REVIEW

CHROMATOGRAPHY OF PORPHYRINS AND METALLOPORPHYRINS

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CONTENTS

1. INTRODUCTION

In recent years, chromatographic methods have played an essential role in many of the diverse aspects of pyrrole pigment research: in the elucidation of the structure of haem *a*, the prosthetic group of cytochromes *a* and a_3; in the elucidation of the structure of haem a_2, the iron-chlorin prosthetic group of bacterial cytochrome a_2; in the definition of a number of the intermediate steps in porphyrin and haem biosynthesis and in the elucidation of the structure and isomeric form of a number of porphyrins including hepta-, hexa- and penta-carboxyl porphyrins, which have long been known to occur both in pathological materials and in biosynthetic experiments.

The chromatography of porphyrins was reviewed by the author in 1954[1].

Considerable advances have since been made, and the time seems opportune for a more detailed account of the techniques available. In view of the rapid expansion of research in this field, and of the needs of the non-specialist, an account is included below of some of the properties of these pigments, of methods for their isolation from natural sources and of various treatments which are often necessary preliminaries to their chromatography.

Some other review articles and books may be found useful: the chemistry and biochemistry of the porphyrins and metalloporphyrins is discussed fully by FALK[4] and their physical chemistry by PHILLIPS[5]; BÉNARD, GAJDOS AND GAJDOS-TÖRÖK[2] have treated in some detail the porphyrias and other aspects of the clinical pathology of these compounds; the comprehensive treatise of LEMBERG AND LEGGE[6] is invaluable in all aspects of the field up to 1949, and lastly, the organic chemical aspects of most of the pigments mentioned, and many others, are treated by FISCHER AND ORTH[7] and FISCHER AND STERN[8].

The chromatography of chlorophylls was reviewed in this Journal by ŠESTÁK[3] in 1958.

2. PROPERTIES, METHODS OF ISOLATION AND PRELIMINARY TREATMENTS

(a) *Acid–base properties and solubility*

1. *Free porphyrins*

The naturally-occurring porphyrins all possess carboxyl side-chains, and are thus ampholytes, soluble in alkali by virtue of the carboxyl groups and in mineral acids by virtue of the ring nitrogens. The isoelectric points of most common porphyrins lie between pH 3 and 4.5; in this pH range they may be precipitated from aqueous solutions. All porphyrins are soluble in glacial acetic acid, strong mineral acids, pyridine and related solvents and in dioxan, and less soluble in methyl and ethyl alcohols, acetone and ether. They are freely soluble in polar organic solvents containing acids, *e.g.*, acetone–HCl, ether–acetic acid or ethyl acetate–acetic acid mixtures, and they are soluble in aqueous solutions of anionic, cationic or neutral detergents. Porphyrins do not dissolve readily in ether from the solid state, and transference from aqueous phases to ether depends on vigorous dispersion with the ether immediately after bringing to the isoelectric point, before flocculation occurs. They may be extracted from ether by aqueous alkali or by aqueous mineral acids. Of the common porphyrins, uroporphyrins are the most water-soluble, and in fact can be brought into organic solvents under special conditions only (see Section 2. b).

HCl number or "Willstätter number". FISCHER AND ORTH[7] have recorded HCl numbers for many porphyrins. The HCl number is defined as that concentration of HCl (in % w/v) which from an equal volume of an ether solution of a porphyrin extracts two thirds of the porphyrin[10].

The "pH" number is defined as the pH of a buffer solution which extracts half of a porphyrin from four volumes of its ether solution[9].

The HCl number depends on both the dissociation of the porphyrin as a base,

and on the distribution coefficient of the free porphyrin and its hydrochloride between water and the organic solvent[11]. HCl numbers are commonly used to separate mixtures of porphyrins, and some approximate values are given in Table I.

TABLE I

HCl NUMBERS

	Free porphyrin	*Methyl ester*
Uro	*	1.5
Copro	0.08	1.5
Haemato	0.1	—
Deutero	0.3	2.0
Meso	0.5	2.5
Proto	2.5	5.5
Aetio	3.0	—
Chlorocruoro	4.6	—

* Uroporphyrins are not soluble in ether.

Some studies have been made[12–14] of the behaviour of porphyrins in countercurrent distribution between organic solvents and dilute hydrochloric acid solutions. Distributions coefficients have been determined for several porphyrins. These values are more precise than the HCl numbers.

2. *Porphyrin esters*

Porphyrins with carboxyl side-chains are readily esterified, and the esters hydrolysed, without degradation. The esters are more lipophilic than the free porphyrins, as indicated by their HCl numbers and their solubility in organic solvents. Unlike the free porphyrins, they are soluble in $CHCl_3$, CCl_4, C_6H_6, CS_2, etc. Purification of porphyrins is often carried out via the methyl esters, particularly when column chromatography is required. The esters crystallise well, having characteristic, though high, melting-points[7]. Mixed melting-point curves are available for the methyl esters of coproporphyrins I and III[15] and uroporphyrins I and III[16].

3. *Metal complexes*

The iron complexes of porphyrins have no basic nitrogen function and are thus insoluble in aqueous acids. They are soluble in alkali, pyridine, dioxan, and less so in alcohol; they can be brought into strong solution in ethyl acetate or ether via their ready solubility in acidified organic solvents. Protohaem and protohaemin (ferro- and ferri-protoporphyrin respectively) dissolve readily in cold concentrated sulphuric acid, which removes the iron. This treatment does not alter the porphyrin nucleus, but causes hydration of the vinyl side-chains to hydroxyethyl; thus protohaemin is converted to haematoporphyrin. Copper is removed from Turacin (copper-uroporphyrin) by similar treatment.

Esters of metalloporphyrins may be prepared in two ways: the carboxyl side-

chains of metalloporphyrins may be esterified, or the metal may be introduced into the esterified porphyrin. The esters have lost their alkali-solubility, have gained solubility in $CHCl_3$, CCl_4, etc., but otherwise have similar solubilities (*e.g.* pyridine, dioxan) to the unesterified compounds.

(*b*) *Extraction from natural materials*

Though metal-free porphyrins may associate with proteins, the binding is usually by electrostatic and van der Waals forces, and is easily broken; acidified organic solvents, such as mixtures of ethyl acetate or ether with glacial acetic acid, acetone with HCl, extract most free porphyrins quantitatively from tissues, at the same time precipitating the protein in a form which may be removed by filtration or at the centrifuge. The bonds between the apoprotein and the haem iron are also broken by these solvents, and most haems are extracted from tissues in the same way as porphyrins. Covalent bonds between haem and apoprotein, such as the thioether bonds in mammalian cytochrome *c*, are of course not broken by these treatments and other methods are required (*cf.* ref.[4]).

The grinding of biological material with glacial acetic acid, followed by admixture with ether and removal of the solids, was used extensively by FISCHER[7] and is particularly useful for extraction of porphyrins from tissues such as bile or faeces. For extraction of both haems and porphyrins from blood, body tissues and plant tissues, the extracting agents most widely used are either acetone containing from 1 to 5 % (v/v) of concentrated HCl, or ethyl acetate mixed with 25 % (v/v) of glacial acetic acid. The latter method was introduced by SCHWARTZ AND WIKOFF[17] and has been used extensively. The isolation of uro-, copro- and protoporphyrins, and haem, following initial extraction with ethyl acetate–acetic acid has been described in detail by DRESEL AND FALK[18]. Briefly, the protein-free extract is washed free of acetic acid, buffering during the washings with sodium acetate. If the pH of the aqueous phase is not permitted to drop below about 4, so that porphyrins of low HCl number (*e.g.* coproporphyrin) are not extracted, copro- and proto- and related porphyrins remain in the organic phase. Uroporphyrins are insoluble in most neutral organic solvents, and are found in the aqueous phase, from which they are recovered by extraction with cyclohexanone after acidification to pH 1.8[18,19]. They are eventually extracted from the cyclohexanone with aqueous HCl, and may then be chromatographed after evaporation of the HCl. The porphyrins remaining in the original organic phase are removed by extraction with HCl, transferred to ether, and removed from this stepwise with increasing concentrations of HCl, according to their Willstätter numbers. These fractions may then be transferred again to ether, when they are ready for chromatography. It is important that the ether used for these purposes be peroxide-free. Any haems extracted remain in the original organic phase after these treatments, and may be recovered, by evaporation, in a relatively pure state. Thus from blood, protohaem recovered in this way may be crystallized[20] without further purification, as may protoporphyrin dimethyl ester prepared from it[18].

