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CHANGES IN CHLOROPHYLLS RESULTING FROM VARIOUS CHEMICAL AND PHYSICAL TREATMENTS OF LEAVES AND LEAF EXTRACTS

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Abstract—Chlorophylls *a* and *b* are easily altered chemically when leaves or leaf extracts are exposed to treatments which include heating and the action of organic solvents. Monitoring by thin-layer chromatography assists in the detection of the chlorophyll derivatives. In addition to loss of magnesium, formation of chlorophyllides and the *a'* and *b'* isomers, and loss of colour, chlorophylls *a* and *b* can each be converted into two other derivatives, with absorption spectra almost identical with those of the parent compounds. The conditions of formation, purification and some of the properties of these derivatives are described, and possible structures are discussed.

INTRODUCTION

WHILE investigating the disappearance of chlorophyll from fresh leaf discs that were kept floating on water in the dark, boiled leaf discs were used as controls. In the unheated discs the chlorophylls disappeared slowly, forming unidentified colourless compounds and only traces of coloured breakdown products such as chlorophyllides and pheophorbides. In the boiled discs, under certain conditions, chlorophylls *a* and *b* were each converted into at least two additional pigments. Strain [1–3] found that altered chlorophylls were formed when leaves were kept in organic solvents, and these are probably identical with some of the pigments we found. However, such pigments do not seem to have been previously observed under entirely aqueous conditions. The present paper describes the conditions under which these "changed" chlorophylls are formed, their chromatographic separation, and some of their properties. The formation of other chlorophyll derivatives when leaves or isolated chlorophylls are exposed to heat or organic solvents is also described.

RESULTS

Two contrasting types of leaves were used for this work: a dicotyledon, Hogweed (*Heracleum sphondylium* L.), with high chlorophyllase activity and a monocotyledon, Barley (*Hordeum vulgare* cv. Maris Badger), with exceptionally low chlorophyllase activity.

Effects of Heating Leaves in Water

Immediate effects. (*a*) *Pheophytin formation.* It is well known that pheophytin is formed when green leaves are boiled. When unheated leaves were ground in acetone a trace of pheophytin *a* was always present, even when calcium carbonate was added to neutralize acids during the grinding, but it represented only about 1 per cent of the chlorophyll *a*. When leaves were heated, the amount of pheophytin produced increased with increasing temperature.

[1] H. H. STRAIN, *J. Agr. Food Chem.* **2**, 1222 (1954).
[2] H. H. STRAIN, *Chloroplast Pigments and Chromatographic Analysis*. 32nd Annual Priestley Lectures, The Pennsylvania State University, University Park, Pa. (1958).
[3] F. C. PENNINGTON, H. H. STRAIN, W. A. SVEC and J. J. KATZ, *J. Am. Chem. Soc.* **86**, 1418 (1964).

Thus, in an experiment with barley heated for 5 min in distilled water, 2 per cent of pheophytin *a* was produced at 61°, 6 per cent at 75°, and 31 per cent at 100°. Pheophytin *b* was not observed at 20° and 61°, but there was 3 per cent at 75° and 14 per cent at 100°. *Heracleum* leaves gave similar results. As with most of the other immediate effects, pH and duration of heating are also important.

(*b*) *Chlorophyllide formation.* In unheated or in boiled leaves, even those of species with exceptionally high chlorophyllase activity such as *Heracleum*, only traces of chlorophyllides were detected. Larger amounts were formed in whole leaves or discs of *Heracleum* heated to temperatures between about 50 and 85°, with most conversion occurring between 60 and 75°. Some pheophorbide was also produced under these conditions. The results with different batches of leaves varied considerably, the maximum conversion ranging from 10 to 70 per cent for the same temperature and time of heating. It seems that the age and condition of the leaf may be important in determining the amount of chlorophyllide formed.[4]

We found that chlorophyllase was largely inactivated by grinding *Heracleum* leaves, with or without added water or buffer solution. Ground leaves therefore formed only traces of chlorophyllide when heated between 50 and 85°.

(*c*) *Formation of isomers.* When *Heracleum* discs were heated in boiling distilled water for 5 min, there was about 10 per cent conversion of chlorophylls *a* and *b* to the *a'* and *b'* isomers described by Strain and Manning.[1, 2, 5] There was also slight conversion at 77°, but the isomers were not formed at 64° unless the heating time was extended. They were not present in extracts from unheated leaves. Similar results were obtained with barley. The rate of formation is probably dependent on pH, and traces of the isomers were observed when leaves were heated at 50° and then left at room temperature on an alkaline solution. Pheophytins *a'* and *b'* were probably produced in the heated leaves, but were not separated from pheophytins *a* and *b* on our chromatograms.

(*d*) *Loss of pigment.* In addition to pheophytin and isomer formation, the total amount of pigment was diminished by about 20 per cent when *Heracleum* discs were boiled for 5 min. With barley, there was very little loss at 100°, although there seemed to be some bleaching (about 10 per cent) at 60°, which may have been enzymic in nature.

(*e*) *Solubility of pigments in light petroleum.* The amount of chlorophyll extractable by light petroleum from leaves of most species is a very small proportion of the total present, although Faludi-Dániel *et al.*[6] found that nearly one-third of the chlorophyll in a barley mutant "xantha-3" was petrol-soluble. With freeze-dried, unheated *Heracleum* leaves, repeated grinding with the solvent removed only about 5 per cent of the green pigments. From discs that had been heated for 5 min at 70° and then freeze-dried, about 40 per cent of the "chlorophyll" was extractable by light petroleum; from discs boiled for 5 min and freeze-dried about 70 per cent was extractable.

Later effects—formation of "changed" chlorophylls. When leaves were boiled and then left at room temperature (21°) in the dark on an alkaline aqueous medium, chlorophylls *a* and *b* were each converted into two pigments that differed from any of those produced immediately on heating. These new pigments had the same colours as the parent compounds, but lower R_f values. We shall use the names "changed" chlorophylls *a*-1, *a*-2, *b*-1 and *b*-2 for these pigments; this nomenclature will be discussed later. Figure 1 shows the positions of these "changed" chlorophylls on a typical chromatogram.

[4] C. A. WEAST and G. MACKINNEY, *J. Biol. Chem.* **133**, 551 (1940).
[5] H. H. STRAIN and W. M. MANNING, *J. Biol. Chem.* **146**, 275 (1942).
[6] A. FALUDI-DÁNIEL, A. NAGY, I. GYURJÁN and B. FALUDI, *Photochem. Photobiol.* **4**, 359 (1965).

Pheophytin that is produced when leaves are boiled is also partly converted into "changed" pigments during the treatment at alkaline pH. Grey zones of "changed" pheophytins *a* are then observed on either side of the lutein zone. In Fig. 1, for clarity, the barley was only heated to 70°, to minimise formation of pheophytins and "changed" pheophytins. In addition to the above compounds, traces of a blue–green and of a yellow–green pigment were sometimes noticed just ahead of "changed" chlorophylls *a*-2 and *b*-2 respectively, and small amounts of greyish-green pigments were usually present between *b*-2 and the baseline.

The "changed" chlorophylls were first observed with leaves that had been boiled and left on tap-water (final pH 8). There was much less conversion into "changed" chlorophylls in boiled leaves left on distilled water, unless a plant having alkaline leaves such as marrow[7] was used. Barley leaves, boiled for 5 min in distilled water and left for 1 day on buffer solutions of pH between 5 and 9, showed most conversion at pH 9 and none below pH 7. After 4 days there were traces of "changed" pheophytins and chlorophylls at pH 6·7. We conclude that, although the reaction occurs fairly rapidly at an alkaline pH, it can still proceed slowly at neutrality. Other reactions, such as formation of chlorophyllins, may take place at pH values greater than 9.

