

A STUDY OF THE EXCRETED

PORPHYRINS IN HUMAN PORPHYRIA

With particular reference to the isolation and identification of
the porphyrin-peptide complexes

by

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A Thesis

submitted to the University of Cape Town.

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This thesis is dedicated
to the memory of Chris.

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ABBREVIATIONS

EDTA	Ethylene diamine tetraacetic acid
Ca(OH)_2	Calcium hydroxide
BuOH	Butanol
Mg	Magnesium
Ker	Kerosene
CHCl_3	Chloroform
Prop	Propanol
'PPO'	2,5-diphenyl oxazole (Primary scintillant)
'POPOP'	p-bis-(2-(5-phenyl-oxazolyl) - benzene
HOAc	Glacial acetic acid
Lut	lutidine
BF_3	boron trifluoride
MeOH	methanol
aa	Amino acid
ALA	Delta aminolaevulinic acid
PBG	porphobilinogen
Ala	alanine
Asp	aspartic acid
CySO_3H	Cysteic acid
CySH	Cysteine
Glu	Glutamic acid
Gly	Glycine
His	histidine

Leu	Leucine
Ileu	Isoleucine
Lys	lysine
Orn	ornithine
Pro	proline
Ser	serine
Thr	threonine
Val	valine
Phe	phenylalanine
Cys	cystine
Arg	arginine
Meth	methionine
Tyr	tyrosine
Try	tryptophan
UV	ultra violet
TLC	thin layer chromatography
Rf	chromatographic distance travelled relative to the solvent front
dpm	disintegrations per minute
Vol	volume
O.D.	optical density
diam	1.5 m
pH	negative log of the hydrogen ion concentration
DNP	dinitro phenyl
VP	variegate porphyria
SP	symptomatic porphyria

AIP acute intermittent porphyria
ME methyl esters
PTMC pepsin trypsin modified cytochrome c
PMC pepsin modified cytochrome c
PMC porphyrin pepsin modified porphyrin c
Porphyrin c cytochrome c after the removal of Fe.

INTRODUCTION.

It is only during the last 30 years that the biosynthetic sequence leading to heme has been elucidated and has paved the way to a better understanding of the disorders of porphyrin metabolism in man.

Thudichum (1867) was the first to recognise the natural porphyrins and their red fluorescence and designated them cruentins; in 1874 Schultz described the first case of porphyria in man - a typical example of congenital erythropoietic porphyria. Baumstark (1874) was able to isolate 2 pigments from the excreta of this patient and suggested that they were precursors of heme. The first to recognise the porphyrin nature of the pigment was Poulsen in 1910. The term porphyrin was introduced by Hans Fischer in 1912, but until the 1930's the terms "porphyrin" and "hematoporphyrin" were used indiscriminately.

Gunther in 1911 was the first to classify the porphyrias clinically, since which time much literature has been published on the subject of classification. (Gunther, 1925; Dean, 1953; Schmidt et al., 1954; Waldenström, 1957; Rimington, 1958; Dean and Barnes, 1958; Dean and Barnes, 1959; Watson, 1960; Eales, 1961; Goldberg and Rimington, 1962; Gray, 1964; Tschudy, 1965).

Characterisation of the porphyrins and hemes in terms of chemical constitution and structural configuration was essential to the development of a knowledge of their functions. Major contributions to this field were made in the early part of the century by Hans Fischer, who established the fact that the major pigments of both plants and animals are built on the same tetrapyrrole structure. It has since been shown that the same biosynthetic sequence operates in all forms of life.

The fact that South Africa has the highest incidence in the world of 2 of the 9 currently recognised porphyrias has provided a unique opportunity for studying these diseases and the manner in which they present in man.

As in most other fields of biochemical study, advances in the knowledge of the porphyrias have been largely dependent on improved techniques for the isolation, purification and identification of the chemical compounds involved. The original analytical techniques for the study of the porphyrins involved, in the main, solvent extraction procedures and the techniques of classical 19th century organic chemistry.

The solvent extraction procedures in use today are based on the work of Zeile and Reuter (1933). The modifications introduced by Rimington and Sveinsson (1950), Rimington (1968), and With (1968) provided quantitative methods for porphyrin analysis. Although these methods achieved, by current standards, indifferent separation of the various porphyrins, they nevertheless provided data of diagnostic nosological value.

Solvent partitioning of porphyrins on the basis of their HCl numbers was achieved much more efficiently by the use of countercurrent distribution techniques developed by Craig et al. in 1945. These methods, although giving excellent resolution, are impractical for routine laboratory use.

The advent of chromatography and electrophoresis, so valuable in other fields of biochemistry, had a similar impact on porphyrin technology. Methods for the isolation, identification and quantitation of porphyrins based upon these techniques provided sensitive and precise procedures for defining hitherto undefined porphyrins. Refinement of chromatographic techniques leading to present thin layer chromatographic methods has provided the means for rapid and accurate determination of porphyrin present in the excreta of patients with porphyria.

The first description of thin layer chromatography appears in a publication by Zechmeister (1938), whose main object was to develop a microchromatography method. This he did by changing from a "closed" to an "open" column method,

i.e. thin layer chromatography. During the early stages of development of the method many variations were used, such as the impregnation of filter paper with silicic acid or alumina; pressed plates of sintered glass; clay plates; and plates of anodally oxidised aluminium foil, but most suitable of all were the thin layers of very fine grained silica gel. There was little enthusiasm for this procedure due to lack of reproducible Rf's. Stahl felt this could be overcome by standardisation of the method and in 1958 presented the findings which were to lead to more general acceptance of the technique.

In 1963 while working in Professor Eales' laboratory I became acquainted with the technique and attempted to apply it to the resolution of porphyrin methyl esters, in the hope that I might exploit the advantages of the method. Until this time only 2 reports (Demole, 1958, and Jensen, 1963) dealing with the separation of porphyrins by TLC had been published. The procedures described gave results which were difficult to reproduce or yielded indifferent separation. In 1966 I applied a modification of Chu and Chu's (1951) reversed phase paper chromatographic method to the separation of porphyrin methyl esters on glass plates coated with silica gel. This gave very satisfactory results (Grosser and Sweeney, 1967).

Since this time many publications of this nature have appeared. (Ellfolk and Sievers, 1966; Chu and Chu, 1966; Koskelo and Toivonen, 1966; Formanek, 1966; Mundschenk, 1966; Chu and Chu, 1967; Scott et al., 1967; With, 1967; Doss, 1967; Doss, 1968 (a) (b); Cardinal et al., 1968; With, 1969 (a) (b), Doss and Bode, 1968).

The first part of this thesis describes the results I obtained when I applied these methods to the investigation of faeces and urine from patients with porphyria.

Three further observations stimulated the subsequent work that constitutes the remainder of this thesis.

During the chromatographic separation of the urinary and faecal porphyrin esters from cases of Variegate and Symptomatic porphyria, new, hitherto undescribed fractions with low Rf's were detected on TL plates. These fractions had been observed by some workers but had been dismissed as "unesterified material". (Cardinal et al., 1968). Since the relative amounts of these fractions varied in different patients I undertook a closer study of these porphyrins and performed a number of experiments which indicated firstly that they behaved chromatographically in a heterogenous manner suggesting that they were probably a complex mixture of porphyrin containing compounds, and secondly that they incorporated radioactivity very rapidly following the oral administration of ^{14}C -labelled ALA indicating that they were probably endogenously derived and possibly of significance in the pathway of heme synthesis or degradation.

This material proved to be in many respects identical to the hydrophilic porphyrin described by Sweeney (1963) and probably to the "ether insoluble" fraction described by Rimington in 1947. Both Sweeney and Rimington detected the unusual hydrophilic porphyrin in cases of porphyria associated with jaundice. We were fortunate in having the opportunity of studying a further case of this nature who provided sufficient quantities of the hydrophilic porphyrin for further study.

In the meantime Rimington directed intensive investigations towards the elucidation of the nature of the hydrophilic porphyrin and between 1966 and 1968 he published a series of papers identifying the hydrophilic porphyrin in urine and faeces as porphyrin-peptide complexes. (Rimington and Lockwood, 1966; Rimington and Benson, 1967; Rimington and Belcher, 1967; Rimington et al. 1968). These he named collectively "Porphyrin X" and likened them to the porphyrin of cytochrome c because of the thio-ether bond linking the porphyrin and peptide moieties. He also suggested the possibility that they may be related

to the protein bound intermediate observed in the coproporphyrinogenase transformation of coproporphyrinogen III to protoporphyrinogen IX. Rimington felt that the determination of porphyrin 'X' may be of particular interest in the diagnosis of Variegate Porphyria in which he found raised levels.

In October 1970, Professor Sano of Kyoto University visited our laboratories in Cape Town and introduced me to the techniques that I have used to study these hydrophilic porphyrins. His interest and expertise in the biochemistry of the cytochromes dictated, for the most part, my approach to the problem and accounts for my deviation in many respects from the more conventional methods of porphyrin chemistry employed by others. (Gray et al., 1947; Rimington and Belcher, 1967; Rimington et al., 1968; Rimington and Lockwood, 1966; Rimington and Benson, 1967; Sweeney, 1963; Smith et al., 1968; Smith et al., 1969; Belcher and Smith, 1970; Belcher et al., 1969; Riley and Belcher, 1968; Elder and Chapman, 1970; Elder, 1971).

The problem, at first seemingly straightforward, grew progressively more formidable as the work proceeded. With each new procedure further components were resolved and a picture of considerable complexity has emerged. The results obtained and the assessment of their value are presented in Section 3 of this thesis.

Except where I have felt it necessary for coherence I have avoided any lengthy discussions of such general topics as the classification of the porphyrias or the biochemistry of common porphyrins or their precursors. There is, in general, little controversy about them and they are completely dealt with in many reviews and books. (Fischer and Orth, 1937; Lemberg and Legge, 1949; Vanotti, 1954; Blackburn, 1960; Granick and Mauzerall, 1961; Goldberg and Rimington, 1962; Lascalles, 1964; Falk et al., 1963, Falk, 1964, Tschudy, 1965; Levere and Kappas, 1968; Goodwin, 1968; Heilmeyer, 1966).

Aim of Thesis.

In the first part of this thesis a comparison has been made between the solvent extraction procedures and the thin layer chromatographic methods which may be used for the separation of porphyrins. An account has been given of the results obtained and an assessment of their value for the routine diagnosis of porphyria.

The second section of this thesis deals with the preliminary examination of the properties of the hitherto undescribed hydrophilic porphyrins; and thirdly, methods for the isolation and purification of the porphyrin peptides have been suggested and the amino acid and porphyrin composition of the hydrophilic porphyrins determined and their role in the porphyrin pathway discussed.

7-1

SECTION I.

PATTERNS OF PORPHYRIN EXCRETION IN
HUMAN PORPHYRIA AS REVEALED BY TLC
OF THE PORPHYRIN METHYL ESTERS.

Differences in the solubility of porphyrins in various solvents have, for many years, been the basis of routine diagnostic biochemical investigations.

Reference to those books concerned with laboratory techniques for porphyrin estimations reveal a generally accepted approach in which porphyrins in biological samples are divided according to their solubility in solvents of differing polarity and subsequently measured under acid conditions either spectrophotometrically or fluorometrically. In most cases these techniques offer poor resolution of the porphyrins present and in general reflect only the predominance of a porphyrin by virtue of its solubility characteristics. This is exemplified in the following experiment :-

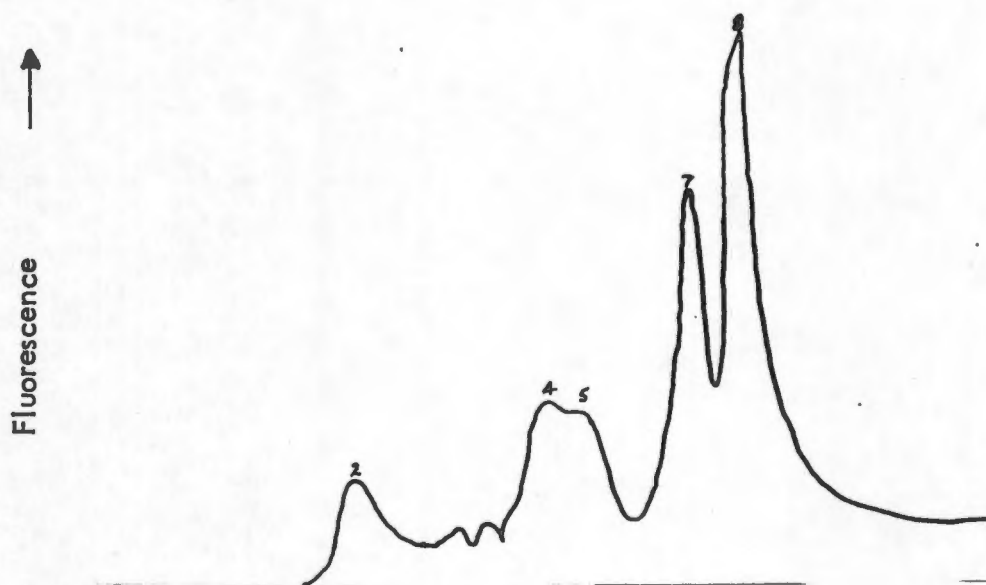
A sample of faeces from a patient with variegate porphyria was processed for quantitative porphyrin determination by the commonly used method of Holti et al. (1958). Briefly, this involved extraction to completion with acetic acid : ether (1 : 10). The pooled ethereal extracts were then extracted, first with 0.1 N HCl to give a "copro" fraction and subsequently with 1.5 N HCl to give a "proto" fraction. These acid extracts were taken to dryness separately, esterified with BF_3/MeOH and these esters examined by thin layer chromatography on silica gel using the system of Doss (1967). As can be seen from fig. 1 (a) these fractions each consist of a mixture of porphyrins. The "proto porphyrin" fraction was shown to contain 2,4,7 and 8 carboxyl porphyrins but with a predominance of the proto porphyrin; the "copro porphyrin" fraction showed all the porphyrins to be present, namely 2, 4, 5, 6, 7 and 8 carboxyl porphyrins but with coproporphyrin dominating.

The same procedure of esterification and thin layer chromatography was applied to the "copro" and "uro" fraction isolated from the urine of a patient

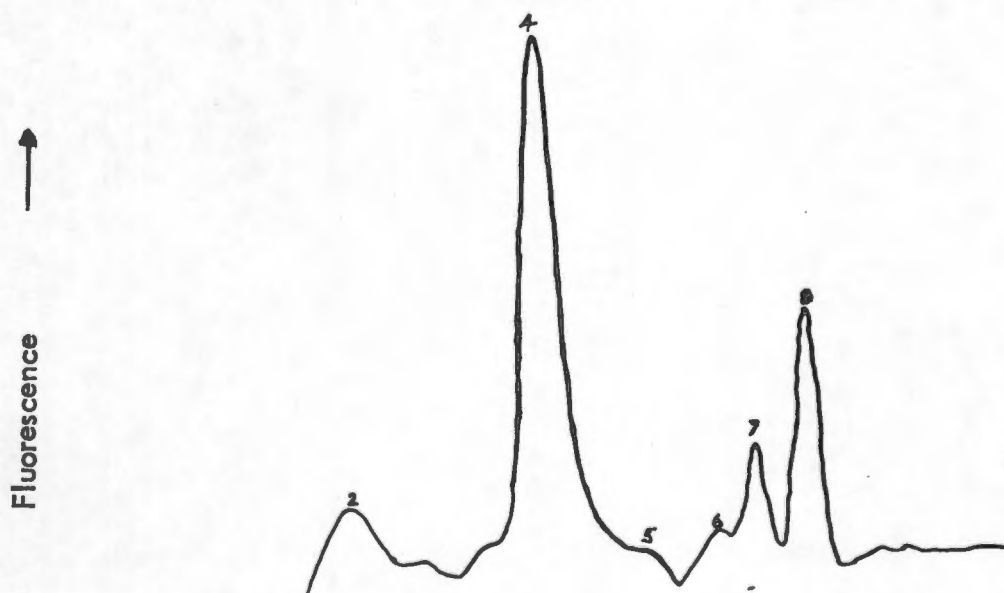
Fig. 1(a)

TLC PATTERN OF FAECAL PORPHYRIN EXTRACTS

PROTOPORPHYRIN FRACTION

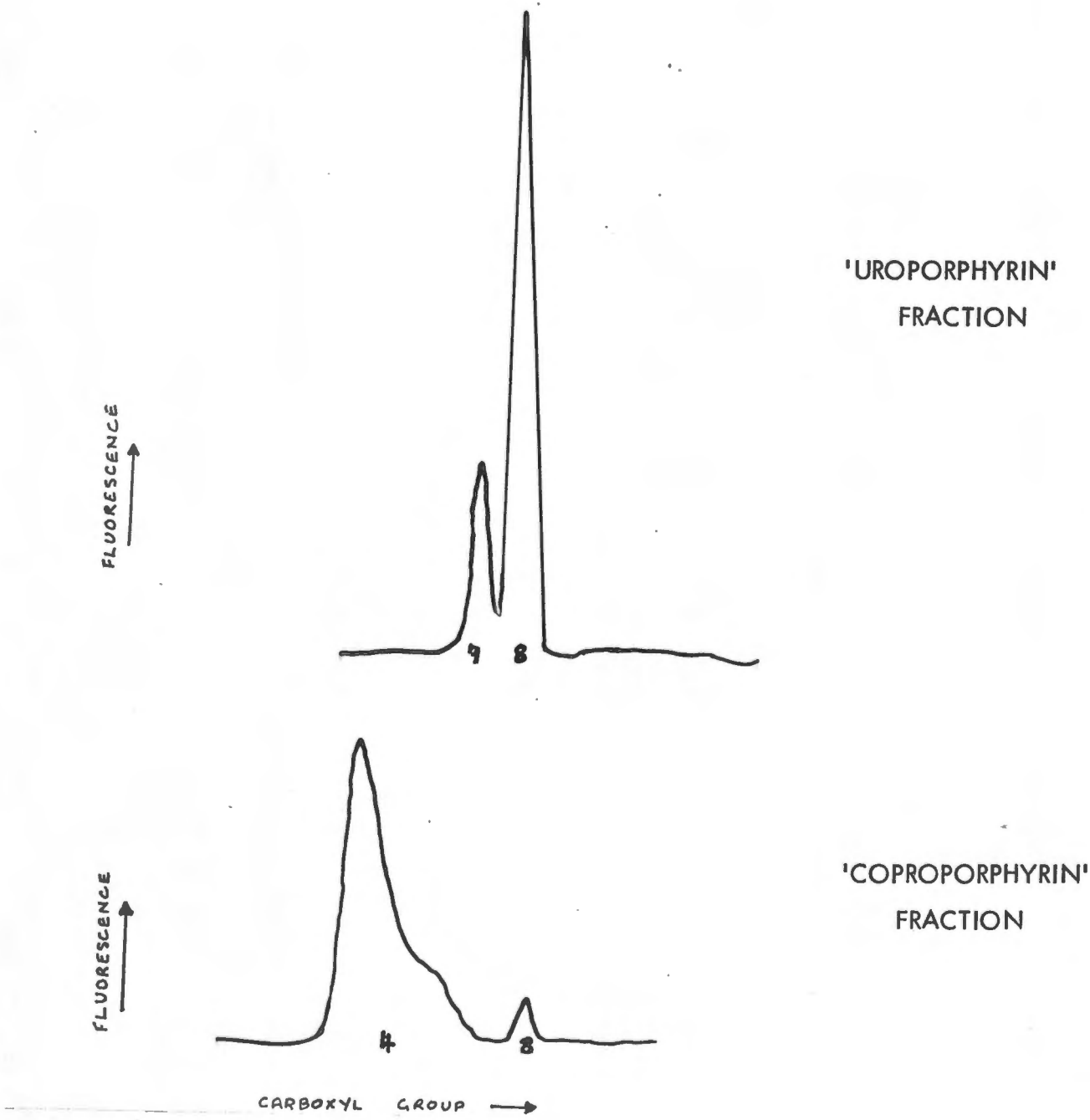


COPROPORPHYRIN FRACTION



TLC pattern of the 'protoporphyrin' and 'coproporphyrin' fractions extracted according to the method of Holti et al (1958).

Fig. 1(b) TLC PATTERN OF URINARY PORPHYRIN EXTRACTS



TLC pattern of the 'uroporphyrin' and 'coproporphyrin' fractions extracted according to the method of Rimington & Sveinsson (1950)

with symptomatic porphyria by the method of Rimington and Sveinsson (1950).

The urine fractions (fig. 1 b) appear to be more consistent in content, with the "copro" fraction containing mainly coproporphyrin and relatively little uroporphyrin. The "uro" fraction incorporated both 7 and 8 carboxyl porphyrins.

Similar findings were obtained in respect of free porphyrins by the talc thin layer chromatography method of With (1969).

There is no justification for assuming, therefore, that quantitative methods based on solvent extraction procedures do in fact extract the porphyrins they claim to with any accuracy.

It was therefore felt desirable to develop a more satisfactory procedure for defining the pattern of porphyrin excretion in human porphyria that would achieve better resolution without sacrificing the ease and rapidity of the conventional solvent extraction methods.

An extension of the solvent extraction procedure is the counter-current distribution method in which multiple extractions of the porphyrins are made according to their HCl numbers. This method is more accurate but an extremely lengthy procedure. (Craig, 1945; French and Thonger, 1964).

Paper chromatography offers further advantages. Nicholas and Rimington (1949) developed the first solvent system suitable for the chromatography of free porphyrins on paper and Eriksen's modifications of this method (1953, 1958) allowed the separation of the coproporphyrin isomers. Chu and Chu (1951) introduced a way of separating the porphyrin esters which is more rapid than that of the free porphyrins but has the disadvantage of poorer resolution. Many other alternative solvent systems and supporting media have been used by other workers with equal success. See Table I.

Column chromatography has been found excellent for the qualitative separation of large quantities of porphyrins but quantitative recoveries are poor. Electrophoresis

has also been applied to porphyrins on paper with great success and a quantitative assay using this method could probably have been devised. All techniques involving the separation of free porphyrins on paper had the intrinsic disadvantage, for quantitative purposes, of the instability of dry free porphyrins adsorbed to a chromatography paper.

The advantages of TLC over other methods already mentioned seemed considerable. There are several solvent systems and supporting media that give equally good resolution. In the initial stages of this work only 2 methods for porphyrin determinations by TLC had been published, i.e. Demole (1958) and Jensen (1963). I devised an adaptation of Chu and Chu's reverse phase paper chromatography to thin layer chromatography and used this system for the earlier chromatograms. At a later stage Doss (1967) published a superior method for the separation of porphyrin methyl esters and the system may be modified according to the porphyrins of particular interest, namely the methanol concentration may be increased in order that the Rf's of the higher carboxyl porphyrins and other more hydrophilic porphyrin fractions may be increased. Another system deserving particular mention is With's method (1967) which has the added advantage that the porphyrins are run in their free form and, provided the plates are scanned immediately after removing them from the solvents (since the porphyrin fluorescence fades within hours) and a standard run with each plate, this system is one of the most rapid and accurate methods of porphyrin estimation.

A few comments on the suitability of other methods that are applicable for quantitation.

The method of Cardinal et al. (1968) uses unactivated air-dried silica gel plates. This results in slight tailing of the samples. Activation of the silica gel led to some improvement but separation of the various porphyrins was still imperfect.

TABLE 1

METHODS OF PORPHYRIN SEPARATION

I. SOLVENT EXTRACTION TECHNIQUES

(a) pH dependent simple extraction

Mundschenk (1966), (1969)
With (1968)
Sweeney et al (1963)
Fernandez et al (1966)
Rimington and Sveinsson (1950), (1961)
Mingiolo (1968)
Heilmeyer (1966)
Beard (1967)
Schwartz and Wikoff (1952)
Schwartz et al (1960)
Zieve et al (1953)

(b) Counter Current

Smith et al (1970)
Barnes (1963)
Falk et al (1956)
Granick and Bogorad (1953)
Paul (1953)
Craig et al (1945)

2. COLUMN CHROMATOGRAPHY

Gainstein et al (1945)
Nicholas (1951)
Eriksen (1957)
Chu and Chu (1959)
Schlenker et al (1965)
Scott et al (1967)
Martina and Mills (1971)

3. PAPER CHROMATOGRAPHY

Nicholas and Rimington (1949), (1951)
Eriksen (1953), (1958)
Corwin and Orten (1954)
Chu and Chu (1951), (1957)

3. PAPER CHROMATOGRAPHY (Continued)

Falk and Benson (1953)
Falk et al (1956)
With (1957)
Del-Battle and Benson (1966)
Belcher et al (1970)

4. THIN LAYER CHROMATOGRAPHY

Cardinal et al (1968)
Belcher et al (1970)
Del Battle and Benson (1966)
Ellfolk and Sievers (1966)
Lynnco and Schanderl (1967)
Elder (1971)
Mundschenk (1966)
Schaltegger (1965)
Henderson and Morton (1967)
Doss (1970), (1968), (1971)
Grosser et al (1967)
With (1969)
Koskelo and Toivonen (1966)
Scott et al (1967)
Chu and Chu (1966), (1967)

5. ELECTROPHORESIS

Papastamatis and Kench (1952)
Eriksen (1958)
With (1956)
Heikel (1955)

6. GAS CHROMATOGRAPHY

Karayannis and Corwin (1968)

When porphyrin is present in small amounts, the fluorescence may be enhanced by the conversion of the porphyrin to a metal complex (Doss, 1968).

Dextran gels and Polyacrylamide have also been investigated but the resolution is inferior to that obtained with silica gel.

OF THE PORPHYRINS.

Four years ago the following method for the routine analysis of urine and faecal porphyrins was devised and applied to the study of porphyrins from patients with various types of porphyria.

Method.

Although all solvents and reagents used were of analytical grade, further purification of certain items was necessary (see Appendix A).

(i) Extraction of porphyrins.

(a) Urine was collected over a 24hour period and, if not extracted immediately, stored without preservation at 4°C until processed. The pH of a 100 ml aliquot was adjusted to pH 3.5 with saturated aqueous Na acetate solution. Talc was then added to the urine and the mixture stirred magnetically for 1 hour. The talc was allowed to settle and the urine decanted. A second quantity of talc was added and this was repeated until no more fluorescence could be detected, when 3 ml of the urine was mixed with 0.5 ml solution (consisting of equal quantities of amyl alcohol, ether and glacial acetic acid), and exposed to a UV light source. The talc was washed with 100 ml water and then dried in a vacuum desiccator over calcium chloride for 24 hours. The porphyrins were eluted from the talc with an esterification mixture of 3% BF_3/MeOH complex and allowed to stand stoppered in the dark for 12 hours. The esterification mixture was then poured off the talc and the talc mixed with more esterification mixture and filtered. This was performed several times until the talc was negative upon screening. The mixture was then neutralized with saturated Na acetate and extracted into chloroform and the latter washed with distilled water 3 - 4 times and finally dried under a current of nitrogen and

taken up in a known quantity (1-2 ml) of chloroform. An aliquot of this was applied to a thin layer plate. The size of the aliquot depended upon the porphyrin fluorescence present.

(b) Faeces. Between 0.5 - 5.0 g of faeces were placed in a ground glass stoppered test tube. The tube was left open in a vacuum desiccator over night in order to remove as much moisture as possible. 30 ml of esterification mixture was then added and the tube stoppered and left for 12 hours. The remainder of the procedure was identical to that for urine. The dry weight of faeces was determined after drying an aliquot at 105°C to constant weight (+ 4 hours).

(ii) Esterification

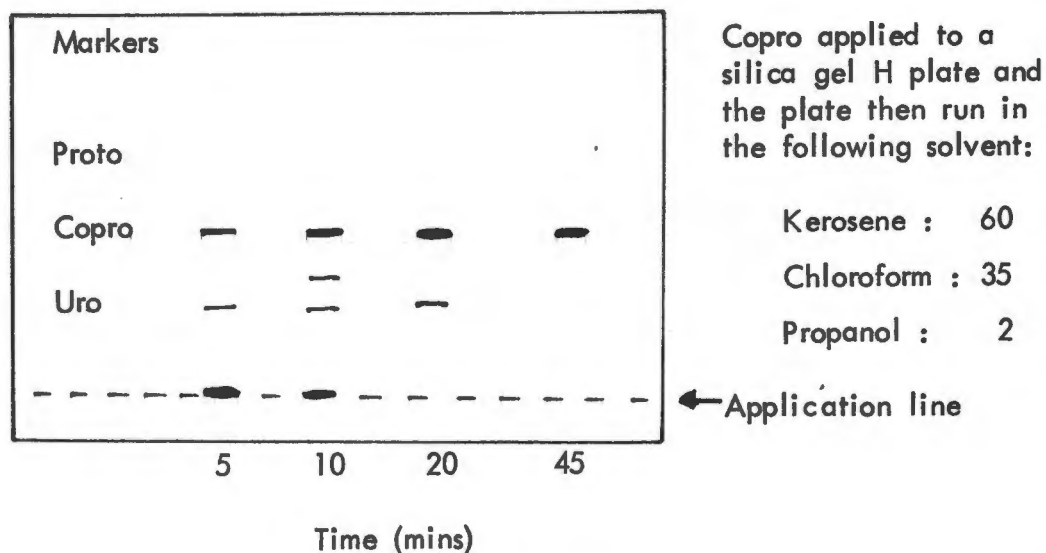
Various methods of esterification are available. The use of 5% sulphuric acid in Methanol is simple and the most commonly used method to esterify porphyrins. Equally effective is the use HCl gas passed into methanol, but it was noted that the recovery of the porphyrin from talc was more complete with $H_2SO_4/MeOH$ than with $HCl/MeOH$.

Diazomethane is recommended by Falk(1964) for porphyrins that are labile to mineral acids. The method of 3% $BF_3/MeOH$ is not widely used, but we found this to be an extremely convenient and slightly more consistent method of esterification, recoveries being approximately 10% higher than with 5% $H_2SO_4/MeOH$.

Time required for Esterification:

Standard solutions of porphyrins were treated with a 5% $H_2SO_4/MeOH$ mixture and samples taken at intervals and spotted on TLC plates. Complete esterification was assumed when one clear band of the porphyrin tested could be seen on the plate. (Fig. 2)

FIG. 2



Esterification was found to be complete for Proto, Copro and Uro after 1 hour. With (1971) has recently published a paper illustrating the same times. Similar results were seen with the use of BF_3/MeOH . An overnight esterification time as was used in this study proved to be more than sufficient time for maximal recoveries.

(iii) Thin Layer Chromatography.

The application of thin layer chromatography to the analysis of biological samples has made it possible to determine the ratio of the individual porphyrins to each other. The total porphyrin content was determined by the use of solvent extraction procedures.

The chromatograms reported in this study were effected on 0.3 mm thick silica gel H (nach Stahl). The plates were spread with a Shandon Thin Layer Apparatus and then activated at 110° for 1 hour and stored in a desiccator for a period not exceeding 5 days.

The porphyrin solutions were spotted on a plate with a Hamilton microsyringe;

each sample was applied over a distance of 1 cm and markers consisting of 2, 4, 5, 6, 7 and 8 carboxyl porphyrins were also spotted on the same plate.

The chromatograms done in the first year of study were run in the following solvent system (Grosser ^{et al.}, 1967) :

Kerosene :	60
Chloroform :	35
Propanol :	2

and more recently in the system (Doss, 1967) :

Benzene :	85
Ethyl Acetate :	13
MeOH :	3

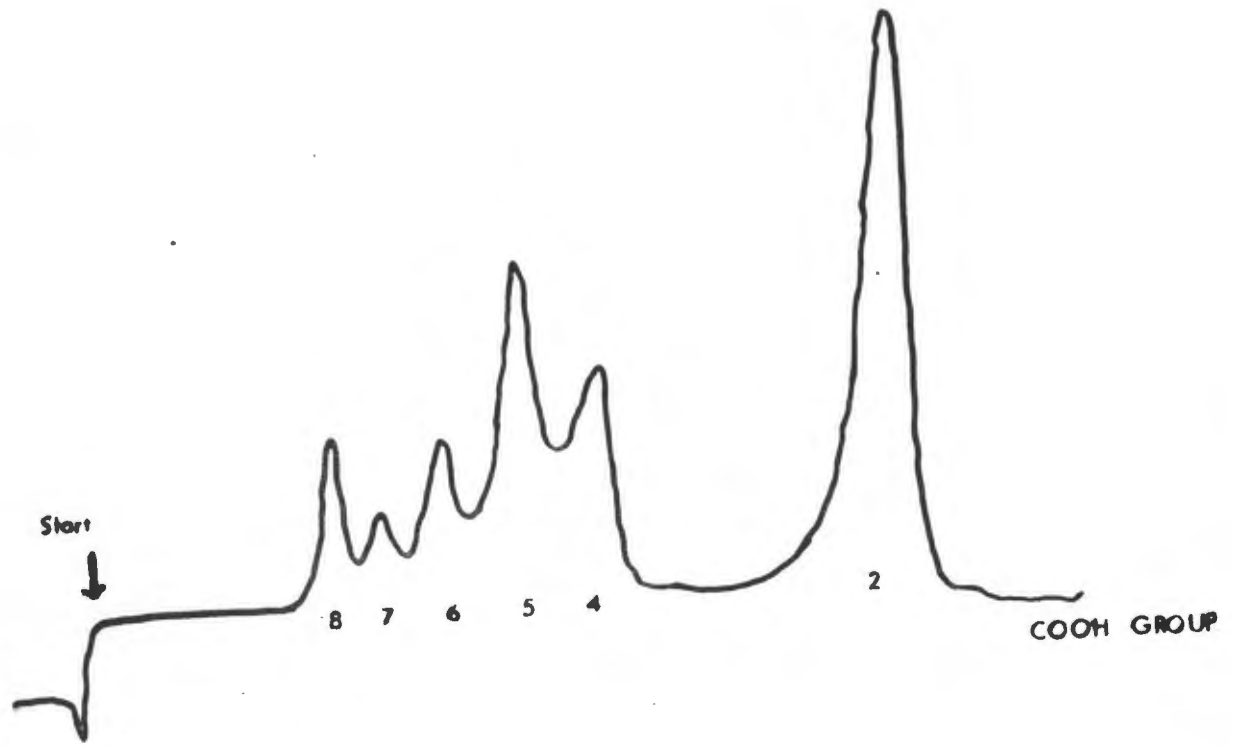
These plates were scanned initially on a home made fluorescence scanner and at a later stage on a Joyce-Lobel Chromoscan Apparatus with thin layer and fluorescence attachments.

The resolution of the method was tested (fig. 3) and its reproducibility investigated by scanning the same spot 5 times (fig. 4).

Since porphyrin fluorescence is due to the excited state of the Pi electron molecules, whose density varies according to electron attracting and repelling groups, it can be envisaged that the different porphyrins will fluoresce to varying degrees. Therefore, if equal quantities of each are applied to a plate, the plate scanned (fig. 5) and the area under the curve integrated, factors may be obtained to convert the area to a percentage of the total porphyrin present. (Table 2 and 3). The intensity of fluorescence varies (Jope, 1945, Chu and Chu 1959) with pH and the ionic strength of the solution and is greatly influenced by impurities; therefore it is necessary to standardise the method completely and under such conditions it was found that a constant relationship between the different porphyrins was present when quantities estimated lay between .01 μ g and 0.08 μ g (fig. 6).

Fig. 3.

FLUORESCENT SCAN OF THIN LAYER PLATE



Faecal porphyrin extract from a patient with variegate porphyria was applied to a thin layer plate, after development, the separated bands were scanned for fluorescence. Good resolution of the individual components was achieved.

Fig. 4.

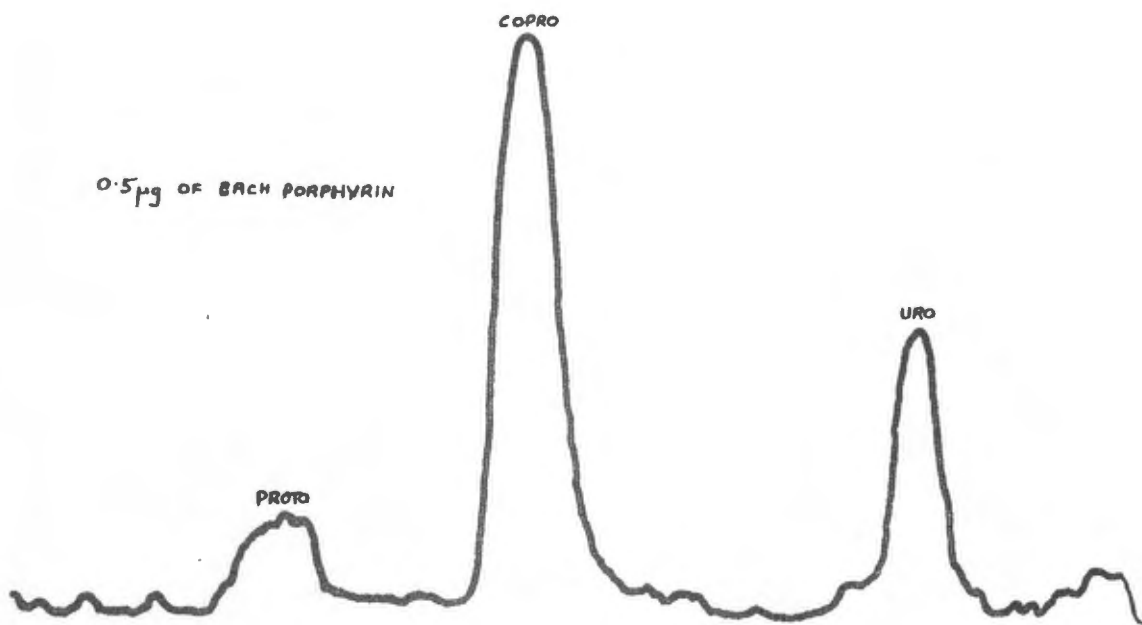
REPRODUCIBILITY OF FLUORESCENT SCANNING TECHNIQUE



A single fluorescent band on a thin layer plate was scanned several times, and the area under the curve integrated. The reproducibility of the scanning apparatus may be assessed from values of the integrated areas.

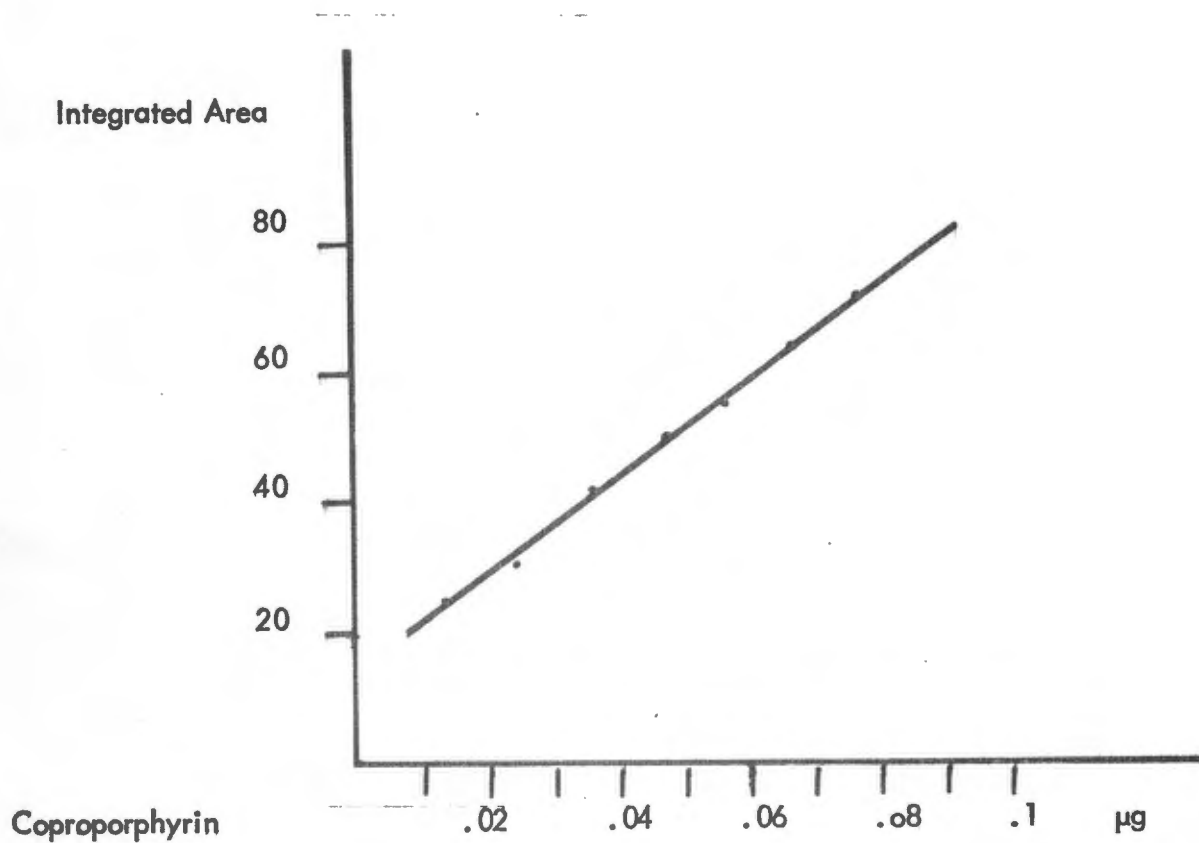
Fig. 5.

FLUORESCENT SCAN OF THIN LAYER PLATE



Equal quantities (0.5 μg) of protoporphyrin, coproporphyrin and uroporphyrin were applied quantitatively to a thin layer plate. The integrated areas under the curves may be used to calculate conversion factors between the individual porphyrins.

Fig. 6 QUANTITATION OF PORPHYRINS BY TLC.



The integrated area is a linear function of the amount of porphyrin present between the quantities of 0.01 µg and 0.08 µg.

TABLE 2.

Conversion factors for the Joyce-Lobel Chromoscan.

No. of porphyrin COOH groups	Porphyrin conc. (μg)	.02	.04	.06	.08
Average ratio* (area in brackets represents the av. of 5 readings)					
	↓				
2	2.09	2.00 (23)	1.99 (30)	2.14 (35)	2.13 (41)
4	1	1 (46)	1 (59)	1 (75)	1 (87)
5	0.77	.79 (58)	.79 (74)	.77 (98)	.74 (118)
6	1.14	1.21 (38)	1.05 (56)	1.15 (65)	1.14 (76)
7	1.11	1.12 (41)	1.09 (54)	1.04 (65)	1.19 (73)
8	1.24	1.28 (36)	1.21 (49)	1.27 (59)	1.21 (72)

* A similar study was performed with the fluorescence scanner assembled in our laboratories by Dr. Sweeney, and the following conversion factors obtained :-

TABLE 3.

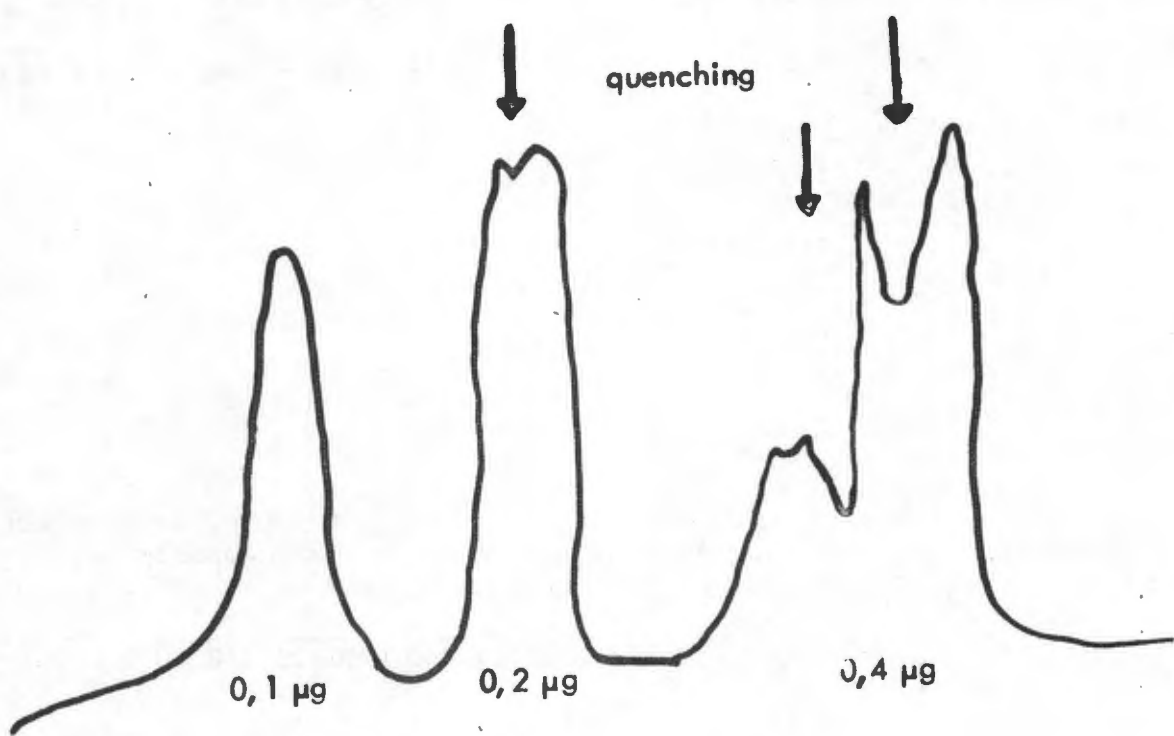
Conversion factors for the laboratory-built fluorescence
chromoscan.

