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Initial Stages in the Onset of Senescence in Tobacco Leaves

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ABSTRACT

A marked loss of leucine ^{14}C incorporation occurred in chloroplasts isolated from *Nicotiana rustica* L. leaves exposed to 24 hours of darkness. This loss is not due to an initial decline in RNA-synthesis potential of the chloroplasts, as was inferred from the extent of UTP incorporation by the isolated chloroplasts. Upon reillumination of the leaves, leucine incorporation by the isolated chloroplasts reverted to its original level within 3 to 4 hours, hence it is doubtful whether the period of 24 hours after detachment should be regarded as the initial phase of leaf senescence.

After 48 and 72 hours of darkness, however, complete recovery of the incorporation activity was not achieved by reillumination of the leaves, representing the apparent onset of an irreversible process. Treatment with kinetin, which markedly delayed the symptoms of senescence in these tobacco leaves, did not prevent the dark-induced decline in chloroplast protein synthesis activity. Nor, up to 24 hours of darkness, did it have any effect on the light-induced complete recovery of this synthesis. Nevertheless, after reilluminating kinetin-treated leaves that had been exposed to darkness for 48 and 72 hours, leucine incorporation in the isolated chloroplasts was resumed at a faster rate and reached a higher level than did the untreated controls.

Detached leaves maintained in darkness provide a standard system for the study of leaf senescence. Various senescence-induced modifications in such leaves have been documented. These include structural alterations in the volume and shape of the chloroplast (5, 6, 9) as well as changes in several metabolic activities. The latter are reflected in the well known decline in the contents of RNA, protein, and Chl of leaf cells (3, 8). These senescence symptoms become clearly observable usually some 48 hr after leaf detachment. However, Richmond *et al.* (8) reported that in detached tobacco leaves kept in darkness, a marked change in the protein synthesis potential of the chloroplasts isolated from these leaves takes place within 24 hr.

This study aims at a more detailed characterization of the decline in the protein synthesis activity of chloroplast isolated from senescing leaves.

MATERIALS AND METHODS

Leaves of *Nicotiana rustica* L. grown in a greenhouse in half-strength Hoagland solution were washed thoroughly under a stream of running tap water and sterilized in a 70% ethanol solution for 10 sec. They were then dipped in 1% calcium hypochlorite for 60 sec and finally washed in sterile H_2O. The major veins were removed, and the pieces of the blade were placed in a plastic bag, weighed, and cooled in ice for 5 min. The leaf pieces were scissors-cut into an ice-cooled mortar and the chloroplasts were extracted according to Spencer and Wildman (10). Two ml Honda solution (containing 25 mg of Ficoll, 50 mg of Dextran T-40, 250 μmoles of sucrose, 100 μg of BSA, 42 μmoles mercaptoethanol fatty-acid poor [Calbiochem] 100 μmoles of magnesium acetate, 100 μmoles of manganese acetate) were added for each g of leaf tissue. This mixture was then chopped with a razor blade for 40 sec and ground with a pestle for an additional 10 sec. The mixture was filtered through Miracloth and centrifuged at 1000g for 1 min at 2 to 4 C. The pellet was dispersed in Honda solution, 0.1 ml for every 1 g of leaf tissue. The incorporation medium was as described by Spencer and Wildman (10); the total volume in each test tube was 0.18 ml and included 2.3 μmoles of tris, pH 7.8; 12 mM Mg^{2+}; 13.8 μmoles of KCl; 1.25 μmoles of phosphoenolpyruvate; 10 μmoles of pyruvate kinase (Boehringer, Mannheim, Germany); 0.02 μmole of each of UTP, CTP, and GTP; 0.3 μmole ATP, 0.0125 μmole each of 19 amino acids except leucine; 0.25 μCi of ^{14}C-L-leucine, specific radioactivity 335 Ci/mole (Radio-chemical Centre, Amersham.); and 0.02 ml of chloroplast suspension. The tubes were placed in a shaker at 23 C, and the reaction was terminated after 30 min, by adding 0.1 ml of 5 mM DL-leucine to each tube, followed by shaking for 10 sec. This procedure substantially reduced the radioactive background due to absorption of free leucine to filter paper discs. The contents of each tube was pipetted onto Whatman No. 3 paper discs, dried quickly by exposure to hot air, washed twice in 70% ethanol saturated with DL-leucine (completely removing the Chl), and left overnight in 5% trichloroacetic acid and then in 100% ethanol; radioactivity was determined in a Packard Tricarb liquid scintillation spectrometer. UTP incorporation by isolated chloroplasts was measured according to Wollgiehn (12). Leaves were pretreated with 20 μM kinetin and 4 μM Actinomycin D by being placed between layers of cheesecloth, thoroughly wetted with the hormone or the inhibitor, and then kept in darkness at room temperature for 20 min and 5 min, respectively.

