Plant Physiol. (1975) 55, 1009–1015

Loss of Ribulose 1,5-Diphosphate Carboxylase and Increase in Proteolytic Activity during Senescence of Detached Primary Barley Leaves

Received for publication December 3, 1974 and in revised form February 13, 1975

LARRY W. PETERSON AND RAY C. HUFFAKER
Department of Agronomy and Range Science, University of California, Davis, California 95616

ABSTRACT

Symptoms typical of senescence occurred in green detached primary barley (*Hordeum vulgare* L.) leaves placed in darkness and in light. Chlorophyll, total soluble protein, ribulose 1,5-diphosphate carboxylase protein and activity each progressively decreased in darkness and to a lesser extent in light. In all treatments most of the total soluble protein lost was accounted for by a decrease in ribulose 1,5-diphosphate carboxylase protein, suggesting that the chloroplast was a major site of degradation early in senescence.

Loss of ribulose 1,5-diphosphate carboxylase protein was negatively correlated with an increase in proteolytic activity measured against azocasein. Both rates were exponential, with about a 30% difference in apparent rate constants. Cycloheximide essentially prevented the loss of chlorophyll, ribulose 1,5-diphosphate carboxylase protein, and activity and completely inhibited the increase in proteolytic activity against azocasein. Since chloramphenicol had little effect on the loss of ribulose 1,5-diphosphate carboxylase protein or chlorophyll, or on proteolytic activity against azocasein, it is suggested that the proteolytic activity was developed on cytoplasmic 80 S ribosomes.

Kinetin greatly retarded the onset of such symptoms of senescence by inhibiting the losses of chlorophyll and ribulose 1,5-diphosphate carboxylase protein and protected against inactivation of enzymic activity. It also prevented the increase in proteolytic activity measured against azocasein. Incorporation of labeled amino acids into ribulose 1,5-diphosphate carboxylase during its rapid degradation showed that the enzyme was under turnover. The changes in ribulose 1,5-diphosphate carboxylase protein and activity, chlorophyll, soluble protein other than ribulose 1,5-diphosphate carboxylase, proteolytic and esterolytic activity during senescence indicate that senescence is a selective, sequential process.

The process of senescence has been studied in intact plants, detached leaves, and leaf discs in both light and dark (31, 37). In general, senescence is speeded by detaching the leaves and/or imposing darkness. Perhaps the most striking changes in senescence are losses of Chl, nucleic acids, and protein (39), accompanied by a general increase in proteolytic activity as measured *in vitro*. Cytokinins (32, 33, 37) or light (7) delay the appearance of several symptoms of senescence in detached leaf tissue. Some studies indicate that cytokinin affects leaf senescence through slowing the breakdown of protein (20, 24, 30, 32, 33) rather than by stimulating protein synthesis (32). Trewavas (35) measured both synthesis and degradation rates accurately in *Lemna minor*, showing that cytokinin could affect either synthesis or degradation depending on growth conditions. In some plants IAA also inhibits senescence, though the mechanism seems to be different from that for cytokinin (28, 29).

Recent reports have shown that L-serine accelerated the senescence of oat (23, 30) and tobacco leaves (11). The effect was most evident in the presence of kinetin (23). The antagonism between serine and kinetin did not appear to be the result of opposing actions at the same site. Rather, it was suggested that serine might be incorporated into the active center of one or more proteolytic enzymes participating in the senescence process, thereby allowing increased degradation (11, 23, 31).

Much work has been done showing the loss of total soluble protein during senescence, but comparatively little of it has dealt with isolated fractions, and even less with purified proteins. Several investigations have shown that fraction I protein was the main protein component lost in senescing tobacco (5, 12, 13, 14) and *Perilla* (10) leaves. Fraction I protein as usually isolated can probably be considered to be crude RuDPCase[1] since it often contains several other enzymatic activities in addition to that of RuDPCase (15, 18, 36). We recently reported that intact barley seedlings placed in extended darkness lost soluble protein, almost all of which was accounted for by the enzyme RuDPCase (9, 25). The results of these investigations are not surprising, since this enzyme is considered to be the major storage protein of many plant leaves (18, 26). Since RuDPCase is also principally responsible for net fixation of CO_2 in plants, its fate during senescence is important. This study is a further extension to show the effects of some chemicals and light on the loss of RuDPCase and to correlate the concurrent development of proteases and esterases during senescence. Evidence is also presented for the turnover of RuDPCase under these conditions.

MATERIALS AND METHODS

Plant Materials. *Hordeum vulgar* L. var. Numar was grown in 28 × 33 cm plastic pans. Moisture was supplied by cotton wicks connecting the vermiculite to a full strength nutrient supply (8). Prior to treatments the plants were grown for 7 days in continuous light (500 μeinsteins cm^{-2} sec^{-1}) at 27 C and 55% relative humidity.

[1] Abbreviations: RuDPCase: ribulose 1,5-diphosphate carboxylase; CTN: N-carbobenzoxy-L-tyrosine-P-nitrophenyl ester; ANA: α-naphthyl acetate.

Plant Treatments. The top 10 cm of the first leaves of barley were detached, and two replicate samples of 80 leaves each were placed in 25 ml of treatment solutions in 200-ml beakers in growth chambers in either light or dark under the same temperature (27 C) and relative humidity (55%) mentioned above. Under these conditions, the detached leaves remained turgid throughout the test periods. Ten leaves were removed from each of two replicated treatments at the indicated times for assay. Treatment solutions were replenished as required. Each experiment was replicated and the results shown are the averages for the two experiments. Losses of RuDPCase and soluble protein as well as Chl and proteolytic activity were followed in plants treated with the chemicals listed below.

