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Effects of Ethephon on Aging and Photosynthetic Activity in Isolated Chloroplasts[1]

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ABSTRACT

Chloroplasts, isolated from the primary leaves of 7-day-old seedlings, were incubated *in vitro* at 25°C with 2-chloroethylphosphonic acid (ethephon) under light (0.16 milliwatts per square centimeter) and dark conditions. Ethephon at 1 micromolar (0.1445 ppm), 0.1 and 1 millimolar, or 5 microliters ethylene promoted the deterioration of chloroplasts, increased proteolysis, and reduced the chlorophyll content and PSI and PSII during 72 hours under both light and dark conditions. The decline in PSI and PSII occurred prior to a measurable loss of chlorophyll. The loss of photosynthetic activity affected by ethephon was initiated prior to 12 hours of incubation. After 24 hours in light, 0.1 millimolar (1.445 ppm) epthephon significantly reduced PSI and PSII and promoted the total free amino acid liberation in isolated chloroplasts. In darkness the rate of loss of PSI activity was about 50% of that in light. After 24 hours, in light at 1 millimolar epthephon, PSII activity was 55% of the control, yet nearly 90% of the chlorophyll remained, which indicates that the loss of thylakoid integrity was promoted by ethephon. Ethylene injected in the chloroplast medium at 5 microliters (0.22 micromolar per milliliter) reduced PSI by nearly 50% of the initial in 12 hours. In leaf sections floated in 5 microliters per milliliter suspension medium, a 36% loss of chlorophyll of the control in 36 hours was observed. Cycloheximide at 0.5 millimolar masked the effect of 1 millimolar ethephon and maintained the initial chlorophyll content during the 72 hour period.

To promote maturity or color of apples, blackberries, blueberries, cranberries, pineapple, and tomatoes, foliar sprays of an ethephon solution ranging from 200 to 1000 actual ppm have been the recent field practice (24). Ethylene evolved from ethephon has been known to hasten maturity and the senescence phenomena in plant tissues. Ethylene released from ethephon promotes the loss of Chl (4, 18, 19), hydrolysis of polymers (12, 14), climacteric rise in respiration (1, 14), and early maturation or accelerated senescence (1, 2, 13). Gepstein and Thimann (8) in 1981 showed that ethylene indeed exerts a dominant regulatory role in initiation of senescence in oat leaves. Although the loss of Chl after ethephon application to leaves and fruits has been observed, few studies have examined the subsequent loss of photosynthetic activities by the leaves sprayed with ethephon. Pallas and Kays (17) in 1982 reported that 1 μl ethylene applied for 6 h inhibited 68% of the net photosynthesis in plants but there was no inhibition in ribulose 1,5-bisphosphate activity, *in vitro*. The ethylene effect on peanut leaf photosynthesis may be on stomatal control since leaf stomatal resistance was influenced by ethylene (6). Our preliminary results showed that, within hours of detachment of leaves of oat seedlings and their suspension in ethephon solution, proteolysis increased and photosynthetic activity of leaf and chloroplasts declined. This paper examines the relationship between the effects of ethephon on the loss of Chl, PSI, and PSII of isolated chloroplasts, as well as on proteolysis and thylakoid membrane deterioration in an isotonic medium under light and dark conditions, and the Chl loss by the leaves being floated on varying concentrations of ethephon solutions. A simultaneous investigation on ethylene gas along with the ethephon concentrations was also carried out to verify the direct and spontaneous effect of ethylene on the photosystem as it contrasts to the gradual effect of ethephon, due to its slow release into the medium.

