trichloroacetic acid, diazotized *p*-nitraniline and NaOH solutions remains the same.

To estimate the drug in urine, preliminary dilution in distilled water may be necessary. The diluted urine is then treated in the same way, the ratio of the reagents being kept as above.

Corresponding standard curves in blood and urine of final concentration range 0–1.0 mg./100 ml. are prepared similarly, using dilutions of the standard solution.

The recoveries of PAS estimated by this method in the presence of various sulphonamides (Table 7) show that it is equally efficient in the three mixtures.

Determination of PAS-sulphonamide mixtures

A differential method, similar to that described for the estimation of mixtures of PAS and sulphetrone, may be applied to the determination of mixtures of PAS and the sulphonamides.

The 'total diazotizable substance' is determined by the naphthylethylenediamine method, the PAS is estimated independently by coupling with diazotized *p*-nitraniline, and the correction for the PAS contribution then calculated as described on p. 304.

Recoveries of 91–108% were obtained in the estimation of mixtures of PAS and sulphanilamide, sulphadiazine or sulphaguanidine.

SUMMARY

1. Methods for determining *p*-aminosalicylic acid (PAS) in body fluids are reviewed.

2. Convenient methods for estimating *p*-aminosalicylic acid in the presence of sulphetrone and other sulphone derivatives, using *p*-dimethylaminobenzaldehyde, and in the presence of sulphonamides by coupling with diazotized *p*-nitraniline, are described.

3. A differential method for the determination of mixtures of *p*-aminosalicylic acid and sulphetrone is described.

4. A similar method for the determination of mixtures of *p*-aminosalicylic acid and sulphonamide drugs is described.

I wish to thank Miss I. Secker for valuable technical assistance.

REFERENCES

- Bratton, A. G. & Marshall, E. K., jun. (1939). *J. biol. Chem.* **128**, 537.
 Brownlee, G., Green, A. & Woodbine, M. (1948). *Brit. J. Pharmacol.* **3**, 15.
 Newhouse, J. P. & Klyne, W. (1949). *Biochem. J.* **44**, vii.
 Short, E. I. (1949). *Biochem. J.* **44**, li.
 Street, H. V. (1949). *J. clin. Path.* **2**, 230.
 Tennent, D. M. & Leland, M. L. (1949). *J. biol. Chem.* **177**, 873.
 Venkatamaran, A., Venkatamaran, P. R. & Lewis, H. B. (1948). *J. biol. Chem.* **173**, 641.
 Way, E. L., Smith, P. K., Howie, D. L., Weiss, R. & Swanson, R. (1948). *J. Pharmacol.* **93**, 368.

Paper Chromatography of Porphyrins: some Hitherto Unrecognized Porphyrins and Further Notes on the Method

By RACHEL E. H. NICHOLAS AND C. RIMINGTON

Department of Chemical Pathology, University College Hospital Medical School, London

(Received 28 June 1950)

Filter-paper chromatography, using a lutidine-water system, has been applied to the separation of the porphyrins by Nicholas & Rimington (1949). The R_f values of free porphyrins are inversely proportional to the number of carboxyl groups in the molecule; hence with the aid of a reference graph one may deduce from the R_f values obtained in any paper chromatographic analysis of a mixture of porphyrins what classes of porphyrins are present, e.g. dicarboxylic porphyrins, coproporphyrins or uroporphyrins.

The technique has proved of value in demonstrating the presence in normal urine of small quantities of uroporphyrin together with coproporphyrin (Nicholas & Rimington, 1949), and also in

identifying the porphyrins of the 'pearl shells' *Pteria (Pinctada) radiata* and *Pteria vulgaris* as uroporphyrin and coproporphyrin (Nicholas & Comfort, 1949). No support was obtained from paper chromatograms for the existence in marine molluscan shells of the pentacarboxylic porphyrin, termed 'conchoporphyrin' by Fischer & Hofmann (1937).

When the total urinary porphyrins of some cases of acute porphyria were examined by this method, it was discovered that in addition to known porphyrins containing two, four or eight carboxyl functions, there were frequently present appreciable quantities of porphyrins giving discrete fluorescent spots, the R_f values of which indicated groups with three,

five, six or seven carboxylic functions in the molecule.

