SEPARATION OF CHLOROPHYLLS AND RELATED PLANT PIGMENTS BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

Investigation of the biodegradation of chlorophylls requires rapid micromethods for the separation of chlorophylls and related pigments, minimizing pigment destruction and permitting detection of derivatives occurring in very low concentrations in the plant extract.

Chromatography of a plant extract on a powdered sugar column has been used. HOLDEN[1] described methods for the paper chromatographic separation of chlorophylls and some of their breakdown products. More recently, STRAIN *et al.*[2,3] discussed the "one-way" and "two-way" paper chromatography of chloroplast pigments and the "zonation effects" of other substances present in the extract. Several chlorophylls besides standard chlorophyll *a* and *b* were separated from chlorella extracts on paper by MICHEL-WOLWERTZ AND SIRONVAL[4]. Two-dimensional paper chromatography was also used for separating chlorophylls and carotenoids from marine algae[5]. The solvent systems and conditions used in the paperchromatographic separation of chloroplast pigments were reviewed by ŠESTÁK[6].

COLMAN AND VISHNIAC[7] used proven column material to prepare plates from powdered sugar for two-dimensional thin-layer chromatography of chloroplast pigments. NUTTING *et al.*[8] using the same material, separated chlorophylls and pheophytins one-dimensionally. BACON[9] separated chlorophylls, chlorophyllides and pheophytins on cellulose plates. HAGER AND BERTENRATH[10] used Kieselguhr with admixtures, and EGGER[11,12] used Kieselguhr and cellulose treated with fat. SCHNEIDER[13] evaluated these methods and, in addition, developed a method, also using cellulose plates, which separated chlorophylls *a* and *b* and pheophytins *a* and *b* when successively treated by two solvent systems in the same direction. Kieselguhr G was also used by BUNT[14] for the separation of algal pigments, and, more recently, silica gel was used by GROB *et al.*[15]. SCHALTEGGER[16], using silica gel for one-dimensional separation of chloroplast pigments in cherry leaves, was able to separate four carotenoids, three chlorophylls *a* and two chlorophylls *b*, pheophytin and porphyrin. KIM[17] used Gas-Chrom P for column and thin-layer chromatography (TLC), and Kieselguhr G coated with trioleine for the complete fractionation of bacteriochlorophyll and its degradation products on TLC.

Most of the previous work was based on the one-dimensional separation or two developments in the same direction.

In this study, an effective two-dimensional separation of chlorophylls and their

related pigments on silica gel thin-layer plates with different solvent systems is reported and evaluated.

EXPERIMENTAL

Preparation of pigment solutions

Chlorophylls. Market-purchased fresh spinach leaves were extracted with cold acetone in a mortar with glass sand. Approximately 0.3–0.5 g of $MgCO_3$ was added before extraction to neutralize the acids. The crude extract was dried under vacuum and redissolved in petroleum ether (P.E., b.p. 30–60°), then chromatographed on a powdered sugar column following the procedure of STRAIN[18]. The zones corresponding to chlorophylls *a* and *b* were collected separately.

Pheophytins. A portion of chlorophyll *a* or *b* solution obtained as above was dried in vacuum and redissolved in 5 ml acetone and 0.1–0.2 g of oxalic acid was added. The mixture was allowed to stand at room temperature for one hour for complete conversion to the metal free compounds. The pigments were transferred to diethylether and washed with water to remove the oxalic acid.

Chlorophyllides. Chlorophyllides were prepared according to HOLT AND JACOBS[19]. *Ailanthus altissima* leaves were ground with acetone in the proportion of 3:7 (w/v). The ground mixture was allowed to stand in the dark at room temperature for 12 h. After centrifugation at 2000 r.p.m. for 10 min, the green supernatant solution was transferred to ether, dried further, and redissolved with P.E. The pigment solution was chromatographed on a powdered sugar column, and the zone containing chlorophyllides *a* and *b* was collected. This was repeated once.

Pheophorbides. The pheophorbides were obtained from the chlorophyllides by treatment with oxalic acid as described above for the preparation of pheophytins.