Boiled leaves left on buffer at pH 8·6 showed no conversion into "changed" chlorophylls *b* after 2 hr, but about 7 per cent after 7 hr and about 70 per cent conversion after 1 day. The percentage of chlorophyll *a* converted into "changed" chlorophylls *a* was only about one-half of these values during this period and, unlike conversion into pheophytins,[8] chlorophyll *a* probably reacts more slowly than chlorophyll *b*. After 3 days, conversion of chlorophyll *a* was only a little less than that of chlorophyll *b*, which by then was almost complete. Approximately equal amounts of "changed" chlorophylls *b*-1 and *b*-2 were formed throughout, but there was about twice as much "changed" chlorophyll *a*-1 as *a*-2. In addition to the formation of the "changed" chlorophylls, there was loss of total green pigment in boiled leaves left on an alkaline medium. But if the "changed" chlorophylls have lower molar absorption coefficients than the parent compounds this loss may not be real. The loss was much smaller when the boiled leaves were left on distilled water.

Unheated leaves, or leaves that were frozen at −20° or freeze-dried before being left on the alkaline medium, did not give the "changed" chlorophylls. The extent to which they were formed after heating barley to various temperatures is shown in Fig. 2. The threshold for appreciable conversion was about 50°; 70° was sufficient for maximum formation of the "changed" chlorophylls. With *Heracleum*, results were similar except that conversion continued to increase between 70° and 100°.

When boiled barley leaves or *Heracleum* discs were kept in an evacuated Thunberg tube containing alkaline buffer, no "changed" chlorophylls were formed. They were produced, however, if air was re-admitted to the system before the tubes were left to stand. It seems therefore, that oxygen is necessary for formation of the "changed" chlorophylls.

Jones, White and Gibbs[9] have reported that chlorophyllase action can take place in unheated plant material left in brine. It seemed possible that sodium chloride might also assist in the formation of "changed" chlorophylls in unheated leaves. When fresh barley leaves were kept in 7·5 per cent (w/v) sodium chloride solution buffered with tris to pH 8·5, at 21° in the dark, there was about 10 per cent conversion into the "changed" chlorophylls or similar

[7] M. Holden, *Biochem. J.* **42**, 332 (1948).
[8] S. H. Schanderl, C. O. Chichester and G. L. Marsh, *J. Org. Chem.* **27**, 3865 (1962).
[9] I. D. Jones, R. C. White and E. Gibbs, *J. Food Sci.* **28**, 437 (1963).

pigments after 10 days. There was little or no conversion with unheated barley left in unbuffered sodium chloride solution.

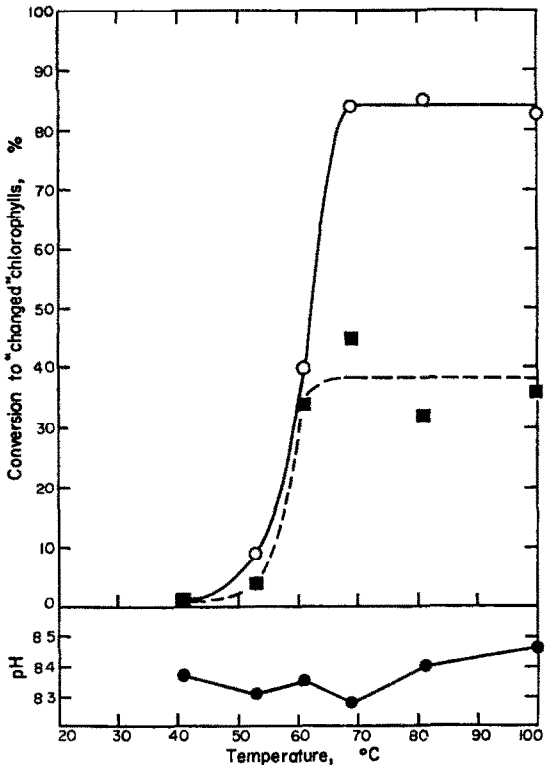

FIG. 2. EFFECT OF INITIAL HEATING TEMPERATURE ON EXTENT OF CONVERSION OF CHLOROPHYLLS TO "CHANGED" CHLOROPHYLLS.

Portions of barley seedlings were heated for 5 min in distilled water at the temperatures indicated, and then left in the dark at room temperature for 2 days on tris buffer, pH 8·6. The amounts of "changed" chlorophylls *b*-1 and *b*-2 (or *a*-1 and *a*-2) have been added together and expressed as a percentage of the total yellow-green (or blue-green) pigments at the end of this period. (○——○) = % conversion of chlorophyll *b*, (■– – –■) = % conversion of chlorophyll *a*. The pH values are those of the tris buffer solutions at the end of the test.

Purification and Properties of "Changed" Chlorophylls a-1, a-2, b-1 *and* b-2

Purification. A concentrated extract in acetone was prepared from *Heracleum* discs that had been heated at 100° for 5 min and left on 0·01 M sodium bicarbonate for 2 days. After adding water (3 vol.) to the extract (7 vol.), it was applied to a column of wet-packed powdered polyethylene [10–12] (Dow Chemical Company, Bay City, Michigan; Resin No. QX-917.6). The column was developed with 70% aqueous acetone, to remove oxygen-containing carotenoids, and then with 80% acetone to elute the green pigments. The pheophytins and carotenes were left on the column. The green pigments were transferred to a water-free solvent which was then applied as a streak to a series of 20 × 20 cm cellulose TLC plates.

[10] A. F. H. ANDERSON and M. CALVIN, *Nature* **194**, 285 (1962).
[11] H. J. PERKINS and D. W. A. ROBERTS, *Biochim. Biophys. Acta* **79**, 20 (1964).
[12] M. HOLDEN, In *Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. GOODWIN), p. 461. Academic Press, New York (1965).

After development with light petroleum spirit–acetone–*n*-propanol (90:10: 0·45 by vol.: solvent A), the middle portion of each pigment zone was removed and eluted with acetone.

Some of the pigments needed further purification by cellulose TLC. "Changed" chlorophyll *b*-1 was freed from "changed" chlorophyll *a*-2 with the same solvent mixture; the magnesium-free derivative of *b*-2 was removed from *a*-1 with light petroleum–acetone (80:20, v/v: solvent B); and *a*-2 was freed from another blue–green pigment and from chlorophyll *b* with light petroleum–ethyl acetate (65:35, v/v: solvent C).