<u>COOH group</u>	<u>Conversion factor</u>
2	4.77
4	1.00
5	2.67
6	2.73
7	2.73
8	2.62
x	2.60

A maximum of 0.1 μg per component, spread over 1 cm, may be resolved and scanned with accuracy but amounts in excess of this cause quenching to occur as shown by a dip at the height of the peak (fig. 7).

Fig. 7.

ILLUSTRATION OF THE QUANTITATIVE LEVEL AT WHICH FLUORESCENT QUENCHING OCCURS.



Fluorescent quenching on a thin layer plate may be observed as a dense area in the centre of a band. A fluorescent scan of this shows depressions of the peak tip.

It must be noted that considerable differences are observed when slight variations in the optical system are made.

In order to test the reproducibility of the method varying amounts of porphyrins (Proto, Copro, Uro 0.1 $\mu\text{g}/10\text{ ml}$) were applied to plates. The various fractions were resolved in solvent systems already mentioned and the plates then scanned. The areas under the curves were integrated and multiplied by the appropriate factors to convert the area to a percentage of the total porphyrin present. Results are tabulated in Table 4.

TABLE 4.

	Proto (0.01 $\mu\text{g}/\mu\text{l}$)	Copro (0.01 $\mu\text{g}/\mu\text{l}$)	Uro (0.01 $\mu\text{g}/\mu\text{l}$)
Amt. applied	10 μl	10 μl	10 μl
% area	35	39	26
Ratio	1	1.1	0.74
Amt. applied	10 μl	7 μl	7 μl
% area	42	32	26
Ratio found	1	0.76	0.62
Ratio expected	1	0.77	0.52
Amt. applied	10 μl	3 μl	3 μl
% area	63	21	15
Ratio found	1	0.30	0.24
Ratio expected	1	0.33	0.21

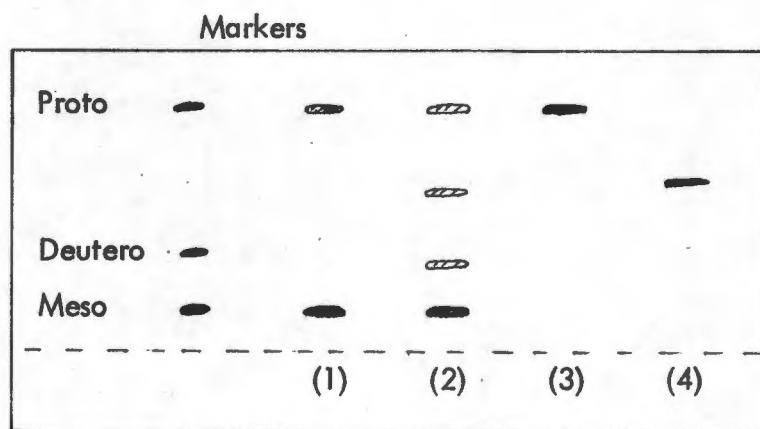
It was therefore shown that the ratios of the porphyrins to each other remained constant to within 4% within the range 0.03 - 0.1 μg of porphyrin.

(iv) The Differentiation of the Dicarboxylic Porphyrins.

The dicarboxyl porphyrins may be separated on TLC using the method of Henderson and Morton (1967). The plates are impregnated with $FeSO_4$ and must be used immediately after preparation. It was found that the substitution of $FeCl_3$ for $FeSO_4$ enabled the plates to be kept indefinitely, but there was also an added disadvantage in that more fluorescent quenching occurred. Other methods attempted include separation on paper (Chu and Chu, 1954), column chromatography (Nicholas, 1951), solvent extraction (Herbert, 1966), counter current partitioning (Sweeney, 1963, Belcher et al., 1970), but the TLC has been found to be the most satisfactory method.

The faeces from 4 patients were studied (fig. 8).

FIG. 8 TLC. Silica gel G slurried in 0.3% (w/v) $FeSO_4 \cdot 7H_2O$.
Dried and then run in the solvent Benzene : 100
Methanol: 5



- (1) S.de J. - Variagate Porphyria
- (2) P.C. - Variagate Porphyria
- (3) S.de L. - Acute Intermittent Porphyria
- (4) B.C. - Symptomatic Porphyria.

It appeared that the major dicarboxylic fraction from the faeces of the 2 Variagate Porphyrics was mesoporphyrin.

Absorption spectra were run on the porphyrin methyl esters of the 4 bands observed in specimen (2) - P.C. and have been recorded in table 5.

TABLE 5. Dicarboxyl Porphyrin from the Faeces of a Variegated Porphyrin.

	Soret	IV	III	II	Ia	I
Band 1	403	507	539	561	603-4	626-7
Band 2	403	502	537	567		620
Band 3	398	496	527	562	593	616
Band 4	397-8	498	529	564		618

Insufficient material prevented me from recrystallising these porphyrins and since it was felt that the presence of traces of Fe and solvents would affect the spectra pure standard porphyrins were put through the same procedure and their spectra recorded (Table 6). As expected slight variations were seen between these readings and those quoted by Falk (1964). (Table 7).

TABLE 6.

	Soret	IV	III	II	Ia	I
Proto	405	504	536	569		623
Deutero	398	495	530	566		613
Meso	398	494	529	564		618
Hemato	402	501	533	567		621

TABLE 7. (According to Falk)

	Soret	IV	III	II	Ia	I
Proto	407	505	541	575	603	630
Deutero	399.5	495	530	566	593	621
Meso	400	499	533	567	594	621
Hemato	402	499.5	532	569.2	596	623

These figures confirmed the chromatographic finding of mesoporphyrin.

The dicarboxylic porphyrins of the faeces have generally been accepted to be protoporphyrin but very little attention has been paid to the differentiation of the dicarboxylic porphyrins.

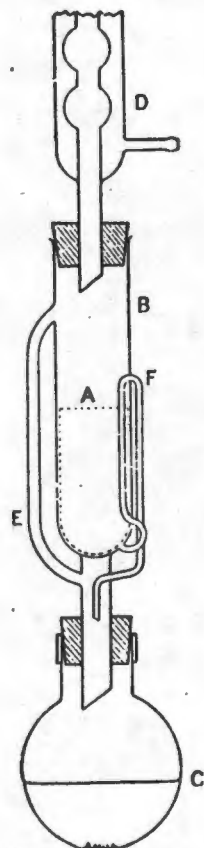
French et al. (1966) noted the occurrence of mesoporphyrins and only very small amounts of protoporphyrin in the bile. The faeces contained proto-, meso- and deuteroporphyrins. Watson (1937) and Vigham (1938) could detect no protoporphyrin in human bile. Smith et al. (1968, 1969) noted the presence of protoporphyrin in bile but also another unidentified dicarboxyl porphyrin with a soret maximum at 404.5 m μ . The concept that proto-porphyrin in faeces is of endogenous origin and that the mesoporphyrin and deuteroporphyrin are degradation products of the proto-porphyrin does not agree with their findings. But this concept is supported by the fact that both deuteroporphyrin and mesoporphyrin are reduced after the administration of antibiotics (Barnes, 1963). Herbert (1968) studied 42 specimens and reports the presence of protoporphyrin, constituting 29-100% of all the faecal dicarboxylic porphyrins, deuteroporphyrin 0-68% and mesoporphyrin 0-25%. Sweeney (1963) likewise noted deutero-, meso- and protoporphyrins in varying amounts, as found in the 4 specimens studied on this occasion.

(v) Total Porphyrin content of the Faeces.

Various methods of extraction were investigated.

(a) The use of a Sock's apparatus should theoretically yield complete extraction of the porphyrins (fig. 9).

FIG. 9 Sock's Apparatus



However recoveries were found to be poor (approximately 50%), probably because the whole process takes several hours and minute traces of peroxide in the glacial acetic acid and ether would destroy the porphyrin over such a long period.

(b) The faeces was extracted to completion with 2.5 N glacial acetic acid and then with 10% NH_4OH . The extracts were pooled, and acidified to pH 1-2 if necessary, and applied to a fluorosil column, 5 cm x 1 cm, (activated at 240° for 3 hrs.).

(Schlenker et al., 1965). The porphyrins were eluted with \pm 30 ml acetone/ether and 2.5 N HCl applied alternatively. Virtually 100% recovery was attained provided sufficient acetone/ether and acid were used to elute the porphyrin off the column.

(c) The Holti (1958) solvent extraction procedure was applied and the various porphyrin fractions extracted separately; the total porphyrin was then estimated. The reproducibility of this method was tested, namely 6 samples were taken from a

TABLE 8.

<u>Specimen no.</u>	<u>Amt. of stool taken</u>	(0.1 N HCl) <u>supposedly Copro $\mu\text{g/g}$</u>	(1.5 N HCl) <u>supposedly Proto $\mu\text{g/g}$</u>	<u>Total $\mu\text{g/g}$</u>
1	0.3103	25.82	73.89	99.71
2	0.2989	30.42	60.65	91.07
3	0.2855	24.19	69.68	93.87
4	0.2691	23.17	53.76	76.93
5	0.2787	26.84	63.50	90.34
6	0.2783	33.40	51.05	84.45

The overall standard error on the total porphyrin is 0.19 which was considered sufficiently reliable to accept as a method for total ether soluble porphyrin determination.

(vi) Uroporphyrin in Faeces.

Very little attention has been paid to the determination of uroporphyrin in faeces. Watson (1960) and Rimington (1961) used solvent extraction procedures which were essentially extensions of the Holti method. Watson's initial extraction was with ethyl acetate instead of ether and the faecal residue was extracted with 10% NH_4OH . This plus the 3% Na acetate-washing were applied to an alumina-chromatography column and the uro eluted with 1.5 N HCl. This method gives excellent recoveries when pure uroporphyrin is applied, but similar to all solvent extraction systems the isolated fractions from the faeces are not pure, and therefore not acceptable when a detailed study of the faecal porphyrins is required. The same applies to Rimington's method which makes use of different solvents. Sweeney (1963) used a method which removed the ether soluble porphyrins and then by means of esterification extracted the more polar components and finally

separated them as free porphyrins by electrophoresis. We attempted to simplify this procedure by esterifying the whole stool and extracting all the porphyrins together and then separating them on TLC. The individual components were measured fluorometrically.

Since the Holti (1958) method of extraction has been shown to contain the higher carboxyl group porphyrins as well as coproporphyrin, and protoporphyrin, these estimations have been taken to represent the total porphyrin (2 - 8 COOH) of the faeces. In this study the specimens were not extracted with the more polar solvents as discussed above, as these fractions were found to contain more porphyrin peptide components than uroporphyrin.

The individual 2 - 8 carboxyl porphyrins are estimated from the thin layer chromatograms.

The method of biochemical classification is as set out by Sweeney (1963 (a)).

See Table 9.

TABLE 9.

<u>Classification</u>	<u>Urine</u>			<u>Faeces</u>		
	ALA	PBG	UP	CP	CP	PP
Variegate Porphyria (remission)	±	±	±	++	+++	+++
Swedish genetic	++	++	variable	±	N	±
Symptomatic	±	N	++	+	± to ++	N

N = normal values

± = sometimes slight elevation.

(vii) Patients studied.

Records of analytical quantitative work on the faeces from patients with porphyria and from 22 normals will be presented. With the exception of the normals, these patients were either in-patients of Groote Schuur Hospital, or

seen at the Out-Patients' Clinic.

Faecal specimens were stored deep frozen if delay in analysis was unavoidable. Urines were collected in amber Winchester bottles and refrigerated, and the porphyrin extracted as soon as possible after receipt.

The patients studied may be grouped as follows : -

<u>No. of patients studied</u>	<u>Disease</u>
22	Normals
22	Symptomatic Porphyria
33 - 24	Remission phase Variegate Porphyria
- 9	Acute phase " "

(viii) Results.

The total faecal porphyrins and other relevant data of normals, symptomatic porphyrics and variegate porphyrics have been listed in tables 10 - 12, and the faecal porphyrin patterns obtained by TLC have been listed in tables 13 - 15.

TABLE 10

NORMALS

Patient	Sex	Age yrs	Urine		Faecal Porphyrins				
			ALA mg/L	PBG mg/L	%	Copro $\mu\text{g/G}$	%	Proto $\mu\text{g/G}$	Total $\mu\text{g/G}$
A.B.	F	20	1.9	0.9	35	7	65	17	24
I. du P.	F	28	2.3	1.3	28	5	72	13	18
A.K.	F	29	3.1	1.9	35	41	65	76	117
B.L.	M	59	2.2	1.3	37	49	63	84	133
B.L.	M	24	3.9	0.9	19	21	81	88	109
M.O.	M	-	-	-	22	16	78	58	74
M.T.	F	27	2.2	1.5	23	12	77	42	53
E.T.	M	23	0.5	0.5	31	30	69	67	97
M. du M.	F	66	2.1	1.9	11	4	89	33	37
J. v. R.	F	19	2.9	3.0	40	23	60	35	58
V. R.	F	45	1.8	1.5	43	10	57	13	23
L. v. R.	F	19	4.1	1.6	33	13	67	26	39
S. V.	F	24	2.4	2.8	37	52	63	89	141
M. V.	M	28	1.5	3.3	29	5	71	12	17
J. B.	M	25	1.3	0.6	21	13	79	49	62
D. v. R.	M	21	2.8	0.9	45	47	55	58	105
M. R. O.	F	44	2.5	6.4	30	11	70	26	37
M. B.	F	16	2.1	4.1	32	34	68	72	106
G. B.	F	6	3.1	3.0	17	1	83	5	6
J. R.	F	16	3.0	0.6	22	20	78	72	92
T. S.	M	9	3.7	1.5	22	21	78	73	94
V. S.	F	7	5.3	4.2	56	72	44	56	128
Va. S.	F	9	4.1	2.9	24	8	76	26	34

TABLE 11.

SYMPTOMATIC PORPHYRIA

Patient	Sex	Age yrs	Urine		Faecal Porphyrins				
			ALA mg/L	PBG mg/L	Copro %	Copro $\mu\text{g/G}$	Proto %	Proto $\mu\text{g/G}$	Total $\mu\text{g/G}$
J.P.	M	57	3.3	3.9	27	14	73	38	52
E.M.	F	43	5.1	1.4	33	14	67	29	43
B.M.	F	34	0.3	0.5	31	144	69	64	208
M.K.	F	35	3.1	1.5	75	179	25	60	239
B.de K.	M	7	3.0	1.0	19	5	81	22	27
J.P.	M	65	2.1	0.8	80	88	20	22	110
J.Z.	M	57	1.0	0.8	26	9	74	26	35
G.R.	M	61	4.8	2.3	67	108	33	53	161
F.S.	M	44	2.7	1.3	20	46	80	188	234
P.T.	M	35	6.1	0.8	74	37	26	13	50
H.B.	M	63	2.7	1.7	41	63	39	89	152
A.J.	F	41	2.5	2.8	29	60	71	145	205
M.M.	M	50	4.9	2.1	65	290	35	159	449
K.J.	F	30	3.7	1.0	63	176	37	102	278
W.de F.	M	67	2.0	1.8	33	3	67	6	9
T.M.	M	46	1.7	0.6	51	79	49	75	154
L.M.	M	60	0.1	0.5	42	24	58	33	57
M.T.	M	37	2.3	2.2	55	62	45	51	113
E.B.	F	50	0.6	1.8	65	178	35	97	275
B.C.	M	21	3.8	3.9	78	180	22	51	231
K.K.	M	53	1.5	1.5	68	99	32	46	145
J.H.	F	43	4.4	1.0	31	8	69	18	26

TABLE 12.

VARIEGATE PORPHYRIA.

Patient	Sex	Age yrs	Urine		Faecal Porphyrins				
			ALA	PBG	Copro		Proto	Total	
			mg/L	mg/L	%	µg/G	%	µg/G	µg/G
I.A.	F	20	5.1	2.3	72	1331	28	514	1845
M.A.	F	66	3.1	1.8	62	629	38	386	1015
M.B.	F	42	6.8	5.4	62	589	38	366	985
J.B.	F	32	11.2	12.1	53	761	47	683	1444*
E.de V.	M	48	4.1	2.1	64	466	36	259	725
J.de V.	F	42	4.5	2.4	64	489	36	272	761
W.V.	M	46	1.1	1.6	80	146	20	37	183
E.E.	F	50	7.7	9.7	67	1232	33	611	1843
D.J.	F	24	4.4	3.8	62	277	38	169	446
A.L.	F	26	16.8	32.9	47	577	53	660	1237*
E.O.	F	29	2.7	1.1	75	114	25	39	153
N.O.	M	22	140	108.5	40	367	60	552	919*
A.S.	F	26	5.0	10.6	67	517	33	253	770*
M.S.	F	30	5.0	4.1	63	191	37	113	304
E.S.	F	28	9.3	6.6	73	732	27	269	1001*
J.v.W.	F	19	4.0	1.1	69	200	31	89	289
C.v.H.	F	41	0.8	1.5	49	932	51	966	1898
N.v.R.	M	41	3.2	4.6	58	999	42	872	1717
M.v.S.	F	39	8.6	5.2	69	1013	31	453	1466*
E.v.Z.	M	35	9.6	5.2	61	313	39	203	516
M.V.	F	26	70	212	55	653	45	536	1189*
A.Y.	F	41	62.4	158	20	363	80	1471	1834*
J.v.D.	F	19	5.3	2.4	52	102	48	94	196
M.A.	F	33	2.3	1.3	48	120	52	128	248
M.v.M.	F	22	7.8	3.8	47	111	53	127	238
S.B.	F	48	7.8	5.3	56	417	44	326	743
E.de J.	F	51	3.2	1.4	72	1001	28	397	1398
J.H.B.	M	25	2.7	6.1	54	148	46	125	273
S.de J.	F	23	5.1	1.5	74	567	26	203	770
P.C.	M	67	1.7	5.0	77	4665	23	1387	6022
M.Q.	F	30	33.3	116.0	50	905	50	889	1794*

* Patients in acute phase. (see next to last column)

TABLE 13

FAECAL PORPHYRIN PATTERNS FROM TLC.

NORMALS.

COOH Grp	2	3	4	5	6	7	8
Patient	% $\mu\text{g/G}$	% $\mu\text{g/G}$	% $\mu\text{g/G}$	% $\mu\text{g/G}$	% $\mu\text{g/G}$	% $\mu\text{g/G}$	% $\mu\text{g/G}$
A.B.	90 22	0 0	5 1	0 0	0 0	0 0	5 1
I.P.	73 13	0 0	23 4	0 0	0 0	0 0	4 1
A.K.	74 86	0.3 0	3 3	0 0	8 10	7 8	6 7
++ B.C.	62 82	0 0	10 13	0 0	0 0	0 0	28 37
"	72 78	2 2	10 10	2 2	4 3	5 5	7 6
M.O.	72 53	0.4 0	7 5	0 0	6 4	7 5	11 7
+ J.R.	89 82	1 1	4 4	0 0	0 0	0 0	6 5
++ T.S.	62 58	0 0	1 1	0 0	0 0	0 0	35 32
V.S.	98 125	0 0	2 3	0 0	0 0	0 0	0 0
M.T.	64 34	0 0	10 5	0 0	0 0	0 0	26 14
E.T.	80 78	1 1	2 2	0 0	0 0	0 0	17 16
++ M.v.M.	77 28	0 0	6 2	0 0	0 0	0 0	17 6
++ J.v.R.	67 39	2 1	3 2	0 0	0 0	0 0	28 16
M.v.R.	57 13	0 0	13 3	0 0	0 0	0 0	56 13
+ L.v.R.	59 23	0 0	3 1	0 0	0 0	0 0	38 15
S.V.	88 124	0 0	2 3	0 0	0 0	2 4	7 10
M.V.	13 2	0 0	43 7	0 0	0 0	0 0	43 7
M.B.	81 50	6 4	5 3	0 0	3 2	2 1	2 1
D.v.R.	92 96	0 0	3 3	0 0	0 0	0 0	4 5
M.R.O.	24 9	0 0	4 2	0 0	0 0	0 0	71 26
M.B.	96 101	0 0	4 4	0 0	0 0	0 0	0 0
G.B.	71 4	0 0	21 1	14 1	0 0	0 0	0 0
V.S.	98 33	0 0	2 1	0 0	0 0	0 0	0 0

+ refers to the relative amounts of porphyrin-peptide complexes present.

(see margin)

TABLE 14

FAECAL PORPHYRIN PATTERNS FROM TLC.
SYMPTOMATIC PORPHYRIA.

COOH Grp	2		3		4		5		6		7		8	
Patient	%	µg/G	%	µg/G	%	µg/G	%	µg/G	%	µg/G	%	µg/G	%	µg/G
J.P.	62	32	3	1	9	5	7	4	1	1	1	1	15	8
E.M.	18	8	0	0	11	4	0	0	0	0	0	0	71	31
B.M.	65	133	2	4	17	35	3	6	2	4	5	10	5	10
M.K.	0	0	0	0	37	86	20	47	15	36	16	36	13	28
B.K.	26	7	0	0	21	6	0	0	0	0	0	0	53	14
++ J.P.	20	22	2	2	23	25	9	10	10	11	17	18	20	22
J.Z.	1	1	1	1	58	21	0	0	0	0	26	9	13	4
G.R.	0	0	0	0	48	76	26	42	8	12	5	8	14	21
F.S.	48	110	0	0	8	17	7	16	2	5	8	19	28	64
P.T.	18	8	0	0	44	22	0	0	9	5	9	4	20	10
++ H.B.	18	27	0	0	29	42	3	4	4	6	15	22	31	47
A.J.	72	145	0	0	22	45	0	0	0	0	0	0	6	12
++ M.M.	9	40	0	0	31	138	17	75	13	59	16	72	15	65
+ K.J.	8	22	0	0	32	89	0	0	16	43	25	68	19	54
++ W.de F.	0	0	5	0.5	10	1	0	0	1	0	1	0	82	7
T.M.	25	38	0	0	22	33	25	38	14	21	12	19	4	6
L.M.	24	13	5	3	25	14	3	1	1	1	5	3	37	21
M.T.	49	54	0	0	23	25	8	9	1	6	1	1	19	20
E.B.	26	71	0	0	47	129	3	8	3	8	3	8	5	13
B.C.	0	0	0	0	51	115	17	37	14	32	16	38	3	6
+ K.K.	4	6	5	7	45	64	10	15	11	16	19	26	5	79
++ J.H.	47	12	0	0	31	8	0	0	9	2	0	0	12	3

+ refers to the relative amounts of porphyrin-peptide complexes present.
(see margin)

TABLE 15.

FAECAL PORPHYRIN PATTERNS FROM TLC.

VARIEGATE PORPHYRIA.

COOH Grp	2		3		4		5		6		7		8	
Patient	%	$\mu\text{g/G}$	%	$\mu\text{g/G}$	%	$\mu\text{g/G}$	%	$\mu\text{g/G}$	%	$\mu\text{g/G}$	%	$\mu\text{g/G}$	%	$\mu\text{g/G}$
I.A.	92	1704	0	0	8	140	0	0	0	0	0	0	0	0
M.A.	76	771	3	25	19	194	0	0	0	0	0	0	3	25
M.B.	89	846	1	9	10	95	0	0	0	0	0	0	0.5	4
+ J.B.	78	1112	3	36	12	163	1.8	24	0.7	10	6	80	0.3	4
E.de V.	78	567	3	22	16	114	0	0	0	0	0.6	4	2	17
J.V.	89	677	0	0	7	54	0	0	0	0	0	0	4	30
W.V.	82	150	0	0	13	24	0	0	0	0	0	0	5	9
++ E.E.	66	1225	4	76	14	258	1.5	25	0	0	0.2	4	14	252
D.J.	68	305	2	11	17	74	0	0	0.5	2	0.8	4	11	50
++ A.L.	77	950	4	49	7	82	5	61	0	0	0	0	5	61
E.O.	95	144	1	2	4	6	0	0	0	0	0	0	0	0
N.O.	45	410	2	20	16	149	4	34	0.5	4	11	105	22	197
A.S.	83	639	2	13	14	105	0	0	0	0	0	0	2	12
N.S.	73	222	0	0	18	53	0	0	3	10	0.7	2	6	17
+ E.S.	80	803	3	27	10	102	0.3	3	1	12	4	41	1	10
++ J.v.W.	96	278	0.3	1	2	4	0	0	0	0	0	0	2	6
C.v.H.	12	304	0	0	54	1015	9	164	9	168	2	46	15	265
+ N.J.R.	55	940	0	0	28	479	0	0	0	0	6	101	11	194
M.S.	73	1072	5	75	12	178	1	10	0	0	0.1	2	9	130
E.v.Z.	66	340	3	17	15	78	0.5	3	5	25	4	20	6	32
M.V.	50	593	3	31	12	147	14	165	5	55	7	84	10	110
++ A.Y.	66	1210	6	113	8	141	2	33	0	0	4	72	14	262
J.v.M.	76	148	6	12	9	17	3	7	0.5	1	0.7	1	4	8
M.A.	89	219	2	5	2	4	0	0	1	2	5	13	0	0
M.v.M.	72	170	4	10	14	32	0.3	1	3.8	9	2	5	5	12
S.B.	90	669	2	15	6	43	0	0	0	0	2	16	0	0
E.de J.	86	1202	2	23	7	96	0	0	5	74	0	0	0	0
J.B.	52	141	3.7	10	25	68	9.8	26	9.0	25	0	0	0	0
++ S.de J.	59	446	0	0	20	153	3	21	9	72	0	0	9	69
P.C.	81	4853	0	0	6	337	0	0	9	511	7	403	21	1264
++ M.Q.	30	546	2	30	19	339	0	0	17	304	13	229	9	157

+ refers to the relative amounts of porphyrin-peptide complexes present.
(see margin)

(ix) Discussion.

It is only during the last decade that much attention has been paid to faecal porphyrins. It was generally considered that urinary porphyrin excretion reflected porphyrin synthesis and that faecal porphyrin excretion was the result of too many factors to be of diagnostic value, but, as pointed out by Eales (1968) the faeces are quantitatively by far the most important route of excretion. It should be remembered that other factors besides porphyrin formation also affect the urinary porphyrin excretion such as liver and kidney function, and therefore in spite of the fact that the faecal porphyrins are derived from both endogenous and exogenous sources, they may be equally useful for diagnosis; and detailed studies of them have proved to be of value. (Eales and Saunders, 1962; Eales et al., 1963; Eales, 1960; Eales et al., 1966; Eales and Dowdle, 1968; Dowdle et al., 1970; Sweeney and Eales, 1963; Chu and Chu, 1967 (a) and (b); Herbert, 1966 (b); Doss, 1969; Aziz and Watson, 1969; Rimington, 1963; Barnes, 1963; Haeger-Aronsen, 1963; Elder, 1971; Lemberg and Legge, 1949; Barnes, 1958; Stathers, 1968). Most previous studies have made use of the Holti method of solvent extraction and Eales (1963) was able to show a distinct difference in the faecal porphyrin excretion pattern between Variegate Porphyria and Symptomatic Porphyria. In the former the protoporphyrin excreted always exceeded the coproporphyrin, and the protoporphyrin was invariably more than 100 µg/g (dry wt.). In Symptomatic Porphyria the coproporphyrin excretion was usually higher than the protoporphyrin. By the use of TLC a similar pattern was shown here for variegate porphyria, but in symptomatic porphyria the 4-8 carboxyl porphyrins taken as a whole exceed the protoporphyrin, but coproporphyrin itself is not always in excess of protoporphyrin. The TLC we feel gives a more accurate estimation of the 2-8 carboxyl porphyrins (see figs. 10 and 11.)

98% of the normals show an upper limit for the total faecal porphyrin of 107 µg/g (i.e. 2 S.D.) which agrees well with that of Eales (1960) and Cochrane

and Goldberg (1968) and similar to the latter is slightly higher than previously quoted normals (Barnes, 1958; Holti et al., 1958; Taddeini and Watson, 1968), but this may be due to variations in diet since the ingestion of meat certainly increases faecal porphyrin (Eales and Saunders, 1962).

It may be seen from fig. 11 that the intermediate carboxyl porphyrins are present in the faeces in approximately the same proportion as in the urine of cases with Symptomatic Porphyria, i.e. $4 \text{ COOH} > 7 \text{ COOH} > 8 \text{ COOH} > 5 \text{ COOH} > 6 \text{ COOH}$, the only variation being that the $8 \text{ COOH} > 7 \text{ COOH}$ in 63% of the cases studied.

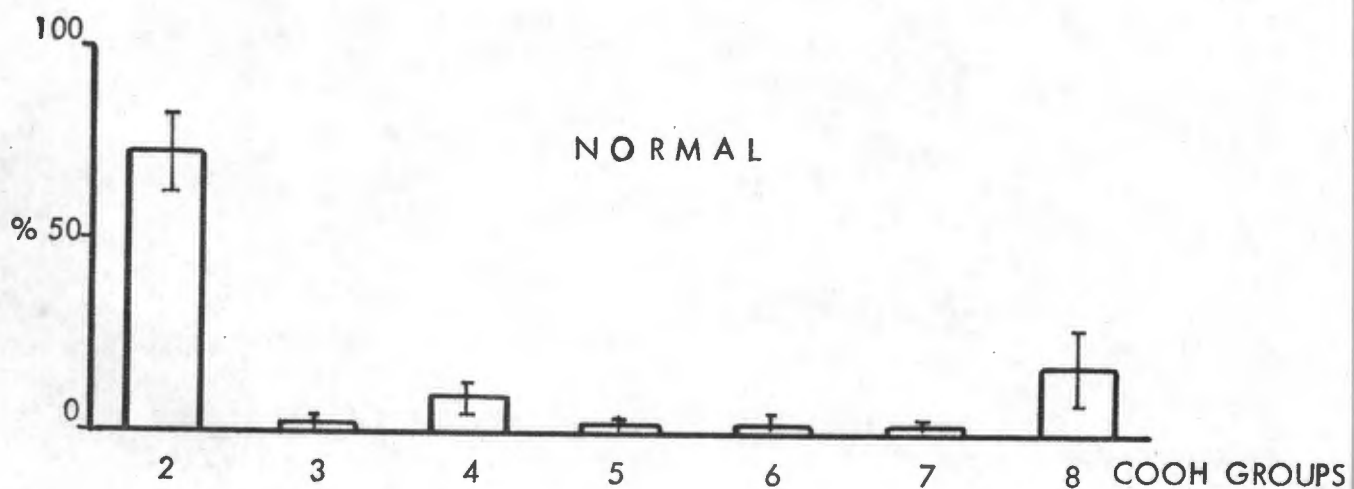
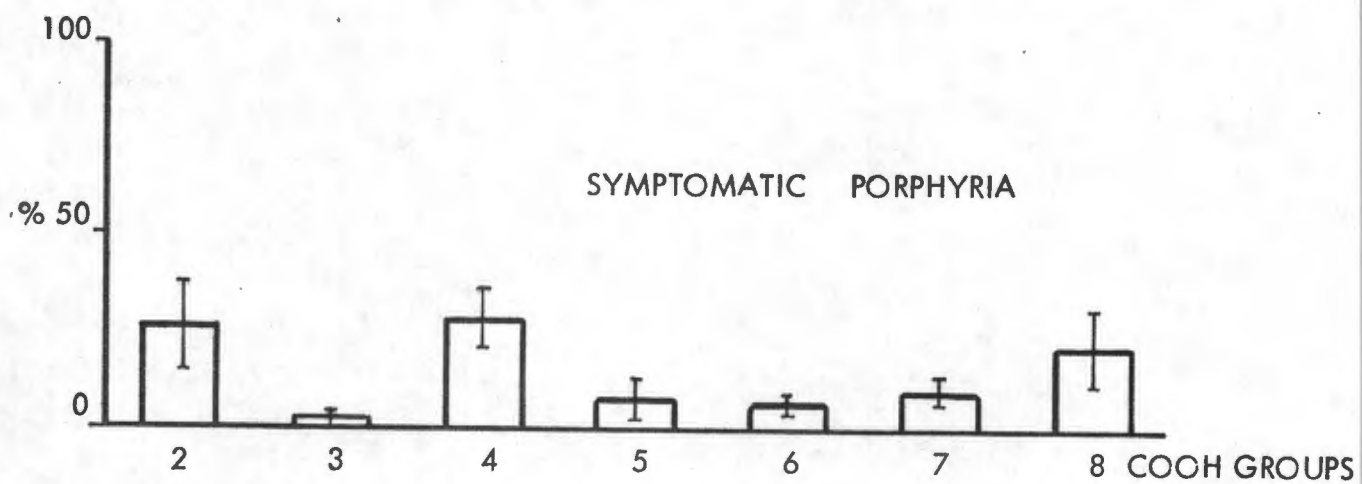
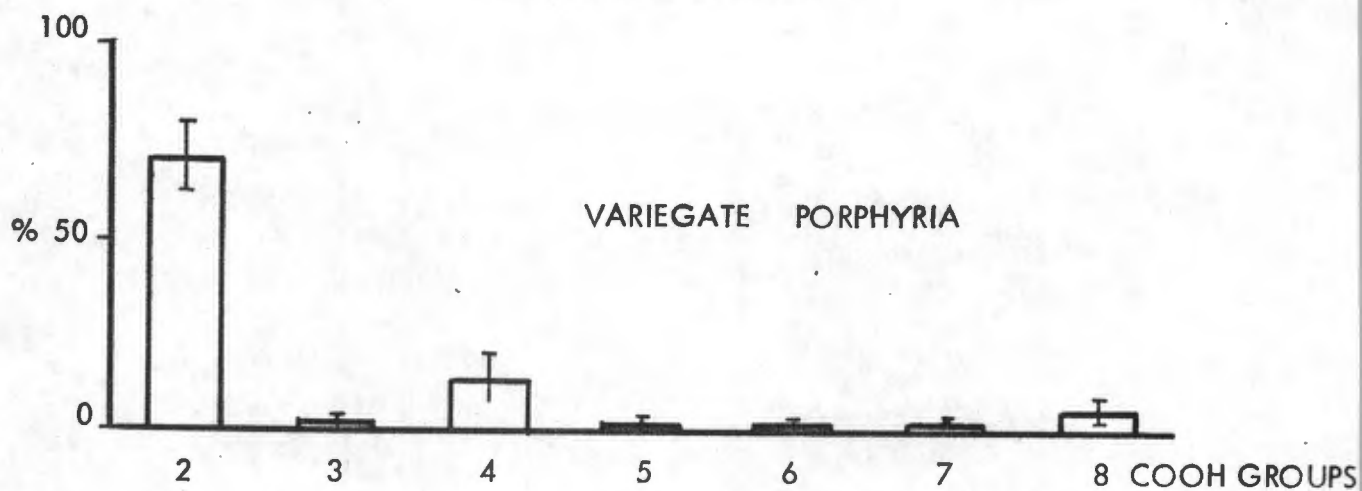
The 4 COOH porphyrin of the symptomatic porphyrics may contain some of Elder's porphyrin 1. (1971). His two-dimensional system was not applied in these studies.

A 3 COOH porphyrin was noted in many of the chromatograms more especially amongst the scans of the variegate porphyria where 75% exhibited the presence of this porphyrin. Chu and Chu (1967) and Doss (1969) noted its presence in some of their chromatograms, and Eriksen (1962) was able to demonstrate that a certain fraction of the 3 COOH porphyrin is a coproporphyrin precursor, but the remaining fraction was unstable and easily lost, a fact which we have likewise noted.

Another interesting point illustrated here is the consistent presence of uroporphyrin, i.e. 89% of all cases studied showed uroporphyrin, the upper limit (2 SD) of the 22 normals studied being $30 \mu\text{g/g}$. In symptomatic porphyria every specimen contained UP but the total amount present never exceeded $80 \mu\text{g/g}$. A wider range was seen in variegate porphyria where a value of $1264 \mu\text{g/g}$ was seen in a case during remission (ALA 1.7 mg/l and PBG 5.0 mg/l), but this is an unusual finding and values normally range between $0 - 270 \mu\text{g/g}$ for VP.

Fig. 10.

FAECAL PORPHYRINS

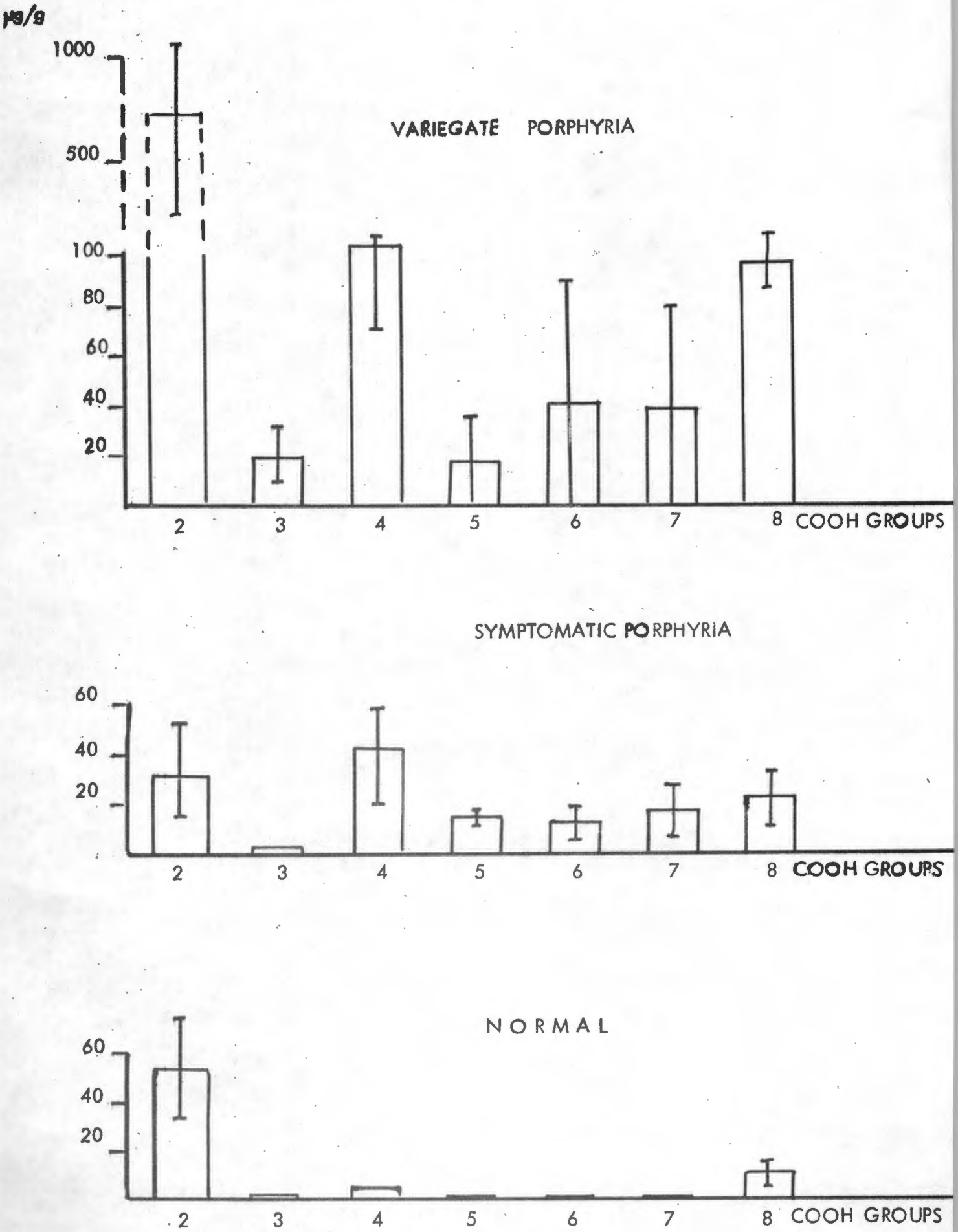


The individual porphyrins have been expressed as a percentage of the total porphyrins

Fig. 11

FAECAL PORPHYRINS

Quantitative pattern of 2 - 8 carboxyl porphyrins



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The majority of other workers who have noted the presence of uroporphyrin in the faeces have found lower values than these (Eales and Dowdle, 1968; Chu and Chu, 1967; Herbert, 1966, Aziz and Watson, 1969; Grinstein et al., 1945; Swartz et al., 1960; Balfour, 1956; Taddeini and Watson, 1968). Watson (1960) reported a case of symptomatic porphyria where the faecal uroporphyrin excretion was three times that of the coproporphyrin and he emphasized the necessity of determining the ether insoluble porphyrins. Such cases were also observed here.

It would seem that the preferential mode of excretion of uroporphyrin is via the urine. Rimington (1963) was able to demonstrate that 80-90% of uroporphyrin introduced intravenously is found in the urine and is not taken into the hepatic cells from the sinusoidal blood. Fischer (1923) gave uroporphyrin orally and found it unchanged in the faeces. In symptomatic porphyria where hepatic ALA synthetase activity is increased (Dowdle et al., 1967) with overproduction of uroporphyrin and the intermediate carboxyl porphyrins the excess of porphyrins are eliminated via both the bile and the kidneys. The fact that the 8 and 7 carboxyl porphyrins are excreted in the oxidised state and that the 4, 5 and 6 carboxyl porphyrins are excreted in the reduced state (Herbert, 1966) rather supports the idea of Heikel et al. (1958) that the increased rates of oxidation are responsible for the excretion of the intermediate porphyrins. It still remains to be shown whether there is impairment of cell permeability. Whether intestinal reabsorption occurs or not has not been conclusively determined and Stathers (1968) points out that absorption of porphyrins is not expected as a result of size and pH of the intestinal porphyrins, but Neubauer (1900) was able to demonstrate the absorption of hematoporphyrin by the intestine, but possibly a special mechanism exists for hematoporphyrin absorption. This will be discussed in a later section.

I think it may be conclusively stated that the thin layer chromatography scans of the faecal porphyrins show a sufficiently distinctive pattern as to aid differential diagnosis of the two forms of porphyria studied.

SECTION II

SECTION II

THE HYDROPHILIC PORPHYRIN - PEPTIDE COMPLEXES.

Chapter 3.

THE ORIGIN OF PORPHYRIN "X".

The presence of hydrophilic porphyrins other than uroporphyrin was first detected in cases of variegate porphyria associated with jaundice. Several workers have detected the presence of unusual hydrophilic porphyrins (MacGregor et al., 1952; Merkelbach, 1943; Sweeney, 1963; Gray and Rimington, 1947). Gray et al. (1947) found an ether insoluble porphyrin in the urine and after esterification obtained an unstable porphyrin methyl ester with an absorption spectrum in chloroform of 623.5, 573.2, 536.6, 503.0, which differed from that of uroporphyrin. He considered the possibility of a coproporphyrin-protein complex. This hydrophilic porphyrin might well have resembled that described by Sweeney in 1963. Sweeney found an ether insoluble porphyrin which on chromatography appeared to be a mixture of porphyrins containing from 2 to 4 carboxyl groups and suggested that under such abnormal conditions the excess protoporphyrin and coproporphyrin which could no longer be excreted via the bile may be conjugated as glucuronides becoming water soluble.

In 1963 we observed hydrophilic porphyrin fractions on our thin layer chromatograms. In isolating these hydrophilic porphyrins it was noted that the molecular weight was significantly greater than that of the free porphyrin and that the isolated product was ninhydrin positive, suggesting the possibility that we were dealing with a porphyrin-peptide complex. The procedures normally used for the isolation of faecal and urinary porphyrins, such as column chromatography using Al_2O_3 and isoelectric precipitation etc., did not give satisfactory separation of the hydrophilic porphyrins and methods normally used for the separation of peptides

proved more appropriate.

