Phytochemistry, 1972, Vol. 11, pp. 2393 to 2402. Pergamon Press. Printed in England.

EFFECTS OF EDTA AND OTHER COMPOUNDS ON CHLOROPHYLL BREAKDOWN IN DETACHED LEAVES

MARGARET HOLDEN

Rothamsted Experimental Station, Harpenden, Hertfordshire

(*Received* 17 *February* 1972)

Key Word Index—*Triticum aestivum*; Gramineae; wheat leaves; chlorophyll breakdown; EDTA; herbicides; kinins.

Abstract—Degradation of chlorophyll was inhibited in the dark in detached leaves floating on neutral EDTA solutions, but was stimulated in the light. Salts of inorganic and organic acids, surface active agents and herbicides, in different ranges of concentrations, likewise prevented breakdown of chlorophyll in the dark and caused it to be photo-oxidised in the light. Kinins inhibited degradation in the dark but did not promote bleaching in illuminated leaves.

INTRODUCTION

SOME chemical and physical treatments affecting the degradation of pigments in detached leaves were described in earlier papers.[1,2] Observations reported here and elsewhere show that several compounds which are routinely used in extraction mixtures for preparing plant organelles have deleterious effects on chlorophylls and carotenoids under some conditions. One of these, ethylene diamine tetra-acetic acid (EDTA) was found by Kotaka and Krueger[3] to cause chlorophyll to bleach in detached barley leaves floated on a 0·05 M solution at pH 7 in the light, but to retard its breakdown in the dark. This result was confirmed and many other compounds that are of interest for various reasons were tested for their effects on chlorophyll in detached wheat leaves in light and dark. The effects of EDTA are far from unique for they resemble those of other compounds, in different ranges of concentrations, and the present paper correlates these findings.

RESULTS

Effects of EDTA on the Chlorophyll in Detached Leaves in Light and Dark

Leaves of wheat seedlings floated on EDTA solution 0·05 M pH 7·2 and illuminated at 15 000 lx, lost nearly 80% of their chlorophyll in 20 hr; Fig. 1 shows the results of a typical experiment. On distilled water there was no loss of chlorophyll and in most experiments a definite gain was found. Carotenoids were also stable under these conditions. On sodium acetate solution (0·05 M, pH 7·1) about 15% of the chlorophyll was lost. The extent of bleaching depended on the concentration of EDTA (Table 1). Varying the pH between 6·5 and 8·5 had little or no effect on the loss of chlorophyll. It was also unaffected by variations in size of the leaf pieces.

When leaf samples on EDTA were transferred from light to dark before much of the chlorophyll had disappeared the bleaching did not continue, indicating that it is a photochemical process. Bleaching on EDTA was much diminished when leaves were illuminated

[1] M. HOLDEN, *Phytochem.* **9**, 1771 (1970).
[2] M. F. BACON and M. HOLDEN, *Phytochem.* **6**, 193 (1967).
[3] S. KOTAKA and A. P. KRUEGER, *Plant Physiol.* **44**, 809 (1969).

in an atmosphere of nitrogen instead of air, but it was not completely prevented. In a typical experiment, 70% of the chlorophyll was lost in air and 30% under nitrogen when illuminated for 18 hr. Because the vessel was evacuated before flushing with nitrogen the EDTA solution penetrated the leaves more rapidly than it penetrated those in air. This might have increased the effect of the EDTA in samples so treated, but there was clearly less pigment in leaves in air than in those with much of the oxygen removed.

TABLE 1. EFFECT OF EDTA CONCENTRATION ON THE CHLOROPHYLL CONTENT OF DETACHED WHEAT LEAVES IN LIGHT AND DARK

Concentration of EDTA (M)	% of chlorophyll retained			
	Light		Dark	
	18 hr	32 hr	2 days	4 days
Water	108	97	59	21
0·005	87	64	65	31
0·01	88	40	74	53
0·02	90	0	96	91
0·05	28	0	104	97

All the EDTA solutions were at pH 7·2 at the start, but during incubation in the dark the pH rose to about 8 in the more dilute solutions.