Twenty-four hr before initiation of the experiment, whole plants were transferred into a growth chamber, with temperatures at 22 C, relative humidity at 70%, light intensity 8000 lux, and a photoperiod of 12 hr light and 12 hr darkness. Dark treatment began after 4 hr of a new photoperiod. Detached leaves taken from growth-chamber plants were washed and arranged on plastic mesh trays kept in glass containers. The sides of these containers were lined with wet paper and there was a layer of water on the bottom. Thick black cloth over the containers kept the light out. For reillumination, trays bearing the leaves were transferred into the illuminated growth

chamber. "Light" chloroplasts were extracted before the dark treatment began. Many experiments indicated that the incorporation activity of such chloroplasts was essentially identical with that of chloroplasts isolated 24 hr later, after an additional photoperiodic cycle.

The quantity of Chl in a chloroplast preparation was determined by taking a 0.02-ml aliquot from the chloroplast suspension and extracting it with 5 ml of 80% ethanol. Afterwards the absorbance of the extract was determined at 665 nm.

RESULTS

Figure 1 shows the incorporation of leucine as a function of time of incubation. The incorporation into "dark" chloroplasts, *i.e.* those isolated from leaves exposed to 24 hr of darkness, was some 25% of that observed in "light" chloroplasts, *i.e.* those isolated from comparable leaves exposed to light. Except for the marked difference in total incorporation, the curve describing incorporation was essentially similar for both light and dark chloroplasts. In both preparations we observed a linear rate for the first 30 min of the reaction, which subsequently flattened out and then reached a plateau some 60 min after the beginning of incubation.

In considering the possible reasons for the sharp decline in the incorporation of leucine by isolated chloroplasts, we tested for a possible effect of various factors. Direct illumination of the isolated chloroplasts showed that neither the dark nor light chloroplasts showed any response (data not shown). Since ATP in the incubation medium was a mandatory requirement for incorporation of amino acids into the chloroplasts, we queried whether dark-induced reduction in leucine incorporation resulted from a deficiency in endogenous ATP. We premised that if this possibility was valid, dark chloroplasts would respond more vigorously to ATP than would light chloroplasts. Figure 2 relates incorporation activity to the concentration of ATP in the incubation medium. Optimal ATP concentration for maximal incorporation activity was identical for both dark and light chloroplasts. Similarly, preliminary probes indicated that within the limits of our experiments, incorporation activity was a function of the total concentration of leucine in the incubation medium. Varying permeability of the chloroplasts to the amino acid would thus in itself result in corresponding modifications in incorporation. As indicated in Figure 3, light and dark chloroplasts responded identically to increasing concentration of leucine in the incubation medium, maximal incorporation activity being reached at the same concentration. Similar results were obtained in the response of light and dark chloroplasts to the concentration of Mg^{2+}, GTP, and amino acids.

