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CHLOROPHYLL BLEACHING SYSTEMS IN LEAVES

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Abstract—Chlorophyll was bleached when leaves of various species were incubated in aq. acetone solutions at pH 6 in the dark, but not when they were suspended in solutions of several surface-active compounds. Leaves that bleached in aq. acetone were all rich in lipoxidase but a few with high activity did not bleach appreciably. Although leaves with low lipoxidase activity did not bleach, pheophytin or pheophorbide was sometimes formed and decreased the absorption of pigment-containing extracts. Adding glycollate to chloroplast-containing aqueous extracts of wheat and barley leaves caused chlorophyll to bleach, but glycollate oxidation seems not to be involved in the bleaching in leaves in aq. acetone.

INTRODUCTION

WHILE investigating the formation of "changed" chlorophylls in leaves, Bacon and Holden[1] observed that most of the chlorophyll disappeared from chopped barley leaves that were suspended in 50% aq. acetone at room temp. overnight in the dark; discs of *Heracleum* leaves bleached much less under the same conditions. Extracts from legume seeds bleached chlorophyll during the breakdown of hydroperoxides of unsaturated fatty acids formed by lipoxidase action.[2] It seemed possible that a similar system might be involved in the bleaching of leaf tissue in aq. organic solvents. Fatty acid peroxidizing activity was reported in extracts from leaves of barley[3] and wheat seedlings[4] and in several other species.[5] In another paper[6] it is shown that the formation of linoleic acid hydroperoxides by extracts from cereal and other leaves is catalysed by a lipoxidase-type enzyme. The relation between lipoxidase activity and chlorophyll bleaching was studied in detail using cereal seedlings and the investigation was extended to a wide range of other species.

Kolesnikov,[7] and Tolbert and Burris[8] found that chlorophyll bleaching in extracts of barley seedlings was connected with the oxidation of glycollic acid, but did not elucidate the mechanism. The possibility that glycollate oxidation was wholly or partly responsible for the bleaching in aqueous organic solvents was therefore also investigated.

RESULTS

Chlorophyll Bleaching in Leaves of Cereal Seedlings

The leaves of three barley cultivars, "Maris Badger", "Impala" and "Rika", all showed bleaching of chlorophyll when suspended in aq. acetone solutions; up to 80% of the chlorophyll disappeared during incubation at about 21° in the dark overnight. The amount of

[1] M. F. BACON and M. HOLDEN, *Phytochem.* **6**, 193 (1967).
[2] M. HOLDEN, *J. Sci. Food Agri.* **16**, 312 (1965).
[3] P. A. KOLESNIKOV, *Dokl. Akad. Nauk. SSSR* **71**, 1085 (1950).
[4] K. V. PSHENOVA and P. A. KOLESNIKOV, *Biokhimiya* **26**, 1008 (1961).
[5] K. S. RHEEI and B. M. WATTS, *J. Food Sci.* **31**, 664 (1966).
[6] M. HOLDEN, *Phytochem.* **9**, 507 (1970).
[7] P. A. KOLESNIKOV, *Dokl. Acad. Nauk. SSSR* **60**, 1353 (1948); *Biokhimiya* **14**, 124 (1949).
[8] N. E. TOLBERT and R. H. BURRIS, *J. Biol. Chem.* **186**, 791 (1950).

bleaching depended on the concentration of acetone; most occurred when it was between 40 and 50%, v/v (Fig. 1). In aq. methanol and ethanol solutions the result was similar.

Three wheat cultivars, "Kloka", "Opal" and "Cappelle", all bleached strongly (50%); rye, "Tetragorzow" and "Lovaszpotonai", bleached less (about 30%). In oats, "Blenda", "Powys" and "Astor", there was little or no bleaching with either chopped (1 cm pieces) or ground leaves.

