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Biochemical Changes that Occur during Senescence of Wheat Leaves[1]

I. BASIS FOR THE REDUCTION OF PHOTOSYNTHESIS

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ABSTRACT

Changes in activities of photosynthetic enzymes and photochemical processes were followed with aging of vegetative and flag leaves of wheat (*Triticum aestivum* L. cv Roy). Activities of stromal enzymes began to decline prior to photochemical activities. In general, total soluble protein and the activities of ribulose-1,5-bisphosphate carboxylase and NADP-triose-phosphate dehydrogenase declined in parallel and at an earlier age than leaf chlorophyll (Chl), leaf photosynthesis, and photosynthetic electron transport activity. Leaves appeared to lose whole chloroplasts as opposed to a general degradation of all chloroplasts based on three lines of evidence: (a) electron transport activity calculated on an area basis declined much earlier than the same data expressed on a Chl basis; (b) Chl content per chloroplast was similar for mature and senescent tissue; and (c) the absorbance at 550 nanometers (light scattering) per unit of Chl remained essentially constant until the end of senescence. Chloroplasts did, however, undergo some modifications before they were lost (*e.g.* loss of stromal enzyme activities), but the reduction in leaf photosynthesis was apparently caused by a loss of whole chloroplasts.

Senescence is a coordinated, deteriorative growth process that is initiated at full maturity and ultimately leads to the death of a cell, organ, or organism. To characterize a tissue that is undergoing senescence, it is necessary to identify the sequential order in which various parameters change. Butler and Simon (5) reported from ultrastructural studies that aging is first expressed in the chloroplast. Thus, changes in the physiology of this organelle are important to our understanding of senescence.

In leaves, senescence is typically characterized by a decline in photosynthesis. The cause for this decline, however, is unknown. Loss of RuBPCase[2] in relation to photosynthesis during senescence in wheat (*Triticum aestivum* L.) has been reported. In wheat, a close correlation between loss of RuBPCase and photosynthetic activities was found by Wittenbach (22), whereas Hall *et al.* (6) found that RuBPCase protein declined at a different rate than photosynthesis. Few studies have investigated loss of other stromal enzymes or studied electron transport (3, 4, 8, 18, 21), and simultaneous investigations of stromal enzymes and electron transport have not been performed. Parallel investigations of stromal enzymes and electron transport are needed to elucidate if these losses occur simultaneously or if one decreases prior to the other.

Wittenbach *et al.* (24) suggested that a sequential degradation of whole chloroplasts in wheat occurred because the number of chloroplasts per protoplast declined during senescence. Ultrastructural work by Peoples *et al.* (16) also showed a reduction in chloroplast number per cell. However, Jenkins and Woolhouse (8) in *Phaseolus vulgaris* L. observed a continual decline in electron transport per mg Chl, which suggested a gradual degradation of all chloroplasts. Studies of electron transport as well as other physiological parameters are needed before definitive conclusions can be made as to the loss of whole chloroplasts from wheat leaves.

In the present study, the time course of leaf development from full expansion through senescence was investigated in vegetative and flag leaves of wheat to determine the basis for reduction of photosynthesis. Specific objectives of this study were to determine (a) if electron transport activities or stromal enzyme activities declined first and (b) if there was a general decline in the photosynthetic functioning of all chloroplasts or if there was a preferential degradation of whole chloroplasts.

MATERIALS AND METHODS

Plant Material. *Triticum aestivum* L. cv Roy was grown in a growth chamber with a photon fluence rate of 3×10^{-4} mol/m^2·s (400–700 nm) and a 15-h photoperiod (22°C day/17°C night). For vegetative leaves, the second leaf to emerge was studied from the time of full expansion (about 14 d from planting) through senescence. Flag leaves were sampled from the point of emergence of the head from its sheath (arbitrarily designated day 0) through senescence. Flag leaves were fully expanded at this stage. Reproductive development was induced by vernalization of 12-d-old seedlings for 6 weeks at 2 to 4°C. All plants were fertilized twice weekly with Hoagland solution.

Two separate plantings of both vegetative and flag leaf material were used. Two to four replications of each parameter for each age were performed and the results from both plantings were averaged.

Leaf Area, Chl, and Protein Assays. Leaf area was measured with a Li-Cor[3] area meter (model LI-3000, Li-Cor, Inc.). Chl and Chl *a*/*b* ratios were determined by the procedure of Arnon (1).

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[2] Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase; RuBP, ribulose-1,5-bisphosphate; MV, methyl viologen.

