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Some Observations on the Bleaching Effect of Ethylenediaminetetraacetic Acid on Green Barley Leaves[1,2]

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Abstract. Green barley leaves (*Hordeum vulgaris*) floated on the surface of 0.05 M ethylenediamine disodium tetraacetate, EDTA-2Na, pH 7.0 and exposed to light (5000 lux) at 25° exhibited a marked bleaching (EDTA-bleaching) visible to the naked eye and paralleled by a striking reduction in content of chlorophylls *a* and *b*. This loss of color did not occur in controls which were treated with H_2O instead of EDTA (water controls). In darkness the leaves in the water controls were bleached while EDTA-treated leaves retained all their color.

EDTA bleaching was observed only in intact leaves. When leaves were boiled EDTA protected their pigment against photodecomposition. Without EDTA boiled leaves were bleached completely in light.

When intact green leaves which had been floated on water and exposed to light for 48 hr were treated with boiling ethanol or acetone, the chlorophylls extracted by this procedure did not undergo bleaching if EDTA were present in solution. Under these conditions a green fine grain precipitate formed which was insoluble in ethanol or acetone and was stable in light or darkness.

EDTA bleaching of green barley leaves was inhibited by KCN, and by the addition of casein hydrolysate.

During the past 8 years the authors have investigated the effect of gaseous ions on higher plants and have found that moderate concentrations of positive and negative ions markedly stimulate plant growth and increase oxygen consumption (2, 3, 6, 7). They increase both the rates of shrinking and swelling of isolated chloroplasts and the utilization of ATP in the process (4). They enhance iron uptake and in plants suffering from iron chlorosis increase the "residual" iron pool (including cytochromes and other Fe-containing enzymes) while the "active" iron fraction of the tissues decreases and the chlorophyll content is diminished (2, 7). Active iron, contained in the chloroplasts, participates in the production of chlorophyll while residual iron is not associated with chloroplasts nor is it involved in the biosynthesis of chlorophyll (1, 8).

In studying the effect of air ions on chlorophyll biosynthesis attempts have been made to use etiolated seedlings for the preparation of isolated chloroplast suspensions which had retained their ability to produce chlorophyll. Unfortunately such isolates bleach when exposed to light; even green chloroplasts become decolorized during light treatment for 72 hr. In our experience retention of the green color of chloroplasts is closely linked to their retention of catalytic activity. Therefore it seemed reasonable that if one knew how to prevent the bleaching of chloroplasts, he might hope to obtain etiolated chloroplast suspensions capable of synthesizing chlorophyll in the light. These considerations led us to study the mechanism of decolorization of isolated green chloroplasts in a defined medium.

In preliminary experiments it was noted that EDTA, a component of the isolation and suspension medium, was responsible for the decolorization of green chloroplasts. It appeared to us that an understanding of this phenomenon would be useful not only in defining the conditions requisite for physiological maintenance of isolated chloroplasts but also in providing further insight into the nature of the chlorophyll-lipoprotein complex—the minimal biochemical unit essential for the full performance of photosynthesis. We are reporting here experimental observations on the EDTA bleaching of barley seedlings.

Materials and Methods

Barley seedlings were routinely raised under laboratory conditions. Barley seeds (*Hordeum vulgaris* var. Mariout) were soaked in distilled water for about 24 hr with vigorous aeration. The soaked seeds were placed in a tray containing Dacron wool moistened with 0.1 mM $CaCl_2$ and 3 μM chloram-

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[2] A part of this paper was presented before the Annual Meetings of Plant Physiologists at the University of Massachusetts, Amherst, Massachusetts, August 20 to 23, 1968(5).

phenicol. This concentration of chloramphenicol discouraged the propagation of bacteria and apparently did not affect either seedling growth or EDTA-bleaching.

The seeded tray was placed in an incubator at 25° and the seeds were allowed to germinate in darkness for 3 days. Then the seedlings were placed in the open room, exposed to indirect light (300 lux) for 48 hr and then to direct light (5000 lux) for 48 hr. When the seedlings had developed a full green color, the green leaves were harvested. Five grams of green leaves were cut transversely into small pieces (*ca.* 5 mm) with scissors and placed in a clear plastic chamber containing 100 ml of the medium specified in the text.