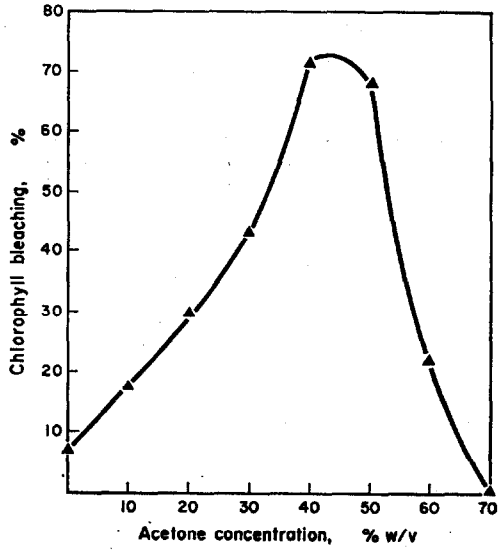

FIG. 1. EFFECT OF ACETONE CONCENTRATION ON CHLOROPHYLL BLEACHING IN CHOPPED BARLEY LEAVES.

The pH optimum for the reaction was near 6; above pH 7 little chlorophyll was bleached and below pH 5 the formation of pheophytin made interpretation of results difficult.

Leaves that had been previously boiled or steamed did not bleach when suspended in aq. acetone; leaves kept overnight at −20° bleached to the same extent as fresh ones. Chopped leaves usually bleached more than leaves ground finely before incubation. When leaf suspensions were incubated under nitrogen, chlorophyll bleaching was much diminished but was not completely prevented. Purified chlorophyll was bleached when added to ground leaves of etiolated seedlings suspended in aq. acetone.

For convenience, samples were often incubated overnight, but 6 hr was usually long enough to get maximum bleaching. Bleaching was speeded by shaking the suspension on a mechanical shaker. For example, in one experiment 49% of the chlorophyll was lost in a sample that was shaken for 3 hr compared with 22% from one that was not shaken. Adding the anti-oxidant nordihydroguaiaretic acid to the incubation fluid diminished the bleaching and with 10 mg/g leaf there was none. Bleaching was increased by adding linoleic acid; 2 mg of acid added to 1 g of ground wheat leaf in 40% acetone nearly doubled the bleaching (19–34%) when incubated for 2 hr.

Pigments Formed during Incubation in aq. Acetone

The small amount of green pigment that remained in barley leaves was a mixture of chlorophyll *a* and *b* and "changed" chlorophylls. Chlorophyllides were not detected because

the chlorophyllase activity of barley leaves is exceptionally small. In wheat leaves, the remaining pigments were mostly chlorophyllides because wheat seedlings are rich in chlorophyllase. In rye, which has less chlorophyllase activity, "changed" chlorophylls and chlorophyllides were formed. In oat, the chlorophylls were almost unchanged; chlorophyllides were not formed and chlorophyllase activity could not be detected with the conditions of assay used.

Chlorophyll Bleaching in Leaf Extracts

In extracts containing chloroplasts and fragments, made from barley and wheat leaves with water or buffer solutions, chlorophyll was bleached on adding acetone. It was also bleached when sodium glycollate was added instead of acetone, confirming the results of earlier workers. Figure 2 shows the effect of various concentrations of acetone, in the presence and absence of glycollate, using a barley leaf extract. The pH used (6·9) was near the

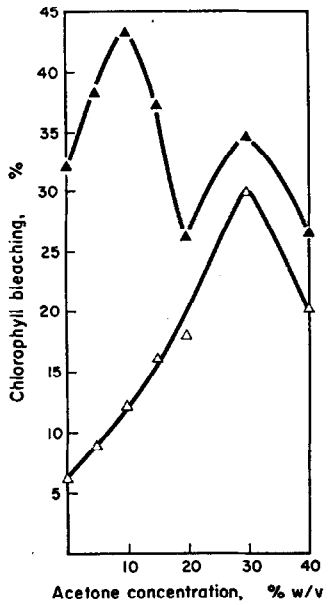

FIG. 2. CHLOROPHYLL BLEACHING IN BARLEY LEAF EXTRACT IN PRESENCE AND ABSENCE OF SODIUM GLYCOLLATE.

△ Without glycollate; ▲, 0·05 M sodium glycollate present. All samples contained sodium citrate 0·033 M, pH 6·9, and were incubated 4 hr in dark at 22°.

optimum for "glycollate bleaching" but rather higher than that for "acetone bleaching". Acetone up to 10% stimulated bleaching with glycollate present but when the concentration increased to 20% bleaching was inhibited. With 30% acetone the bleaching without glycollate became considerable and the "glycollate bleaching" was diminished still further. In this experiment the total bleaching was slightly greater with 0·05 M glycollate present than without glycollate, and this happened in about half the experiments although in the others there was no difference. With 40% acetone the total bleaching was decreased, whereas in chopped leaves maximum bleaching occurred at this concentration, or even slightly higher.