(c) *Removal of iron from haemins*

A variety of methods[6,7] is available for the removal of iron; they all depend upon the fact that in these complexes, ferrous iron is more readily replaced by protons than is ferric iron. Most iron–porphyrin complexes and their derivatives are readily autoxidized, so that most processes for the removal of iron feature a reducing agent (*e.g.* iron-powder, stannous chloride, sodium amalgam) and a proton-source (formic, oxalic, acetic, hydrochloric acids). On the small scale, a convenient and widely-used method is that of WARBURG AND NEGELEIN[21], in which the solvent is glacial acetic acid, the reducing agent ferrous acetate, and the proton-source concentrated HCl. The process has been modified and much improved by making use of ferrous sulphate in place of ferrous acetate[22].

(d) *Esterification and saponification*

Porphyrins readily form esters with alcohols; of these, methanol is most commonly used. The porphyrin is dissolved in anhydrous methanol saturated with gaseous HCl or, much more conveniently, in anhydrous methanol to which 5 % (v/v) concentrated sulphuric acid has been added. The solution is allowed to stand in the dark for about 24 hours at room temperature. This time and temperature are required for full esterification of, *e.g.*, uro- and coproporphyrins, but protoporphyrin in particular is fully esterified in 24 hours, and suffers less decomposition, at 0°. To the solution an equal volume of crushed ice is added, and as quickly as possible the ester is extracted into chloroform and the chloroform solution washed with water, with 2 *N* ammonia and finally with water until the washings are neutral. The chloroform is removed *in vacuo* and the porphyrin ester crystallized from a methanol–chloroform mixture. Traces of unesterified porphyrin, though they should be extracted by ammonia, sometimes persist. Free porphyrins do not move from the origin in paper chromatography under some conditions (see Section 3.a.2) and may thus be detected, as may also partly esterified porphyrins, which are sometimes soluble in chloroform. The methyl esters may be prepared also very conveniently, by reaction with diazomethane in ethereal solution.

The esters are saponified by standing at room temperature for about 40 hours in 7 *N* HCl.

(e) *Detection and determination*

1. *Spectra*

Some extinction coefficients for the more common compounds are given in Table II.

The spectrum of most common porphyrins in neutral solvents consists of four relatively sharps bands, increasing stepwise in intensity from band I, in the region of 620 mμ, to band IV, in the region of 500 mμ. Between bands I and II a very small band, Ia, occurs in some porphyrins. This type of spectrum in the visible region is called aetio-type. In addition, a band much more intense than band IV is found in the region of 400 mμ; this is the "Soret" band, characteristic of all conjugated

TABLE II

a. POSITIONS AND EXTINCTION COEFFICIENTS OF SORET BANDS

In chloroform Porphyrin ester	*Soret max. (mμ)*	$10^{-5}\varepsilon_M$	*Mol. wt.*
Uroporphyrin III octamethyl ester	405–6	2.17	942
Coproporphyrin III tetramethyl ester	399.5	1.80	710
Deuteroporphyrin dimethyl ester	399	1.75	538
Protoporphyrin dimethyl ester	407.5	1.71	590

In aqueous HCl Porphyrin	*Concn. of HCl (N)*	*Soret max. (mμ)*	$10^{-5}\varepsilon_M$	*Mol. wt.*
Uroporphyrin III	0.5	405.5	5.41	830
Coproporphyrin III	0.1	399.5	4.89	654
Deuteroporphyrin	0.1	398	4.33	510
Protoporphyrin	1.37	408	2.75	562

The values of λ and ε for uro- and copro-porphyrins I are identical to those of the corresponding isomers of series III. The data quoted are from RIMINGTON[23], who gives also correction factors, for use when porphyrins in tissue extracts are determined by measurement at the Soret band. The position and intensity of the Soret band vary with HCl concentration[24,25].

b. POSITIONS AND EXTINCTION COEFFICIENTS OF BANDS IN THE VISIBLE REGION (NEUTRAL SPECTRA)

Porphyrin	*Band: I*		*Ia*		*II*		*III*		*IV*	
	λ_{max} (mμ)	$10^{-4}\varepsilon_M$	λ_{max} (mμ)	$10^{-4}\varepsilon_M$	λ_{max} (mμ)	$10^{-4}\varepsilon_M$	λ_{max} (mμ)	$10^{-4}\varepsilon_M$	λ_{max} (mμ)	$10^{-4}\varepsilon_M$
Uroporphyrin	624	0.412	596	0.137	569	0.711	532	0.957	499	1.567
Coproporphyrin	621	0.515	595	0.132	567	0.672	529	0.997	497	1.470
Deuteroporphyrin	618	0.433	593	0.129	565	0.680	525	0.859	495	1.595
Protoporphyrin	630	0.558	603	0.141	575	0.678	537	1.158	503	1.464
Mesoporphyrin	620	0.541	594	0.133	567	0.659	528	0.982	496	1.424

The values given, for the methyl esters in dioxan solution, are from the papers of STERN AND WENDERLEIN (*cf.*[26]). For use with porphyrins dioxan must be purified[26].

tetrapyrroles, but lacking when the conjugation is broken as in the bile pigments.

Solutions of porphyrins in aqueous mineral acids have two bands in the visible region, characteristic of the porphyrin dication. The Soret band also is present, its maximum being found at longer wavelengths as the proton concentration increases[24,25].

Complexes of porphyrins with metals such as Ni, Co, Cu, have a Soret and two visible bands, with intensities I>II, while in complexes such as those with Zn, Cd, Mg, the relative intensities are reversed (*cf.* ref.[27]).

2. *Fluorescence*

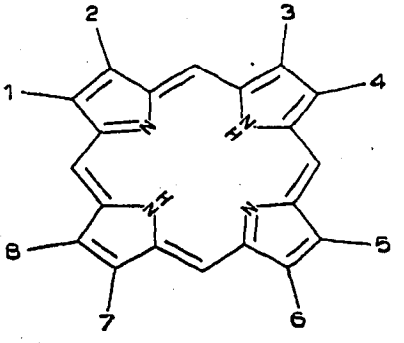
On irradiation with light at about 400 mμ (*e.g.* u.v. light filtered through Woods glass) porphyrins have a characteristic, very intense orange to red fluorescence. The fluorescence is quenched by some organic solvents, and by a variety of biological

compounds, and thus for quantitative determinations strict control of the conditions is necessary. The fluorescence is best observed in aqueous HCl solutions, in which less than 0.1 μg/ml is quite visible to the eye; very much lower concentrations are detectable by fluorimetry[28]. Metal complexes such as those with Cd, Zn, Mg fluoresce in some solvents, but the Co, Ni, Fe, Cu complexes, for example do not[5,27].