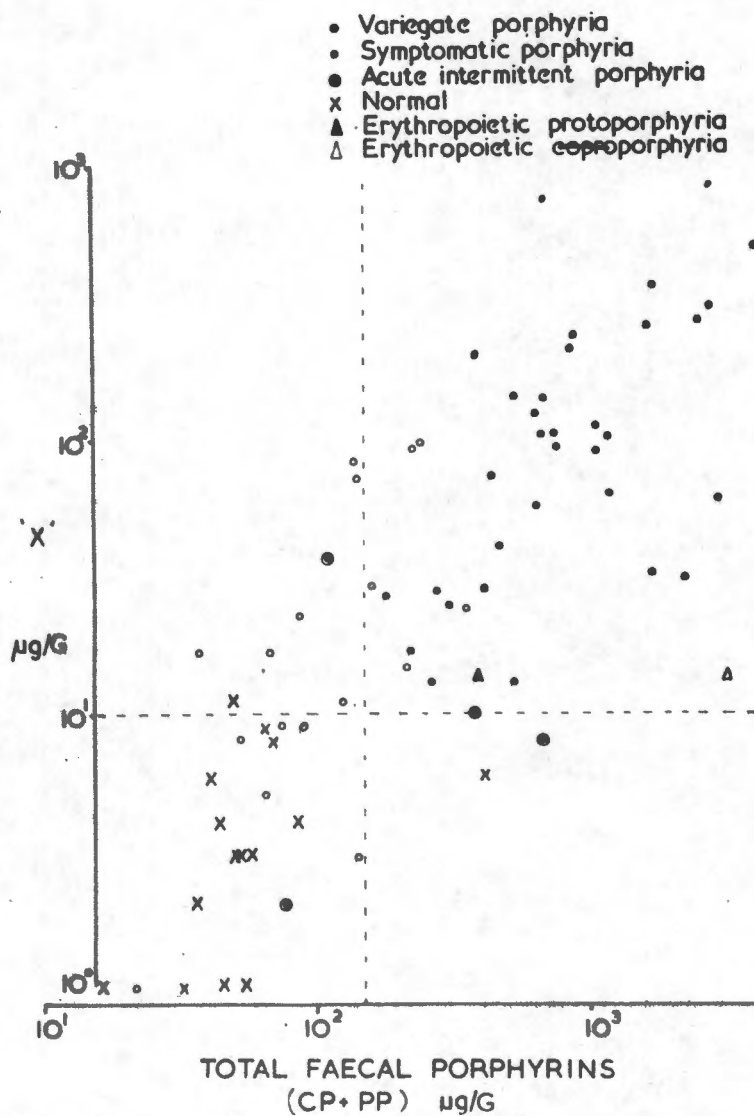
Rimington has exhaustively investigated the characteristics of the hydrophilic porphyrins. His coworkers and others in this field, notably Belcher, Smith and Elder have used solvent extraction procedures which, as discussed earlier, have certain disadvantages; nevertheless the values obtained are sufficiently consistent for comparative and diagnostic investigations.

More sophisticated methods of purification are required for structural analysis. Belcher (1969) has carried the solvent extraction procedures further and applied counter current analysis and has demonstrated the close similarity between the biliary and faecal porphyrins. This excluded the possibility that the hydrophilic porphyrins are exogenously produced in the intestine by bacterial decomposition. Also in favour of an endogenous origin is that bile pigments and vinyl substituted porphyrins are normally reduced by micro-organisms and not hydrated (Elder, 1970). Furthermore, following the administration of C^{14} labelled ALA it was possible to show radioactivity in the hydrophilic porphyrins and thereby demonstrate that they formed part of the porphyrin biosynthetic pathway.

One of the possibilities was that the levels of hydrophilic porphyrins in urine and faeces were merely reflections of the levels of copro and protoporphyrin, but evidence against this theory is that the hydrophilic porphyrins are not increased in the excreta of normal subjects, nor in patients with erythropoietic porphyria, and only reach really high levels in variegate porphyria. In a scatter diagram of "X" plotted against total porphyrin (fig. 12) a straight line could possibly be drawn if all the various types of porphyria are represented on the same graph, but if one considers each group separately, the normals almost exclusively occupy the lower left quadrant, showing no correlation amongst themselves between "X" and total porphyrin. The cases of variegate porphyria occupy the upper right quadrant exclusively and likewise show no correlation between "X" and total porphyrin present.

Fig. 12

THE RELATIONSHIP BETWEEN THE TOTAL
FAECAL PORPHYRINS AND FAECAL
PORPHYRIN 'X' VALUES



Whether porphyrin and peptide are only loosely associated with the formation of ionic or hydrogen bonds or whether true covalent or dative bonding exists needs to be established because if covalently bound, there is the possibility that the hydrophilic porphyrin may be involved in either the catabolic or anabolic processes of cytochrome c.

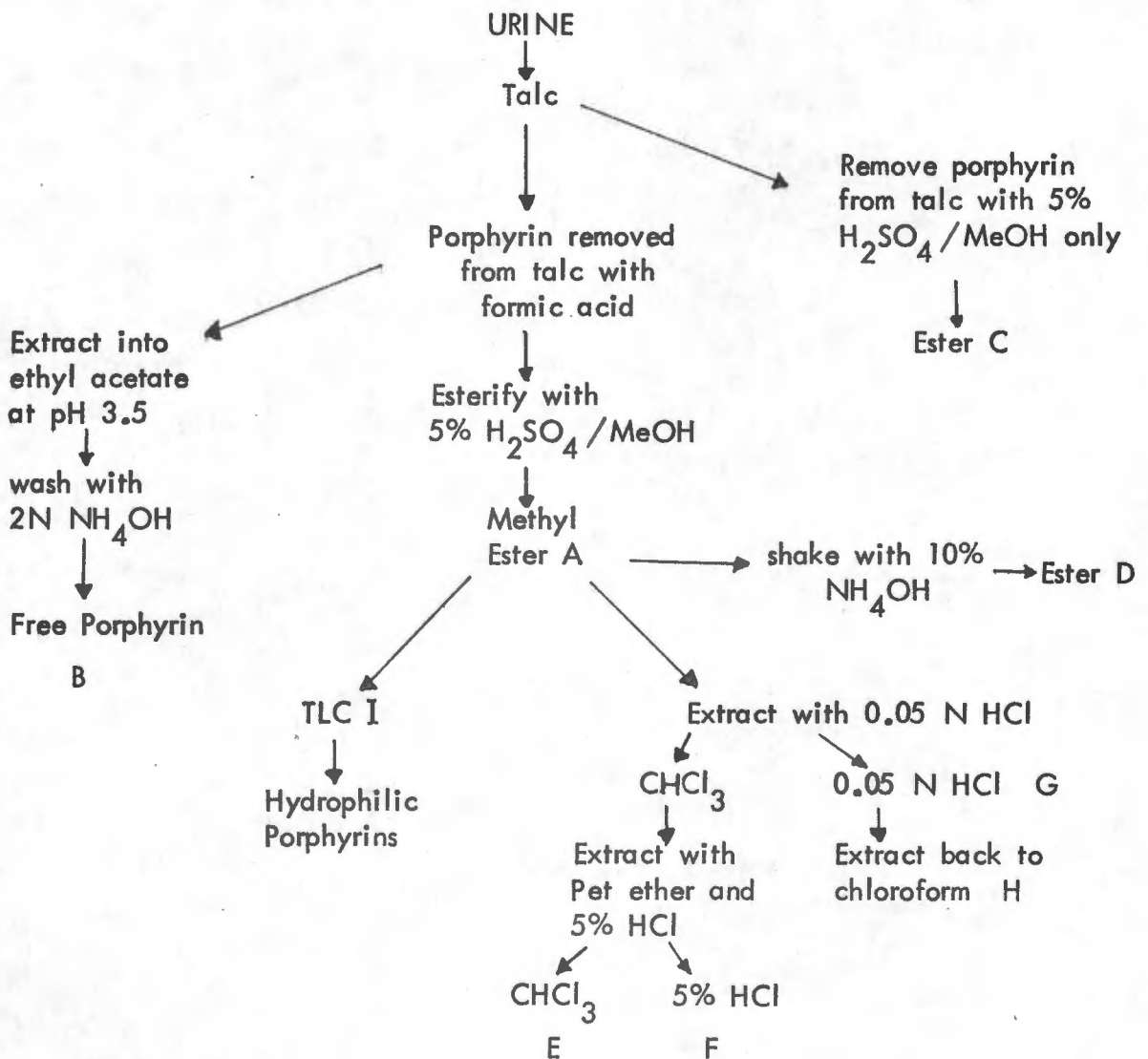
Rimington (1968) suggested that porphyrin "X" (his collective term for the hydrophilic porphyrins) may be the protein bound intermediate observed in the enzymic transformation of copro III to proto IX, involving coproporphyrinogenase.

Part of the preliminary work was to repeat the scheme proposed by Rimington and Lockwood (1966) and Rimington et al. (1968) for the isolation of porphyrin "X". It has been possible to confirm that our material is similar to the porphyrin "X" reported in these papers. This work was followed by a quantitative survey of porphyrin "X" in the hope that it might be of diagnostic value. The measurement and quantitation of an unknown group of substances is highly unsatisfactory and therefore attempts were made to purify the material as far as possible and to obtain some information on its structure.

Initial Extraction Scheme according to the method of Rimington and Lockwood (1966)

An abbreviated summary of the methods employed is shown in the following flow diagram; individual procedures have been given in appendix I.

FIG. 13 EXTRACTION SCHEME ACCORDING TO RIMINGTON AND LOCKWOOD (1966)

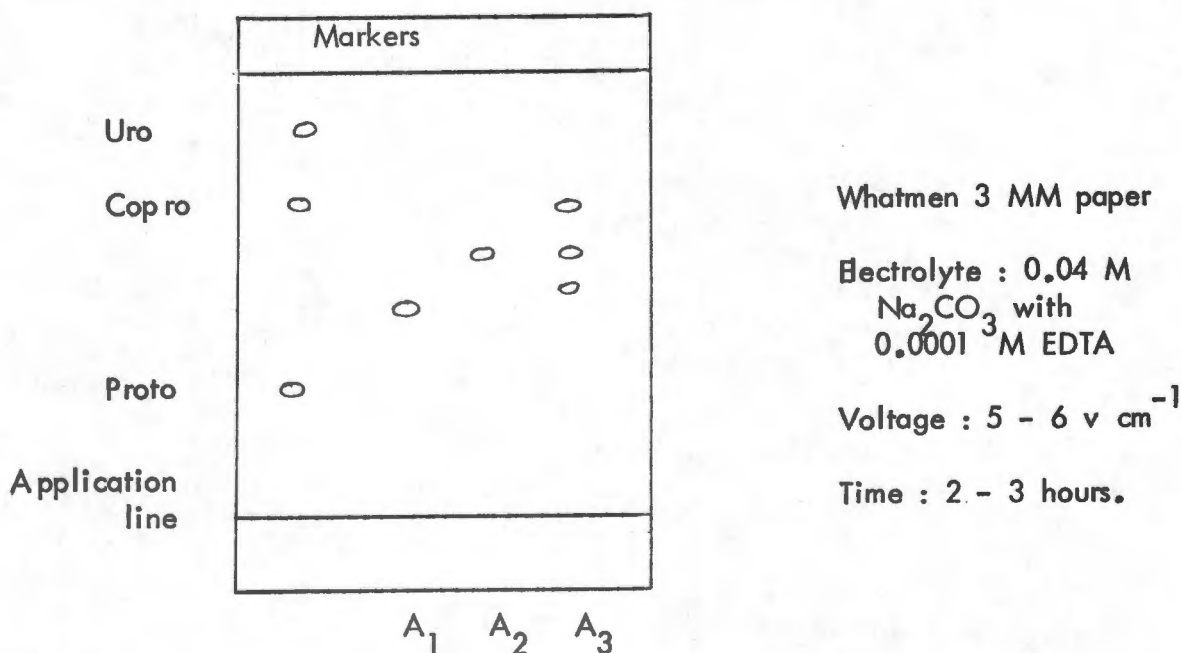


with 10% MeOH/CHCl₃. These fractions now designated A₁, A₂, A₃ were then run in several other systems (figs. 15 to 21).

It was first necessary to establish that no free porphyrins remained in these fractions. Therefore the material was re-esterified and run in the same system again. Three consistent bands were still found in the same positions as before and a small amount of the material now ran between the 4 and 8 carboxyl porphyrin markers, therefore showing only a minor amount of unesterified material.

Electrophoresis was performed on fractions A₁, A₂, A₃, after they had been hydrolysed (fig. 15).

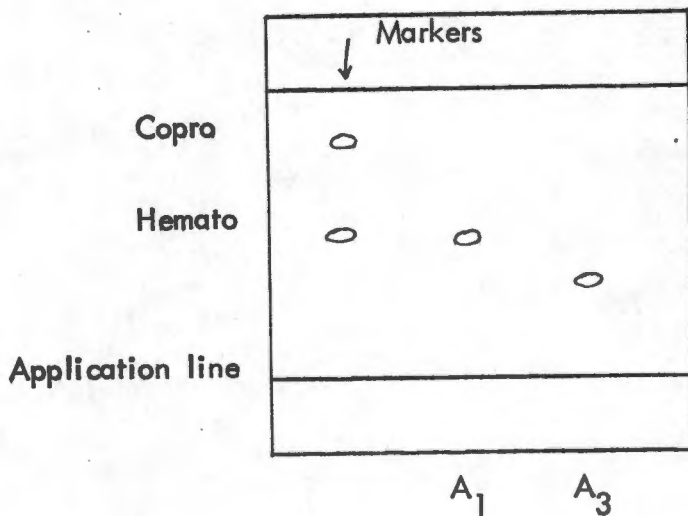
FIG. 15. ELECTROPHORESIS (LOCKWOOD AND DAVIES, 1962)



Fractions A₁, A₃ were applied to paper and chromatographed according to Chu, Green and Chu (1951), fig. 16.

FIG. 16

PAPER CHROMATOGRAPHY.



Paper : Whatman No. 1

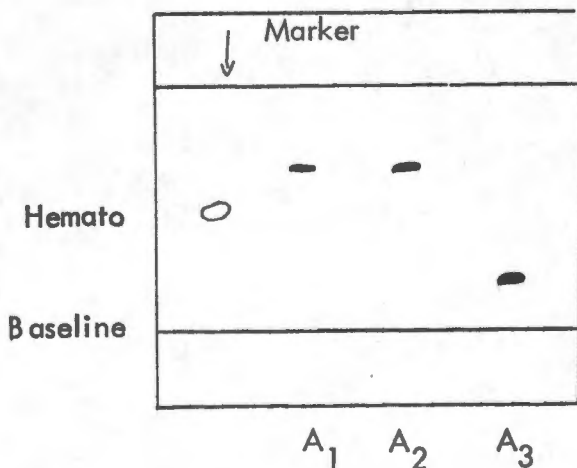
Solvent : kerosene : 35
chloroform : 30
n-propanol : 2

Time : 3 hours.

Since one fraction - A₁ corresponded to the hemato marker, they were run in another system (fig. 17)

FIG. 17

TLC FOR THE SEPARATION OF DICARBOXYLIC PORPHYRIN
(HENDERSON AND MORTON, 1967).



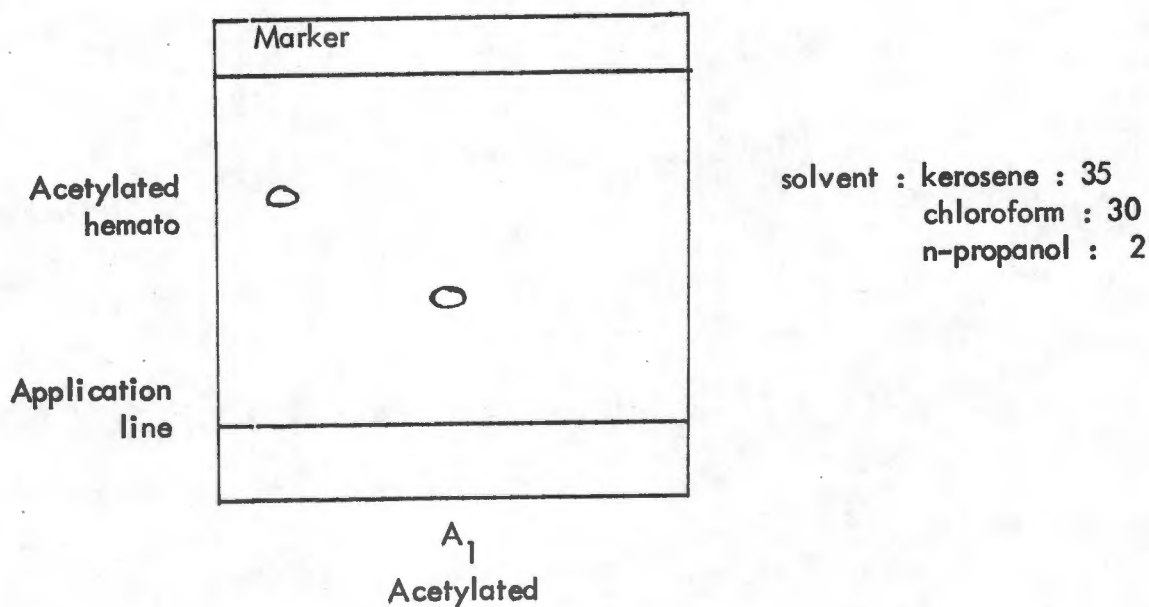
TLC : silica gel G
slurried in 0.3% (w/v)
FeSO₄ - 7 H₂O

Solvent : Benzene 100
Methanol 5

Fraction A₁ was acetylated and subjected to paper chromatography (fig. 18)

FIG. 18

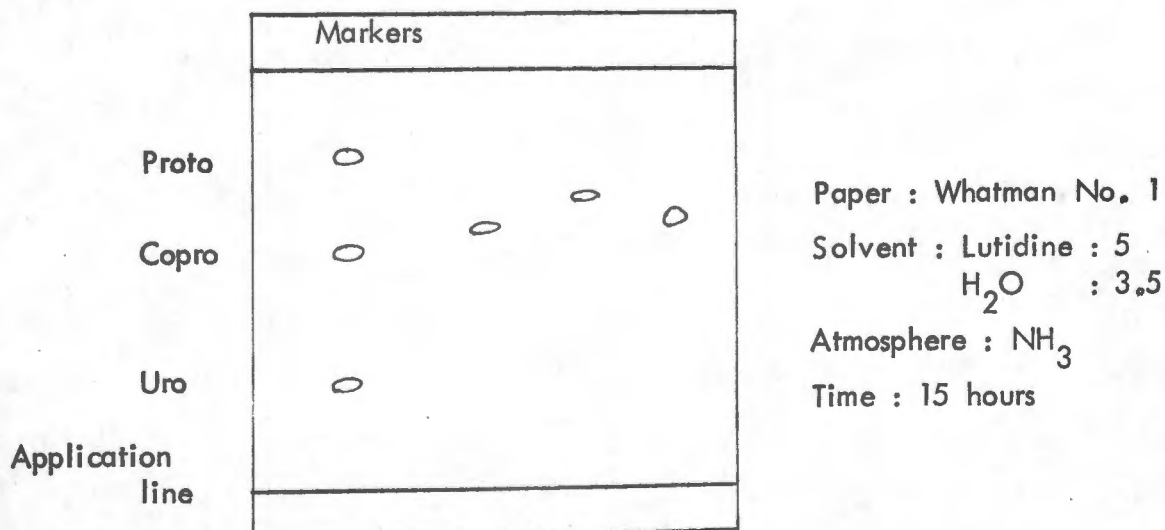
PAPER CHROMATOGRAPHY OF AN ACETYLATED URINARY HYDROPHILIC PORPHYRIN (CHU, GREEN AND CHU, 1951).



Fraction A₁ and A₃ were hydrolysed with 7.5 N HCl. A small amount was taken for chromatography in Eriksen's system (1953) and the remainder extracted with ether at pH 3.5. The pH of the remaining solution was then raised to 4.5 and the porphyrin extracted into cyclohexanone and then into 1.4 HCl. This was analysed by paper chromatography (fig. 19).

FIG. 19

PAPER CHROMATOGRAPHY OF THE ACID SOLUBLE HYDROPHILIC PORPHYRIN.

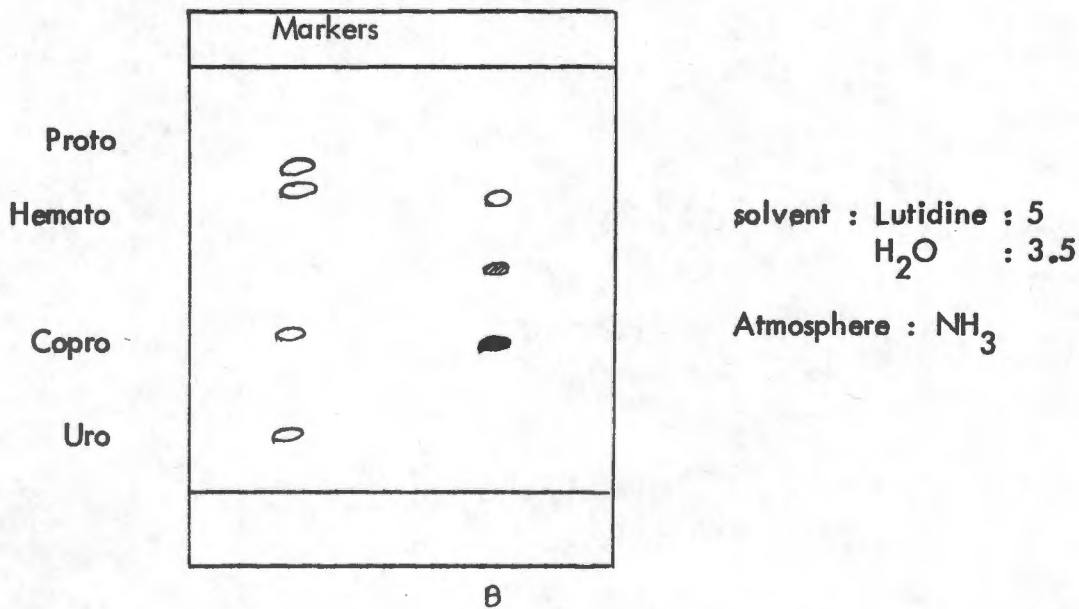


These investigations showed the presence of a hydrophilic porphyrin behaving like a 3 carboxyl porphyrin in several systems but which could not be positively identified with any of the standard markers.

To return to the flow diagram (fig. 13).

(ii) B was chromatographed in the system for free porphyrins (fig. 20).

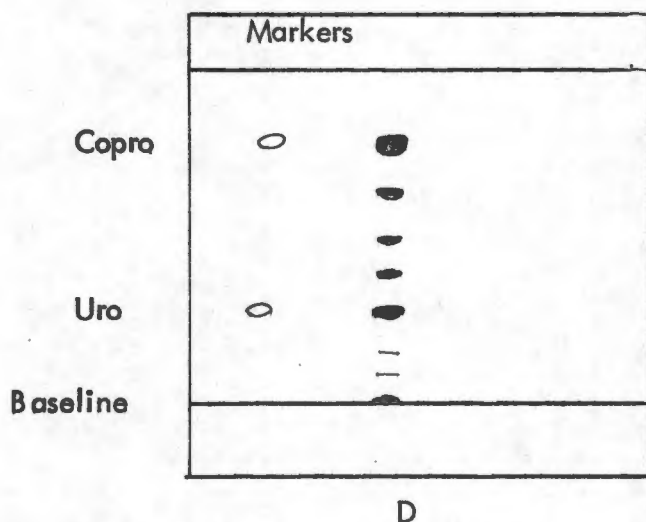
FIG. 20 PAPER CHROMATOGRAPHY (ERIKSEN, 1953).



(iii) D (see fig. 13) was chromatographed in a system for porphyrin esters (fig.21).

FIG. 21

PAPER CHROMATOGRAPHY OF THE FRACTION OF THE
PORPHYRIN M.E. THAT IS EXTRACTED BY 10% NH₄OH
(CHU, GREEN AND CHU, 1951)



The fractions between the baseline and uroporphyrin were not completely removed by the NH₄OH.

(iv) A (see fig. 13) was extracted with 0.05 N HCl. This acid extract showed a *soret* peak at 400 m μ ; this was extracted back into chloroform and when chromatographed on TLC (Grosser et al., 1967) the porphyrin remained on the baseline and did not move. After the initial extraction with 0.05 N HCl, the remaining chloroform was extracted with petroleum ether and 5% hydrochloric acid and, similar to the 0.05 N HCl fraction, this likewise showed the hydrophilic porphyrins only.

These preliminary studies have all shown the presence of hydrophilic porphyrins, Rimington and Lockwood (1966) have identified one of their fractions as hematoporphyrins, but we were unable to detect any free hematoporphyrin in the urine under study; the fractions which corresponded to the hematoporphyrin markers in figs. 16 and 20 were shown to behave differently in other systems (figs. 17 and 18)

and are therefore not free hematoporphyrin; the possibility of hematoporphyrin being bound to a protein moiety and being released during manipulation had to be considered.

In a later paper (Rimington et al., 1968) he systematically worked through a complicated ramifying system in an attempt to identify porphyrin "X". He divided the urinary and faecal ether insoluble porphyrins according to their solubilities in acid or alkali solutions; the former he considered to be a smaller molecule than the latter. For the main part we were able to reproduce the same results, but with variations in certain respects which have been summarised in figs. 22 and 27 in order to facilitate comment on the method employed.

(a) URINARY EXTRACTS.

(i) Quantitation of Porphyrin "X".

Scheme I (fig. 22) is an outline of the method adopted for routine quantitative investigations of porphyrin X. We have adhered to this explicitly in order that our results might be compared with those of other workers. Chapter 5 contains a quantitative survey performed using this method for the urine, and scheme III of fig. 27 for the faeces.

The following comments on scheme I for the urine are relevant as follows :-

1. It was found that the sodium acetate washings did not contain hydrophilic porphyrins and therefore it was found not necessary to add these to the aqueous extraction remaining after ether extraction.
2. The division between uroporphyrin and porphyrin "X" is very crude, each fraction contained other porphyrins.

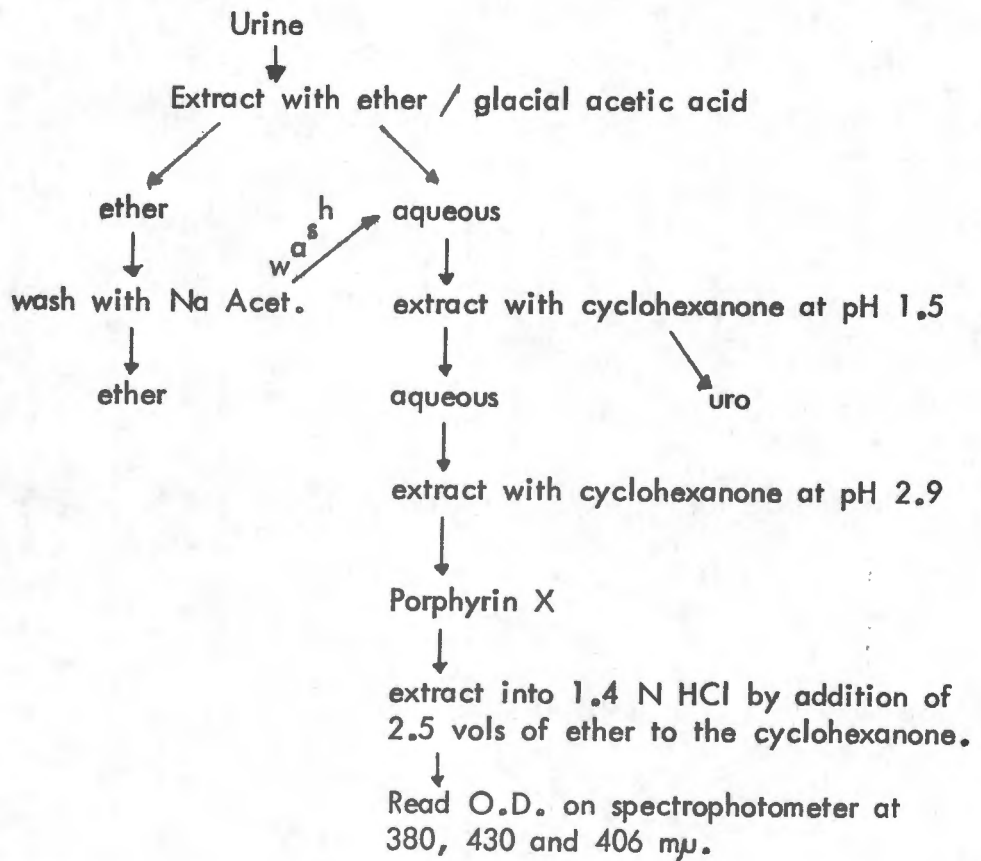
In another paper of Rimington and Benson (1967) porphyrin "X" was extracted first, using 50% saturation of sodium acetate and pH 4.5.; it was found that 60% of the porphyrin "X" was extracted into the cyclohexanone and only 5% of the

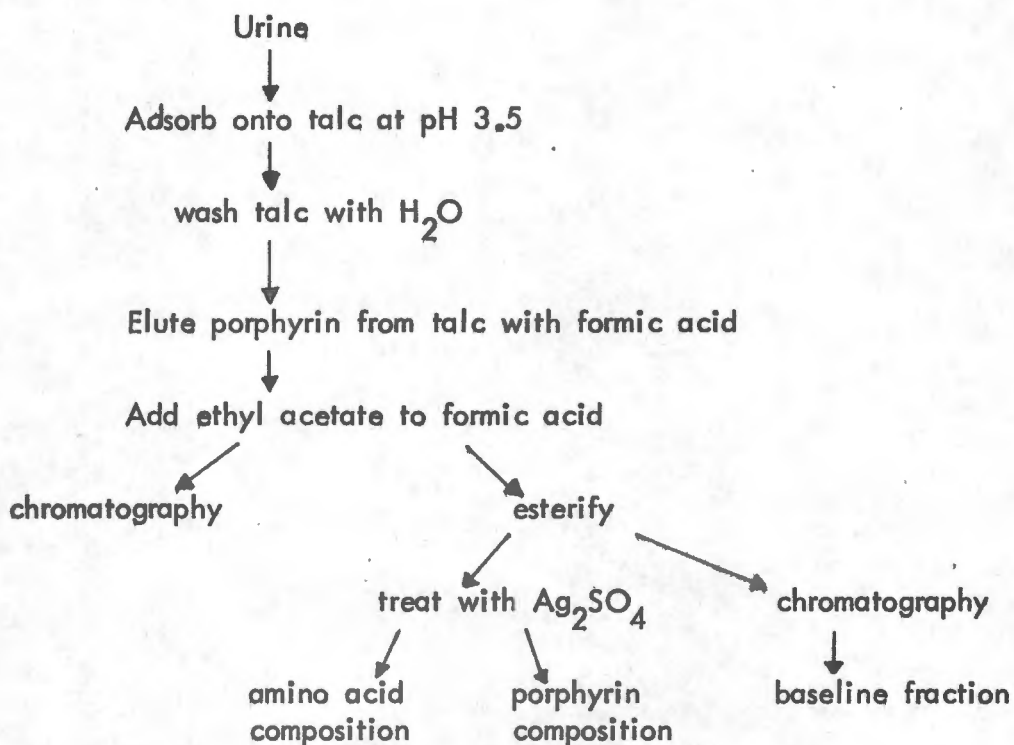
uroporphyrin. For uniformity of results scheme I mentioned in fig. 22 was adhered to in this study; it is not claimed in any way to represent a true quantitative measurement of the hydrophilic porphyrins but merely a reproducible reliable method by which to obtain comparable results.

FIG. 22 METHODS FOR THE EXTRACTION OF THE HYDROPHILIC PORPHYRINS.

(System according to Rimington et al., 1968).

SCHEME I

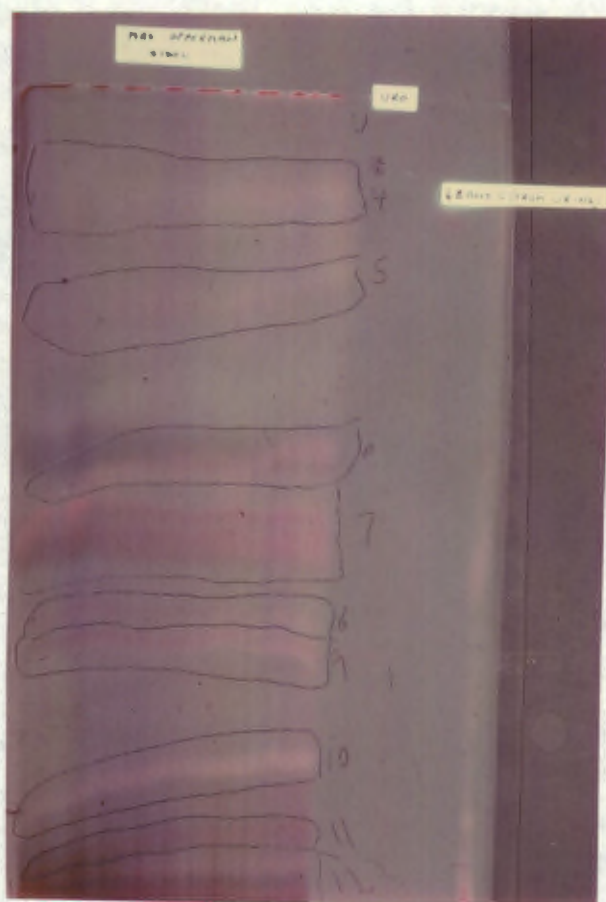


SCHEME II(ii) Qualitative Recovery of Porphyrin "X".Scheme II (fig. 22).

This method was used for the recovery of the hydrophilic porphyrins from large quantities of urine. Absorption of porphyrins onto the talc is complete, but a major problem arises in trying to elute the porphyrins off the talc. Rimington recommended the use of formic acid which in most cases removes 95% of the porphyrins, but a small amount is always left behind. We found the use of acetone/HCl 0.5 N (7 : 3) followed by trifluoroacetic acid to be more efficient. The washing of the talc prior to elution must be carried out with a minimal amount of water, since some of the hydrophilic porphyrins are easily lost at this stage, but if quantitative recovery is not essential, then the talc may be washed with several litres of

distilled water in order to remove the free peptides present in the urine. The urinary amino acids are not adsorbed by the talc. Esterification of porphyrin "X" results in many fractions and it has been found possible to separate up to twenty bands on TLC (Fig. 23).

FIG. 23. ESTERIFIED HYDROPHILIC PORPHYRINS RUN ON A SILICA GEL G PLATE.



Solvent : Benzene : 85
 Ethyl Acetate : 13
 Methanol : 10

The multiplicity of fractions is probably due to varying stages of esterification of the peptide moiety of the molecule, apart from the possibility of there being molecules where the lengths of the peptide chains differ from each other. From later studies it would appear that the porphyrin moiety is a constant component.

The identification of the amino acids present after treatment with

61

Ag_2SO_4 was found to be very similar to that of the free peptides present in urine. (TABLE 16)

It was also found that the free peptides were readily extracted along with the hydrophilic porphyrins in this scheme. It was clear that amino acid analysis without further purification was unsatisfactory.

(b) FAECAL EXTRACTS.

The scheme for faecal extraction according to Rimington et al. (1968) (Fig. 24) requires comment.

(i) Quantitation of Faecal Porphyrin "X".

Scheme III has been used for routine investigation of the hydrophilic porphyrins again for comparative reasons. The acid soluble fraction may not always be a true reflection of the relative amount of porphyrin "X" present, since the primary residue fraction varies in each specimen and has on one occasion exceeded that of the acid soluble fraction. Also the porphyrin extracted by the urea/triton mixture is not always completely soluble in the butanol and therefore varying amounts are lost at this stage, too. It was also found that the fraction being measured as porphyrin "X" contained some of the higher carboxyl porphyrins as well.

(ii) Qualitative recovery of Faecal Porphyrin "X".

In Scheme IV (fig. 24) one of the purification steps is molecular sieving. We found the use of Sephadex at this stage to have limited application. The use of G 25, 50 or 100 with the buffers recommended by Rimington et al. (1968) did not afford good separation of the porphyrins. Fig. 25 illustrates the elution profile obtained by using Sephadex G 25 (Rimington, 1968); 2 peaks may be seen but separation is poor and from the pattern displayed by TLC of the fractions, all that can be said is that

TABLE 16.

AMINO ACID ANALYSIS

Relative Amounts.

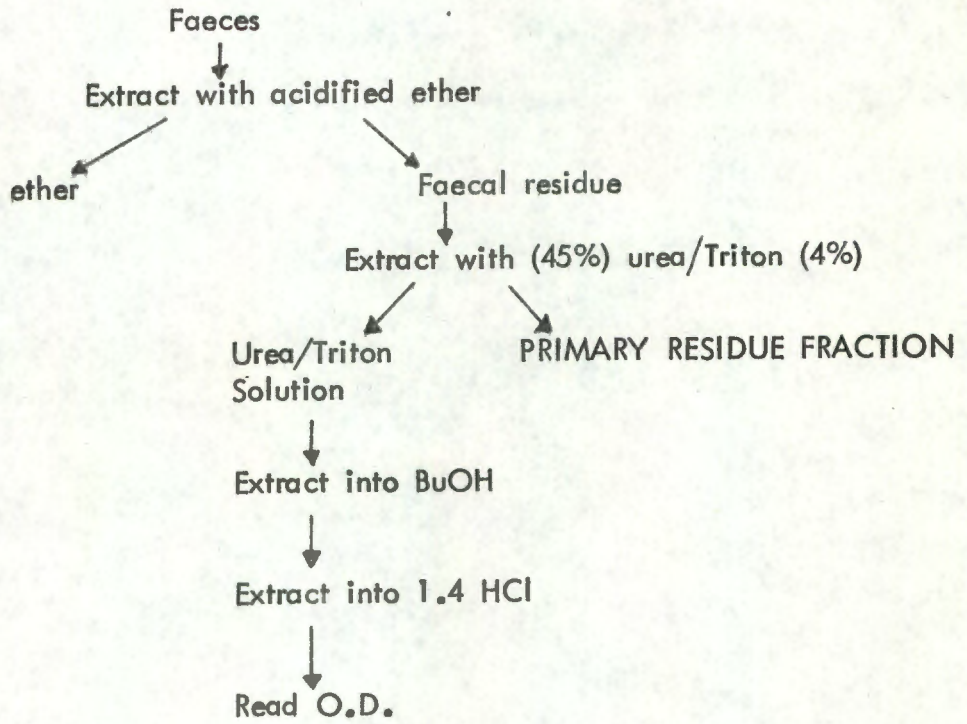
	<u>Free Peptides</u>	<u>Crude Porphyrin Peptide</u>
Asp	0.9	0.9
Thr	0.5	0.4
Ser	0.5	0.6
Pro	0.4	1.0
Glu	1.0	1.0
Gly	1.0	1.0
Ala	0.6	0.5
Val	0.5	0.4
Met	0.1	
Ile	0.3	0.3
Leu	0.6	0.4
Tyr	0.3	
Phe	0.3	0.1
Lys	0.3	0.6
His	0.1	trace
Arg		
CySO ₃ H	+	+

FIG. 24

FAECES

(System according to Rimington et al., 1968).

SCHEME III for quantitative faecal hydrophilic porphyrin analysis.



SCHEME IV for further purification :

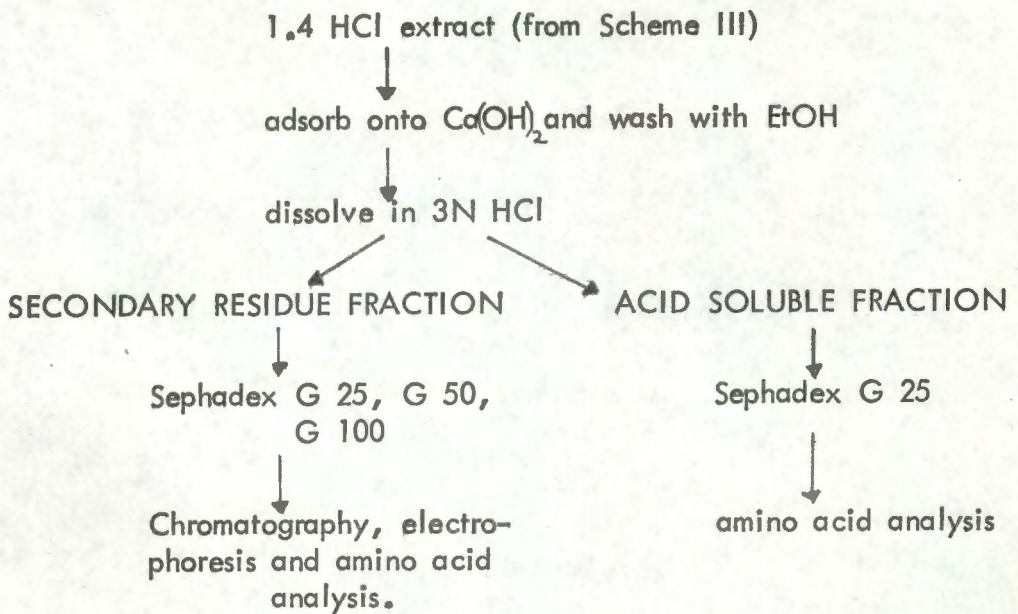
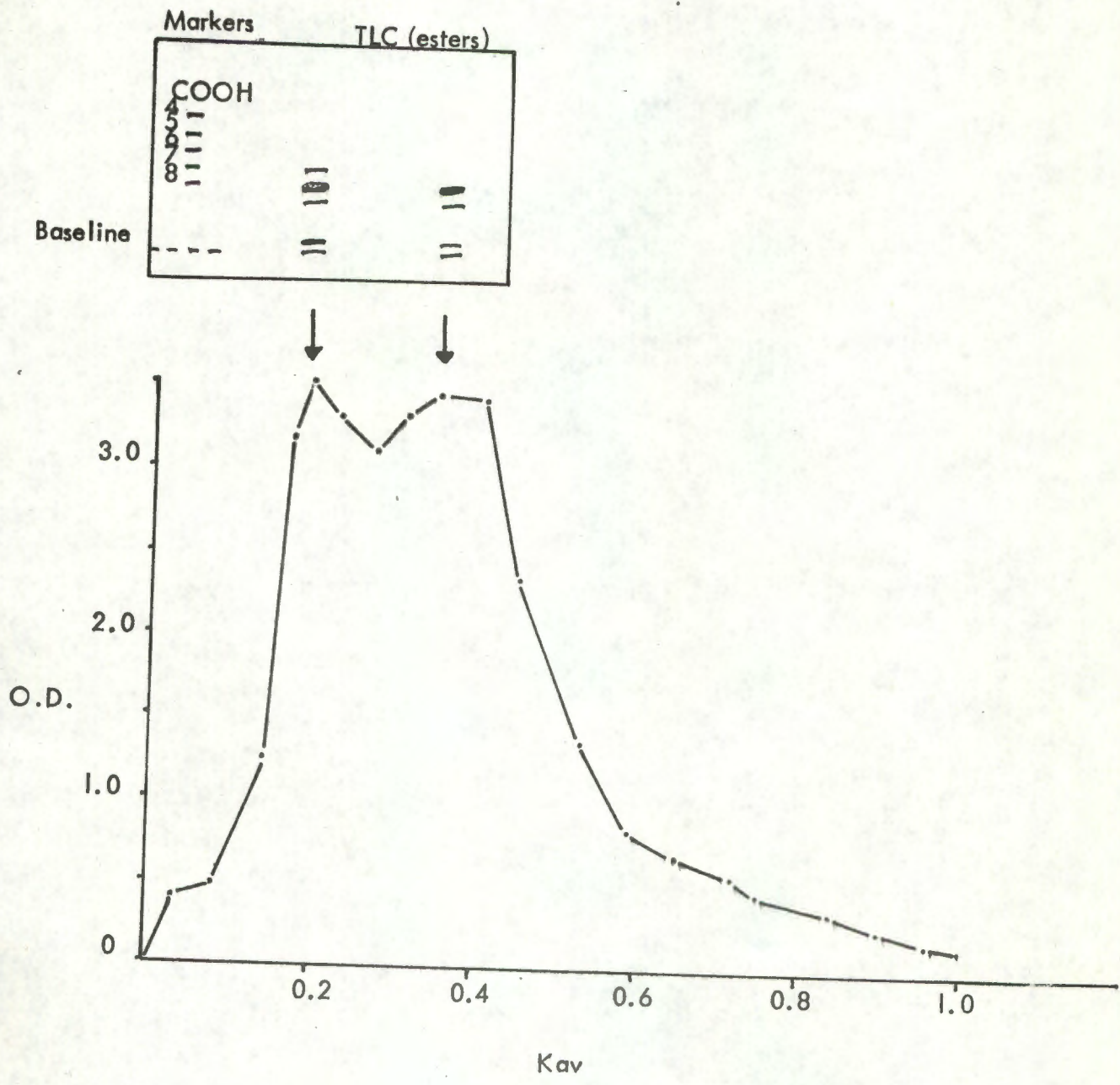


Fig. 25.