The amount of chlorophyll bleached in leaves suspended in aq. acetone was slightly but consistently increased by adding soya lipoxidase; with 4 hr incubation in 33% acetone, 1 mg lipoxidase increased the bleaching of a wheat-leaf extract from 27 to 38%.

Chlorophyll Bleaching in Solutions of Surface-active Agents

To see whether chlorophyll was bleached when the aq. organic solvent solution was replaced by a solution of a surface-active compound, several detergents and dimethyl sulphoxide were each tested with a range of concentrations. With dimethyl sulphoxide and the non-ionic detergents Triton X-100 and Tween-20, there was no sign of chlorophyll bleaching in wheat or barley leaves. Solutions of cetyl trimethyl ammonium bromide (cationic detergent) were acid and the pH was therefore adjusted to 6·5 before use; no bleaching occurred. With anionic detergents Manoxol OT (sodium dioctylsulphosuccinate) and sodium lauryl sulphate, there seemed to be some bleaching. However, although the pH did not fall below 6·2, these detergents caused enough pheophytin to be formed to account for the loss of absorption of pigment-containing extracts.

Lipoxidase Activity of Cereal Seedlings

Wheat leaves had the highest lipoxidase activity of the cereal seedlings tested, with "Cappelle" considerably more active than the other cultivars. Barley was intermediate between wheat and rye. Oat cultivars all had very low lipoxidase activity.

In chopped wheat leaves suspended in 50% acetone, 65% of their lipoxidase activity was inactivated in 1 hr and only 5% remained after 4 hr.

Chlorophyll Bleaching and Lipoxidase Activity in Leaves of Other Species

To investigate whether bleaching of chlorophyll in leaf material suspended in aq. acetone is a general phenomenon, species from a range of other families were tested. The leaves were usually from mature plants so are not strictly comparable to the cereal-seedling leaves, but where possible young and old leaves were tested. With more than half the species examined (Table 1) there was little (< 10%) or no chlorophyll bleaching in discs or in ground leaves. The other species showed more than 10% bleaching but few exceeded 50% (Table 2). In this group, bleaching in discs was nearly always greater than in ground leaves but discs of *Digitalis purpurea* bleached less than ground leaves. In some species, e.g. *Iris* and *Paeonia*, the bleaching seemed to be almost entirely due to formation of pheophytins and pheophorbides, rather than to actual loss of pigments by conversion to colourless compounds.

Lipoxidase activity was measured in extracts from samples of all the leaves tested for chlorophyll bleaching and the values are given in the tables. With some exceptions, fatty-acid peroxidizing activity was correlated with the bleaching of chlorophyll in leaves suspended in aq. acetone, and there was usually little or none when the lipoxidase activity was small. With moderate to high activity, the bleaching in discs ranged from large in *Doronicum plantagineum* to very small in *Arctium lappa* and *Cucumis melo*. There was, however, some bleaching in ground leaves of the two latter species.

DISCUSSION

The results of experiments with sodium glycollate and acetone added to wheat and barley leaf extracts suggest that glycollate oxidation is probably not responsible for chlorophyll bleaching in these leaves when they are suspended in aq. acetone. The evidence points to a connexion between fatty-acid peroxidation and chlorophyll breakdown, but with the information available it is not possible to say whether the mechanism resembles that in legume-seed extracts, with chlorophyll being bleached during the breakdown of fatty-acid hydroperoxides.