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Total soluble protein was measured by the method of Murphy and Kies (14). Leaf tissue was collected 5 to 7 h after the beginning of the photoperiod for protein and Chl determinations. Tissue (about 0.3 g/4 ml buffer) was ground in 10 mM Tricine-NaOH (pH 8.0) and centrifuged at 13,000*g* for 5 min. A 0.05-ml aliquot was then diluted into 3 ml of water and the absorbance difference between A_{225} and A_{215} determined. This simplified procedure gave results similar to Lowry determinations (13) on acid-precipitated samples. Lysozyme (grade I, Sigma Chemical Co.) was used as the standard.

Enzyme Assays. Leaf tissue was collected 3 to 5 h after the beginning of the photoperiod unless noted otherwise, and leaf areas and fresh weights determined so that enzyme activities could be expressed either on a leaf area or fresh weight basis. Leaf tissue (about 0.3 g/4 ml buffer) was ground in a mortar and pestle (2°C) in a solution that contained 50 mM Tricine-NaOH (pH 7.9), 0.1% PVP-40, and 5 mM $MgCl_2$ followed by centrifugation at 13,000*g* for 5 min. One ml of the supernatant was desalted on a 4-ml Sephadex G-25-300 column preequilibrated with 50 mM Tricine-NaOH (pH 7.9) and 2 mM $MgCl_2$.

RuBPCase measurements were made essentially as described by Lilley and Walker (12). Enzyme extract was added to a final 1 ml volume reaction mixture that contained 50 mM Tricine-NaOH (pH 7.9), 10 mM KCl, 1 mM EDTA, 2 mM DTT, 0.2 mM NADH, 5 mM ATP, 15 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM phosphocreatine, 2 units/ml creatine phosphokinase, 4 units/ml each of NAD-dependent glyceraldehyde 3-P dehydrogenase, and 3-P glycerate kinase and incubated for 5 min at 25°C. Reactions were initiated by addition of 0.5 mM RuBP.

NADP-glyceraldehyde-3-P dehydrogenase was assayed by the procedure of Latzko and Gibbs (11). Enzyme extract was added to a reaction mixture that contained 100 mM Tricine-NaOH (pH 7.9), 10 mM $MgCl_2$, 2 mM DTT, 5 mM ATP, 1 unit/ml glycerate 3-P kinase, and 0.2 mM NADPH (final volume of 1 ml) and incubated for 5 min at 25°C. The reaction was initiated with 4 mM 3-P glycerate and the decrease in A_{340} was measured.

Photosynthetic Measurements. Photosynthetic measurements were made with leaf segments (9) cut from tissue harvested 2 and 3 h after the beginning of the photoperiod. From a minimum of three leaves, three (1 × 5 mm) segments from the second vegetative leaves and three (1 × 10 mm) segments from flag leaves were cut from a region 8 to 12 cm from the leaf tip. Each group of three segments was incubated at 25°C with or without illumination (6×10^{-4} mol/cm^2·s) in a reaction mixture that contained 0.5 ml 50 mM Hepes-NaOH (pH 7.5) and 10 mM $NaH^{14}CO_3$ (1.25 μCi/μmol). Photosynthesis was linear for at least 50 min, and an illumination time of 30 min was chosen for experiments reported in this paper. Reactions were terminated by the addition of 0.1 ml 10 N formic acid and 2.4 ml 100% ethanol The segments were then ground in a tissue homogenizer and an aliquot removed and evaporated to less than 0.2 ml for determination of the acid-stable radioactivity incorporated. Dark samples showed less than 8% of the light-rate incorporated throughout senescence.

Electron Transport. Thylakoid membranes were isolated by grinding leaf tissue (about 2 g/50 ml buffer) in a Waring Blendor in a solution that contained 0.4 M sorbitol, 0.1 M Tricine-NaOH (pH 7.8), 0.1% PVP-40 (w/v), and 0.05% β-mercaptoethanol (v/v) followed by sequential filtration through 4 and 12 layers of cheesecloth and centrifugation at 1000*g* for 6 min. The resulting pellet was washed in resuspension medium [100 mM sorbitol, 100 mM Tricine-NaOH (pH 7.8), 5 mM $MgCl_2$, and 10 mM NaCl] and centrifuged at 10,000*g* for 5 min. The pellet was again resuspended in resuspension medium.

