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Separation of phosphate esters and algal extracts by thin-layer electrophoresis and chromatography

For the study of the photosynthesis in isolated chloroplasts carried out in our laboratory[6], we needed a suitable method for the fractionation of radioactive chloroplast extracts. Using various thin-layer chromatographic methods reported previously[1,7], we did not obtain the results expected. We then tested a variety of different solvents, but without great success. Especially the first separation in two-dimensional thin-layer chromatography was consistently unsatisfactory. We finally chose thin-layer electrophoresis for the first dimension and used the best solvent mixture found for the chromatography in the second dimension.

With this fingerprint technique, described here, we got a very good separation of the radioactive CO_2 fixation products isolated from chloroplasts as well as from whole algal cells (*Chlorella pyrenoidosa*).

Experimental

Preparation of plates. The preparation of very homogeneous thin layers proved to be of the utmost importance. 15 g of Cellulose MN 300 (Macherey, Nagel and Co.), 1 ml of a 0.1 *M* EDTA diNa-solution and 85–88 ml water are homogenized for 1 min at full speed with a Tornado-Rührstab (Emmendinger Maschinenfabrik). To obtain better reproducibility and smoother surfaces, this slurry, which is enough for 5 plates (20 cm × 20 cm), is allowed to stand for at least 12 h (ref. 2). After that time the slurry is mixed again by simply shaking and the glass plates are coated with a 0.5 mm thick layer, using a Quickfit equipment and air-dried overnight.

Electrophoretic and chromatographic procedure. On to the dry plates, 5–20 μl of an acidic chloroplast[6] (or aqueous algal) extract are applied (reaction mixture of JENSEN AND BASSHAM[3]; fixation stopped with 0.3 *N* HCl according to WALKER[8]) as a small spot, 2.5 cm from the edges. The plates are then carefully sprayed (until the surface appears shiny) with a pyridine–acetic acid buffer (10 ml pyridine + 35 ml acetic acid + 955 ml water; pH 4.0), and electrophoresis in the first dimension is started immediately. During spraying, diffusion of the sample is avoided by placing a small piece of parafilm over the point of application[5].

Electrophoresis is carried out at 0° and 900 V, approx. 32 mA (LKB 3371 C, DC Power Supply), for 1 h in an electrophoresis chamber with an aluminum cooling plate built by us. The starting point is placed on the side of the cathode. Two Whatman No. 1 paper wicks (6 × 20 cm) connect the plate with the buffer troughs, each of which is filled with 15 ml of buffer solution.

After electrophoresis in the first dimension, the plates are thoroughly dried in a stream of cold air and then developed in the second dimension (twice) with the solvent system 2-butanol–formic acid–water (6:1:2, v/v) according to the method of METZNER[4]. The chromatography is stopped when the solvent front has moved 14 cm (after about 3 1/2 h) and 17 cm (after about 5 1/2 h), respectively, from the starting point. The distance of 17 cm is marked by a line scratched into the cellulose layer. The sides where the paper wicks had overlapped are scraped off.

Detection and identification. The radioactive spots are detected by autoradio-

graphy (Osray DW, X-ray film, Agfa-Gevaert). Their radioactivity is determined by liquid scintillation counting. The identity of the different compounds is ascertained by cochromatography with authentic substances.

Results and discussion

The method described allows a good and reproducible separation of all the products of chloroplastid photosynthesis (Fig. 1A) and of most of the substances formed during photosynthesis of intact algal cells (Fig. 1B). Fig. 2 shows a schematic diagram of the two-dimensional separation of the major substances found.