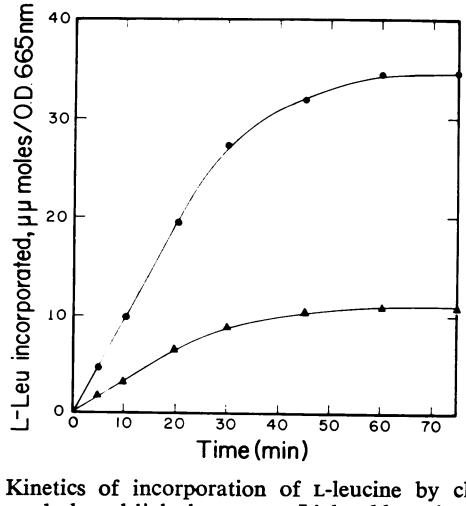

FIG. 1. Kinetics of incorporation of L-leucine by chloroplasts isolated from dark and light leaves. ●: Light chloroplasts (isolated before the beginning of the exposure to darkness); ▲: dark chloroplasts (isolated from leaves exposed to 24 hr of darkness.

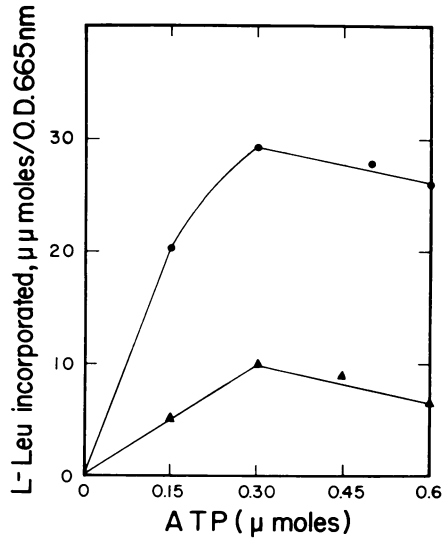

FIG. 2. Effect of the ATP concentration in the incubation medium on incorporation of L-leucine by chloroplasts isolated from dark and light leaves. Symbols are the same as those defined for Fig. 1.

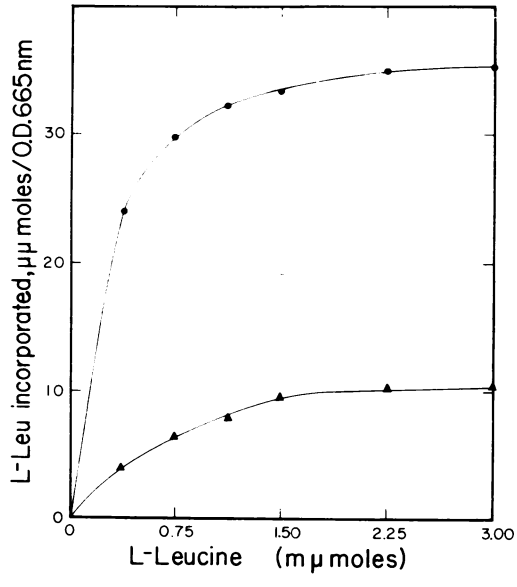

FIG. 3. Effect of the concentration of L-leucine on the extent of its incorporation by chloroplasts isolated from dark and light leaves. Symbols are the same as those defined for Fig. 1.

We tested the possibility that the observed decline in leucine incorporation reflected an artifact caused by isotopic dilution of the radioactive precurser. Accordingly, the leucine pool would be increased in the dark chloroplast, resulting in an apparent decline in leucine ^{14}C incorporation. To verify this possibility, the effect of diluting the specific radioactivity of the label in the incubation media on the incorporation activity into dark and light chloroplast was tested. Specific radioactivity was modified by increasing the concentration of leucine, maintaining radioactivity at a fixed (0.25 μCi) level. Measurement of the radioactivity (dpm) incorporated into an acid-insoluble

Table I. *Effect of Modifying Specific Radioactivity of L-leucine in Incubation Medium on Extent of Incorporation of Light and Dark Chloroplast*

Leucine Incorporation		Specific Radioactivity	Dilution Factor	Factor of Relative Change in Incorporation[1]
Light	Dark			
dpm		*Ci, mole*		
4482	947	335	2.1	2.1
2101	450	160		2.1

[1] In light *versus* dark chloroplasts.

material indicated that doubling the isotopic dilution of leucine resulted in a similar effect on light and dark chloroplasts (Table I), indicating that the curtailed incorporation into dark chloroplasts was not a pool effect.