At stated intervals 0.5 to 1.0 g of leaves were collected, washed with a 500 ml volume of distilled water, blotted dry and weighed. The washed leaves were extracted 3 times with 50 ml of boiling 80 % (v/v) ethanol, and once with boiling ethyl ether. All alcoholic fractions were combined to form a chlorophyll extract and the ethereal fraction was discarded. After evaporating the ether, the residues were dried in an oven at 50° and their dry weights were determined. The alcoholic extract was kept in the refrigerator overnight and then centrifuged at 10,000*g* for 10 min. The precipitate formed was discarded. The extinction of the supernatant was determined with a Beckman DU spectrophotometer at 650 mμ and 665 mμ and the contents of chlorophyll *a* and *b* were calculated by the method of Mackinney as modified by Vishniac (11). For the acetone extract, green leaves (1 g) were ground with a mortar and pestle with 10 ml of 85 % (v/v) acetone. The homogenate was centrifuged at 10,000*g* for 10 min, the supernatant was saved, and the residue was extracted with 85 % acetone until no green color remained in the residue. All supernatants were combined to form a chlorophyll acetone extract. The acetone extract was kept in the refrigerator overnight and centrifuged at 12,000*g* for 10 min. Both the ethanol and acetone extracts were adjusted to contain 400 μg total chlorophyll/ml.

The trichloroacetic acid soluble fraction, consisting mainly of nucleic acids, was extracted from the dried residue according to the method of Schneider (9) with a slight modification. One gram of the dried residue was homogenized in 5 ml of distilled water using a mortar and pestle. To each 1 ml of the tissue homogenate were added 1.3 ml cold 10 % (w/v) trichloroacetic acid. The mixture was centrifuged at 12,000*g* for 10 min. The supernatant was saved and the residue was resuspended in 5.0 ml of 5 % (w/v) trichloroacetic acid and centrifuged at 12,000*g* for 10 min. All trichloroacetic acid fractions were combined to form the nucleic acid extract and nitrogen content was determined.

The residue remaining after trichloroacetic acid extraction was suspended in 20 ml of 5 M H_2SO_4, transferred quantitatively into a 120 ml Erlenmyer flask, placed in an oven at 150°, and digested with a trace of $CuSeO_3$ and with occasional addition of a few drops of 30 % (w/v) H_2O_2. After adjustment of the total volume of the digest to 20 ml with distilled water, 1 ml of the digest was taken into a larger test tube and diluted to 10 ml with distilled water. The nitrogen content of the diluted digests was determined by Nesslerization based on the procedure of Seligson and Seligson (10). The color intensity was determined by either the Klett-Summerson photoelectric colorimeter with a No. 42 filter (blue) or the Beckman DU Spectrophotometer at 500 mμ against a reagent blank.

Experimental Results

When green barley leaves were floated on 0.05 M EDTA-2Na, pH 7.0, exposed to light (5000 lux) at 25°, the leaf tissues were completely bleached to a yellow color within 40 hr. Fig. 1 shows the EDTA-bleaching in terms of decrease in chlorophylls *a* and *b* in EDTA-treated leaves. The chlorophyll *a* and *b* concentrations in the H_2O control remained at a constant level throughout the experimental period. EDTA-treated leaves showed a drastic decrease in chlorophyll *a* concentration as early as 20 hr after beginning exposure to EDTA and light. The chlorophyll *b* concentration followed a similar trend but its rate of decrease was lower than that of chlorophyll *a*. The ratio of chlorophyll *b*/chlorophyll *a* exceeded 1 after 20 hr of exposure.