MATERIALS AND METHODS

Chloroplasts were isolated from oat (*Avena sativa* cv. 'Garry Spring') seedlings which were grown for 7 d in vermiculite at 25°C. Cool white fluorescent tubes provided light for 14 h/d at approximately 0.81 mW cm^{-2} at the plant level. A bundle of 10 g of apical 5-cm leaves were chilled, cut, surface sterilized, finely minced with a razor blade, and homogenized in a freshly prepared chilled isolation medium A (5). This medium A contained 30 mM Tes, 0.33 M sorbitol, 1 mM EDTA, 1 mM $MgCl_2$, 5 mM sodium ascorbate, and 0.1% BSA fraction V and was adjusted to pH 7.4 with 1 N NaOH. Chloroplasts were aseptically isolated at 0 to 4°C from the homogenate as previously described (6), and they were homogenized with medium A lacking 0.1% BSA fraction V and washed until no succinate dehydrogenase activity was observed. The chloroplasts were then incubated either in the dark or light in a suspension medium B which contained 30 mM Tes, 0.44 M mannitol, and 0.1 mM streptomycin adjusted to pH 7.4. To each of 3 sets of 8 concave wells in a porcelain spot plate, 1.0 ml of chloroplast suspension containing about 200 μg of Chl was added to medium B which contained ethephon at final concentrations of 1 mM, 0.1 mM, and 1 μM. In addition, 22 nM (5 μl) of ethylene was slowly injected into a separate well with chloroplasts in the medium for comparison. To prevent the chemical acid formation by ethephon in the lower pH media, all chloroplast suspension media B containing ethephon were adjusted to pH 7.4 with Tes buffer. At this pH ethylene is liberated at a much higher rate than at the lower pH in a medium (27). Each sterilized porcelain plate was rested on moist paper in a 14 cm diameter Petri dish with cover, and left in the dark at 25°C or illuminated with fluorescent light at 0.16 mW cm^{-2} (600 lux) until assayed at 0, 12, 24, 36, 48, and 72 h. Twenty 3-cm long apical leaf sections were floated on the same ethephon concentrations as for the chloroplast test solutions. Additionally, after 20 oat leaf sections were placed in the Petri dish containing 10 ml of water, 50 μl (0.22 μM) of ethylene was very slowly injected into the water with a syringe. Care was taken so that the Petri dish cover was lifted at one side as little as possible and then immediately covered after injection. No bubbles were visible

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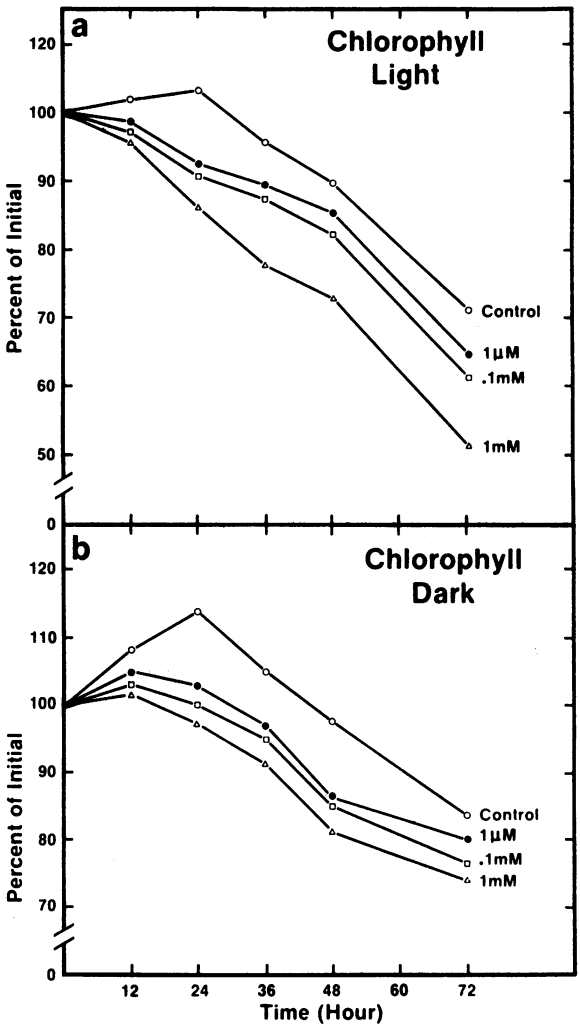