Cycloheximide and chloramphenicol were included in the treatments to determine whether differential effects on the symptoms of senescence might be detected on the basis of the intracellular location of the response. Serine was used to determine whether it would speed the onset of the symptoms. Ammonium nitrate was used to determine whether an external source of N might delay senescence. Earlier observations showed that protein was not lost from detached leaves supplied with N (40). Kinetin was included because of its well known effects as a retardant of symptoms of senescence. The following concentrations were used: chloramphenicol, 25, 50, and 100 μg/ml; cycloheximide, 5 μg/ml; kinetin, 1 and 10 μg/ml; IAA, 0.01 and 0.1 μg/ml; 6 mM NH_4NO_3; or 50 mM serine. Cycloheximide at 5 μg/ml produced no visible toxicity or wilting symptoms. All treatments, including controls, contained 0.2 mM $CaSO_4$. $CaSO_4$ was supplied since calcium ions help maintain normal membrane function (6, 22, 27) in both root (6) and leaf (27) tissue.

The concentrations of cycloheximide and chloramphenicol were chosen on the basis of previous studies with detached leaves. Cycloheximide from 2 to 8 μg/ml and chloramphenicol at 100 μg/ml differentially inhibited the incorporation of amino acids into RuDPCase in detached barley leaves (4). Since the present experiments lasted up to 3 days it was judged that chloramphenicol at 100 μg/ml might be toxic; therefore, the inhibitor was used at 25, 50, and 100 μg/ml.

A mixture of ^{3}H-labeled (G) amino acids was supplied to the excised leaves where indicated in both light and dark treatments. Each treatment received the same amount, 1 μCi/ml at an average specific radioactivity of 18.82 mCi/μmole.

Preparation of cell-free extracts and assays. Cell-free extracts were prepared as described previously (19). RuDPCase activity was determined by the method of Kleinkopf *et al.* (19) by following the conversion of $KH^{14}CO_3$ into acid-stable products at 28 C. The reaction mixture (0.1 ml) contained the following, in μmoles: ribulose 1,5-diP, 0.5; $KH^{14}CO_3$, 2.5 (with a specific radioactivity of 0.455 μCi/μmole); $MgCl_2$, 3; tris-SO_4 buffer (pH 8), 6. The reaction was initiated by adding to the reaction mixture 0.1 ml of an appropriately diluted extract. After 10 min the reaction was stopped by adding 50 μl of 1 N HCl. Aliquots of 0.1 ml were dried on strips of filter paper, placed in a toluene-base scintillator solution, and then counted in a scintillation counter. Ribulose 1,5-diphosphate was prepared as described previously (8).

Azocasein (3) was used to measure proteolytic activity. The reaction mixture contained 0.3 ml of 10 mg/ml azocasein, 0.5 ml of 0.2 M sodium phosphate buffer, pH 6, and 0.2 ml of the cell-free extract. After 2 hr at 40 C the reaction was stopped by adding 2 ml of 12% perchloric acid. The precipitated protein was removed by centrifugation, and the absorbance of the supernatant solution was read at 340 nm. One unit of azocasein activity is the amount of enzyme which results in an increased absorbancy of 0.01 per min.

Esterase activity was measured against the synthetic substrates CTN and ANA. Used to measure activity against CTN was a modification of the procedure of Klein and Harpaz (17). The reaction mixture contained 1.4 ml of 0.1 M sodium phosphate, pH 6.2, and 0.2 ml of cell-free extract, and was started by the addition of 0.2 ml of 2 mM CTN in acetone. After 10 min at 28 C the reaction was stopped with 0.2 ml of 50% trichloroacetic acid, the precipitated protein was removed by centrifugation, and the absorbance of the supernatant solution was measured at 320 nm (17). Activity against ANA was followed in a spectrophotometer at 310 nm at 28 C by the procedure of Burger *et al.* (2). The reaction mixture (3 ml) contained 2.9 ml of 5×10^{-4} M ANA in 0.05 M phosphate buffer, pH 7.5, and 0.1 ml of cell-free extract. All reactions were linear with respect to time and enzyme concentration.

Purification of RuDPCase (18), development of the rabbit antibody against RuDPCase (18), determination of its specificity for RuDPCase by double immunodiffusion in agar (18), and quantitation (and its validity) of the anti-RuDPCase-precipitable material (19) were all done exactly as previously described. In the present study, double immunodiffusion of the purified RuDPCase and the antibody showed one band of precipitation. This line showed serological identity with the line that developed between the crude enzyme homogenate and the antiserum. The anti-RuDPCase precipitated 100% of the RuDPCase activity from solution. In addition, the precipitated RuDPCase retained significant enzymic activity when tested for its ability to fix CO_2 in a standard reaction mixture.

Incorporation of radioactive label into RuDPCase and total protein was determined by precipitation with, respectively, RuDPCase antiserum or trichloroacetic acid (final concentration 5%) in the presence of unlabeled amino acids. The precipitates were collected by centrifugation. The precipitates were resuspended and washed 3 times with unlabeled amino acids. The precipitates were then collected over Whatman glass-fiber filters (GF-83), and washed with hot 10% trichloroacetic acid, then with 95% ethanol, and, finally, with ethanol and ether (1:1). The filters were dried and counted in a Tri-Carb scintillation spectrometer using a toluene-omnifluor solution.