Spectra. The absorption spectra of the purified "changed" chlorophylls were measured in acetone and, after evaporation to dryness under reduced pressure at about 20°, in diethyl ether and in methanol. The positions and the relative intensities of the principal absorption maxima in the blue and the red are compared in Table 1 with those of chlorophylls *a* and *b* isolated from French bean (*Phaseolus vulgaris*) leaves and purified in a similar manner. Although only small differences in the peak positions were detected, we consider some of these to be significant. Thus, relative to the parent compounds, the maxima are shifted to

TABLE 1. COMPARISON OF POSITIONS AND RELATIVE INTENSITIES OF PRINCIPAL ABSORPTION MAXIMA, FOR CHLOROPHYLLS AND "CHANGED" CHLOROPHYLLS IN ACETONE (a), DIETHYL ETHER (e) AND METHANOL (m)

Compound	$\lambda_{max\ (red)}$ (nm)			$\lambda_{max\ (blue)}$ (nm)			$\frac{A\lambda_{max\ (blue)} - A_{700\ nm}}{A\lambda_{max\ (red)} - A_{700\ nm}}$		
	a	e	m	a	e	m	a	e	m
Chlorophyll *a*	662·5	661·5	666·0	430·0	429·5	432	1·24	1·28	0·96
"Changed" chlorophyll *a*-1	661·5	661·0	665·5	429·0	428·0	431	1·25	1·28	1·04
"Changed" chlorophyll *a*-2	662·0	662·0	665·5	429·5	429·0	432	1·21	1·26	1·02
Chlorophyll *b*	645·0	643·0	653·0	455·5	453·0	469	2·87	2·82	2·83
"Changed" chlorophyll *b*-1	644·0	642·0	652·0	453·5	451·5	468	3·02	2·98	3·05
"Changed" chlorophyll *b*-2	644·0	642·5	651·0	452·5	451·0	466	3·48	3·21	3·49

A = Absorbancy.

shorter wave-lengths by about 1 nm for "changed" chlorophylls *a*-1 and *b*-1, and by about 2 nm for *b*-2; there was no shift for *a*-2. The blue/red peak height ratios of the *a* pigments are similar to each other in acetone and diethyl ether, but those of the "changed" *b* compounds, especially of *b*-2, show an increase over the ratio for chlorophyll *b*. In methanol, all the "changed" pigments have higher ratios than the parent compounds, the effect again being greatest with *b*-2.

The spectra in diethyl ether are shown in Figs. 3 and 4. There is little difference between the curves for the *a* pigments, except that "changed" chlorophyll *a*-2 absorbs strongly below 350 nm. Chromatography in the presence of ethyl acetate was used in the final stage of purification of this compound, but not of the others. The eluate was then evaporated to dryness twice under reduced pressure before the spectrum was run in ether. It seems unlikely that any ethyl acetate was still present but possibly some impurity from it may account for the greater absorption below 350 nm. There is also some extra absorption at about 455 nm with "changed" chlorophyll *a*-2, perhaps because of slight contamination with chlorophyll *b*.

The curves for the *b* pigments are also similar to each other, the greatest deviation being with "changed" chlorophyll *b*-2 (Fig. 4). This compound shows extra absorption at about 630 and 440 nm, and also in the u.v. region particularly below 260 nm. These differences may

be real, but are more probably due to contamination by another compound, which might also account for the shifts in the positions of the absorption maxima and for the increase in the blue/red ratio.

Absorption spectra of the "changed" chlorophylls were also measured in light petroleum (40–60°). Our results were only approximate, because some of the pigments were not very soluble, but the maxima were at about 663 and 428 nm for *a*-1 and *a*-2, and at about 644 and 452 nm for *b*-1 and *b*-2.

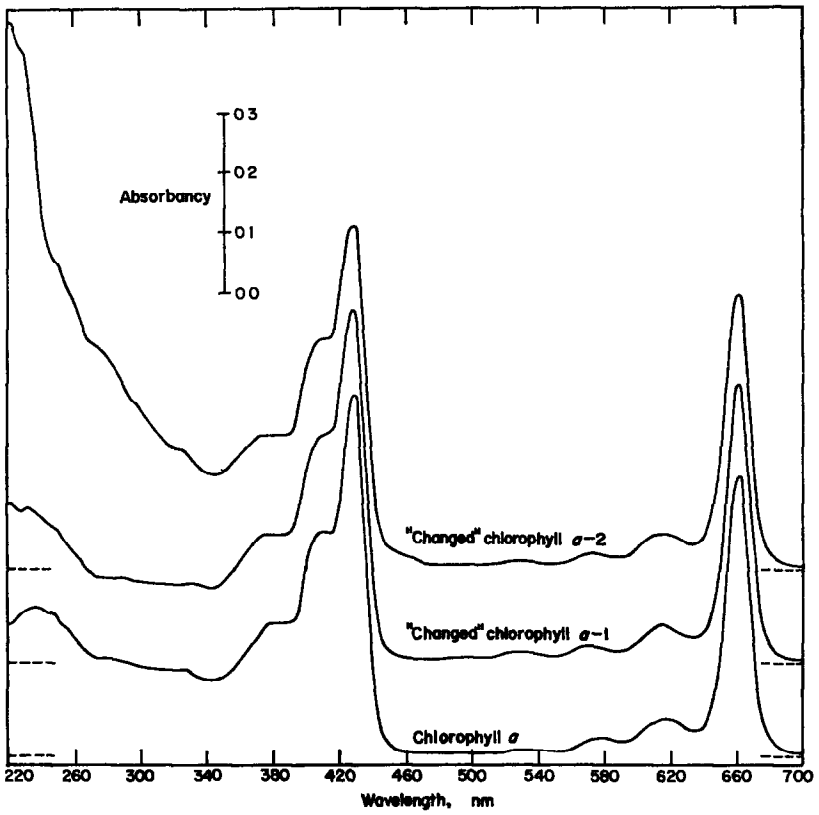

FIG. 3. ABSORPTION SPECTRA OF THE *a* PIGMENTS IN DIETHYL ETHER.

Phase test. Diethyl ether solutions of the pigments were treated with 30% (w/v) potassium hydroxide in methanol, as described by Smith and Benitez,[13] to test for possible changes in the cyclopentanone ring. Chlorophylls *a* and *b* gave positive reactions; "changed" chlorophylls *a*-1, *a*-2, *b*-1 and *b*-2 all gave negative results.

Partition between diethyl ether and dilute aqueous potassium hydroxide. When ether solutions of the pigments were shaken gently for 10 sec with an equal volume of 0·02 N KOH, chlorophylls *a* and *b* and the "changed" chlorophylls all remained in the ether phase. Under the same conditions, pheophorbides *a* and *b* and chlorophyllides *a* and *b* went into the aqueous alkaline phase.

[13] J. H. C. SMITH and A. BENITEZ, In *Modern Methods of Plant Analysis* (Edited by K. PAECH and M. V. TRACEY), Vol. 4, p. 142. Springer-Verlag, Berlin (1955).

Test for the presence of phytol. When "changed" chlorophylls *a*-1, *a*-2, *b*-1 and *b*-2 were hydrolysed each produced a compound that did not separate from phytol on co-chromatography. The amounts detected were of the order expected for phytol:phorbin ratios of 1. Methyl chlorophyllides *a* and *b* when treated in the same way, as a control, gave only a trace of material with the same R_f as phytol. It seems, therefore, that "changed" chlorophylls *a*-1, *a*-2, *b*-1 and *b*-2 are all phytyl esters.

Action of chlorophyllase. An acetone-dried powder with chlorophyllase activity was prepared from Honesty (*Lunaria biennis*) leaves. The "changed" chlorophylls were incubated separately in 50% (v/v) aqueous acetone for 15 hr at room temperature with portions of this powder. The pigments were then extracted into diethyl ether and chromatographed by