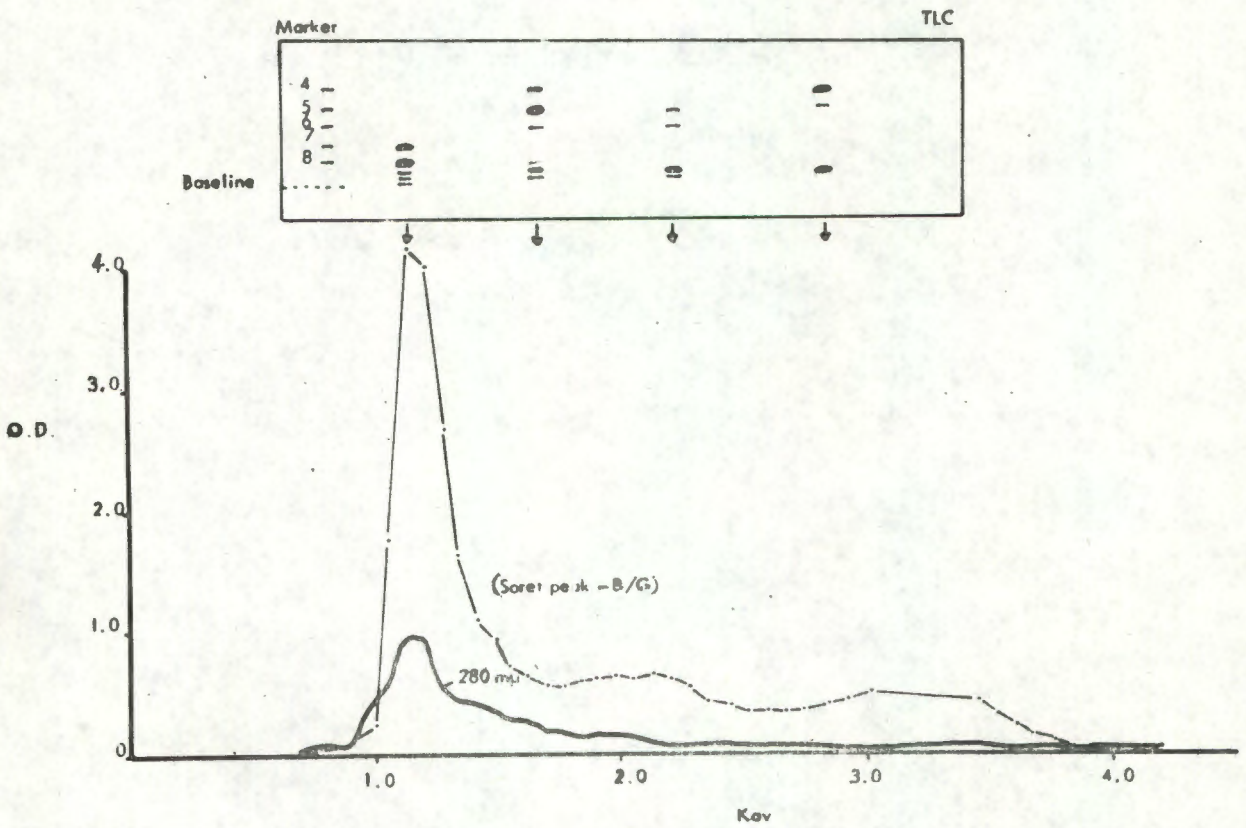
ELUTION PROFILE OF FAECAL PORPHYRINS FROM AN AIP PATIENT



Sephadex G 25 column equilibrated with 0.05 M barbital buffer pH 8.6. The TLC porphyrin patterns of the 2 peaks are shown above the elution profile from the Sephadex Column.

Fig. 26

ELUTION PROFILE OF URINARY PORPHYRINS FROM A CASE OF VARIEGATE PORPHYRIA



Sephadex G100 equilibrated with 0.05M barbital buffer pH 8.6. The elution profile shows the presence of an initial peak absorbing both at 280 mμ and 400 mμ.

a high molecular weight porphyrin is present. The use of Sephadex for urinary porphyrins gave similar results.

Fig. 26 shows one large peak emerging very close to the void volume; this also showed absorption at 280 μ . The fractionation from this column was a little better than the G 50 but still not good. As may be seen from fraction 2 large amounts of 4 and 5 carboxyl porphyrins are present. This is probably due to ionisation of the carboxyl groups at pH 9 with a resulting electrostatic attraction between them and the protein moiety of porphyrin "X". When these bands are run by themselves on the same Sephadex column they are eluted at a later stage corresponding to their correct markers. The absorption at 280 μ may in part be due to protein material being present, but the presence of a dark brown pigment also contributed towards this peak. The use of Sephadex G 25 at a later stage in the purification procedure gave far better results (see section III), but for separation of the crude porphyrin X material from contaminating free peptides and free porphyrins the use of Sephadex LH 20 proved superior.

In this instance the porphyrin extract was prepared as shown in fig. 27.

Figs. 29 and 30 show elution profiles from this column and as may be seen there was a distinctive separation of the hydrophilic porphyrins, copro and proto. Fig. 29 shows the monitoring of a column eluate by means of fluorescence (activation wavelength of 400 and emission wavelength of 600) which is better than the absorption measurements in that the interference by the brown pigment is small. The first peak representing the high molecular weight fraction was pooled, evaporated to dryness and then rerun on Sephadex LH 20, using MeOH/CHCl₃ (9 : 1) for elution. This separated off more impurities, namely yellow and pink pigments; the peaks from this

run were then pooled and subjected to Ag_2SO_4 - treatment to release any bound porphyrin. These fractions were then again chromatographed (fig. 31) where it may be seen that a large amount of coproporphyrin appeared with minor amounts of 6, 7 and 8 carboxyl group porphyrins and hematoporphyrin.

The unbound porphyrins were not adsorbed onto the LH20 Sephadex to the same extent as to the G series Sephadex. The latter is shown in a calibration run on Sephadex G 100 where coproporphyrin has a K_{AV} of greater than 1. (fig. 32). The use of Sephadex columns has been discussed fully in appendix A. (The G series has been used for the separation of porphyrins with free carboxyl groups and the LH20 series for their methyl esters).

This method for the isolation of porphyrin "X" by the use of Sephadex LH20 was used in the procedure for the preliminary identification of amino acids present in the peptide moiety of the porphyrin-peptide complex (fig. 27).

The amino acid composition of the hydrophilic porphyrins from 8 porphyric patients were studied according to the scheme A \rightarrow F (fig. 27). Only one sample was analysed on the automatic amino acid analyser, all the others being analysed by paper chromatography and electrophoresis, due to lack of equipment in the earlier part of this study (fig. 28). The results of the two different methods compared very favourably in the one case studied.

A summary of the qualitative results from the chromatograms is shown in figs. 33 and 34. The urinary porphyrins were adsorbed onto talc and after elution from the talc as described previously, were esterified and the procedure continued the same as for the faeces.

As may be seen from these two figures a relatively consistent pattern

emerges and is comparable with those shown by Rimington (fig. 35).

The clinical status of the individual patients may be deduced from the biochemical data represented in table 17.

These amino acid patterns do not appear to show any striking variations between the acute phase and the remission phase of Variegate Porphyrria. The only difference noted was the increase in serine and aspartic acid seen in both the urine and faeces during the acute attack.

Glutamic acid and Glycine are dominant in all the specimens and Leucine in all the cases except the urine of the variegate porphyria associated with jaundice.

End group amino acid determination by means of the formation of dansyl derivatives was performed on the isolated hydrophilic porphyrins. The dansyl derivatives were chromatographed on TLC. (Morse and Horecker, 1965; Zdenck and Rosmos, 1965; Gros and Labonesse, 1969). Glycine and threonine were detected as the end group amino acids.

TABLE 17.

BIOCHEMICAL DATA OF PATIENTS STUDIED

Patient	Diagnosis	Faeces		Urine				
		Proto ug/G	Copro ug/G	vol. ml.	PBG mg/d	ALA mg/d	Copro ug/L	Uro ug/L
S.v.T.	VP with jaundice	84	43	500	2	3	3324	3131
E.B.	VP acute phase	1996	989	1000	79	45	2927	2276
P.E.	VP remission phase	561	454	1200	5	6	291	64
J.M.	VP acute phase	1336	1198	720	60	8	3357	2936
	20 days later remission phase			520	2	1	280	149
G.S.	SP.	95	129	1400	1	2	96	1704
H.M.	SP	64	56	1960	2	4	145	986
Y.F.	SP	63	191	1340	1	4	245	2590
I.A.	SP	55	46	1470	89	29	733	2085

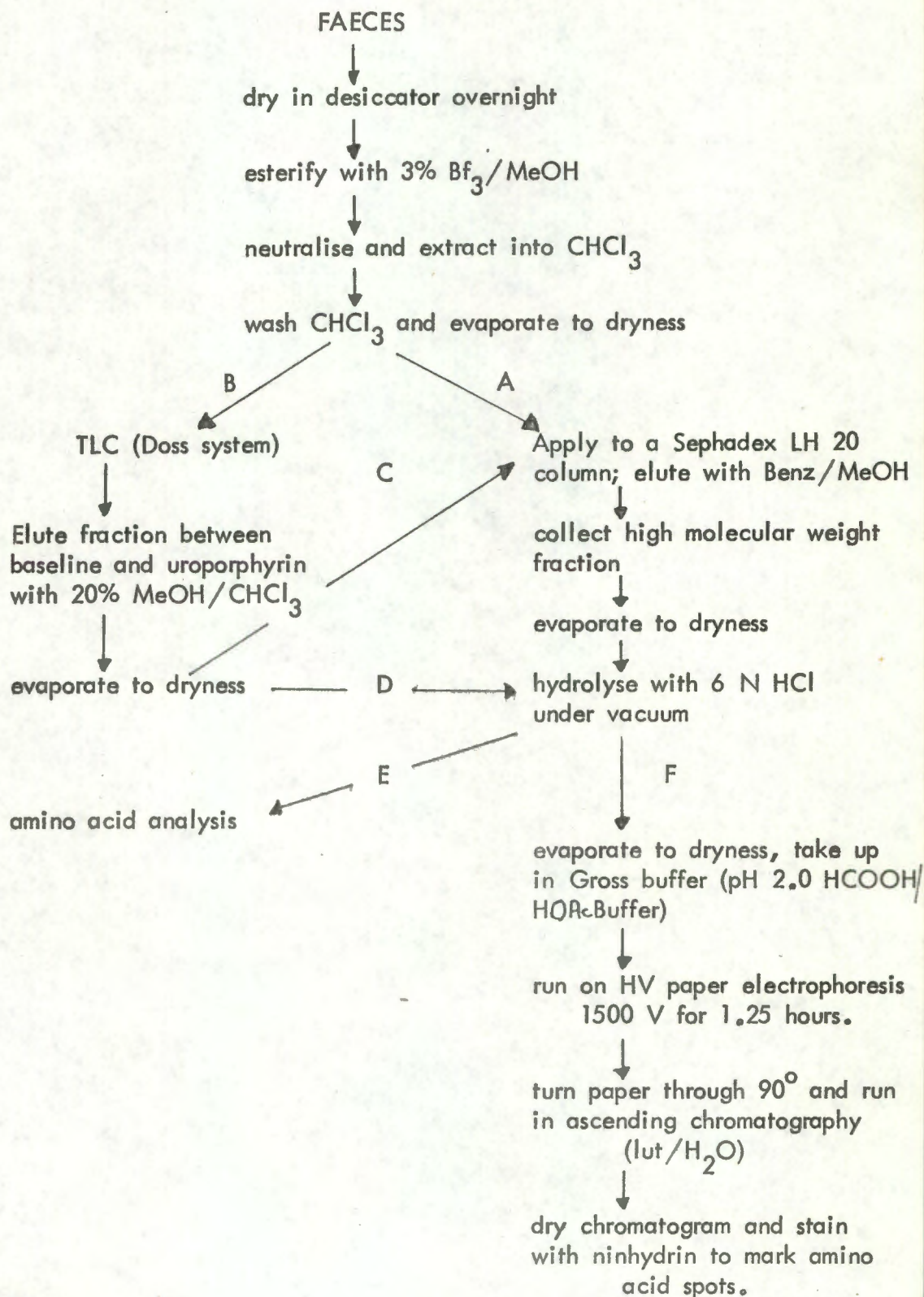
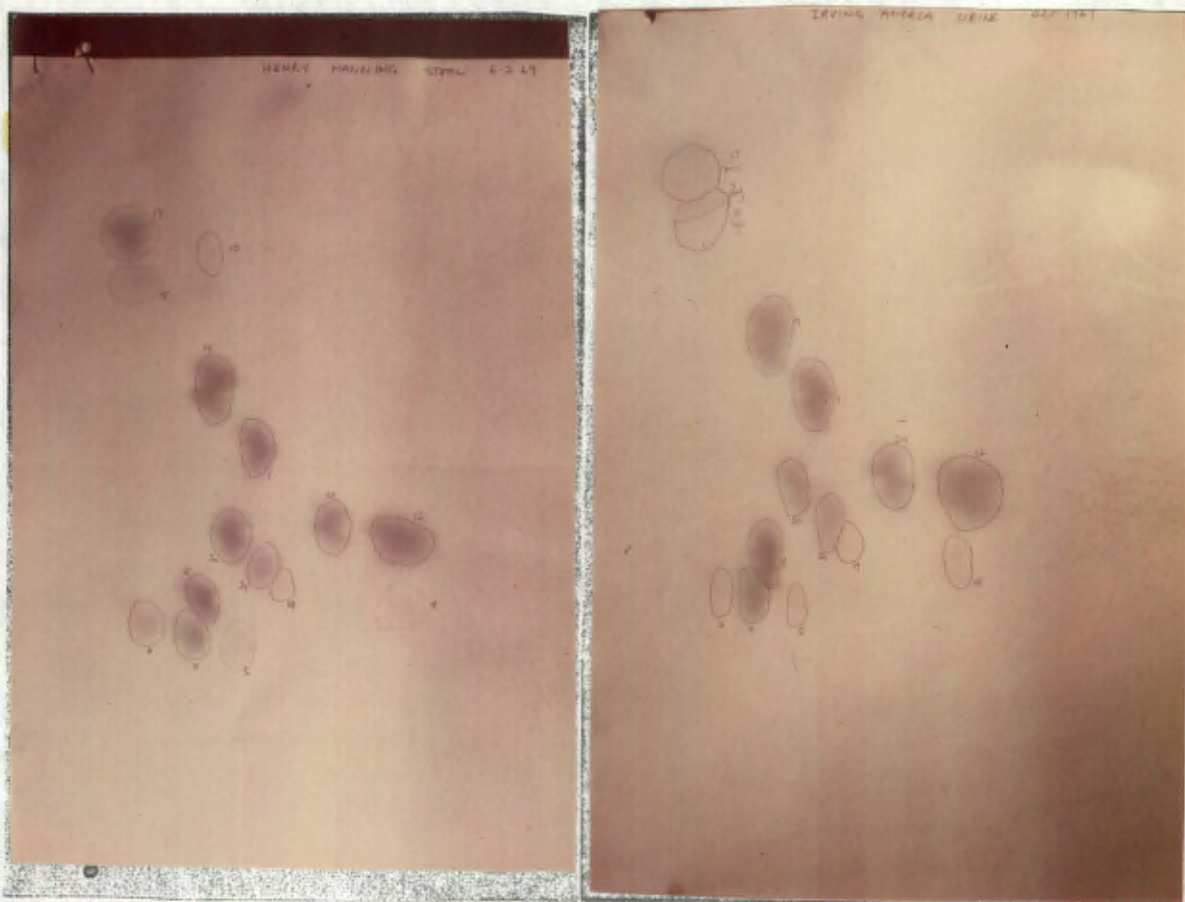


Fig. 28.

THE FINGERPRINT PATTERN OF THE AMINO ACIDS LIBERATED FROM PORPHYRIN 'X' AS EXEMPLIFIED BY PAPER CHROMATOGRAPHY AND H.V. ELECTROPHORESIS.

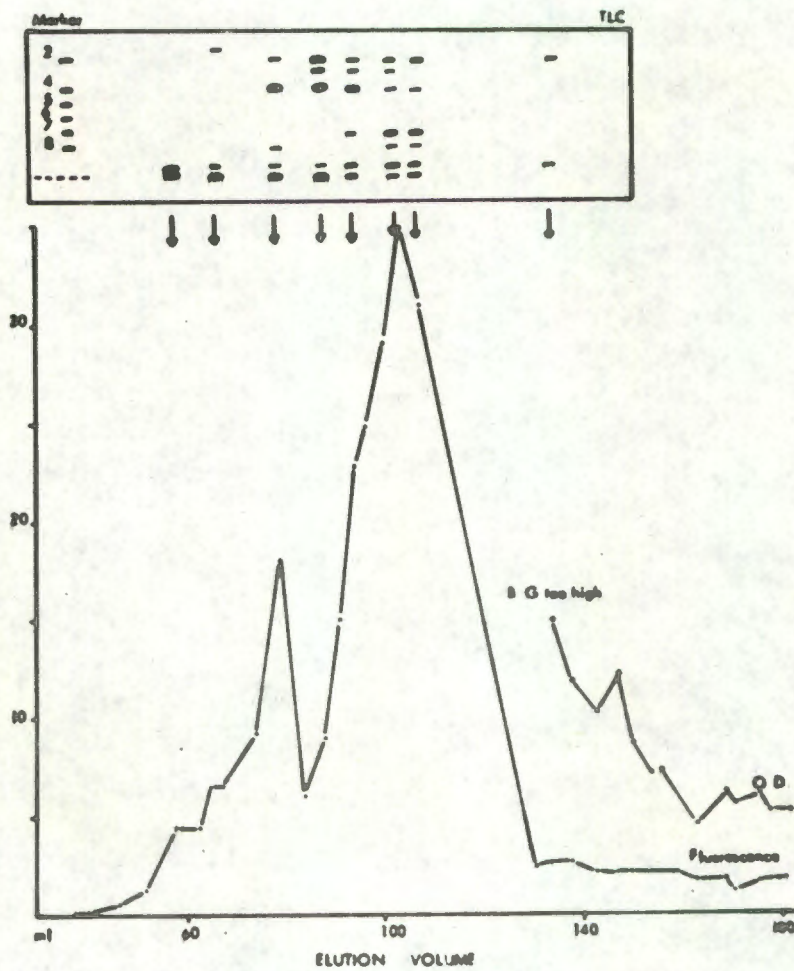


aa pattern of porphyrin 'X' from faeces of a case of SP.

aa pattern of porphyrin 'X' from the urine of a case of SP.

Fig. 29.

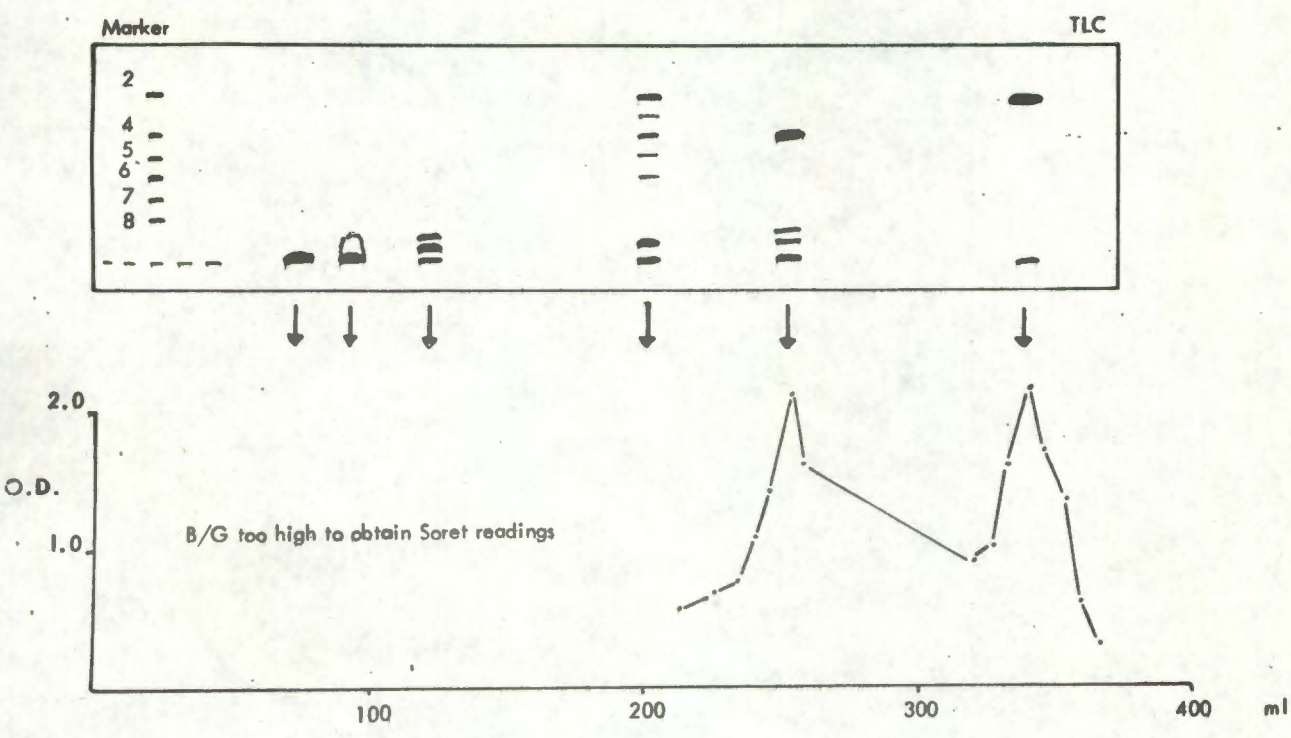
THE ELUTION PROFILE OF FAECAL PORPHYRINS FROM
A SEPHADEX COLUMN AS DEPICTED BY FLUORESCENCE



Sephadex LH 20 equilibrated with Benz : MeOH (1 : 1). The first
fluorescent peak shows a large amount of porphyrin 'X' on TLC.

Fig. 30.

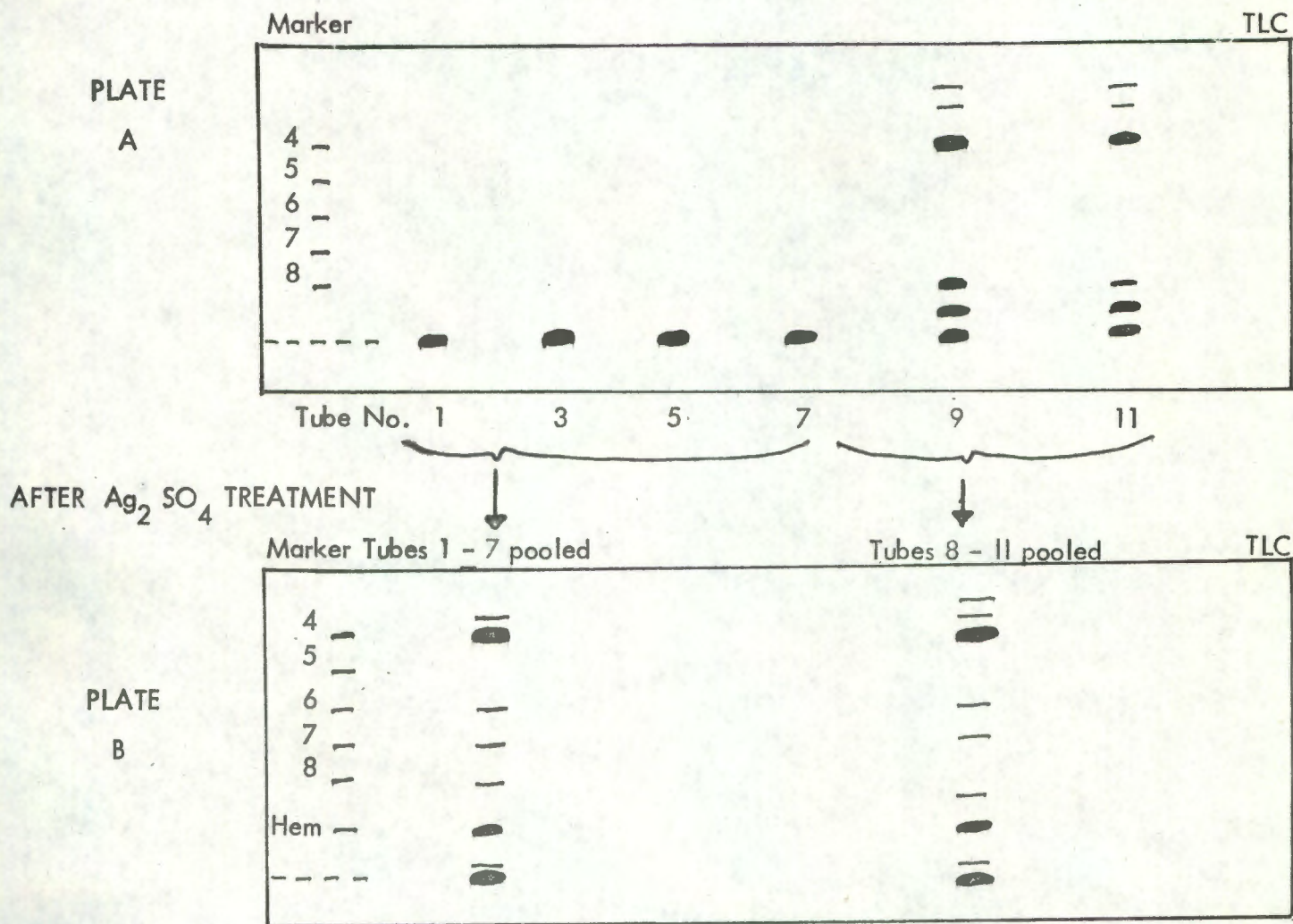
ELUTION PROFILE OF THE FAECAL PORPHYRIN (M.E.)
FROM A VARIEGATE PORPHYRIA PATIENT.



Sephadex LH 20 equilibrated with MeOH : Benz (1 : 1). The initial fractions off the column contained large amounts of brown pigment which masked the porphyrin absorption. The thin layer plate shows this material to contain large amounts of porphyrin 'X'.

Fig. 3I

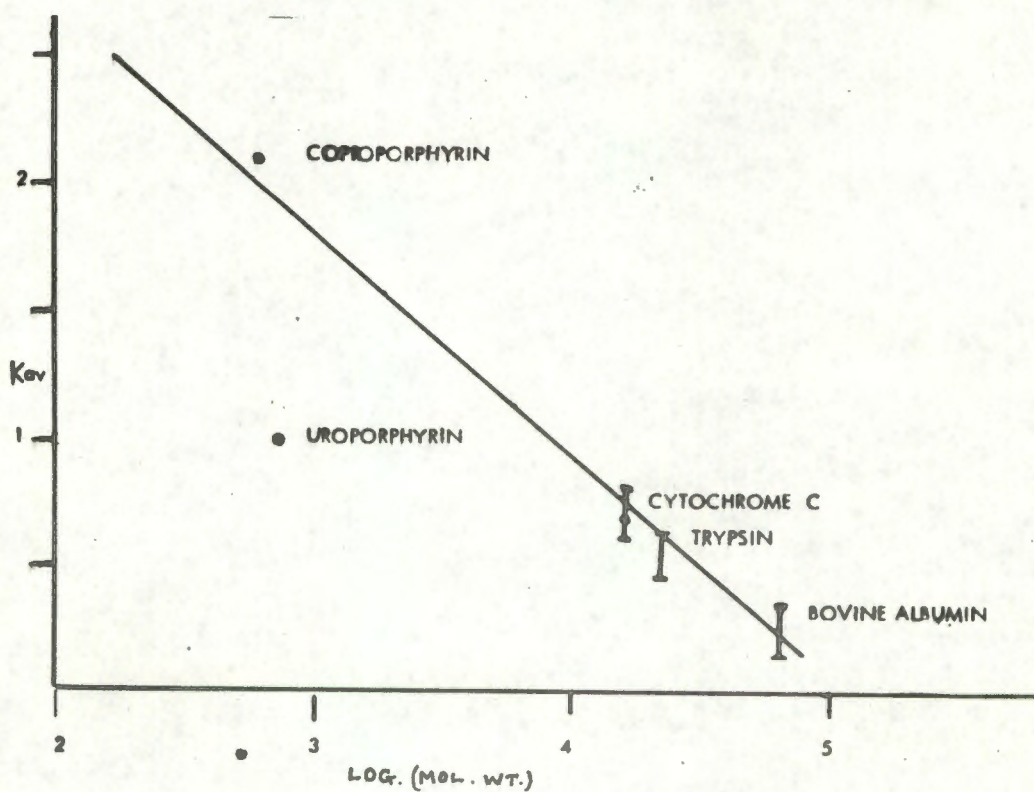
THIN LAYER CHROMATOGRAPHY OF THE PORPHYRIN
FRACTIONS SEPARATED ON A SEPHADEX LH 20 COLUMN
MeOH/CHCl₃ (9 : 1)



The fractions from the Sephadex column were subjected to treatment from a heavy metal to split any thio-ether bonds present. The free porphyrins were then chromatography on TL plates.

Fig. 32

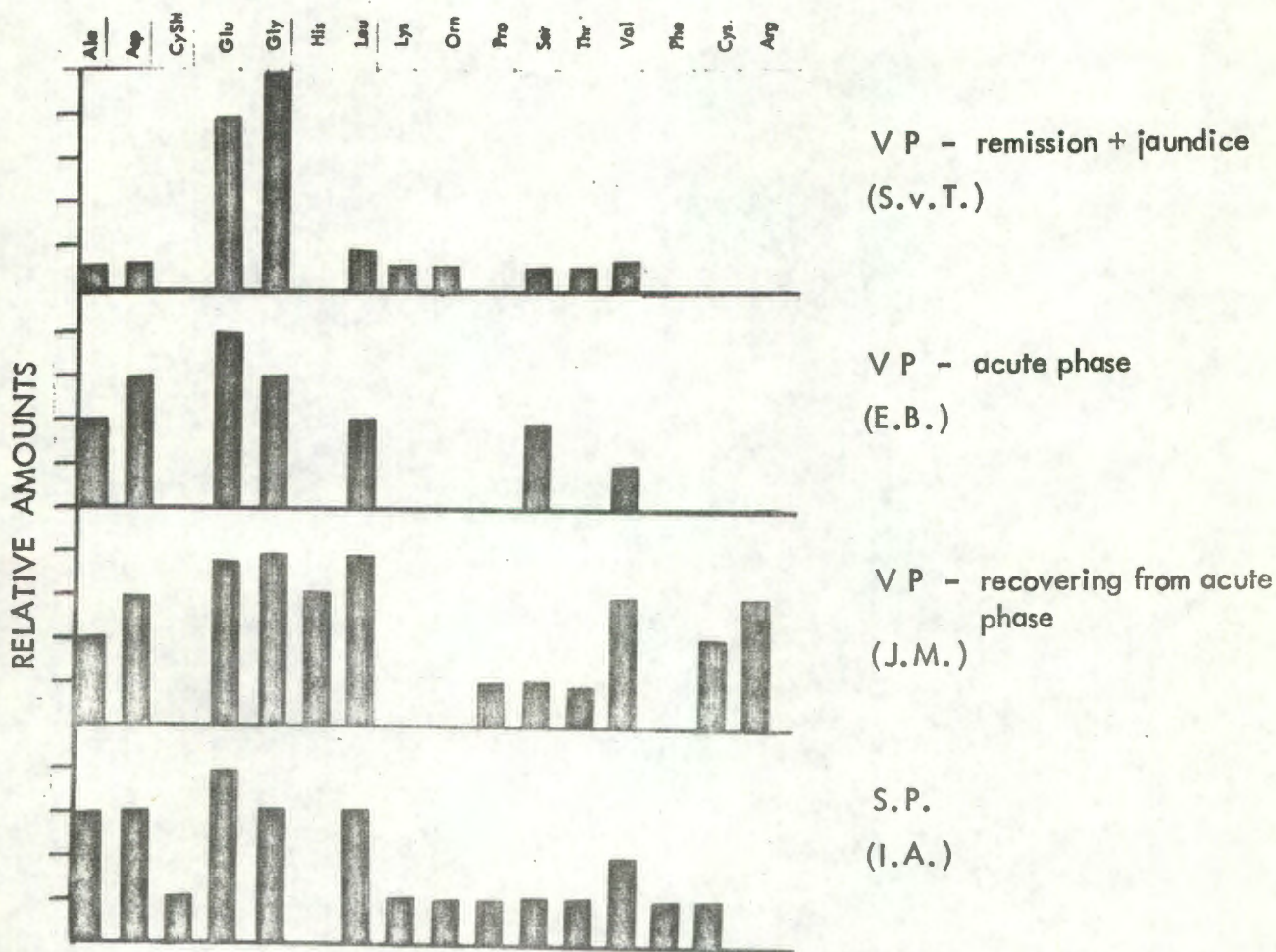
CALIBRATION OF SEPHADEX G 100 COLUMN



Separation of protein markers on Sephadex G 100 column using 0.05 M barbital buffer pH 8.6. Retention of the coproporphyrin and uroporphyrin on the column may be noted by a K_{av} value of greater than 1.

Fig. 33.

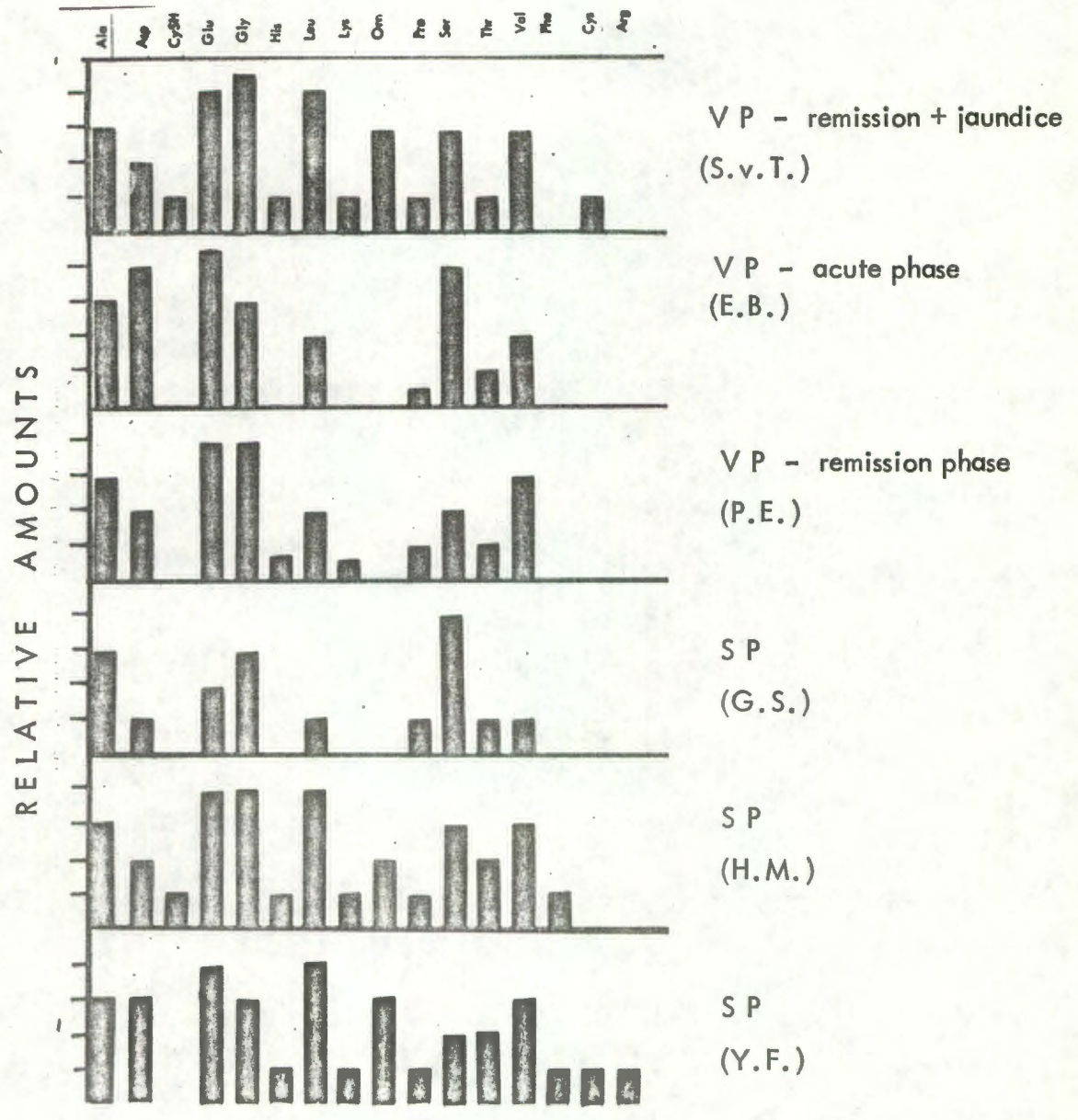
THE AMINO ACID COMPOSITION OF URINARY HYDROPHILIC PORPHYRINS



The urinary porphyrin-peptide complexes were grouped together and subjected to acid hydrolysis. The amino acid compositions were determined by paper chromatography and HV electrophoresis.

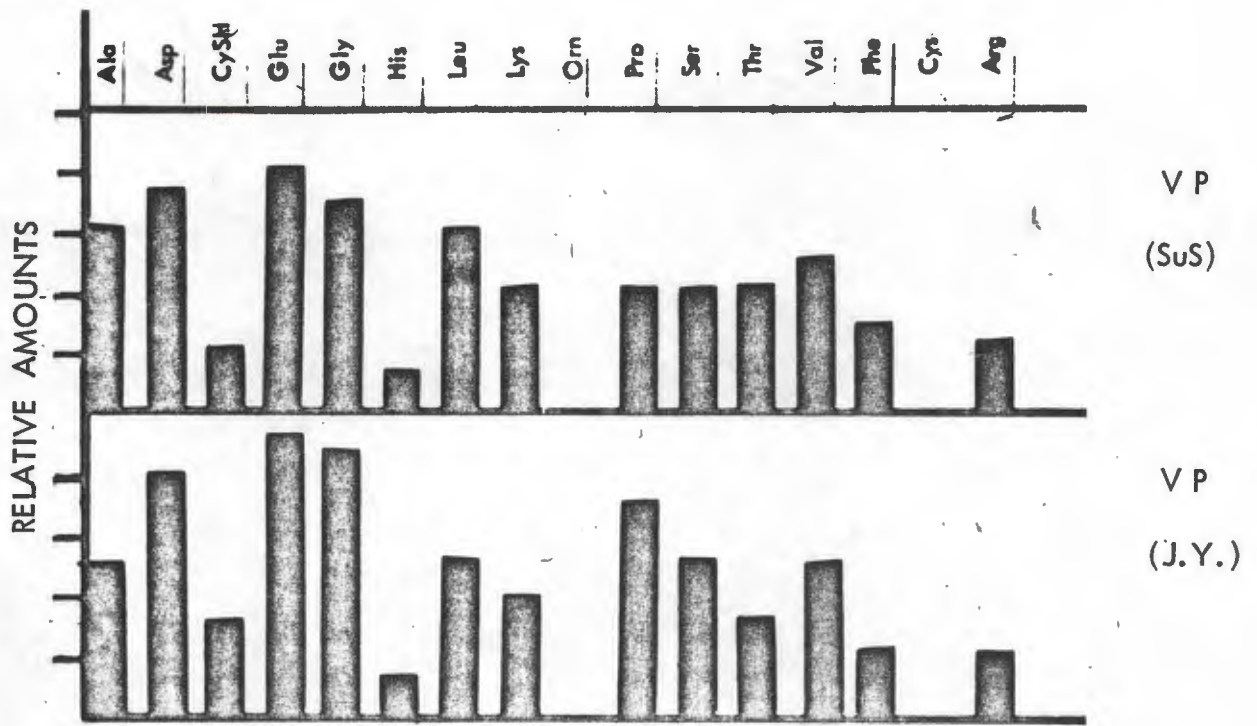
Fig. 34

THE AMINO ACID COMPOSITION OF THE FAECAL HYDROPHILIC PORPHYRINS



The faecal porphyrin-peptide complexes have been combined and hydrolysed. The amino acid compositions were determined.

Fig. 35 THE AMINO ACID COMPOSITION OF THE FAECAL SECONDARY RESIDUE FRACTION AS INVESTIGATED BY RIMINGTON (1968)



For comparative purposes the amino acid compositions of porphyrin 'X' (secondary residue fractions) taken from variegate porphyric faeces which were reported by Rimington (1968), have been depicted in a similar manner.

Shortly after the first description of the hydrophilic porphyrins or porphyrin "X" in the excreta of cases of porphyria Rimington et al. (1968) suggested that the determination of this "compound" in the excreta constituted a diagnostically valuable procedure in a number of respects. Firstly it was claimed by Rimington et al. (1968) that elevated faecal porphyrin "X" levels are characteristic of variegate porphyria and that moderately raised levels may be found in acute intermittent porphyria but not in other forms of the disease. If this were the case faecal porphyrin X determinations would have nosological value. Secondly Rimington reported several cases from relatives of porphyric patients where the faecal coproporphyrin and protoporphyrin levels were within normal limits and the porphyrin X levels raised. From family studies he was able to show these members to be carriers of the disease. Riley and Belcher (1968) felt that if a single dominant gene were responsible for the disease, porphyrin "X" should present as a bimodal distribution; since this was not the case they postulated the production of porphyrin "X" to be bimodal but the excretion to be affected by a different gene, and that only individuals with a homozygous recessive complement at this second locus would excrete much of the porphyrin, and that the remaining individuals would retain it. They also felt that those individuals who retained the formed porphyrin would be more light sensitive.

Rimington et al. (1968) found raised porphyrin X values in 2 younger members of his family under study. We felt that if porphyrin X became raised at an earlier age than the protoporphyrin and coproporphyrin, determinations of porphyrin "X" would help in an earlier diagnosis than that afforded by currently available techniques.

In order to assess the validity of this assertion and to obtain a general idea of the diagnostic role of faecal porphyrin "X" determinations in Variegate Porphyria as seen in Cape Town, I undertook a quantitative study of faecal porphyrin X values, on patients available to us at G roote Schuur Hospital. This constituted analysis of the acid soluble fraction from the faeces according to the method of Rimington laid out in fig. 27, Scheme III. Specimens from a total of 55 porphyric patients were examined. In all cases the diagnosis could be established beyond doubt on the basis of clinical presentation and conventional faecal and urinary porphyrin determinations (Holti et al. 1958).

In addition I examined the excreta of children from porphyric parents. On genetic grounds one would expect half of these to be prospective porphyrics.

The survey comprised the following sections:-

	No. of cases
Variegate porphyria	33
Symptomatic porphyria	17
Acute Intermittent porphyria	4
Erythropoietic Protoporphyria	1
Erythropoietic Congenital porphyria	1
Normal adults from porphyric families	4
Normal children from porphyric families	41
Normal adults (over 20 years of age)	12
Normal children (under 20 years of age)	11

Table 18-25 have been drawn up to show the faecal hydrophilic porphyrins together with faecal protoporphyrin and coproporphyrin. The urinary porphyrins have also been listed when determined. The upper limit (2.S.D.) of porphyrin "X" in normal adults was found to be 10 $\mu\text{g}/\text{G}$.