The detection[48] of spots on paper containing as little as 0.005 μg of porphyrins and 0.04 μg of non-fluorescent metallo-porphyrins, is described in Section 3.a.2, and the detection[82] of $3 \cdot 10^{-4}$ μg of haemins, by the use of a special benzidine spray, in Section 4.a.

TABLE III

THE SIDE-CHAINS OF SOME COMMON PORPHYRINS



	Side-chains							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Aetioporphyrin I	M	E	M	E	M	E	M	E
Coproporphyrin I	M	P	M	P	M	P	M	P
Coproporphyrin II	M	P	P	M	M	P	P	M
Coproporphyrin III	M	P	M	P	M	P	P	M
Coproporphyrin IV	P	M	M	P	M	P	P	M
Uroporphyrin I	A	P	A	P	A	P	A	P
Uroporphyrin III	A	P	A	P	A	P	P	A
Protoporphyrin IX	M	V	M	V	M	P	P	M
Deuteroporphyrin IX	M	H	M	H	M	P	P	M
Haematoporphyrin IX	M	B	M	B	M	P	P	M
Mesoporphyrin IX	M	E	M	E	M	P	P	M

Side-chain abbreviations: A = $-CH_2COOH$; B = $-CH(OH)CH_3$; H = $-H$; M = $-CH_3$; P = $-CH_2CH_2COOH$; V = $-CH=CH_2$.

The structures of many other porphyrins are given by FISCHER AND ORTH[7] and of chlorophyll derivatives by FISCHER AND STERN[8].

3. CHROMATOGRAPHY OF PORPHYRINS

There exist now a variety of methods for column- and paper-chromatography of porphyrins and haems. In some methods the free porphyrins are used, and in some the porphyrin esters. Column chromatograms serve most commonly for separations on the preparative scale, and paper chromatograms for identifications; the capacity of paper for porphyrin separations is rather low—spots of from 0.005 to 0.5 μg are

usually employed and are quite easily visible under ultraviolet light filtered through Wood's glass.

(a) *Paper chromatography*

1. *Free porphyrins*

One method of basic importance is the separation of free porphyrins in lutidine–water mixtures, when their R_F's bear a more or less linear relationship to the number of carboxylic acid side-chains they possess. The method, which was introduced by NICHOLAS AND RIMINGTON[29] in 1949, may be used with crude materials (blood fluids, urine, tissue and plant extracts; FALK, unpublished observations) and is thus useful for preliminary surveys of porphyrin content, though for more critical identifications isolation and some purification of the porphyrins is required. The solvent system used originally by NICHOLAS AND RIMINGTON[29] was the organic phase obtained by saturating a mixture of 2,4- and 2,5-lutidines (dimethylpyridines) with water at 21°, and the atmosphere was saturated with ammonia vapour (but see ref.[30]). The virtually linear relationship of R_F to the number of carboxyl groups in a porphyrin is illustrated in Fig. 1a. 2,4-Lutidine has been used similarly[33,34].

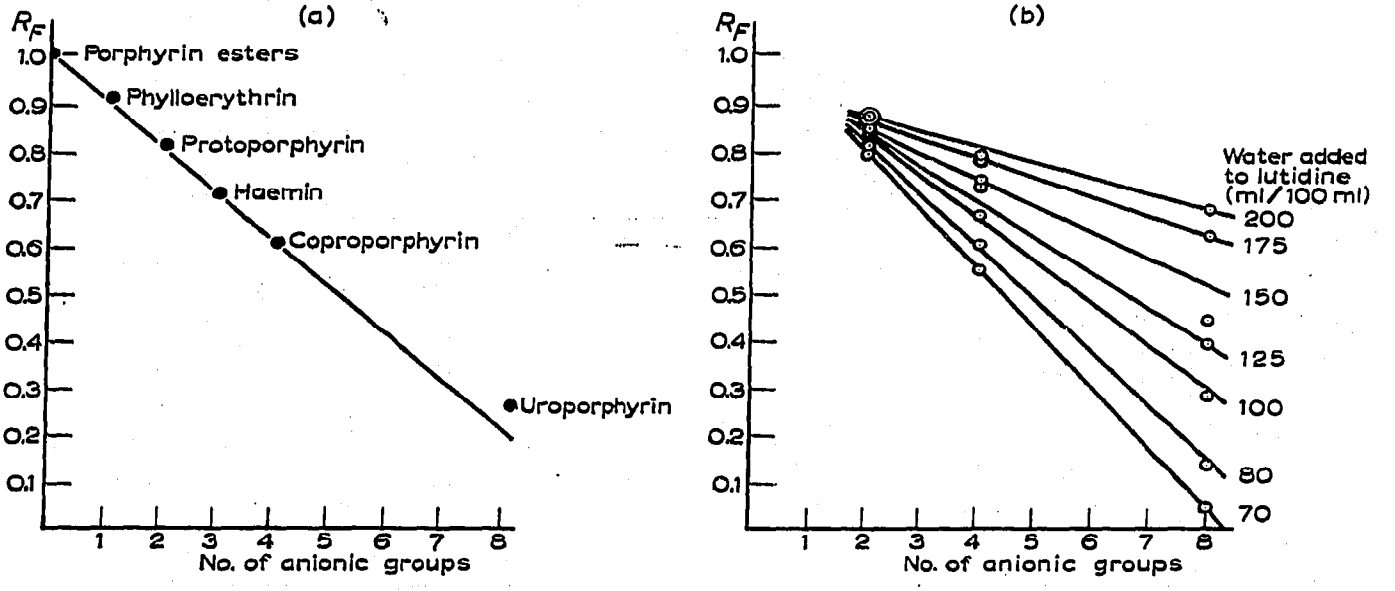


Fig. 1. Paper chromatography with 2,4-,2,5-lutidine–water systems. (a) Relationship between R_F and number of dissociable anionic groups per molecule, at 21°. (b) Effect of quantity of water present upon R_F, at 5°.

NICHOLAS AND RIMINGTON[30] have studied the effect of water content in this developing solvent, at a temperature (5°) below that at which 2,4-, 2,5-lutidine becomes miscible with water (17°). The relationship of R_F to number of carboxyl groups holds good over a wide range of water concentrations, R_F decreasing as water concentration decreases (Fig. 1b).

For the use of this method at temperatures around 20°, well-controlled constant temperature rooms are essential, since the solubility of water in the lutidine phase is very temperature-sensitive. This difficulty was overcome by KEHL AND STICH[31]

TABLE IV

R_F VALUES IN 2,4-, AND 2,4-,2,5-LUTIDINE–WATER SYSTEMS

	R_F*	R_F**
Uroporphyrin	0.3	0.19
Coproporphyrin	0.6	0.47
Haemin	0.68	0.63
Protoporphyrin	0.8	0.75
Haematoporphyrin	0.8	0.77
Deuteroporphyrin	0.8	0.79
Mesoporphyrin	0.8	0.81
Phylloerythrin	0.8	—
Porphyrin esters	1.0	1.0

* NICHOLAS AND RIMINGTON[29], 2,4-,2,5-lutidine–water, Whatman No. 1 paper, 19°, descending, NH_3 vapour (but *cf.* ref. [30]).
** KEHL AND STICH[33], 2,4-lutidine–water, Schleicher & Schüll paper 2043b, 15°, ascending, NH_3 vapour.

who in 1951 introduced the use of 2,6-lutidine, which is miscible with water up to 40°. The method was studied further by ERIKSEN[32]; ascending chromatograms were used, at 21°, with NH_3 vapour in the atmosphere, and the solvent was a mixture of 5 vols. of 2,6-lutidine with 3 vols. of water. The R_F values found by KEHL AND STICH and by ERIKSEN are shown in Table V.