In the present paper we record the occurrence of new porphyrins, as judged by paper chromatograms, in a variety of materials which we have investigated. Some further notes upon paper chromatography are also included.

METHODS

Porphyrins were extracted from urines, faeces, etc., by the well known procedures. They were usually purified by chromatography in acid solution upon small columns of talc (Nicholas & Comfort, 1949), eluted by dilute NH_4OH and applied to the paper strips in this solvent. Esters were hydrolysed by contact with approx. 7N-HCl for 36 hr. at room temperature, the acid removed by vacuum desiccation over KOH and the residue dissolved in 2N- NH_4OH .

Filter-paper chromatography was performed using a lutidine-water system, and to provide markers a mixture of uroporphyrin I, coproporphyrin I and deuteroporphyrin IX was always placed upon the same strip adjacent to the unknown. The paper used was 'Whatman no. 1 for chromatography'. Better results were obtained when the chromatograms were run in an atmosphere of lutidine and water only, without NH_3 vapour as originally described (Nicholas & Rimington, 1949). The R_F values were, however, slightly lower.

Fig. 1. Paper chromatography of porphyrins with different proportions of water to lutidine. Temp. 5°. Effect of quantity of water present upon R_F .

Effect of temperature. Equally good results are obtainable using lutidine saturated with water at temperatures varying from 18 to 25° and a similar ambient temperature during the run. The R_F values become lower with increase of temperature, but the linear relationship between the number of carboxyl functions and R_F still holds. The R_F value appears to depend upon the proportion of water present in the solvent system.

Effect of water content. Lutidine (a mixture of 2:4- and 2:5-dimethylpyridines) and water become completely miscible at a temperature of 17° and below. Good chromatograms may be run with the solvent not completely saturated with water, and experiments were carried out at 5° to

determine the R_F values obtaining with lutidine-water mixtures of known composition (see Fig. 1). The linear relation between R_F and number of carboxyl functions holds good over the range of lutidine-water mixtures studied and, in the case of uroporphyrin at any rate, R_F appears to be linearly related to the quantity of water present.

At a temperature of 5° and with lutidine containing 40% (v/v) of water, the R_F values obtained on paper chromatograms are the same as those obtained at 21° with the water-saturated solvent. The lutidine-water phase at this higher temperature contains, however, the same proportion of water, namely, 40% (v/v).

In the present study, a temperature of $21 \pm 1^\circ$ has been used and lutidine saturated with water at this temperature.

Effect of light. The best chromatograms with porphyrins are obtained by running them in the absence of light.

RESULTS

A summary of our findings is presented in Table 1.

DISCUSSION

It is clear from Table 1 that hitherto unrecognized porphyrins occur frequently in pathological and in some normal materials. The technique of paper chromatography has, in fact, revealed an 'intermediary metabolism of the porphyrins' which was unsuspected. The isolation of those pigments which stand midway between uroporphyrin with eight carboxyl groups and coproporphyrin with four carboxyl groups, and the determination of their exact structure, may be expected to throw further light upon the relation of the copro- and uroporphyrins to one another and to the erythropoietic system in general. We have commenced this task of isolation, using standardized methods of adsorption chromatography (Nicholas, 1951).

Indications have not been lacking in previous work that unidentified porphyrins existed. Thus, Watson (1935) isolated the crystalline ester (m.p. 202–203°) of a porphyrin resembling deuteroporphyrin from the faeces of a case of familial haemolytic jaundice; Grinstein, Schwartz & Watson (1945) claimed to have separated the Waldenström ester from urines of acute porphyria patients into uroporphyrin I and a heptacarboxylic porphyrin, presumably belonging to the isomeric series III; McSwiney, Nicholas & Prunty (1950) found in the faeces of a case of acute porphyria a porphyrin which upon chromatography displayed evidence of five carboxyl groups. More recently, Grinstein, Wikoff, de Mello & Watson (1950) have drawn attention to the presence of a porphyrin, which they term porphyrin α (ester m.p. 210–215°), in the faeces of rabbits experimentally poisoned by lead. Our finding of a tricarboxylic porphyrin in lead poisoning is interesting in this connexion.