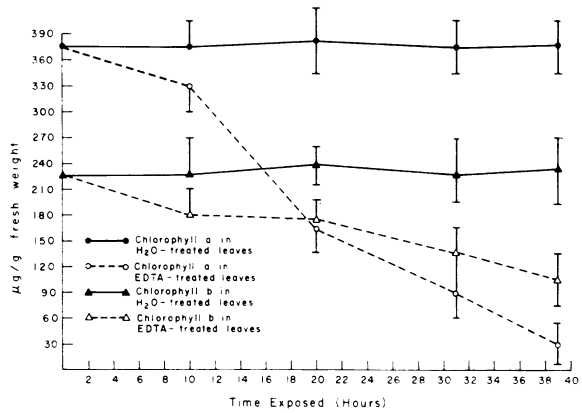

FIG. 1. Effect of EDTA on chlorophyll content of green barley leaves in light. Green barley leaves were cut into small pieces and floated on the surface of 0.05 M EDTA (pH 7.0) and on H_2O as controls. They were exposed to light (5000 lux) at 25°. At stated intervals the leaf pieces were sampled and extracted with boiling 80 % (v/v) ethanol. Chlorophyll content was determined photometrically. The vertical bars indicate 95 % confidence intervals.

When green leaves were treated with EDTA at 25° in darkness, no bleaching was observed. In this

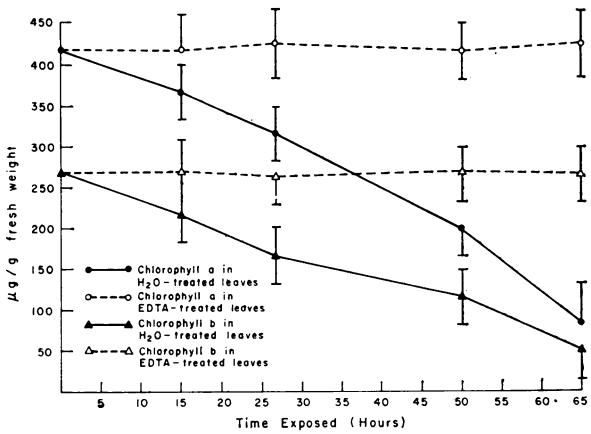

FIG. 2. Effect of EDTA on chlorophyll content of green barley leaves in darkness. The conditions were identical with those prevailing in Fig. 1 except that the experiment was conducted in darkness.

case, however, the H_2O control exhibited bleaching. Fig. 2 shows the results obtained from a dark experiment. The decrease in concentration of both chlorophyll *a* and chlorophyll *b* took place at a lower rate than was observed in the case of EDTA-treated leaves in light (Fig. 1). Complete bleaching of water-treated leaves was observed after 65 hr of incubation whereas EDTA-treated leaves retained all their color during this period. In this case the ratio of chlorophyll *b*/chlorophyll *a* did not exceed 1.

If green barley leaves were boiled for 1 min, cooled in ice cold water, then treated with 0.05 M EDTA in light, no bleaching was observed. Comparable treatment of leaves with water instead of EDTA produced a marked bleaching (Fig. 3). The total chlorophyll content of boiled leaves treated with EDTA was not altered, whereas very little chlorophyll remained in untreated boiled leaves.

In order to test the direct effect of EDTA on chlorophyll extracts, boiling 80 % (v/v) ethanol and 85 % (v/v) acetone extracts were prepared from 7-day-old green barley leaves which had been exposed to light in H_2O containing 3 μM chloramphenicol for 48 hr. The green leaves (1 g) were extracted with a 50 ml portion of boiling 80 % (v/v) ethanol and the residue was extracted twice with 30 ml volumes of boiling 80 % (v/v) ethanol. The alcohol fractions were combined, kept in the refrigerator overnight and centrifuged at 12,000*g* for 10 min. The supernatant obtained was used as a chlorophyll alcohol extract. An acetone extract was prepared as described in Materials and Methods section.

