Artifacts from Chromatography of Chlorophylls

By M. F. BACON

Rothamsted Experimental Station, Harpenden, Herts.

(Received 19 August 1966)

Recent reports (Michel-Wolwertz & Sironval, 1965; Schanderl & Lynn, 1966) describe the separation by two-dimensional chromatography of unusual chlorophyll-like compounds from extracts of *Chlorella* and of ripening green peppers. The similarity of these pigments to 'changed' chlorophylls produced when, for example, leaves are treated with aqueous organic solvents (Bacon & Holden, 1966) prompted an investigation of the chromatographic methods used. The results show that chlorophylls *a* and *b* are readily altered, probably by oxidation, during chromatography on certain adsorbents and that at least some of the *Chlorella* and pepper pigments may have been artifacts.

Materials and methods. Chlorophylls *a* and *b* were purified by chromatography on wet-packed columns of polyethylene powder (Dow QX-917.6; see Holden, 1965) and of Whatman CC41 cellulose powder [development with light petroleum (b.p. 60–80°)–acetone–propan-1-ol (90:10:0.45, by vol.)]. The method described previously (Bacon, 1965; Bacon & Holden, 1966), with Macherey Nagel MN 300 as adsorbent, was used for thin-layer chromatography on cellulose. Thin layers of other adsorbents (silica gel G, Merck; kieselguhr G, Merck; CaSO₄-free silica gel, Whatman SG 41) were prepared by slurring in water, drying at 104° for 1 hr. and cooling for 15 min. Chromatography was done at room temperature in darkness or dim artificial light. Solvents were A.R. grade.

Results and discussion. To simulate the conditions used by Schanderl & Lynn (1966), an acetone solution of purified chlorophyll *a* or *b* was streaked on to a thin layer of silica gel G and developed for 16 cm. with benzene–light petroleum (b.p. 40–60°)–acetone (10:2.5:2, by vol.). Five zones were produced from each pigment; the faint leading one probably included any unchanged chlorophyll *a* or *b*. The zones were scraped off separately and eluted with acetone, after the chromatogram had been allowed to dry in the dark for 10 min. The principal pigments in each zone were purified by thin-layer chromatography on cellulose, and characterized (see Table 1). Six of the pigments were identical with the 'changed' chlorophylls described by Bacon & Holden (1966). These were also rapidly formed, with more than 90% conversion of chlorophylls *a* and *b*, when a water-free acetone extract of barley

seedlings was chromatographed in the same way on silica gel G. Development in a second direction, as used by Schanderl & Lynn (1966), would probably have resulted in further conversion. The 'changed' chlorophylls are very similar to the pigments described by these authors, so it seems almost certain that most, if not all, of their compounds were artifacts of chromatography, rather than products of the degradation of chlorophyll in the plant.

To test the conditions used by Michel-Wolwertz & Sironval (1965), water-free acetone extracts of barley were streaked on to Whatman no. 1 paper and developed by ascending chromatography with toluene for 15 cm. After the chromatograms had been allowed to dry in the dark for 10 min., the chlorophylls were eluted with acetone and analysed by thin-layer chromatography on cellulose. About 15% of the chlorophyll *a* had been converted into 'changed' chlorophyll *a*-1, 7% into *a*-2 and 4% into *a*-3. Only about 3% of the chlorophyll *b* had been converted, into 'changed' chlorophylls *b*-1, *b*-2 and, probably, *b*-3.

The 'changed' chlorophylls were not observed when a barley extract was subjected to thin-layer chromatography on cellulose, and the chlorophylls were eluted immediately and again subjected to thin-layer chromatography on cellulose. This shows that the barley extract was free from the 'changed' chlorophylls, and that these pigments had not been formed during the thin-layer chromatography on cellulose.

'Changed' chlorophylls *a*-1, *a*-2, *a*-3, *b*-1, *b*-2 and *b*-3 all gave negative reactions in the phase test, whereas some of the pigments observed by Michel-Wolwertz & Sironval (1965) were phase-positive. This discrepancy can perhaps be explained by the following observations. During the present investigation, diethyl ether solutions of small amounts of phase-negative pigments, concentrated by evaporation, sometimes gave ambiguous phase reactions. These were caused by traces of a non-volatile non-peroxidic impurity in certain samples of ether, which after concentration gave a magenta-coloured positive reaction. In spite of some other discrepancies, it seems probable that the 'changed' chlorophylls are identical with the pigments found by Michel-Wolwertz & Sironval (1965). This does not necessarily mean that their pigments were

Table 1. *Pigments produced by chromatography of chlorophylls a and b on silica gel G*