Whole chain electron transport activity ($H_2O \rightarrow MV$) was measured by recording O_2 uptake with a water-jacketed Clark-type O_2 electrode (25°C). Assay mixtures (2) for coupled electron transport activity contained 100 mM sorbitol, 100 mM Tricine-NaOH (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM MV, and chloroplast thylakoids. Uncoupled electron transport activities were determined by the addition of 10 mM NH_4Cl to the assay mixture. Sodium azide had no effect on the rate of O_2 uptake, which indicated that the thylakoids were relatively free of catalase.

Light Scattering and Chl/Chloroplast. Light scattering was measured in resuspension medium by determining the A_{550} of an aliquot (25 μg Chl) of the thylakoid membranes used to study electron transport. Preparations of chloroplasts from mature and senescent leaf tissue were also used to determine if there was a change in the amount of Chl per chloroplast. The number of chloroplasts (2.5 μg Chl/ml) was determined with a hemocytometer at × 10 magnification, and Chl content was measured as described above. Chloroplast intactness was not a factor in these measurements, because both intact and envelope-free chloroplasts could be resolved easily.

Gel Procedures. Analysis of chloroplast proteins by SDS-polyacrylamide gel electrophoresis was conducted with the discontinuous buffer system of Laemmli (10). Electrophoresis was performed with a continuous 12 to 16% (w/v) polyacrylamide separating gel and a 5% (w/v) stacking gel in a slab gel apparatus (20). Samples containing equal amounts of Chl were solubilized in a 65 mM Tris-HCl (pH 6.8) sample buffer that contained 10% (v/v) glycerol, 1% (v/v) β-mercaptoethanol, and 2% (w/v) SDS. Electrophoresis was conducted at a constant current of 35 mamp. The gels were stained for protein in a solution that contained 0.2% (w/v) Coomassie blue, 50% (v/v) methanol, and 7% (v/v) glacial acetic acid for 30 min followed by destaining in 20% methanol and 7% acetic acid. Coupling factor from wheat leaves was purified by the procedure of Strotmann *et al.* (19).

RESULTS

Leaf Chl content, uncoupled electron transport activity in isolated thylakoid membranes, and leaf photosynthesis showed generally similar patterns of loss during senescence of the second vegetative leaf (Fig. 1A). Initially, photosynthesis declined whereas leaf Chl content and electron transport activity increased. However, all three parameters remained relatively constant from day 17 until day 23, after which the parameters declined continually. Total soluble protein, RuBPCase, and NADP-triose-P dehydrogenase activities also had similar patterns of decline (Fig. 1B). From the time of full expansion, maximum levels were observed at day 17 and declined continually thereafter. The various parameters measured were expressed on a leaf area basis because, unlike other parameters, area remained constant throughout senescence. Expression of the data on a fresh weight or dry weight basis, as opposed to an area basis, produced similar trends (data not shown). Activities of the stromal enzymes, RuBPCase and NADP-triose-P dehydrogenase, as well as total soluble protein declined at an earlier age than photochemical processes (leaf photosynthesis or electron transport activity) as shown by comparison of Figures 1A and 1B. For example, at age 26 d where 65% of the stromal enzymes had been lost, photosynthesis had decreased only 36% and uncoupled electron transport activity only 23%.

In general, similar trends were observed during senescence of flag leaves. The photochemical parameters of flag leaves reached maximum levels at 2 weeks and declined thereafter (Fig. 2A). Stromal enzyme activities and total soluble protein, on the other hand, were maximal at emergence and declined thereafter (Fig. 2B). Total soluble protein did not follow stromal enzyme activities as closely in flag leaves as in vegetative leaves. As shown by comparison of Figures 2A and 2B, stromal enzyme activities once again decreased at an earlier age than photochemical activities.

Changes in Chl *a*/*b* ratios during senescence were measured in vegetative and flag leaves. In both cases, a gradual decline was observed throughout the senescence process. Flag leaves declined

