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Twin-film technique for de-salting and chromatographing amino acids in two dimensions

Multiple-film (usually twin-film) laminar chromatography has two main fields of application. First, for isolating compounds which are heavily contaminated with substances having no interest for the analyst; for example, for separating unwanted iodides from organic iodo-compounds[1], or for isolating traces of pesticides from foodstuffs[2,3]. Secondly, for resolving mixtures of compounds, the components of which have widely different polarities[4]. Recently, ion-exchange resin–cellulose twin films have been used for examining urinary amino acids[5], but the procedure seems unnecessarily complicated. In addition to the labour of grinding and sieving the resin, the process requires five elutions for one-dimensional chromatography. We describe below the much simpler, two-dimensional method which has been in use in this laboratory for the past two years.

Mixtures of amino acids are easily resolved in the absence of other electrolytes, but if salts are present, the quality of the resolution depends on the salt–amino acid ratio. At low ratios (up to 4:1, molar) the amino acids form trails but their identification presents no real difficulty. At higher ratios (*e.g.*, 45:1) identification of the amino acids is impossible (Fig. 1). Natural polypeptides and the amino acids derived

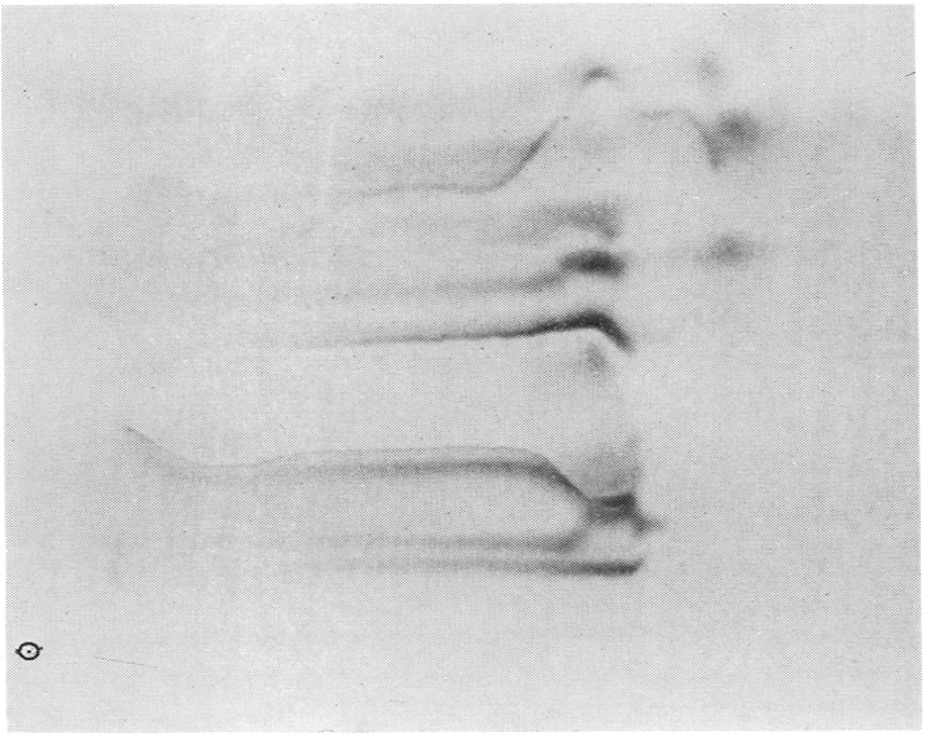

Fig. 1. Two-dimensional chromatogram of a mixture of potassium chloride and 22 amino acids (87:1, molar) on cellulose.

from them are usually accompanied by salts (chlorides). If the material has been fractionated, then buffer salts (*e.g.*, phosphates and citrates) or density gradient generators (*e.g.*, caesium sulphate or chloride) are present in relatively large amounts. Clearly, these salts must be removed before the amino acids can be chromatographed satisfactorily. On an ion-exchange resin–cellulose twin film, the amino acids may be separated from one another on the cellulose portion after the salts have been removed on the resin band. In this way the need for a separate de-salting process is eliminated.

