shows the effect of nutrient starvation in light on protein degradation. The comparison shows that the presence or absence of light has little effect on the overall protein degradation which occurs in response to nutrient starvation.

Effect of Stress Conditions on the Degradation of RuBPCase. RuBPCase separated by ion exchange chromatography on the Mono Q column was resolved into its subunits by SDS-PAGE using a 12.5% (w/v) polyacrylamide gel to prevent the small subunit of RuBPCase migrating with the front (15). The bands corresponding to the large and small subunits of RuBPCase were cut out and counted for ^{14}C and ^{3}H. The results are presented in Figure 8 together with data on RuBPCase degradation obtained by ion exchange chromatography and by gel filtration, for each of the three stress conditions investigated. The data show that RuBPCase undergoes little or no degradation under conditions of nitrogen starvation, but there is significant degradation under conditions of total nutrient starvation in both light and dark. It

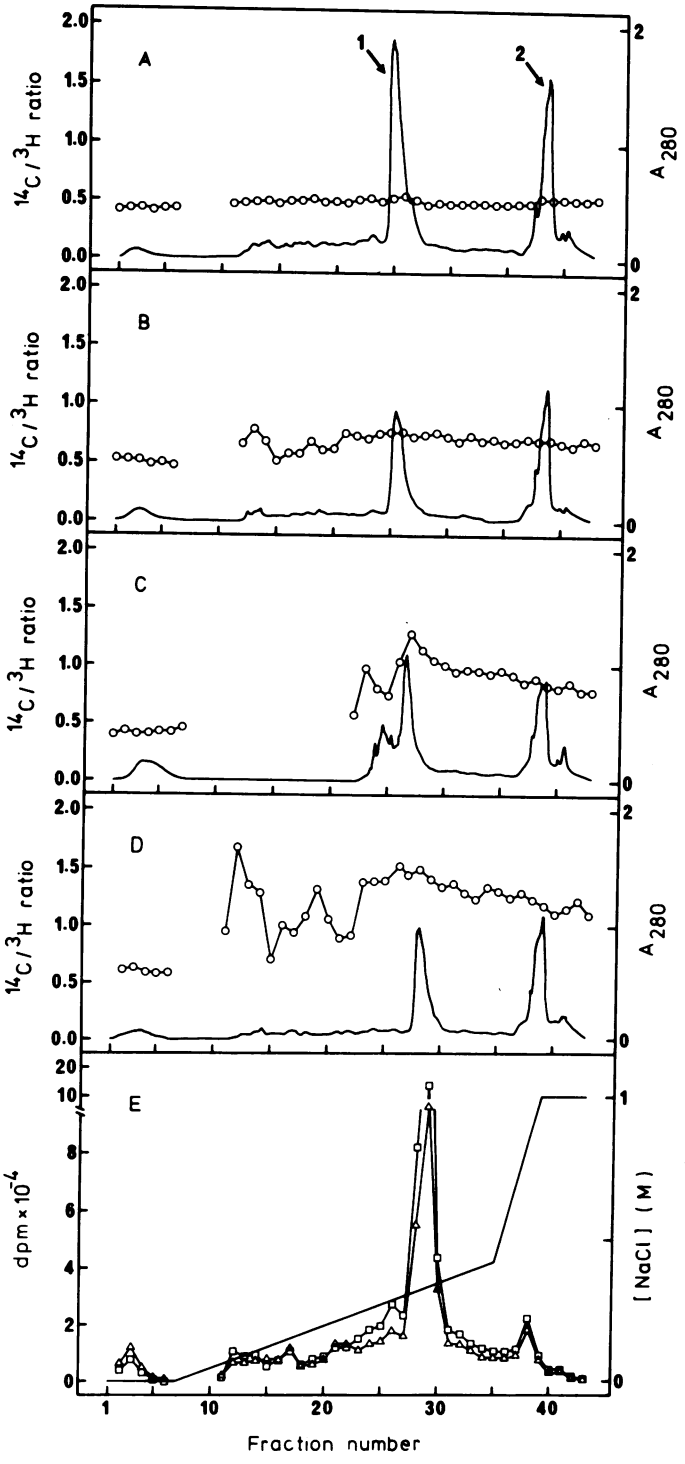

FIG. 4. Relative degradation rates of *Lemna* total soluble proteins fractionated by anion exchange chromatography, after 0, 3, 6, and 9 d in water (total nutrient starvation). Double labeled soluble proteins from *Lemna* were isolated, loaded into the FPLC Mono Q column equilibrated with 20 mM-Tris-HCl buffer (pH 7.5), and eluted with a gradient of NaCl; 1 ml fractions were collected. A high ($^{14}C/^{3}H$) ratio indicates a high rate of degradation. A, B, C, and D, Degradation of soluble proteins after 0, 3, 6, and 9 d in water, respectively. E, ^{3}H and ^{14}C dpm per fraction, corresponding to degradation after 9 d (D), and shape of the NaCl gradient used. Note break in ordinate. O, ($^{14}C/^{3}H$) ratio; Δ, ^{3}H dpm; □, ^{14}C dpm. Arrows: 1, RuBPCase; 2, nucleic acids, as judged by the absorption spectrum, binding properties to the anion exchanger and level of incorporation of label.

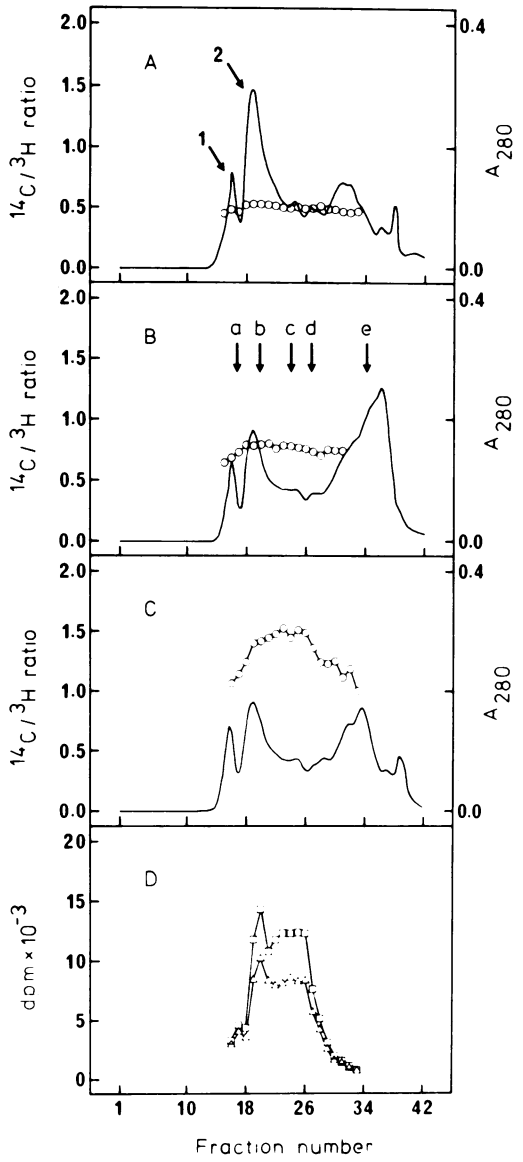

FIG. 5. Relative degradation rates of *Lemna* total soluble proteins fractionated by gel filtration, after 0, 3, and 9 d in water (total nutrient starvation). Double labeled soluble proteins from *Lemna* were isolated and loaded into the FPLC Superose 12 column equilibrated with 100 mM-Tris-HCl buffer (pH 7.5); 0.5 ml fractions were collected. A high ($^{14}C/^{3}H$) ratio indicates a high rate of degradation. A, B, and C, Degradation of soluble proteins after 0, 3, and 9 d in water, respectively. D, ^{3}H and ^{14}C dpm per fraction, corresponding to degradation after 9 d, (C). O, ($^{14}C/^{3}H$) ratio; Δ, ^{3}H dpm; □, ^{14}C dpm. Arrows: 1, nucleic acids, as judged by the absorption spectrum, mol wt and level of incorporation of label; 2, RuBPCase. 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.

should be noted that under all conditions the RuBPCase subunits degrade at the same rate, as judged by the ($^{14}C/^{3}H$) ratios.