Chlorophyll was determined spectrophotometrically from 80% (v/v) acetone extracts of the pelleted cell-debris by the method of Arnon (1). Protein was measured by the method of Lowry *et al.* (21). The standard was BSA, fraction V.

RESULTS

Symptoms of senescence appeared within 24 hr in detached barley leaves placed in darkness. Chlorophyll concentration (Fig. 1), total soluble protein (Fig. 2), and RuDPCase activity (Fig. 3) decreased similarly in plants treated with $CaSO_4$ (control), and serine. Chloramphenicol tended to decrease the loss of RuDPCase activity between 24 and 60 hr. Chloramphenicol was used at 25, 50, and 100 μg/ml. Since little difference was observed among the three concentrations, only the results from 25 μg/ml are shown. Ammonium nitrate slowed the loss of Chl somewhat but had very little effect on the loss of soluble protein and RuDPCase activity. Cycloheximide markedly inhibited the loss of Chl, total soluble protein, and RuDPCase activity. Kinetin was as effective as cycloheximide in inhibiting the loss of Chl and total soluble protein, but not quite as effective as cycloheximide in reducing the loss of RuDPCase activity. Kinetin was less effective at 1 μg/ml (data not shown) than at 10 μg/ml in retarding loss of Chl and RuDPCase activity. Since IAA at 0.1 and 0.01 μg/ml had no effect on any

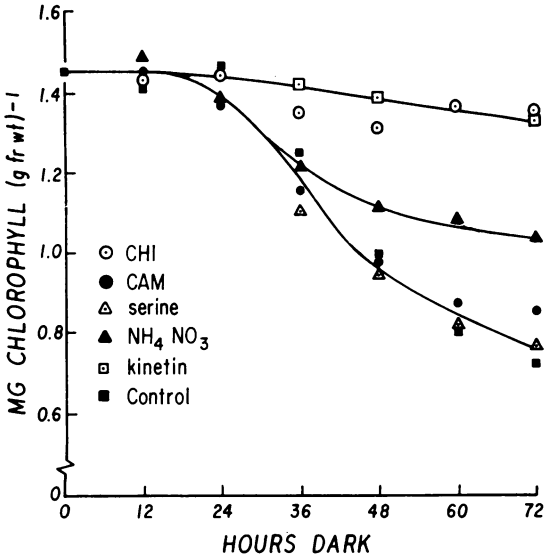

FIG. 1. Time course for the loss of Chl in detached barley leaves in the dark. The top 10 cm of 80 of the first leaves of 7-day-old barley seedlings were placed in 200-ml beakers containing 25 ml of 5 μg/ml cycloheximide (CHI), 25 μg/ml chloramphenicol (CAM), 0.05 M serine, 6 mM NH_4NO_3, 10 μg/ml kinetin, or 0.2 mM $CaSO_4$ control. At given intervals, 10 leaves were removed from each replicated treatment for analysis.

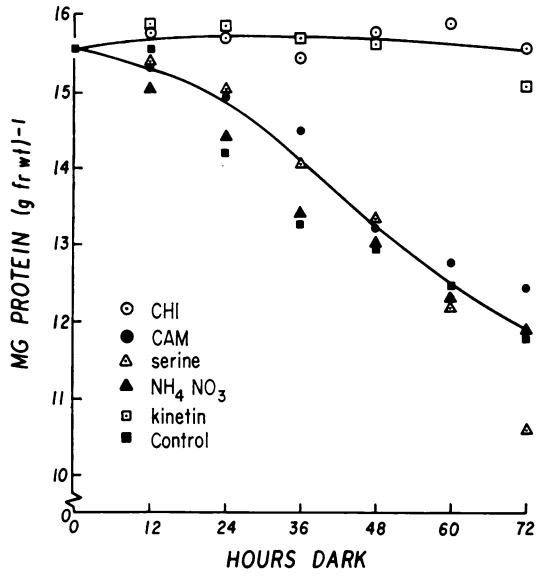

FIG. 2. Change in total protein with time in detached barley leaves in the dark. See Fig. 1 for treatment conditions.

of the factors measured, these data were not shown in the figures.

Similar treatment effects occurred with RuDPCase protein (Fig. 4). There was little loss of RuDPCase protein in plants treated with cycloheximide; kinetin inhibited the loss to a lesser extent. Compared with the $CaSO_4$ control, serine, ammonium nitrate, and chloramphenicol had little effect on the loss of RuDPCase protein. The difference between the concentration of total soluble protein (Fig. 2) and that of RuDPCase protein (Fig. 4) showed little change in the amount of non-RuDPCase protein (Table I) in any of the treatments up to 60 hr and only slight decreases (respectively 16 and 18%) in the ammonium nitrate and serine treatments at 72 hr. Thus, in

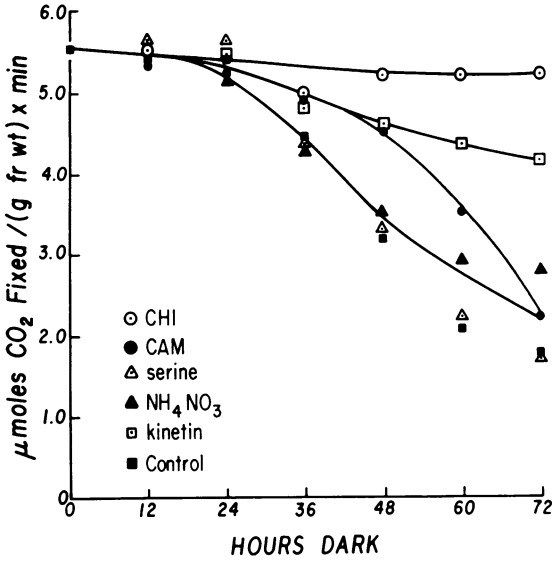

FIG. 3. Change in ribulose diphosphate carboxylase activity with time in detached barley leaves in the dark. See Fig. 1 for treatment conditions.