TABLE 18

VARIEGATE PORPHYRIA

Name	Sex	Faecal Porphyrins ug/G dry wt.			Urinary Porphyrins ug/L			
		Copro	Proto	Total	X	Copro	Uro	X
1. M.v.W.	F.	182	453	635	58	N/D	N/D	N/D
2. M.H.	F.	188	500	688	141	"	"	"
3. M.K.	F.	433	715	1148	102	"	"	"
4. S.W.N.	M.	1231	916	2147	31	4524	1757	"
5. L.de J.	F.	1732	2294	4026	152	2414	1711	"
6. Y.M.	F.	757	892	1649	33	4926	1558	"
7. M.C.	F.	739	994	1173	65	102	-	"
8. E.B.	F.	989	1995	2985	62	5864	2949	"
9. K.R.	F.	180	229	409	29	143	1470	"
10. I.A.	M.	78	102	180	27	725	1653	296
11. J.M.	F.	363	301	664	109	101	207	52
12. M.P.	F.	604	1031	1635	262	N/D	N/D	N/D
13. P.E.	F.	454	561	1015	93	2 91	64	"
14. G.A.	F.	52	227	279	28	14	-	"
15. E. A.	F.	125	135	260	13	31	-	"

TABLE 18 continued

VARIEGATE PORPHYRIA

Name	Sex	Faecal Porphyrins ug/G dry wt			Urinary Porphyrins ug/L			
		Copro	Proto	Total	X	Copro	Uro	X
16. F.Y.	M.	392	123	515	13	N/D	N/D	N/D
					124			
					32			
17. M.S.	F.	237	241	478	41	"	"	"
18. M.Se.	F.	1225	1458	2683	312	"	"	"
19. W.C.	M.	302	520	822	217	"	"	"
20. M.S.	F.	219	320	539	149	244	51	"
21. M.A.	F.	110	199	309	25	33	-	"
22. M.H.	F.	173	269	442	76	122	112	"
23. M.C.	F.	400	629	1029	215	1054	2327	"
24. M.G.	M.	325	427	752	94	250	40	"
25. R.G.	M.	472	255	727	103	225	19	"
26. H.C.	F.	869	1687	2556	285	658	90	"
27. C.C.	M.	561	1138	1699	382	1179	354	"
28. M.I.	M.	135	83	218	18	6	-	"
					24			
29. G.L.	F.	344	285	629	122	199	22	"

TABLE 18 continued - 3

VARIEGATE PORPHYRIA

Name	Sex	Faecal Porphyrins ug/G dry wt			Urinary Porphyrins ug/L			
		Copro	Proto	Total	X	Copro	Uro	X
30. M.C.	F.	402	473	875	244	179	109	N/D
31. S.v.T.	F.	105	161	266	N/D	6714	3138	"
		180	200	380	202	3378	4346	1505
32. L.R.	M.	1383	1229	2612	805	691	69	N/D
33. J.V.	F.	408	255	663	704	N/D	N/D	"
						1907	1190	60

TABLE 19

SYMPTOMATIC PORPHYRIA

Name	Sex	Faecal Porphyrins ug/G dry wt			Urinary Porphyrins ug/L		
		Copro	Proto	Total	Copro	Uro	X
1. J.P.	M.	39	13	52	1391	6659	N/D
2. A.C.	M.	75	65	140	403	3379	"
3. M.A.	F.	87	66	153	596	2725	"
		75	81	156	1232	7342	"
4. G.S.	M.	129	95	224	96	1704	"
5. F.T.	M.	237	118	355	186	3505	"
6. M.D.	F.	89	49	138	1366	6394	"
7. J.M.	M.	11	63	74	309	240	"
8. G.R.	M.	51	34	85	411	4466	"
9. J.v.N.	M.	32	36	68	56	196	"
10. H.M.	M.	56	64	120	145	986	"
11. E.L.	M.	80	56	136	532	2203	"
12. J.V.	M.	7	15	22	47	7	"
13. D.H.	M.	121	89	210	286	4139	"
14. J.C.	M.	17	19	36	201	176	"

TABLE 19 continued

SYMPTOMATIC PORPHYRIA

Name	Sex	Faecal Porphyrins ug/G dry wt.			Urinary Porphyrins ug/L			
		Copro	Proto	Total	X	Copro	Uro	X
15. M.T.	M.	32	31	63	5	118	320	N/D
16. D.S.	M.	114	127	241	98	172	1553	"
17. E.M.	M.	14	73	87	9	10	-	"

TABLE 20

ACUTE INTERMITTENT PORPHYRIA

Name	Sex	Faecal Porphyrins ug/G dry wt.			Urinary Porphyrins ug/L			
		Copro	Proto	Total	X	Copro	Uro	X
1. R.W.	F.	40	126	166	8	90	-	N/D
2. M.B.	F.	24	51	75	2	393	174	"
3. M.P.	F.	204	176	380	10	N/D	N/D	"
4. A.G.	M.	58	59	117	37	480	2211	"
						1184	35558	1082
						595	13677	289
						243	546	90
						210	228	10
						79	131	B/G too hig
						189	262	8
						137	171	1

TABLE 21.

ERYTHROPOIETIC PROTOPORPHYRIA

Faecal Porphyrins ug/G dry wt.

		Faecal Porphyrins ug/G dry wt.		
Name	Sex	Copro	Proto	Total

P.H.

M

30

364

394

14

		Faecal Porphyrins ug/G dry wt.		
Name	Sex	Copro	Proto	Total

M.A.

F

2209

841

3050

14

ERYTHROPOIETIC CONGENITAL PORPHYRIA

TABLE 22.

NORMAL ADULTS FROM PORPHYRIC FAMILIES (over 20 years of age)

Name	Sex	Faecal Porphyrins ug/G dry wt.			Urinary Porphyrins ug/L			
		Copro	Proto	Total	X	Copro	Uro	X
1. R.R.	M	13	22	35	2	57	-	N/D
2. J.R.	M	11	47	58	4	76	-	"
3. J.A.	F	13	36	49	11	8	-	"
4. E.J.		19	45	64	9	N/D	N/D	"

TABLE 23.

NORMAL CHILDREN FROM PORPHYRIC FAMILIES (under 20 years of age)

Name	Sex	Age	Faecal Porphyrins $\mu\text{g}/\text{G}$ dry wt			Urinary Porphyrins $\mu\text{g}/\text{L}$			
			Copro	Proto	Total	X	Copro	Uro	X
1. J.P.	M.	16	14	45	59	4	57	-	N/D
2. R.B.	F.	10	13	27	40	2	88	-	"
3. C.R.	F.	6	25	50	75	5	insufficient urine	-	"
4. K.M.	F.	8½	76	98	174	3	356	79	"
5. P.M.	M.	5	10	29	39	2	123	-	"
6. F.A.	F.	16	30	33	63	4	86	-	"
7. P.H.	F.	10	25	43	68	9	45	-	"
8. C.H.	F.	4	20	88	108	28	59	-	"
9. S.H.	M.	14	8	70	78	5	73	-	"
10. N.P.	F.	12	5	8	13	2	145	-	"
11. P.S.	M.	10	8	24	32	9	55	-	"
12. J.S.	F.	16	18	50	68	12	47	-	"
13. R.S.	M.	18	11	50	61	16	64	-	"
14. M.S.	M.	9	17	35	52	0	9	-	"
15. S.S.	F.	5	3	9	12	0	5	-	"

TABLE 23 NORMAL CHILDREN FROM PORPHYRIC FAMILIES (under 20 years of age) Continued

Name	Sex	Age	Faecal Porphyrins $\mu\text{g}/\text{G}$ dry wt			Urinary Porphyrins $\mu\text{g}/\text{L}$			
			Copro	Proto	Total	X	Copro	Uro	X
16. P.B.	F.	10	55	21	76	3	22	-	N/D
17. D.B.	M.	4	12	51	63	5	32	-	"
18. S.A.	M.	10	82	82	164	9	38	-	"
			6	2	8	3	69	-	"
19. C.S.	F.	3	2	7	9	1	28	-	"
20. S.M.	F,	9½	8	14	22	0	N/D	N/D	"
21. R.M.	M.	7	8	13	21	7	162	-	"
22. M.M.	M.	8	27	67	94	40	9	-	"
23. L.A.	F.	12	129	143	272	37	82	-	"
			39	49	88	14	15	-	"
24. R.W.	F.	9	22	84	106	10	34	-	"
25. A.W.	M.	7	14	44	58	24	31	-	"
26. L.G.	M.	9	7	14	21	4	78	-	"
27. D.G.	M.	7	6	5	11	17	62	-	"
28. M. du P.	F.	5	18	88	106	2	N/D	N/D	"
			10	31	41	8	N/D	N/D	"
29. K.M.	F.	9	14	29	43	5	271	-	"
30. A.P.	M.	7	12	21	33	3	45	-	"

NORMAL CHILDREN FROM PORPHYRIC FAMILIES (under 20 years of age)

TABLE 23

Name	Sex	Age	Faecal Porphyrins $\mu\text{g}/\text{G}$ dry wt.				Urinary Porphyrins $\mu\text{g}/\text{L}$.		
			Copro	Proto	Total	X	Copro	Uro	X
31. M.G.	M.	3	7	19	26	1	38	-	N/D
32. A. du P.	M.	9	18	54	72	5	N/D	N/D	"
33. D.A.	M.	6	28	21	49	10	52	-	"
34. P.C.	F.	10	7	22	29	11	122	-	"
35. R.C.	M.	4	17	72	89	4	30	-	"
36. P.L.C.	M.	7	6	32	38	5	93	-	"
37. S.L.	M.	3 months	7	18	25	7	N/D	N/D	"
38. L.L.	M.	5	8	4	12	3	96	-	"
39. Se.L.	M.	7	6	18	24	12	92	-	"
40. C.			17	20	37	4	N/D	N/D	"
41. J.J.	F.	11	3	6	9	6	N/D	N/D	"
			22	44	66	38	N/D	N/D	"

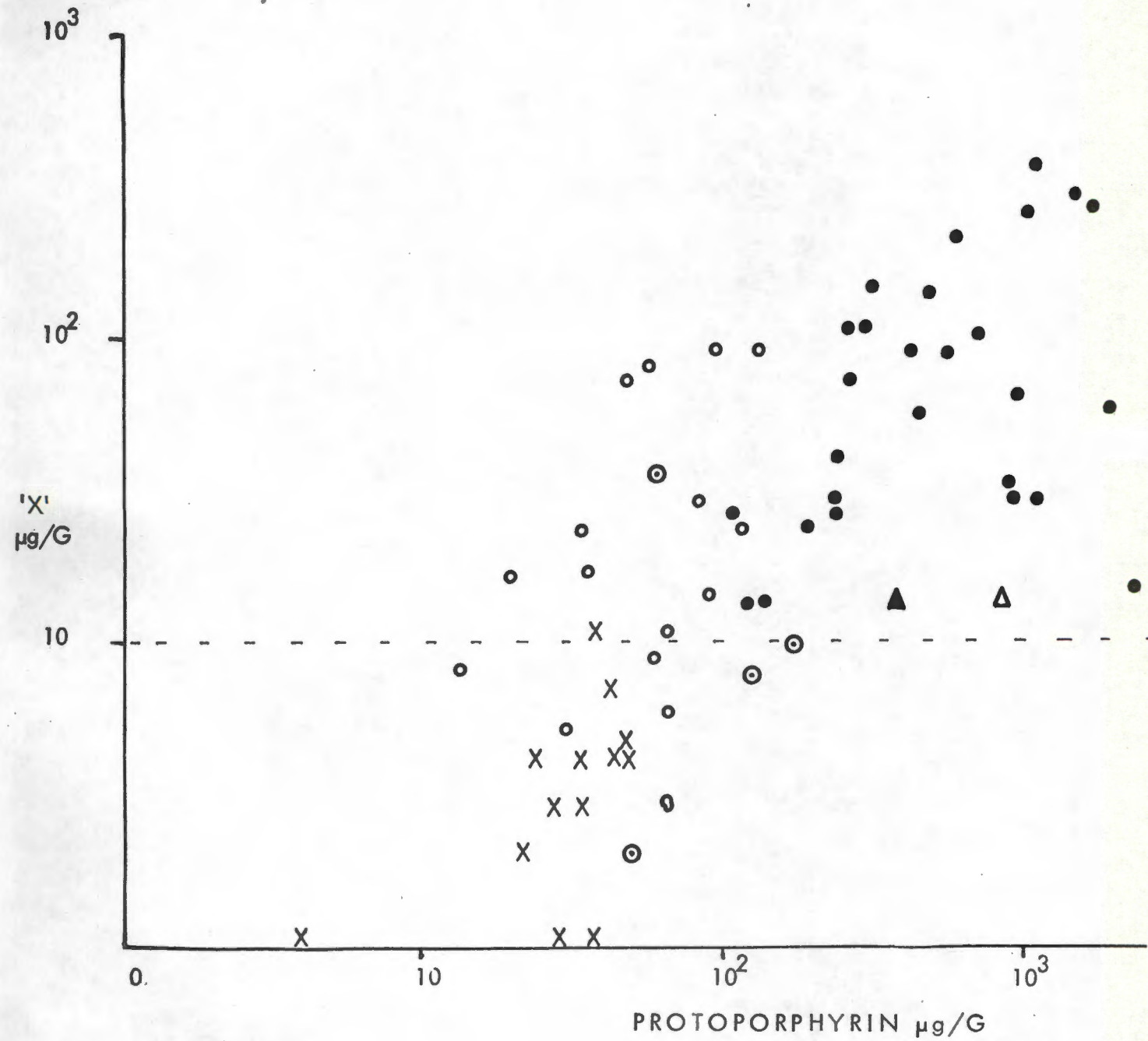
TABLE 24

NORMAL ADULTS (over 20 years of age)

Name	Sex	Faecal Porphyrins ug/G dry wt.			Urinary Porphyrins ug/L			
		Copro	Proto	Total	X	Copro	Uro	X
1. S.S.	F.	39	46	85	5	N/D	N/D	N/D
2. M.A.	M.	15	37	53	1	20	-	N/D
3. M.W.	M.	15	29	44	0	18	-	N/D
4. M.G.	F.	19	24	43	4	47	-	N/D
5. M.du P.	F.	25	33	58	3	43	-	N/D
6. M.T.	M.	23	28	51	3	-	-	N/D
7. M.v.R.	M.	10	4	14	0.4	112	-	N/D
8. C.G.	M.	21	29	50	3	N/D	N/D	N/D
9. M.C.	F.	9	60	69	8	"	"	1.8
10. M.L.	F.	13	19	32	0.4	"	"	N/D
11. Y.G.	F.	23	44	67	8	"	"	N/D
12. D.M.	F.	82	137	219	20	"	"	1.5
		47	94	141	6	"	"	N/D

Fig. 36.

THE RELATIONSHIP BETWEEN PORPHYRIN 'X' AND PROTOPORPHYRIN IN CASES OF PORPHYRIA

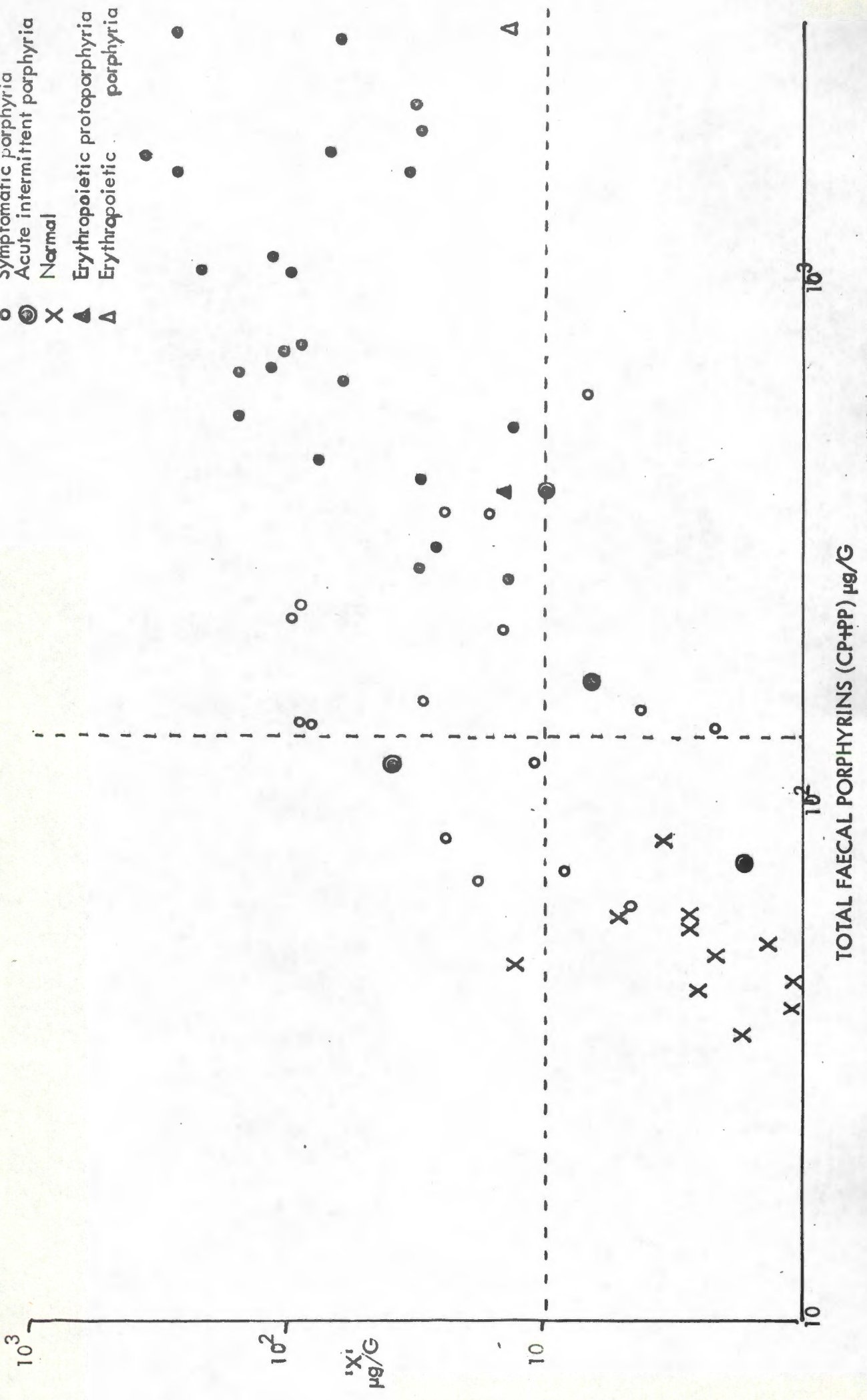


- Variegate porphyria
- Symtomatic porphyria
- ⊙ Acute intermittent porphyria
- X Normal
- ▲ Erythropoietic protoporphyria
- △ Erythropoietic congenital porphyria

Fig. 37.

FAECAL PORPHYRIN 'X' VALUES

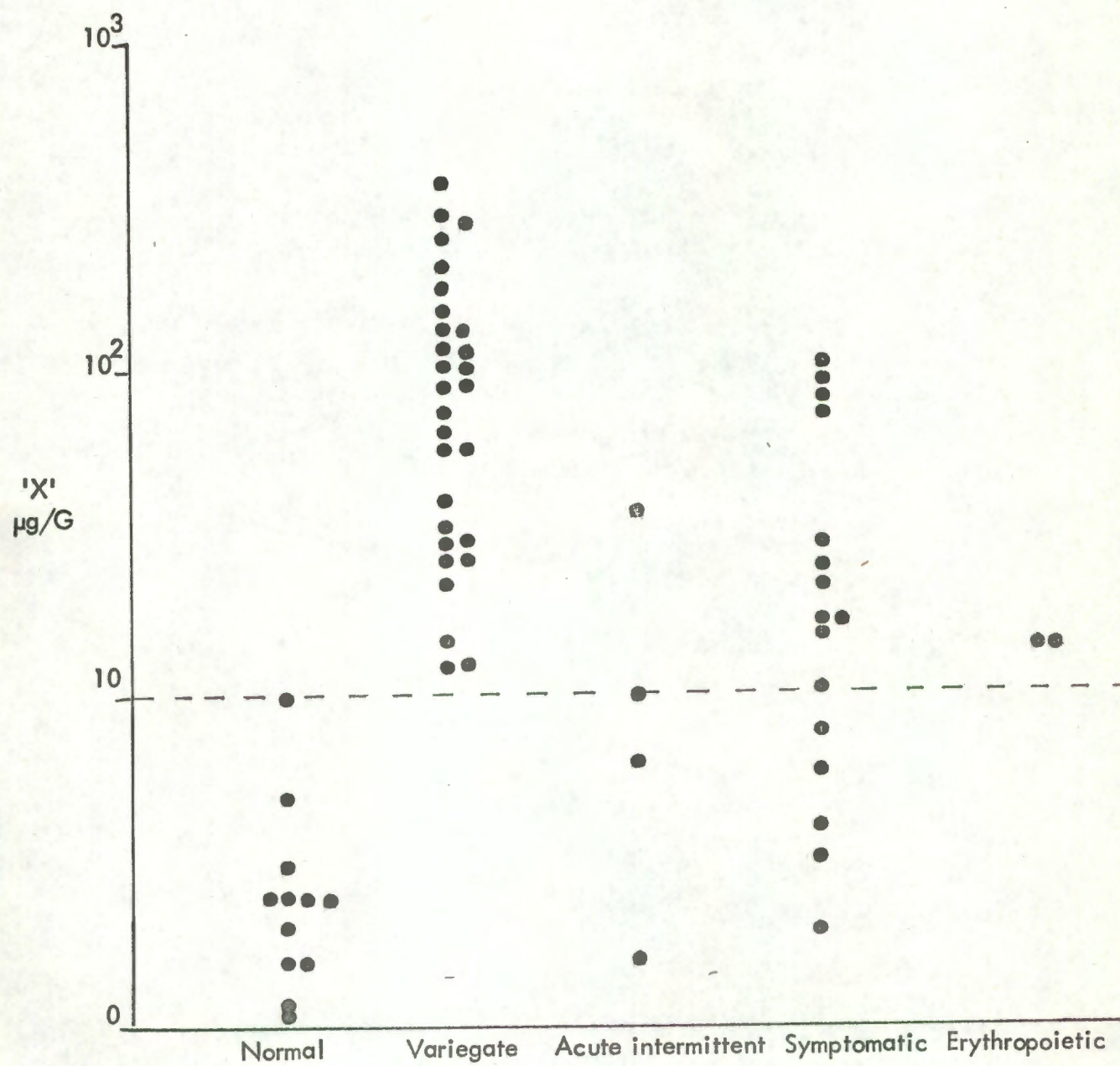
- Variegate porphyria
- Symptomatic porphyria
- ⊙ Acute intermittent porphyria
- X Normal
- ▲ Erythropoietic protoporphyria
- △ Erythropoietic porphyria



TOTAL FAECAL PORPHYRINS (CP+PP) µg/G

Fig. 38

FAECAL PORPHYRIN 'X' VALUES IN 56 CASES OF PORPHYRIA



FAECAL PORPHYRIN 'X' VALUES IN CHILDREN.

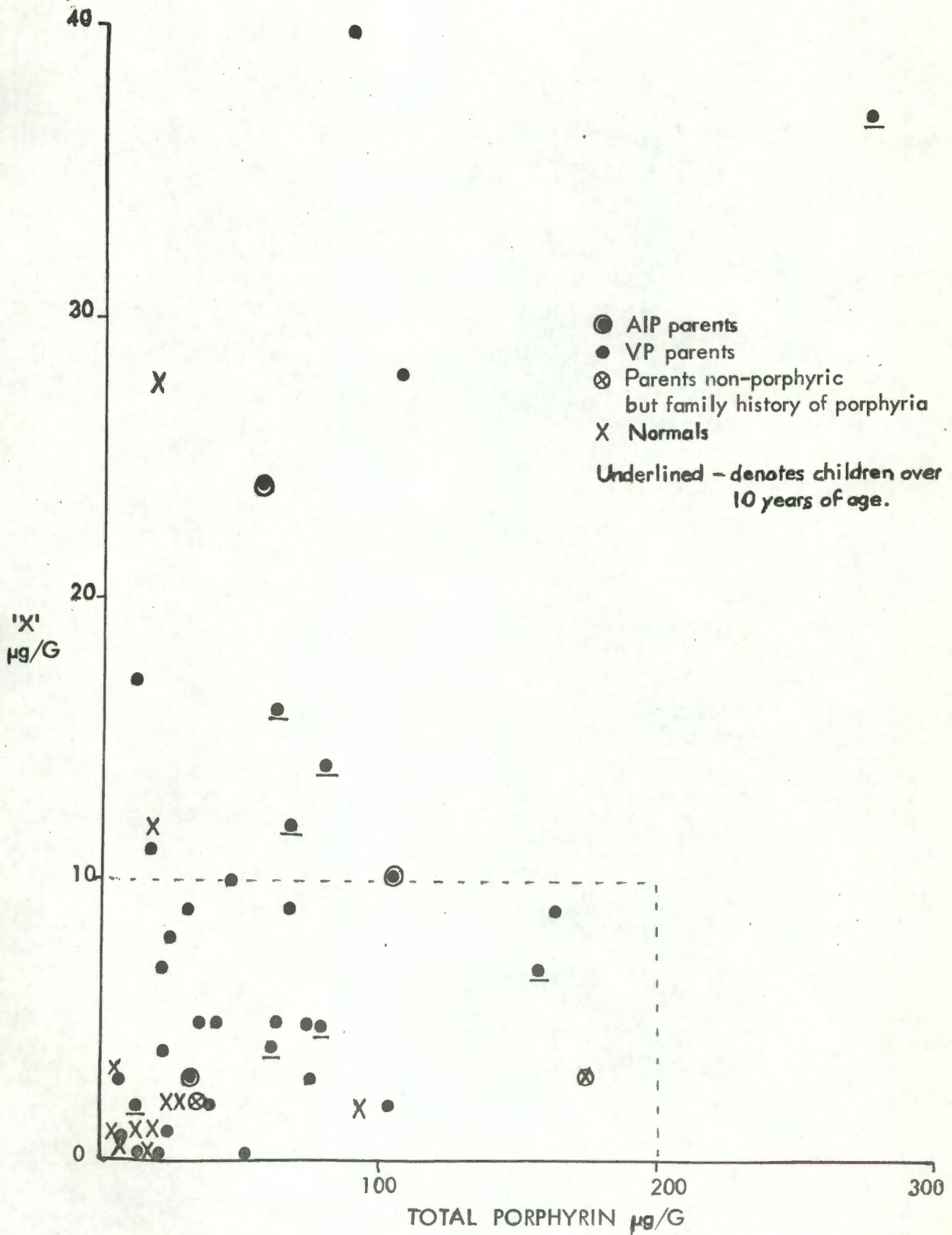
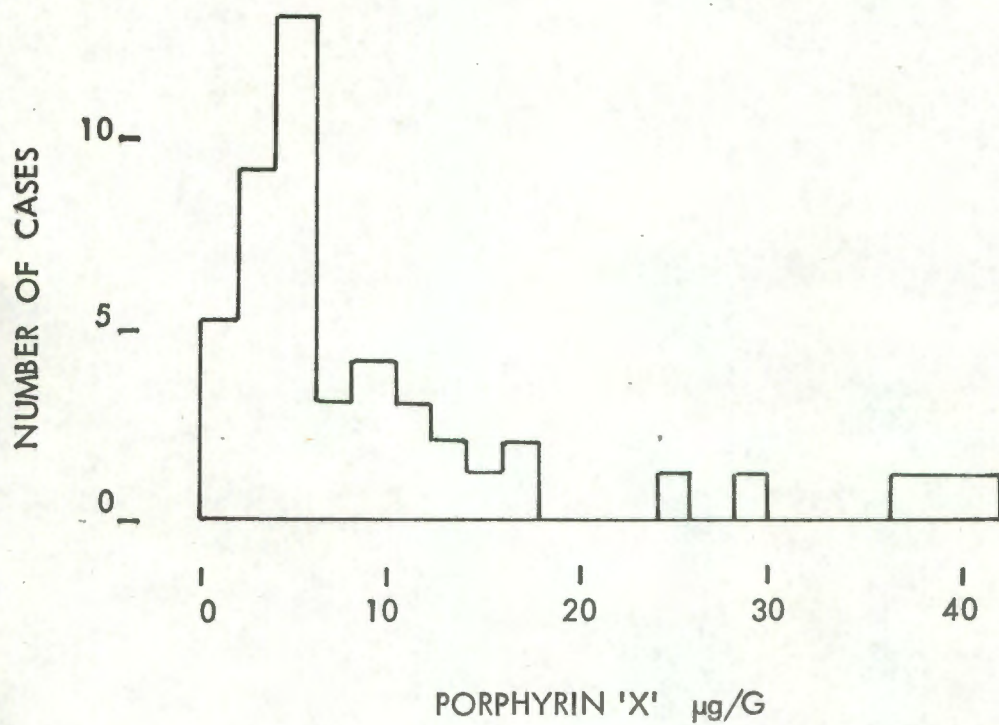


Fig. 40.

HISTOGRAM OF THE DISTRIBUTION OF PORPHYRIN 'X' OUTPUT IN THE SAMPLED POPULATION OF CHILDREN FROM PORPHYRIC FAMILIES



Discussion.

At one stage it was considered possible that the hydrophilic porphyrin values merely reflected the protoporphyrin values, but as may be seen from fig. 36 the scatter is too great for any correlation to exist. It may in fact be noticed that the entire graph can be divided into 4 quadrants, with the symptomatic porphyrics occupying the upper and lower left quadrants only and 88% of the variegate porphyrics occupying the upper right hand quadrant. The one congenital erythropoietic porphyric studied had only a very slightly raised porphyrin "X" associated with a high protoporphyrin value which does not support the concept of a correlation between the two porphyrins. In a similar manner no correlation can be found between porphyrin "X" and the total faecal porphyrins (fig. 37).

From fig. 38 it is evident that a raised faecal porphyrin "X" is definitely not specifically characteristic of variegate porphyria but may be considerably raised in symptomatic porphyria. Statistical analysis shows a highly significant difference at a 0.1% level between the faecal porphyrin "X" values in symptomatic porphyria and those in normals. This is contrary to the findings of Rimington et al (1968).

There is the possibility that porphyrin "X" may aid in the early diagnosis of porphyria but this can only be proven by a series of studies over the next 10 to 20 years. If the extreme upper limit of faecal total porphyrin is taken as 200 $\mu\text{g}/\text{G}$ it may be seen from fig. 39 that, with the exception of one individual, all those determined lie within this range and yet 10 children exhibit a raised porphyrin "X" value. Statistical analysis of the faecal porphyrin values, shows the children from porphyric families to be significantly different from normal children with respect both to

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porphyrin "X" and protoporphyrin.

Porphyrin "X" shows a p value of 0.0018, protoporphyrin a p value of 0.0005 and coproporphyrin 0.014. A non bimodal pattern of porphyrin "X" excretion similar to that found by Riley and Belcher (1968) was observed (fig. 40).

Among the normal children studied, one child showed a consistently raised faecal porphyrin "X". The paternal parent could not be traced, but was reported to be a non-porphyric.

It is difficult to allocate any significance to these findings at present, but it presents a situation definitely warranting further investigation at a later stage.

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Chapter 6. THE INCORPORATION OF C¹⁴ INTO THE HYDROPHILIC
PORPHYRINS.

The necessity to detect whether these hydrophilic porphyrins are artifacts due to procedures, or whether they are an integral part of the anabolic or catabolic processes of porphyrin metabolism was at this stage essential. We therefore decided to administer carbon-14-labelled ALA to a variegate porphyric and then to isolate the hydrophilic porphyrins from the excreta.

PROCEDURE

500 ug (2 uM) of ALA - ¹⁴C in 0.1 N HCl with an activity of 45.4 uc was administered orally to a 34 year old white woman. The urine was collected in 3-hourly samples for the first 12 hours; then in 6-hourly collections during the next 12 hours and thereafter in 24-hour collections. Only one stool was passed during the period of investigation. Total porphyrin estimations were performed as discussed in section 1. (Rimington and Sveinsson, 1950; Holti et al. 1958) (See Table 25). The remainder of the samples were esterified and run as esters on TLC (Doss, 1967). The individual bands were scraped off the plate and the porphyrins eluted from the silica gel and subjected to combustion (see Appendix A) and the ¹⁴C counted in a scintillation counter. The radioactivity was expressed as dpm per mmole of porphyrin and these figures for the urine were plotted against time (fig. 41). For the urine the hydrophilic porphyrins were grouped together, but for the faeces, where more material was available, the individual bands on the thin layer chromatograms have been treated separately and labelled 1 through to 8. The brown pigment present in the chromatograms may possibly be a dipyrrole but no confirmatory work was done on this apart from

solubility tests and an absorption spectrum recording, which showed no specific peaks but a flat area between 485 mu and 495 mu as expected (Siedel and Möller, 1939; Siedel et al. 1948; Gilbertson et al., 1959). The radioactivity of these samples was expressed as dpm per optical density unit since no molar extinctions are known. The results are shown in fig. 42. Absorption spectra were run on the more unusual porphyrins (Table 26). They all showed aetiotype spectra but differed from the known porphyrins.

From figure 41 it may be seen that the highest activity for both uro, copro and the dipyrrole occurred in urine I. Possibly, if urines had been collected over 1 hour periods instead of 3 hour periods, we would probably have been able to show separate peaks. Nevertheless, the purpose of the exercise was to determine the presence of radioactivity in the hydrophilic porphyrins and this has been achieved, the peak occurred after the uro and copro peak indicating their formation after the decarboxylation of the uroporphyrin has taken place, which is compatible with the idea of the porphyrin moiety being hematoporphyrin. The molar extinction coefficient of uroporphyrin has been used to quantitate the hydrophilic porphyrins and therefore the dpm/umole shown on the graph really only represent relative amounts present, but one would expect a smaller amount of radioactivity per umole, as in fact is the situation here if the porphyrin and peptide were combined after the formation of hematoporphyrin. The specific activity maximum for uroporphyrin was found to be less than that for coproporphyrin, which is similar to that found in Symptomatic porphyria by Goldswain et al. (1970).

TABLE 2S.

Urine	Vol.	Time hrs	Copro		Uro		PBG		ALA	
			mg %	total μ g	mg %	total μ g	mg/L	total mg	mg/L	total mg
1.	410	3 hrs (3.0)	.0033	13.7	.0026	10.47	1.64	0.67	1.28	0.52
2.	54	3 (6.0)	.0274	14.8	.0139	7.5	2.90	0.16	7.53	0.41
3.	20	3.3 (9.33)	.0648	13.0	.0285	5.7	9.07	0.18	16.52	0.33
4.	112	3.6 (12.99)	.0695	77.8	.0113	12.7	8.06	0.90	10.56	1.18
5.	90	10.6 (23.65)	.0277	24.9	.0527	47.5	21.42	1.93	24.10	2.17
6.	585	24 (47.65)	.0271	158.5	.0084	49.2	4.66	2.73	4.77	2.79
7.	880	24 (71.65)	.0311	274	.01003	88.2	17.64	15.52	13.77	12.12

TABLE 26

FAECES (E.O.)Absorption Spectra of Faecal Hydrophilic Porphyrin.

		Soret	IV	III	II	I
Preproto		405				
3 COOH		399	too little to detect			
1	Hydrophilic Porphyrin	403	504	535		
2	" "	403	499	535	566	
3	" "	402	500	532		
4	" "	402	503	537	573	612-5
5	" "	401	498	532	567	610
6	" "	403	504	539	570	
7	" "	403-4	504	538	572	623
8	" "	405	503	537		
9	" "	404	504	535	570	

Fig. 41

THE RELATIONSHIP BETWEEN TIME AND SPECIFIC ACTIVITY OF URINARY PORPHYRINS FOLLOWING ALA - C¹⁴ ADMINISTRATION TO A SUBJECT WITH VARIEGATE PORPHYRIA

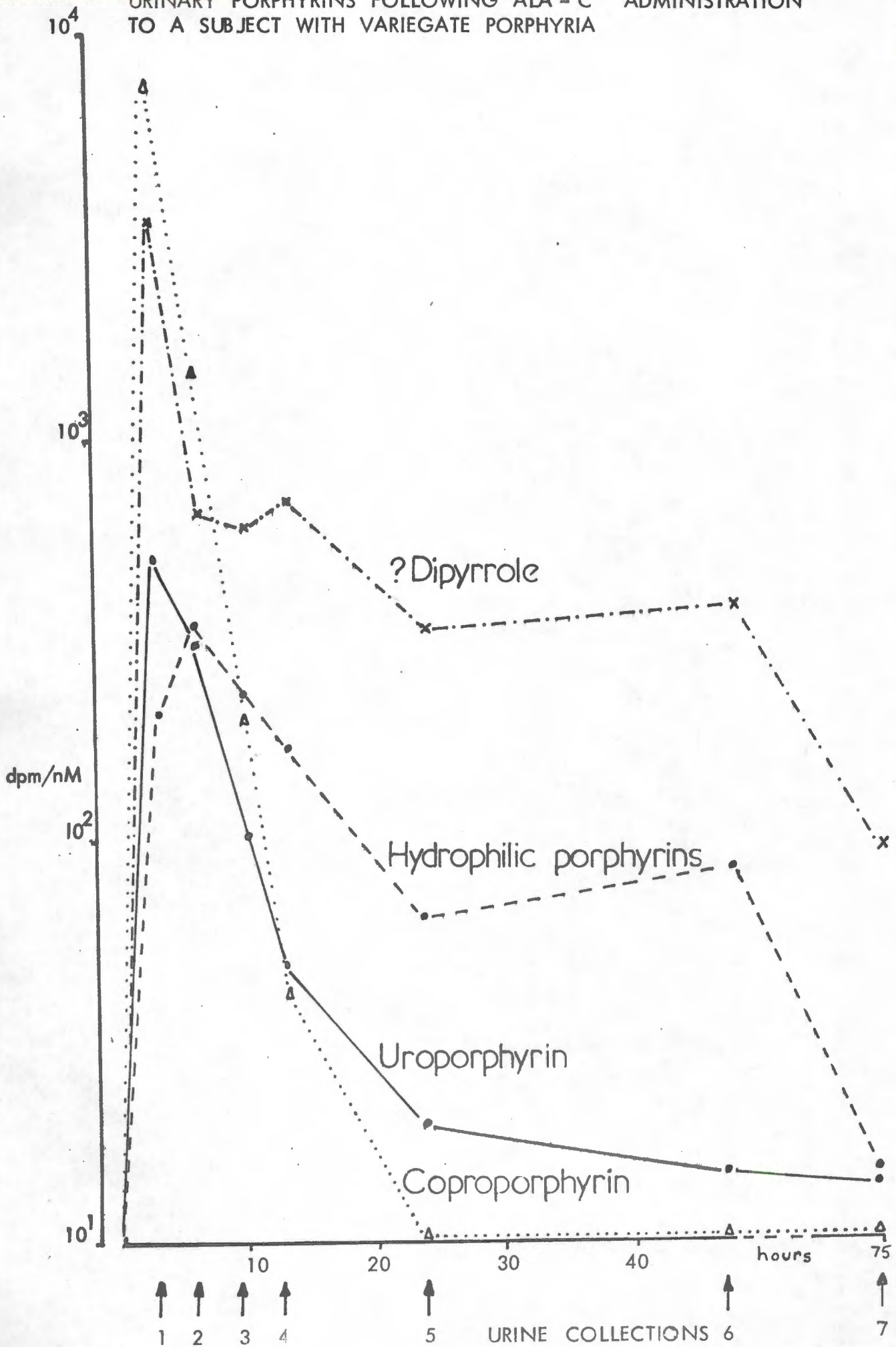
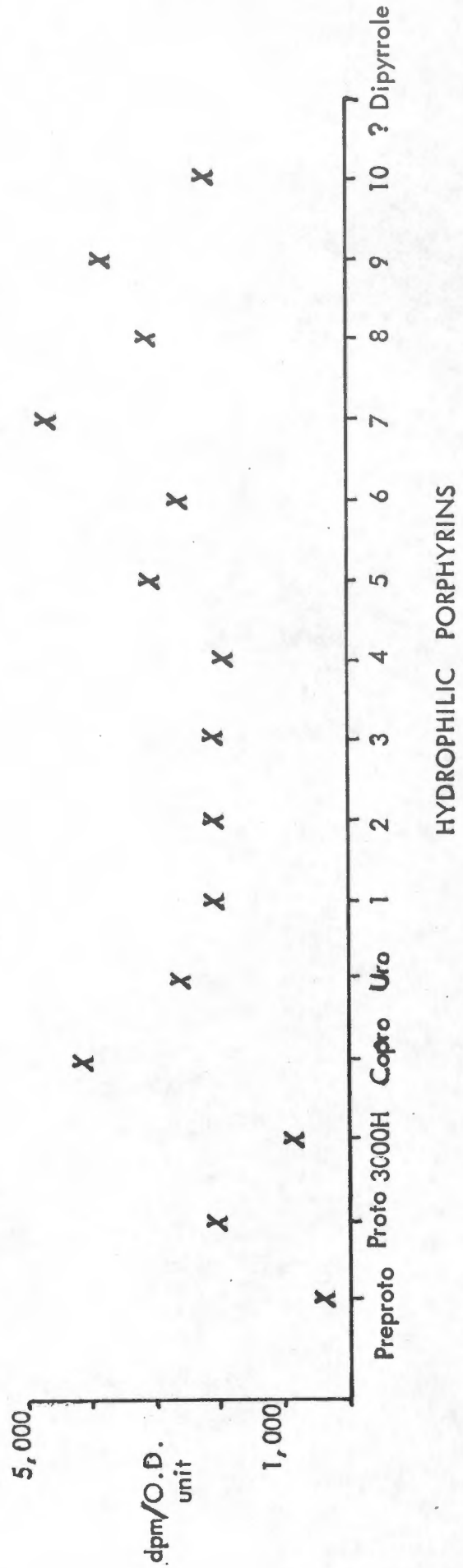


Fig. 42

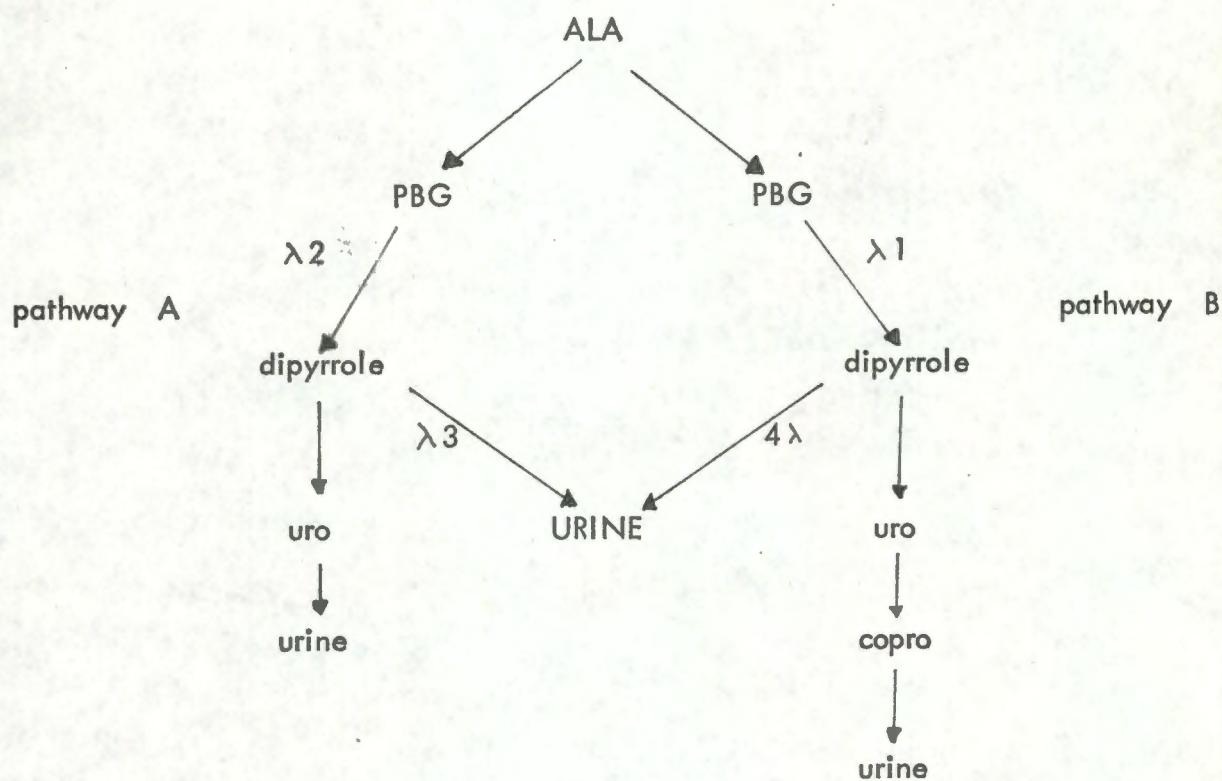
THE RELATIONSHIP BETWEEN THE RADIOACTIVITY IN THE VARIOUS FAECAL PORPHYRIN-PEPTIDE COMPLEXES FOLLOWING ALA-C¹⁴ ADMINISTRATION TO A SUBJECT WITH VARIEGATE PORPHYRIA X

FAECAL PORPHYRINS



HYDROPHILIC PORPHYRINS

FIG. 43

PROPOSED PATHWAY FOR PORPHYRIN SYNTHESIS

If 2 pathways for coproporphyrin formation exist, as postulated by Dowdle et al. (1970) (fig. 43) and if the overall rate constant $\lambda 2$ is less than that of $\lambda 1$, it is the rate limiting step for pathway A.

The appearance of three peaks in the specific activity of the dipyrrole would appear to demonstrate the dipyrrole arises from both pathway A and B, the first peak representing pathway B, the third small peak which may be seen in the dipyrrole and hydrophilic fractions may arise due to enterohepatic recycling of the porphyrins.

Fig. 42 demonstrates the presence of carbon - 14 in all 10 of the hydrophilic porphyrin bands observed on the thin layer chromatogram; whether the graph is a true reflection of the differences in the level of radioactivity between the different bands or whether the variation may be accounted for by differences in their molar extinction coefficients is not yet known. An interesting feature is the consistent presence of

bands 5 and 7 in practically all faeces studied and in this study they appeared to be a little more radioactive than most of the others. This could possibly represent preferential binding of the porphyrin with certain peptides possibly due to structure or basicity.

From this experiment it was conclusively shown that all the hydrophilic fractions isolated contained radioactivity and therefore probably participate in porphyrin biosynthesis.

SECTION III

SECTION III

THE ISOLATION AND CHARACTERISATION OF THE HYDROPHILIC PORPHYRINS.

Chapter 7.

INTRODUCTION

Urine and faeces were obtained from a patient with variegate porphyria associated with hepatitis. During the jaundiced phase the patient's faecal porphyrins fell to very low levels and the urinary porphyrins which were greatly elevated contained large quantities of hydrophilic porphyrins. This presented a unique opportunity to obtain large amounts of porphyrin "X" for identification and analysis.