FIG. 1. Loss of Chl on addition of ethephon to chloroplasts in medium B at pH 7.4 and 25°C. Chl concentration at 0 h, 27.3 μg/ml. See "Materials and Methods". a, Under light at 60 μE m^{-2} s. b, In darkness with all conditions same as in a. Note: Approximate C_2H_4 (in nl) released into the chloroplast suspension medium from 1 μM ethephon ml^{-1} (parentheses indicate time in h aged: 23 (12), 65 (24), 99 (36), 145 (45), 181 (60), 221 (72), respectively, at pH 7.4 ml^{-1}); 0.36 μl corresponds to 100% of ethylene released from 1 μM ethephon (27).

during the injection. Thus the ethylene was apparently totally absorbed into the water. PSI activity was measured using the methyl viologen (electron acceptor) Mehler reaction as described by Choe and Thimann (5) using the following modifications. The assay medium included 25 mM Tes adjusted to pH 8.0, 2 mM TMPD[2] as an electron donor, 1 mM methyl viologen, 1 μM DCMU, and 16 mM Na-ascorbate. For PSII oxygen release, the assay medium contained 25 mM Tes at pH 8.0, 15 mM methyl viologen as an uncoupler, and 0.25 mM KCN. Reactions were carried out in a 3 ml reaction flask containing 20 to 30 μg Chl which was connected to a Lauda K-12 Thermoregulator maintained at 25 ± 0.1°C. O_2 uptake (PSII) was continuously monitored on a chart recorder using an O_2 electrode (Yellow Spring Instrument model 53). The energy incident on the reaction flask in all cases was kept to 15 k lux (4.05 mW cm^{-2}) using a 300-W reflector flood light with a 1-inch thick water glass filter between the sample and the light source. The O_2 changes were measured

[2] Abbreviation: TMPD, *N,N,N,N*-tetramethyl-*p*-phenylenediamine hydrochloride.

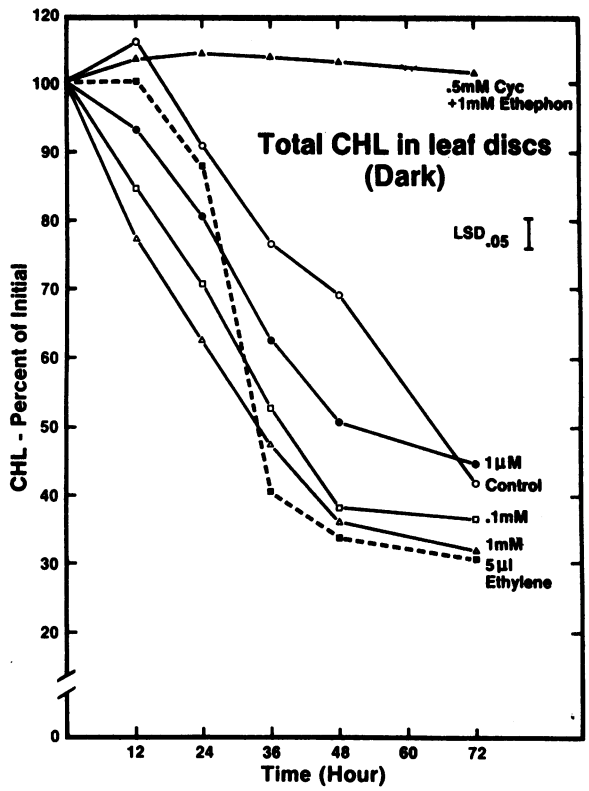

FIG. 2. Retention of total Chl in leaf discs by ethephon concentrations. All conditions are as in Figure 1 except those in darkness. Initial rate is 35.9 μg Chl in each 3-cm long leaf disc at 0 h as 100%. $LSD_{0.05}$ 2.2 μg = 6.2%.

at 730.5 mm Hg. Protein content was measured by the method of Martin and Thimann (12) and 80% acetone chloroplast precipitates following Chl determination (3) were used for free amino nitrogen analysis by the Moore and Stein (15) method. For Figures 1, a and b, through 4, a and b, each point is the mean of at least three replications or more, each in duplicate.