Barley and oat seedling leaves behaved like wheat, but in discs from bean leaves that were illuminated chlorophyll was broken down more readily than in cereal leaves. On water, more than 20% was lost in 20 hr and the breakdown on 0·05 M sodium acetate (70% loss) was nearly as great as on EDTA (85% loss).

During EDTA bleaching the ratio of chlorophyll *a* to chlorophyll *b* changed from about 3 at the start to near 1 when only 20% of the chlorophyll remained; thus chlorophyll *a* was degraded more rapidly than *b*. In wheat leaves floated on water in the light for up to 48 hr there was either no change or a slight increase in the ratio. In the dark for up to 5 days there was no consistent change in the ratio.

The decrease in the chlorophyll content of bleached leaves is mostly accounted for by the formation of colourless breakdown products, but coloured products such as pheophytins, with smaller absorption coefficients than chlorophyll, may also be formed.[2] Neither pheophytins nor pheophorbides were, however, usually detected during EDTA bleaching, but a 'changed' chlorophyll *a* (which is probably an oxidation product), lying just ahead of chlorophyll *b* on TLC plates, often occurred in extracts of partly bleached leaves. There was also some pigment that did not move far from the origin. Carotenoids were also broken down; the carotene and lutein contents were about half the starting values when 80% of the chlorophyll had disappeared.

Cereal leaves floated on water in alternating light (16 hr) and dark (8 hr) for 4 days retained 80–90% of their chlorophyll, whereas those in the dark for the whole period retained only about 20%. Figure 2 shows that in wheat leaves in the dark on 0·05 M EDTA, pH 7·2, chlorophyll breakdown was inhibited and after 4 days only 10% had disappeared. In another experiment with longer incubation times, 90% of the chlorophyll was still retained after 10 days in the dark on EDTA at 22°, although on water all the chlorophyll had disappeared after 7 days.

In other tests, leaves were removed from EDTA after 3 days in the dark, blotted dry, transferred to water and again kept in the dark. The effect of the EDTA persisted and after 3 days on water the leaves were dark green whereas those that had been on water for the whole 6 days were completely yellow. Leaves illuminated after being kept on EDTA in the dark for several days bleached on EDTA as fast as fresh leaves; they also bleached rapidly on water, presumably because EDTA had been absorbed.

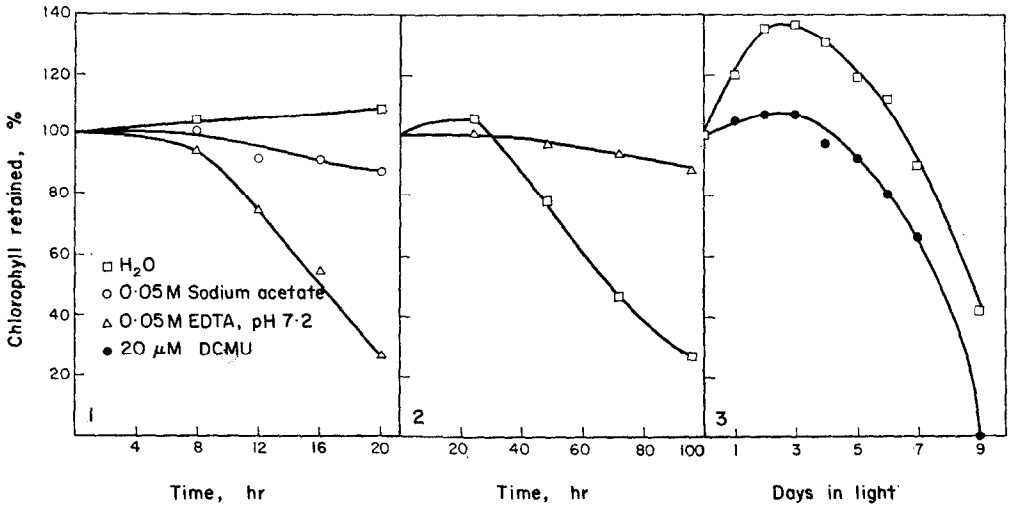

FIG. 1. EFFECT OF EDTA ON THE CHLOROPHYLL CONTENT OF DETACHED WHEAT LEAVES IN THE LIGHT.