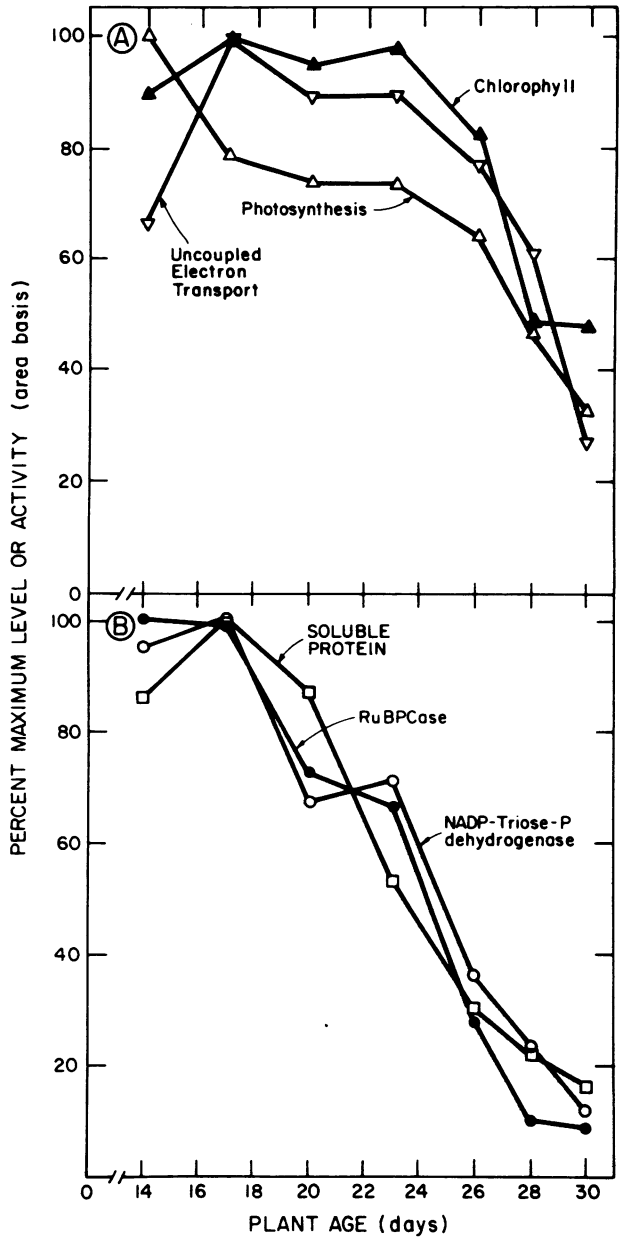

FIG. 1. Changes in total soluble protein, photochemical parameters, and activities of selected chloroplast enzymes during senescence of the second vegetative leaf of wheat. A, Changes in: Chl, (▲, 44 $\mu g/cm^2$); uncoupled electron transport rates, (▽, 6.6 μmol $O_2/cm^2 \cdot h$); leaf photosynthesis, (△, 2.6 μmol $CO_2/cm^2 \cdot h$). B, Changes in: total soluble protein (□, 3.08 mg/cm^2); RuBPCase activity, (●, 19.2 μmol product/$cm^2 \cdot h$); and NADP-triose-P dehydrogenase activity, (○, 51.2 μmol NADPH/$cm^2 \cdot h$) with age. Absolute values corresponding to 100% in the figure, are expressed parenthetically following each parameter. Age represents days from time of planting.