TABLE V

R_F VALUES IN 2,6-LUTIDINE–WATER SYSTEMS

	R_F*	R_F**
Uroporphyrin	0.26	0.06
Pentacarboxylic porphyrin	—	0.42
Coproporphyrin	0.54	0.56
Haemin	0.7	—
Tricarboxylic porphyrin	—	0.68
Protoporphyrin	0.84	0.83
Mesoporphyrin	0.86	—
Haematoporphyrin	0.87	—
Deuteroporphyrin	0.88	—
Aetioporphyrin	1.0	—
Porphyrin esters	1.0	—

* KEHL AND STICH[31], Schleicher & Schüll paper 2043b, 25°, ascending, NH_3 vapour.
** ERIKSEN[32], Whatman No. 1 paper, ~ 20°, ascending, NH_3 vapour.

It was found by FALK AND BENSON[49] that the 2,6-lutidine method, in addition to separating porphyrins in accordance with the number of carboxyl groups, is capable of separating mixtures of the coproporphyrin isomers (Fig. 2), and it has been used for this purpose[1,14,35,36]. The method has been modified further by ERIKSEN[37]. Isomers I and III, which occur in nature, and isomer II are well separated. Isomer IV which, like isomer II, is not known to occur in nature, has an R_F identical to that of isomer III.

Similar isomer-separation was then found with the NICHOLAS AND RIMINGTON method; these separations, though intrinsically very useful, of course preclude strict interpolation of the R_F's of unknown porphyrins in the curve of R_F against number of carboxyl groups. Happily, the isomers of uroporphyrins I and III do not separate under these conditions, and isomers of dicarboxylic (proto-, deutero-) and of tricarboxylic porphyrins, other than those related to coproporphyrin III, are not known

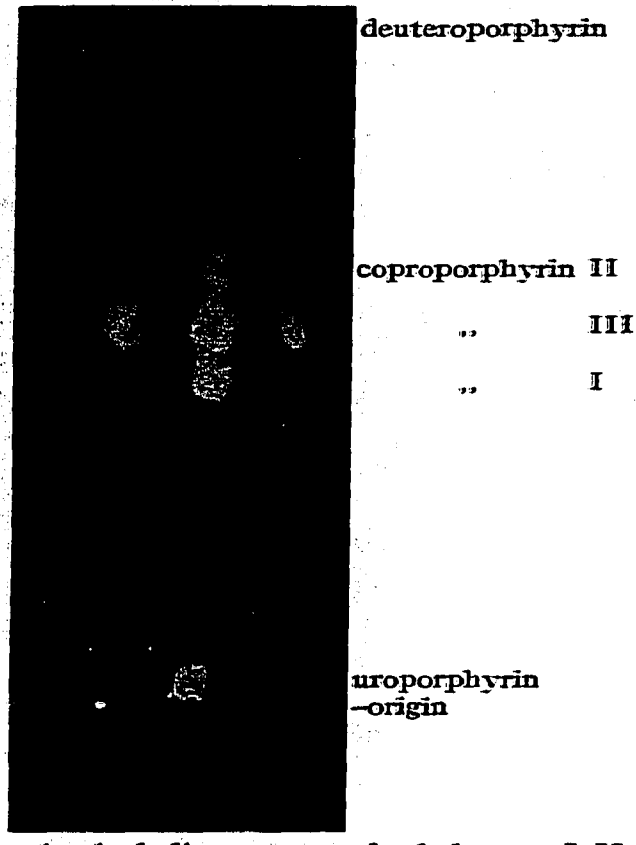


Fig. 2. Separation of porphyrins, including coproporphyrin isomers I, II and III, by 2,6-lutidine[14]. A photograph of a chromatogram under u.v. light.

to occur in nature. The region of the chromatograms in which interpretations must be made with most caution is thus where R_F values appear to correspond to penta-, hexa- or heptacarboxyl porphyrins; the identification of isomers among these porphyrins is discussed in Sections 3.a.2 and 3.b, below.

The paper used most widely for these separations has been Whatman No. 1; a number of investigators in Europe have found that Schleicher and Schüll paper 2043b gives similar results. Spots containing about 0.1 to 0.3 μg of porphyrin are applied to the paper by a micropipette, usually from a solution in dilute NH_4OH, though solutions in ether or acetone are equally suitable. High salt concentrations interfere with the chromatography.

WITH[38] has used instead of lutidine–water mixtures, neutral aqueous salt solutions as developing solvents. In an ascending method, with Whatman No. 1 paper,

using 0.1 *M* LiCl as developing solvent with an atmosphere containing NH_3 vapour, he has found the R_F's shown in Table VI. The separation according to number of carboxyl groups occurs in the reverse order to that found with lutidine developments and the times required are much shorter.

TABLE VI

R_F VALUES IN A LiCl–WATER SYSTEM

	R_F*
Haematoporphyrin	0.05–0.09
Coproporphyrin	0.20–0.30
Hexacarboxylic porphyrin	0.45–0.55
Uroporphyrin	0.85–0.95

* WITH[38], Whatman No. 1 paper, 0.1 *M* LiCl, ~ 20°, ascending, NH_3 vapour.

Some other methods have been reported. Thus absolute methanol has been used as developing solvent by NISHIKAWA[39], who reported separation of coproporphyrins I and III, and that the logarithm of the concentration was proportional to the area and diameter of the porphyrin spots.

A simplified procedure, adapting the lutidine method for use with paper strips in test tubes, and using simple HCl-eluates of lead acetate precipitates of urines, has been described by CORWIN AND ORTEN[40]. Several authors have adapted the methods to radial (circular) paper chromatography, for which a very convenient apparatus has been described by RAPPOPORT, CALVERT, LOEFFLER AND GAST[41]; R_F values have been given (Table VII) for a number of chlorophyll derivatives chromatographed in this way with mixtures of acetone, benzene and hexane, by HENDRICKSON, BERUEFFY AND MCINTYRE[42].

The paper chromatographic identification of some porphyrins from a petroleum aggregate has been described by DUNNING AND CARLTON[43]. A mixture of butanol, glacial acetic acid and water (4:1:1) has been found useful by URATA AND KIMURA[44] for paper chromatographic studies of the porphyrins formed enzymically from porphobilinogen. An apparatus for paper chromatography of porphyrins has been described by KENNEDY[45]; a number of paper strips may be run simultaneously, and the positions of spots may be monitored, during development, by their fluorescence in u.v. light.

2. *Porphyrin esters*

In 1951, CHU, GREEN AND CHU[46], using the porphyrin methyl esters and development first by a chloroform–kerosene mixture, then by a mixture of kerosene and *n*-propanol, achieved the first separation of porphyrin isomers on paper. The coproporphyrins I and III separated as shown in Table VIII, and by the same method the esters of uroporphyrin I, protoporphyrin and mesoporphyrin also were separated. As in the separation of the free porhyrins by lutidine (above) there is again an approximate