FIG. 2. EFFECT OF EDTA ON THE CHLOROPHYLL CONTENT OF WHEAT LEAVES IN THE DARK.

FIG. 3. EFFECT OF DCMU ON THE CHLOROPHYLL CONTENT OF WHEAT LEAVES IN CONTINUOUS LIGHT.
For Fig. 3 only. The samples were kept in the dark for 48 hr on either water or DCMU before being illuminated (4000 lx).

The amount of chlorophyll retained in the dark depended on the EDTA concentration (Table 1). The effective range of concentrations was the same as for promoting chlorophyll bleaching in the light.

Effect of EDTA on the Liberation of Non-protein Nitrogen

Kotaka and Krueger[3] found that protein N and nucleic acid N decreased in leaves illuminated on EDTA. In the present study there was an increase in the amount of TCA-soluble N in leaves floated on 0·05 M EDTA, pH 7·3 in the light; in addition, nitrogen was leached out into the solution on which the leaves were floated. During incubation of leaf samples on water in the light for 16–20 hr there was no increase, but sometimes a slight decrease, in the TCA-soluble N. By contrast, in leaves in the dark for several days the amount of TCA-soluble N increased greatly in leaves floated on water but not on EDTA. Table 2 gives the results of a typical experiment using wheat seedling leaves.

Effect of EDTA on Solubility of Pigments in Light Petroleum

Little chlorophyll can be extracted by light petroleum (b.p. 40–60°) from fresh leaves, but much of it extracts from boiled leaves because heating breaks the link between pigments

and lipoprotein. Evidence was sought for EDTA treatment having a similar effect. Leaves that had been, (a) partly bleached by EDTA in the light, or (b) kept on EDTA in the dark, were extracted with light petroleum and petroleum–acetone mixtures. The proportion of light petroleum-soluble chlorophyll was not increased in leaves treated with EDTA in light or dark.

TABLE 2. EFFECT OF EDTA ON THE TCA-SOLUBLE N OF WHEAT LEAVES IN LIGHT AND DARK

	mg N/g wet wt leaf			
	Light, 20 hr		Dark, 4 days	
Fraction	water	EDTA	water	EDTA
N in incubation fluid	<0·10	0·36	<0·10	0·10
TCA soluble-N in leaves	0·42	1·57	2·17	1·00

1 g lots of leaf (containing 142 mg dry matter, 4·70 mg total N, 0·62 mg TCA-soluble N) floated on either water or 0·05 M EDTA solution pH 7·3.

Effect of EDTA on Chlorophyll in Boiled and Frozen Leaves and in Extracts

Chlorophyll bleaches quickly on exposure to light in leaves that have been boiled, macerated or frozen. Kotaka and Krueger[3] claimed that boiled leaves incubated on 0·05 M EDTA did not bleach, but this observation could not be confirmed. Leaves that were either boiled in water, or steamed over boiling water, bleached just as fast on EDTA as on water. Leaves boiled in EDTA solution lost their chlorophyll equally rapidly whether illuminated on water or EDTA. Suspensions of leaves ground in water or 0·05 M EDTA, pH 7·3, bleached completely when illuminated for 11 hr. Furthermore, EDTA did not stabilise the chlorophyll in frozen leaves kept in the light. Kotaka and Krueger[3] also stated that 0·025 M EDTA stabilised chlorophyll in solvent extracts exposed to 5000 lx. This effect was not confirmed in tests using acetone and ethanol extracts of leaves illuminated at 4000 or 15 000 lx.