The chlorophyll bleaching phenomenon is shown convincingly by comparatively few of the many species tested. In several species, coloured breakdown products of chlorophyll, with lower

TABLE 1. CHLOROPHYLL BLEACHING 0–10% IN LEAF DISCS SUSPENDED IN 40% ACETONE, OVERNIGHT IN DARK

Plant studied (family, genus and species)		Linoleic-acid peroxidizing activity (units/g wet wt. leaf)
AMARYLLIDACEAE	*Narcissus* cv, Carlton	26
ARACEAE	*Arum maculatum*	4
CAPRIFOLIACEAE	*Sambucus nigra*	1
CHENOPODIACEAE	*Chenopodium album*	4
COMPOSITAE	*Arctium lappa*	45[a], 85
	Calendula officinalis	1
CUCURBITACEAE	*Bryonia dioica*	3
	Cucumis melo	23, 28[b]
	Cucurbita pepo	4, 24[c]
EUPHORBIACEAE	*Euphorbia peplus*	< 1
	Mercurialis perennis	6
GRAMINEAE	*Avena sativa* cv. Blenda	10
LABIATAE	*Lamium album*	6
	Stachys sylvatica	12
LEGUMINOSAE	*Phaseolus vulgaris*	10 (mature)–124 (young)
LILIACEAE	*Endymion hispanicus*	3
ONAGRACEAE	*Chamaenerion angustifolium*	5
	Circaea lutetiana	2
POLYGONACEAE	*Rumex sanguineus*	2
SOLANACEAE	*Nicotiana tabacum*	2
UMBELLIFERAE	*Aegopodium podagraria*	4

Bleaching in ground material: a, 20%; b, 39%; c, 33%.

specific absorption coefficients, were formed and there was no real bleaching. The leaves that bleached most were from barley and wheat seedlings, young leaves of *Phaseolus mungo*, young and mature leaves of nettle (*Urtica dioica*) and a few species of Compositae. All these had high lipoxidase activity, but in leaves of other species whose activity was similar the chlorophyll either did not bleach at all or bleached only a little. If a chain reaction is involved in the bleaching, one of the stages may be blocked because the amounts of enzyme or substrate are insufficient, or alternatively enzyme inhibitors may be present.

The amount of free fatty acids in leaves is small, but the first stage might be their liberation from lipids by lipases, thus providing the substrate for lipoxidase. For example, linolenic acid will be formed from mono- and digalactosyldiglycerides by galactolipases. These enzymes have been found in the leaves of several plants: high activity in species of *Phaseolus* and low in *Spinacia*[9] and also in barley.[10] However, activity was not detected in other tissues in which it was sought. There seems to be no information about galactosyldiglycerides as substrates for lipoxidase, but peroxidation of bound fatty acids is not improbable, although it might take place less readily than with free fatty acids. If, due to low lipase activity, little substrate is available for the lipoxidase, the formation of hydroperoxide will be blocked. Alternatively, the breakdown of hydroperoxide could be slow because of low "lipoperoxidase" activity.

In leaf discs or pieces, substrates and enzymes are certainly separated from each other and the role of the acetone may be to loosen the substrates and bring them in closer contact with the enzymes. However, too great a concentration of organic solvent will inactivate the

[9] P. S. SASTRY and M. KATES, *Biochem.* **3**, 1280 (1964).
[10] L. A. APPELQUIST, P. K. STUMPF and D. VON WETTSTEIN, *Plant Physiol.* **43**, 163 (1968).

TABLE 2. CHLOROPHYLL BLEACHING GREATER THAN 10% IN LEAF DISCS SUSPENDED IN 40% ACETONE OVERNIGHT IN DARK

	Plant studied (family, genus and species)	% Bleaching	Linoleic-acid peroxidizing activity (units/g wet wt. leaf)
COMPOSITAE	*Centaurea* sp.	50–57 (4)	11–22 (4)
	Cirsium arvense	56	25
	Doronicum plantagineum	27–57 (6)	31–72 (4)
	Helianthus tuberosus	12–33 (5)	4, 9
CRUCIFERAE	*Alliaria petiolata*	35–52 (5)*	1–4 (3)
	Lunaria biennis	16–27 (3), 39	3, 7, 18
GRAMINEAE	*Hordeum vulgare* cv. Maris Badger	50–85 (>10)	26–64 (3)
	Triticum aestivum cv. Cappelle—green	50–80 (>10)	85–136 (6)
	etiolated		120–200 (6)
IRIDACEAE	*Iris* unknown cv.	36*	4
LEGUMINOSAE	*Lupinus*, Russell strain	42*	22
	Phaseolus mungo (young)	47–84 (5)	35, 48
	Trifolium repens	47*, 22	8–14 (4)
LILIACEAE	*Convallaria majalis*	14–31 (3)	13
OLEACEAE	*Syringa vulgaris*	8–38 (4)*	10–12 (3)
PAEONIACEAE	*Paeonia officinalis*	27–38 (3)*	1, 2
SCROPHULARIACEAE	*Digitalis purpurea*	9, 26†, 46	20, 31, 93
SOLANACEAE	*Solanum dulcamara*	24	91
UMBELLIFERAE	*Heracleum sphondylium*	9–28 (5)	4–6 (3)
	Sanicula europaea	22–26 (3)*	8, 11
URTICACEAE	*Urtica dioica*	39–51 (5)	68–120 (6)