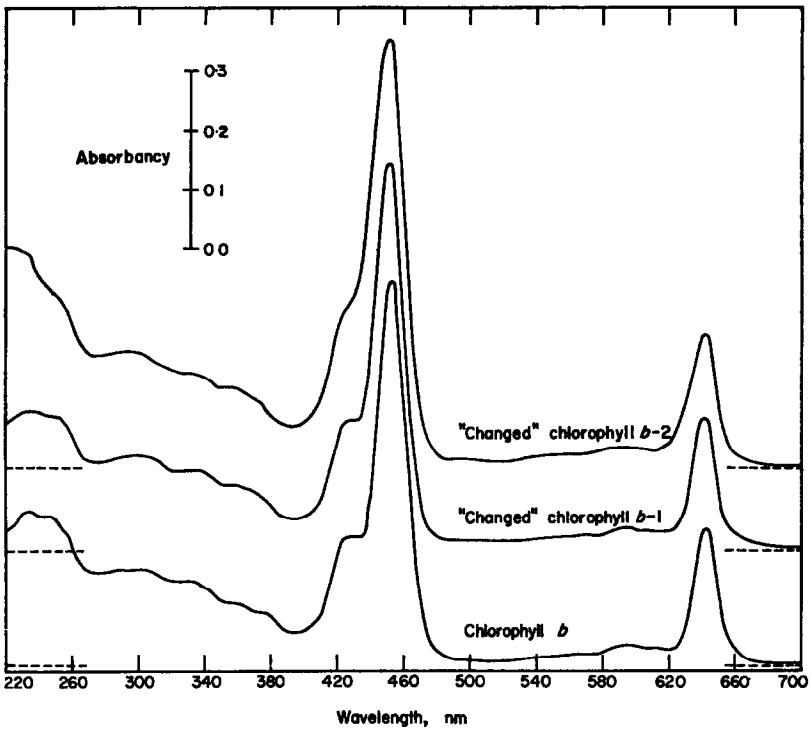

FIG. 4. ABSORPTION SPECTRA OF THE *b* PIGMENTS IN DIETHYL ETHER.

cellulose TLC with light petroleum (60–80°)–acetone (80:30 v/v). "Changed" chlorophylls *a*-1 and *b*-1 were each largely converted into single, slower-running pigments with lower R_f values than chlorophyllides *a* and *b*, respectively. The enzyme seemed, however, to have little or no effect on "changed" chlorophylls *a*-2 and *b*-2. This is being studied further.

Magnesium-free derivatives. Acetone solutions of chlorophylls *a* and *b* and of the "changed" chlorophylls were treated for a few minutes with 0·1 N HCl (1/20th vol.). After removing water and acid by evaporation under reduced pressure, the residues were taken up in acetone and chromatographed by cellulose TLC with solvent A (Fig. 5). The six compounds formed in this way all differed from each other; each *a* pigment gave a single grey spot and each *b* pigment a single yellow or yellow-brown spot.

It is likely from their R_f values that the "changed" pheophytins formed in boiled leaves

left on an alkaline medium are the same as the magnesium-free compounds obtained from the purified "changed" chlorophylls. An extra grey pigment, with an R_f slightly lower than that of magnesium-free "changed" chlorophyll *a*-2, is also formed in the boiled leaves.

Comparison with pyrochlorophyll a. Some of the properties of "changed" chlorophylls *a*-1 and *a*-2 are similar to those of pyrochlorophyll *a*, in which the C-10 methoxycarbonyl group of chlorophyll *a* is substituted by hydrogen.[3] Pyrochlorophyll *a* was prepared by heating chlorophyll *a* in pyridine (A.R., freshly distilled over KOH) for 48 hr at 100°, as described by Pennington *et al.*[3] Co-chromatography with low loadings showed that pyrochlorophyll *a* was slightly more adsorbed than "changed" chlorophyll *a*-1, but ran appreciably faster than *a*-2, on cellulose plates developed with solvent A. The magnesium-free derivative of pyro-

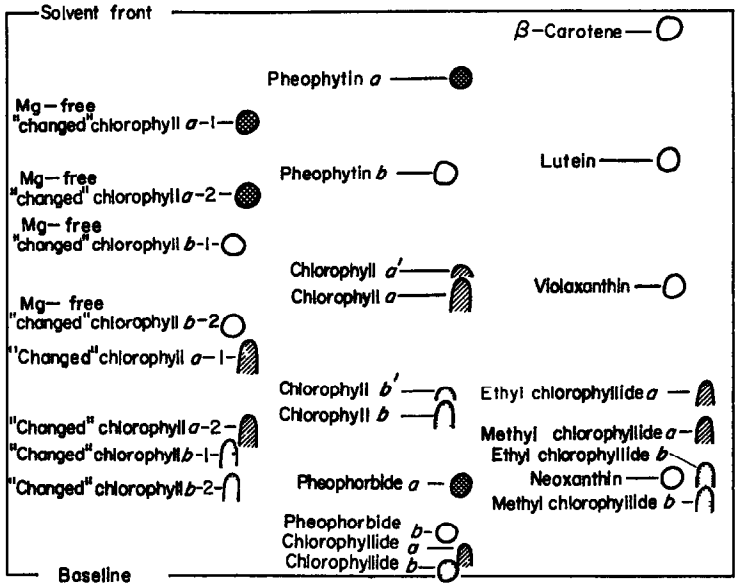

FIG. 5. RELATIVE POSITIONS OF CHLOROPHYLLS, CHLOROPHYLL DERIVATIVES AND CAROTENOIDS AFTER TLC ON CELLULOSE WITH PETROLEUM SPIRIT–ACETONE–*n*-PROPANOL (90:10:0·45, by vol.).

The positions are only approximate and correspond to those obtained when small amounts (about 0·2 μg) of the substances are spotted separately. Colours: = blue–green, = yellow–green, = yellow, orange–yellow or yellow–brown, = grey.

chlorophyll *a* ran at about the same R_f as pheophytin *a*, and separated easily from magnesium-free "changed" chlorophyll *a*-1. Pyrochlorophyll *a* is therefore not the same as either "changed" chlorophyll *a*-1 or *a*-2.

Identity of precursors of the "changed" chlorophylls. On the basis of their spectra, we have assumed above that "changed" chlorophylls *a*-1 and *a*-2 are derived from chlorophyll *a*, and that *b*-1 and *b*-2 are derived from chlorophyll *b*. To test this assumption, the formation of "changed" chlorophylls in a brown alga (*Fucus serratus*) was examined. This alga contains chlorophylls *a* and *c* instead of *a* and *b*. Portions were boiled in distilled water for 1 min and left for 2 days in tris buffer, pH 8·6. Four extra pigments were formed in appreciable amounts: pheophytin *a*, a grey pigment running just ahead of chlorophyll *a* on cellulose TLC chromatograms in solvent A, and two blue–green pigments more adsorbed than chlorophyll *a*. On

co-chromatography with an extract of "changed" pigments from barley, the last three did not separate from one of the grey "changed" pheophytins and from "changed" chlorophylls *a*-1 and *a*-2, respectively. About 2 per cent only of a yellow–green pigment was also present, with an R_f similar to, although not necessarily identical with, that of "changed" chlorophyll *b*-2. Of the two extra blue–green pigments produced in the alga, the slower-running compound was present in the greater amount, whereas in barley and *Heracleum* the faster-running compound predominated.

Chlorophyll *c* was strongly adsorbed, with an R_f and colour similar to that of chlorophyllide *b*, and had apparently been largely converted into one or more substances of unchanged colour that did not move at all from the baseline. It is most unlikely that the much less adsorbed blue–green pigments could have been formed from chlorophyll *c*.

Thus in the absence of chlorophyll *b*, compounds are formed with the same R_f values and colours as "changed" chlorophylls *a*-1 and *a*-2. We conclude that "changed" chlorophylls *a*-1 and *a*-2 are derived from chlorophyll *a*. Only a trace of yellow–green pigment was produced, and chlorophyll *b* is almost certainly the precursor of "changed" chlorophylls *b*-1 and *b*-2.

Effects of Organic Solvents on Leaves

Formation of chlorophyllides. Chlorophyllase action in leaves treated with organic solvents is well known[14] and is only mentioned here for completeness. Chlorophyllides *a* and *b* are formed in the presence of aqueous acetone solutions; methyl or ethyl chlorophyllides *a* and *b* are produced in addition when leaves are treated with methanol or ethanol. The corresponding pheophorbides may also be observed.