Samples of standard mixtures containing up to twenty four amino acids were mixed with potassium chloride or sodium citrate or sodium dihydrogen phosphate; the ratio is the total weight of salt: total weight of the amino acids, where the weight may be expressed in grams or in molar terms. Chromatoplates (20 × 20 cm) carrying a sulphonated ion-exchange resin (in the H^+ form) on a 35 mm wide band along one edge, the rest of the plate being coated with cellulose, were used. It was very important to ensure that there were no flaws in the junction between the two substrates.

Samples were spotted on to the resin band near one corner of the plate, at a distance 5–10 mm from the film junction. If the sample was placed too far from the film junction, some of the amino acid sample, especially arginine, was not transferred to the cellulose. Conversely, if the sample was placed too near the junction, amino acids and salts were carried on to the cellulose and subsequent chromatography was unsatisfactory. The chromatogram was then developed, at 40°, with water, from the resin band to the cellulose, the front being allowed to travel to the top of the film. This elution carried cysteic acid and the salt anions to the top of the cellulose and left the amino acids and the salt cations on the resin.

After the chromatogram had been dried, the second elution, at 40°, was carried out in the same direction as the first, using a basic eluent. After the chromatogram had again been dried, a line was cut along the junction of the substrates to isolate the resin band. A second line was cut in the cellulose at the opposite edge of the plate, to isolate the band of dirt that occurs in the solvent front. Subsequently, the third elution, at right angles to the first, was carried out, at 40°, in an acidic solvent. Finally, the chromatogram was dried, sprayed with a solution of ninhydrin and stored in the dark for several hours.

Fig. 2 shows the result for a potassium chloride–amino acid ratio of 87:1 (molar). Equally good results were obtained when the salt was sodium citrate (23:1, molar) or sodium dihydrogen phosphate (50:1, molar). Samples having a higher ratio may be processed if they are placed further from the film junction, but there is a progressive loss of arginine from the pattern, and of resolution between cystine and lysine. For a caesium sulphate–amino acid ratio exceeding 200:1 (w/w) the pattern of the non-polar amino acids was fair but those amino acids which are more polar than glycine were not easily distinguishable[6].

The basic eluent used here, a modification of one described earlier[7], is less susceptible to the formation of a purple band near the acidic front.

Experimental

Preparation of chromatoplates. Shandon apparatus was used for coating batches of six plates (20 × 20 cm); the spreader-gap was set at 250 μ. The spreader was divided into two compartments by a rectangular cork wedge (5 mm at the thick end) wrapped in polythene. The wedge fitted snugly into the spreader and was placed about 35 mm