Number of determinations given by figures in brackets.
* Pheophorbide formation.
† 52% Bleaching in ground material.

enzymes. The fact that the optimum concentration of acetone for chlorophyll bleaching in discs is between 40 and 50% whereas in extracts it is only 30%, suggests that inactivation occurs more quickly in extracts.

There is no reason to think that the way in which chlorophyll is bleached in leaves suspended in aq. acetone is related to normal chlorophyll breakdown in senescing tissues. For example, oat leaves show negligible bleaching when kept in aq. acetone overnight but lose their chlorophyll faster than most other leaves when kept moist and dark at room temp.

EXPERIMENTAL

Plant Material

Samples of cereal seeds were kindly given by Dunns Farm Seeds, Salisbury. Seedlings were grown on moist filter paper in glass tanks and usually harvested when 8–10 days old. Leaves of other plants were from various sources, both cultivated and wild; leaves from mature plants were mostly used.

Chlorophyllase Determinations

Leaves (1 g) were ground rapidly in 10 ml 50% aq. acetone, the suspension kept at room temp. in the dark for 1 hr, then diluted to 25 ml with acetone and filtered. The pigments in the filtrate were transferred to diethyl ether and this solution used for TLC on a microscope slide. The relative amounts of chlorophyll *a* and chlorophyllide *a* were estimated by eye.[11]

[11] M. F. BACON and M. HOLDEN, *Phytochem.* **9**, 115 (1970).

Thin Layer Chromatography

Extracts containing chlorophyll pigments were chromatographed on cellulose layers with a mixture of light petroleum (b.p. 60–80°) and acetone, 80:20, v/v.[11]

Lipoxidase Determinations

The formation of linoleate hydroperoxide was followed by a slight modification[6] of the ferric thiocyanate method.[12] Leaves were ground in a mortar with sodium acetate buffer 0·2 M, pH 5·9, 4 ml/g wet wt. and a small amount of fine sand. The extract was squeezed out through cloth and the sand and leaf debris removed by centrifuging briefly at low speed. The amount of chlorophyll etc. in the small volume of extract usually needed for determining the activity did not interfere with the colorimetry of the red pigment formed.

One arbitrary unit of enzyme activity is the amount that gives a reading of 1·0 on the EEL colorimeter, with filter 623, with 10 min incubation, after deduction of the blank reading given by the linoleic acid plus the reagents.

Determination of Chlorophyll Bleaching

Weighed leaf material (0·5–1 g), consisting either of discs (1 cm) cut with a cork-borer or of short lengths (1 cm), was suspended in 10 ml aq. acetone (usually 40%) and kept at room temp. in the dark. After a suitable interval the fluid was filtered into a 25 ml volumetric flask, the leaf material was then ground in acetone and this extract and acetone washings added to the flask to make up to vol. The reading on an EEL colorimeter (A), using filter 607, was compared with that given by the extract of a similar sample that was not incubated and was ground directly in 80% acetone (A_0).

$$\% \text{ bleaching} = \frac{(A_0 - A)100}{A_0}$$

When ground leaf samples were used these were made by grinding rapidly in some of the aq. acetone that was to be used as incubation fluid. After incubation, the volume was made up to 25 ml with acetone and the suspension filtered before colorimetry.

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

[12] R. B. Koch, B. Stern and C. G. Ferrari, *Arch. Biochem. Biophys.* **78**, 165 (1958).