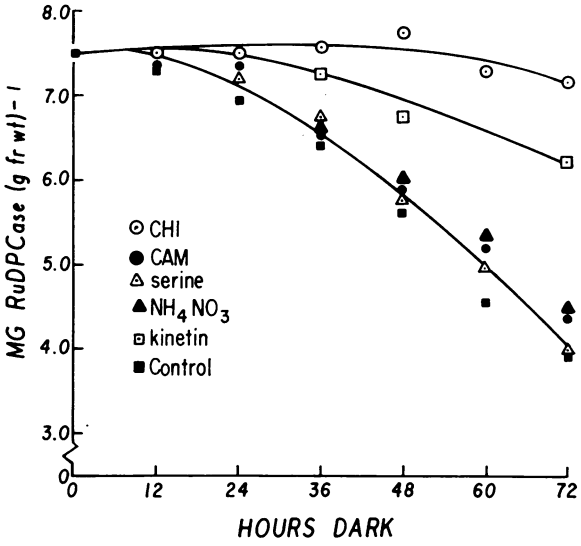

FIG. 4. Change in antibody-precipitable ribulose diphosphate carboxylase protein with time in detached barley leaves in the dark. See Fig. 1 for treatment conditions.

Table I. *Non-RuDPCase Protein in Detached Barley Leaves in Dark*

Non-RuDPCase protein is the difference between total soluble protein (Fig. 2) and RuDPCase protein (Fig. 4).

Time of Darkness	Control ($CaSO_4$) (0.2 mm)	CHI (5 μg ml^{-1})	CAM (25 μg ml^{-1})	Serine (50 mm)	NH_4NO_3 (6 mm)	Kinetin (10 μg ml^{-1})
hr	*mg protein/g fresh wt*					
0	8.10	8.10	8.10	8.10	8.10	8.10
12	8.38	8.56	7.98	7.95	7.46	8.48
24	7.22	8.40	7.47	7.79	7.11	8.47
36	7.79	7.75	8.01	8.24	7.33	8.38
48	7.48	8.16	7.42	7.52	7.48	8.98
60	7.90	8.94	7.47	7.89	7.32	
72	7.89	8.42	8.11	6.67	6.82	7.84

most of the treatments the loss of soluble protein could be accounted for almost entirely by the loss of RuDPCase protein.

To determine whether protein synthesis occurred while RuDPCase was being degraded, detached leaves were treated with tritiated amino acids in both dark and light. Labeled amino acids were incorporated into RuDPCase even though it was being rapidly degraded (Fig. 5). Because transpiration rates were higher, amino acids were taken up more rapidly in light than in dark, resulting in more incorporation and a higher specific radioactivity of proteins in tissue in the light (Fig. 5). In both light and dark the proportion of the label going into RuDPCase protein represented about one-fifth of the label being incorporated into total protein. Thus, during senescence, RuDPCase protein was synthesized more slowly than was non-RuDPCase protein. Even though the amount of label incorporated into RuDPCase protein and total protein differed between light- and dark-treated leaves, the ratio of radioactivity in the protein to that taken up was the same for both treatments (Fig. 6).

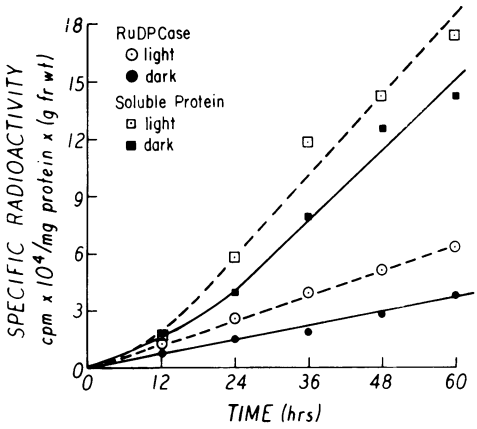

FIG. 5. Specific radioactivity of RuDPCase protein and total soluble protein in detached barley leaves in the dark and light. One μCi/ml of an ^{3}H-amino acid mixture (G) was added to the $CaSO_4$ treatment solution at the beginning of each treatment at an average specific radioactivity of 18.82 mCi/μmole. Other experimental details as in Fig. 1.

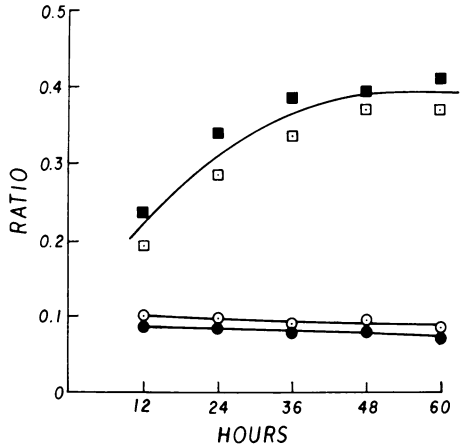

FIG. 6. Ratio of the labeled amino acids in ribulose diphosphate carboxylase protein (●,○) and non-RuDPCase protein from Table I (■, □) to the total amount of labeled amino acids taken up by detached leaves in the dark (●,■) and light (○, □). The total uptake of labeled amino acids was determined by counting an aliquot of the initial cell-free extract. Other experimental details as in Figs. 5 and 1.

