Nucleoside mono-, di-, and triphosphates are separated in 10–30 min with neutral electrolyte solutions on the poly-EI-paper, whereas on the commercial DEAE-, AE-, and ECTEOLA-papers* this separation cannot be obtained under these mild conditions.

In some cases repeated chromatography yields better separations: after the first chromatography the paper is washed with distilled water, dried and developed a second time with the same or with a stronger solvent. This procedure can be repeated a third time and so on.

Table I shows the R_F values of ribonucleotides on poly-EI-paper obtained with 1.0 M sodium chloride solution.

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1 H. WILFINGER, *Papier*, 2 (1948) 265.
2 H. WILFINGER, *Angew. Chem.*, 62 (1950) 405.
3 K. RANDERATH, to be published.
4 K. RANDERATH, *Dünnschicht-Chromatographie*, Verlag Chemie, Weinheim a.d. Bergstr., 1962.

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* H. Reeve Angel, London, E.C. 4, England.

Notes

Separation of the coproporphyrin isomers I and III by thin layer chromatography*

Ascending paper chromatography[1] was used for isomer analysis of coproporphyrin (COPRO), produced by a heme-requiring *Staphylococcus aureus* (JT/52)[2,3]. Though this method gave satisfactory results under standard conditions, it became desirable to develop a procedure that was more rapid, more sensitive, and less subject to disturbances due to slight temperature changes, contaminating salts or organic materials, and heavy sampling. An attempt was made therefore to adapt ERIKSEN'S method[1] to thin layer chromatography (TLC). While TLC was suggested for the separation of the tetramethyl esters of COPRO I and III[4], no mention of TLC applied to the chromatographic analysis of *free* porphyrins could be found in the literature.

Materials and methods

Only small glass plates (50 mm × 200 mm) in small, cylindrical developing chambers (58 mm × 230 mm) with plastic closures were used throughout this study. Thus, 3 or 4 samples could be conveniently accommodated while considerable amounts of materials were saved and the equilibration time in the chamber was reduced.

The plates were coated with silica gel G (30 g in 60 ml H_2O) using a 250 micron

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spreader. The dried and activated plates (110° for 45 min) were stored in a desiccator over $CaSO_4$ at room temperature. It was found that better separation was achieved if the plates were not used immediately after activation but rather after overnight storage in the desiccator or after approximately 1 h exposure to the room air (25°, rel. humid. 50 %).

Reference porphyrins were chromatographically pure COPRO I and COPRO III tetramethyl esters which were hydrolysed in 7 *N* HCl at room temperature in the dark. The bacterial porphyrins were dissolved in 1.5 to 2 *N* HCl. The samples were dried in an air current at room temperature. For application the solvent mixture suggested by ERIKSEN[1] proved most satisfactory: ammonium hydroxide (30 % NH_3) + water + acetone 1 + 2 + 7 volumes respectively. The samples were applied in quantities of 0.002 to 0.004 ml containing from 0.01 μg porphyrin to amounts readily visible in daylight.

The developing mixture was 2,6-lutidine (Eastman 95 % Practical) and water. The lutidine–water proportion was found to be very critical. The mixture used in paper chromatography[1] was not satisfactory since the R_F values of the I and III isomers were too similar to give good separation. After many trials the best mixture proved to be 10 ml 2,6-lutidine + 3 ml H_2O. Thus, 20 ml lutidine and 6.0 ml H_2O were poured into the chamber, and thoroughly mixed. Then a small cylindrical glass container (50 mm × 22 mm), filled 2/3 with NH_4OH (30 % NH_3), was placed on the bottom of the jar with the aid of a glass rod. The jar was then allowed to equilibrate for at least 30 min before the plates were inserted. Attempts to omit the ammonia vessel by adding ammonia directly to the water, as suggested for paper chromatography[5], failed.