The presence of a pentacarboxylic porphyrin, together with coproporphyrin in calf meconium, is

Table 1. *Partition chromatographic analysis of porphyrins present in some natural and pathological materials*

Material	(COOH) ₂	(COOH) ₃	(COOH) ₄ (copropor- phyrins)	(COOH) ₅	(COOH) ₆	(COOH) ₇	(COOH) ₈ (uropor- phyrins)
Urine of ground squirrel (<i>Sciurus niger</i>)	.	++	(+)	(+)	.	.	+++
Meconium of calf	.	.	+++	+	.	.	.
Meconium of rabbit (<i>Lepus cuniculus</i>)	.	.	+	.	.	.	.
Meconium of civet cat (<i>Civettictis civetta</i>)	.	.	+	.	.	.	.
Meconium of bactrian camel (<i>Camelus bactrianus</i>)	.	(+)	+	.	.	.	.
Meconium of sea lion (<i>Zalophus californianus</i>)	.	.	+	.	.	.	.
Meconium of human baby	.	.	+	+	(+)	.	.
Urine from case of industrial lead poisoning	.	+	++	.	.	.	+
Coproporphyrin preparation from urine of lead-poisoned rabbits	.	+	++	.	.	.	.
Urine of acute porphyria case 'Glasgow'	+	.	.	+	.	++	+++
Urine of acute porphyria case 'Liverpool'	.	(+)	.	.	+	.	++
Urine of acute porphyria case 'S'	.	.	.	+	.	(+)	++
Urine of acute porphyria case 'Weaver'	.	.	.	.	.	.	++
Porphyrin methyl ester preparation, m.p. 143–162°, from case 'C' of porphyria cutanea tarda (Gray, Rimington & Thomson, 1948)	.	+	++	.	+	.	.
Urine from case 'L.W.' of porphyria cutanea tarda	+	+	+	+	.	+	++
Faeces from same case	.	+	++	.	.	.	(+)
Urine from case 'W' of porphyria cutanea tarda	.	.	.	.	(+)	+	++

Very strong spot, +++; strong spot, ++; moderately strong spot, +; faint spot, (+).

of no little interest. Such pigments must definitely be products of the foetus and cannot have arisen as the result of bacterial activity in the gut. We have isolated this pentacarboxylic porphyrin and will shortly report upon its constitution. It may be recalled that Grinstein *et al.* (1945) encountered a pentacarboxylic porphyrin among the products of the decarboxylation of the Waldenström ester. They give a carbon and hydrogen analysis of the pentamethyl ester, which had m.p. 221–224° and the α -band in chloroform at 623.5 m μ . Further decarboxylation with hydrochloric acid at 180° led to coproporphyrin I.

SUMMARY

1. A further study has been made of the technique of paper chromatography of the porphyrins.

2. The method has been applied to the examina-

tion of the porphyrins present in the meconium of several animal species, urines in industrial and experimental lead poisoning and the urine and faeces from a number of cases of porphyria.

3. The frequent presence has been revealed of hitherto unrecognized porphyrins possessing three, five, six or seven carboxylic functions as determined by their chromatographic behaviour.

We wish to thank the Medical Research Council for a personal grant which supported this investigation, and Miss A. Benson, Mr P. Bliss and Miss P. A. Miles for their valuable assistance.

We are greatly indebted to Dr R. E. Rewell and Dr W. E. Lawrence of the Zoological Society of London for kindly placing at our disposal the specimens of animal meconium. The porphyrin from the urine of lead-poisoned rabbits was kindly supplied by Dr M. Weatherall of the London Hospital Medical College.