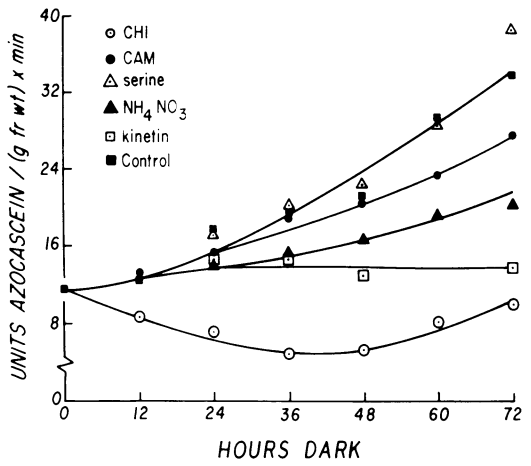

FIG. 7. Time course for the development of proteolytic activity measured by the hydrolysis of azocasein in detached barley leaves placed in dark. See Fig. 1 for treatment conditions.

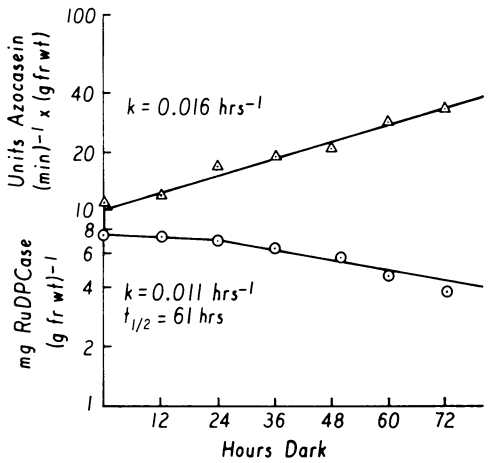

FIG. 8. Data from Figs. 4 and 8 plotted semilogarithmically.

The loss of RuDPCase protein and activity was correlated negatively with the appearance of proteolytic activity toward azocasein. Protoeolytic activity was highest in control and serine-treated leaves after 72 hr (Fig. 7). Kinetin inhibited the development of proteolytic activity, and some inhibition was noted with ammonium nitrate. Chloramphenicol had a negligible effect (less than 20% at 60 and 72 hr). Cycloheximide completely prevented the increase in activity against azocasein.

After an initial lag, RuDPCase in the $CaSO_4$ control was lost at a constantly increasing rate (Fig. 4) that gave an apparent rate constant of 0.011 hr^{-1} when plotted as the log of the respective quantities (Fig. 8). The appearance of proteolytic activity as measured against azocasein increased exponentially (Fig. 7), yielding an apparent rate constant of 0.016 hr^{-1} when plotted (Fig. 8). In the present experiments the amount of proteolytic activity measured with CTN (Fig. 9) differed from that measured with azocasein. In contrast to that observed with azocasein no significant increase in activity occurred in any of the treatments. Most noteworthy is the continual decrease in activity after 12 hr in plants treated with cycloheximide. No differences were detected among any of the treatments when esterolytic activity was assayed with ANA (Fig. 10). All treatments gave a similar slow decline in activity.

Senescence of detached leaves was slowed but still occurred if they were placed in the light (Table II). In light, Chl, total

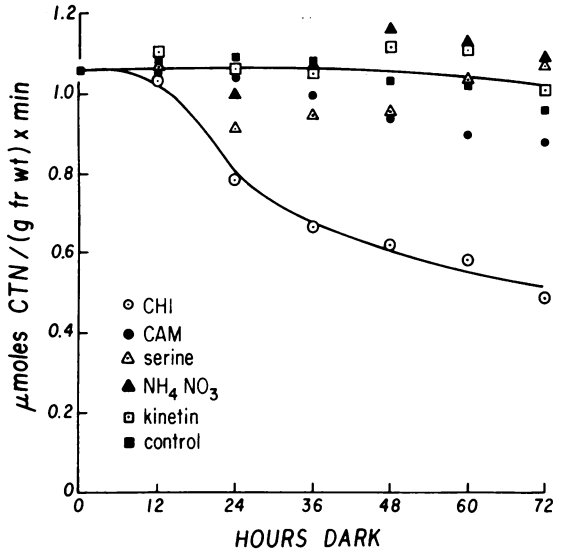

FIG. 9. Time course for the development of proteolytic activity as measured by the hydrolysis of CTN in detached barley leaves in the dark. See Fig. 1 for treatment conditions.