Effects of Ethephon on Chl Loss in Chloroplasts. Ethephon at 0.001 mM (1 μM = 0.1445 ppm) to 1 mM as well as 22 μM (5.2 μl) ethylene promoted the loss of Chl from the chloroplasts in suspension medium B in both light and darkness (Fig. 1, a and b). In light in 24 h, Chl losses were 7, 10, and 14% respectively, at 0.001, 0.1, and 1 mM when compared with initial growth, but there was over 7% Chl synthesis in the control under light. After 72 h, Chl losses were 7, 11, and 21% at 0.001, 0.1, and 1 mM, respectively, over the control. In dark in 24 h, ethephon at 1 mM promoted Chl loss by 3% compared to a 13% synthesis in the control. In light after 72 h, Chl losses were 18, 24, and 25%, respectively, at 0.001, 0.1, and 1 mM as compared to the control. At 10 mM ethephon, chloroplasts became yellow in less than 12 h in the dark. At this ethephon concentration the pH of the medium without buffer showed 2.5, thus experiments at 10 mM or higher were discontinued. Addition of varying concentrations of ethephon every 24 h to the chloroplasts *in vitro* had no further effect on Chl loss. Figure 2 shows that all ethephon solutions significantly promoted a loss of Chl in the leaf discs submerged in the Petri dishes. The leaf sections in the Petri dish with 50 μl ethylene showed a drastic drop of Chl content from 87% at 12 h to 41% in 36 h. Thus a slower response than with ethephon was observed initially; yet, there was a speedy Chl loss after 24 h as compared to the ethephon treatment, which indicates a lag period before ethylene diffused through the oat leaf sections. Cycloheximide at 0.5 mM masked the effects of 1 mM ethephon, thus maintained the level of Chl at higher than original for the 72 h