Effect of Other Chelators

Three chelating compounds of the same type as EDTA were tested at 4 g/l., pH 7·2 for their effect in light and dark. After 32 hr in the light, leaves on hydroxyethylene diamine triacetic acid (HEEDTA) were, like those on EDTA, completely bleached, but those on diethylene triamine penta-acetic acid (DTPA) and cyclohexane*trans*-1,2-diaminotetra-acetic acid (CDTA) were only slightly bleached. In the dark, chlorophyll breakdown was almost completely prevented in leaves on HEEDTA but not at all on the other compounds. Perhaps DTPA and CDTA penetrated the leaves less well than EDTA and HEEDTA. The effect of other metal-binding compounds such as citrate and oxalate is discussed in the next section on the effect of salts.

Effect of Salt Solutions

The loss of chlorophyll from leaves illuminated on 0·05 M NaOAc solution suggested further studies on the effects of salt solutions. Table 3 shows the effects of varying the concentration of NaOAc. The amount of chlorophyll bleached on 0·2 M NaOAc in the light

was similar to that on neutralized 0·05 M EDTA; 0·2 M acetate prevented chlorophyll degradation in the dark. Tris-HCl buffer and citrate–HCl buffer, both about 0·1 M and pH 7·2, caused more bleaching in the light than 0·1 M acetate and partly prevented breakdown in the dark. In contrast, Na_2HPO_4 0·1 M adjusted to pH 7·2 with HCl had no effect on the chlorophyll content in light and dark. NaCl and NH_4Cl less concentrated than 0·1 M did not affect dark breakdown in wheat leaves; this agrees with the results of Thimann and Sachs[4] with oat leaves. In the light, solutions weaker than 0·2 M had no effect but with 0·5 M about half the chlorophyll was lost in 17 hr. The following organic acids, neutralised to about pH 7·2 with NaOH, were tested in light and dark: glycollic, malic, succinic, tartaric, oxalic and benzoic. With concentrations of 0·1 M and less, only oxalate and benzoate partly inhibited breakdown in the dark and caused slight bleaching in the light. The effects were bigger with 0·2 M oxalate and benzoate and small effects were found with the other acids at this concentration.

TABLE 3. EFFECT OF SODIUM ACETATE ON THE CHLOROPHYLL CONTENT OF WHEAT LEAVES IN LIGHT AND DARK

Concentration of sodium acetate (M)	(pH)	% of chlorophyll retained		
		Light		Dark
		18 hr	26 hr	3 days
Water		114	102	46
0·01	6·5	—	99	48
0·02	6·9	103	80	48
0·05	7·0	86	70	55
0·1	7·2	58	38	68
0·2	7·4	12	0	100

Effect of Sucrose

Khudairi[5] studied the effect of sucrose on chlorophyll degradation in leaf discs of *Xanthium* in light and dark. In the light with sucrose more concentrated than 0·01 M, more chlorophyll was lost than on water, whereas in the dark slightly less was lost. It is, however, difficult to compare his results with mine because he used longer incubation times and less illumination. With wheat leaves illuminated for 19 hr, sucrose at concentrations between 0·005 and 0·1 M did not cause bleaching and the rate of chlorophyll breakdown in the dark was also not affected within this range. With 0·5 M sucrose detectable bleaching occurred in the light. Goldthwaite and Laetsch[6] found that 0·01–0·1 M sucrose accelerated the breakdown of chlorophyll in bean leaf discs in the dark but 1 μM–1 mM slightly retarded the loss. Experiments using bean leaves did not confirm this observation.