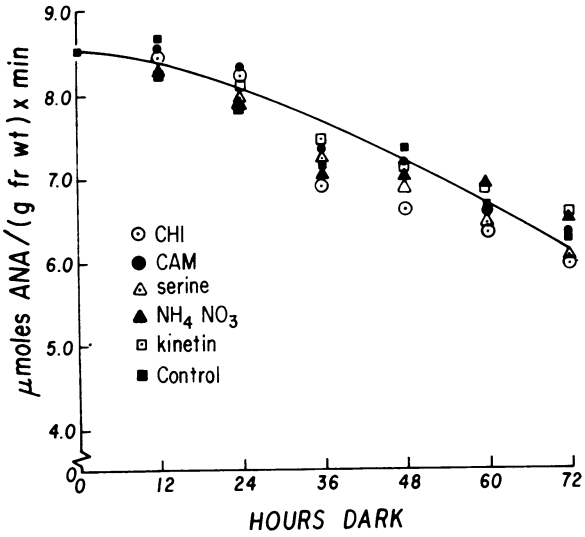

FIG. 10. Change in the esterolytic activity measured by the hydrolysis of ANA in detached barley leaves in the dark. See Fig. 1 for treatment conditions.

soluble protein, and RuDPCase protein were lost less rapidly, whereas RuDPCase activity was lost almost as fast as in dark controls. In light as well as dark, RuDPCase essentially accounted for the loss of soluble protein. The reduced loss of protein in the light corresponded with a reduction in proteolytic activity measured against azocasein. Table II also shows that kinetin depressed the appearance of azocasein activity and maintained the other constituents more effectively than did light.

DISCUSSION

RuDPCase and Senescence. Detached barley leaves showed typical symptoms of senescence by progressive reductions in Chl, RuDPCase protein and activity. In all treatments most of the total soluble protein lost was accounted for by the decrease in RuDPCase protein. RuDPCase protein also accounted for almost all of the soluble proteins lost from leaves of intact barley seedlings placed in darkness to induce senescence (23).

Kinetin had very important effects during senescence of the detached leaves. It almost totally inhibited losses of Chl and RuDPCase protein and significantly decreased the loss of enzymic activity. Kinetin also inhibited the increase in proteolytic activity azocasein.

Other investigations have shown that light retarded losses of Chl (7) and soluble protein (7, 38) in senescing tissue mainly by allowing photosynthesis (7). In the present study, light protected against a loss of soluble protein by greatly retarding the loss of RuDPCase protein (Table II). Concurrently, light also inhibited the increase in proteolytic activity against azocasein.

Other investigators have shown that, during the senescence of tobacco (4, 14) and *Perilla* (10) leaves, fraction I protein, *i.e.*, crude RuDPCase (15, 18, 36), was lost more rapidly than low mol-wt fraction II proteins, and during the curing of tobacco leaves (12) and senescence of kikuyu grass and oats (40) the chloroplast protein disappears more rapidly than cytoplasmic proteins. Those results, taken with ours, suggest that the chloroplast is a major site of proteolysis early in leaf senescence and that RuDPCase is the major protein hydrolyzed. It has been suggested that RuDPCase could be considered a major leaf storage protein (9, 18, 26) since it occurs in large quantities and is not rapidly turned over (9, 26). Perhaps the loss of RuDPCase during senescence is a plant response utilizing this "stored" protein so as to maintain itself.

During greening (19, 25) or leaf expansion (8), when RuDPCase is actively synthesized, large amounts of amino acids are incorporated into the enzyme. After those periods of synthesis, however, the enzyme seems remarkably stable unless the plant is put under conditions inducing leaf senescence. We previously reported that little if any turnover (simultaneous synthesis and degradation) of RuDPCase protein was detected in intact green barley leaves (25) under optimal growing conditions. Zucker (41) also observed the stability of RuDPCase in green xanthium leaf discs. He found that little radioactivity was detected in fraction I protein even though significant amounts were incorporated into phenylalanine am-

Table II. *Comparison between Detached Leaves Placed in Continuous Light or Dark*

Experimental conditions were 500 μeinsteins cm^{-2} sec^{-1}, 27 C, and 55% relative humidity. All treatment solutions contained $CaSO_4$. Treatment results are given as a per cent of the original remaining after 60 hr of treatment.

	Original	Light	Dark	Dark + 10 μg/ml Kinetin
			%	
Chlorophyll, mg (g fresh wt)$^{-1}$	1.37	83	67	95
Azocasein activity, units/min × (g fresh wt)	15.2	150	195	122
Soluble protein, mg (g fresh wt)$^{-1}$	16.0	91	62	96
RuDPCase protein, mg (g fresh wt)$^{-1}$	7.5	81	48	95
RuDPCase activity, μmoles/min × (g fresh wt)	4.98	48	41	74

monia lyase. Likewise, when Kannangara and Woolhouse (10) fed radioactive CO_2 to fully expanded *Perilla* leaves only 5% of the activity was in fraction I protein. In our previous work observance of turnover depended upon changes in the specific radioactivity of previously highly labeled RuDPCase protein (25). Under this condition it is possible that a relatively small amount of synthesis (less than 10% of the total) could have gone undetected. The present study was designed to detect small amounts of incorporation of radioactive amino acids into RuDPCase protein while it was under active degradation during senescence. The results showed that some synthesis did occur in the detached leaves while RuDPCase was being rapidly degraded.

Other differences were detected between non-RuDPCase and RuDPCase protein in the incorporation of amino acids. The specific radioactivity of RuDPCase protein increased linearly with time, while that of non-RuDPCase protein showed a lag of 12 (light treatment) to 24 hr (dark treatment) before linearity occurred. A linear increase in specific radioactivity indicates that the precursor pool(s) are saturated (9). On this basis, then, the precursor pool(s) for synthesis of RuDPCase protein were saturated much faster than those of non-RuDPCase protein.