To 10 ml portions of each extract were added an equal volume of 0.05 M EDTA-2Na, pH 7.0, or the same amount of H_2O as a control. Both solutions contained 3 μM chloramphenicol. The mixtures were

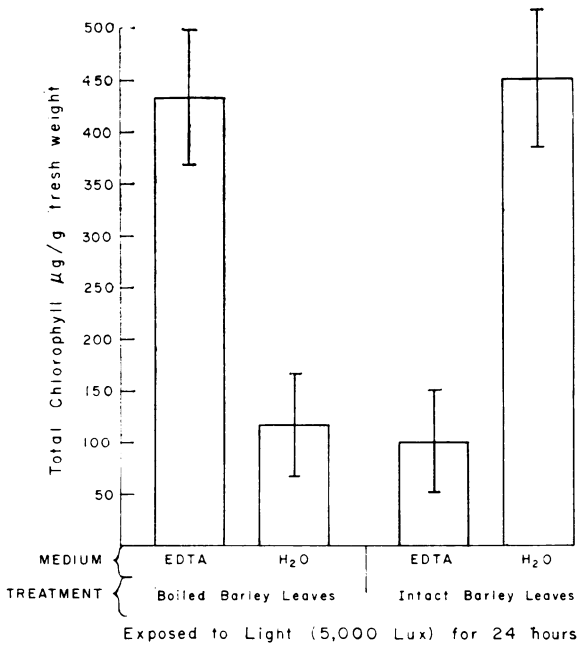

FIG. 3. Effect of boiling on EDTA-bleaching. Green barley leaves were heated in boiling water for one minute and cooled in ice-cold water. The boiled leaves were blotted dry, weighed, cut into small pieces, and floated on the surface of 0.05 M EDTA-4 Na pH 7.0 or on H_2O as controls. After 24 hr of exposure to light (5000 lux) the treated leaves were extracted with boiling 80 % ethyl alcohol. The vertical bars indicate 95 % confidence intervals.

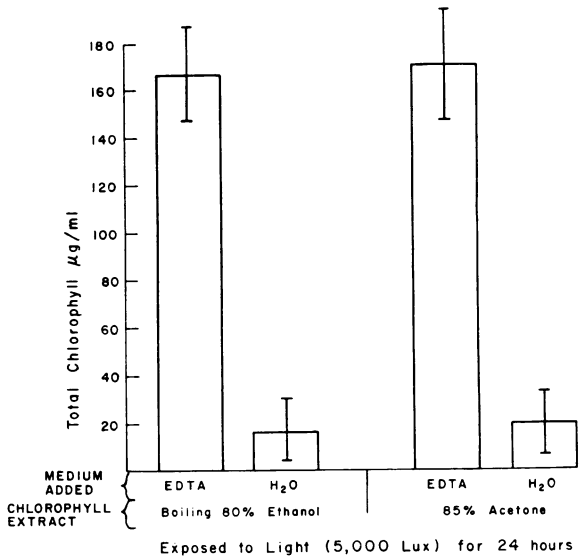

FIG. 4. Effect of EDTA on bleaching of barley chlorophyll extracts. Chlorophylls were extracted either with boiling 80 % (v/v) ethanol or with 85 % (v/v) acetone. The concentration of the extracts was adjusted to 400 μg/ml. To 50 ml of each chlorophyll preparation were added 50 ml of 0.05 M EDTA-2 Na, pH 7.0 or 50 ml of H_2O as a control. The mixtures were exposed to light (5000 lux) for 24 hr. Chlorophylls in the mixtures were extracted with diethyl ether and determined spectrophotometrically. The vertical bars indicate 95 % confidence intervals.

exposed to light (5000 lux) at 25°. After a 24-hr exposure period, an equal volume of H_2O was added to each mixture and chlorophylls in the mixture were extracted with an equal volume of diethyl ether. The total chlorophyll concentration was determined spectrophotometrically. Fig. 4 shows the results. In this case the addition of EDTA protected against the bleaching of either alcoholic or acetone chlorophyll extracts. EDTA-treated extracts formed a green fine grain precipitate which remained green permanently in either light or darkness.

The data cited above show that in order to induce EDTA-bleaching leaf tissues must be intact, suggesting that an enzyme may participate in this phenomenon. In order to assess this possibility several enzyme inhibitors and activators were tested for their effect on EDTA bleaching. Normally when barley leaves are floated in EDTA in the light a marked bleaching occurs. In the presence of KCN, the EDTA bleaching was inhibited (Fig. 5). As yet there is no conclusive evidence for the involvement of a respiratory system in the EDTA-bleaching. Inhibitors (each at 10 mM) such as antimycine A, 2,3-dimercapto-1-propanol and naphthoquinone did not prevent EDTA-bleaching. Also EDTA-bleaching experiments were performed under aerobic and anaerobic conditions created by bubbling 100 % O_2 and 100 % N_2 gas respectively into the media contained in an Erlenmeyer flask.