Chromatographic plates

The adsorbents used for thin-layer plates were silica gel with binder (silica gel G from E. Merck, Darmstadt, Germany), or without binder (from Joseph Crossfield & Sons, Ltd., Warrington, England) as well as fluorescent silica gel G (from E. Merck, Darmstadt, Germany). Five grams of silica gel powder were mixed with approximately 15 ml of deionized water in a screw-cap vial. After vigorous shaking for 20 sec, the slurry which had a pH of 6.2–6.5 was poured on a grease-free glass plate (20 cm × 20 cm). Care was taken that the glass surface was very clean. The plate was tilted in different directions to obtain a uniform coating, and dried at room temperature on a level surface overnight. For comparisons, some coated plates were partially dried at room temperature for 20 min, then in an oven at 85° for 30 min and cooled in a desiccator. This method gives a layer of evenly distributed silica gel with a known quantity per unit area and the thickness of the plate could be adjusted readily by varying the amount of silica gel used. An average thickness of the silica gel G coating was 0.24–0.25 mm. Only freshly prepared plates were used.

Chromatography

One-dimensional chromatography

The pigment samples were applied immediately after preparation approximately 1.5 cm from the bottom edge of the plate with capillary tubes under dim light.

The plate was developed in the dark at 16° with the first modified BAUER solvent[20], described below, in a glass chamber saturated with P.E. After 40 min, the solvent front had moved about 19 cm. The plate was viewed under ultra-violet (U.V.) radiation and photographed immediately.

Two-dimensional chromatography

The pigment preparations were successively applied in one spot at one corner of the plate about 2 cm from the edges, under dim light. Replicate plates were developed in the dark in a glass chamber saturated with P.E. at 16° using three different solvent systems.

Solvent system 1 (modified Bauer solvents). 1st dimension: benzene–P.E.–acetone (10:2.5:2, by vol.) 40 min; 2nd dimension: benzene–P.E.–acetone–MeOH (10:2.5:1:0.25, by vol.) 40 min.

Solvent system 2. 1st dimension: benzene–P.E.–acetone–MeOH (10:2.5:1:0.25, by vol.) 40 min; 2nd dimension: P.E.–acetone–*n*-propanol (8:2:0.5, by vol.) 40 min.

Solvent system 3. 1st dimension: benzene–P.E.–acetone (10:2.5:2, by vol.) 40 min; 2nd dimension: P.E.–acetone–*n*-propanol (9:1:0.45, by vol.) 2 h. Immediately following development the chromatograms were photographed under daylight and U.V.

For paper chromatography, the same pigment samples as used on TLC were spotted on Whatman No. 1 paper and chromatographed one-dimensionally with HOLDEN's solvent mixture of P.E.–benzene–acetone (4:1:0.5, by vol.) in a P.E. saturated chamber at room temperature. The time required for a satisfactory separation was 12 h. Photographs were taken immediately after development.

The comparison of several duplicate developments showed no difference in either the separation or the nature of the pigments when using either oven-dried or room-dried plates or silica gel with or without binder. The fluorescent silica gel powder showed no particular advantage in the detection of the chlorophyll derivatives studied here.

For these reasons, room-dried silica gel G (with binder) was used to prepare the thin-layer plates throughout all succeeding experiments.

RESULTS AND DISCUSSION

The results show that two-dimensional thin-layer chromatography (TLC) with appropriate solvent systems permits the separation of 8 major and 8–10 minor tetrapyrrole pigments.

Fig. 1 presents photographs of chromatograms of the pigments separated on TLC (left) and paper (right). Both chromatographic methods show that more than one compound can be separated from the single zone obtained from a sugar column. On TLC, chlorophyllides remained at the origin, and pheophorbides began to move only after 40 min of development. Pheophytin *a* moved most rapidly and separated well from *b*, which followed it closely. The chlorophylls had R_F values of about 0.4, chlorophyll *a* slightly ahead of chlorophyll *b*. A number of pigments moved ahead of chlorophyll *a*, and some partially separated spots can be seen behind chlorophyll *b*. As will be seen later, by two-dimensional development, more compounds can be separated from each zone. Paper chromatography with HOLDEN's solvent shows very good separation, but at least 12 h were needed to achieve satisfactory results. This is a

disadvantage compared to the fast TLC method, because it may allow the formation of the pheophytin observed. However, the chlorophyll *a* band from the sugar column separated into at least four compounds and the chlorophyll *b* into three compounds, excluding pheophytins. Also, the separation of pheophorbides and chlorophyllides was superior to that obtained with TLC.