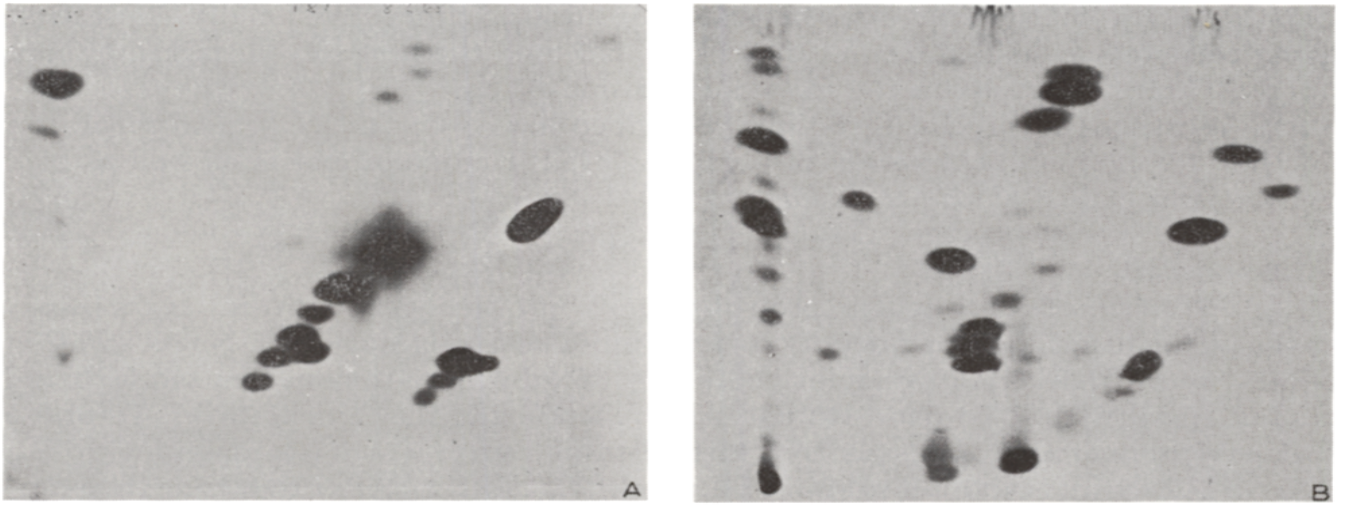

Fig. 1. Radioautograms (^{14}C) after two-dimensional separation of a chloroplast (A) and an algal (B) extract. Cellulose layer, 0.5 mm. Electrophoresis: pH 4.0, 900 V, 60 min, 0°. Chromatography: 2-Butanol–formic acid–water (6:1:2), developed twice, to 14 and 17 cm, respectively.

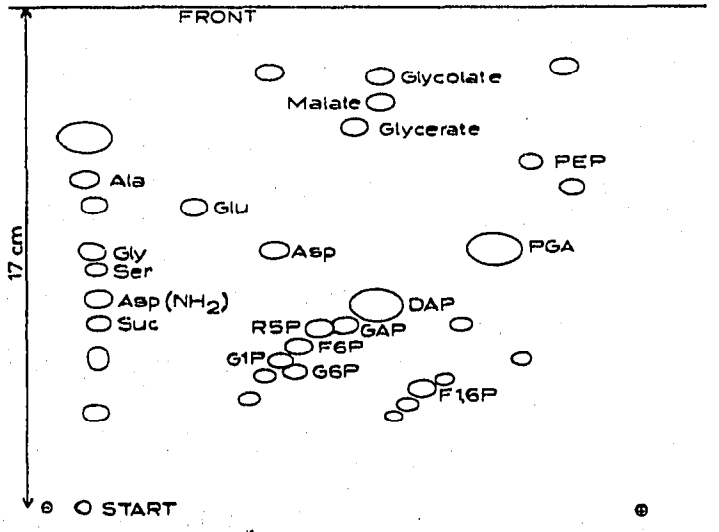

Fig. 2. Schematic diagram of the two-dimensional separation of the major substances found. The front line (17 cm) is scratched into the thin layer of cellulose. Ala = alanine; Gly = glycine; Ser = serine; Asp(NH_2) = asparagine; Asp = aspartic acid; Glu = glutamic acid; PEP = phosphoenolpyruvic acid; PGA = phosphoglyceric acid; DAP = dihydroxyacetone phosphate; GAP = glyceraldehyde phosphate; R5P = ribose 5-phosphate; F6P = fructose 6-phosphate; G1P = glucose 1-phosphate; G6P = glucose 6-phosphate; F1,6P = fructose 1,6-diphosphate; Suc = sucrose.

By varying the time of electrophoresis and the distance at which the chromatograms are developed, the separation of some very interesting compounds can be improved.

To check the electrophoresis, a small strip of X-ray film was placed overnight, before the chromatography, on the plate where electrophoretic separation had taken place. The electrophoretic separation was almost always very good, and the substances were recovered as well-defined spots.

The chromatographic separation varied to a greater extent. Although all possible variables had been standardized, the distribution pattern was sometimes distorted.

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