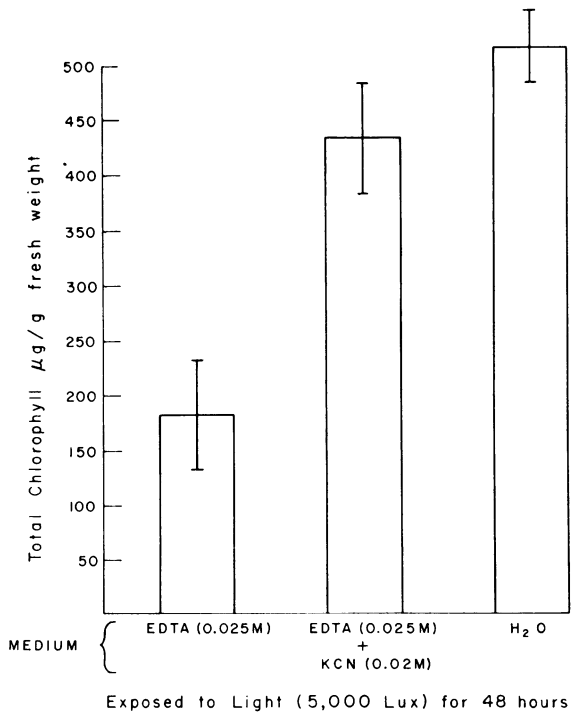

FIG. 5. Effect of cyanide on EDTA-bleaching. Intact green barley leaves (8 days old) were floated on the surface of the media indicated. After 48 hr of exposure to light, the chlorophylls were extracted with boiling 80 % (v/v) ethanol and determined spectrophotometrically. The vertical bars indicate 95 % confidence intervals.

Table I. *Effect of Nitrogen Atmosphere on EDTA-Bleaching in Light*

Seven-day-old green barley leaves (3 g) were floated on the surface of 200 ml of the specified medium in a 500 ml Erlenmeyer flask, nitrogen gas was bubbled through the medium and the leaves were exposed to light (5000 lux) for 48 hr.

Condition of leaves	Intact	Intact	Boiled	Boiled
Medium	0.05 M EDTA	H_2O	0.05 M EDTA	H_2O
Chlorophyll *a* µg/g fresh wt.	239.8	695.6	492.2	467.7
Chlorophyll *b* µg/g fresh wt.	240.9	275.4	337.4	259.6

Table I shows that after 48 hr exposure to light under anaerobic conditions no apparent bleaching occurred. Intact green barley leaves treated with EDTA yielded bright green colored residues after extraction with boiling 80 % (v/v) ethyl alcohol. The green pigments in the residues could not be extracted even after boiling with 70 % ethyl alcohol for 30 to 60 min. As a result marked bleaching was recorded in terms of boiled alcohol soluble chlorophylls (table I). In the case of boiled leaves their initial dark green color altered to a slightly brownish green during exposure to light in EDTA solution or in water. This green pigment present in boiled leaves was extracted completely with boiling 80 % (v/v) ethanol within 10 min, leaving a residue of a faintly yellowish tinge. Accordingly no bleaching was recorded in terms of chlorophyll content (table I).

Table II. *Effect of Oxygen and Aeration on EDTA-Bleaching in Light*

Experimental conditions in this series duplicated those established in table I except that oxygen or air replaced nitrogen.