R_F values are for development with benzene–light petroleum (b.p. 40–60°)–acetone (10:2.5:2, by vol.) on silica gel G, with light petroleum (b.p. 60–80°)–acetone–propan-1-ol (90:10:0.45, by vol.) on cellulose. Identification with the ‘changed’ chlorophylls $a-1$, $a-2$, $b-1$ and $b-2$ of Bacon & Holden (1966) and with two other pigments observed by them (termed here ‘changed’ chlorophylls $a-3$ and $b-3$) was based on the spectra in diethyl ether, the phase test (Smith & Benitez, 1955) and co-chromatography on cellulose thin layers with the pigments produced by the action of aq. 50% (v/v) acetone on barley (*Hordeum vulgare*). The spectra of ‘changed’ chlorophylls $a-1$, $a-2$, $b-1$ and $b-2$ have been published by Bacon & Holden (1966); spectra given by Schanderl & Lynn (1966) (compound F) and by Michel-Wolwertz & Sironval (1965) (a_4 and b_3) were the same as those of ‘changed’ chlorophylls $a-3$ and $b-3$, except that ‘changed’ chlorophyll $b-3$ absorbed slightly less at 631 m μ and not at all at 686 m μ . Tentative identifications with the pigments described by Schanderl & Lynn (S & L) and by Michel-Wolwertz & Sironval (M-W & S) are in parentheses.

Identity	Approx. R_F ($\times 100$)		Relative amounts	Spectra in diethyl ether			Phase test
	On silica gel G	On cellulose		$\lambda_{\max.}$ (red) (m μ)	$\lambda_{\max.}$ (blue) (m μ)	$\frac{E \text{ at } \lambda_{\max.} \text{ (blue)}}{E \text{ at } \lambda_{\max.} \text{ (red)}}$	
Chlorophyll a derivatives							
(chlorophyll a ...		55		662	429	1.3	(+)
Chlorophyll a or unknown* (C of S & L)	61	57	+	similar to chlorophyll a			
Unknown* (D_a of S & L)	41	49	+	666	431	1.8	
‘Changed’ chlorophyll $a-1$ (a_1 of S & L; a_3 of M-W & S)	36	44	++++	661	428	1.3	–
‘Changed’ chlorophyll $a-2$ (a_2 of S & L; a_5 of M-W & S)	33	28	+++	662	429	1.3	–
‘Changed’ chlorophyll $a-3$ (F of S. & L; a_4 of M-W & S)	15	31	++	653	417	1.9	–
Chlorophyll b derivatives							
(chlorophyll b ...		31		643	453	2.8	(+)
Unknown* (D_b of S & L, or D_b may be chlorophyll b)	43	33	+	642	449	3.4	
Unknown*	35	28	+	646	454	3.5	
‘Changed’ chlorophyll $b-1$ (b_1 of S & L; b_2 of M-W & S)	33	24	++++	643	452	3.1	–
‘Changed’ chlorophyll $b-2$ (b_2 of S & L; b_5 of M-W & S)	31	18	+++	643	452	3.1	–
‘Changed’ chlorophyll $b-3$ (G of S & L; b_3 of M-W & S)	9	18	++	631	443	4.6	–

* Just possibly present in the original chlorophyll solutions and not therefore chromatographic artifacts.

artifacts from chromatography, because they claim that chlorophyll a was not altered by successive chromatography under their conditions, which may have been more rapid. Perhaps the altered chlorophylls were formed during preparation of the extracts (see Bacon & Holden, 1966), or were truly present in the cells extracted.

Various adsorbents were compared for their ability to promote the formation of the ‘changed’ chlorophylls. Spots of purified chlorophyll a in acetone were dropped on to thin layers of the adsorbents, which were then left in the dark for 11 min. After elution of the pigment, quantitative analysis by thin-layer chromatography on cellulose showed that there had been 89% conversion into ‘changed’ chlorophylls with silica gel (with or without CaSO_4 binder), 31% with kieselguhr G, 17% with

Whatman no. 1 paper and 4% with cellulose powder. This shows that ‘changed’ chlorophylls can be formed even with thin-layer chromatography on cellulose when working slowly. A similar test with a loose unheated layer of Celite 545 (Johns–Manville) gave about 50% conversion into ‘changed’ chlorophylls; this may be important when diatomaceous earth is used during the purification of chlorophylls (cf. Broyde & Brody, 1965). These experiments indicate that it is the adsorbent, and not a particular developing solvent, that causes the ‘changed’ chlorophylls to form. With chromatography of plant extracts on the less-active adsorbents, most conversion probably occurs after development, but before elution, when the pigments are spread out and probably in addition separated from any protective substances in the sample.