It became apparent at an early stage that porphyrin "X" was in fact a complex mixture of heterogenous compounds with solubility in water as their only obvious physico-chemical characteristic in common. Therefore, the methodology for the resolution of the mixture could not rely solely on the solvent extraction procedures that gave porphyrin "X" its "identity" and it was clearly necessary to devise a more elaborate approach to the analytical problem that confronted us.

Based on published data regarding porphyrin "X", our own experience with the hydrophilic porphyrins and porphyrin extraction procedures and the suggestion again of porphyrin "X" as a degradation product of cytochrome c, a series of procedures was developed that will be described in detail in this section. The following initial experimental observations were made to formulate the detailed experiments. Details of the individual methods may be found in Appendix A.

PRELIMINARY EXPERIMENTS

(a) The Extraction of Porphyrin - Peptide from the Excreta.

Several methods were attempted for the initial extraction of the porphyrin-peptide from the urine and faeces.

(i) Urine. The primary concentration of the urinary porphyrins was achieved by adsorbing them onto talc at pH 3.5 or onto Ca(OH)₂ at pH 6. The talc was more commonly used as this could be thoroughly washed with water to remove any free peptides present. This washing resulted in a slight loss of the hydrophilic porphyrins but proved to be worthwhile since the main problem in the purification procedures was to irradiate the free peptide. The water washings when lyophilised and separated by ion exchange chromatography were found to contain 16 free peptides. It is therefore advantageous to eliminate these free peptides in the first purification step despite the slight loss of material. The extraction of porphyrin from talc is normally performed with the esterification mixture of 5% H₂SO₄ in MeOH. This extraction is only 95% complete. Rimington (1968) suggested the use of formic acid which affords a slight improvement. Various other solvents were tried, namely

- (1) Acetone/HCl 0.5 N (7 : 3)
- (2) Acetone/H₂O/NH₄OH (7 : 2 : 1)
- (3) Lut/H₂O (5 : 2)
- (4) Dimethyl sulphoxide
- (5) Trifluoroacetic acid.

The Dimethyl sulphoxide and Trifluoroacetic acid proved to be the most efficient. The use of the latter is rather expensive and therefore primary extractions with acetone/HCl were performed and the Trifluoroacetic acid used to remove the remaining porphyrin from the talc. The chromatographic picture of the porphyrin

extraction at this stage is shown in figs. 44 and 45, and as may be noted large amounts of porphyrin are present that correspond to the PTMC (pepsin-trypsin modified cytochrome c) marker.

FIG. 44

TLC OF PORPHYRIN "X"

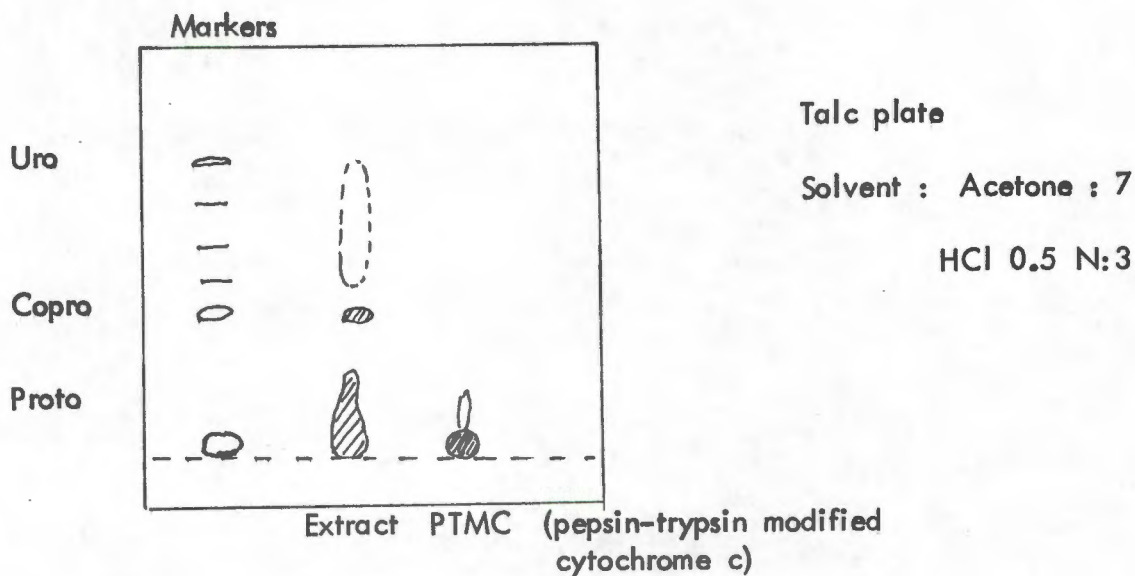
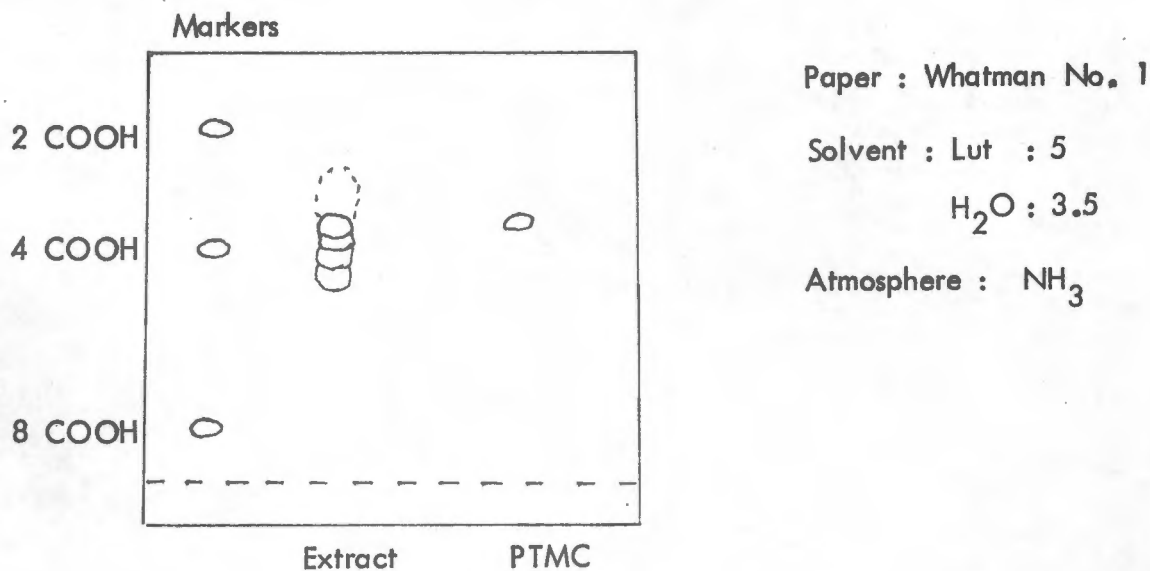


FIG. 45

PAPER CHROMATOGRAPHY



This chromatographic method proved to be the most rapid and reliable way of detecting porphyrin "X" in various extracts during the purification procedures.

(ii) Faeces.

The amount of porphyrin-peptide present in the faeces was far less than that in the urine which meant that large quantities (approx. 800 gms) had to be extracted in order to obtain sufficient material to work with. Initially an attempt was made to extract all the porphyrins simultaneously with acetone/HCl 0.5 N (7 : 3) and then to separate off the ether soluble porphyrins as was performed for the urinary porphyrins but with the faecal extractions this resulted in the formation of emulsions, and it was found better to extract the faeces first with ethyl acetate/glacial acetic acid (3 : 1) to remove the less polar porphyrins and then with acetone/HCl 0.5 N. 45% urea/triton 4% as recommended by Rimington et al. (1968) was not used because of the high salt content it introduced and extraction with this was not found to be any more complete than with the acetone/HCl.

From this stage onwards the urinary and faecal extracts were treated in the same manner and will be discussed together.

(b) Membrane Dialysis.

Membrane dialysis was attempted in order to remove any 6, 7 and 8 carboxyl porphyrins or small free peptides from the ether insoluble porphyrin mixture; but the molecular weight retention limit is approx. 12,000 which meant the loss of the lower molecular weight unpolymerised hydrophilic porphyrins. This step was later abandoned in favour of better methods.

(c) Ion exchange column chromatography.

(i) Anion exchange resins, i.e. Dowex 1 x 2 and amberlite CG 400 were used

with pyridine buffers of increasing concentrations. If the crude ether insoluble porphyrin extract was applied to such a column an elution profile consisting of 1 major fluorescent peak with a further 3, rather diffuse, bands at a later stage and many free peptide peaks emerged (fig. 46). This method served only in removing some of the free peptides from the porphyrin "X".

(ii) Cation ion exchange resins, i.e. Amberlite CG 50, Dowex 50 x 2 and Dowex 50 x 8.

The cation exchange resin Amberlite CG 50 was prepared in the NH_4^+ form and equilibrated with 0.125 M ammonium acetate. The elution profile from this column showed on most occasions a single large fluorescent peak emerging first, followed rapidly by a further 5 ninhydrin positive peaks (fig. 47). On 2 occasions the porphyrin-peptide peak split into two, probably due to polymerisation of the sample (Okunuki et al., 1968).

Stepwise elution using pyridine buffers was attempted primarily because of the ease with which the pyridine may be removed after elution. The pH was changed in 5 steps from pH 3.1 to 5.1 and the molarity simultaneously increased from 0.2 M to 2 M (fig. 48). The fluorescence was spread out over many tubes with only 1 major peak. Bile pigment was always present with the porphyrin, and many ninhydrin positive peaks were present. The Dowex 50 x 2 aspherical resin used with the same buffers gave only 1 fluorescent peak, this time emerging after the elution of the free peptides (fig. 49).

The separation achieved was no better than that on the Amberlite columns and therefore a Dowex cation exchange resin with a chosen cross linkage was tried. Dowex 50 x 8 was prepared according to the method of Stein and Moore (1951). The column was equilibrated with a citrate buffer pH 3.28. The sample was dissolved in citrate buffer pH 2.2 and applied to the column. The column was

washed for 3-4 days with citrate buffer gradually increasing the pH until all the bile pigments and free peptides had been removed (fig. 50). Small amounts of porphyrin-peptide were eluted at pH 5.20 and 8.25 and a further fraction when water was passed through the column but the major portion remained at the top of the column, which provided us with a relatively large amount of porphyrin "X" to work with. This could be removed from the resin with lutidine/water/acetic acid (10 : 10 : 1.5) at pH 7. Calibration of the Dowex 50 x 8 column was performed using pure uroporphyrin and hematoporphyrin as markers. The uroporphyrin was eluted by the pH 5.28 citrate buffer and the hematoporphyrin by the 8.25 citrate buffer.

(d) Molecular Sieving.

The use of molecular sieving used as the initial purification step gave results similar to those found by the use of Amberlite resins, i.e. only one large porphyrin-peptide peak emerged (as shown in Section II, fig. 26). The use of Sephadex as a second purification step, i.e. after the initial use of an ion exchange column, proved particularly gratifying. Sephadex G 25 super fine was used. The results obtained from 2 columns with different buffers showed excellent separation. In trial run, a sample containing cytochrome c that had been digested with pepsin (PMC) was applied to the system. The PMC was first run on a Sephadex G 25 column equilibrated with a phosphate/borate buffer 0.05 M pH 8.4. The PMC peak was pooled lyophilised, a small amount was hydrolysed and the amino acid composition compared with the expected theoretical ratios; the remainder of the material was applied to the second Sephadex G 25 column equilibrated with 30% acetic acid. The PMC peak from this column was lyophilised and hydrolysed. The results are shown in Table 27.

Fig. 46. FAECAL PORPHYRINS SEPARATED FROM FREE PEPTIDES ON AMBERLITE CG 400 (anion exchange resin)

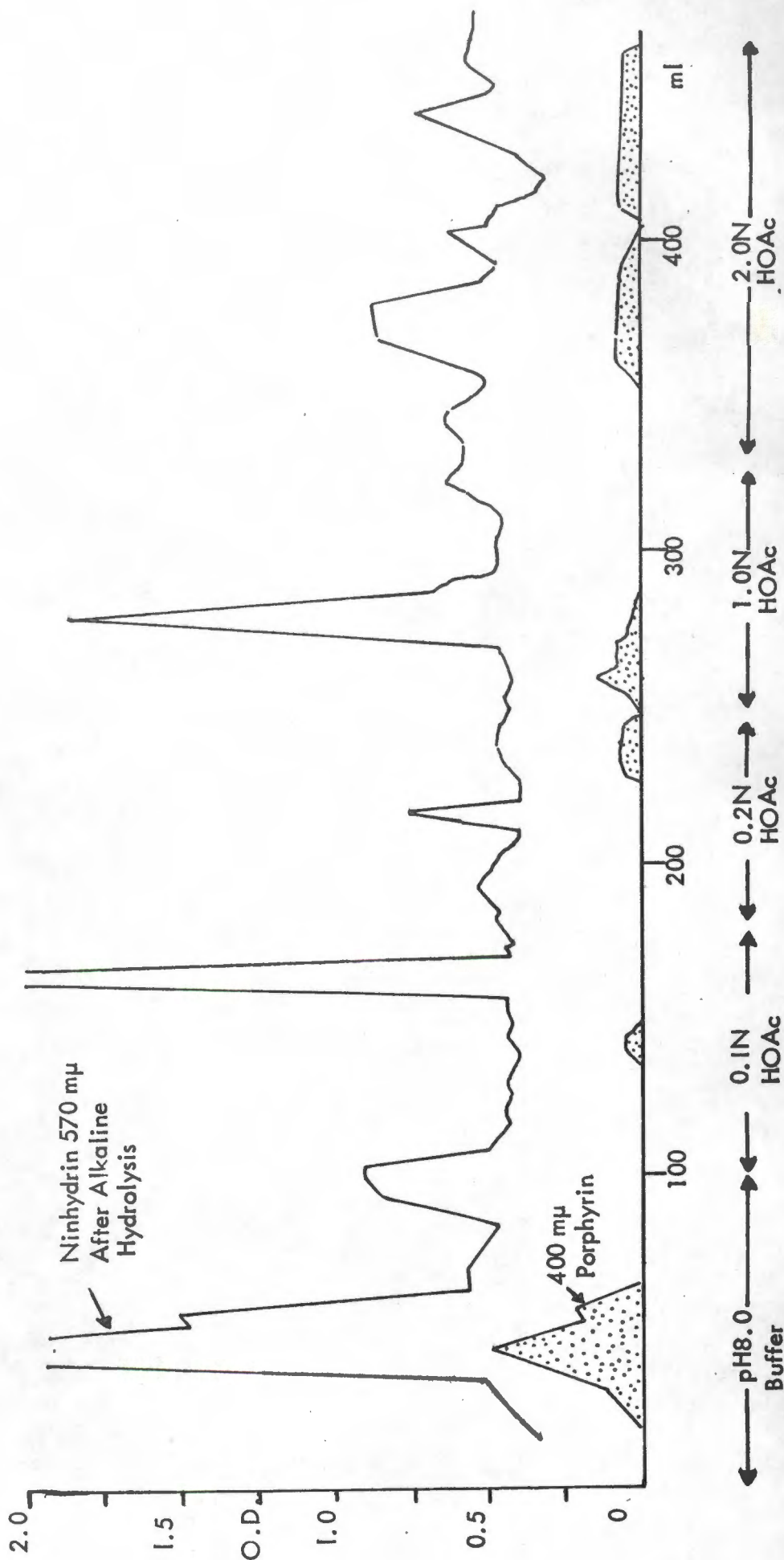
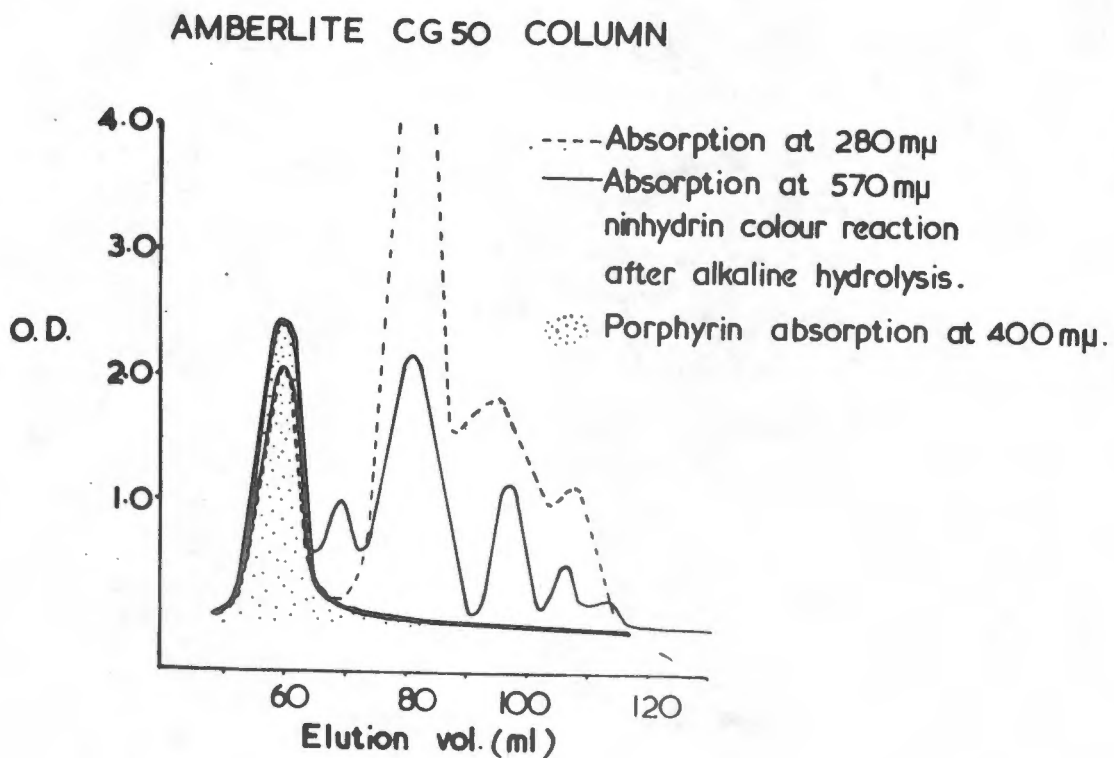


Fig. 47.

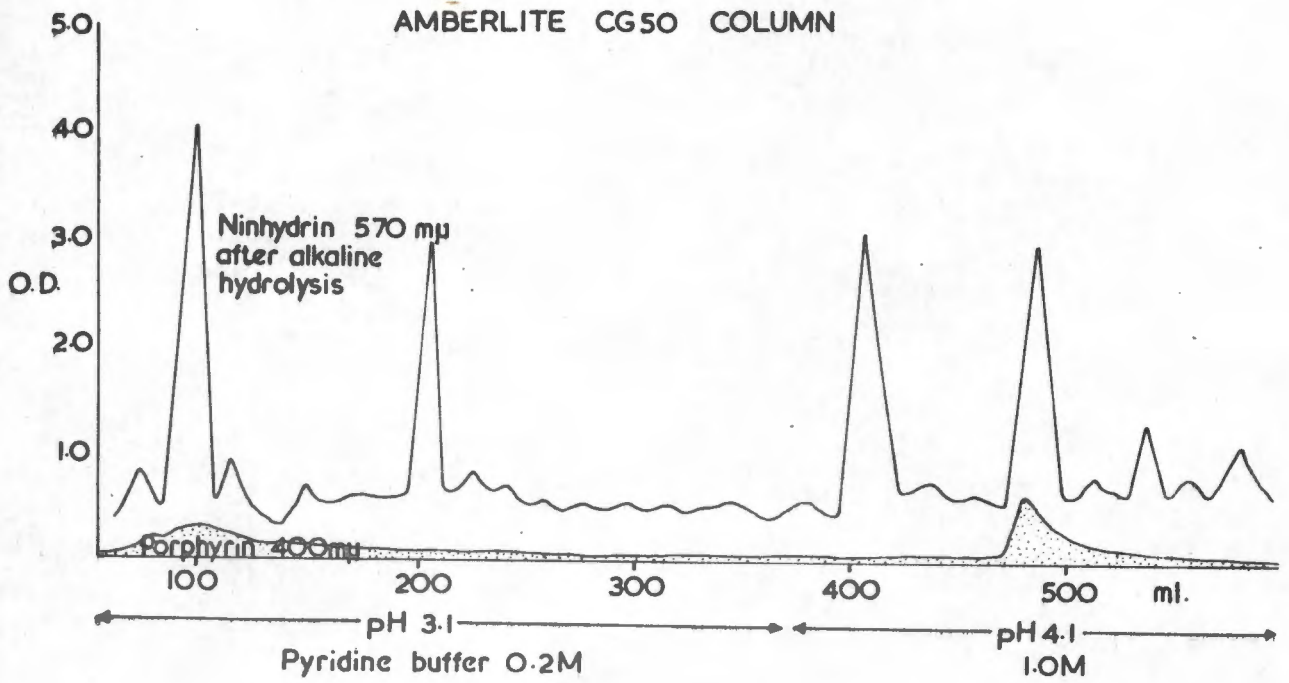
THE ELUTION PROFILE OF THE URINARY HYDROPHILIC PORPHYRIN FROM A CATION EXCHANGE COLUMN
(Amberlite CG 50 - 0.125 M Ammonium Acetate)



Several free peptides of varying sizes were removed from the porphyrin-peptide complex by means of separation on the amberlite CG 50 column.

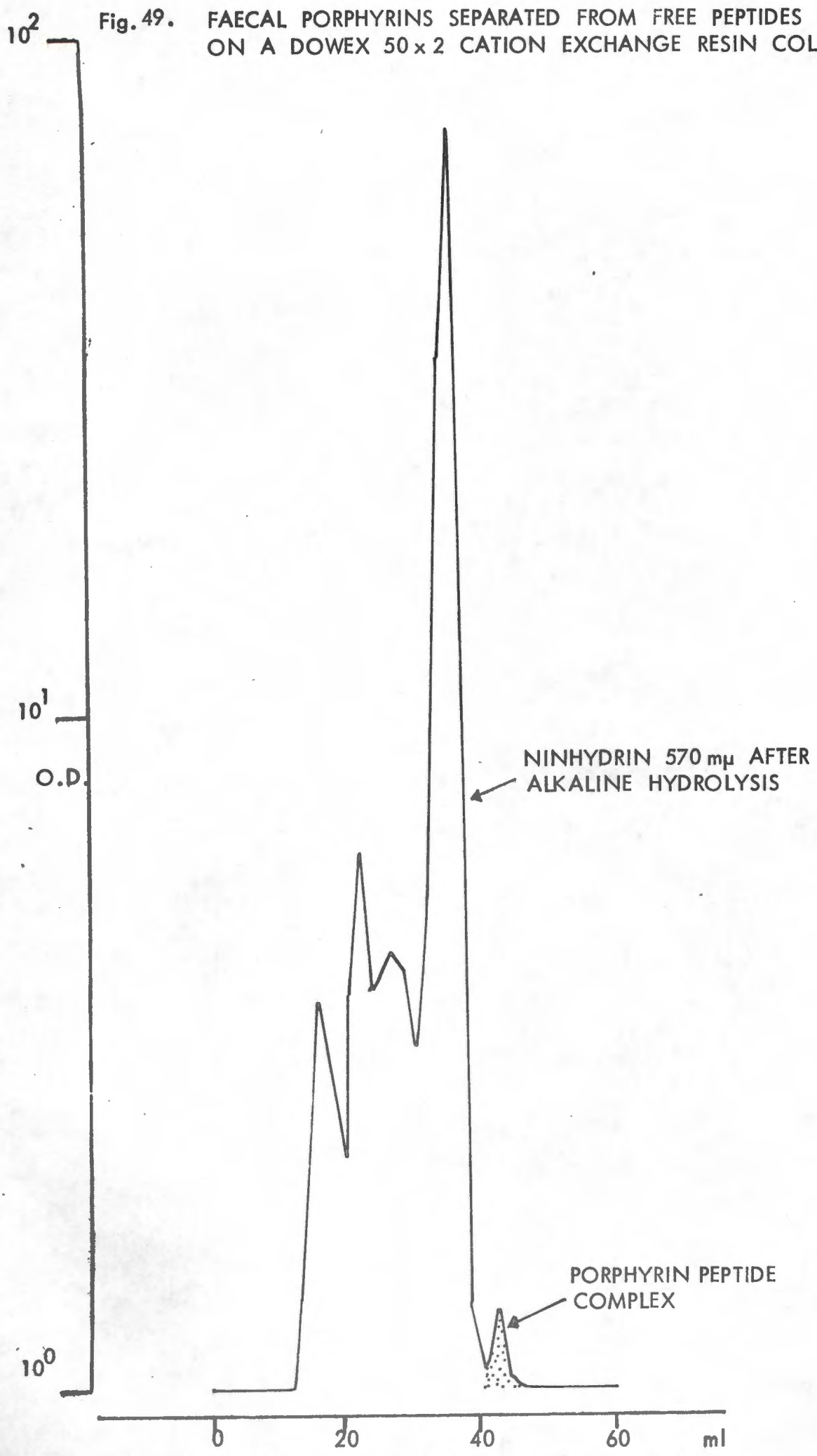
Fig. 48.

THE SEPARATION OF THE HYDROPHILIC PORPHYRINS
ON AMBERLITE CG 50 USING PYRIDINE BUFFERS



The porphyrin-peptides were spread over many tubes, and several free peptides were isolated.

Fig. 49. FAECAL PORPHYRINS SEPARATED FROM FREE PEPTIDES ON A DOWEX 50x2 CATION EXCHANGE RESIN COLUMN



The sample was eluted with a 2M pyridine-acetate buffer pH 4.9

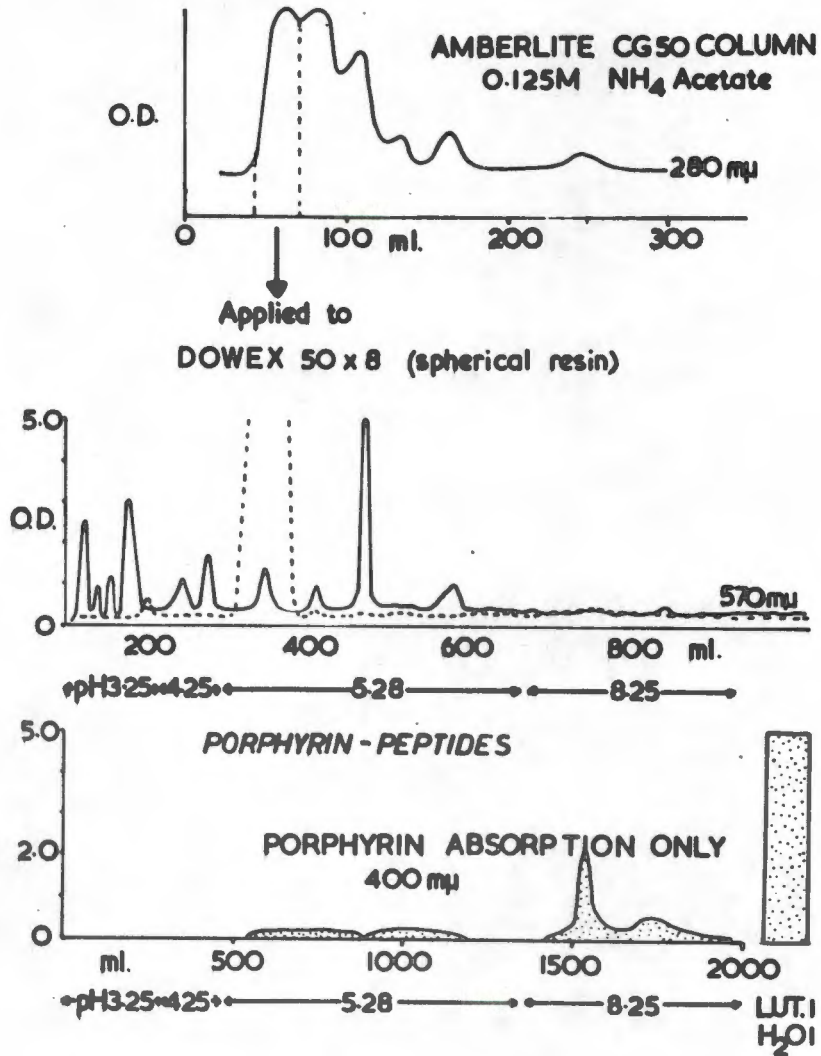
TABLE 27

THE AMINO ACID COMPOSITION OF PMC AFTER
PURIFICATION OF SEPHADEX G 25 SUPERFINE COLUMNS.

	Theoretical ratio expected	Amino acid composition after passing through SG 25 at pH 8.4.		Amino acid composition after 1. SG 25 with PO_4/bor 0.05 M 2. SG 25 with 30% HOAc	
		<u>mmoles</u>	<u>ratio .</u>	<u>mmoles</u>	<u>ratio</u>
Asp		0.19			
Thr	1	0.48	1	0.43	1
Ser		0.09		-	
Glu	3	1.54	3	1.36	3
Pro		-		-	
Gly		0.18		-	
Ala	1	0.55	1	0.42	1
Cys/2	2	0.93	2	-	
Val	2	0.93	2	0.81	2
Met		-		-	
Ileu		0.08		-	
Leu		0.09		-	
Tyr		-		-	
Phen		-		-	
Lys	1	0.90	2	0.61	1.1
Hist	1	0.80	1.5	0.56	1.1
Arg		-		-	

Fig. 50.

THE SEPARATION OF THE PORPHYRIN-PEPTIDE
COMPLEXES FROM CONTAMINATING FREE PEPTIDES



The first peak eluted from the amberlite CG 50 column (0.125 M NH₄ Acetate) was lyophilised and applied to the Dowex 50 x 8 column, as may be seen many free peptides were removed from the porphyrin 'X'.

As may be seen from this table, the amino acid ratios gave perfect correlation with the theoretical ratio expected when both columns were used. When only the phosphate/borate buffered column was used a fair amount of contamination existed.

It was hoped that the molecular sieving would give an approximate indication of the molecular weight of the porphyrin-peptide as well as merely serving as a purification procedure. The two columns were therefore calibrated and results recorded in Table 28.

TABLE 28. CALIBRATION OF SEPHADEX G 25 (SUPER FINE) COLUMN
(0.9 x 150 cm)

	Phosphate/Borate Buffer pH 8.4 Elution vol. (ml)	Acetic Acid 30% Elution vol. (ml)
PTMC	46	
PMC	42	57
PMC porphyrin	57	65
Cytochrome c	63	44
Hematoporphyrin	420	73

With the use of the phosphate/borate buffer the order of elution was not according to the molecular size and free porphyrins were strongly adsorbed.

(e) Enzyme Digestion.

In order to determine the amino acid composition of the peptide core immediately surrounding the porphyrin moiety of porphyrin "X" the complex was digested with trypsin and pepsin. Preliminary runs were performed using cytochrome c with completely successful results as shown in Table 27.

(f) The isoelectric precipitation of porphyrin "X".

If the ether insoluble porphyrins were allowed to stand at pH 3.5. for several days a black precipitate formed. We initially thought that this precipitate might be porphyrin "X". After purification on ion exchange columns, the amino acid composition was determined (see Table (i) Appendix B) and found to be essentially similar to that noted on the H.V. electrophoretograms reported in Chapter 4, the only variation being the high content of lysine.

(g) Isoelectric focusing.

It was thought that isoelectric focusing could be used for 2 purposes, namely as a purification step as well as the determination of this of the isoelectric point of the purified product. A 110 ml LKB apparatus was used with a pH range of 3-10. A preliminary run using porphyrin c was carried out (fig. 51). Both fluorescence and absorption at 400 mu were recorded to ascertain the peak positions. Absolute readings could not be taken directly since it was not possible to prepare a blank for each tube that would have an identical sucrose concentration and pH. For absolute readings each tube was adjusted to pH 3.5 with glacial acetic acid and the porphyrin peptide extracted into butanol which was then washed with water. For this method to be of use as a purification procedure it was necessary to remove the ampholytes from the sample. This presented a problem since the ampholytes were of approximately the same molecular weight and carried the same electrostatic charge as the porphyrin peptide under investigation; solvent partitioning did not prove entirely satisfactory either. This method was therefore only used for the determination of the isoelectric point of the purified porphyrin "X". Fig. 51 shows the presence of 2 peaks. The first peak was entirely ether soluble and therefore represented the free porphyrin present in the sample.

(h) Disc electrophoresis using Polyacrylamide gels.

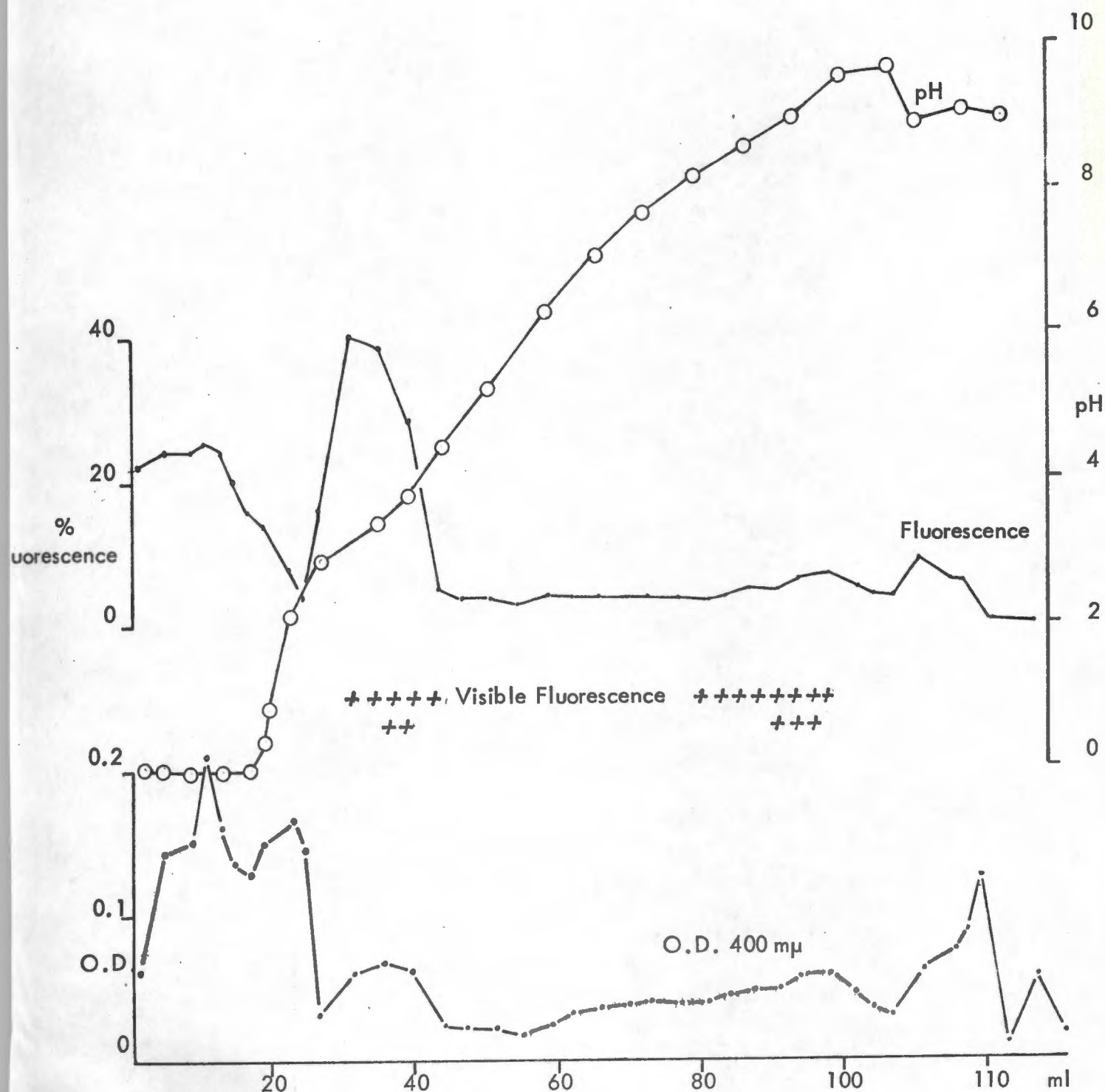
A sample of the crude ether insoluble porphyrin peptide was run on disc

electrophoresis.

A small pore gel pH 9.5 was used (7.5% acrylamide) (see Appendix A). The sample was adjusted to pH 9.5, sucrose was added and the solution directly layered on top of the small pore gel. The sample was run towards the anode. At the end of the run one gel was fixed immediately and stained for protein. Another gel was taken for scanning in a gel attachment on a Gilford spectrophotometer. The gel was scanned at 280 mu and 400 mu and in each instance a single band was detected. The stained gel likewise showed a single band (fig. 52).

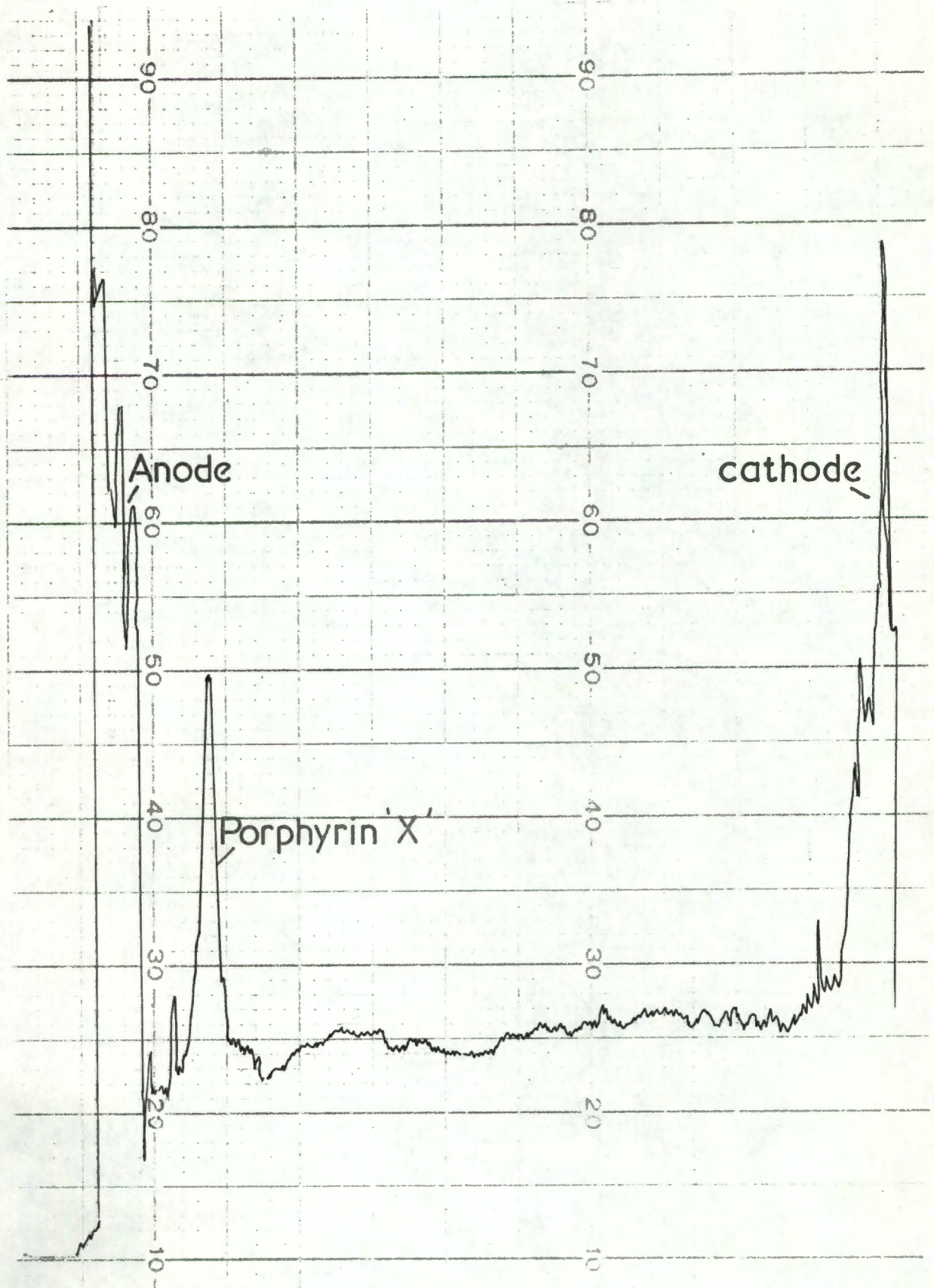
Polyacrylamide disc electrophoresis could obviously not be used as a criterion for purity since only one band was detected, and yet previously it had been shown by other methods that a complex mixture of substances existed in the crude extract.

Fig. 51. ISOELECTRIC FOCUSING IN A pH GRADIENT



Isoelectric focusing was performed on porphyrin C
the column was divided up into 3 ml fractions and the O.D. and fluorescence recorded.

Fig. 52. DISC GEL ELECTROPHORESIS OF CRUDE FAECAL PORPHYRIN EXTRACT



The crude faecal porphyrin extract was applied at the cathode and a voltage of 300V applied across the terminals.

An outline of the final scheme adopted for the isolation of the porphyrin-peptides from faeces has been given in fig. 53. The scheme for urine varied only in so far as the initial extraction was concerned. The urine (\pm 15 L) was adsorbed onto talc and the talc washed with 10 L of distilled water. The porphyrin-peptides and free porphyrins were eluted from the talc with acetone/HCl and trifluoroacetic acid as discussed earlier and the procedure continued as for faeces from Step III, fig. 53. (p128)

The isolation procedure became a ramifying process with each new step that was taken and a picture of immense complexity resulted. In order to clarify the situation the isolation procedure has been divided into steps and the porphyrin and peptide moieties of the compound will be discussed separately.

(a) The Heterogeneity of the Hydrophilic Porphyrins.

From Step VI (fig. 53) onwards the scheme begins to ramify. Each step has been discussed in order.

Step VI. The porphyrin-peptides were eluted from the Dowex 50 x 8 column in a stepwise manner by the use of citrate buffers pH 3.25 to 8.25; water was then passed through the column and finally 0.1 N NaOH (fig. 50) (p121)

Step VII. The eluate was monitored at 280 m μ and showed the presence of 25 peaks, 14 of which fluoresced red under UV light. 2 of these were eluted at pH 3.25, 1 at pH 5.28, 8 at pH 8.25, 2 were eluted with water, and 1 with NaOH. Of these 14 fluorescent peaks eluted from the Dowex 50 x 8 column only 3 were in sufficient quantity to allow further purification, i.e. peak II (pH 3.25) and peak XVI (pH 8.25) and XXIII (H₂O). These will be described later in fig. 53 (c).