Condition of leaves	Intact	Intact	Boiled	Boiled
Medium	0.05 M EDTA	H_2O	0.05 M EDTA	H_2O
Chlorophyll *a* µg/g fresh wt	229.6	585.5	429.0	139.0
Chlorophyll *b* µg/g fresh wt	240.9	375.4	245.0	115.0
Atmosphere		Oxygen		
Condition of Leaves	Intact	Intact	Boiled	Boiled
Medium	0.05 M EDTA	H_2O	0.05 M EDTA	H_2O
Chlorophyll *a* µg/g fresh wt	325.0	750.0	635.4	335.0
Chlorophyll *b* µg/g fresh wt	230.6	385.5	365.7	265.0
Atmosphere		Air		

In the presence of oxygen marked bleaching was observed both in EDTA-treated intact leaves and in H_2O-treated boiled leaves. Concomitantly there occurred a marked decrease in the chlorophyll content in both instances (table II). The residues remaining after a 10-min period of extraction with boiling alcohol were white to gray in color without any visible greenish cast.

Table III. *Effect of EDTA-Bleaching on TCA Soluble Nitrogen and Total Protein Nitrogen*

Seven-day-old green barley leaves were floated on the surface of the medium indicated and exposed to light (5000 lux) for the periods indicated. Nitrogen was determined by Nessler's method after wet digestion of the sample.

Time exposed	24 hr		48 hr	
Medium treated	EDTA (0.05 M, pH 7.0)	H_2O	EDTA (0.05 M, pH 7.0)	H_2O
Trichloroacetic acid soluble N				
μg/mg dry wt	3.2	4.2	2.9	5.3
Total protein N				
mg/g dry wt	32.9	54.2	32.1	62.1

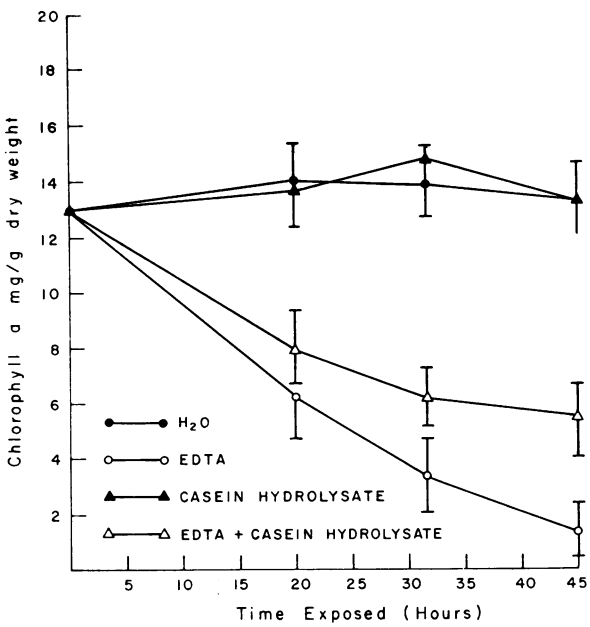

FIG. 6. Effect of casein hydrolysate on chlorophyll *a* content in EDTA-treated green barley leaves. Green barley leaves were floated on media contained in clear plastic containers and exposed to light (5000 lux). The final concentration of the media was 0.05 M EDTA (pH 7.0), and 0.025 M EDTA (pH 7.0), and 0.025 M EDTA (pH 7.0) plus 2 % (w/v) casein hydrolysate aqueous solution. Chlorophylls were extracted with boiling 80 % ethanol and determined spectrophotometrically. The vertical bars indicate 95 % confidence intervals.

In order to determine whether protein metabolism is involved in EDTA-bleaching, the trichloroacetic acid soluble nitrogen (total nucleic acid fraction) and total protein nitrogen (8) were determined by Nesslerization after wet digestion of samples. Table III presents the results obtained. It is clear that a significant difference developed between EDTA-treated leaves and the water controls with respect to both trichloroacetic acid soluble nitrogen and protein nitrogen suggesting the participation of protein metabolism in the phenomenon. To test this possibility, the effects of casein hydrolysate on EDTA-bleaching were observed. Green barley leaves were floated in 200 ml aliquots of the following media: (1) H_2O, (2) 2 % (w/v) casein hydrolysate, (3) 0.025 M EDTA-2Na at pH 7.0, and (4) 2 % (w/v) casein hydrolysate in 0.05 M EDTA-2Na at pH 7.0. All media contained 3 μM chloramphenicol to prevent microbial propagation. Fig. 6 shows the effects of