TABLE VII

CHROMATOGRAPHIC CONSTANTS OF SELECTED CHLOROPHYLL DERIVATIVES*

Compound	Solvent**	R_R (3000 sec)	R_F (3000 sec)
Pheophytin *a*	10:20:70	0.966	0.933
Pheophytin *b*	10:20:70	0.855	0.731
Pheophorbide *a*	30:70	0.784	0.614
Pheophorbide *a* methyl ester	10:20:70	0.908	0.824
Pheophorbide *b*	30:70	0.570	0.324
Pheophorbide *b* methyl ester	10:20:70	0.753	0.568
Chlorin e_6	30:70	0.355	0.126
Chlorin e_6 trimethyl ester	10:20:70	0.933	0.871
Chlorin p_6 trimethyl ester	10:20:70	0.939	0.881
Cu-chlorin p_6 trimethyl ester	10:20:70	0.879	0.772
Purpurin 7*a* trimethyl ester	10:20:70	0.878	0.771
Purpurin 7*b*	30:70	0.613	0.375
Purpurin 7*b* trimethyl ester	10:20:70	0.642	0.412
Cu-purpurin 7*b* trimethyl ester	10:20:70	0.584	0.341
Purpurin 18*a*	30:70	0.755	0.569
Cu-purpurin 18*a* methyl ester	10:20:70	0.722	0.521
"Unstable" rhodin (rhodin *k*) trimethyl ester	10:20:70	0.852	0.725
Rhodin *g* trimethyl ester	10:20:70	0.771	0.595

* HENDRICKSON *et al.*[42], circular chromatography, Whatman No. 3 paper, ~23°. For definitions of R_R and R_F see original paper. For the structures of the chlorophyll derivatives referred to here and in Table IX, the original papers, and also FISCHER AND STERN[8] should be consulted.
** 10:20:70, proportions of acetone–benzene–hexane. 30:70, proportions of acetone–hexane.

TABLE VIII

R_F VALUES OF PORPHYRIN METHYL ESTERS*

	Second run, *n*-propanol with:				
	kerosene**	*n*-decane***	*n*-dodecane***	*n*-tetradecane***	*n*-hexane***
Uroporphyrin I	0.17	0.14	0.20	0.13	0.15
Coproporphyrin I	0.47	0.42	0.52	0.45	0.47
Coproporphyrin III	0.67	0.70	0.76	0.74	0.66
Protoporphyrin IX	0.84	0.86	0.92	0.92	0.89
Mesoporphyrin IX	0.89	0.92	0.96	0.95	0.93

Whatman No. 1 paper, 24°, ascending; the paper was dried at 105–110° for about 4 min after completion of the first run. The atmosphere was saturated with the same solvents as used for development.
* CHU *et al.*[46].
** First run, chloroform–kerosene, 2.6:4.0.
*** First run, chloroform with the same alkane as used in the second run. Propanol–alkane, 1:5.

inverse relationship between R_F and the number of carboxyl groups present. In place of kerosene, *n*-decane, *n*-dodecane, *n*-tetradecane and *n*-hexadecane were all found to effect satisfactory separations, but the intensity of fluorescence of the spots was found to be less than when kerosene was used. It was found that isopropanol may be used in place of the *n*-propanol, that changes in temperature affect the R_F values

but not the relative sequence of R_F's of the different porphyrin esters, and that the porphyrin free acids do not move with these organic developing solvents. The papers were not dried before use, and in the writer's experience it is not necessary to dehydrate the solvents rigorously—ordinary distilled, pure solvents are suitable. It is thus possible that the separations involve both partition on the hydrated cellulose and adsorption. CHU *et al.* used Whatman No. 1 paper; the method has been found[47] to work similarly with paper 2043b of Schleicher & Schüll.

This method has been modified by BLUMER[48], for the purpose of studying the porphyrin compounds occurring in petroleum and in sedimentary rocks. BLUMER found that using Whatman No. 3 paper, running with the grain, and developing with a mixture of iso-octane (3 vols.) and carbon tetrachloride (7 vols.), the preliminary development introduced by CHU *et al.* was not necessary for good resolution. Porphyrin esters and porphyrin metal complexes, and porphyrins lacking carboxyl groups (*e.g.* aetioporphyrin) all move away from the origin and separate adequately. Porphyrins with free carboxylic acid groups (*e.g.* proto-, meso-porphyrins) remain at the origin; this spot may be esterified on the paper with diazomethane, and then developed in the second dimension with the same solvent. Some R_F values found by BLUMER are shown in Table IX. It was found also that spraying the completed

TABLE IX

R_F VALUES OF FREE PORPHYRINS, THEIR METAL COMPLEXES AND METHYL ESTERS*

	R_F
Aetioporphyrin III	0.63
Deoxophylloerythroaetioporphyrin–nickel	0.54
Deoxophylloerythroaetioporphyrin	0.47
Deoxophylloerythroaetioporphyrin–vanadium	0.20
Deoxophylloerythrin ester–nickel	0.23
Deoxophylloerythrin ester	0.15
Deoxophylloerythrin ester–vanadium	0.08
Mesoporphyrin ester–nickel	0.12
Mesoporphyrin ester	0.10
Protoporphyrin ester–copper	0.13
Protoporphyrin ester–nickel	0.10
Protoporphyrin ester	0.07

* BLUMER[48]; horizontal development on Whatman No. 3 paper, with carbon tetrachloride–iso-octane, 70:30.
See also footnote* to Table VII.

chromatograms with iso-octane before observation under u.v. light increased markedly the sensitivity of the fluorescence, 0.005 μg being observable. For spots of non-fluorescent porphyrin metal complexes, it was found that on spraying with a solution of fluoranthene in *n*-pentane, and then illuminating with light at 366 mμ, the porphyrins and their metal complexes showed as dark spots against a fluorescent background, about 0.04 μg being observable. The maximum amount of porphyrin or metal complex that could be chromatographed without tailing was 0.5 μg. It was found that with care, determinations by measuring the area of spots could be made to

an accuracy of ± 10%, and a calibration curve for the determination of mesoporphyrin in this way is given.

FALK AND BENSON[49] found that isomers I and III of the uroporphyrins (methyl esters) did not separate under the conditions of CHU *et al.*[46], and introduced the use of dioxan for this purpose. Again a double development, kerosene–chloroform followed by kerosene–dioxan, with Whatman No. 1 paper at 22°, was used. As far as the uroporphyrins are concerned, the main function of the preliminary development is to move the esters away from the origin, leaving behind both impurities and any non-esterified materials; the paper is cut off above this level before the second development. This procedure, a single development with kerosene–chloroform, serves incidentally as a convenient test for the presence of unesterified porphyrins in preparations of the esters. Separation by the dioxan method of mixtures of uroporphyrins I and III permits a certain assessment of the proportions of the isomers present (see Fig. 2 of ref.[49]); it was apparent, however, that uroporphyrin I tends to be carried along with isomer III when considerable proportions of the latter are present. It has been found[50] that the resolution is much improved if the amount of porphyrin ester analysed does not exceed about 0.3 μg. Using approximately 1:1 mixtures of ^{14}C-labelled uroporphyrins I and III, BOGORAD AND MARKS[51] have found that not only is uroporphyrin I entrained with uroporphyrin III, but that isomer III is held up in the spot of isomer I, though when chromatographed separately the two isomers moved cleanly to their respective R_F positions. There has long been evidence for some kind of "molecular compound" formation between porphyrins of this type[16], and some controversies in the literature have resulted. While entrainment in paper chromatograms of a slower-moving substance in the spot of a faster-moving one is not uncommon, the virtually equal partition between the two positions found by BOGORAD AND MARKS appears to be a new phenomenon. The method of FALK AND BENSON[49] has been used widely and successfully, but there is real need for a new method which does not suffer from this complication. In addition, there is need for a method which separates the uroporphyrin isomers II and IV; under the conditions described above, isomers I and II have identical R_F values, as do isomers III and IV, and no conditions have yet been found to separate them. Though it is unlikely that isomers III and IV should occur in nature, this is not impossible (*cf.* ref.[52]).