Dithiothreitol, a potent reducing agent, reportedly reactivates various enzyme systems previously inactivated by darkness (1, 2), hence our query whether DTT[1] could restore the incorporation activity of dark chloroplasts. Although dark chloroplasts responded to some extent to increasing concentration of DTT, the response of light chloroplasts to DTT was significantly greater (Fig. 4). As with the response curve to ATP and to leucine concentration however, maximum incorporation for both dark and light chloroplasts was reached at the same concentration of DTT in the incubation medium (Fig. 4).

The only treatment that was found to restore fully the initial incorporation activity of isolated chloroplasts was reillumination of the leaves for 3 to 4 hr prior to extraction of the chloroplasts (Fig. 5). It is worth noting that the rate of light-induced restoration of leucine incorporation was several times faster than the rate of dark-induced decline in incorporation. In addition, the extent and the rate of decline in incorporation activity and its full (light-induced) resumption after 24 hr of darkness was identical in detached and attached leaves. Since the decline in chloroplast incorporation activity may be the result of a previous decline in RNA synthesis in this organelle, we tested the effect of darkness on the RNA synthesis potential of isolated chloroplasts. Figure 6A indicates that concomitant with the decline in incorporation of amino acid, there was a parallel decline in incorporation of ^{14}C UTP into an acid-insoluble material after the leaves were exposed to darkness. While reillumination resulted in complete resumption of the leucine-incorporation activity, the increase in UTP incorporation of the isolated chloroplasts was more sluggish: following a lag of about 1 hr, a level of only 50% of the original was reached after 4 hr. Treatment of the leaves with actinomycin D before transfer to light inhibited the light-induced rise in incorporation of UTP into RNA although not affecting the full light-induced increase in protein synthesis. Only after some 6 hr of light, actinomycin D affected a sharp decline in leucine incorporation (Fig. 6B).

Reillumination of the leaves after longer exposure to darkness, *i.e.* 48 to 96 hr, did not result in full restoration of the incorporation potential (Table II). In addition, maximal restoration could be achieved only by a longer period of leaf reillumination. Hence, after the initial 24 hr of darkness, a different or additional process is apparently involved in the dark-induced loss of leucine incorporation activity.

Kinetin did not affect the extent of incorporation activity of chloroplasts isolated from leaves exposed to 24 hr of darkness (Fig. 7A). However, after 48 and 72 hr of darkness, when reillumination of the leaves only partially restored the incorporation activity of the isolated chloroplasts, chloroplasts from reilluminated, kinetin-treated leaves showed a much improved response. Not only was the maximal incorporation activity higher in these chloroplasts but also the rate of light-induced resumption of activity was higher (Fig. 7, B and C). Nevertheless, leaf treatment with kinetin could not restore the incorporation activity to the initial level, and the incorporation of chloroplasts from kinetin-treated leaves was higher only as compared with that of the control chloroplasts, *i.e.* those extracted from nontreated leaves. In some experiments, kinetin was added directly into the incubation medium of the isolated chloroplasts but no effect was obtained.