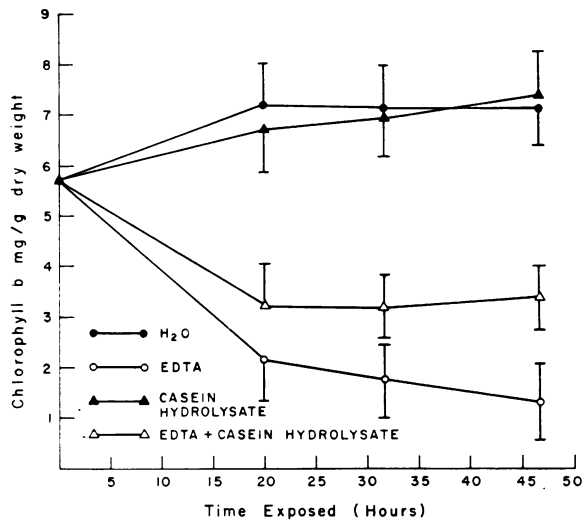

FIG. 7. Effect of casein hydrolysate on chlorophyll *b* content of EDTA-treated green barley leaves. See the legend for Fig. 6. Vertical bars signify 95 % confidence intervals.

casein hydrolysate on the chlorophyll *a* content of EDTA-bleached leaves. The H_2O and casein hydrolysate controls maintained a constant level of chlorophyll *a* throughout the entire exposure period, while EDTA-treated leaves underwent a marked decrease in chlorophyll *a* content. The rate of EDTA bleaching was appreciably reduced in the presence of 2 % (w/v) casein hydrolysate. A similar trend was noted in chlorophyll *b* content (Fig. 7).

Discussion

When green barley leaves are floated on the surface of water containing EDTA and are exposed to light, they undergo a pronounced bleaching.

Leaves floated on water in the absence of EDTA retain their color. This phenomenon is reversed in darkness, *i.e.*, the EDTA-treated leaves remain green while the leaves floated on H_2O are bleached.

One might be inclined to conclude that the bleaching effect of EDTA is produced by the chelating action of the EDTA molecule, *i.e.*, the magnesium atom in the chlorophyll molecule may be removed in the form of an EDTA-Mg chelate complex with the rest of the pyrrole rings of the chlorophyll molecule undergoing decomposition either by spontaneous photolysis or by an enzyme-linked photodecomposition. However, this concept is not compatible with the fact that alcohol or acetone extracts of water-treated leaves are bleached completely by exposure to light while in the presence of EDTA the chlorophyll preparations retain their green color permanently (Fig. 4). In the latter case a fine grain green precipitate forms which, like the supernatant, is not decolorized in light. The green color of either the precipitate or the supernatant is easily transferred to diethyl ether and displays the characteristic spectra of chlorophylls *a* and *b*.

It can be postulated alternatively that EDTA may inhibit a certain enzyme system required for chlorophyll formation. In this event chlorophyll production would cease and photodecomposition would account for the bleaching without involving direct action of EDTA on the chlorophyll molecule. This possibility has been explored by observing the effects of chlorophyll precursors such as CO_2, glycine, and δ-aminolevulonic acid on EDTA-bleaching. None of these substances produces any measurable inhibition of EDTA bleaching. Actually δ-aminolevulonic acid accelerates the rate of bleaching.

Also relevant is the experimental fact that the visible EDTA-bleaching of intact leaves does not occur in an atmosphere of nitrogen. Subsequent extraction of the leaves with hot alcohol, however, produces a green residue which remains insoluble even after 30 min of extraction. Thus a marked decrease in the hot-ethanol extractable chlorophyll content is observed in EDTA-treated but not in H_2O-treated intact leaves under anaerobic conditions (table I). Neither apparent bleaching nor the formation of hot-alcohol-insoluble green pigment is observed in boiled leaves in an atmosphere of nitrogen. Essentially the same amounts of chlorophylls *a* and *b* are extracted from both EDTA- and H_2O-treated boiled leaves (table I). In the presence of oxygen, more marked bleaching is observed in EDTA-treated intact and H_2O-treated boiled leaves than occurs in room air (table II).