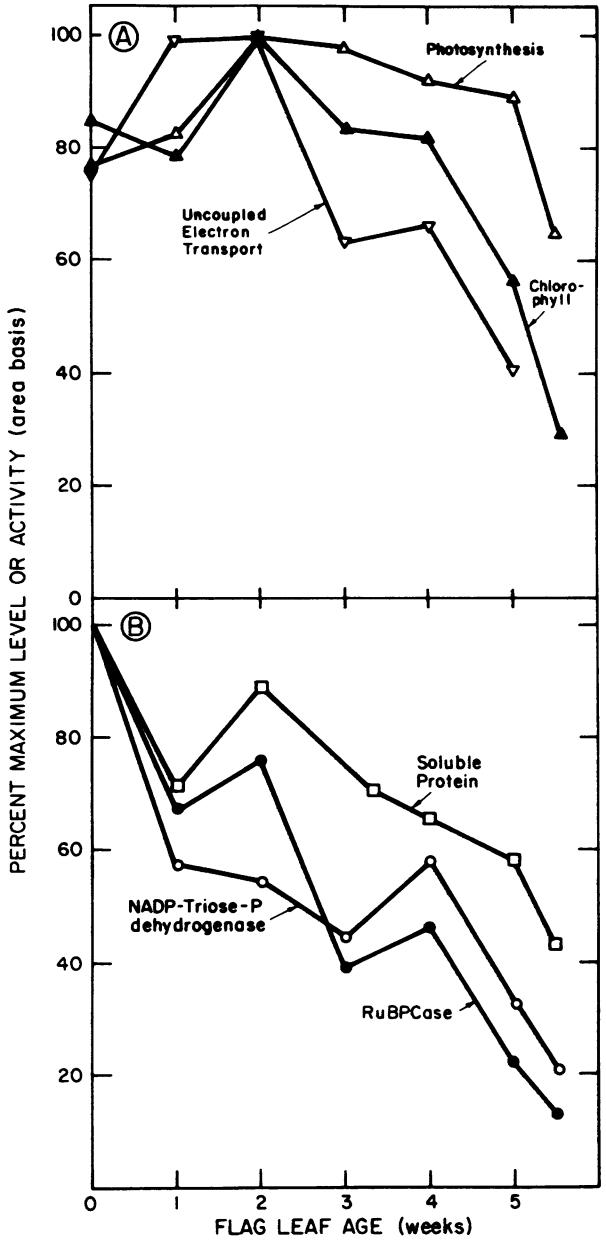

FIG. 2. Changes in total soluble protein, photochemical parameters, and activities of selected chloroplast enzymes during senescence of the flag leaf of wheat. A, Changes in: Chl, (▲, 77 $\mu g/cm^2$); uncoupled electron transport rates, (▽, 11.4 μmol $O_2/cm^2 \cdot h$); leaf photosynthesis, (△, 2.4 μmol $CO_2/cm^2 \cdot h$). B, Changes in: total soluble protein, (□, 1.84 mg/cm^2); RuBPCase activity, (●, 33.5 μmol product/$cm^2 \cdot h$); and NADP-triose-P dehydrogenase, (○, 91.2 μmol NADPH/$cm^2 \cdot h$) with age. Absolute values corresponding to 100% in the figure, are expressed parenthetically following each parameter. Age in weeks is from time of opening of the leaf sheath from around the head.

from an *a/b* ratio of 4.47 at 0 weeks to 2.9 at 5.5 weeks, whereas the ratio in the second vegetative leaf declined from 3.87 at 14 d to 3.00 at 30 d. Thus, flag leaves had a much higher initial *a/b* ratio, but declined to approximately the same value as the vegetative leaf by the end of senescence (data not shown).

The apparent pattern of loss of chloroplast thylakoid electron transport activity during senescence depended upon whether activity was expressed on an area or Chl basis. Whole chain electron transport activity expressed on a leaf area basis (Fig. 3) declined after 23 d of age. The same data expressed on a Chl basis remained relatively constant from 17 to 28 d of age, after which it declined. The results corresponded to a loss of 44% of the activity after 28 d based on area and only a 9% loss of activity at the same age on a Chl basis. Similar results were obtained with thylakoids obtained from flag leaves (data not shown). At 4 weeks, 53% of electron transport activity was lost on an area basis whereas only 23% was lost on a Chl basis. The results suggested that whole chloroplasts were lost from the tissue. This postulate was supported by the observation that with age there was no loss in the amount of Chl per chloroplast in vegetative tissue (mature leaves had 4.30×10^4

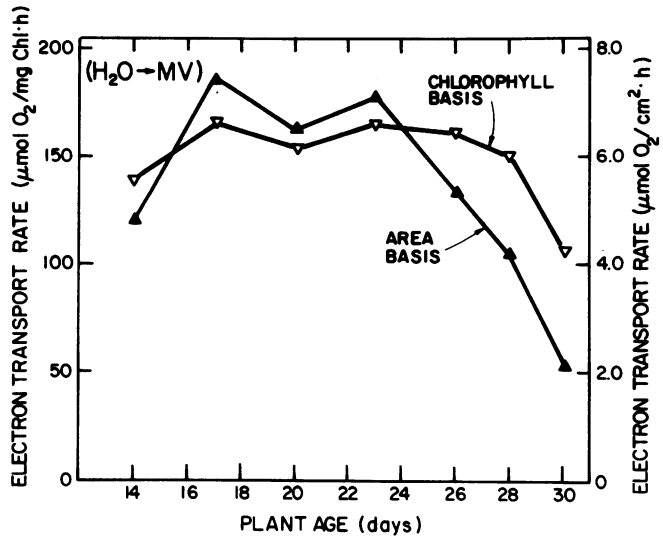

FIG. 3. Changes in the rate of uncoupled whole chain electron transport expressed on a Chl (▽) and leaf area (▲) basis during senescence of the second vegetative leaf of wheat. Water served as the electron donor and MV as the electron acceptor.