DISCUSSION

Our data indicate that the loss in protein synthesis potential after 24 hr of darkness was apparently not due to damage to

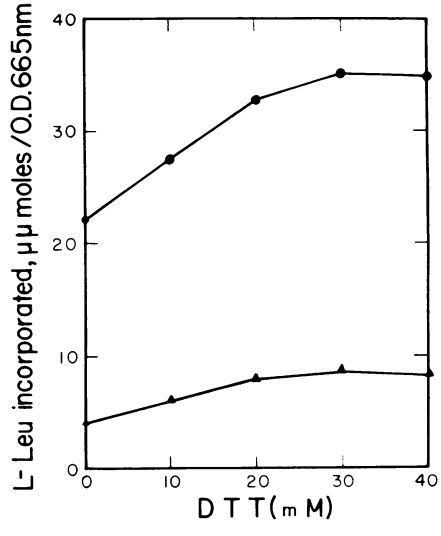

FIG. 4. Effect of DTT on the incorporation of L-leucine by chloroplasts isolated from dark and light leaves. Symbols are the same as those defined for Fig. 1.

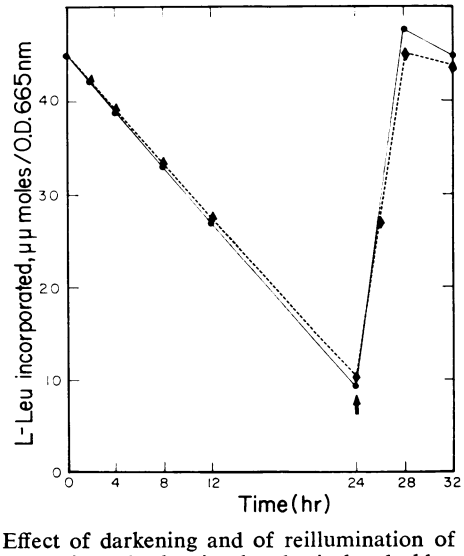

FIG. 5. Effect of darkening and of reillumination of the leaves on the incorporation of L-leucine by the isolated chloroplasts. ●: Chloroplasts isolated from attached leaves; ▲: chloroplasts isolated from detached leaves. At the time indicated by the arrow, leaves were transferred to the illuminated growth chamber.

[1] Abbreviation: DTT: dithiothreitol.

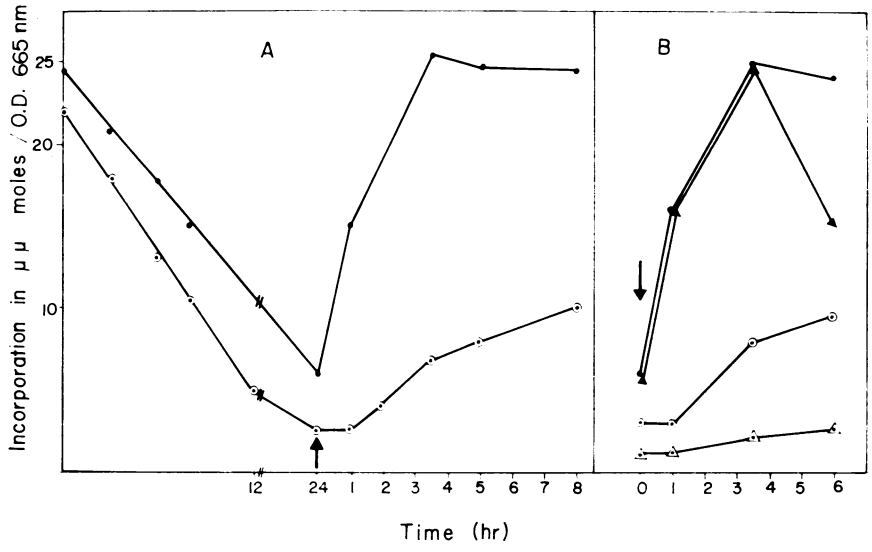

FIG. 6. Effect of the light regime on leucine and UTP incorporation by isolated chloroplasts. A: Effect of darkness and re-illumination of the leaves; B: effect of pretreatment of leaves with actinomycin D on light-induced restoration of incorporation. At the time indicated by the arrow, leaves were transferred to the illuminated growth chamber. ●: L-Leucine incorporation; ⊙: UTP incorporation; ▲: L-leucine incorporation in chloroplasts isolated from actinomycin D-treated leaves, △: UTP incorporation in chloroplasts isolated from actinomycin D-treated leaves.