DISCUSSION

RuBPCase is widely considered to be a leaf storage protein (17, 18) being synthesized early in the development of a leaf and undergoing little degradation until senescence occurs. We take leaf senescence to involve a programmed degradation of proteins, thereby providing amino acids for transport to other parts of the plant. In the previous paper (15) we noted that in leaves from a

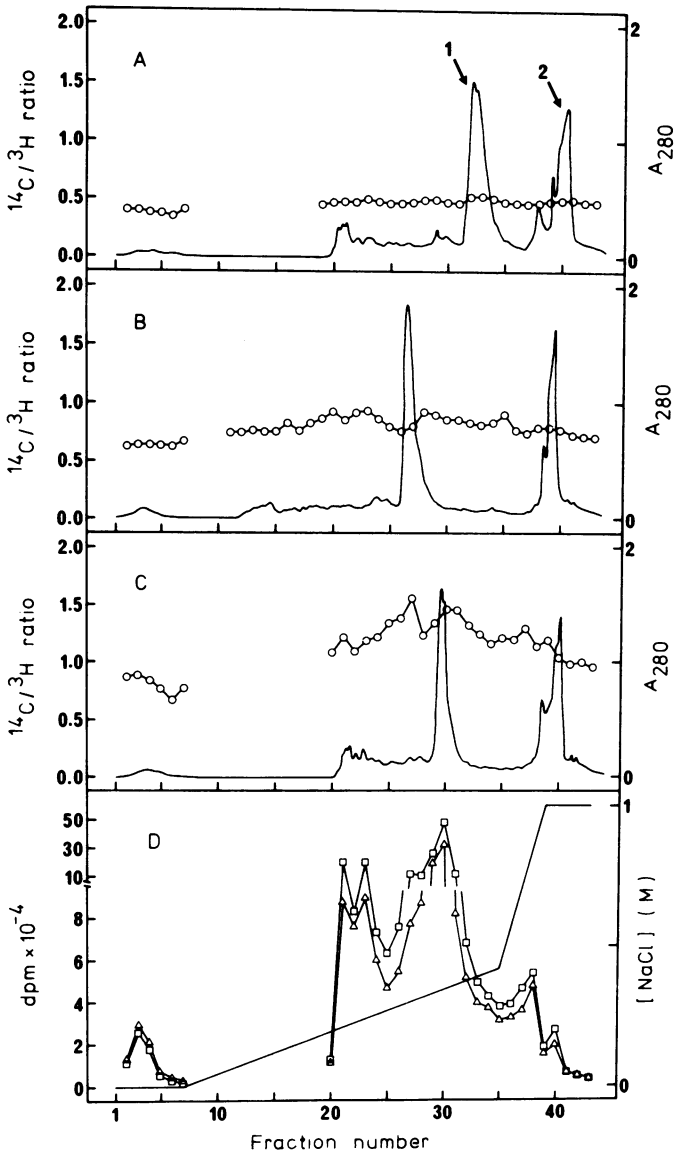

FIG. 6. Relative degradation rates of *Lemna* total soluble proteins fractionated by anion exchange chromatography, after 0, 5, and 10 d in water in the dark. Double labeled soluble proteins from *Lemna* were isolated, loaded into the FPLC Mono Q column equilibrated with 20 mM-Tris-HCl buffer (pH 7.5), and eluted with a gradient of NaCl; 1 ml fractions were collected. A high ($^{14}C/^{3}H$) ratio indicates a high rate of degradation. A, B, and C, Degradation of soluble proteins after 0, 5, and 10 d in water in the dark, respectively. D, ^{3}H and ^{14}C dpm per fraction, corresponding to degradation after 10 d, (C), and shape of the NaCl gradient used. Note break in ordinate. O, ($^{14}C/^{3}H$) ratio; Δ, ^{3}H dpm; □, ^{14}C dpm. Arrows: 1, RuBPCase; 2, nucleic acids.