Effect of Surface-active Agents

Triton X100 (non-ionic detergent) buffered with 0·025 M sodium acetate caused marked bleaching in the light (Table 4). In the dark, chlorophyll breakdown was apparently completely prevented by concentrations of 200 mg/l. and greater. However, some pigment was

[4] K. V. THIMANN and T. SACHS, *Am. J. Bot.* **53**, 731 (1966).
[5] A. K. KHUDAIRI, *Physiol. Plant.* **23**, 613 (1970).
[6] J. J. GOLDTHWAITE and W. M. LAETSCH, *Pl. Physiol.* **42**, 1757 (1967).

leached out of the leaves into the fluid on which they were incubated. Manoxol OT (dioctyl sodium sulphosuccinate, anionic detergent) causes pheophytin to be formed in leaves in the dark, although the pH of the suspension remains above 6.[1] In the light there was obvious loss of chlorophyll on a solution 0·5 g/l., buffered at pH 7, but this too was mainly due to pheophytin formation. The pheophytin is degraded more slowly than chlorophyll to colourless compounds.

Cetyl trimethylammonium bromide (cationic detergent) caused slight bleaching in the light with solutions more concentrated than 2 g/l. buffered at pH 7. Solutions of dimethylsulphoxide up to 100 g/l. had no effect on the rate of chlorophyll breakdown in the dark; in the light, 50 g/l. caused loss of chlorophyll.

TABLE 4. EFFECT OF TRITON X100 ON THE CHLOROPHYLL CONTENT OF WHEAT LEAVES IN LIGHT AND DARK

Concentration of Triton X100 (mg/l.)	% of chlorophyll retained	
	Light 15 hr	Dark 4 days
0·025M NaOAc	86	33
100	63	43
200	19	69*
500	13	79*

* Some pigment lost to Triton solution.
The Triton solutions contained 0·025 M sodium acetate giving a pH of 7·2.

Effect of Herbicides and Growth Substances

Diquat (1,1-*ethylene*-2,2 *dipyridylium dibromide*) *and paraquat* (1,1-*dimethyl*-4,4-*dipyridylium dichloride*). The bleaching of chlorophyll by EDTA in the light has obvious similarities to the effects of some herbicides. Baldwin *et al.*[7] studied the effect of the bipyridylium herbicides, diquat and paraquat, on detached cotyledons of flax (*Linum usitatissimum*) in the light. They found that during bleaching chlorophyll *a* was partly converted into pheophytin *a* and was broken down faster than chlorophyll *b*, leading to a smaller *a* to *b* ratio. They also found that the chlorophyll *a* to *b* ratio decreased in cotyledons kept for many days on water in the light. This was part of the evidence that led them to conclude that changes caused by herbicides resemble hastened senescence. Chlorophyll *a* to *b* ratios are, however, probably meaningless when only a small fraction of the chlorophyll remains in detached leaves or cotyledons kept for long periods. Stokes *et al.*[8] studied the disorganisation of chloroplast structure and simultaneous chlorophyll bleaching in *Chlorella vulgaris* treated with diquat. They suggested that the effects seen could be caused by the continual production of a free radical and by H_2O_2 resulting from oxidation of this radical, or more directly to photooxidation in a pigmented system in which photosynthesis is inhibited. Diquat and paraquat were tested for their effects on the chlorophyll content of detached wheat leaves in light and dark and the results of one experiment are given in Table 5. These compounds at 20 mg/l. not only caused bleaching in the light but inhibited chlorophyll breakdown in

[7] B. C. BALDWIN, A. D. DODGE and N. HARRIS, *Proc. 9th Brit. Weed Control Conf.* p. 639 (1968).
[8] D. M. STOKES, J. S. TURNER and K. MARKUS, *Aust. J. Biol. Sci.* **23**, 265 (1970).

the dark. No pheophytin or pheophorbide could be detected in extracts from wheat leaves floated on paraquat in the light, but there was some brown pigment that was immobile in the solvent systems used for separating the pigments. In agreement with Baldwin *et al.*[7] the chlorophyll *a* to *b* ratio diminished during bleaching by paraquat; in one experiment it changed from 3·0 at the start to 1·5 when 70% of the chlorophyll had disappeared.