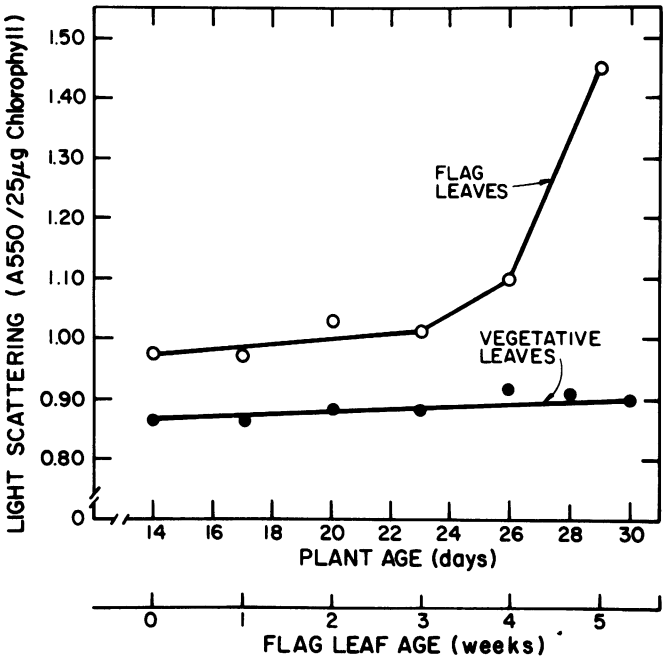

FIG. 4. Changes in light scattering as determined by A at 550 nm of vegetative (●) and flag leaf (○) thylakoids isolated from wheat of different ages.

chloroplasts/μg Chl ± 0.06 SE and senescent tissue had 4.33×10^4 chloroplasts/μg Chl ± 0.17 SE).

The results led to a study of the light scattering properties (measured as A_{550}) of thykaloids from both vegetative and flag leaves. As shown in Figure 4, light scattering by thylakoid membranes from vegetative leaves remained relatively constant with leaf age (A_{550} = 0.90/25 μg Chl). Light scattering of thylakoid membranes from flag leaves was essentially constant until after the third week, when an increase in A_{550} from 1.0 to a final value of 1.45/25 μg Chl occurred. The thylakoids isolated were representative of the leaf plastid population at every leaf age, because Chl a/b ratios of leaves and isolated thylakoids declined similarly during senescence. Assuming thylakoid membranes remain stacked with age, an increase in A_{550} (increased light scattering)

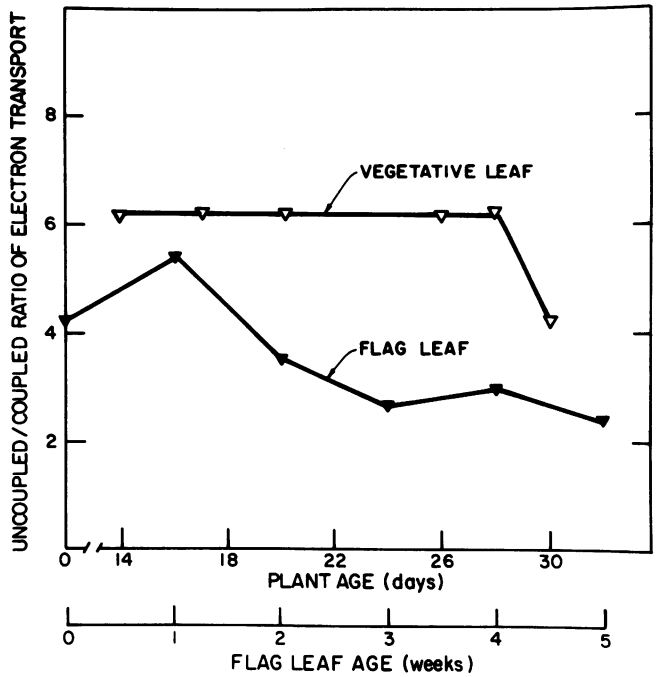

FIG. 5. Changes in the ratio of uncoupled/coupled whole chain electron transport rates in vegetative (▽) and flag leaf (▼) thylakoids of wheat with age. Electron transport rates were determined as described in "Materials and Methods."

per unit of Chl would suggest that chloroplasts were gradually losing Chl. If, however, whole chloroplasts were lost from the tissue, light scattering would remain constant. The results supported the postulate that whole chloroplasts were lost from leaves, although some general degradation of chloroplasts from flag leaves may occur in the latter stages of senescence (*i.e.* 4th and 5th weeks). The light scattering technique was useful only because wheat chloroplasts accumulated little starch that might interfere with the assay.

The ratio of uncoupled to coupled electron transport activity in isolated thylakoids was determined for vegetative and flag leaves of increasing age (Fig. 5). The ratio for vegetative leaves remained constant at 6.2 until 28 d of age when the ratio declined to a final value of 4.2. Thylakoid membranes from flag leaves, on the other hand, exhibited a gradual decline from a maximum ratio of 5.4 at 1 week to a final value of 2.4 at 5 weeks. The constant ratio of uncoupled to coupled electron transport suggests that thylakoid membranes of vegetative leaves remained tightly coupled, whereas in flag leaves the degree of coupling was gradually reduced throughout senescence.