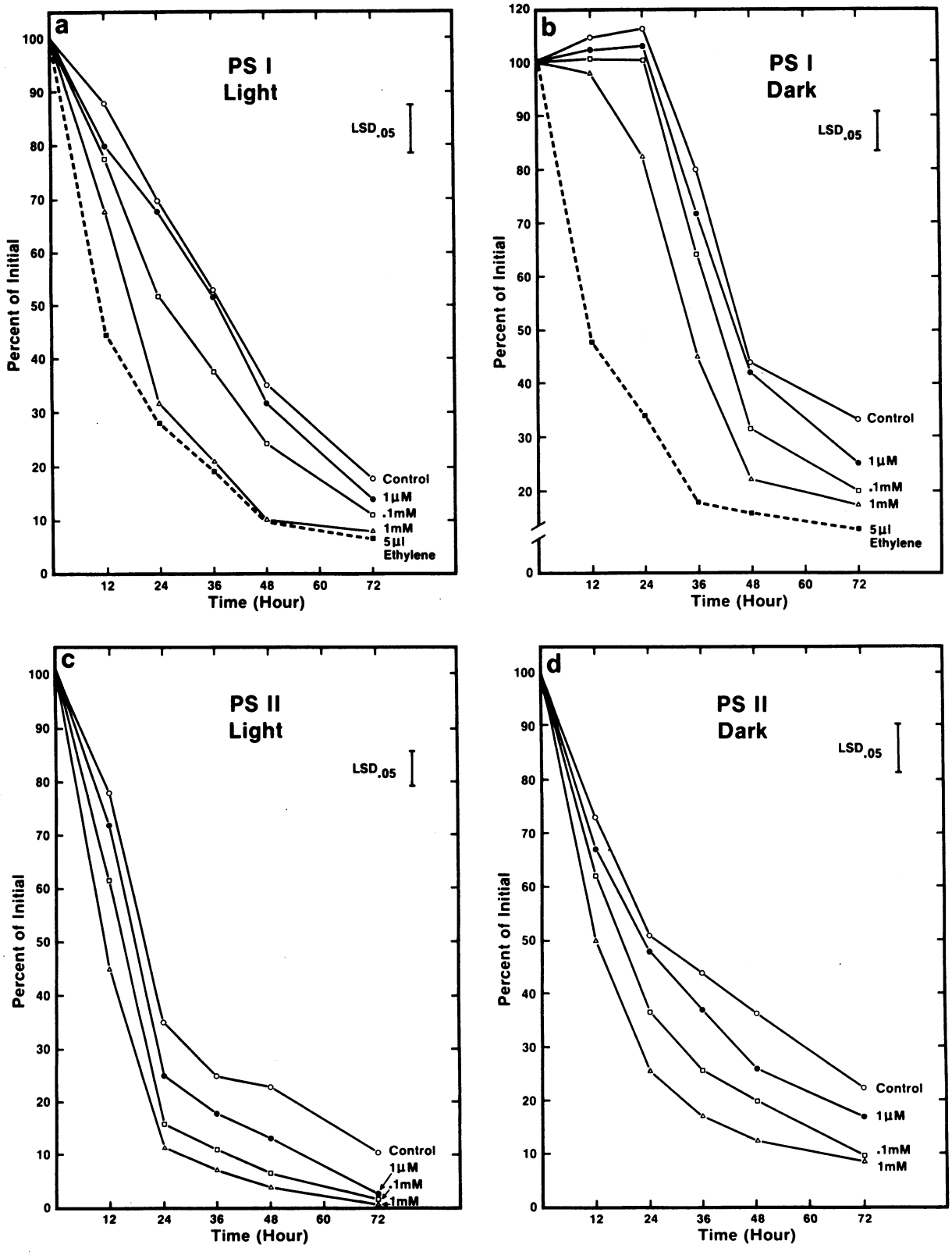

FIG. 3. The effects of ethephon on retention of photosystem activities in chloroplasts suspended in medium B at pH 7.4. See under "Materials and Methods". a, PSI in light. Initial rate: 1,360 μM O_2 mg^{-1} Chl based on 20 μg Chl as 100%. Each point is the average of six duplicates based on the Chl remaining in the suspension medium. $LSD_{0.05}$ 115 μM O_2 = 8.5%. b, PSI in darkness. All conditions are same as in a, except those in the dark. $LSD_{0.05}$ 77 μM = 5.6%. c, PSII in light. All conditions are same as in a and b. Initial rate: 245 μM O_2 mg^{-1} h^{-1} at 0 h based on 27 μg ml^{-1} as 100%. $LSD_{0.05}$ 14 μl = 5.7%. d, PSII in darkness. All conditions are same as in c. $LSD_{0.05}$ 21 μl = 8.7%.

Table I. *Effects of Ethephon on Amino Acid Liberation in Chloroplasts in Light and Darkness*

Percent of initial value: total amino-N 0.75 mM as 100% at 0 h.

Concentration	Light or Darkness Treatment			
	24 h		48 h	
	Dark	Light	Dark	Light
μM	*percentage ± SE*			
0	105 ± 2.3[a]	113 ± 3.4	135 ± 2.2	158 ± 3.0
1	110 ± 2.5	150 ± 3.2	157 ± 4.0	198 ± 3.1
100	115 ± 3.0	173 ± 3.1	168 ± 3.1	215 ± 4.0
1000	140 ± 3.4	255 ± 4.6	218 ± 2.2	308 ± 5.0

[a] Data average of six duplicates.