With species rich in chlorophyllase, conversion into chlorophyllides can be extensive and rapid and may sometimes occur during extraction of pigments from plant material. Thus when *Heracleum* discs (0·5 g) were left in 80% acetone (25 ml) at 1° (cf. Ref. 15), the pigments in the acetone solution were almost completely in the form of chlorophyllides *a* and *b* and pheophorbide *a* after 15 hr. However, only about 1 per cent of chlorophyllide was present in acetone extracts made from *Heracleum* by the procedure described in the experimental section.

Barley leaves left in aqueous organic solvents gave only a trace of chlorophyllide because their chlorophyllase activity is so small. This species was therefore particularly suitable for demonstrating the formation of chlorophyll derivatives other than chlorophyllides.

Formation of "changed" chlorophylls. When unheated barley leaves were suspended in a 50% (v/v) aqueous acetone solution for 3 days in the dark at 21°, blue–green and yellow–green pigments were formed that resembled the "changed" chlorophylls. After removing carotenoids by column chromatography on polyethylene powder, the purified extract was co-chromatographed on cellulose thin layers with the mixture of pigments obtained from barley which had been boiled and left on an alkaline medium. The four main pigments produced by the treatment with 50% acetone were not separated in the three solvent systems (A, B and C) from "changed" chlorophylls *a*-1, *a*-2, *b*-1 and *b*-2, respectively. The absorption maxima, measured in acetone, were the same as or very close to those of these "changed" chlorophylls. Pigments with the same R_f values were also formed when barley leaves were treated with 50% aqueous methanol. We shall assume, therefore, that "changed" chlorophylls

[14] R. WILLSTÄTTER and A. STOLL, *Investigations on Chlorophyll* (Translated by F. M. SCHERTZ and A. R. MERZ). Science Press, Lancaster, Pa. (1928).
[15] S. VENKETESWARAN, *Physiol. Plantarum* **18**, 776 (1965).

a-1, *a*-2, *b*-1 and *b*-2 are also produced by the action of aqueous acetone or methanol on unheated leaves.

In addition to these pigments, a small blue–green zone ran just ahead of "changed" chlorophyll *a*-2 in light petroleum–acetone–*n*-propanol. Contamination by chlorophyll *b* prevented accurate spectral measurements, but this compound probably absorbed maximally at about 657 and 418 nm in acetone. Two or three extra yellow–green pigments were separated just ahead of "changed" chlorophyll *b*-2, and other green and grey pigments moved more slowly than *b*-2. When solvent B was used as developing solvent, traces of a further blue–green compound were observed in the tail of "changed" chlorophyll *b*-1; this pigment probably ran with "changed" chlorophyll *b*-2 in solvent A. The chlorophyll *a'* and *b'* isomers also appeared to be formed.

TABLE 2. EFFECT OF ACETONE OR METHANOL CONCENTRATION ON EXTENT AND RATE OF CONVERSION OF CHLOROPHYLL *a* INTO "CHANGED" CHLOROPHYLLS *a*-1 AND *a*-2 IN BARLEY

Solution	Percent conversion of chlorophyll *a* into "changed" chlorophylls *a*-1 and *a*-2		
	After 2 hr	After 7 hr	After 28 hr
Acetone %			
10	0	0	3
25	<1	4	8
50	5	15	50
75	5	15	5
80	<1	4	5
Methanol %			
10	0	0	<1
25	<1	2	4
50	<1	15	50
75	0	1	3

Barley leaves (0·5 g) were left in the dark at 22° in aqueous solutions (10 ml) containing the percentages (v/v) of acetone or methanol shown. Aliquots were extracted at intervals, and the relative amounts of chlorophyll *a* and "changed" chlorophylls *a*-1 and *a*-2 were estimated by visual examination of chromatograms of the extracts.

Table 2 shows the apparent extent and rate of conversion of chlorophyll *a* to "changed" chlorophylls *a*-1 and *a*-2 in barley leaves left in distilled water containing various concentrations of acetone or methanol. The results for chlorophyll *b* were similar. The values given are only approximate, because the analyses were not quantitative and because bleaching (see below) might have affected each pigment differently. It is likely, nevertheless, that conversion becomes appreciable at about 10 per cent of acetone or 20 per cent of methanol, is at a maximum with about 50–60 per cent of either solvent, and is small at concentrations greater than 80 per cent. Barley leaves left at 1° in 80% acetone showed less than 1 per cent of "changed" chlorophylls after 7 hr, but about 4 per cent after 28 hr.

Strain and his co-workers[1–3] have reported the formation of "oxidized chlorophylls" in leaves exposed to methanol, acetone, ethanol or diethyl ether. These compounds were isolated by chromatography on powdered-sugar columns developed with 0·5 per cent of *n*-propanol in light petroleum. Only one oxidized chlorophyll similar spectroscopically to

chlorophyll *a*, and one similar to chlorophyll *b*, were apparently found. Experiments we made with a similar chromatographic system suggested that Strain's oxidized chlorophylls *a* and *b* may have been mixtures of "changed" chlorophylls *a*-1 and *a*-2, and *b*-1 and *b*-2, respectively.

When *Heracleum* discs were left in 50% acetone, the chlorophylls were mainly converted into chlorophyllides, and only small amounts of "changed" chlorophylls were formed. We do not know if the chlorophyllides (or methyl chlorophyllides) can be converted subsequently into compounds analogous to the "changed" chlorophylls but lacking the phytyl group.

Loss of pigment. In addition to the changes described above, there was a large and rapid loss both of green pigment and of carotenoids, when barley leaves were left in aqueous solutions of acetone or methanol. This bleaching is probably enzymic and is being investigated further. With *Heracleum* the bleaching was much less than with barley.

Formation of "Changed" Chlorophylls in Extracts of Leaves and from Purified Pigments

In leaf extracts. When fresh barley leaves were ground with light petroleum (40–60°)–methanol (approx. 1:2 v/v), the extract filtered, the pigments transferred to the petroleum layer by shaking with concentrated sodium chloride solution, and the petroleum solution washed three times with water,[1] large amounts of pigments similar to, and probably identical with, the "changed" chlorophylls were formed rapidly in the dark at 21° or at 0°. One or more blue pigments running more slowly than "changed" chlorophyll *b*-2 were also characteristically produced under these conditions. Strain [2] has reported that some pigments are not stable in solutions prepared by this method, but did not give further details. The rate of conversion of chlorophylls *a* and *b* in the light petroleum solutions varied, but usually about half had been converted after 4 hr. In one experiment, about 20 per cent of a pigment similar to "changed" chlorophyll *a*-1 had even been formed by the time the extraction and washing process was complete.

The "changed" chlorophylls or similar pigments were also formed when light petroleum was not added until after barley had been extracted with methanol or acetone and the extract had been filtered. They were formed only slowly or not at all, however, when the extracts were shaken with water instead of with sodium chloride solution, or when water was removed from the final petroleum solutions by evaporation or with sodium sulphate. Nor were they formed rapidly when chlorophylls, purified by chromatography on polyethylene and on cellulose, were dissolved in light petroleum–methanol and transferred to the light petroleum by washing with sodium chloride solution and with water. Therefore not only the moisture in the petroleum solutions and the treatment with sodium chloride, but also material extracted from the leaves, may hasten the formation of these altered chlorophylls.

These pigments were not formed after 2 days at 21° in filtered acetone extracts of barley, even when sodium chloride was added to the extract or the acetone concentration was brought to about 70 per cent by adding water. Nor were they produced after 4 days in acetone extracts that had been freed from water by evaporation as described in the Experimental (TLC Section). Small amounts were, however, found after 4 days in diethyl ether solutions, either moist or dried, obtained as described in the Experimental section. Such diethyl ether solutions should, therefore, be used within a few hours of preparation.