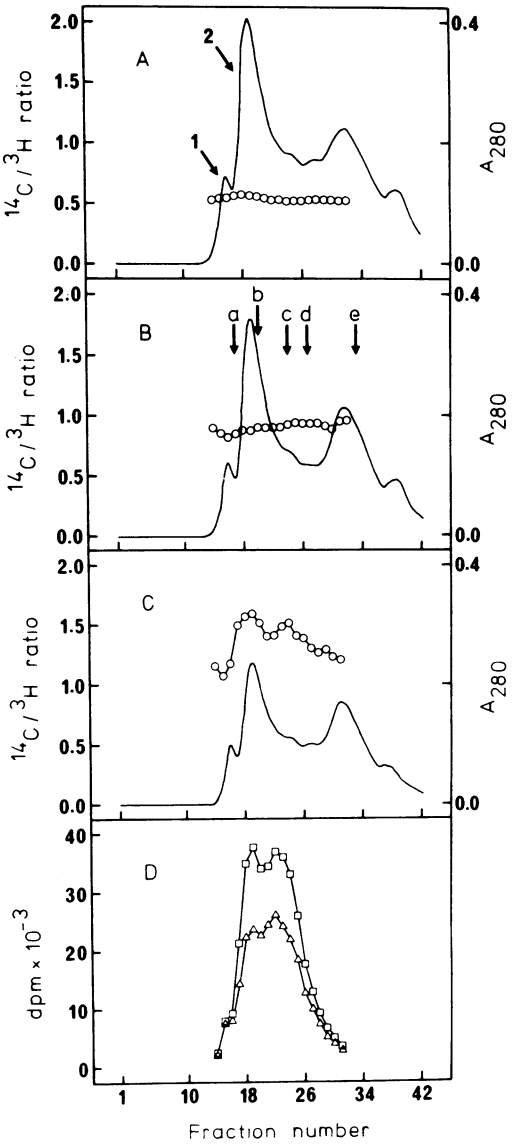

FIG. 7. Relative degradation rates of *Lemna* total soluble proteins fractionated by gel filtration, after 0, 5, and 10 d in water in the dark. Double labeled soluble proteins from *Lemna* were isolated and loaded into the FPLC Superose 12 column equilibrated with 100 mM-Tris-HCl buffer (pH 7.5); 0.5 ml fractions were collected. A high ($^{14}C/^{3}H$) ratio indicates a high rate of degradation. A, B, and C, Degradation of soluble proteins after 0, 5, and 10 d in water in the dark, respectively. D, ^{3}H and ^{14}C dpm per fraction, corresponding to degradation after 10 d (C). O, ($^{14}C/^{3}H$) ratio; Δ, ^{3}H dpm; □, ^{14}C dpm. Arrows: 1, nucleic acids; 2, RuBPCase. Mol wt markers as in Figure 5.

number of species (28) darkness stimulates the degradation of both RuBPCase and Chl, whereas in the case of *Lemna* darkness had little or no effect on the degradation of either. We now report that nitrogen starvation did not stimulate the degradation of RuBPCase, although there was a marked increase in the rate of Chl degradation. Total nutrient starvation resulted in the degradation of RuBPCase although not at an enhanced rate compared with other leaf proteins and, interestingly and surprisingly, darkness inhibited the degradation of Chl which occurs with total nutrient starvation in the light.

It therefore appears that *Lemna* fronds are not programmed to senesce, a conclusion supported by our observation (unpublished) that the dramatic increase in glutamate dehydrogenase which occurs when cereal leaves are kept in the dark does not occur when *Lemna* fronds are kept in the dark for periods up to 2 weeks.