The kerosene–dioxan chromatograms have proved valuable for the study of a porphyrin which may be important for the elucidation of the biosynthetic pathway[14,53], and which has been called *pseudo*uroporphyrin because of its chemical similarity to a uroporphyrin[14,36]. Chromatographically, it differs from all four uroporphyrin isomers; further decarboxylation of it led to coproporphyrin III, thus establishing its isomeric form[36]. A porphyrin behaving similarly, and occurring in certain porphyric urines, has been studied by CANIVET AND RIMINGTON[54], and another by GRINSTEIN, SCHWARTZ AND WATSON[55]. The methyl ester of the latter has a melting point of 208°, and chromatography has shown that it differs from *pseudo*uroporphyrin[36,56]. A recent study[57] suggests that these porphyrins may be mixtures of isomeric heptacarboxylic porphyrins.

3. *Dicarboxylic porphyrins*

While most of the methods described above separate porphyrins or their esters according to the number of carboxyl side-chains they possess, they do not effect very useful separations from each other of the common dicarboxylic compounds such as meso-, proto-, haemato- and deutero-porphyrins. A method which serves this purpose, found by CHU AND CHU[58], involves development of the porphyrin esters in a first direction, against the grain of Whatman No. 1 paper, with a mixture of kerosene, tetrahydropyran and methyl benzoate. The paper is then dried, treated with silicone in petroleum ether solution, dried again, and developed in the second direction with a mixture of water, acetonitrile, *n*-propanol and pyridine. The second development is "reverse phase" chromatography, silicone serving as the stationary phase and the atmosphere being saturated with water vapour. For some purposes, the second development may be carried out with a mixture of water, acetonitrile and dioxan. Very good separations of a number of dicarboxylic porphyrins were effected, as shown in Table X.

TABLE X

R_F VALUES OF THE METHYL ESTERS OF SOME DICARBOXYLIC PORPHYRINS*

Methyl esters of	R_F			*Minimal detectable amount*	
	*KTM***	*WAPP****	*WAD*†	*Two-dimensional development* μg	*Single development* μg
Haematoporphyrin IX dimethyl ether	0.59	0.63	0.46		
Deuteroporphyrin IX	0.59	0.45	0.46	0.01	0.005
Mesoporphyrin IX	0.59	0.23	0.23	0.01	0.005
Protoporphyrin IX	0.51	0.23	0.24	0.02	0.01
Monovinylmonohydroxyethyl-deuteroporphyrin IX	0.20	0.74	0.61		
Haematoporphyrin IX	0.03	0.80	0.71	0.005	0.003

* CHU AND CHU[58], Whatman No. 1 paper, 22°, ascending. For vapour phases, see original paper.
** KTM, 5:1.4:0.35, kerosene–tetrahydropyran–methyl benzoate.
*** WAPP, 3.8:1:2:0.5, water–acetonitrile–*n*-propyl alcohol–pyridine (with silicone as stationary phase).
† WAD, 2.3:2.8:0.8, water–acetonitrile–dioxan (with silicone as stationary phase).

4. *Porphyrins with hydroxyl groups in the side-chains*

This method was devised by BARRETT[59] for studies of porphyrin *a* and chlorin a_2, obtained by removal of iron from the prosthetic groups of the cytochromes *a* and a_2 respectively, and its use revealed that each of these porphyrins contains one side-chain with a hydroxyl group. The methyl esters of the porphyrins, and the same compounds after acetylation, were chromatographed by the method of CHU, GREEN AND CHU[46]. As may be seen in Table XI, the differences in R_F between free hydroxyl compounds and their acetylation products give some indication of the number of hydroxyl side-chains.

TABLE XI

R_F VALUES OF HYDROXYLATED PORPHYRINS AND CHLORINS, AND THEIR ACETYLATED PRODUCTS*

	Chloroform–kerosene, 2.6: 4		*Propanol-kerosene, 1: 5*	
	Alcohol	*Acetate*	*Alcohol*	*Acetate*
Monohydroxyethyl deuteroporphyrin	0.34	0.64	0.38	0.68
Monovinyl-monohydroxyethyl deuteroporphyrin	0.29	0.60	0.34	0.66
Haematoporphyrin	0.03	0.56	0.18	0.57
Monohydroxymethyl deuteroporphyrin	0.19	0.54	0.24	0.57
Monohydroxymethyl monovinyl deuteroporphyrin	0.22	0.54	0.31	0.56
Dihydroxymethyl deuteroporphyrin	0.01	0.56	0.14	0.56
2-Formyl-4-hydroxyethyl deuteroporphyrin	0.16	0.58	0.26	0.64
Porphyrin *a*	0.10	0.56	0.26	0.62
2-Ethyleneglycol deuteroporphyrin	0.30	0.64	0.32	0.61
2-α-Hydroxymesorhodochlorin	0.31	0.80	0.34	0.78
2-α-Hydroxymesochlorin p_6	0.33	0.82	0.35	0.80
2-α-Hydroxymesophaeophorbide *a*	0.10	0.78	0.34	0.72
Chlorin a_2	0.30	0.65	0.40	0.60

* BARRETT[59]; chromatography by the method of CHU *et al.*[46]. R_F values for some further chlorins are given by BARRETT[60].

(*b*) *Column chromatography*

Talc columns were used for purification of porphyrin esters by FISCHER AND HOFMANN[61], and for free porphyrins by COMFORT[62], who used aqueous HCl for the elution.

Columns of Al_2O_3 were used with some success for the separation of porphyrin esters by WALDENSTRÖM[63], PRUNTY[64], LEDERER AND TIXIER[65] and MCSWINEY, NICHOLAS AND PRUNTY[66], while $CaCO_3$ columns were used by GRINSTEIN, SCHWARTZ AND WATSON[55], WATSON, SCHWARTZ AND HAWKINSON[67] and GRAY AND HOLT[68].

The technique was studied systematically by NICHOLAS[69] in 1951. The adsorbants Al_2O_3, MgO, $MgCO_3$ and $CaCO_3$ were each prepared in several grades of adsorptive power, classified by the method of BROCKMANN AND SCHODDER[70] (*cf.* WILLIAMS [71]). The separation upon such columns of the methyl esters of uroporphyrins, coproporphyrins, protoporphyrin and some other dicarboxylic porphyrins was shown to be reproducible provided that the conditions are standardized. As in paper chromatography, porphyrins separate on columns in order of the number of carboxyl side-chains they possess. Thus on Al_2O_3 or $CaCO_3$, the ease of elution decreases from proto- to copro- to uroporphyrin (2-, 4-, 8-carboxyl groups respectively). This order is reversed on MgO or $MgCO_3$ columns. It is very convenient in practice to have the choice of reversal of order of elution; thus with a mixture of porphyrins, it is convenient to choose for the first separation that column from which the porphyrin(s) required pure are eluted easily, leaving others behind; the fractions so obtained are then purified separately on columns on which they are more strongly adsorbed, so that traces of easier-eluted impurities may be removed. A wide choice of organic solvent systems is available for the different columns, as indicated in Fig. 3.

Columns of $CaCO_3$ were used by GRINSTEIN *et al.*[55] for the isolation of the porphyrins from pathological urines, including their "208" porphyrin. The same sample of

$CaCO_3$ (kindly made available by Dr. C. J. WATSON) allowed the separation from uroporphyrin III of the *pseudo*uroporphyrin of DRESEL AND FALK[53], but no other samples tried were effective. The constitution of these two porphyrins has been discussed in Section 3.a.2.

Calcium carbonate columns, developed with benzene, are used also in a standard purification of coproporphyrin III tetramethyl ester[72].