Loss of coupling was correlated with a loss of coupling factor protein from vegetative leaf chloroplasts, as evidenced by gel electrophoresis of isolated thylakoid membranes (Fig. 6). Purified coupling factor (2nd and 5th lanes) was included in the gel for comparison with thylakoid preparations. On a Chl basis, chloroplasts from mature leaves (Fig. 6, lane 3) had more α- and β-subunits of coupling factor (approximately 55 kD) than chloroplasts from senescent tissue (Fig. 6, lane 4), thereby showing a selective loss of coupling factor protein from the older tissue. Similar correlations between loss of coupling and coupling factor were also observed for flag leaves (gel not shown). Subunits of coupling factor were not detected in gels of soluble fractions (gel not shown) obtained during preparation of the thylakoid membranes, which indicated that coupling factor was not removed during isolation.

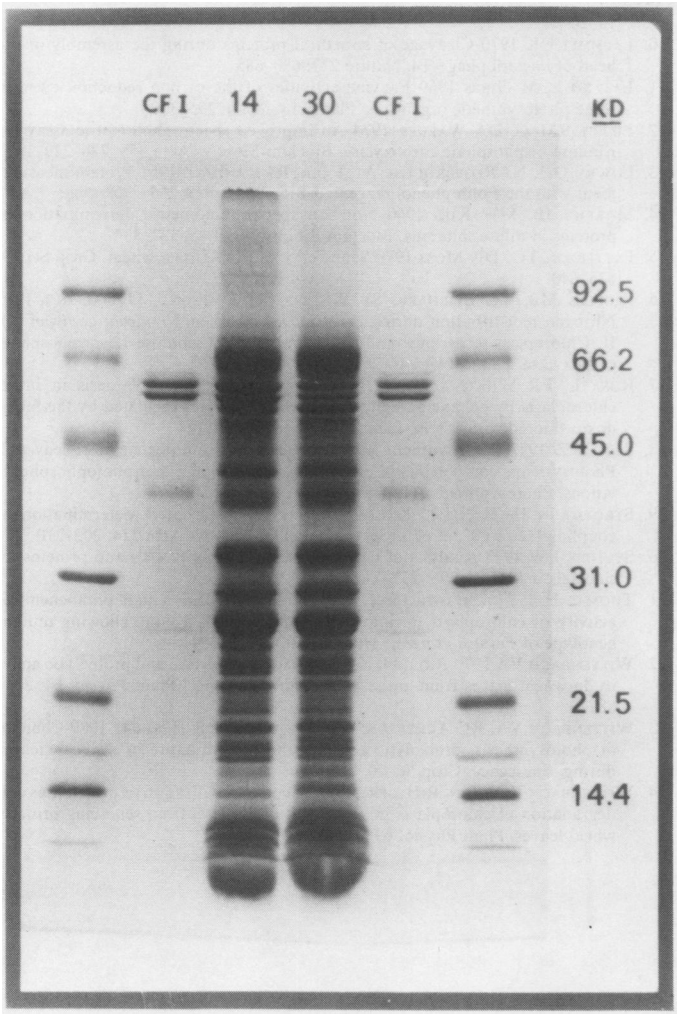

FIG. 6. Polyacrylamide slab gel of wheat thylakoid membranes that shows a loss of coupling factor in the latter stage of senescence. Lanes 1 and 6 are standards of known mol wt; lanes 2 and 5 are purified coupling factor from wheat; and lanes 3 and 4 are thylakoid membranes from 14- and 30-d-old wheat plants, respectively. The thylakoid samples (lanes 3 and 4) contained equal amounts of Chl. The mol wt standards (obtained from Bio-Rad) used in this study were as follows: phosphorylase B (92,500); BSA (66,200); ovalbumin (45,000); carbonic anhydrase (31,000); soybean trypsin inhibitor (21,500); and lysozyme (14,400).