Step VIII. The major portion of the porphyrin-peptide which had not moved on the cation exchange resin was manually removed from the top of the column, and the porphyrin-peptide removed from the resin with lut/H₂O/HOAc (10 : 10 : 1.5)

FIG. 53 (a)

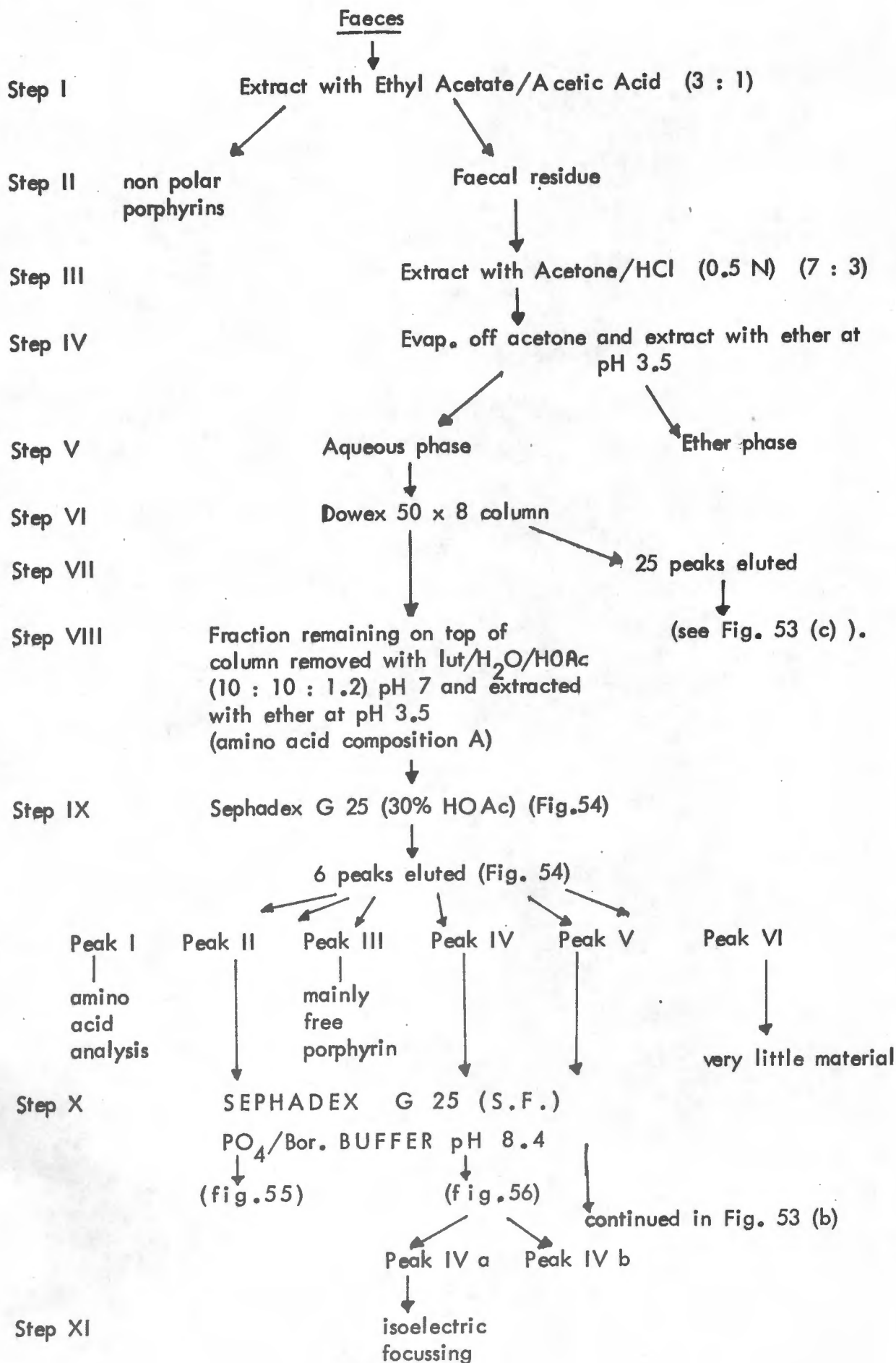


FIG. 53 (b)

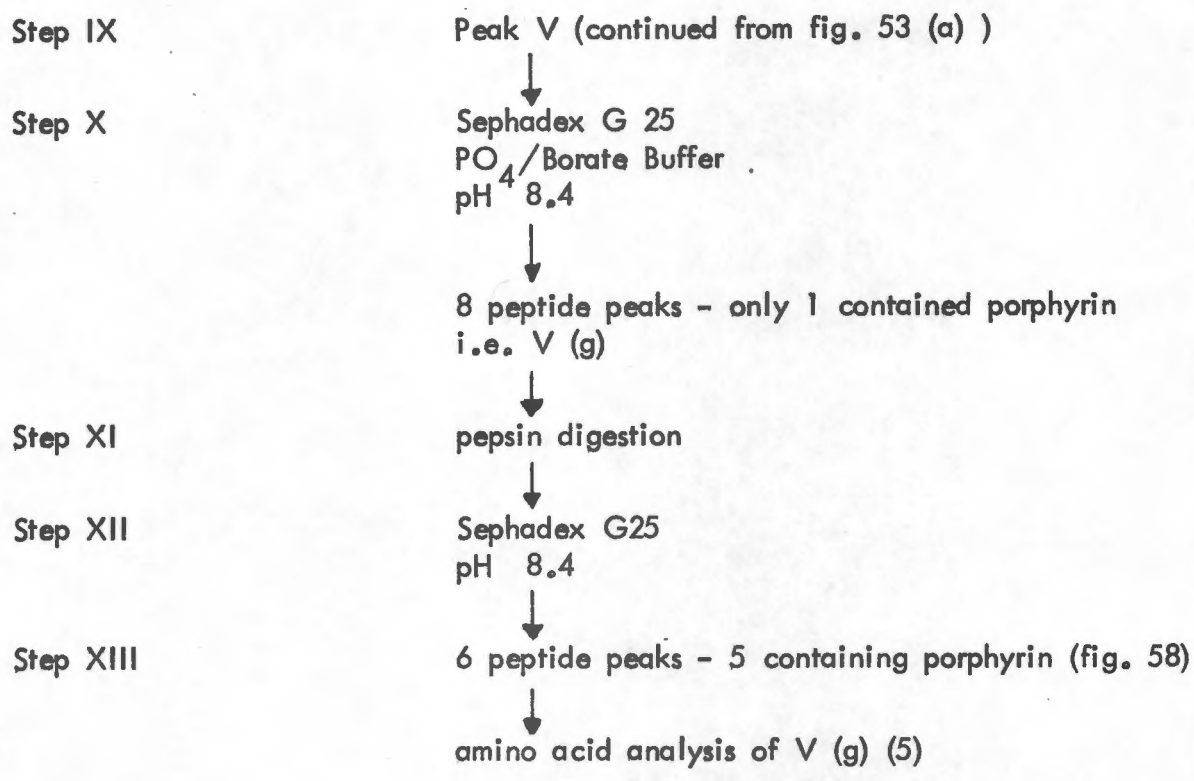
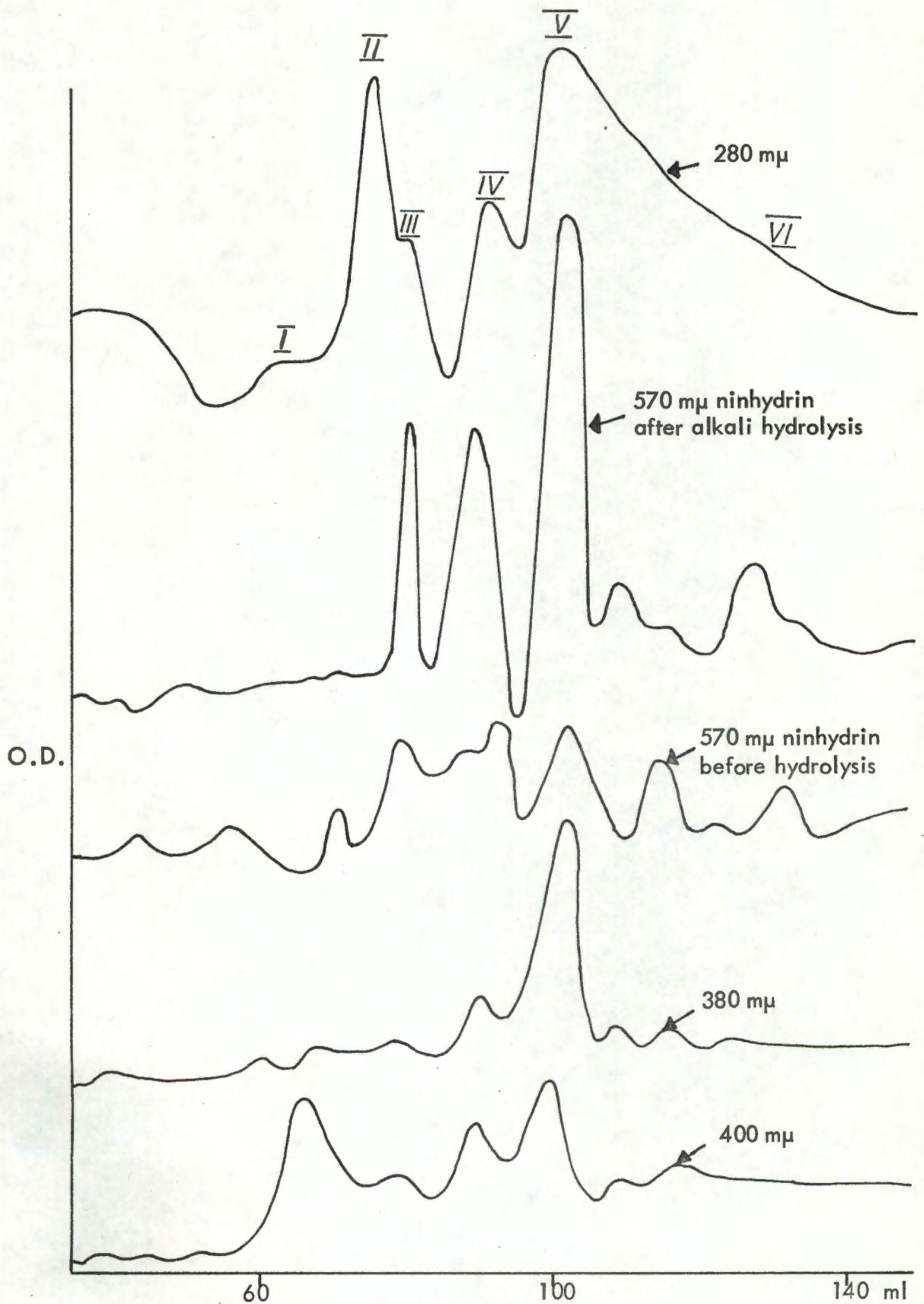


Fig. 54. ELUTION PROFILE OF HYDROPHILIC PORPHYRINS ON SEPHADEX G25



The fractions were eluted with 30% HOAc.

Fig.55. ELUTION PROFILE OF PEAK II TAKEN FROM A SEPHADEX G25 COLUMN (30% HOAc) AND NOW APPLIED TO A SEPHADEX G25 COLUMN AND ELUTED WITH A 0.05 M PHOSPHATE/BORATE BUFFER pH 8.4.

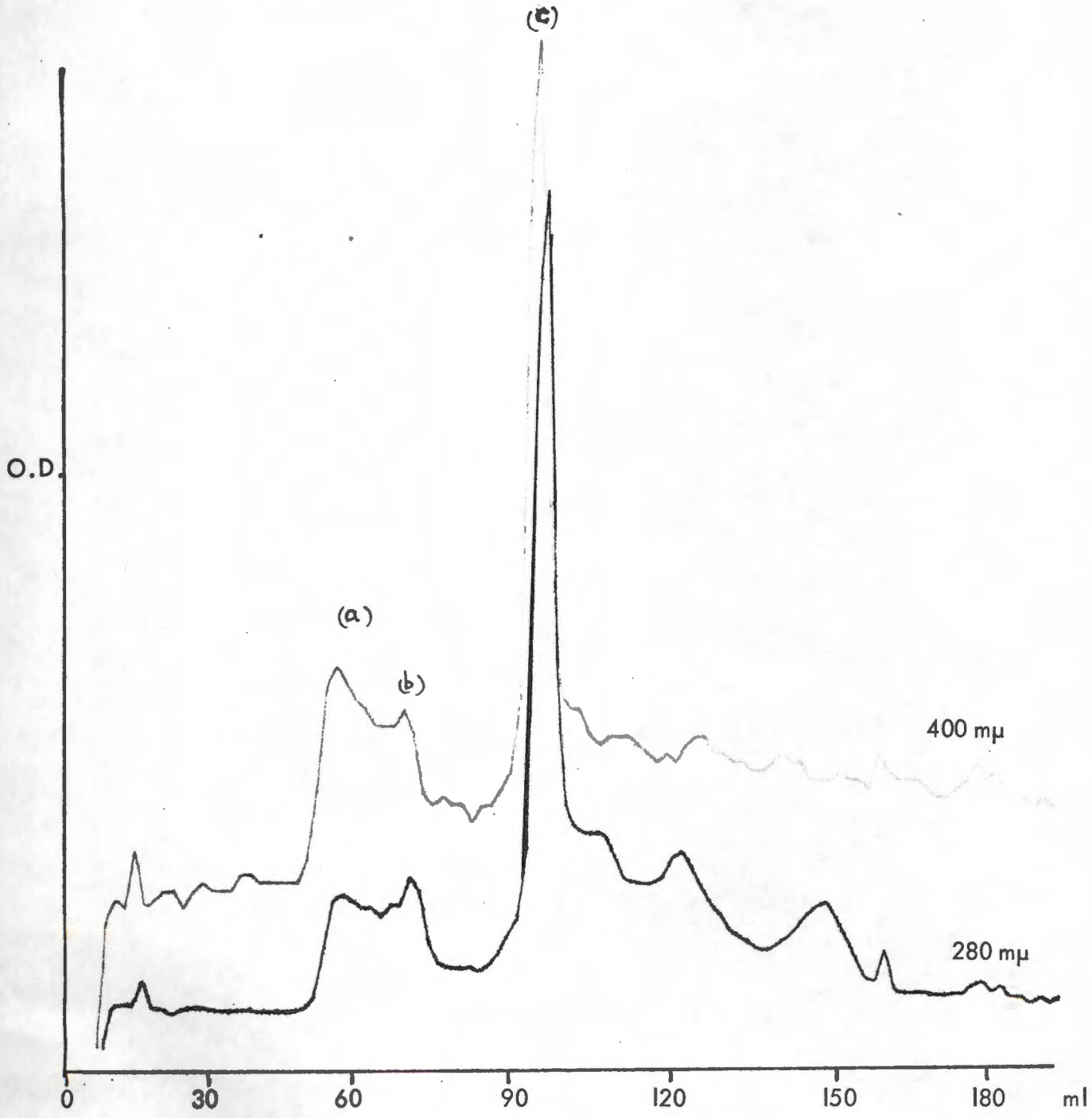


Fig. 56. ELUTION PROFILE OF PEAK IV TAKEN FROM A SEPHADEX G25 COLUMN (30% HOAc) AND NOW APPLIED TO A SEPHADEX G25 COLUMN AND ELUTED WITH A 0.05 M PHOSPHATE/BORATE BUFFER pH 8.4.

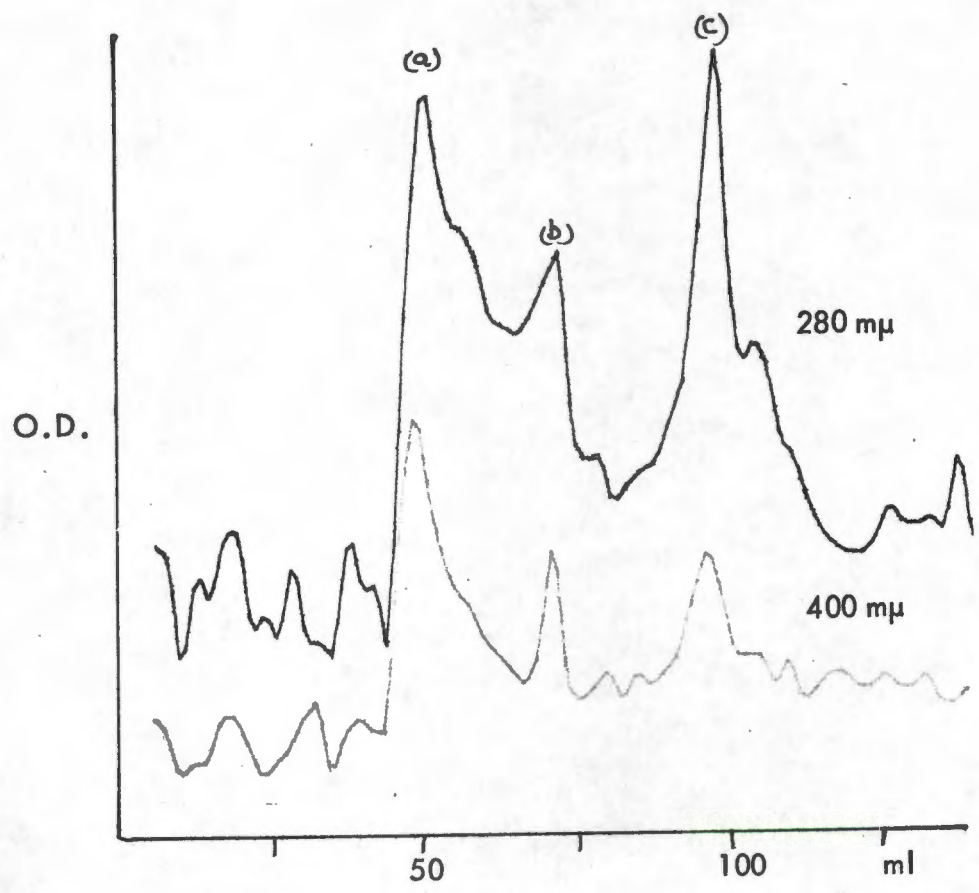


Fig. 57. ELUTION PROFILE OF PEAK V (SEE FIG. 54) TAKEN FROM A SEPHADEX G25 COLUMN (30% HOAc) AND NOW APPLIED TO SEPHADEX G25 COLUMN AND ELUTED WITH A 0.05 M PHOSPHATE/BORATE BUFFER pH 8.4.

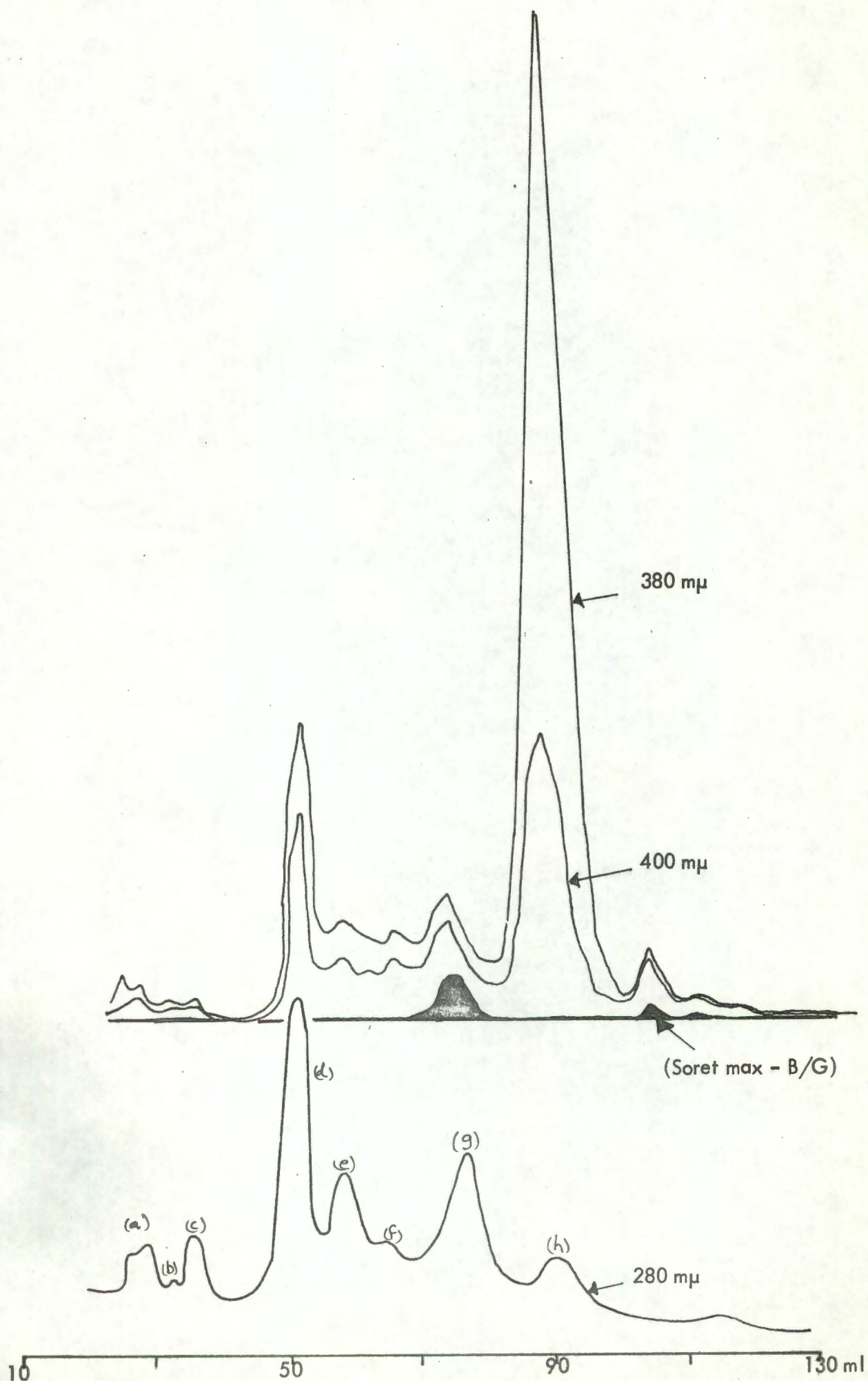


Fig. 58 ELUTION PROFILE OF PEAK (g)(Fig. 57) AFTER PEPSIN DIGESTION. THE PORPHYRIN-PEPTIDES WERE ELUTED WITH A 0.05M PHOSPHATE/BORATE BUFFER pH 8.4.

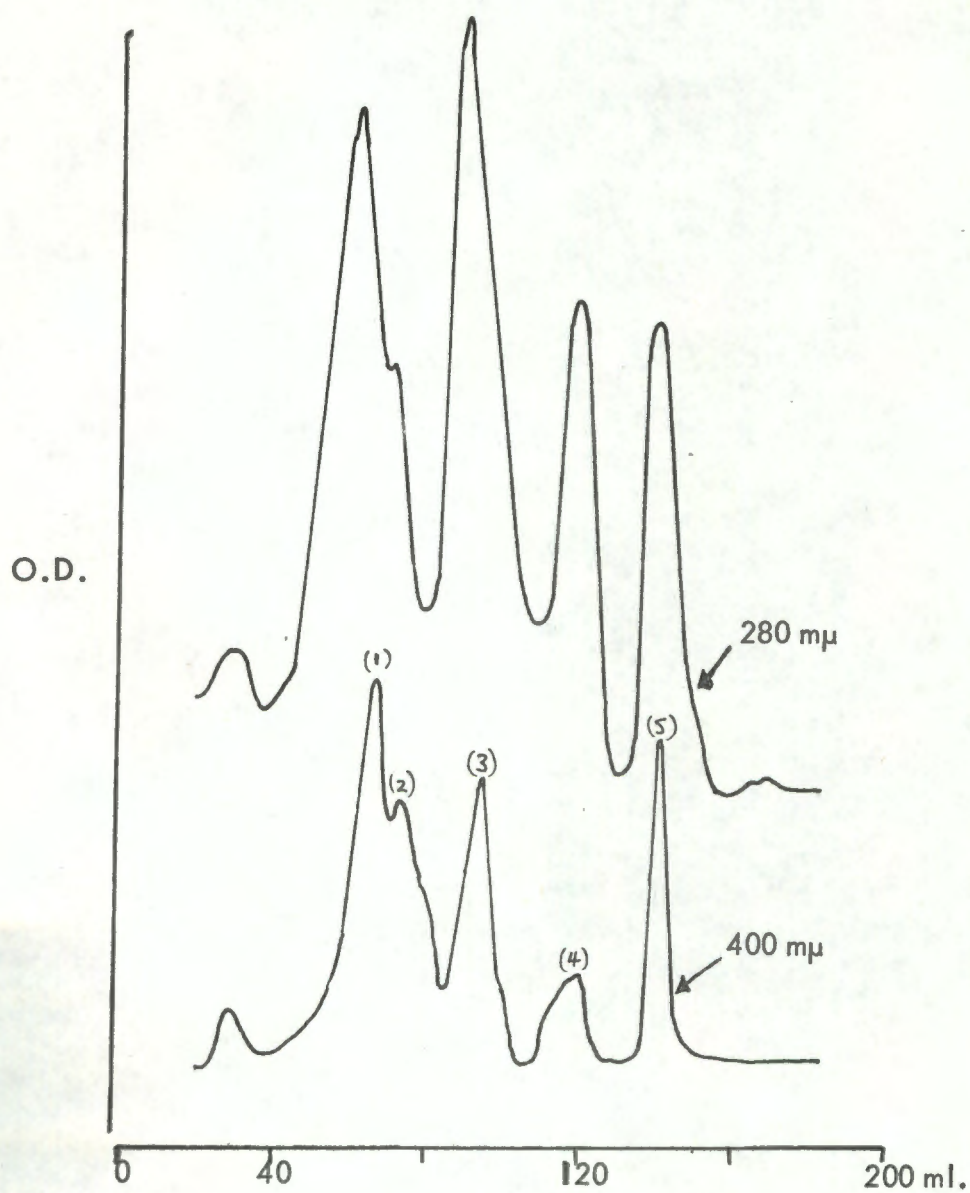
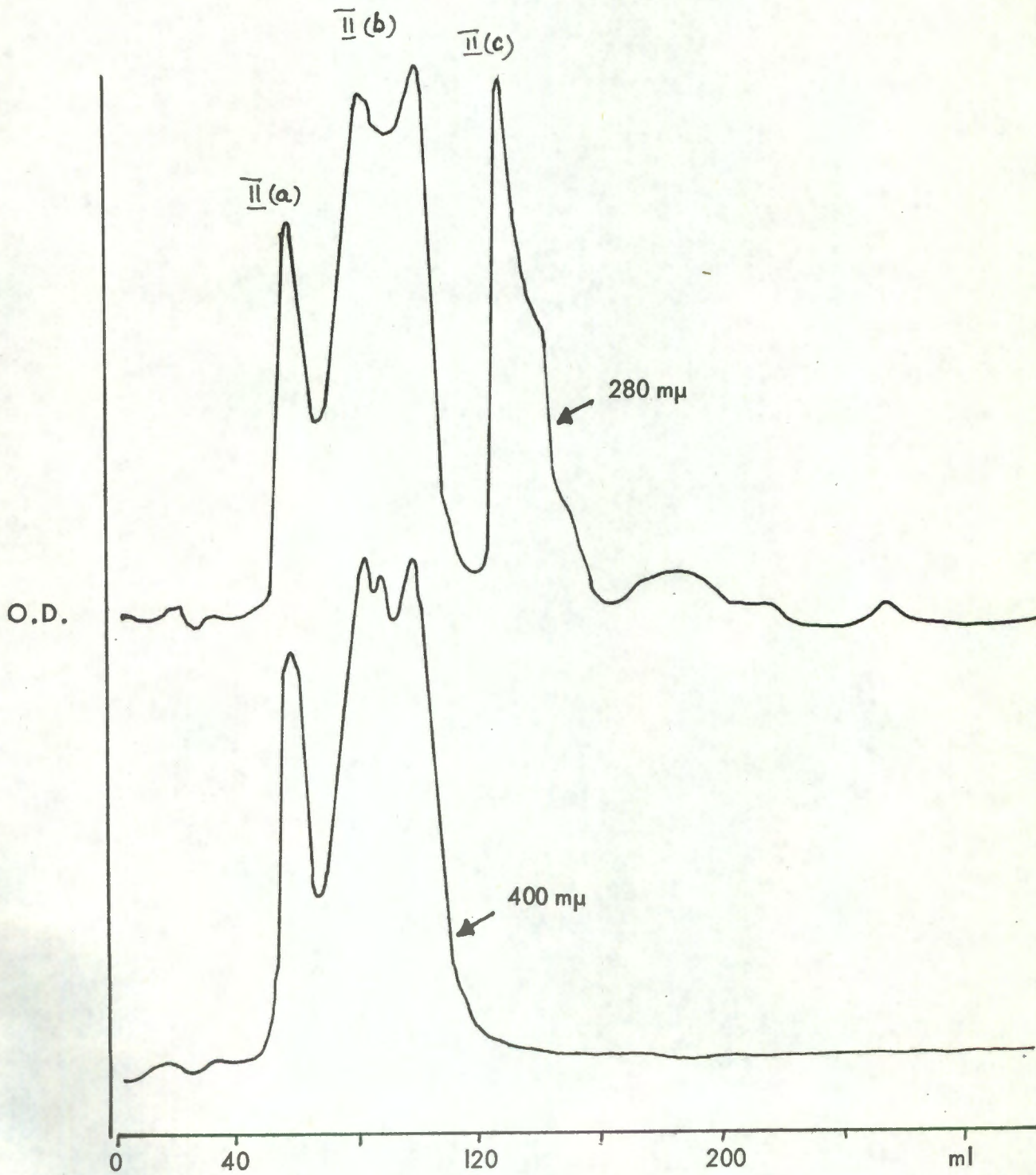
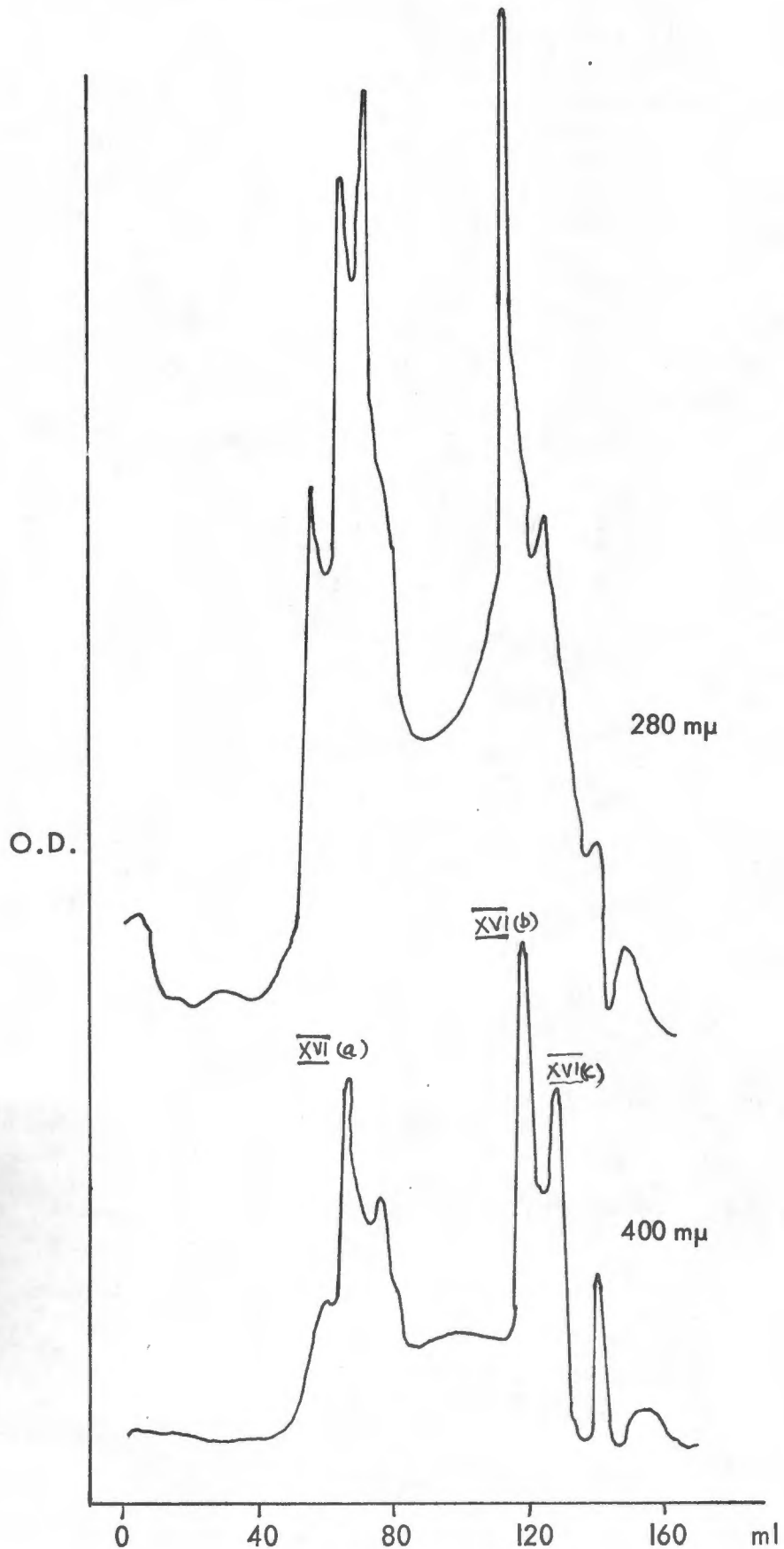


Fig.59. ELUTION PROFILE FROM SEPHADEX G25 COLUMN
0.05 M PHOSPHATE/BORATE BUFFER pH 8.4.



The second peak that was eluted from the Dowex 50 x 8 column was subjected to pepsin digestion, and has been applied to the Sephadex column.

Fig. 60. ELUTION PROFILE FROM A SEPHADEX G25 COLUMN - 0.05 M PHOSPHATE/BORATE BUFFER pH 8.4.



Peak XVI that was eluted from the Dowex 50 x 8 column at pH 8.25 has been subjected to pepsin digestion and applied to the Sephadex column.

Fig. 61 PEAK (b) FROM FIG. 59 HAS BEEN SEPARATED ON SEPHADEX G25 USING 30% HOAc.

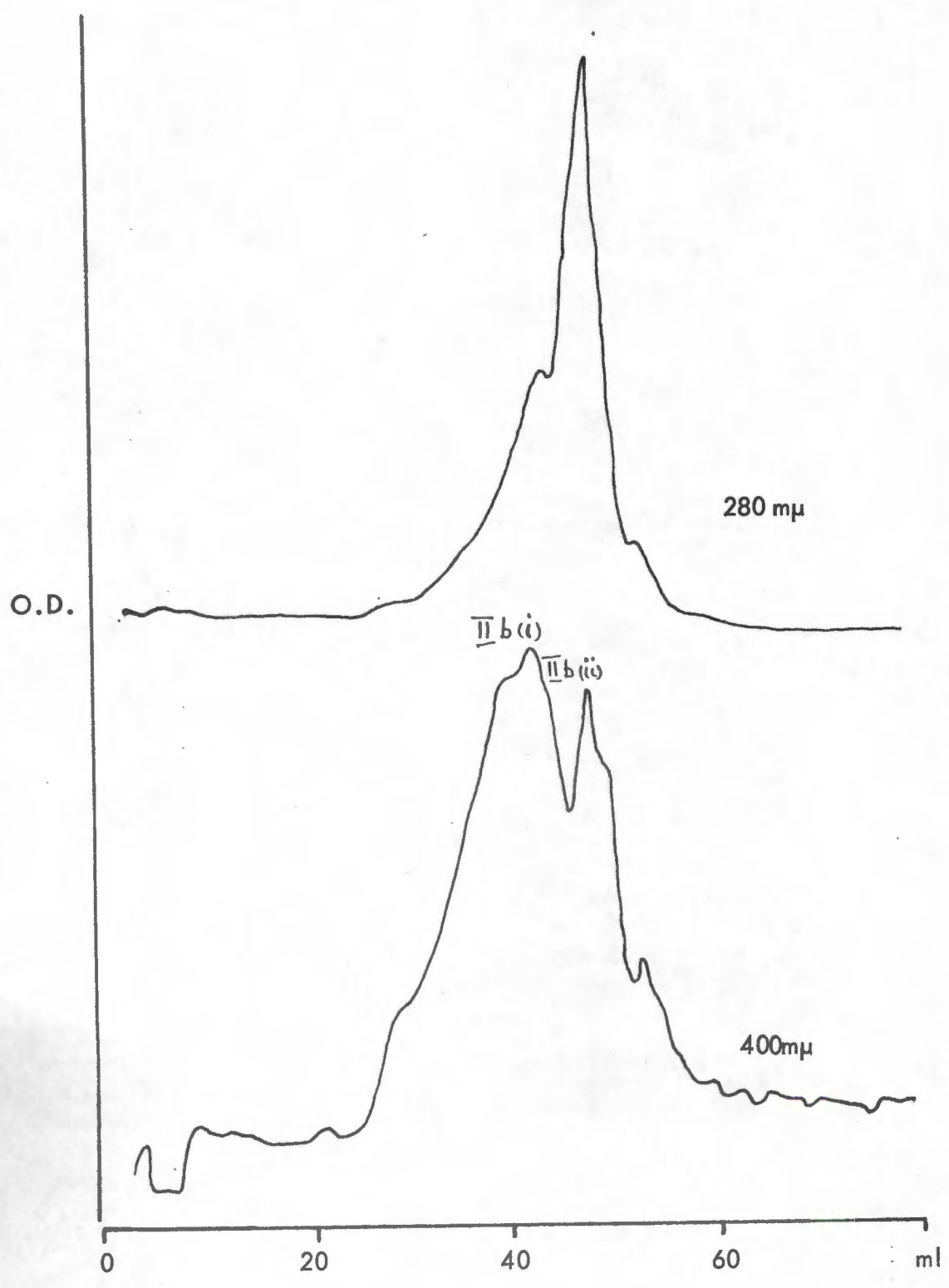
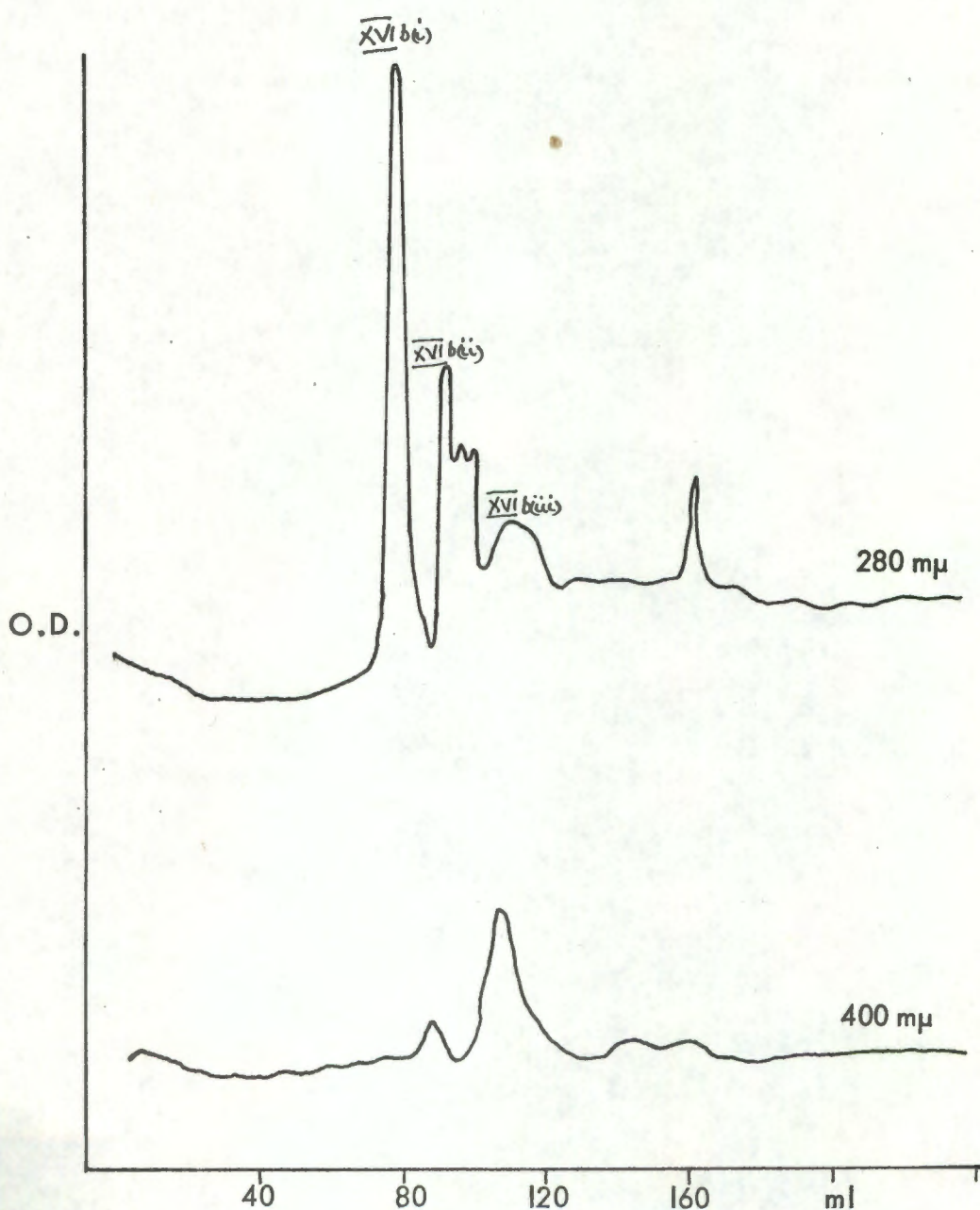


Fig. 62 ELUTION PROFILE FROM A SEPHADEX G25 COLUMN - 30% HOAc.



Peak XVI(b) (see Fig. 60) was lyophilised and then applied to a Sephadex column and the porphyrin-peptide eluted with 30% HOAc.

at pH 7. This was lyophilised several times to remove all traces of the lutidine. At this stage the material was found to be readily soluble in water and a small amount of it became ether soluble.

Step IX. 80% of the ether insoluble fraction from the Dowex column was taken up in 30% acetic acid and applied to a Sephadex G 25 super fine column, equilibrated with the same. This separated the porphyrin peptide material into 6 distinct peptide peaks that absorbed at 280 μ (fig. 54). Ninhydrin determinations were performed before and after alkali hydrolysis. Peak V showed the presence of a particularly large peptide as exemplified by the difference between the special density readings before and after hydrolysis. It also showed a large amount of bile pigment present that absorbed at 380 μ . Most of the porphyrin in peak III became ether soluble at this stage. Peak I showed the best separation and was taken for amino acid analysis. Peaks II, IV and V were lyophilised several times and applied individually to the Sephadex G 25 column that had been equilibrated with the phosphate/borate buffer 0.05 M pH 8.4.

Step X. The elution profiles from these three samples may be seen in figs. 54, 55 and 56.

In fig. 54 it may be seen that 2 further free peptides were removed and 2 fluorescent peaks were present. Fig. 55 depicting Peak IV showed a similar pattern but with the peak emerging at 50 ml now being greater than that at 94 ml. Peak V (fig. 56) showed a slightly more complicated pattern with the presence of 8 peaks absorbing at 280 μ , peaks (d) and (h) (fig. 56) contained large amounts of bile pigment and peak g was the only one containing fair amounts of porphyrin and was taken for further study.

Much of the similarity seen between the elution profiles depicted in figs. 54, 55 and 56 is due to overlap of the initial peaks I \rightarrow IV in step IX. An attempt to separate these peaks in a better manner was made by placing two 150 cm columns in series with each other. The improvement attained was offset by the

loss of material and therefore this idea was abandoned.

Step XI. (fig. 53 (b)). Peak V (g) was found to contain 4.5. μ Moles of protein as determined by the ninhydrin method. This was subjected to pepsin digestion in order to determine the amino acid core around the porphyrin.

Step XII. The digested sample was applied to a Sephadex G 25 column and eluted with phosphate/borate buffer pH 8.4 (fig. 58). Six peptide peaks were seen, five of which contained porphyrin. This was not expected and the only explanation for the five porphyrin-peptide components is the possibility of there being incomplete cleavage by the pepsin. One of these fractions was taken for amino acid analysis.

Step VII. To return to fig. 53 (a) ^{P128} 25 peptide peaks were eluted from the Dowex 50 x 8 cation exchange resin column; of these 14 showed red fluorescence. The scheme for these has been continued in fig. 53 (c). Only 3 peaks were in sufficient quantity to allow further purification.

Step XIV. Peak II, that was eluted with the pH 3.25 citrate buffer and Peak XVI, which was eluted with the 8.25 buffer, were both subjected to pepsin digestion. The products of digestion were then run on a Sephadex G 25 column with a phosphate/borate buffer. Peak XXIII was found in relatively larger amounts in the urine than in the faeces. It was noted that the porphyrin-peptide could be divided into butanol soluble, and butanol insoluble fractions. These were hydrolysed separately and the amino acid composition determined.

Step XV. The elution profile from Peak II has been shown in fig. 59 and that from peak XVI in fig, 60. As on the previous occasion more than one porphyrin-peptide product was noted after pepsin digestion. Only the major component was taken for further purification, i.e. Peaks II (b) and XVI (b).

Step XVI. These two peaks (II (b) and XVI (b)) were purified further on a Sephadex G 25 equilibrated with 30% HOAc (fig. 61 and 62 respectively).

Peak XVI (b) yielded a single porphyrin-peptide peak and 2 free peptide peaks. Amino acid analysis was performed on all three, in the hope that we might have detected a particular component that was responsible for the porphyrin binding.

Peak II (b) yielded two porphyrin-peptide peaks. The resolution achieved in fig. 60 was excellent and therefore peak II (b) (i) and (ii) were not due to overlap between peak II (a) and II (b).

Step XVIII. Peak II (b) (i) was subjected to trypsin digestion, the products of which were separated on the Sephadex at pH 8.4 and an amino acid analysis determination performed.

As may be seen from fig. 53 (a), (b) and (c), an extremely ramifying pattern emerged, which illustrated the heterogeneity of the porphyrin-peptide complexes and simultaneously stressed the necessity for the isolation of a pure compound before structure analysis could be performed. The peptide and porphyrin moieties of these various fractions have been discussed separately.

(b) The amino acid composition of the peptide components of the porphyrin "X" complexes.

Study directed at the elucidation of the composition of the peptide moiety of the porphyrin-peptide complexes was attempted with a number of points in view. It was felt that such a study might contribute information regarding the relationship between porphyrin "X" and cytochrome c. If it could be shown that the peptide, associated with the porphyrin in the excreta, had an amino acid composition consistent with that of cytochrome c, the latter might be considered the parent substance and the porphyrin-peptide complexes forming part of the cytochrome c pathway.

If a distinct relationship between the porphyrin-peptide and cytochrome c could not be established, was it possible that there are discrete peptides that

complex preferentially to porphyrin by virtue of their amino acid composition and if so are these particularly elevated in the excreta of variegate and symptomatic porphyrics.