Table II. *Effect of Reillumination of Leaves Exposed for Varying Times to Darkness on Restoration of Protein Synthesis Potential of Isolated Chloroplasts from Such Leaves*

Time in Darkness before Reillumination	Restoration of Original[1]	Time of Reillumination Needed for Maximal Restoration
hr	%	*hr*
24	102	4
48	60	8
72	37	10
96	20	12

[1] 100% incorporation refers to incorporation of L-leucine by isolated chloroplasts before exposure of leaves to darkness.

the integrity of the chloroplast. Thus, the kinetics of the incorporation was similar for both dark and light chloroplasts wth the only change being the magnitude of the incorporation. Neither direct illumination of the isolated chloroplasts nor addition of DTT eliminated the effect of darkness. Moreover, there was no change in the response of the dark chloroplasts to ATP, leucine, GTP, amino acids, or Mg^{2+}—upon which factors the reaction was dependent. The effect of increasing concentrations of the L-leucine on its incorporation by chloroplasts isolated from dark and light leaves, as well as the effect of isotopic dilution on the relative extent of incorporation into light and dark chloroplasts were tested. No support was found for the possibility that the dark-induced decline in leucine incorporation reported here merely reflected light-induced modification of the endogenous leucine pool. It seems clear that the decline did not result from a lack of those factors reportedly required for resumption of activity in other systems inactivated by darkness (1, 2, 4, 11).

After a 24-hr exposure to darkness, illumination of the leaves for 3 to 4 hr completely restored incorporation activity of the isolated chloroplasts without any lag period. This indicated that for the initial 24 hr the dark-induced decline in synthesis was not associated with the over-all degradative process that takes place in leaves after detachment. Pretreatment of the leaves with actinomycin D did not affect the complete light-induced restoration of leucine incorporation, although inhibiting the light-induced restoration of UTP incorporation, thus indicating that the dark-induced decline in protein synthesis did not result from the decline in RNA synthesis.

Exposure to a 48-hr or 72-hr period of darkness apparently initiated some irreversible process in the chloroplasts, for the full synthesis potential could not be achieved upon reillumination of the leaves. Treatment with kinetin, which we found to be a very effective retardant of tobacco leaf degradation,

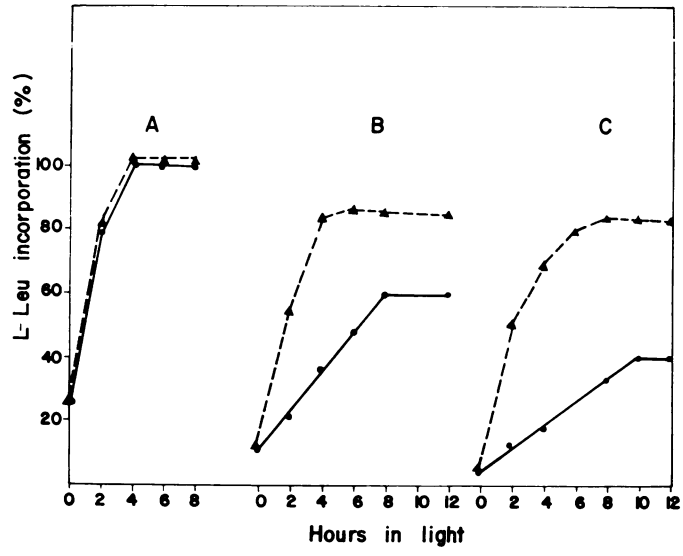

FIG. 7. Effect of kinetin on the pattern of dark-induced decline and light-induced restoration of L-leucine incorporation by isolated chloroplasts. A: Chloroplasts isolated from leaves exposed to 24 hr of darkness before reillumination; B: chloroplasts isolated from leaves exposed to 48 hr of darkness before reillumination; C: chloroplasts isolated from leaves exposed to 72 hr of darkness before reillumination. ▲: Chloroplasts isolated from leaves pretreated with 20 mM kinetin; ●: chloroplasts isolated from untreated (control) leaves. 100% incorporation refers to incorporation of L-leucine by isolated chloroplasts before exposure of the leaves to darkness.