TABLE 5. EFFECT OF DIQUAT AND PARAQUAT ON THE CHLOROPHYLL CONTENT OF WHEAT LEAVES IN LIGHT AND DARK

	% of chlorophyll retained			
	Diquat		Paraquat	
Concentration (mg/l.)	Light 16 hr	Dark 4 days	Light 10 hr	Dark 4 days
0·025 M NaOAc	94	28	97	27
2	79	55	—	—
5	59	89	84	49
10	33	92	77	64
20	26	92	57	82
50	10	95	18	94

All solutions contained 0·025 M NaOAc giving a final pH of about 7.

Ioxynil (3,5-*diiodo*-4-*hydroxybenzonitrile*). Wain[9] stated that leaf discs placed in aqueous ioxynil solution (1 g/l.) lost their chlorophyll in the light though not in the dark, but gave no further details about breakdown in the dark. He suggested that free radicals are formed when plants are treated with ioxynil. Table 6 shows that in detached wheat leaves ioxynil, at greater concentrations than with diquat and paraquat, inhibited breakdown in the dark and promoted bleaching in the light. However, it seemed that the ioxynil was not completely penetrating the pieces of leaf because they were striped. The leaves that had been kept in the dark had green bands on the ends of the strips and a pale area in the middle, whereas those in the light had bleached ends with a green area in the middle.

TABLE 6. EFFECT OF IOXYNIL ON THE CHLOROPHYLL CONTENT OF WHEAT LEAVES IN LIGHT AND DARK

	% of chlorophyll retained	
Concentration (mg/l.)	Light 22 hr	Dark 3 days
0·025 M NaOAc	91	35
19	88	29
38	80	33
75	67	45
188	36	62
375	24	78

TABLE 7. EFFECT OF 8-QUINOLINOL SULPHATE ON THE CHLOROPHYLL CONTENT OF WHEAT LEAVES IN LIGHT AND DARK

	% of chlorophyll retained	
Concentration (mg/l.)	Light 13 hr	Dark 5 days
Water	110	0
100	96	14
200	75	24
500	56	70
*1 g/l.	36	95

* 0·00246 M.

[9] R. L. WAIN, *Proc. 7th Brit. Weed Control Conf.* 306 (1964).

DCMU (3-(3,4-*dichlorophenyl*)-1,1-*dimethylurea*). Goldthwaite and Laetsch[6] found that bean leaf discs on DCMU (0·75 μM) in the dark for 8 days lost chlorophyll at the same rate as discs on water. In the light, chlorophyll was lost more slowly than in the dark from discs on water, but it disappeared from DCMU treated discs at the same rate as in the dark. That is, DCMU abolished the effect of light in preventing chlorophyll breakdown. DCMU is slightly soluble in water and was tested on wheat leaves at concentrations of 50 μM and less. In agreement with Goldthwaite and Laetsch[6] there was no effect on the rate of dark breakdown. It was mentioned earlier that the chlorophyll content of detached wheat-seedling leaves increases when they are illuminated on water for about 20 hr. With continuous illumination (4000 lx) the increase continued for at least another day, sometimes reaching over 140% of the starting value and the dry weight remained constant. After another day the chlorophyll content started to diminish and seven days after being detached the leaves usually had a value similar to the original. Figure 3 shows the effect of DCMU on leaves illuminated for several days. In this particular experiment there was a small increase in the chlorophyll content during the first three days in the light, but in others the increase was eliminated by 20 μM DCMU. The rate of chlorophyll decrease on DCMU was similar to that in leaves on water in the light. After 9 days illumination the leaves on DCMU were colourless, whereas those on water still had 40% of their original chlorophyll content. On water and DCMU in the dark all the chlorophyll was lost in 7 days. In the light therefore, even with DCMU present, senescence was delayed for several days.