DISCUSSION

The results obtained in the present study suggest that the loss of photosynthetic activity during senescence in wheat is related to the loss of whole chloroplasts from leaves as opposed to a general degradation of the entire chloroplast population. The postulate is supported by several lines of evidence. First, electron transport rates in isolated thylakoids prepared from both vegetative and flag leaves began to decline at mid-senescence when activities were expressed on a leaf area basis, whereas on a Chl basis, the rates declined only at the latter stages of senescence. The data suggested that the chloroplasts remaining in the leaf were photochemically active until late senescence. Second, thylakoid membranes remained coupled to the proton gradient generated during electron transport (Fig. 5). Loss of apparent coupling that did occur corresponded to a loss of coupling factor as evidenced by SDS-gel electrophoresis (Fig. 6). A loss of coupling factor from thylakoid membranes, would be accompanied by an increased permeability of the membrane to protons and a decreased ratio of uncoupled to coupled electron transport would result (7). Third, the amount of Chl per chloroplast was constant throughout senescence of vegetative leaves, which implied that loss of Chl from leaf tissue was the result of a loss of whole chloroplasts from the leaf. Further support was provided by light scattering studies where no change in absorbance per unit of Chl was observed in chloroplasts from vegetative leaves throughout senescence. In flag leaves, light scattering increased at the last two sampling dates. Hence, a general degradation of chloroplasts may have occurred at the latter stages of senescence, whereas a preferential degradation of whole chloroplasts occurred prior to the final stages of senescence. Evidence from other studies that involved wheat and support a loss of whole chloroplasts includes a loss of chloroplasts per protoplast (24) and a decrease in chloroplast number per cell (16).

Even though wheat leaves lost whole chloroplasts, there were changes that apparently occurred in the chloroplasts prior to complete degradation. A change in chloroplasts was evidenced by the earlier decline of the activities of the stromal enzymes NADP-triose-P dehydrogenase and RuBPCase compared to photosynthetic electron transport activity, leaf photosynthesis, or Chl. The loss of stromal enzymes did not, however, greatly limit photosynthesis as shown by comparison of Figures 1 and 2. Photosynthesis did not begin to continually decline until after day 23, at which point the stromal enzymes had already decreased by almost 50%. Maximum activities of RuBPCase and NADP-triose-P dehydrogenase in leaf extracts were 2 to 5 times greater than needed to account for carbon fixation. Changes in photosynthesis most closely paralleled changes in Chl content considering results obtained with both vegetative and flag leaves. Other investigators have also reported correlations between loss of Chl and photosynthesis in both wheat and soybeans (22, 23).

Several investigations of photosynthetic electron transport activities during senescence have been attempted. However, as pointed out in a review by Sestak (18), chloroplast isolation procedures and reaction conditions were not always optimal. In the present study, electron transport activities were relatively high and were similar to those of other workers (2, 17). As previously mentioned, rates of electron transport activity, expressed on a Chl basis, did not decrease until the final stages of senescence. This is in contrast to results obtained by Jenkins and Woolhouse (8) with *Phaseolus vulgaris* in which electron transport decreased throughout senescence on a Chl basis. Their data (8) would be consistent with a general degradation of the entire chloroplast population and is in contrast with the results of the present study. The discrepancy could perhaps be caused by differences in genotypes or growth conditions.

Total soluble protein losses paralleled losses of RuBPCase and NADP-triose-P dehydrogenase activities. The correlation was greater in vegetative leaves than in flag leaves. Because RuBPCase constitutes 40 to 50% of the total soluble protein in wheat flag leaves and an even greater percentage in vegetative leaves (22), it is not unreasonable that a closer correlation of protein and stromal enzymes should occur in vegetative tissue. The data also suggest that the activities of RuBPCase and NADP-triose-P dehydrogenase were degraded at the same rate as other soluble proteins in vegetative leaves and at a slightly greater rate than other soluble proteins in flag leaves. The suggestion of a preferential loss of RuBPCase in flag leaves is supported by previous investigations (16, 22).

Results from flag leaves were, in general, more variable than results obtained with vegetative leaves. Several factors may contribute to this variability. First, the vegetative leaves all emerged at the same time and senesced over approximately a 2-week period. Flag leaves, on the other hand, emerged at asynchronous times and senesced over a much longer time course (5½ weeks). Also, flag leaf size was highly variable with lengths that ranged from 8 to 20 cm, whereas vegetative leaves were all similar in length. Despite these differences, parameters that varied together

in vegetative leaves generally showed similar patterns in flag leaves.

A decline in Chl *a/b* ratios was observed in both vegetative and flag leaves during senescence. This loss may have been caused by a shading effect in vegetative leaves and a continual downward decline in leaf angle away from light in flag leaves as suggested by the work of Patterson and Moss (15). They found Chl *a/b* ratios to vary with leaf position in the canopy and the amount of light that reached the leaf. Regardless of the reason for decline, changes in *a/b* ratios indicated that changes were occurring in the chloroplast membranes, but it is important to note that these changes did not seem to affect photosynthesis in general.

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