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Technical Methods

Thin-layer chromatography for amino-acids and sugars

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Although thin-layer chromatography is now an established and advancing technique in biochemistry (Demole, 1959, 1962; Stahl, 1962) it has not come into general use in chemical pathology. The procedures described below are suggested as simple and rapid means of differentiating amino-acids and sugars in the laboratory. Many commercial firms sell excellent but expensive equipment for thin-layer chromatography but this is not essential.

SPECIAL APPARATUS

GLASS PLATES Good quality window glass can be obtained locally and cut in the workshop. The conventional size is 20 × 20 cm. which may be subdivided into 20 × 10 cm. (half-plate) and 20 × 5 cm. (quarter-plate). The plates must be grease-free and thoroughly clean before use.

GLASS CHROMATOGRAPHY JARS Morbid anatomy museum jars are suitable. A convenient size is about 25 × 25 × 10 cm. and this takes two plates back to back. A well-fitting lid is necessary and if the bottom is not flat a layer of paraffin wax can be used, and this is insoluble in the solvents described.

REAGENTS

SILICA GEL This is supplied by many manufacturers; Silica gel G (Merck), which contains calcium sulphate as binding agent appears the best. A silica gel without binding agent (Woelm) will also give good thin layers but this material is a little more difficult to handle.

SOLVENTS For amino-acids, n-butanol/acetic acid/water (3/1/1) and phenol/water (3/1) were used, and for sugars n-butanol/acetic acid (1/).

LOCATION REAGENTS For amino-acids see Moffat and Lytle (1959).

Solution I Ninhydrin, 0·25 g., 100 ml. methanol, 20 ml. glacial acetic acid, 4 ml. 2,4,6-collidine.

Solution II Cupric nitrate trihydrate in methanol (1% w/v).

To 25 ml. solution I add 1·5 ml. solution II immediately before use.

For sugars see Smith (1960).

Solution I Aniline, 1 g., diphenylamine 1 g., acetone to 100 ml.

Solution II Phosphoric acid (85%).

To 10 ml. solution I add 1 ml. solution II immediately before use.

PREPARATION OF PLATES

A clean plate is placed on a flat level surface; a strip of adhesive plaster is stuck down both sides of the plate with about 0·5 cm. on the plate and 0·5 cm. fixing the plate to the table (Lees and De Muria, 1962). A strip of Sellotape is then struck over each strip of plaster and smoothed down. The silica gel G is prepared: for amino-acids 1 part gel/2 parts water (w/v); for sugars 1 part gel/2 parts 0·1M boric acid (w/v). The materials are mixed rapidly to a smooth homogenous cream with care to avoid bubbles. It is convenient to prepare gel for four whole plates at once, *i.e.*, 30 g. gel + 60 ml. liquid, and the plates must be spread within four minutes of adding the water (Fig. I).

About 15 ml. of gel cream is poured on to the far end of the plate. As if spreading a blood film, a half-plate is firmly held at an angle of 30° resting on the side-strips at the far end, and is pulled steadily to the near end. This spreads the gel evenly over the plate and makes a layer about 250 μ thick. The plates are dried or activated (see below), and may be stored in a desiccator.

AMINO-ACIDS

Thin-layer plates dried in air for at least three hours are used. A tank is prepared by lining two sides with filter

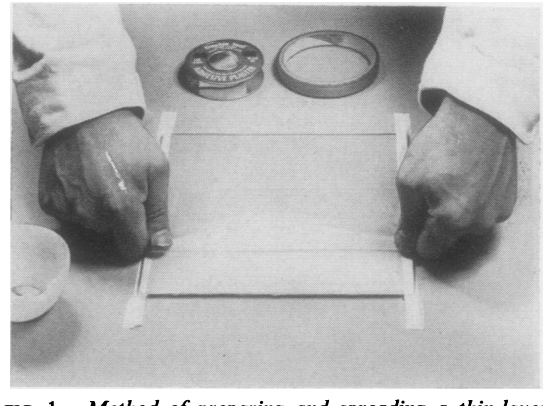

FIG. 1. *Method of preparing and spreading a thin-layer plate.*

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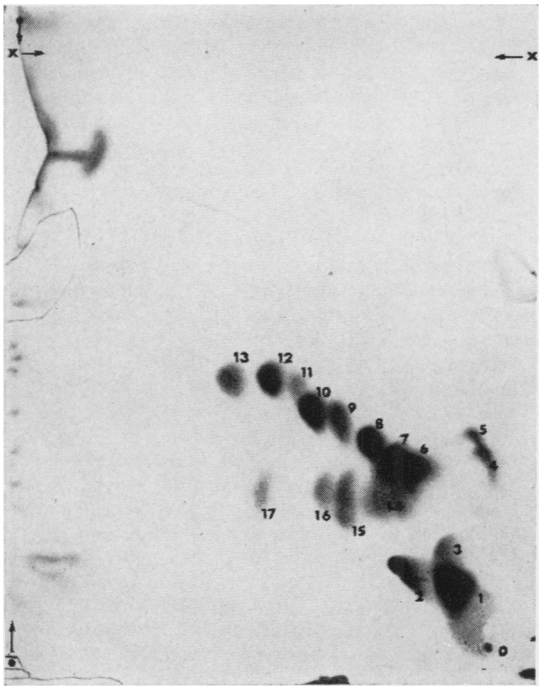