yielded some interesting observations. Kinetin did not affect the rate or the extent of dark-induced decline in leucine incorporation. Also, kinetin had no effect on the light-induced restoration of synthesis after 24 hr of darkness, when full restoration of the synthesis potential in the chloroplasts was ordinarily obtained. Only when complete resumption of leucine incorporation could not be achieved—possibly owing to irreversible degradative processes—could an effect of kinetin on the protein synthesis activity in the chloroplasts be observed.

We have described the course of leucine and UTP incorporation activity in chloroplasts isolated from detached, darkened leaves—a system commonly used by many workers studying leaf senescence (3, 5–9). Initially, after a 24-hr exposure, the sharp decline in chloroplast protein synthesis potential may be fully restored upon reillumination of the leaves. Since this phase seems fully reversible, as indicated by the complete restoration of the incorporation activity, it is doubtful whether it can be regarded as the initial stage in leaf senescence. Later, after a longer exposure to darkness, full restoration of protein synthesis activity may no longer be achieved. However, up to 72 hr of darkness, treatment with kinetin maintained the protein synthesis potential of isolated chloroplasts at almost the predarkness level. We propose that the full reversibility in the initial phase results from the alleviation of some dark-induced mechanism of inactivation, while the irreversible phase may be due to the onset of a kinetin-responsive degradative process in the chloroplast.

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LITERATURE CITED

1. Anderson, L. E. 1973. Dithiothreitol activation of some chloroplast enzymes of etiolated pea seedling. Plant Sci. Lett. 1: 331–333.
2. Anderson, L. E. and Toh-chin Linn. 1972. Chloroplast glyceraldehyde 3-phosphate dehydrogenase. Light dependent change in the enzyme. FEBS Lett. 27: 189–191.
3. Chibnal, A. C. 1939. Protein Metabolism in the Plant. Yale University Press, New Haven.
4. Lin, D. C. and P. S. Nobel. 1971. Control of photosynthesis by Mg^{2+}. Arch. Biochem. Biophys. 145: 622–632.
5. Mlodzianowski, F. and M. Kwintkiewiez. 1973. The inhibition of chloroplast degeneration by kinetin. Protoplasma 76: 211–226.
6. Mlodzianowski, F. and A. Ponitka. 1973. Ultrastructural changes of chloroplasts in detached parsley leaves yellowing in darkness and the influence of kinetin on that process. Z. Pflanzenphysiol. 69: 13–75.
7. Richmond, A. E. and A. Lang. 1957. Effect of kinetin on the protein content and survival of detached Xanthium leaves. Science 125: 650.
8. Richmond, A. E., B. Sachs, and D. J. Osborne. 1971. Chloroplasts, kinetin, and protein synthesis. Physiol. Plant. 24: 176–180.
9. Shaw, M. and M. S. Manocha. 1965. Fine structure in detached senescing wheat leaves. Can. J. Bot. 43: 747–755.
10. Spencer, D. and S. C. Wildman. 1964. The incorporation of amino acid into protein by cell free extracts from tobacco leaves. Biochem. J. 3: 954–959.
11. Travis, R. L., C. Y. Lin, and J. L. Key. 1972. Enhancement by light of the *in vitro* protein synthesis activity of cytoplasmatic ribosomes isolated from dark grown maize seedlings. Biochim. Biophys. Acta 222: 606–614.
12. Wollgiehn, R. 1972. RNA synthesis in isolated chloroplasts. Symp. Biol. Hung. 13: 201–211.