2,4-*Dichlorophenoxyacetic acid* (2,4-*D*). On wheat leaves 2,4-D behaved much like EDTA, with chlorophyll bleached in the light but breakdown inhibited in the dark with 0·05 M, and smaller effects with lower concentrations.

Kinins

Benzyladenine and 6-furfurylaminopurine (kinetin) were tested in the range 0·5–5 mg/l. In the dark for 5 days, with all concentrations of both compounds, about 80% of the chlorophyll of wheat-seedling leaves was retained compared with only 30% in the control sample on 0·025 M NaOAc. In the light for 17 hr there was no chlorophyll breakdown in any of the test samples nor in the controls. Thus, concentrations that inhibit breakdown of chlorophyll in the dark do not cause bleaching in the light.

Chua[10] observed that 8-quinolinol sulphate had phytokinin-like activity because it retarded chlorophyll degradation in oat leaves in the dark. He did not test its action in the light. Table 7 shows its effects on chlorophyll in wheat leaves in light and dark. Although 1 g/l. prevented breakdown of chlorophyll in the dark, this concentration caused bleaching in the light with only 13 hr illumination. Quinolinol does not therefore behave as a kinin but in a similar way to EDTA and other substances.

DISCUSSION

EDTA is only one of many substances which, in different ranges of concentrations, promote the bleaching of chlorophyll in the light and inhibit its degradation in the dark. Several of these compounds including EDTA, Triton X100 and the herbicides have been tested for their effects on some of the reactions of photosynthesis.[11–13] Reactions may be

[10] S. E. CHUA, *Nature, Lond.* **225,** 101 (1970).
[11] A. T. JAGENDORF and M. SMITH, *Plant Physiol.* **37,** 135 (1962).
[12] L. P. VERNON and E. SHAW, *Plant Physiol.* **40,** 1269 (1965).
[13] M. W. KERR and R. L. WAIN, *Ann. appl. Biol.* **54,** 441, 447 (1964).

inhibited or stimulated depending on the concentration. Most of the compounds are inhibitors of the Hill reaction and some are also uncoupling agents. Although they do not all affect the same reactions, they nevertheless probably all interfere enough to block photosynthesis.

The mechanisms by which chlorophyll is broken down in plant tissues have not yet been elucidated, but breakdown is clearly linked with the degradation of protein and probably also of lipids. In detached leaves floated on water in the light, photosynthesis and protein synthesis can be maintained for a limited period and chlorophyll is retained or even increases in amount. When photosynthesis is inhibited, the chloroplasts become non-functional and catabolic reactions associated with senescence start, leading ultimately to death. Protein synthesis stops and it soon starts to break down; degradation of chlorophyll also starts and, in addition to the normal mechanism of breakdown, irreversible photo-oxidation occurs when the cells die. The speed at which this sequence occurs will depend on a variety of factors, including the nature and concentration of the inhibitor and the rate at which it diffuses into leaf pieces and enters the chloroplasts.

In the dark, even without inhibitors present, photosynthesis stops, protein breaks down and chlorophyll will be broken down. With poisons present in large enough amounts to inhibit enzyme reactions, protein and chlorophyll degradation will probably be prevented until the tissue is broken down by micro-organisms.

The effects of EDTA and other compounds on chlorophyll resemble those caused by irradiation with γ-rays. Haber and Walne[14] found that chlorophyll was bleached in detached wheat-leaf tips when these were illuminated after irradiation; they also found that normal chlorophyll degradation in the dark was prevented. These effects were two of the criteria they used for establishing conditions for killing the tissues.

Some of the compounds tested have been described as having phytokinin-like activity (e.g. 8-quinolinol sulphate by Chua[10]) but they inhibit chlorophyll breakdown in the dark, not by retarding the senescence of living tissue, but by poisoning it. A distinction can be made between compounds such as EDTA and growth substances like kinetin and benzyladenine. Growth substances do not cause chlorophyll to be bleached in the light when tested at concentrations that retard its breakdown in the dark.