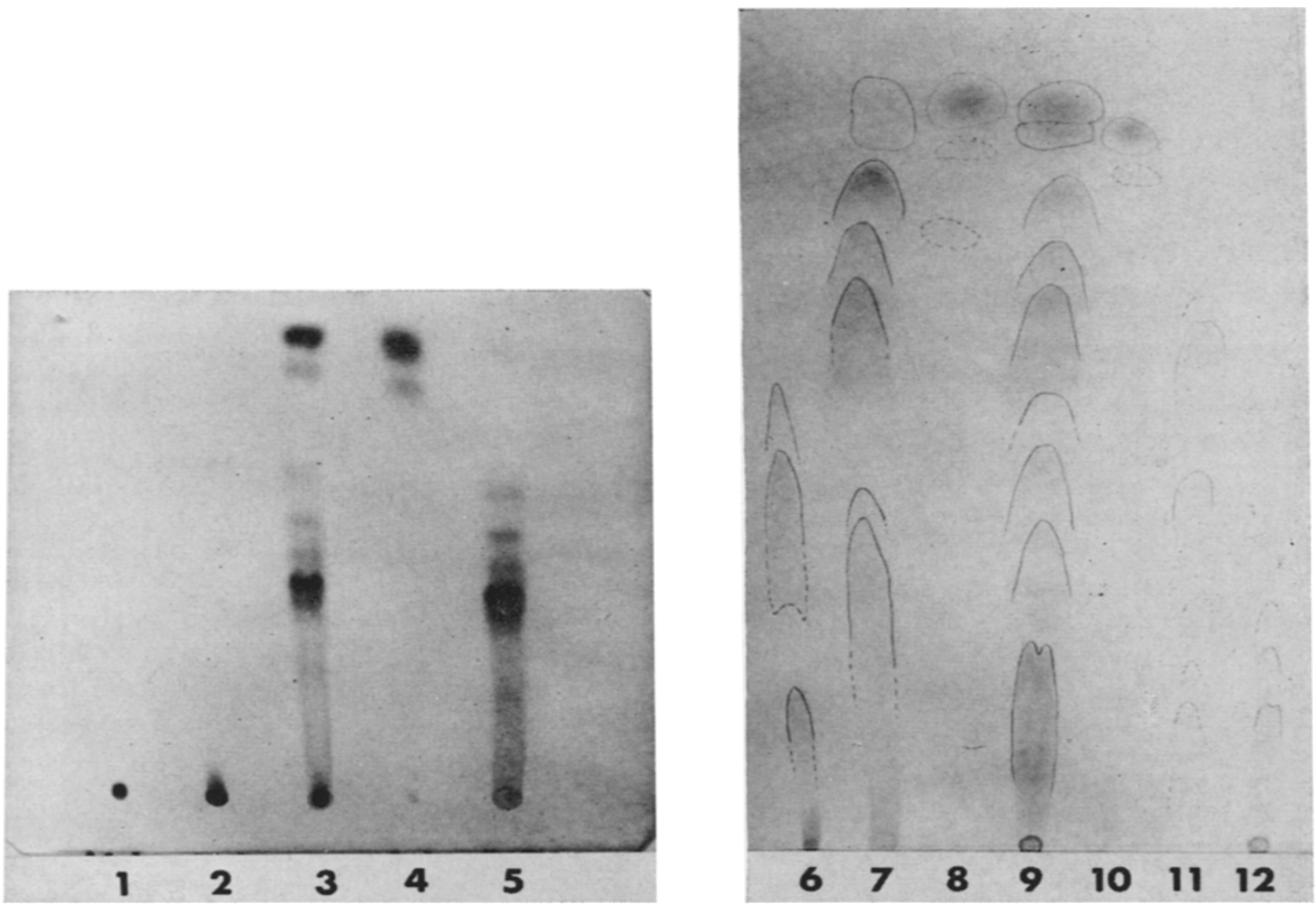

Fig. 1. Separation by one-dimensional TLC using modified BAUER solvent 1 (left) and descending paper chromatography with HOLDEN solvent (right). Prepared pigments applied were: (1) = pheophorbides; (2) = chlorophyllides; (3) = mixture of all pigments; (4) = pheophytins *a* and *b*; (5) = chlorophylls *a* and *b*; (6) = chlorophyll *b*; (7) = chlorophyll *a*; (8) = pheophytin *a*; (9) = mixture of all pigments; (10) = pheophytin *b*; (11) = pheophorbides; (12) = chlorophyllides. Spots visible on the paper chromatogram under daylight are outlined with a solid line and under U.V. with broken lines.