The model for stress induced protein degradation, which is based on the Matile hypothesis of the lysosomal nature of the vacuole, predicts that the selectivity of protein degradation is determined by the physical properties of the proteins being degraded, *e.g.* acidic soluble proteins degrade more rapidly than basic or neutral proteins (16). Earlier experiments with *Lemna* (7) produced data broadly in agreement with this hypothesis, *e.g.* large proteins tend to degrade faster than small proteins. However, more recent data for *Lemna* obtained by separating proteins on the basis of their age, suggested that the correlation between size and degradation was at best weak (6). The FPLC methods

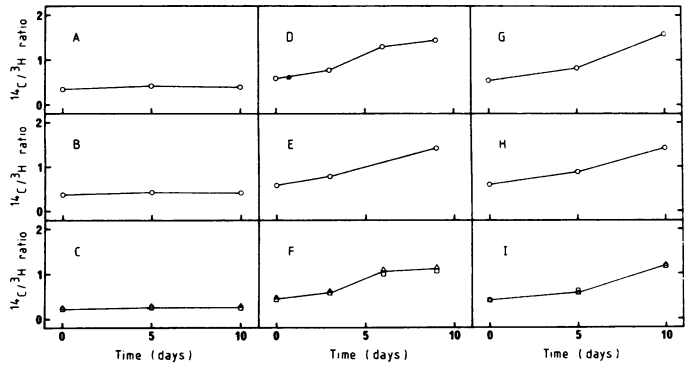

FIG. 8. Relative rate of degradation of RuBPCase from *L. minor* stressed in complete medium lacking nitrogen (nitrogen starvation), in water (total nutrient starvation), and in water in the dark. A, D, and G: O, Relative rate of degradation of RuBPCase, partially purified by ion exchange chromatography (Figs. 2, 4, and 6), from fronds stressed in complete medium lacking nitrogen, in water, and in water in the dark, respectively. B, E, and H: O, Relative rate of degradation of RuBPCase, partially purified by gel filtration (Figs. 3, 5, and 7), from fronds stressed in complete medium lacking nitrogen, in water, and in water in the dark, respectively. C, F, and I: Samples of double labeled RuBPCase partially purified by ion exchange chromatography (Figs. 2, 4, and 6) 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 protein bands were stained, sliced and digested as described in "Materials and Methods." The ($^{14}C/^{3}H$) ratio corresponding to each band was determined by liquid scintillation counting. A high ($^{14}C/^{3}H$) ratio indicates a high rate of degradation. △, □: Relative rates of degradation of RuBPCase large and small subunits, respectively, from *Lemna* fronds stressed in complete medium lacking nitrogen (C), in water (F), and in water in the dark (I).

used in this work give a much better separation of proteins than the methods used in earlier work (7) and using these improved methods we find no correlation between the rate of protein degradation and either the charge or size of proteins. The discrepancy between the results reported in this paper and those reported by Cooke and Davies (7) may be due partly to technical differences in the methods of protein separation and partly to the design of the experiments. The 'control' fronds employed by Cooke and Davies (7) were labeled with a ^{14}C-amino acid, then chased under non-stress conditions for a time equal to the time the ^{3}H-labeled fronds were chased under stress conditions. In the experiments described in this paper the ^{3}H-labeled stressed fronds were mixed with ^{14}C 'control' fronds which had not been chased.

We do not regard the lack of correlation between protein degradation and either protein charge or size as evidence against the Matile-lysosomal hypothesis of protein degradation. Rather we believe that so many properties of proteins contribute to the selectivity of degradation, that a simple correlation between the rate of degradation and a single property is unlikely.

However, the marked differences in the rates of degradation of RuBPCase under different stress conditions suggest that the model for stress induced protein degradation cannot be extended to the degradation of RuBPCase. On the contrary, the data are best explained by a dual system in which cytosolic proteins are degraded by the vacuolar/lysosomal system and chloroplast proteins are degraded within the chloroplasts.

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