The difference in incorporation rate between leaves kept in light and dark suggests a similar difference in rate of protein synthesis (Fig. 6). Such a conclusion would be valid only if there were no change in the specific radioactivity of the protein precursor pool with respect to both leaf treatment and time. Since more labeled amino acids were taken up by leaves maintained in the light it is quite likely that the specific radioactivity of the precursor pool of these plants was also higher. To test this, a ratio was determined of the radioactivity in the soluble fraction to that incorporated into RuDPCase protein and non-RuDPCase protein. The ratio was approximately the same at any given time for both light- and dark-treated leaves (Fig. 6). This suggests that, although the total amount of radioactivity incorporated into protein differed between light and dark, the rate of protein synthesis (the fractional part of the label incorporated per unit time) was the same in both treatments. Kemp and Sutton (16) obtained similar results with tobacco callus. They showed that an apparent 2.3-fold difference in rate of protein synthesis in callus of different ages was the result of differential uptake rates of tritiated leucine with age. Thus, the specific radioactivity of the soluble leucine pool was greater in young tissue than in old. The difference in incorporation was due to a difference in the specific radioactivity of the two pools, not a difference in actual synthesis rate.

Our results present a possible explanation of an interesting observation made by Mizrahi *et al.* (24). They pulse-labeled detached *Tropaeolum majus* leaves with $^{14}CO_2$ for 1 hr, transferred the leaves to $^{12}CO_2$ and to kinetin or water control, and then followed the radioactivity present in amino acids and protein. The specific radioactivity of the leaf proteins increased continuously in both the control and hormone-treated plants as the leaves senesced. They concluded that "the bulk of the proteins that became labeled during the experiment degraded at a slower rate than the bulk of the unlabeled, previously synthesized proteins." A possible explanation is that RuDPCase protein may be synthesized much more slowly than other proteins in the mature green leaves and may be degraded more rapidly in senescing leaves, resulting in the greater specific radioactivity of total protein.

Proteolytic Activity and Senescence. Of the proteolytic and esterolytic activities tested in the study, only the activity against azocasein appeared to be correlated with the loss of RuDPCase. It may be fortuitous; however, it was interesting to note that the increase in extractable activity against azocasein and the rate of loss of RuDPCase with time were both exponential and had apparent rate constants within about 30% of each other (Fig. 8).

Cycloheximide almost completely prevented the loss of RuDPCase protein, prevented the induction of increased activity against azocasein, and allowed a loss of CTN activity. Since chloramphenicol had little effect on any of the above constituents, the proteolytic enzyme(s) responsible for the hydrolysis of RuDPCase may be synthesized primarily on cycloheximide-sensitive ribosomes. If these are the 80 S ribosomes of the cytoplasm, then, after synthesis, the proteolytic enzymes may be transported into the chloroplasts to function in the degradation of RuDPCase. Another possibility is that RuDPCase or its subunits may leak out of the chloroplast into the cytoplasm if the chloroplast membrane changes in property during senescence. Irrespective of the localization of proteolysis, the results indicate that protein synthesis may be required to support the degradation of RuDPCase. The possible requirement for protein synthesis for the inactivation of nitrate reductase in barley leaves has also been reported (34).

The results suggest that changes in hydrolytic activities during senescence are not random events but may have a degree of specificity. Activity against azocasein (Fig. 7) increased, against CTN (Fig. 9) changed little, and against ANA (Fig. 10) decreased exponentially with time. The loss of RuDPCase increased exponentially with time, while the bulk of the remaining soluble protein changed very little.

LITERATURE CITED

1. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
2. Burger, W. C., N. Prentice, M. Moeller, and J. Kastenschmidt. 1970. Hydrolysis of alpha naphthyl acetate and L-leucyl naphthylamide by barley enzymes. Phytochemistry 9: 33–40.
3. Charney, J. and R. M. Tomarelli. 1947. A colorimetric method for the determination of the proteolytic activity of duodenal juice. J. Biol. Chem. 171: 501–505.
4. Criddle, R. S., B. Dau, G. E. Kleinkopf, and R. C. Huffaker. 1970. Differential synthesis of ribulosediphosphate carboxylase subunits. Biochem. Biophys. Res. Commun. 41: 621–627.
5. Dorner, R. W., A. Kahn, and S. G. Wildman. 1957. The proteins of green leaves. VII. Synthesis and decay of the cytoplasmic proteins during the life of the tobacco leaf. J. Biol. Chem. 229: 945–952.
6. Epstein, E. 1961. The essential role of calcium in selective cation transport by plant cells. Plant Physiol. 36: 437–444.
7. Goldthwaite, J. J. and W. M. Laetsch. 1967. Regulation of senescence in bean leaf discs by light and chemical growth regulators. Plant Physiol. 42: 1757–1762.
8. Huffaker, R. C., R. L. Obendorf, C. J. Keller, and G. E. Kleinkopf. 1966. Effects of light intensity on photosynthetic carboxylative phase enzymes and chlorophyll synthesis in greening leaves of *Hordeum vulgare* L. Plant Physiol. 41: 913–918.
9. Huffaker, R. C. and L. W. Peterson. 1974. Protein turnover in plants and possible means of its control. Annu. Rev. Plant Physiol. 25: 363–392.
10. Kannangara, C. G. and H. W. Woolhouse. 1968. Changes in the enzyme activity of soluble protein fractions in the course of foliar senescence in *Perilla frutescens* (L.) Britt. New Phytol. 67: 533–542.
11. Kawashima, N. 1973. Studies on protein metabolism in higher plants. VII. Degradation mechanism of fraction I protein by a protease increased in tobacco leaves during curing. Hatano Fabako Shikenjo-Hokoku. 71: 111–116.
12. Kawashima, N., H. Fukushima, and E. Tamaki. 1967. Studies on protein metabolism in higher plant leaves. II. Variation in amino acid composition of protein in autolyzed tobacco leaves. Phytochemistry. 6: 339–345.
13. Kawashima, N., A. Imai, and E. Tamaki. 1967. Studies on protein metabolism in higher plant leaves. III. Changes in the soluble protein components with leaf growth. Plant Cell Physiol. 8: 447–458.
14. Kawashima, N. and T. Mitake. 1969. Studies on protein metabolism in higher plants. VI. Changes in ribulose diphosphate carboxylase activity and fraction I protein content in tobacco leaves with age. Agric. Biol. Chem. 33: 539–543.
15. Kawashima, N. and S. G. Wildman. 1970. Fraction I protein. Annu. Rev. Plant Physiol. 21: 325–358.