Fig. 2 shows a one-dimensional TLC chromatogram photographed under daylight and under U.V. A number of compounds are barely visible under daylight, but the U.V. clearly locates 2 spots ahead of chlorophyll *a* and 2 spots ahead of chlorophyll *b*. The spots, marked by circling with a pin, can easily be scraped off, and eluted with suitable solvents. Methanol can be used for eluting those components more polar than chlorophylls (lower R_F) and diethyl ether for chlorophylls and the less polar compounds.

Two-dimensional thin-layer separation of the pigments prepared on sugar columns and developed by modified BAUER solvent systems is demonstrated in Fig. 3. The chromatogram was photographed under daylight (left) and U.V. (right). As in

the U.V. photograph of the single dimension chromatogram, the heavier pheophytin and chlorophyll spots appear dark though they fluoresce pink (light spots) in lower concentrations. The U.V. photograph shows clearly that the single spots which had appeared in one-dimensional chromatography were separated into several more spots. The chlorophylls separated into three spots, chlorophyll *a* on the left, chlorophyll *b* on the right, and a mixture of chlorophyll *a* and *b*, possibly isomers, in the center.

The combination of solvent systems chosen is very important. Numerous solvent systems were tried with silica gel thin-layer plates, but only the two most effective ones are reported here along with the modified BAUER solvent system.

Two modified solvents used did not change the order but accomplished better separation especially in the chlorophyll region. Fig. 4 (left) shows good separation of chlorophylls into four spots, two of the chlorophyll *a* and two of the chlorophyll *b* spectrum. The spots appear even more compact than with solvent system 1, and the time required was the same. Fig. 4 (right) shows the best separation of the chloro-