When chlorophyll *a* was left on silica gel G under an atmosphere of nitrogen, conversion into the 'changed' chlorophylls was greatly diminished. This suggests that these compounds are oxidized chlorophylls. The chlorophyll C-10 hydrogen may be replaced in 'changed' chlorophylls *a*-1 and *b*-1 by a hydroxyl group (not a methoxyl as in certain chlorophyll 'allomers'), and 'changed' chlorophylls *a*-2 and *b*-2 may be the optical isomers at C-10 of 'changed' chlorophylls *a*-1 and *b*-1 respectively (Bacon & Holden, 1966). 'Changed' chlorophyll *a*-3, which is perhaps a mixture of isomers, may in addition have oxygen between C-9 and C-10 in view of its similarity to pigments described by Holt (1958). The structure of 'changed' chlorophyll *b*-3 is probably analogous to that of 'changed' chlorophyll *a*-3.

There have been many recent reports of the use of silica gel or kieselguhr for the chromatography of chloroplast pigments. The identities and purity of pigments separated on such adsorbents and assumed to be chlorophylls *a* and *b* should be checked by a sensitive chromatographic method causing little or no damage to the pigments. Rapid working with thin-layer chromatography on cellulose is suitable for this. The formation of altered chlorophylls may lead not only to errors of interpretation, particularly when these pigments are

made more obvious by chromatography with a second solvent, but also to errors in quantitative analysis due, for example, to differences in molar extinction coefficients. Bleaching of the chlorophylls may also occur and affect recoveries. Carotenoids as well as chlorophylls can be damaged on some inorganic adsorbents (Strain, Sherma, Benton & Katz, 1965).

I am indebted to Mr D. Wilson for his assistance with this work, and to the Dow Chemical Company (U.K.) Ltd. for gifts of polyethylene powder.

Bacon, M. F. (1965). *J. Chromat.* **17**, 322.

Bacon, M. F. & Holden, M. (1966). *Phytochemistry*, (in the Press).

Broyde, S. B. & Brody, S. S. (1965). *Biochem. biophys. Res. Commun.* **19**, 444.

Holden, M. (1965). In *Chemistry and Biochemistry of Plant Pigments*, p. 461. Ed. by Goodwin, T. W. London and New York: Academic Press Inc.

Holt, A. S. (1958). *Canad. J. Biochem. Physiol.* **36**, 439.

Michel-Wolwertz, M.-R. & Sironval, C. (1965). *Biochim. biophys. Acta*, **94**, 330.

Schanderl, S. H. & Lynn, D. Y. C. (1966). *J. Fd. Sci.* **31**, 141.

Smith, J. H. C. & Benitez, A. (1955). In *Modern Methods of Plant Analysis*, vol. 4, p. 142. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.

Strain, H. H., Sherma, J., Benton, F. L. & Katz, J. J. (1965). *Biochim. biophys. Acta*, **109**, 23.

Biochem. J. (1966) **101**, 36c

The Location of Chitin in the Yeast Cell Wall

By J. S. D. BACON, ELIZABETH D. DAVIDSON, D. JONES and IRENE F. TAYLOR
Macaulay Institute for Soil Research, Aberdeen

(Received 23 August 1966)

Although chitin was known to be present in the cell walls of many fungi its occurrence in yeasts was not conclusively demonstrated until Houwink & Kreger (1953) showed that the cell walls of *Saccharomyces cerevisiae* after successive acid and alkali extractions yielded a small residue, granular when examined in the electron microscope, that gave an X-ray diagram characteristic of chitin; this confirmed a tentative chemical identification by Roelofsen & Hoette (1951). The total amount of amino sugar in the wall is small (perhaps as little as 0.8%; Eddy, 1958), and Korn & Northcote (1960) have suggested that only about one-tenth of it can be present as chitin.

The chief difficulty in identifying chitin was the

presence of much larger amounts of glucan, hitherto regarded as 'extremely insoluble' in dilute aqueous alkali (Northcote, 1963) despite its highly branched structure (cf. Manners & Patterson, 1966) and lack of internal hydrogen-bonding (Northcote, 1953a). There appeared to be a contradiction between the failure of aqueous alkali to extract any glucan from whole yeast (Bell & Northcote, 1950) and the undoubted presence of an alkali-soluble fraction of the glucan in cell-wall preparations (Eddy, 1958; Roelofsen, 1953; Falcone & Nickerson, 1956). A possible explanation for this discrepancy could be some degradation by an active laminarinase that we have recently found to be present in cell-wall preparations from baker's yeast; alternatively, the