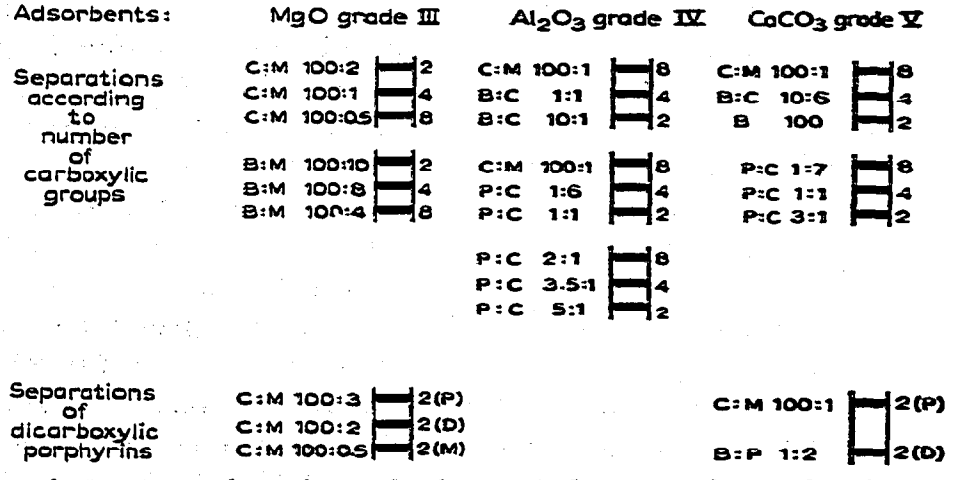


Fig. 3. Column chromatography of porphyrin methyl esters, from the data of NICHOLAS[69]. *Abbreviations.* Solvents: B, benzene; C, chloroform; M, methanol; P, light petroleum. Porphyrins: 8, 4, 2 represent the octa-, tetra- and di-carboxylic uro-, copro- and proto-porphyrins respectively. P, D and M represent the dicarboxylic proto-, deutero- and meso-porphyrins.

It was found by NICHOLAS[69] that the esters of dicarboxylic porphyrins may be separated from each other either on MgO grade III or on $CaCO_3$ grade V, the ease of elution increasing from proto- to deutero- to meso-porphyrin (Fig. 3).

Preparations of porphyrin esters are often contaminated by small amounts of the free (non-esterified) porphyrins, which remain at the top of columns developed with these organic solvents and may be recovered and re-esterified.

It has been found by the writer that metal complexes of the porphyrin esters may be purified conveniently on columns of Al_2O_3 or of $MgCO_3$, on which they are readily separated from metal-free porphyrins. Certain metal complexes, however (*e.g.* Zn, Cd) lose their metal on Al_2O_3 columns (*cf.* ref.[27]).

LEMBERG AND PARKER[73] have reported the separation, on columns of BDH "alumina for chromatographic separation", of proto- (2,4-divinyl-), chlorocruoro- (2-formyl-4-vinyl-) and 2,4-diformyl-deuteroporphyrins, and of the very closely related 2- and 4-monoformyl-deuteroporphyrins. The columns were packed as a slurry in a mixture of equal volumes of chloroform and ether, and the same mixture used for development.

Separations on the above columns are clearly due to adsorptive forces. A method of partition chromatography, in which porphyrin esters are chromatographed on columns of hydrated silica gel, with development by mixtures of chloroform and ligroin, has been worked out by LUCAS AND ORTEN[74]. When 1 mg of a mixture con-

taining equal amounts of the methyl esters of proto-, meso-, copro- and uro-porphyrins was applied to a column made from 1 g of silica gel, complete elution of the protoporphyrin was achieved with 100 ml of ligroin containing 20% (v/v) of chloroform. A further 50 ml of the same mixture then removed the mesoporphyrin; 100 ml of 30% chloroform in ligroin eluted the coproporphyrin, and finally 100 ml of 50% chloroform in ligroin eluted the uroporphyrin.

The order of elution is similar to that described by NICHOLAS[69] (see above) for columns of Al_2O_3 or $CaCO_3$, and again unesterified material remains at the top of the column. The silica gel columns have the advantage, however, of much higher capacity than the adsorption columns; for chromatography of 1 mg of a porphyrin mixture on the latter, a column size of the order of 2 × 20 cm at least, is required.

Silica gel columns, and also cellulose powder columns developed with ether, petroleum ether and acetone, have been used successfully by LEMBERG AND STEWART[75] for the purification of porphyrin *a* from heart muscle.

None of the methods of column chromatography so far described is capable of separating mixtures of porphyrin isomers. Several such separations claimed in the earlier literature have not been confirmed, but recently CHU AND CHU[50,76] have found that on columns of celite (Hyflo supercel) not only may porphyrin esters be separated in order of their carboxyl groups but also, under appropriate conditions, uroporphyrins I and III, and also penta- hexa- and hepta-carboxylic porphyrins of isomer-types I and III may be separated.

For the preliminary separation according to carboxyl groups, a solution of about 0.5 g of a mixture of the porphyrin esters is mixed with a little supercel and dried; this is then packed on the top of a column of supercel of dimensions 1.8 × 14 cm. Development is carried out, with suction, with a mixture of 1 vol. of chloroform and 2 vols. of petroleum ether (b.p. 30°–60°). The fluorescent zones are then removed by spatula from the column and analysed by paper chromatography. The lower the number of carboxyl groups, the faster the migration. For the isomer-separation, a column of about 3 × 45 cm of supercel is used for 2 to 3 mg of porphyrin ester mixture; the column is developed with benzene, then with a mixture of chloroform (1 vol.) and benzene (2 vols.) with increasing additions of ethanol, until the porphyrin front almost reaches the bottom of the column. The fluorescent zone is then removed by spatula in equal arbitrary fractions, the porphyrin eluted from the supercel with chloroform, and analysed by paper chromatography by the method of FALK AND BENSON[49] with some modifications[50]. Pure uroporphyrin III was found towards the top of the column, and pure uroporphyrin I at the bottom, separated by a graded mixture of the two isomers. The purity of the uroporphyrins I and III so obtained was confirmed[77] by their melting points, by melting points and chromatography (CHU *et al.*[46]) of the coproporphyrins obtained from them by decarboxylation and by the use of infra-red spectra.

As has long been evident (see refs. 1–12 of ref.[78]) porphyrins with 3, 5, 6 and 7 apparent carboxyl groups occur in various pathological materials as well as in biosynthetic preparations. CHU AND CHU[57,77,78] have been able to prepare, by stepwise

decarboxylation of uroporphyrins I and III, reference samples of hepta-, hexa- and penta-carboxylic porphyrins of both isomeric types. Natural materials isolated from the urine of patients with porphyria cutanea tarda have been compared with the reference substances in respect to behaviour on the supercel columns, paper chromatography, melting points and infra-red spectra, all the evidence confirming the existence in the natural samples of isomeric types I and III in these "intermediate" porphyrins.

Cellulose powder columns have been used by ERIKSEN[79] for the large-scale separation of porphyrins. A mixture of uro-, copro- and meso-porphyrins, as the free acids, was dissolved in a small volume of acetone containing NH_4OH; the solution was mixed with a little cellulose powder (Whatman "for Chromatography") and dried in a desiccator. The dry powder was packed on top of a 15 cm column of the same cellulose powder, packed dry by tamping. Development by 2,6-lutidine–water (6:2) separated the porphyrins with the approximate R_F values: meso-, 0.95; copro-, 0.53; uro-, 0. Further development with the same solvent, under slight positive pressure, caused elution of the mesoporphyrin. On development now with 2,6-lutidine–water (6:4) the coproporphyrin was eluted, and the uroporphyrin was finally eluted as a narrow, concentrated band, when a drop of concentrated NH_4OH was added to 25 ml of the same solvent. The behaviour of the porphyrins and the R_F values found on these columns are very similar to those found on paper chromatography with similar solvents (see above). Attemps at isomer-separation on such columns have not been reported.