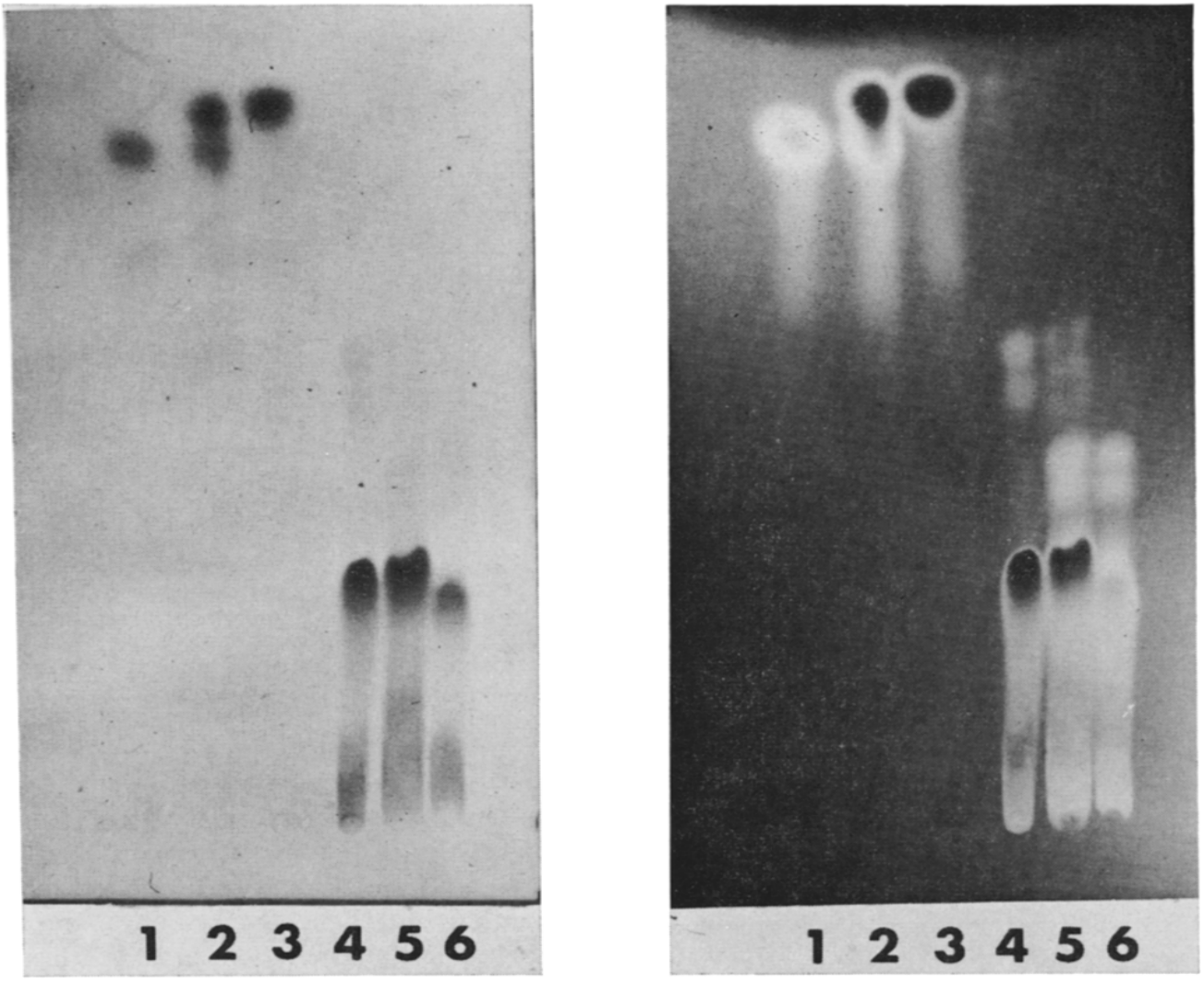

Fig. 2. One-dimensional TLC of prepared pigments developed with modified BAUER solvent 1 photographed under daylight (left) and U.V. (right). The pigments applied are: (1) = pheophytin *b*; (2) = pheophytins *a* and *b*; (3) = pheophytin *a*; (4) = chlorophyll *a*; (5) = chlorophylls *a* and *b*; and (6) = chlorophyll *b*.

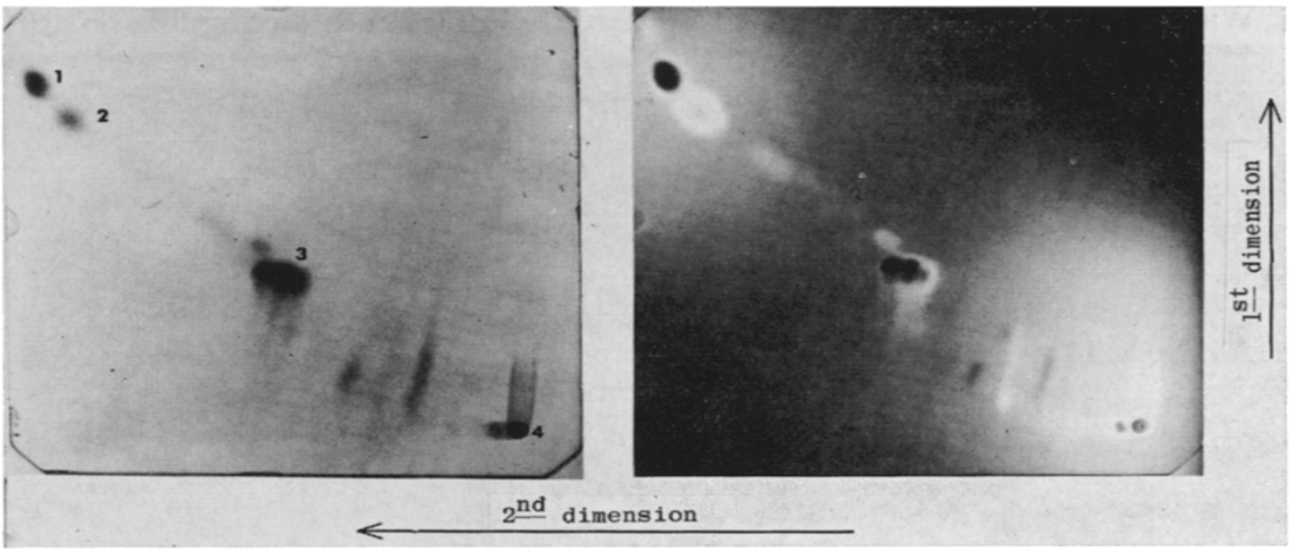

Fig. 3. Two-dimensional thin-layer chromatograms of a pigment mixture developed with solvent system 1 photographed under daylight (left) and U.V. (right). Pheophytins *a* and *b* appear in the upper left corner (1 and 2). The chlorophylls are grouped together near the center (3) and the pheophorbides are unresolved to the left of the origin (4), where the chlorophyllides remained. The separation of smaller spots between these known compounds is enhanced.

phylls into four distinct spots, two of the chlorophyll *a* and two of the chlorophyll *b* spectrum. The time required for the first dimension was also only 40 min, but the second dimension required 2 h for development.