16. KEMP, J. D. AND D. W. SUTTON. 1971. Protein metabolism in cultured plant tissue. Calculation of an absolute rate of protein synthesis, accumulation, and degradation in tobacco callus *in vivo*. Biochemistry 10: 81–88.
17. KLEIN, M. AND I. HARPAZ. 1965. Proteolytic activity in maize leaves. Phytochemistry 4: 327–332.
18. KLEINKOPF, G. E., R. C. HUFFAKER, AND A. MATHESON. 1970. A simplified purification and some properties of ribulose 1,5-diphosphate carboxylase from barley. Plant Physiol. 46: 204–207.
19. KLEINKOPF, G. E., R. C. HUFFAKER, AND A. MATHESON. 1970. Light-induced *de novo* synthesis of ribulose 1,5-diphosphate carboxylase in greening leaves of barley. Plant Physiol. 46: 416–418.
20. KURAISHI, S. 1968. The effect of kinetin or protein level of *Brassica* leaf disks. Physiol. Plant 21: 78–83.
21. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
22. MARINAS, N. G. 1962. Studies on submicroscopic aspects of mintera deficiencies. I. Calcium deficiency in the shoot apex of barley. Amer. J. Bot. 49: 834–841.
23. MARTIN, C. AND K. V. THIMANN. 1972. The role of protein synthesis in the senescence of leaves. I. The formation of protease. Plant Physiol. 49: 64–71.
24. MIZRAHI, Y., J. AMIR, AND A. I. RICHMOND. 1970. The mode of action of kinetin in maintaining the protein content of detached *Tropaeolum majus* leaves. New Phytol. 69: 355–361.
25. PETERSON, L. W., G. E. KLEINKOPF, AND R. C. HUFFAKER. 1973. Evidence for lack of turnover of ribulose 1,5-diphosphate carboxylase in barley leaves. Plant Physiol. 51: 1042–1045.
26. RACUSEN, D. AND M. FOOTE. 1965. Protein synthesis in dark-grown bean leaves. Can. J. Bot. 43: 817–824.
27. RAINS, D. W. 1968. Kinetics and energetics of light-enhanced potassium absorption by corn leaf tissue. Plant Physiol. 43: 394–400.
28. SACHER, J. A. 1967. Senescence: action of auxin and kinetin in control of RNA and protein synthesis in subcellular fractions of bean endocarp. Plant Physiol. 42: 1334–1342.
29. SACHER, J. A. 1969. Hormonal control of senescence of bean endocarp: auxin-suppression of RNase. Plant Physiol. 44: 313–314.
30. SHIBAOKA, H. AND K. V. THIMANN. 1970. Antagonisms between kinetin and amino acids. Experiments on the mode of action of cytokinins. Plant Physiol. 46: 212–220.
31. SIMONS, E. W. 1967. Types of leaf senescence. Soc. Exp. Biol. Symp. 21: 215–230.
32. SKOOG, F. AND D. J. ARMSTRONG. 1970. Cytokinins. Annu. Rev. Plant. Physiol. 21: 359–384.
33. TAVARES, J. AND H. KENDE. 1970. The effect of 6-benzylaminopurine on protein metabolism in senescing corn leaves. Phytochemistry 9: 1763–1770.
34. TRAVIS, R. L., W. R. JORDAN, AND R. C. HUFFAKER. 1969. Evidence for an inactivating system of nitrate reductase in *Hordeum vulgare* L. during darkness that requires protein synthesis. Plant Physiol. 44: 1150–1156.
35. TREWAVAS, A. 1972. Control of the protein turnover rates in *Lemna minor*. Plant Physiol. 49: 47–51.
36. VAN NOORT, G. AND S. G. WILDMAN. 1964. Proteins of green leaves. IX. Enzymatic properties of Fraction-I protein isolated by specific antibody. Biochim. Biophys. Acta 90: 309-317.
37. VARNER, J. E. 1961. Biochemistry of senescence. Annu. Rev. Plant Physiol. 12: 245–264.
38. VICKERY, H. B., G. W. PUCHER, A. J. WAKEMAN, AND C. S. LEAVENWORTH. 1937. Chemical investigations of the rhubarb plant. Conn. Agric. Expt. Sta. New Haven Bull. No. 424.
39. WOLLGIEHN, R. 1967. Nucleic acid and protein metabolism of excised leaves. Soc. Exp. Biol. Symp. 21: 231–246.
40. WOOD, J. G. AND D. H. CRUICKSHANK. 1944. The metabolism of starving leaves. Aust. J. Exp. Biol. Med. Sci. 22: 111–123.
41. ZUCKER, M. 1972. Light and enzymes. Annu. Rev. Plant Physiol. 23: 133–156.