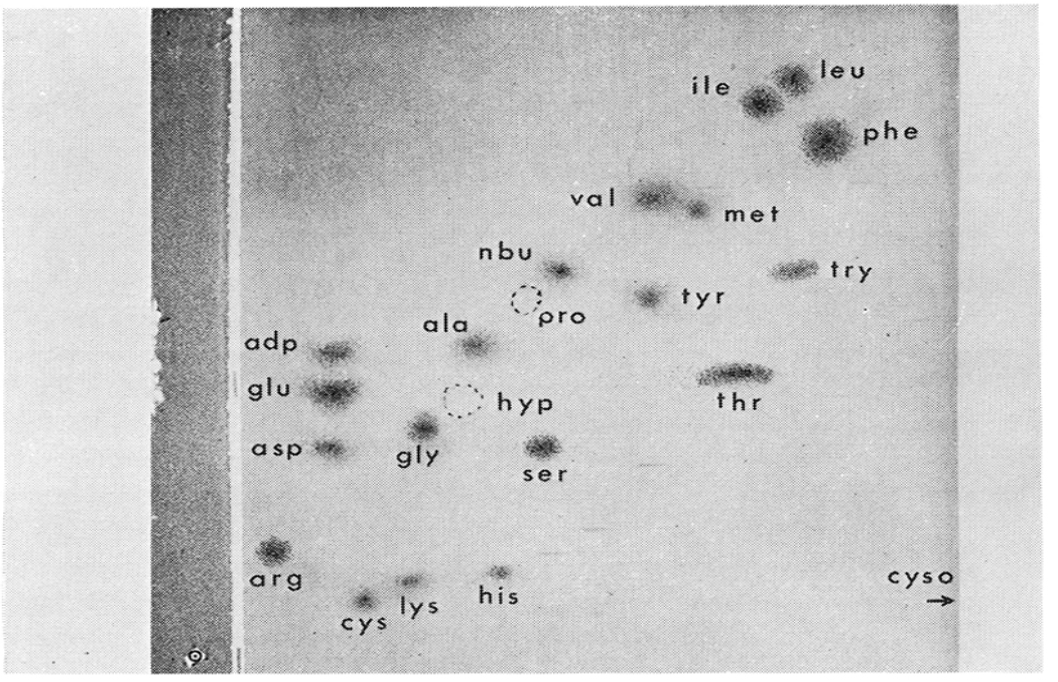

Fig. 2. Two-dimensional chromatogram of a mixture of potassium chloride and 22 amino acids (87:1, molar) on a twin-film of Amberlite CG 120–cellulose.

from one of its ends, with the thin end of the wedge just making contact with the underlying glass plate.

Cellulose slurry. Cellulose (MN 300; 15 g) and water (90 ml) were mixed for 40 sec in a fast mechanical homogeniser.

Ion-exchange resin slurry. Amberlite resin CG 120 (200 mesh; 3 g) in the H^+ form, water (7 ml) and a portion of the homogenised cellulose slurry (3 ml) were stirred by hand. By using a small proportion of cellulose as a binding agent for the resin, the need for grinding and sieving the resin was eliminated.

Each slurry was poured into the appropriate compartment of the spreader, and after the plates had been coated in the usual way, the films were allowed to dry at room temperature for 16 h.

Sample. A solution of 22 amino acids (1.25 μmoles/ml of each) in water–propanol (9:1) was mixed with an equal volume of aqueous potassium chloride (2.4 *M*). In the resulting solution the weight ratio is 45:1 and the molar ratio is 87:1. Similar solutions containing 24 amino acids (0.625 μmol/ml of each) and either sodium dihydrogen phosphate (0.75 *M*) or sodium citrate (0.35 *M*) were also prepared. Samples (8 × 1 μl) of these solutions were placed near one corner of the resin band, at a distance of 5–10 mm from the film-junction.

Development of the chromatogram. Three successive elutions were performed in unlined tanks, all elutions being carried out at 40°.

First elution: with water, from the resin band to the cellulose, the aqueous front being allowed to travel to the top of the chromatoplate (160 mm; time required, 65 min). Subsequently, the chromatogram was dried at 40° for 2 h.

Second elution: with cyclohexanol–acetone–water–1-dimethylaminopropanol-2–diethylamine (10:5:5:1:1) in the same direction as the first elution, for 160 mm

(time required, 200 min). After the chromatogram had been dried (2 h at 40°) two parallel lines were cut in the substrate; one along the film-junction to isolate the resin band, and the other about 10 mm from the opposite edge of the cellulose to isolate the band of dirt in the front.

Third elution: with *tert.*-butanol–acetic acid–water (5:1:1) in a direction at right angles to the first elution, for 160 mm (time required, 210 min). After the substrate had been dried, it was sprayed with a solution of ninhydrin (0.2 % in butanol–acetic acid (4:1)) and the plate was stored in the dark for some hours to allow full development of the colour.

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Gel-electrofocusing in combination with immunodiffusion

With gel-electrofocusing[1–5] being used increasingly in protein chemistry as a new purity criterion for isolated proteins, the question of identification of the different zones focused becomes of great importance.

This communication describes a procedure where isoelectric separation of the proteins in polyacrylamide gel is used and the proteins are then identified by a method based on immunodiffusion with specific antisera[6–8]. Electrofocusing was carried out in tubes according to WRIGLY[4] using a conventional disc-electrophoresis apparatus, and on polyacrylamide plates (18 × 8 cm) using an apparatus described by AWDEH *et al.*[5].

Stock solutions for electrofocusing gels were prepared as follows: Acrylamide solution: 30 g acrylamide; 0.8 g N,N′-methylenebisacrylamide; water to 100 ml. Catalyst solution: 1.0 ml N,N,N′,N′-tetramethyl ethylene diamine; 14 mg riboflavine; water to 100 ml.