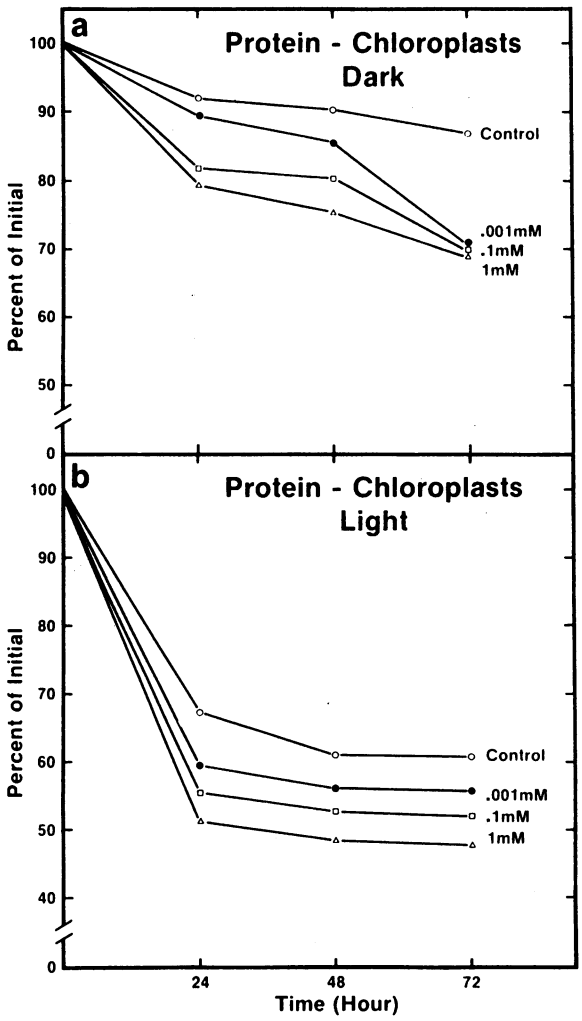

FIG. 4. Proteolysis in chloroplasts *in vitro* medium B with 0.001 mM, 0.1 mM, or 1 mM ethephon for 72 h at 25°C. See "Materials and Methods". a, Protein loss in chloroplasts in darkness. b, Protein loss in chloroplasts in light.

period.

Inhibition of PSI and PSII by Ethephon. Figure 3, a and b shows that ethephon at all concentrations inhibited PSI in both light and dark. In light in 24 h, PSI inhibition at 1 mM was 38%, compared with the control, followed by 17.5% at 0.1 mM and 2% at 0.001 mM, respectively. But the difference between the control and 1 mM narrowed to 11% over the 48 and 72 h period. A significant inhibition was already started at 0.1 and 1 mM ethephon in 12 h (Fig. 3a). But the difference between the control and 1 mM narrowed to 10% over the 48 to 72 h period. Ethephon at 0.1 mM significantly inhibited PSI through 12 to 48 h, but it narrowed to 5 to 7% in 72 h, and at 1 μM it narrowed to 3 to 5% over the control during the 72 h period. Thus ethephon at 1 μM is considered the threshold concentration during the 72 h period. The ethephon inhibition of PSI and 1 mM in light in 24 h was strikingly high, 68% of the initial (Fig. 3a), while over 86% of the initial Chl content remained in the control chloroplasts (Fig. 1a). Figure 3, c and d, shows the sequence of the PSII inhibition by ethephon in light and darkness. PSII in light was already reduced to 45% (Fig. 3c) of the initial in 12 h at 1 mM as compared with the 86% of initial Chl (Fig. 1) remaining. One μM ethephon inhibited about 6% more than the control during 12 h. In general the inhibition of PSII was slightly more rapid at 1 μM and 0.1 mM in 48 h than that of PSI in light. Figure 3d shows that, while about 80% of the Chl remained at 48 h at 1 mM ethephon (Fig. 1b), there was a drastic drop to 21% of PSII in darkness. All ethephon treatments reduced PSII to close to 0 in 72 h, but PSII in control still remained at 10% of the initial after 72 h.