With all three solvent systems, chlorophyllides and pheophorbides could not be separated from each other in the first dimension, and pheophorbides moved only slightly in the second dimension.

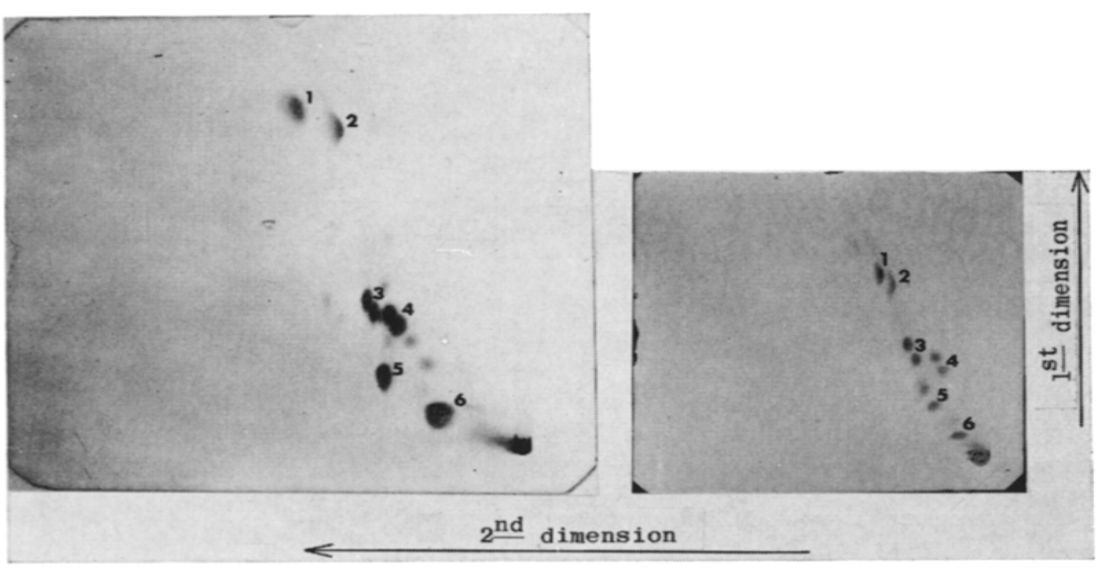

Fig. 4. Two-dimensional thin-layer chromatograms of a pigment mixture developed with solvent systems 2 (left) and 3 (right). Besides a large number of smaller spots, there appear well resolved spots including (1) pheophytin *a* ; (2) pheophytin *b*; (3) two chlorophylls *a*; (4) two chlorophylls *b*; (5) compound "418"; and (6) compound "444". The advantage of the slower solvent 3 is seen in the separation of the chlorophylls.

With the modified BAUER solvent system, the compounds having less polarity than chlorophylls separated quite well. Five are readily visible in daylight and seven can be detected under U.V. Those moving more slowly, *i.e.*, compounds more polar than chlorophylls, also separated reasonably well into four or more spots.

Fig. 4 (left) shows the chromatogram which was developed with solvent system 2 on silica gel G. This method appears most appropriate for work on biodegradation, where the separation of unknown compounds with mobilities similar to the chlorophylls is of importance. These are the spots just ahead of chlorophyll *a* (the "D group"[21]), and the ones behind chlorophyll *b* including two spectrally identified chlorophyll-type pigments[22]. The system used in Fig. 4 (right) shows promise for chlorophylls themselves, but the length of time of development might permit formation of artifacts.

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SUMMARY

Thin-layer chromatography using silica gel G adsorbent and several solvent systems permits rapid separation of small amounts of chlorophylls and related pigments. U.V. radiation is used as a sensitive aid in the detection of very small concentrations of these pigments. The results of the thin-layer chromatography are evaluated and compared with several methods of column and paper chromatography using known compounds. Under the conditions described, as many as 16 compounds can be detected.

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