4. CHROMATOGRAPHY OF IRON COMPLEXES OF PORPHYRINS (HAEMS)

(a) *Paper chromatography*

CHU AND CHU[80] have described methods for the separation of haemins or their methyl esters, in reverse phase systems in which Whatman No. 1 paper coated with silicone furnishes the stationary phase. A number of solvent systems were found to be useful (Table XII), the most interesting involving development of the free haemins with a mixture of water, *n*-propanol and pyridine. This system gave good separation of uro-, copro- and proto-porphyrins according to the number of carboxyl groups, and in addition separated from each other the dicarboxylic haemato-, deutero-, meso- and proto-haemins. Spots as small as 0.05 μg were detectable under u.v. light.

Using a similar silicone-treated Whatman No. 1 paper, MORRISON AND STOTZ[81] were able to separate protoporphyrin, its mono-and di-methyl esters, haem *a* and haematohaem *c*. The two latter were prepared from the prosthetic groups of cytochromes *a* and *c* respectively, and each separated, on the paper chromatograms, into two components. The best solvent system of the four mixtures used by these authors appears to have been a mixture of chloroform, hexane and formic acid, though they found basic solvents (*e.g.* pyridine–water or pyridine–isopropanol–ammonia) to be useful for particular purposes. In 1958, CONNELLY, MORRISON AND STOTZ[82] reported

the use of several other solvent systems for similar separations, a mixture of 8 vols. of glacial acetic acid in 100 vols. of water-saturated toluene having particularly good resolving power for heart muscle haemins.

TABLE XII

R_F VALUES OF HAEMINS*

Haemin	R_F**	R_F***
Proto-	0.34	0.76
Meso-	0.45	0.77
Deutero-	0.62	0.76
Haemato-	0.72	0.77
Copro-	0.88	0.56
Uro-	0.96	0.20

* CHU AND CHU[80], Whatman No. 1 paper treated with silicone, 22°, ascending.
** Developing solvent mixture: water–*n*-propanol–pyridine, 5.5:0.1:0.4; atmosphere, water and pyridine vapours.
*** Developing solvent mixture: 2,6-lutidine–water, 3.3:2.7; atmosphere, water vapour.

By the use of a special benzidine spray, CONNELLY *et al.*[82] were able to detect as little as $3 \cdot 10^{-4}$ μg of haemin. The spray is made up, not more than 3 hours before use, as follows: 25 ml of absolute methanol are shaken for 1 min with excess of benzidine hydrochloride, the solution is decanted, and to it is added 12.5 ml of water, 5.0 ml of glacial acetic acid, 2.5 ml of 3% hydrogen peroxide and 0.5 ml of pyridine.

(*b*) *Column chromatography*

KIESE AND KURZ[83] were able to separate the haemin *a* of heart muscle from protohaemin on columns of Al_2O_3. For the separation of heart-muscle haemins, MORRISON AND STOTZ[84] used partition chromatography on silicic acid columns. After washing, the silicic acid was mixed with 0.15 *N* HCl, chloroform added, and a column packed from the slurry. The haemins were applied in chloroform–hexane solution and the columns developed with this mixture to remove lipides before elution of the haems with chloroform.

For his purification of haem a_2, the iron-chlorin prosthetic group of bacterial cytochrome a_2, BARRETT[60] used silica gel columns. The following procedure was found to leave the particles of the gel well dispersed, giving smoothly working columns: 9 g of silica gel were shaken, under N_2, with 4 ml of a mixture of 7 vols. of methanol with 3 vols. of water. The silica gel particles were then suspended in 60 ml of petroleum ether (b.p. 68°), and a column packed from the slurry. The haemins were applied to the column in benzene solution, lipides were eluted with petroleum ether (b.p. 68°), and development followed by petroleum ether equilibrated with an equal volume of a mixture of 7 vols. of methanol with 3 vols. of water, then by wet benzene to which increasing amounts of methanol were added. The green haem a_2 was eluted when the methanol concentration was 0.5 to 1.0%.

5. ELECTROPHORESIS OF PORPHYRINS

Electrophoresis has been applied to porphyrins both on paper and on other supporting media, by several investigators. While both methods are capable of separating porphyrin free acids with more than 3 carboxyl groups from each other, the dicarboxylic porphyrins are not separated, and no isomer-separations have been achieved.

(*a*) *Media other than paper*

The first report of the application of this method to the separation of porphyrins appears to be that of KENCH AND PAPASTAMATIS[85], who used agar gel as the supporting medium with 0.06 *M* phosphate buffer of pH 8.0 and 4 V/cm. Uro- copro- and proto-porphyrins were found to separate, and 60 to 80% recoveries were obtained at the 1.0 μg level. No separation was found of uro- or copro-porphyrin isomers I and III, or of the dicarboxylic deutero-, meso- and protoporphyrins.

ERIKSEN[86] has tried a number of supporting media, including starch paste, starch gel, paper powder and glass powder, all of which were found to be inferior to agar gel. Troughs 50 × 18 × 1.5 cm were used, with 0.5 or 1.0% agar gel, prepared in veronal buffer of pH 8.6 and ionic strength 0.05. The porphyrins were applied in highly concentrated solution in small holes in the gel, and electrophoresis was carried out at 4°; for a film thickness of 2 to 5 mm, 6 V/cm were applied, and separation was complete after 10 to 12 hours. Excellent separation was found of octa-, hexa-, penta-, tetra- and tri-carboxylic porphyrins.

(*b*) *Paper electrophoresis*

The electrophoretic separation on paper of uro-, copro- and proto-porphyrins, as the free carboxylic acids, was studied by LARSEN, MELCER AND ORTEN[87] in 1955. Separation according to the number of carboxyl groups was achieved in veronal or borate buffers of pH 8.5. The porphyrin precursors δ-aminolaevulic acid and porphobilinogen, were separated from porphyrin mixtures in borate buffer at pH 11 and ionic strength 0.2 to 0.3, and were stable under these conditions. Isomers I and III of copro- and uro-porphyrins did not separate. In the same year HEIKEL[88] published a similar method. The method was studied further by WITH[89], who used Whatman No. 1 paper, 0.05 *M* barbiturate buffer of pH 8.6, and 7.5 to 8 V/cm for 1 to 3 hours. WITH[89] also found that no isomer-separations could be achieved by this method. The distances moved from the origin (near the anode end) under WITH's conditions were: uroporphyrin 10–15 cm, coproporphyrin 0.5–2.0 cm and protoporphyrin 0–0.3 cm.

STERLING AND REDEKER[90] used 30 × 3 cm strips of Whatman No. 3 paper with an EDTA buffer of pH 8.6, and ran for 4 h at 7 V/cm at a temperature of 5°. The distances moved were 6–6.5 cm, 0–0.2 cm and zero for uro-, copro- and proto-porphyrins respectively. Within certain stated limits the amount of porphyrin applied

did not affect the separation or the recovery. After the electrophoresis the fluorescent areas were leached from the paper with aqueous HCl solutions, and recoveries for uro-, copro- and proto-porphyrins were 96 ± 3%, 87 ± 1% and 87 ± 2.5% respectively.

WITH[91] applied the method to investigate whether the commonly-occurring porphyrins are bound to serum proteins, as is bilirubin. Electrophoresis at 400 V, 20 mA, in barbiturate buffer of pH 8.6, on Whatman No. 1 paper, showed that proto- and haemato-porphyrins, coproporphyrins I and III and uroporphyrins I and III move quite independently of the proteins of normal human serum to which they had been added. Bilirubin added to serum in the same way is immediately attached to the albumin.

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