From purified pigments. Chlorophylls *a* and *b*, purified by chromatography on polyethylene powder and on cellulose, but contaminated with *a'* and *b'* isomers formed during storage, were dissolved separately in acetone or methanol, and water was added to portions to bring the concentration of organic solvent to 50% (v/v). The concentration of pigment in

the solutions was 3×10^{-5} M. Most of the chlorophylls *a* or *b* precipitated out later in the 50% acetone samples, but remained in colloidal suspension in 50% methanol. The samples were left in stoppered flasks with air present and were analysed after 20 days in the dark at 27°.

With 50% acetone, about 10 per cent of chlorophyll *a* was converted into a pigment with spectral and chromatographic properties similar to those of "changed" chlorophyll *a*-1. A slightly larger amount of a slower-moving blue–green pigment was also formed. This absorbed maximally at 655 and 417 nm in diethyl ether, with a blue/red ratio of about 2·4, and may have been the same as the extra blue–green pigment, running just ahead of "changed" chlorophyll *a*-2, which was formed by the action of 50% acetone on barley leaves. Traces of a further blue–green compound, possibly "changed" chlorophyll *a*-2, ran just behind it. Chlorophyll *b* showed about 70 per cent conversion into three yellow–green compounds. One of these had spectral and chromatographic properties similar to "changed" chlorophyll *b*-1. The others were not well separated and were eluted together; the spectrum suggested that one may have been "changed" chlorophyll *b*-2 and that the other probably absorbed maximally at about 630 and 440 nm in diethyl ether, with a high blue/red ratio.

In 50% methanol, chlorophyll *a* remained unaltered except for some material which remained on the baseline of the chromatogram. About 30 per cent of chlorophyll *b* was converted into yellow–green pigments with R_f values similar to those of the derivatives formed in 50% acetone.

With methanol containing less than 0·5 per cent of water, most of the chlorophyll *a* had been transformed after 20 days into two major blue–green compounds, one running at about the same speed as chlorophyll *a*, the other slightly faster. Both absorbed maximally at approximately 654 and 417 nm in diethyl ether, with blue/red ratios of 2·0 and 1·9, respectively. A small amount of another blue–green pigment, with an even higher R_f value, was also formed. Chlorophyll *b* was largely converted into a yellow–green compound with an R_f slightly higher than chlorophyll *b* itself, and absorption maxima in diethyl ether at 632 and 442 nm, and a blue/red ratio of 4·7. Two other yellow–green pigments ran just ahead of this, the slower of which absorbed maximally at about 641 and 444 nm in diethyl ether, with a blue/red ratio of nearly 9. Some at least of these pigments are probably the same as those observed by Strain[1, 2] when he treated purified chlorophylls *a* and *b* with absolute methanol.

Apart from the compounds described above, pigments which remained on or close to the baselines of the chromatograms were also formed by the action of methanol, 50% acetone and 50% methanol on the purified chlorophylls. Pigments running faster than the parent compounds were formed in addition with 50% acetone and 50% methanol, and were probably magnesium-free derivatives.

Solutions of chlorophylls *a* and *b* in acetone containing less than 1 per cent of water showed little or no conversion to altered chlorophylls after 20 days in the dark at 27°. However, pigments similar to the "changed" chlorophylls were formed in acetone solutions allowed to evaporate to dryness in an open flask.

DISCUSSION

We have used the term "changed" chlorophylls to describe the pigments produced by alkaline treatment of heated leaves or by the action of aqueous acetone or methanol on unheated leaves. The suffixes *a*-1, *a*-2, *b*-1 and *b*-2 have been added for the "changed" chlorophylls studied in most detail. A more precise nomenclature is desirable, but is not possible until more is known of the structure of these compounds.

Strain[1–3] described the altered pigments produced in leaves by acetone or methanol as

"oxidized chlorophylls". Although our "changed" chlorophylls may have been formed by oxidation of the parent compounds, we intend to be less specific at this stage. The term "allomer" has been widely used for a variety of oxidized chlorophyll derivatives which give negative phase tests.[2, 14, 16] Although the "changed" chlorophylls may be the same as some of these compounds, we prefer not to call them allomers because this term usually implies that the reactions were done in alcoholic solution with purified pigments. Also the prefix "allo-" is often used in chemical nomenclature to distinguish an isomer, whereas the "changed" chlorophylls are almost certainly not isomeric forms of chlorophylls *a* and *b*. Goodwin[17] used the description "changed chlorophylls" for altered chlorophylls found in autumn leaves; these pigments probably differ from our "changed" chlorophylls.

The designations chlorophylls a_1 and a_2 have been used for different *in vivo* forms of chlorophyll *a*,[18] and the same form of suffix was used by Michel-Wolwertz and Sironval[19] for differing chlorophyll molecules found in *Chlorella* extracts. We do not suggest that our "changed" chlorophylls are present *in vivo*, and we use the suffixes *a*-1, *a*-2, etc., to distinguish them from chlorophylls a_1 and a_2 or from derivatives of these.

The properties of "changed" chlorophylls *a*-1, *a*-2, *b*-1 and *b*-2 allow some suggestions to be made about their structures. The similarity of their spectra to those of the parent chlorophylls indicates that the conjugated double-bond system is unaltered. They are unlikely to differ from each other and from the parent compounds by the form of attachment of magnesium, because the magnesium-free derivatives are all different. They are not extracted from diethyl ether by dilute alkali; they therefore probably contain no free carboxylic acid group. A compound with the same R_f as phytol is released from all of them on hydrolysis, suggesting that the phytyl group has not been altered. The necessity for oxygen indicates that they may be oxidized, and therefore more polar, forms of the chlorophylls; this is in agreement with their slower running speeds on cellulose, a polar adsorbent. However, lower R_f values might also be explained by chemical combination with another chlorophyll molecule or with the elements of water, rather than with oxygen. They give negative phase tests; the presence of an intact cyclopentanone ring with both hydrogen and an electron-withdrawing group attached to C-10 are required for a positive phase test.[3, 20] This part of the molecule at least may therefore be assumed to have been altered.

The simplest explanation may be that "changed" chlorophylls *a*-1, *a*-2, *b*-1 and *b*-2 have the C-10 hydrogen replaced by a hydroxyl group, and that *a*-1 and *b*-1 are optical isomers at C-10 of *a*-2 and *b*-2, respectively. The combination of stereoisomerism at C-10 and optical activity at C-7 and C-8 could then account for the differences in chromatographic properties in each pair of isomers. Chlorophyll *a'* is probably the optical isomer at C-10 of chlorophyll *a*,[1, 3] and could be the immediate precursor of either *a*-1 or *a*-2. Similarly, one of the "changed" *b* pigments is perhaps derived from chlorophyll *b'*, rather than directly from chlorophyll *b*.