REFERENCES

- Fischer, H. & Hofmann, H. (1937). *Hoppe-Seyl. Z.* **246**, 24.
 Gray, C. H., Rimington, C. & Thomson, S. (1948). *Quart. J. Med.* **17**, 123.
 Grinstein, M., Schwartz, S. & Watson, C. J. (1945). *J. biol. Chem.* **157**, 323.
 Grinstein, M., Wikoff, H. M., de Mello, R. P. & Watson, C. J. (1950). *J. biol. Chem.* **182**, 723.
 McSwiney, R. R., Nicholas, R. E. H. & Prunty, F. T. G. (1950). *Biochem. J.* **46**, 147.
 Nicholas, R. E. H. & Comfort, A. (1949). *Biochem. J.* **45**, 208.
 Nicholas, R. E. H. & Rimington, C. (1949). *Scand. J. clin. lab. Invest.* **1**, 12.
 Nicholas, R. E. H. (1951). *Biochem. J.* **48**, 309.
 Watson, C. J. (1935). *J. clin. Invest.* **14**, 110.

Chromatographic Methods for the Separation and Identification of Porphyrins

By RACHEL E. H. NICHOLAS

Department of Chemical Pathology, University College Hospital Medical School, London

(Received 28 June 1950)

Reproducible results by chromatographic methods can only be ensured by strict adherence to experimental conditions. Although successful separations of porphyrin esters have been reported by previous workers using columns of alumina (Prunty, 1946; McSwiney, Nicholas & Prunty, 1950; Lederer & Tixier, 1947) or of calcium carbonate (Grinstein, Schwartz & Watson, 1945; Watson, Schwartz & Hawkinson, 1945; Gray & Holt, 1948), the adsorptive power of the commercial materials employed has not been strictly defined and, moreover, little if any reference has been made to the purification or standard of purity of the solvents employed. Repetition of their results, therefore, has often proved difficult.

Filter-paper chromatography has been applied to the porphyrins by Nicholas & Rimington (1949) and in the lutidine-water system used is capable of separating them into groups corresponding to the number of carboxyl functions in the molecule. It can thus be used as an analytical procedure, but appears to be not so well adapted for the preparation of material in quantities suitable for chemical characterization. For this purpose, adsorption chromatography of porphyrin esters upon solid columns is preferable.

Before attempting the isolation of certain hitherto unrecognized porphyrins, which partition chromatography has shown to be present in pathological materials (Nicholas & Rimington, 1951), we deemed it essential to study carefully the chromatographic behaviour of known porphyrin esters in precisely defined systems and to extend, if possible, the range of adsorbents available. This we have done, and our results are presented below. Paper chromatography has proved a very effective means of ascertaining the homogeneity of fractions, and therefore the sharpness of the separations obtained.

METHODS

Adsorbents

The commercial materials used were:

Al_2O_3 , 'Aluminium oxide for chromatographic analysis standardized according to Brockmann', grade I (Savory and Moore Ltd.).

MgO , heavy magnesium oxide (British Drug Houses Ltd.).

MgCO_3 , heavy magnesium carbonate (British Drug Houses Ltd.).

CaCO_3 , calcium carbonate, 'Analar' (British Drug Houses Ltd.).

From these materials, adsorbents of graded adsorptive power were prepared as described below, and all preparations were finally standardized according to the technique of Brockmann & Schodder (1941), whose system of classification for aluminas we have extended to all our materials. Brockmann & Schodder's classification is summarized by Williams (1946).

Graded aluminium oxide. The Al_2O_3 supplied by Savory and Moore (see above) is termed by the manufacturers 'Grade I Brockmann', but we have found that samples can vary from grade I to grade II. The adsorptive power appears to depend upon the amount of water present. Samples can be made more active by heating followed by cooling in a confined atmosphere. Conversely they may be deactivated by the addition of water. Grade II Al_2O_3 can be prepared from any other grade by suspending in water, filtering, drying at 120° and then cooling in a desiccator. Grade IV Al_2O_3 was similarly prepared by wetting followed by drying thin layers in air at 20° .

Graded magnesium oxide. The adsorptive power of the commercial material is rather variable. The grades used in our study were prepared as were the aluminium oxides, the conditions of drying being as follows: grade II, dried at 120° and cooled in a confined space; grade III, dried at 37° in thin layers in a constant temperature room; grade IV, dried in thin layers in air at 20° .

Graded magnesium carbonate. Heavy MgCO_3 as supplied by British Drug Houses was found to be grade III. It behaves chromatographically in all respects as does MgO of similar grade.