Salts of metals such as Ni, Co, Pb, Fe and Zn are known to affect chlorophyll breakdown[15,16] but only a few preliminary tests on them were made in the present study. These suggested that the interpretation of results was likely to be complicated by the effects of these substances on the pigments themselves, in addition to any effects on factors concerned with chlorophyll degradation.

EXPERIMENTAL

Plant material. Cereal seedlings were grown in the laboratory on moist filter paper in glass tanks and harvested when 8–10-days-old. For all experiments with wheat leaves cv. Cappelle was used. Seedlings of *Phaseolus vulgaris* were grown in a glasshouse and the undivided first pair of leaves used. Leaves from cereals were cut into 10–15 mm lengths; from broadleaved plants 10 mm discs were cut with a cork borer.

Conditions of incubation. Leaf samples (0·4–0·8 g) were floated on 10 ml water, or the solution being tested, in plastic Petri dishes. EDTA solutions were made up by dissolving the disodium salt in water, neutralising to about pH 7·2 with NaOH and diluting to the appropriate volume. For dark treatment the dishes were kept in a dark cupboard at room temp. For light treatment they were kept in constant environment cabinets at 25° illuminated by warm-white fluorescent tubes giving a light intensity of either about 4000 or 15 000 lx. For incubation under nitrogen, flat glass vessels that could be evacuated and then flushed with N_2 were used.

[14] A. H. Haber and P. L. Walne, *Radiat. Bot.* **8**, 389 (1968).
[15] D. Wang and E. R. Waygood, *Can. J. Bot.* **37**, 743 (1959).
[16] W. R. Bushnell, *Can. J. Bot.* **44**, 1485 (1966).

Determination of pigments. (1) *Chlorophyll.* Pigments were extracted from weighed samples of leaves (0·4–0·8 g) by grinding in 80% acetone with sand in a mortar. The suspension was made up to 25 ml, filtered and a reading taken on an EEL colorimeter using filter 607. Readings were obtained for samples of untreated leaves (A_1) and for samples after the various treatments (A_2). The value '% of chlorophyll retained' is $A_2/A_1 \times 100$. In some experiments chlorophylls *a* and *b* were determined separately, (a) by spectrophotometry of the crude extracts using the equations given by Arnon,[17] and (b) by TLC on cellulose,[18] scraping off the bands containing the pigments, eluting with acetone and measuring the absorption at 662 nm for chlorophyll *a* and 645 nm for chlorophyll *b*. The amounts of pigments were calculated using the specific absorption coefficients given by Mackinney.[19] (2) *Carotenoids.* Acetone extracts containing both chlorophyll and carotenoids were saponified with methanolic potash and the carotenoids transferred to light petroleum. The same solvent that was used for TLC of chlorophylls gave satisfactory separation of carotenoids. The carotene and lutein bands were eluted with light petroleum and the absorption curves between 350 and 550 nm run.

Determination of acid-soluble nitrogen of leaves. Leaf material (1 g) was ground with 10 ml 10% w/v trichloroacetic acid at room temperature and the extract separated from the residue by filtering through a small sintered glass funnel. Nitrogen was determined in the extract by the Kjeldahl method. N was also determined on samples of the fluids on which the leaves had been incubated because preliminary tests showed that with some treatments much N could be leached out. The N content of a sample of the EDTA solution actually used for the tests was always determined.

Acknowledgement—I thank Mrs L. J. Tollett for technical assistance.

[17] D. I. ARNON, *Plant Physiol.* **24,** 1 (1949).
[18] M. F. BACON, *J. Chromatog.* **17,** 322 (1965).
[19] G. MACKINNEY, *J. Biol. Chem.* **132,** 91 (1940).