Results

The technique described is a direct adaptation of ERIKSEN's method[1] to TLC. It has all the advantages of his method along with the additional ones characteristic of TLC: (1) rapidity, (2) sensitivity, (3) reproducibility, and (4) ease of recovery. (1) After 2 h the solvent front has traveled approximately 100 mm and the COPRO I and III isomers are completely separated into two distinct spots (R_F 0.19 and 0.25 respectively) of uniform density. (2) As little as 0.01 μg of COPRO I or III can readily be detected with the aid of a U.V. lamp. (3) Though R_F values vary slightly from one plate to another, on the same plate, spots containing minute amounts of porphyrin have exactly the same R_F values as those containing 10 to 100 times that amount. The spots are equally distinct and show no "tailing". (4) The spots can be scraped off easily and quantitatively, extracted with, for example, 2 *N* HCl, and rechromatographed or assayed in a spectrofluorometer. The system does not require special insulation; no disturbances have been observed without such provisions in a normally air-conditioned room at room temperatures of approximately 25°. Furthermore, preliminary studies have shown that metal complexes such as Cu–COPRO III as found in our bacterial system[2] have distinct R_F values, and the spots can be made fluorescent by direct treatment with concentrated H_2SO_4.

The U.V. photograph (Fig. 1) shows a typical chromatogram as it is obtained with the technique described in this communication. It shows from left to right COPRO I, COPRO III, and a mixture of the two isomers. The latter two samples were applied rather heavily in order to show (a) the contamination of the III isomer, which appeared

pure on paper chromatography, with traces of I and (b) the independence of the R_F values from the concentration of the sample.

Since two-carboxyl porphyrins and uroporphyrin were found to follow essentially the paper chromatography pattern, it seems that TLC can be applied with advantage to porphyrin chromatography in general.

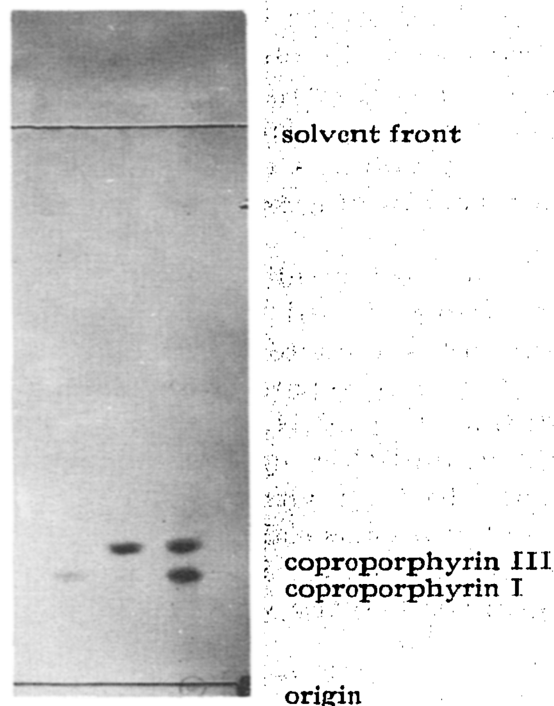

Fig. 1. A photograph of a thin layer chromatogram (silica gel G) under U.V. light showing separation of coproporphyrin isomers I and III by 2,6-lutidine–water–NH_3. Running time 130 min. At extreme left 0.01 µg coproporphyrin I sampled.

As far as our own work is concerned[2], this sensitive method confirmed our former findings: the heme-requiring *Staphylococcus* (JT/52), whose porphyrin production is greatly enhanced by the presence of heme in the culture medium, produces only coproporphyrin of the type III isomer, its metal complexes (Zn and Cu) and uroporphyrin. Neither two-carboxyl porphyrins nor porphyrins with more than four and less than eight carboxyl groups could be detected in the medium.

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1 L. ERIKSEN, *Scand. J. Clin. Lab. Invest.*, 10 (1958) 319.
2 J. JENSEN, *Biochem. Biophys. Res. Commun.*, 8 (1962) 271.
3 J. JENSEN, *Bacteriol. Proc.*, (1962) 116.
4 E. DEMOLE, *J. Chromatog.*, 1 (1958) 28.
5 D. MAUZERALL, personal communication.

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