The fact that inhibitors of the respiratory system (except KCN, Fig. 5) fail to prevent EDTA-bleaching suggests that no essential element in the respiratory system may be involved. The addition of KCN does not prevent the photodecomposition of purified chlorophylls *a* and *b* in organic solvents such as ethanol, acetone, *etc.* and H_2O-treated boiled leaves. KCN is only effective in preventing EDTA-bleaching in intact leaves under aerobic conditions.

Striking reversals of EDTA effects are observed when green leaves are treated with EDTA in light and in darkness (Figs. 1 and 2). A similar reversal is observed in comparing intact and boiled leaves (Fig. 3). This phenomenon is currently interpreted as indicating that EDTA may form a complex, which becomes insoluble in hot alcohol when it forms under anaerobic conditions, and this complex resists decomposition in the dark but in light it readily undergoes photodecomposition. From the fact that the bleaching of boiled leaves in light is protected by adding EDTA, it seems likely that the complex formation may proceed nonenzymatically but the photodecomposition of the complex may be catalyzed by an enzyme. Investigation of this point is presently in progress.

Casein hydrolysate inhibits EDTA-bleaching (Figs. 6 and 7). The total protein content of leaves exposed to EDTA and light declines markedly with bleaching (table III). One would be inclined to assume that calcium contained in casein hydrolysate complex reacts directly with EDTA. This is not likely to be the case, because the addition of $CaCl_2$ did not prevent EDTA bleaching. This fact indicates that only amino acid components of casein hydrolysate participate in the inhibition of EDTA bleaching.

No attempt has been made here to develop a hypothetical mechanism of EDTA action which would account for all the observed phenomena. This will be done in a subsequent paper utilizing the results of additional experimentation now in progress.

Acknowledgments

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Literature Cited

1. Jacobson, L. 1945. Iron in the leaves and chloroplasts of some plants in relation to their chlorophyll content. Plant Physiol. 20: 233–45.
2. Kotaka, S. and A. P. Krueger. 1968. Studies on the air-ion-induced growth increase in higher plants. In: Advancing Frontiers of Plant Sciences, L. Chandra, ed. New Delhi, India. p 115-208.
3. Kotaka, S., A. P. Krueger, and P. C. Andriese. 1965. Some observations on air-ion-enhanced iron chlorosis in barley (*Hordeum vulgaris*) seedlings. Plant Cell Physiol. 6: 233–45.
4. Kotaka, S., A. P. Krueger, and P. C. Andriese. 1968. The effects of air ions on light-induced swelling and dark-induced shrinking of isolated chloroplasts. Intern. J. Biometeorol. 12: 85–92.
5. Kotaka, S., A. P. Krueger, and P. C. Andriese. 1968. Bleaching effects of EDTA on green barley leaves. Plant Physiol. 43: S-4.

6. KOTAKA, S., A. P. KRUEGER, K. NISHIZAWA, T. OHUCHI, M. TAKENOBU, Y. KOGURE, AND P. C. ANDRIESE. 1965. Air ion effects on the oxygen consumption of barley seedlings. Nature 208: 1112–13.
7. KRUEGER, A. P., S. KOTAKA, AND P. C. ANDRIESE. 1966. Studies on the biological effects of gaseous ions. Vol. 1. Special Monograph Series, Biometeorological Research Center, Leiden.
8. OSERKOWSKY, J. 1933. Quantitative relation between chlorophyll and iron in green and chlorotic pear leaves. Plant Physiol. 8: 449–68.
9. SCHNEIDER, W. C. 1945. Phosphorus compounds in animal tissues. I. Extinction and estimation of deoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. 161: 293–303.
10. SELIGSON, D. AND H. SELIGSON. 1951. A microdiffusion method for the determination of nitrogen liberated as ammonia. J. Lab. Clin. Med. 38: 324–30.
11. VISHNIAC, W. 1957. Methods for the study of the Hill reaction. In: Methods in Enzymology. Colowick and Caplan, eds. Vol. 4. Academic Press, New York, New York. p 342–55.