Holt[16] has reported that the positions of the absorption maxima are scarcely altered by introducing a C-10 hydroxyl group into magnesium methyl pheophorbide *a* (methyl chlorophyllide *a*). Barrett and Jeffrey[21] have described an atypical free chlorophyllide formed rapidly by the action of 50% acetone on a marine alga, and consider that this compound

[16] A. S. Holt, *Can. J. Biochem. Physiol.* **36**, 439 (1958).
[17] T. W. Goodwin, *Biochem. J.* **68**, 503 (1958).
[18] W. J. Vredenberg and L. N. M. Duysens, *Biochim. Biophys. Acta* **94**, 355 (1965).
[19] M.-R. Michel-Wolwertz and C. Sironval, *Biochim. Biophys. Acta* **94**, 330 (1965).
[20] S. Aronoff, In *Encyclopedia of Plant Physiology* (Edited by W. Ruhland), Vol. 5, Part 1, p. 234. Springer-Verlag, Berlin (1960).
[21] J. Barrett and S. W. Jeffrey, *Plant Physiol.* **39**, 44 (1964).

contains one or more hydroxyl groups of alcoholic or enolic nature. The spectrum of this atypical chlorophyllide is almost identical with that of the C-10-hydroxy derivative of magnesium methyl pheophorbide *a*. The blue/red ratio of both these compounds is about 9 per cent greater than that of the parent substances, whereas it is almost unaltered with the "changed" chlorophylls *a*-1 and *a*-2. The presence of the phytyl group in the "changed" chlorophylls, but not in the other compounds, may explain the difference in the blue/red ratios. Preliminary NMR measurements by Pennington and co-workers,[3] on the "oxidized chlorophyll *a*" produced by the action of acetone or methanol on leaves, indicate the introduction of a hydroxy group at C-10.

The possibility of a C-10 hydroperoxide group,[20, 22] rather than a hydroxyl, should perhaps not be excluded. Alternatively, hydrolysis of the bond between C-10 and the methoxycarbonyl group might leave both hydrogen and a hydroxyl group on C-10. The "changed" chlorophylls can be formed in the absence of methanol and are unlikely, therefore, to have a methoxyl group attached directly to C-10. A methoxyl on C-10 might be present in the compounds formed by the action of methanol on the purified chlorophylls, however, and there might also be an extra oxygen atom between C-9 and C-10 with some of these derivatives.[16]

The extra blue–green and yellow–green pigments, running close to "changed" chlorophylls *a*-2 and *b*-2, were not studied in detail and suggestions about their structures would be premature. At least some have their principal absorption maxima at shorter wave-lengths than the parent compounds.

The "changed" chlorophylls must be produced non-enzymically in boiled leaves left on an alkaline medium. Some of the altered pigments formed by the action of 50% acetone on purified chlorophylls, or in light petroleum extracts washed with sodium chloride solution, may be the same as "changed" chlorophylls *a*-1, *a*-2, *b*-1 and *b*-2 and must also be products of non-enzymic reactions. We have been unable to decide from preliminary experiments whether or not the "changed" chlorophylls are formed enzymically in unheated leaves treated with aqueous acetone or methanol. Conversion to "changed" chlorophylls was lessened in boiled leaves left in 50% acetone, but not always eliminated. Strain,[1, 2] however, considers that his "oxidized chlorophylls" are produced by an enzymic process.

The "changed" chlorophylls are not formed in leaves left in an alkaline medium unless the leaves are first heated to about 50° or more. The heating probably disrupts the chloroplast structure, to a greater or lesser extent, so that the chlorophylls are no longer adequately protected physically by the lipids or proteins associated with them. Aqueous solutions of acetone, methanol or sodium chloride probably also modify the lamellar structure and hence assist in the formation of the "changed" chlorophylls. Also, the chlorophylls are perhaps protected chemically in the chloroplasts by an oxidation inhibitor, which is destroyed or leached out by the above treatments.

In addition to formation of the "changed" pigments, other changes in the chlorophylls can occur when leaves or leaf extracts are heated or exposed to aqueous organic solvents or sodium chloride solution. These include alterations in the *in vivo* spectra,[14, 23–28] formation

[22] E. I. RABINOWITCH, *Photosynthesis and Related Processes*, Vol. 1, p. 462. Interscience, New York (1945).
[23] P. HAGÈNE, *Compt. Rend. Soc. Biol.* **139**, 159 (1945).
[24] J. B. THOMAS and U. P. VAN DER WAL, *Biochim. Biophys. Acta* **79**, 490 (1964).
[25] J. B. THOMAS, J. W. KLEINEN HAMMANS and W. J. ARNOLDS, *Biochim. Biophys. Acta* **102**, 324 (1965).
[26] E. L. SMITH, *J. Gen. Physiol.* **24**, 565 (1941).
[27] B. KE, *Arch. Biochem. Biophys.* **112**, 554 (1965).
[28] M. B. ALLEN, C. S. FRENCH and J. S. BROWN, In *Comparative Biochemistry of Photoreactive Systems* (Edited by M. B. ALLEN), p. 33. Academic Press, New York (1960).

of chlorophyllides and the *a'* and *b'* isomers, loss of magnesium, and increase in extractability of the chlorophylls with light petroleum. These changes are no doubt also the result of modification of the chloroplast structure.

The results of our experiments and of other work show that certain extraction processes and treatments of leaves can lead to chemical alteration of the chlorophylls. Qualitative monitoring of extracts need take only a few minutes by thin-layer chromatography on microscope slides and would be useful, for example, before making spectrophotometric measurements on solutions assumed to contain only chlorophylls *a* and *b*. It is interesting that, in testing for chlorophyllase activity, "changed" chlorophylls or similar pigments could be formed both during treatment with aqueous organic solvent and on subsequently extracting unhydrolysed pigment into light petroleum and washing with sodium chloride solution.

MATERIALS AND METHODS

Leaf Material

Heracleum leaves were collected from May to September when required. In October young leaves were picked from plants that had been cut back earlier in the season. These kept satisfactorily for several weeks, with little chlorophyll breakdown, when stored in polyethylene bags at 0°. Discs were cut out with a 15 mm cork borer, avoiding large veins. Usually, 10 discs (about 300 mg) were taken for a sample.

Barley seedlings were grown on filter paper moistened with a nutrient solution (Hoagland and Arnon[29]). The top 5–6 cm were harvested after about 10 days and were cut into 1 cm lengths. Samples weighing about 300 mg were used for most experiments.

Solvents

The following solvents were used: light petroleum (A.R. grade, boiling range 60–80°, or G.P.R. grade, boiling range 40–60°); acetone (A.R., water < 1%); diethyl ether (A.R., negative reaction for peroxides, water < 0·5%); *n*-propanol (G.P.R.); methanol (for non-aqueous titrations, water < 0·02%) and ethyl acetate (A.R.). The methanol was supplied by British Drug Houses Ltd., the other solvents by Messrs. Hopkins & Williams, Ltd.

Leaf Treatments

The leaf material was stirred with distilled water (about 200 ml) maintained at the required temperature, usually for 5 min. The leaves were then strained off, washed with cold distilled water, and blotted gently. In subsequent treatments with alkaline media, the leaves were left floating on 10 ml of 0·01 M sodium bicarbonate or tris buffer (0·05 M, pH 8·2, or 0·067 M, pH 8·6) in Petri dishes in the dark at room temperature.

For treatments with organic solvents, fresh leaf material was suspended in 10 ml of solution in a glass-stoppered 50 ml flask that was then kept at room temperature in the dark.

Preparation of Leaf Extracts

Extraction of total pigments with acetone. The leaves or discs were blotted dry and then ground with sand and acetone in a mortar. The acetone was decanted into a volumetric flask and the residue was extracted with further portions of acetone to give a total volume of 25 ml. The extract was filtered under slight vacuum through Whatman No. 1 or No. 42 paper.

[29] D. R. HOAGLAND and D. I. ARNON, *Calif. Agr. Expt. Sta., Circ.* 347, 1 (1938).

The grinding and filtering were done as rapidly as possible in dim light. Samples were taken either for analysis by thin-layer chromatography or for estimating total pigment concentration using an Optica CF4 DR recording spectrophotometer.* When it was necessary to minimize formation of pheophytins, which might result from acid conditions, calcium carbonate was added before making the extract.

In some of the experiments, when leaf material was left in solutions containing large concentrations of acetone or methanol, pigment was leached out of the leaves. The external solution was then added to the acetone extract of the leaves.