FIG. 2

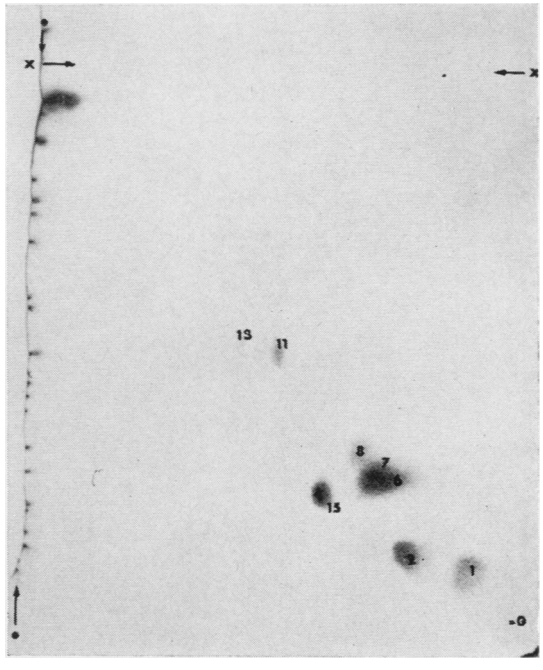

FIG. 3

FIG. 2. *Thin-layer chromatogram of amino-acid standards (procedures as in text).*
O *origin.* ×—× *butanol/acetic acid/water solvent front.* ●—● *phenol/water solvent front.*
1 *Lysine, purple-brown;* 2 *histidine, brown;* 3 *cystine (tailing), grey-brown;* 4 *aspartic acid, blue changing to brown;* 5 *glutamic acid, purple;* 6 *serine (tailing), purple;* 7 *glycine, orange;* 8 *alanine, purple;* 9 *β amino-isobutyric acid, brown;* 10 *valine, purple;* 11 *tyrosine, brown;* 12 *leucine, purple;* 13 *phenylalanine, brown;* 14 *taurine, purple;* 15 *glutamine, purple;* 16 *hydroxyproline, yellow;* 17 *proline, yellow.*

FIG. 3. *Thin-layer chromatogram of 10 μl. normal urine. Procedure and interpretation as in Figure 2.*

paper and 50 ml. of butanol acetic acid solvent poured in. About 15 minutes serves to saturate the tank with vapour.

Urine (a 1 sec. secretion) or amino-acid mixture (5 μ., containing 5 to 10 μg. of each amino-acid) is spotted on to a corner of the plate about 2 cm. from the edges as for paper chromatography. The plate is placed in the tank, and a further 50 ml. of solvent carefully added. The solvent is allowed to ascend at room temperature for two to two and a half hours, when the front has ascended 11 to 13 cm. The plate is withdrawn and gently dried with a fan. It is then run at right angles in a similar manner with phenol as the second solvent for two to two and a half hours, the front again moving 11 to 13 cm. The plate is removed and dried in an oven at 100°C. for about 15 minutes to remove all traces of phenol.

The amino-acids are stained by spraying with the copper-ninhydrin reagent and heated carefully at 105°C. for two to five minutes. The colours develop on a white background. With this technique about 0·025 μg. of glycine can be detected. Figures 2 and 3 show the appearance of a standard amino-acid mixture and of a normal urine.

Brenner and Niederwieser (1960) discuss this and four other solvent systems in detail and list R_F values of many amino-acids. We have found the above combination the most useful, though the R_F values are not absolute. Other amino-acid stains can be used as for paper chromatography.

Ordinary ninhydrin gives mostly pink spots but purple spots on gel without binder. Cadmium-ninhydrin (Heilmann, Barrollier, and Watzke, 1957) gives mainly variants of a vivid crimson, and dicyclohexylamine-ninhydrin (Hardy, Holland, and Nayler, 1955) yields a colour range mostly based on the familiar purple. The copper-ninhydrin stain is recommended for general use.

Silica gel without binding agent (3 parts gel/4 parts water) gives a less satisfactory pattern for amino-acid chromatography: it has the small theoretical advantage that calcium sulphate is slightly soluble in the aqueous solvents (Niederwieser and Pataki, 1960).

