Separation by paper chromatography of chlorophylls *a* and *b* and some of their breakdown products

Chromatography on columns has been used for many years to separate leaf pigments but paper chromatography only recently. BAUER[1] obtained quite good separation of the pigments in leaf extracts on two-dimensional chromatograms with a mixture (I) of "spezial benzin"–petroleum ether–acetone (10:2.5:2) as the solvent for the first run and a mixture (II) of "spezial benzin"–petroleum ether–acetone–methanol (10:2.5:1:0.25) for the second run. More than 50 other papers on chromatographic methods for separating chloroplast pigments which appeared before 1958 were reviewed by ŠESTÁK[2]. Almost all the methods were concerned with the unmodified pigments and the separation of chlorophyll-breakdown products has received little attention. SIRONVAL[3] was, however, able to separate on a one-dimensional chromatogram chlorophylls *a* and *b*, pheophytins *a* and *b* and pheophorbides *a* and *b* leaving the chlorophyllides unresolved at the origin. He substituted benzene for "benzin" in BAUER's solvent I and ran the chromatogram in a tank saturated with petroleum ether. Many solvent systems, including the one used by SIRONVAL, have been tested and the marked effects of small variations in the running conditions, particularly in the atmosphere of the tank, have been noted.

A mixture of chlorophylls *a* and *b* was made by the method of ZSCHEILE AND COMAR[4] from acetone extracts of bean leaves (*Phaseolus vulgaris*). Pheophytins were made by acidifying a chlorophyll solution. Chlorophyllides and pheophorbides were made by incubating an acetone solution of chlorophylls with a partly purified preparation of chlorophyllase from sugar-beet leaves[5] in enough 0.1 *M* sodium citrate solution to give pH 7.8 and a concentration of 45% (v/v) acetone, at room temperature, in the dark for 16 h. The digest was shaken with petroleum ether to remove any intact chlorophyll. The chlorophyllides were transferred from the acetone solution to ether, which was washed with water and dried with anhydrous Na_2SO_4. Chlorophyllides were converted to pheophorbides by acidifying the digest; the brown precipitate was filtered off and dissolved in ether.

Descending chromatograms were run on Whatman No. 1 paper in an all-glass tank 29 cm square and 53 cm high, at room temperature, in the dark. All the pigments were visible as coloured spots on the paper; the chlorophyllide spots bleached rapidly in the light.

A mixture of petroleum ether (b.p. 40–60°)–benzene (4:1) as the running solvent, in a tank saturated with the same mixture, gave good separation of the pheophytins; the chlorophylls did not move far from the origin and though they were partly separated from each other there was no clear zone between them, and they were not separated from the pheophorbides. The addition of acetone, 0.5 parts, to the running solvent increased the R_F values of the pheophytins and also the distance between the spots; the chlorophylls moved further but did not separate (Fig. 1a). When acetone was also added to the mixture used for saturating the tank the pheophytins ran behind the solvent front, only partly separated from each other, but the chlorophylls were well separated with R_F's of about 0.7 and 0.5. Pheophorbides were also separated from each other under these conditions, pheophorbide *a* running close to chlorophyll *b*; chlorophyllides did not move from the origin. When the paper was equilibrated overnight in the tank before adding the running solvent the

pheophytins were unseparated on the solvent front and chlorophyll *a* ran close behind with an R_F of 0.85 (Fig. 1b).

Pheophorbides *a* and *b* were well separated using a mixture of benzene and acetone (9.5:0.5 or 9:1) in an atmosphere of benzene; chlorophylls and pheophytins ran together on the solvent front and chlorophyllides remained at the origin or streaked from it. Increasing the amount of acetone in the running solvent did not separate the chlorophyllides. They could, however, be separated by using benzene as the running solvent on a paper which had been equilibrated overnight in a tank saturated with both benzene and acetone. Under these conditions the pheophorbides ran together on the solvent front, chlorophyllide *a* had an R_F value of 0.8 and chloro phyllide *b* of 0.7. Chromatograms run without equilibration of the paper, or with too little acetone in the atmosphere of the tank, failed to separate the chlorophyllides which formed a long streak from the origin.

SIRONVAL's solvent gave a separation similar to the result shown in Fig. 1b but with chlorophyll *b* and pheophorbide *a* closer together.

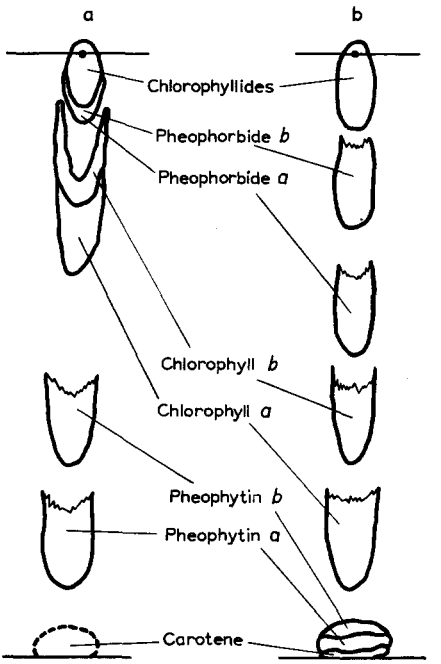

Fig. 1. Separation of pigments with petroleum ether–benzene–acetone (4:1:0.5) as running solvent. a, tank saturated with petroleum ether–benzene (4:1), paper not equilibrated; b, tank saturated with same mixture as running solvent, paper equilibrated.

Although the eight pigments could not be separated with one solvent mixture, any one could be separated from the others by varying the proportions of petroleum ether, benzene and acetone in the solvent and making small alterations in the running conditions. The method is being used in a study of the enzymic breakdown of chlorophyll in leaves.

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