Proteolysis in Chloroplasts. Table I clearly shows that ethephon at all concentrations promoted amino acid liberation in the chloroplasts in both light and darkness, *i.e.* the higher the concentrations the greater the proteolysis for the three concentrations tested. The most rapid proteolysis was at 1 mM followed by 0.1 and 1 μM which was least. In light at 1 mM ethephon in 24 h, about 2.3 times as much amino acid was liberated as the control, but it dropped to 1.9 times in 48 h. In darkness at 1 mM ethephon in 24 h, there was about 1.3 times the level of amino acids formed as compared to the control, but about 1.6 times was liberated in 48 h. The chloroplasts at 10 mM or higher ethephon completely yellowed in 12 h, thus no higher concentration was tested. Figure 4, a and b, shows that proteolysis in chloroplasts *in vitro* was promoted by the three ethephon concentrations in both light and dark conditions during the 72 h period. The rate of proteolysis in light was higher (Fig. 4a) than that of the dark (Fig. 4b). The general trend of proteolysis in terms of the protein breakdown agrees well with the promoted amino acid liberation (Table I).

DISCUSSION

Ethephon concentrations ranging from 1 μM to 1 mM at pH 7.4 were found to be the physiological ranges for the study of chloroplasts *in vitro*. Ethephon at 1 μM was the threshold critical level for both Chl loss, proteolysis, and declines in photosynthetic rate. The liberation of ethylene from ethephon in a solution or *in vitro* had clearly been shown earlier (27, 29), and ethylene was liberated linearly up to 30 h as the pH of solution increased from 5.4 to 8.0 (27). This coincides with the fact that an exogenous application of ethylene to green plant materials induced a decrease in Chl content (17, 19), and an inverse relation between the rapid senescence by Chl loss and an increased ethylene

evolution was also found (8). In light, the rapid loss of Chl shown (Fig. 1a) can be expected because of the photooxidation of Chl (8) through the disintegration of the internal structure of chloroplasts (23): (a) A number of morphological and physiological changes such as a more rapid swelling in light than in darkness (10, 16) cause the grana and intergrana thylakoid to swell up to form a balloon like structure (16). (b) Illumination of the chloroplasts causes proton transport from stroma space into the thylakoid space. Choe and Thimann observed that acidification of oat chloroplasts promoted Chl loss in both light and dark (9). Kelly (11) in 1983 also showed that photosynthetic activity mediated the light-stimulatedd acidification of oat protoplasts suspensions. An illumination of chloroplasts *in vitro* decreased the pH in the thylakoid space by 1.5 and increased the pH in the stroma by almost 1 pH unit. In darkness however, the difference in pH between the thylakoid space and stroma was negligible (28). (c) Chloroplasts are known to contain a number of autocatalytic enzymes (20–22). Ethylene is known to induce several enzymes including catalase and peroxidase which has been thought to have a stabilizing effect on Chl (21). An enhanced solubility of the chloroplast thylakoid membrane (10, 25) along with the increased protease activity and reduced catalase and peroxidase by ethylene (22) may have resulted in the breakdown of chloroplast membrane leading to more exposure of Chl to free radicals under light (7). Thus ethephon at three concentrations consistently inhibited both PSI and PSII more in light and to a less degree in dark. PSI activity determined by the O_2 uptake in light was significantly reduced at all concentrations within 12 h, whereas only 0.1 mM or higher ethephon concentrations significantly reduced the rate in the dark (Fig. 3, a and b). The photosystems have a specific orientation across the chloroplast membrane (4). O_2 produced in the inside of the thylakoid at PSII, and PSI is coupled to the reduction of ferredoxin and $NADP^+$ on the outer face of the thylakoid. Thus in light, the inner face of thylakoid appeared to be damaged faster than the outer face of the thylakoid which is related to PSI (Fig. 3, a *versus* c). Apparently even in the dark, PSII system which is involved in O_2 production to the inside of the thylakoid, was damaged faster than PSI. Proteolysis by an enhanced amino acid liberation in the chloroplast *in vitro* (Table I) coincides well with an accelerated loss of protein caused by ethylene in the aging chloroplasts *in vitro* in both light and dark (Fig. 4, a and b) and in oat leaves (9). Our earlier observations, that ethephon enhanced protease activity and reduced peroxidase and catalase activity in parallel to its concentration, indicate that the rapid drop of PSII followed by the decline in PSI activity in chloroplasts affected by ethephon was mainly due to the deterioration of structure and electron transport systems which precedes measurable Chl loss.

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