SUGARS

Thin-layer plates dried in air for two hours and then

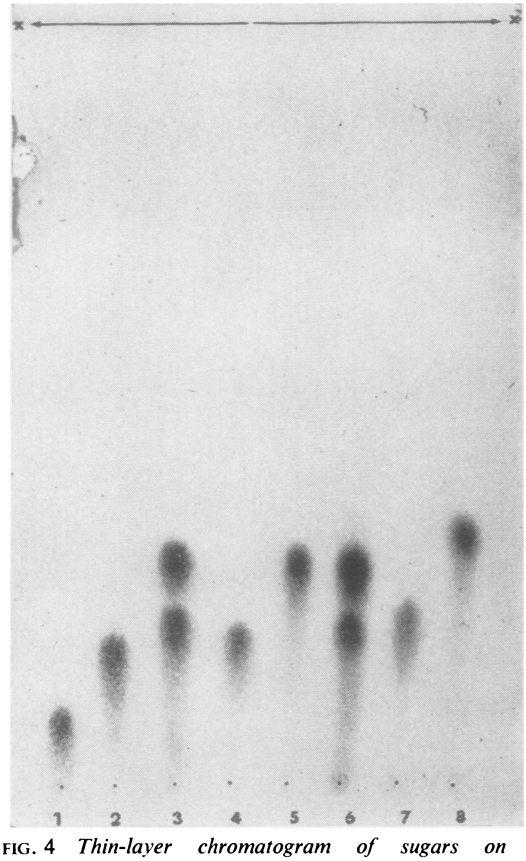

FIG. 4 *Thin-layer chromatogram of sugars on a 20 × 10 cm. plate (procedure as in text).*
×—× solvent front, marked on plate.
1 *lactose, blue-grey;* 2 *sucrose, brown-grey;* 3 *glucose and galactose;* 4 *galactose, mauve-grey;* 5 *glucose, mauve-grey;* 6 *glucose and galactose in urine;* 7 *fructose, brown;* 8 *xylose, blue.*

activated at 105°C. for 30 minutes are used. A quarter-plate will take four or five spots.

A tank is prepared as for amino-acids, using the butanol acetic acid solvent. An application spot of 5 μl. urine (or standard containing 5-10 μg. sugar) is convenient. It is not necessary to de-salt the urine but the presence of salts may slow the sugars slightly. The run is allowed to proceed for two and a half hours, the front advancing 12-15 cm., although separations can be seen in 45 minutes. By performing the chromatography at 37°C. the running times can be reduced by one-third.

The sugars are sprayed with the aniline reagent and heated for about five minutes at 120°C. Figure 4 shows the position of sugars in standards and in urine. It is necessary to include standards as RF values on thin-layer chromatography may vary. Our average RF values were: lactose, 7; sucrose, 16; galactose, 19; fructose, 21; glucose, 28; xylose, 31. Other solvent systems tried were benzene/acetic acid/methanol and methyl ethyl ketone/acetic acid/methanol (Pastuska, 1961) and the usual paper chromatographic systems, but the new solvent described gives the best separation for glucose and galactose. The stain used combines sensitivity to 0·1 μg. glucose with good colour differentiation, and will often detect glucose in normal urine. The other sugar stains familiar to users of paper chromatography can be used for thin-layer chromatography.

Silica gel without binding agent was not found to give good separation in this solvent system.

For small-scale work microscope slides (3 in. × 1 in.) can be used, and a Coplin staining jar serves as the chromatography tank. Satisfactory thin layers can be spread by the blood-film technique without side-strips using 0·5 ml. of gel-cream per slide, or by spraying (Bekersky, 1963). Small spots ($\sim$ 1·5 μl.) can be applied by a tuberculin syringe and an intradermal needle (size 20). Separation of sugars is achieved in 25 min. at 37°C. (in a water-bath) with butanol/acetic acid as the solvent system.

SUMMARY

Simple methods are described for the use of thin-layer chromatography in the chemical pathology laboratory for the separation and identification of amino-acids and sugars in urine and other fluids.

REFERENCES

Bekersky, I. (1963) *Anal. Chem.*, **35**, 261.
Brenner, M., and Niederwieser, A. (1960). *Experientia (Basel)*, **16**, 378.
Demole, E. (1959). *Chromatog. Rev.*, **1**, 1.
—— (1962). *Ibid.*, **4**, 26.
Hardy, T. L., Holland, D. O., and Nayler, J. H. C. (1955). *Analyt. Chem.*, **27**, 971.
Heilmann, J., Barrollier, J., and Watzke, E. (1957). *Hoppe-Seylers Z. physiol. Chem.*, **309**, 219.
Lees, T. M., and De Muria, P. J. (1962). *J. Chromatog.*, **8**, 108.
Moffat, E. D., and Lytle, R. I. (1959). *Analyt. Chem.*, **31**, 926.
Niederwieser, A., and Pataki, G. (1960). *Chimia*, **14**, 378.
Pastuska, G. (1961). *Z. anal. Chem.*, **179**, 427.
Smith, I. (1960). *Chromatographic and Electrophoretic Techniques*. 2nd ed., vol. 1, p. 251. Heinemann, London.
Stahl, E. (1962). *Dünnschicht-Chromatographie*. Springer-Verlag, Berlin.