Extraction of non-bound chlorophylls with light petroleum. This was based on the method of Osipova,[30] but we found that extraction of moist plant material by grinding with light petroleum (40–60°) was unsatisfactory and gave erratic results. To overcome this, leaf discs that had been treated in various ways were freeze-dried before extraction. From untreated discs the small amount of chlorophyll (less than 5 per cent of the total) that could be extracted with petroleum was not increased by the freeze-drying. From discs where the treatment had made some of the chlorophyll petrol-soluble, the pigment was much more readily extracted than from wet material and there was good agreement between duplicates.

Thin-Layer Chromatography

The pigments were separated on thin layers of cellulose. The method has already been described,[31] but further details are given here. Because the pigments are labile, chromatography was done rapidly in a dark-room with a minimum of artificial light.

When the sample contains water, some chlorophyll remains on the baseline after developing cellulose thin-layer chromatograms.[31] Water was therefore removed by repeated evaporation under reduced pressure at about 20°, with the addition of small amounts of acetone after each distillation, until the residue was dry. Three additions of acetone were usually sufficient. The residue was then immediately taken up in a small volume of acetone, and applied to the chromatographic plate.

Removing water by this method occasionally led to the formation of small amounts of pheophytins, particularly pheophytin *a*. Although this conversion was usually not large enough to be important, an alternative procedure was adopted when necessary. This was also used when the amount of water to be removed was inconveniently large. The extract (5 ml) was shaken gently with diethyl ether (2 ml) and sodium chloride solution (nearly saturated, 5 ml). The bottom layer was run off, and the ether layer was washed three times with more salt solution. The ether solution was then dried (Na_2SO_4) and applied to the chromatogram. This procedure is preferable to a method using ammonium acetate suggested earlier by one of us.[31]

Glass plates (20 × 20 cm) were coated with a layer (approx. 250 μm thick) of cellulose powder (MN-300, Macherey, Nagel & Co.,† without binder; 12 g) slurried with water (72 ml), and the layers were dried for 30 min at room temperature and then for 45 min at about 105°. For quantitative, and sometimes for qualitative work, the sample was applied as a 17 cm streak along the baseline of the chromatoplate. This allows sufficient material (about 60 μg of total green pigments) to be applied, without overloading the chromatogram, for spectral measurements to be made on the eluates. Clear separations are obtained, minor components

* Optica U.K. Limited, Gateshead-on-Tyne.

† U.K. Distributor: Camlab (Glass) Ltd., Cambridge.

[30] O. P. OSIPOVA, *Dok. Akad. Nauk SSSR* **57**, 799 (1947).

[31] M. F. BACON, *J. Chromatog.* **17**, 322 (1965).

are obvious and double tailing, due to colourless materials in the extracts,[32] occurs only at the ends of the streak. The R_f values of the chlorophylls vary considerably with the load, and it is therefore essential to apply the sample evenly to avoid ragged edges to the separated pigment bands. A simple streaking device,[33] which uses an Agla micrometer syringe to measure accurately the volume applied, satisfies this requirement. Repeated applications were made along the same line until enough material had been deposited. The plate was fanned gently between applications to evaporate some of the solvent and thus keep the streak narrow.

The pigments tended to concentrate at the periphery of the spot or streak when the sample solution was applied repeatedly at the same position. With the slower-running pigments, this sometimes led to double-zoning of a particular compound, perhaps because of interference by even less mobile, colourless substances inside the periphery. It was overcome, when necessary, by first developing with acetone until the solvent front was just above the

TABLE 3. CELLULOSE TLC: COMPARISON OF R_f VALUES IN TWO SOLVENT SYSTEMS

Compound	R_f value	
	Light petroleum –acetone–*n*-propanol (90:10:0·45, by vol)	Light petroleum –acetone (80:20, v/v)
Pheophytin *a*	0·90	0·95
Pheophytin *b*	0·73	0·91
Chlorophyll *a*	0·54	0·78
Chlorophyll *b*	0·31	0·62
Pheophorbide *a*	0·18	0·51
Pheophorbide *b*	0·08	0·37
Chlorophyllide *a*	0·03	0·25
Chlorophyllide *b*	0·01	0·13

The values are for loads of about 0·4 μg per spot, and are only approximate.

baseline of the chromatogram. The trailing edge of the streak or spot was carried forward by the acetone until it joined with the leading edge. The acetone was allowed to evaporate and the plate was then developed in the usual way.

The chromatograms were usually developed with a mixture of light petroleum (60–80°)–acetone–*n*-propanol (90:10:0·45, by vol.: solvent A) over a distance of 15 cm. For improved separation of chlorophyllides *a* and *b* from each other and from the pheophorbides and chlorophylls (Table 3), a mixture of light petroleum (60–80°)–acetone (80:20, v/v: solvent B) was sometimes used.

For quantitative measurements, the separated pigment zones were scraped into centrifuge tubes and shaken gently with acetone (5 ml). After removal of the cellulose by centrifuging, the spectra of the solutions were recorded from 360 or 610 nm to 700 nm.

When a chlorophyll *a*-type zone was incompletely separated from a *b*-type pigment, the amounts of each pigment could be estimated from the shape of the spectrum. For calculating

[32] H. H. STRAIN, J. SHERMA, F. L. BENTON and J. J. KATZ, *Biochim. Biophys. Acta* **109**, 1 (1965).
[33] M. F. BACON, *J. Chromatog.* **16**, 552 (1964).

pigment concentrations, the reading at 700 nm was deducted from the absorbancy at the wave-length maximum in the red, which could otherwise be exaggerated by absorption due to traces of residual cellulose suspended in the solution. Specific absorption coefficients for chlorophylls and pheophytins in acetone have been noted previously.[31] The molar absorption coefficients of the "changed" chlorophylls were assumed to equal those of the parent compounds. For quantitative estimation of chlorophyllides, 80% acetone may be preferable as eluant.[34]

Cellulose layers on microscope slides[31] were used extensively for rapid qualitative work.

Estimation of Phytol

The method of Shimizu, Fukushima and Tamaki[35] was used with small modifications. The pigment (1×10^{-8} mole, containing 3 μg of phytol if present) was hydrolysed at 65–70° for 30 min in 7 ml of a 2·5% (w/v) solution of KOH in methanol. After cooling, light petroleum (60–80°, 1·5 ml) was added and dissolved by shaking. The solution was mixed with 10 ml of water, the separated petroleum layer removed by pipette, and the bottom layer was extracted with a further 0·7 ml of petroleum. The combined extracts were evaporated to dryness under reduced pressure and the residue was dissolved in one drop of light petroleum. This was applied as two approximately equal spots to a microscope slide coated with a thin layer of silica gel G (E. Merck AG., Darmstadt; approx. 13% $CaSO_4$). A separate spot of phytol (British Drug Houses Ltd., 1·5 μg) was applied to the slide, and the same amount of phytol was also added to one of the extract spots. After developing with benzene–ethyl acetate (19:1 v/v), the slide was sprayed with a 0·25% aqueous solution of potassium permanganate. The R_f value for phytol was about 0·25, and 1·5 μg of this substance gave a pronounced dark brown spot on a background which turned to pale brown on standing at room temperature. When chlorophylls *a* and *b* were treated in this way, recovery of their phytol was almost complete as judged by visual comparison of the spots.

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[34] Z. ŠESTÁK, *Biol. Plant. Acad. Sci. Bohemoslov.* **6**, 132 (1964).
[35] S. SHIMIZU, H. FUKUSHIMA and E. TAMAKI, *Phytochem.* **3**, 641 (1964).