It seemed important to establish whether the apparent heterogeneity and complexity of the porphyrin-peptides reflected a real biosynthetic difference in their origin and whether it reflected random degradation of a common parent porphyrin-peptide molecule in the gut.

To investigate this, amino acid analysis was performed on as many of the fractions isolated as possible. The results have been recorded in Tables 29 and 30. The notation has been taken from figs. 53 (a) (b) and (c).

The amino acid analysis performed on the various fractions isolated could not be positively identified with any known proteins but very close similarities existed between fraction XVI (a) - isolated from the urine (fig. 60) and β_2 microglobulin isolated by Bergg^ord and Bearn (1968) from the urine of patients with tubercular porteinurias. The amino acid compositions of these two proteins have been laid out for ease of comparison in table 31.

Some similarity was also seen between fraction XVI (a) (ii) and II (b) (i) (n) isolated from the faeces (Steps XVI and XVIII respectively, fig. 53 (c)) and the heme binding β globulin hemopexin. These have been set out in table 32. The slight variations seen may be due to the fact that the fractions isolated were subjected to pepsin digestion prior to the amino acid analysis. It is also interesting to note that nearly all the fractions isolated although varying in molar ratios between each other, showed a complete dominance of Glutamic acid, glycine and aspartic acid. Also present in fairly high concentrations in most fractions were alanine and serine. This same amino acid pattern was seen in the preliminary studies done (figs. 33 and 34). The high content of acidic amino acids is consistent with the low isoelectric point found in one isolated fraction. Rimington et al.

TABLE 29. THE AMINO ACID COMPOSITION OF THE PORPHYRIN - PEPTIDES ISOLATED FROM THE URINE, EXPRESSED AS A MOLE RATIO (NOTATION FOR FRACTIONS TAKEN FROM FIG. 53)

<u>Step Ref. No.</u>	VIII	XV	XV	XIV	XIV
<u>Peak Ref. No.</u>	A	XVI (a) (fig. 60)	XVI (b) (fig. 60)	XXIII (i)	XXIII (ii)
Asp.	7	9	4	12	14
Thr.	4	5	8	5	7
Ser.	16	8	15	8	7
Pro.		4	5	13	10
Glu.	12	9	25	12	18
Gly.	15	11	18	16	9
Ala	10	10	10	10	10
Val.	5	6	9	8	8
Cys.	10	2	N/D	13	N/D
Met.	Trace	1	Trace	N/D	2
Ileu.	3	4	6	3	5
Leu.	2	6	12	6	9
Tyr.	N/D	2	17	destroyed	2
Phe.	N/D	3	15	N/D	3
Lys.	15	6	9	5	5
His.	Trace	3	5	Trace	10
Arg.	Trace	2	4	3	3

Permission to quote these figures was kindly given by Professor S. Sano of Kyoto University, Japan.

TABLE 30

THE AMINO ACID COMPOSITION OF THE PORPHYRIN PEPTIDES ISOLATED FROM THE URINE, EXPRESSED AS A MOLE RATIO (NOTATION FOR FRACTIONS TAKEN FROM FIG.53)

Step Ref. No.	XIII	XVII	XVIII	IX	XVI	XVI	XVI	XVI	XVI
Peak Ref. No.	Vg (5)	II b (i)	II b (i) (n)	I	XVI a (i)	XVI a (ii)	XVI b (i)	XVI b (ii)	XVI b (iii)
Asp.	7	3	14	17	24	14	13	10	10
Thr.	-	1	8	7	9	8	9	6	5
Ser.	-	1	11	6	17	10	10	10	7
Pro.	-	3	7	12	7	3	4	0	0
Glu.	5	3	14	14	18	15	10	13	10
Gly.	-	4	16	18	26	17	19	17	17
Ala.	10	10	10	10	10	10	10	10	10
Val.	5.5	16	8	10	-	9	13	2	0
Cys.	N/D	2	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Met.	3	10	20	0.5	12	6	N/D	20	12
Ileu.	4	8	6	6	6	4	20	27	18
Leu.	4.5	14	6	7	9	5	21	27	15
Tyr.	2	0.5	3	-	2	1	1	2	3
Phe.	2	Trace	2	-	4	2	1	3	3
Lys.	3	2	15	24	4	3	Trace	Trace	10
His.	0.5	Trace	2	5	1	1	1	0.5	5
Arg.	2	1	0.5	14	Trace	1	10	0	0

TABLE 31.

THE MOLAR RATIOS OF THE AMINO ACID COMPOSITIONS
OF β_2 MICROGLOBULIN AS COMPARED WITH THOSE OF
PEAK XVI α .

	Peak XVI α	β_2 Microglobulin
CySO ₃ H	2	2
Asp.	9	9
Thr.	5	4
Ser.	8	8
Glu.	9	9
Pro.	4	4
Gly.	11	2
Ala.	10	2
Val.	6	6
Met.	1	1
Ileu.	4	4
Leu.	6	6
Tyr.	2	5
Phe.	3	4
Trp.	trace	2
Lys.	6	6
His.	3	3
Arg.	2	4

TABLE 32.

THE MOLAR RATIOS OF THE AMINO ACID COMPOSITIONS
OF HEMOPEXIN AS COMPARED WITH THOSE OF PEAK XVI
a (ii) - (faeces)

	Fraction II b (i) (n)	Fraction XVI a (ii) (faeces)	Hemopexin (Schultze and Herekans)
Asp.	14	14	14
Thr.	8	8	8
Ser.	11	10	10
Glu.	14	15	15
Pro.	7	3	12
Gly.	16	17	14
Ala.	10	10	10
Val.	8	9	9
Met.	20	6	2
Ileu.	6	4	3
Leu.	6	5	13
Tyr.	3	1	6
Phe.	2	2	6
Trp.	-	-	-
Lys.	15	3	8
His.	2	1	6
Arg.	0,5	1	7

(1968) likewise found high concentrations of glycine and glutamic acid in their alkali soluble extracts from faeces.

The presence of a hemopexin like peptide in the hydrophilic porphyrin molecule is not surprising since free hemopexin has been reported to be present in variegate porphyria (Meiers and Ippen, 1968). It is only occasionally found in symptomatic porphyria and therefore this particular type of hydrophilic porphyrin is probably absent from the excreta in these cases. Hemopexin is probably synthesised in the liver (Hochwald et al., 1964) and possibly binds any free hematoporphyrin in the liver, and the latter are then excreta in the bound form in the bile. Hemopexin has also been reported to be present in the interstitial fluid of the dermis (Zimmer and Dub, 1960; Muller et al. 1962). The porphyrins isolated from the blister fluid of a variegate porphyric were not bound, but possibly the hemopexin only binds hematoporphyrin since the blister porphyrins were tetracarboxylic.

It would appear from these results that the peptide moiety of the porphyrin-peptide complexes bears no relation to the amino acid composition of cytochrome c, but that the porphyrin binds preferentially with peptides of a particular type of composition, which have high proportions of aspartic acid, glycine and glutamic acid.

A comparison of faeces from normals and cases of porphyria showed very little variation between the amino acid composition of the peptides excreted. Tyrosine which showed a variation at the 2% level and phenylalanine at a 6% level were the only differences found. Both these amino acids being lower in the porphyria cases. The one case of variegate porphyria associated with jaundice on whom the major portion of the porphyrin "X" studies were performed, showed a marked increase in the level of peptides excreted in the urine.

As Berman (1964) points out, the peptiduria may merely be due to tissue destruction as found in subjects with hepatitis and skin lesions.

(c) The nature of the chemical bond between the porphyrin and peptide moieties of porphyrin "X".

With the supposition initially that the porphyrin-peptide complexes might be related or similar to cytochrome c, it was necessary to establish the type of bonding that existed between the porphyrin and protein components.

At Step IV (fig. 53 a) the urinary and faecal fractions were extracted to completion at pH 3.5 with ether after which they were subjected to changes in pH and then extracted again with ether at pH 3.5. No further release of free porphyrins was noted. The hydroxylamine test was also performed on the aqueous fractions. Neither method demonstrated the presence of any thio-ester bonds.

At Step VIII the porphyrin peptide solutions were again extracted with ether at pH 3.5. Approximately 26% of the porphyrin present in the urinary extract became ether soluble, and 37% likewise in the faecal extract. A small amount of the ether insoluble material was lyophilised and treated with mercury which binds any -SH groups present thereby splitting thioether bonds. 14% of the urinary sample and 26% of the faecal sample showed the presence of thio-ether bonds. This treatment was carried out twice but there still remained a major portion that resisted cleavage. This same fraction showed the presence of cysteic acid after performic acid hydrolysis, which points to the presence of a thio-ether or thio-ester bond.

C type hemoproteins which show resistance to C-S cleavage by treatment with a heavy metal in acid conditions were found to be denatured or polymerised cytochrome c. In the porphyrin-peptide such a possibility may exist or alternatively there may be a possibility of another peptide configuration around the porphyrin. Robinson and Kamen (1968) were able to demonstrate that only 1.9% of the bonds in pure *Rhodospseudomonas* hemoprotein could be split.

The porphyrin component released by the treatment with a heavy metal was shown to be hematoporphyrin and it would appear that the peptide moiety of porphyrin "X" is attached to the 2 and 4 substituent positions of the porphyrin since permethylation prior to mercury treatment followed subsequently with esterification resulted in the formation of hematoporphyrin dimethyl ester, and a minute amount of hematoporphyrin dimethylester monomethyl ether, but no hematoporphyrin dimethyl ester dimethyl ether, which demonstrated the absence initially of a dihydroxy molecule with the peptide being attached to the propionic groups (Sano, 1972).

(d) The nature of the porphyrin moiety of the porphyrin-peptide complexes.

With the establishment of the presence of a thio-ether bond, a cytochrome c type structure seemed most probable. It was therefore interesting to determine whether the same porphyrin moiety was responsible for the binding of the peptide, and chromatographic and spectral studies were performed.

The non-polar porphyrins extracted initially (Step I, fig. 53) were investigated for the presence of hematoporphyrin as follows.

The ethyl acetate/glacial acetic acid (3 : 1) was extracted with 0.01 N HCl; this was extracted back into ether at pH 3.5 and then into 1.5 N HCl which was chromatographed in 2 systems (fig. 63). The remainder of the extract was esterified and extracted into chloroform and run in a further two chromatographic systems (fig. 64). Only a minute amount of free hematoporphyrin was detected, namely 8×10^{-11} moles/G wet stool.

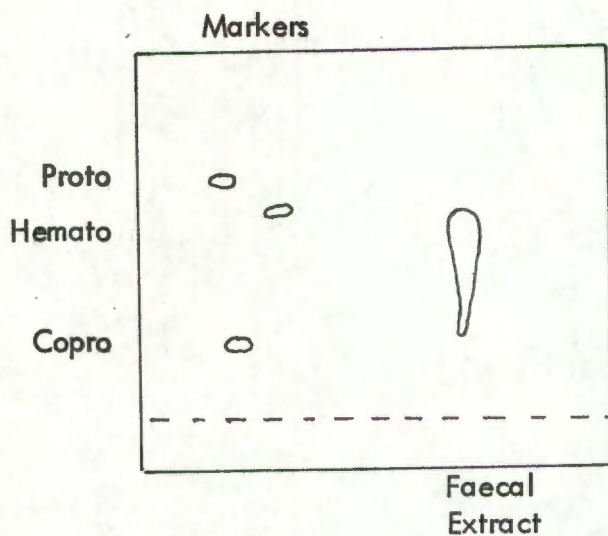
Hematoporphyrin was shown to be present in all these systems.

At Step VII fourteen fluorescent peaks were eluted from the Dowex 50 x 8 column; some of the porphyrin from these became ether soluble, the latter fractions all behaved the same on chromatography and could not be likened to any known marker. On TLC - talc plates with acetone/HCl (7 : 3) as solvent, most of the

FIG. 63

CHROMATOGRAPHY OF FREE HEMATOPORPHYRIN DETECTED
IN THE FAECES FROM A CASE OF VARIEGATE PORPHYRIA.

(i)



Ascending chromatography

Paper : Whatman No. 1

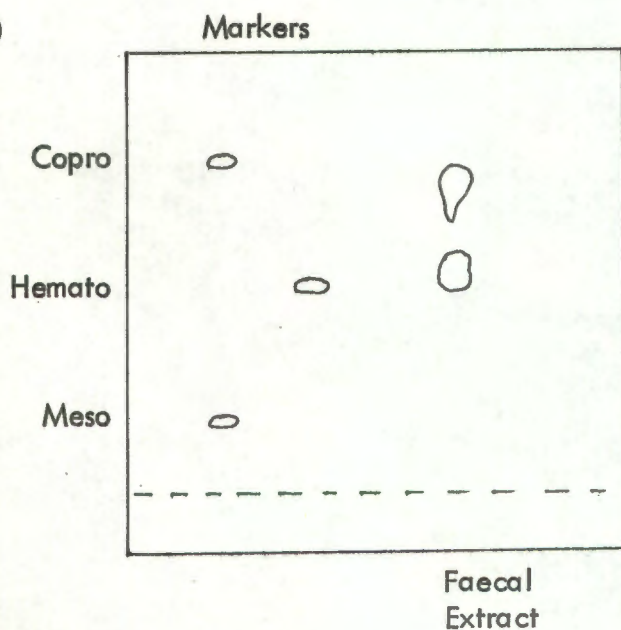
Solvent : Lutidine : 5

Water : 3

Atmosphere : NH_3

Time : 14 hours.

(ii)



Ascending chromatography

Paper : Whatman 3 mm

Solvent : pyridine : 1

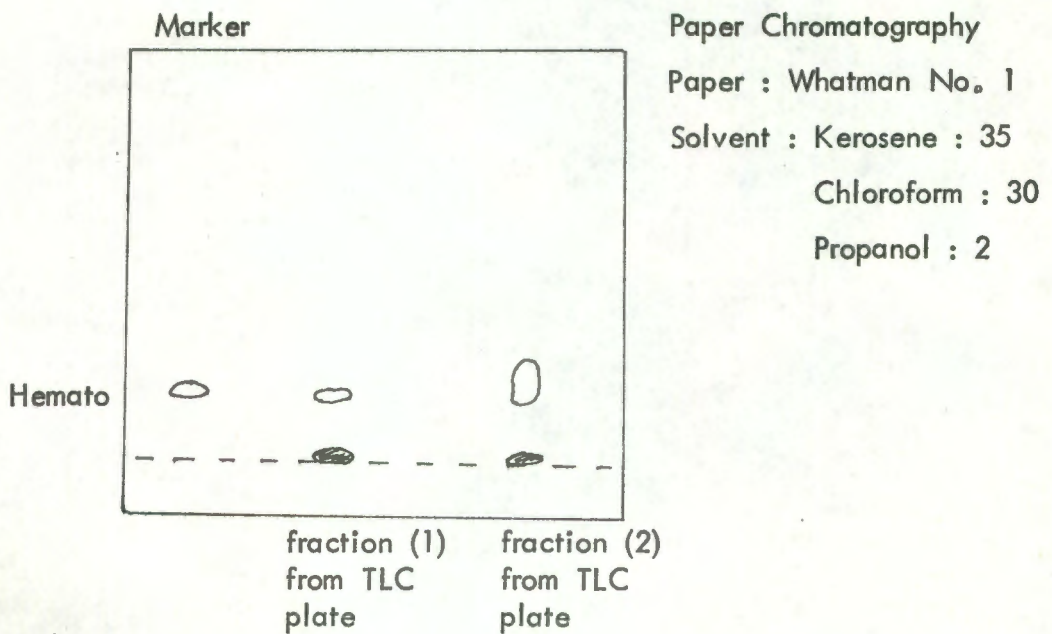
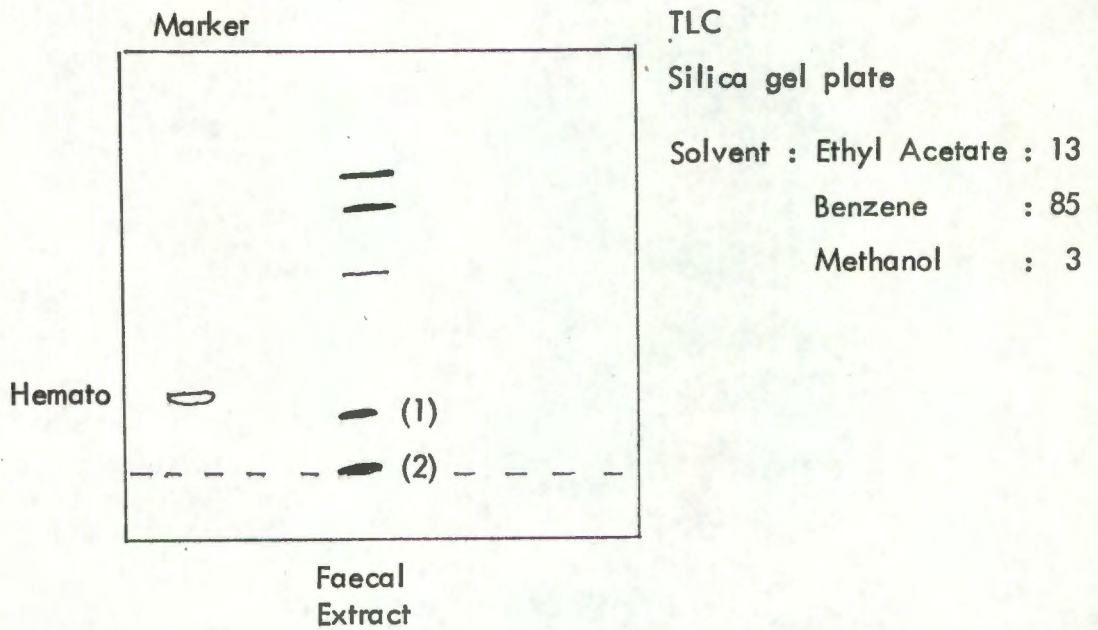
Na citrate
buffer : 9

pH 8.25 (0.35N)

Atmosphere : NH_3

FIG. 64

**CHROMATOGRAPHY OF THE HEMATOPORPHYRIN
METHYL ESTER ISOLATED FROM THE FAECES OF A
CASE OF VARIEGATE PORPHYRIA**



porphyrins remained on the baseline and some ran as uroporphyrin; on paper chromatography with lut./H₂O (5 : 3) as solvent they migrated ahead of the dicarboxylic markers. Some free porphyrins were being held by weak bonds since they were released after changing to pH 11 and then back again to acid. These behaved as 7 and 8 carboxyl porphyrins on TLC, but were ether soluble and could therefore possibly be similar to Elder's porphyrin 2 (Elder, 1971). The ether insoluble fractions were treated with Hg to split any thioether bonds present. Hematoporphyrin was the main component present with traces of uro and copro in some of the samples.

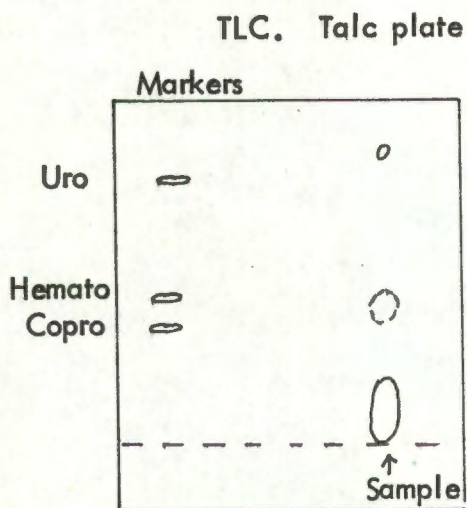
Step VIII. The major portion of the porphyrin-peptide remained at the top of the Dowex column. After removal of the same with lut/H₂O/HOAc (10 : 10 : 1.5) and lyophilisation to remove all traces of the lutidine an absorption spectrum was recorded and showed the presence of peaks at 405 mu, 550 mu and 280 mu. The porphyrin-peptide was then extracted with ether at pH 3.5. The ether soluble fraction was chromatographed on TLC and paper chromatography (fig. 65) and an absorption spectrum recorded on this too (fig. 66).

The ether insoluble fraction was submitted to Hg treatment and the porphyrin released was shown to be hematoporphyrin in all systems studied.

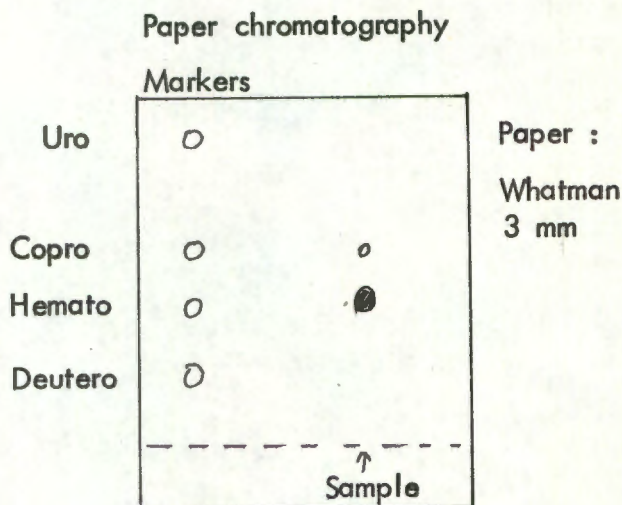
It has been evident throughout these studies that hematoporphyrin has been the dominant porphyrin present in the porphyrin-peptide complexes, but other undefined porphyrins were also observed. The fact that free hematoporphyrin was only found in minute quantities in the faeces but released after manipulations may be due to the presence of weak ionic bonds that were broken by alkali conditions. Alternatively it may be that polymerisation of the peptides trapped the hematoporphyrin. It would seem that hematoporphyrin is only excreted in the conjugated form since Sano and Rimington (1963) found no free hematoporphyrin in the urine even after injection of the same. Meyer-Betz (1913) likewise found very little free

FIG. 65

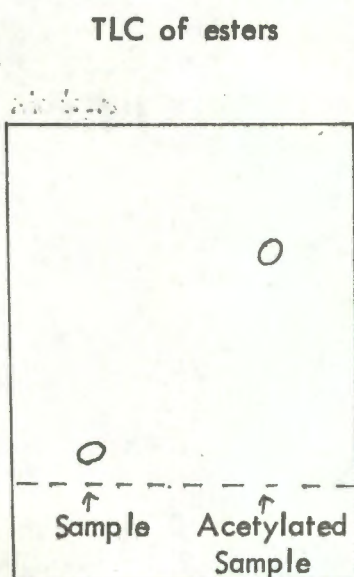
CHROMATOGRAPHY OF THE ETHER SOLUBLE PORPHYRINS THAT REMAINED AT THE TOP OF THE DOWEX 50 CATION EXCHANGE COLUMN



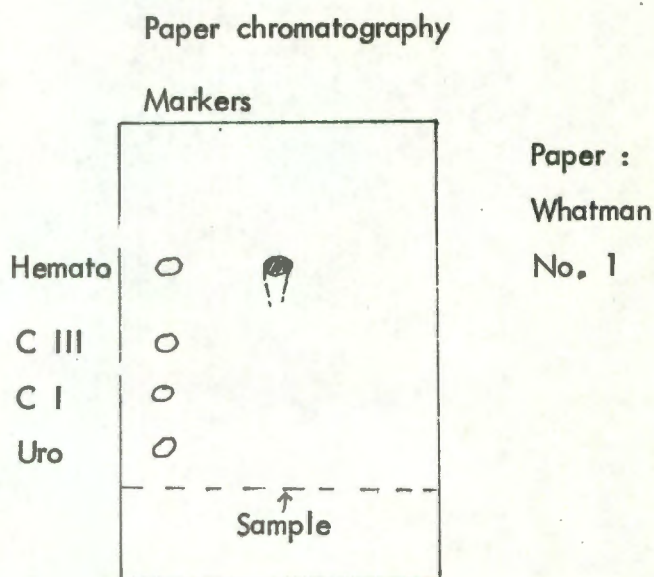
Solvent : Acetone : 7
HCl : 3



Solvent : Pyr : 1
Na Citrate:9
Buffer pH 8.5
Atmosphere : NH₃



Solvent : Ker : 35
CHCl₃ : 30
Prop : 2



Solvent : Lut : 5
H₂O : 3
Atmosphere : NH₃

FIG. 66(a)

ABSORPTION SPECTRA OF (1) THE FREE PORPHYRIN LIBERATED DURING ISOLATION PROCEDURES, AND (2) THE PORPHYRIN - PEPTIDE (ether insoluble fraction) REMAINING.

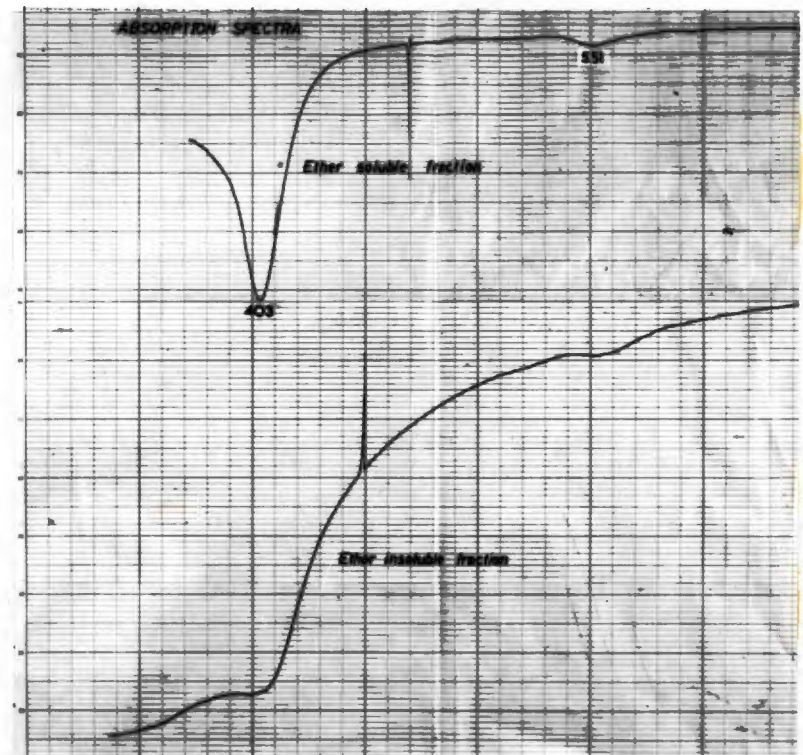
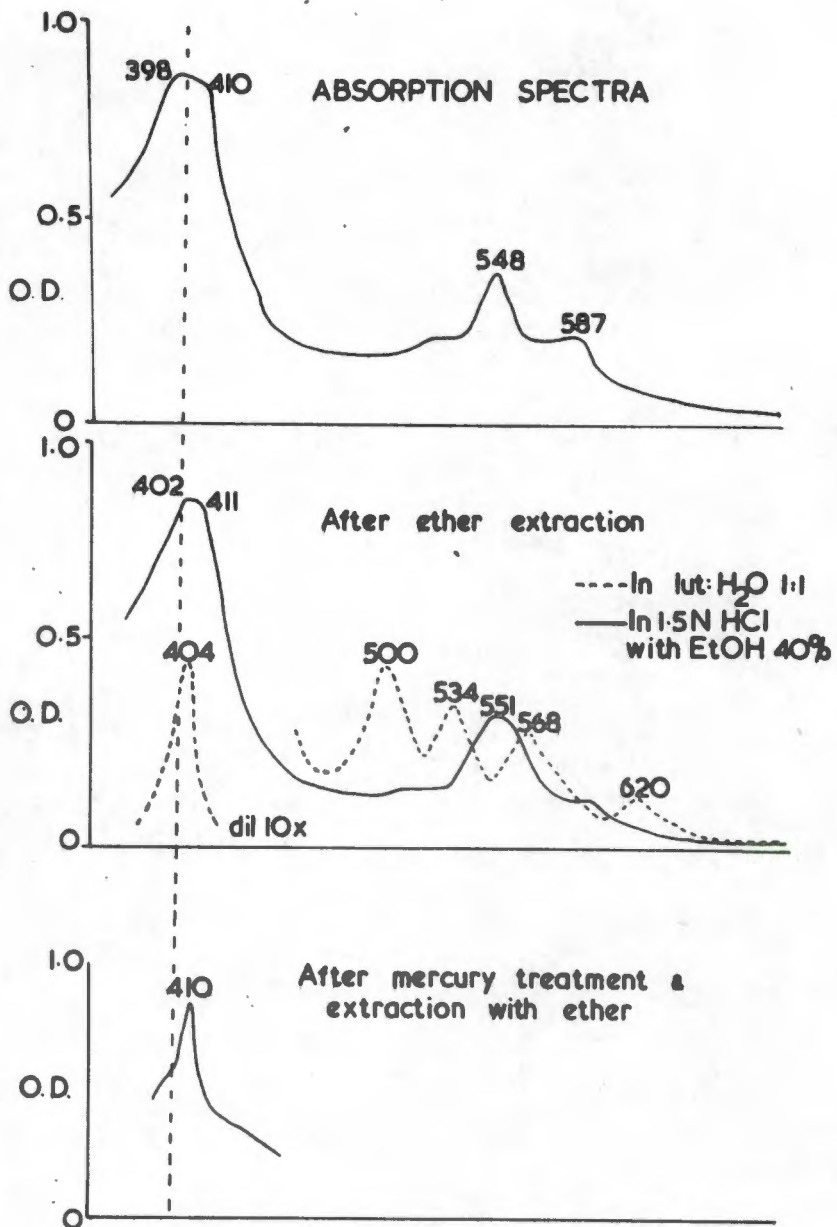


Fig. 66.b)

THE ABSORPTION SPECTRA OF THE ISOLATED PORPHYRIN-PEPTIDE COMPLEXES BEFORE AND AFTER THE REMOVAL OF THE FREE PORPHYRINS



hematoporphyrin in the urine after intravenous injection of 0.2 g of hematoporphyrin. Vanotti (1952) found that hematoporphyrin given orally was absent from the urine but present in the faeces. From our experience it is surprising that this too was not protein bound. It is also possible that hematoporphyrin may have been present in the urine of these various studies but remained undetected if it were protein bound. In support of this are the findings of Hinsberg and Merten (1939) who gave hematoporphyrin to rabbits intramuscularly and noted an increase in the urinary albumin which we have noted avidly binds porphyrin in the serum.

(e) The physical properties of porphyrin "X".

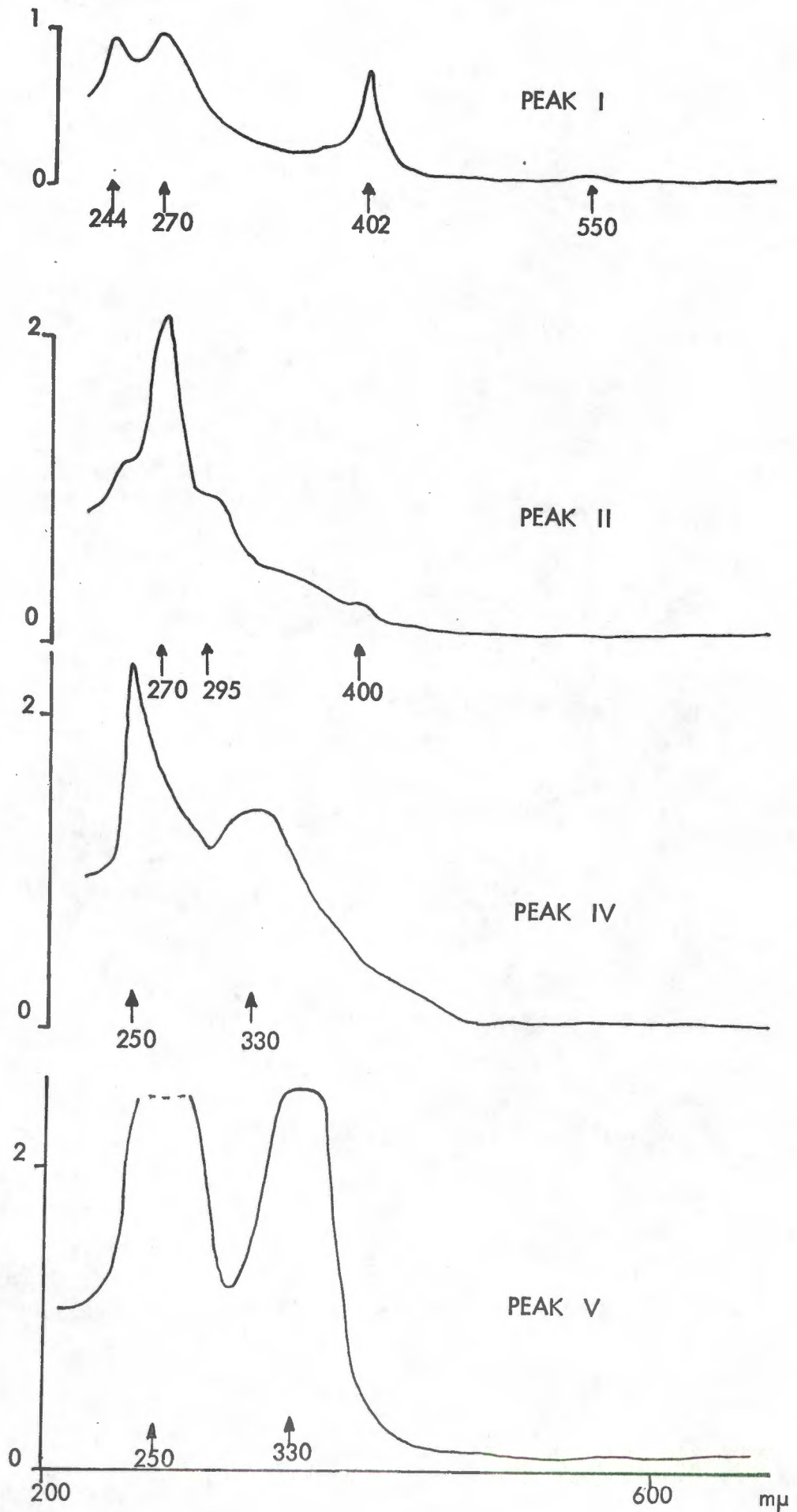
The isolation procedure became so ramifying that very little homogeneous pure material was recovered and therefore only a few properties could be investigated.

If the Sephadex columns may be used as a rough indication of molecular weight, both the G and LH series of Sephadex showed the purified porphyrin-peptide complexes to have molecular weights below 2,000, but the degree of retention on the columns is not known and may be considerable. The crude extract of porphyrin "X" from both urine and stool showed a molecular weight of 9,000. It could be that either the porphyrin-peptide complex was polymerising or that intermolecular bonding was occurring between the free peptides and porphyrin "X". Rimington et al. (1968) found two distinct fractions by ultracentrifugation, i.e. an alkali-soluble fraction with a molecular weight of 8 000 and a smaller acid soluble molecule.

The absorption spectra recorded throughout this work do not agree with any of those published by other authors (Appendix B). This is probably due to varying stages of purification reached by different authors.

The absorption spectra of the individual peaks separated at Step IX (fig. 53) have been shown in fig. 67. Peak I is the only spectrum that shows a solet absorption band. In peaks IV and V the solet band has been completely masked

Fig. 67. ABSORPTION SPECTRA OF FRACTIONS TAKEN FROM SEPHADEX G25 (30% HOAc)



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by the 330 peak. The chromatographic picture of these same fractions has been shown in fig. 68. Peak III was shown to contain mainly free hematoporphyrin. In the other samples the porphyrin was protein bound and behaved in the same chromatographic manner as the crude porphyrin "X". Fig. 70 compares the chromatographic behaviour of porphyrin "X" with known porphyrin-peptide markers. Electrophoresis at pH 6.5 of porphyrin "X" isolated at Step VIII (fig. 53) showed a slight tendency of the sample to move towards the negative pole (fig. 69), and yet the isoelectric point of a sample taken from peak IV a (Step XI, fig. 53) was found to be between 2 and 4. It would therefore seem that Peak IV (a) was a particularly acidic fraction of the group of porphyrin-peptides and yet it seemed strange that the various fractions were not separated on the paper electrophoretogram. The amino acid composition of the porphyrin peptide fractions (tables 29 and 30) show a preponderance of the acidic amino acids in most of the fractions studied, which supports the finding of a low iso electric point.

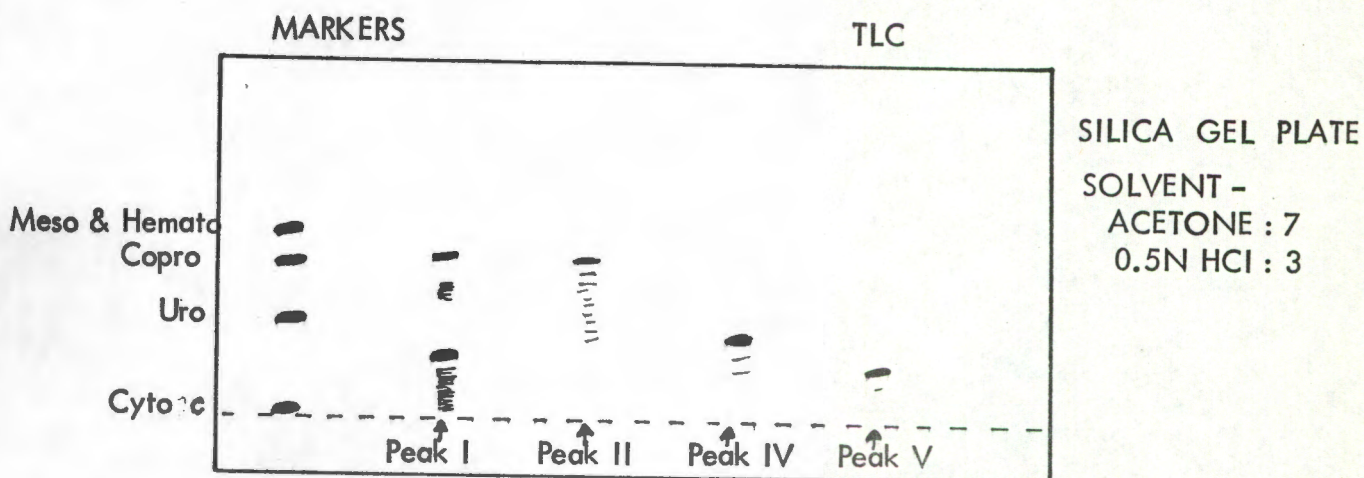
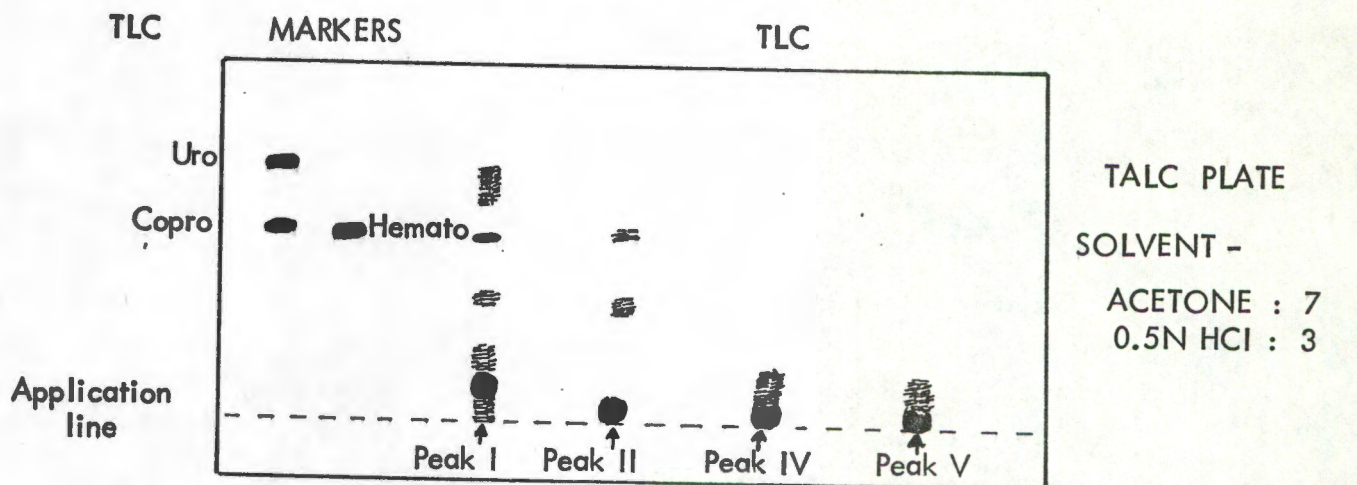
It was thought of interest to note the binding properties of porphyrins with various amino acids and proteins in vitro, with the hope that we might be able to correlate these findings with the properties of porphyrin "X".

The different methods available for binding studies are listed in Appendix A. Measurement by means of precipitation of the porphyrin-protein complex, or by spectral changes proved to be the most satisfactory. Changes in R_f on TLC were also useful as an indication of binding.

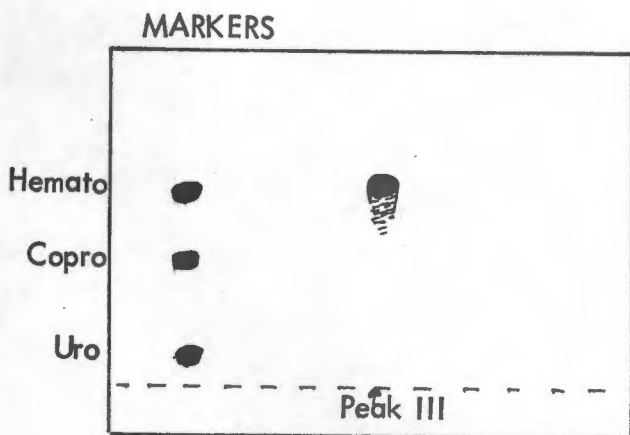
A considerable amount of work has been done on the binding of porphyrins with albumin, globin and free amino acids. Our own experiences have shown that the pH of the initial porphyrin solution as well as the pH of the final solution and the concentration and type of buffer used are critical in determining the presence of binding. Coproporphyrin for example binds readily with albumin in the presence of a Tris buffer pH 7.4 but when dissolved in phosphate buffered saline

Fig. 68.

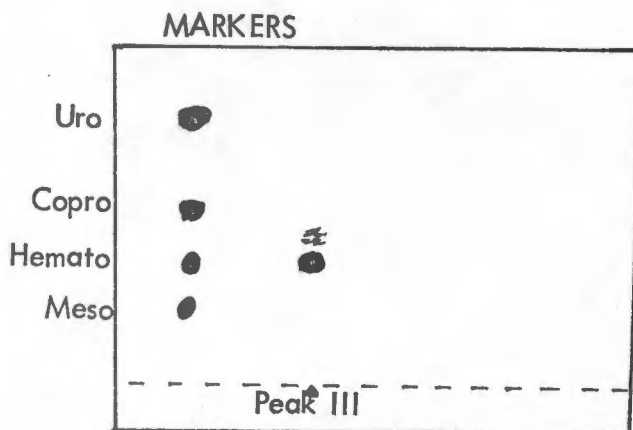
CHROMATOGRAPHY OF FRACTIONS ELUTED FROM SEPHADEX G 25 COLUMN (30% HOAc).



PAPER CHROMATOGRAPHY



Solvent : Lut/H₂O (5 : 3)
Atmosphere : NH₃



Solvent : Pyr/Citrate Buffer
1 : 9

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the same amount of binding takes 5 times as long. Possibly the Tris (Trihydroxymethylaminomethane) is exercising a catalytic effect. Different buffers may also form interchain bridges by ionic attraction.

Most authors who judge binding by the shift in wavelength or change in O.D. generally measure the effects within a few minutes of placing the 2 components together. Fig. 71 illustrates a time study of these 2 parameters during the binding of coproporphyrin and bovine serum albumin. This graph was reproducible in every detail and no isosbestic point was seen. (fig. 72). It would therefore appear to be merely a physical interaction between the absorbing materials and buffer.

Hematoporphyrin in the presence of albumin in Tris buffer does exhibit an isosbestic point and therefore we can assume that a chemical equilibrium has been reached, involving two forms of a chromophore (fig. 73).

This method may be used to determine the binding ratio as demonstrated in fig. 74. 0.02 μ M of albumin in 0.2 M Na_2HPO_4 buffer at pH 6.8 was added to increasing concentrations of protoporphyrin dissolved in 50 μ l of 1N HCl. The sorret peak wavelength was read after 10 minutes. The plot of wavelength shift against porphyrin concentration demonstrates a binding in the ratio of 1 : 1. This takes place in the initial fast reaction; the following slow changes in wavelength and O.D. may be due to conformational changes of the proteins or aggregation of the complexes since spectral changes in the UV region were also seen. Porphyrin polymerisation can cause incomplete binding and therefore the solutions should be prepared immediately before use. Table 33 (a) lists the binding investigation performed in this study and table 33 (b) relevant work of other authors.

The binding studies showed that albumin bound readily with all porphyrins except uroporphyrin I, probably only in a physical manner which is supported by the fact that none of the isolated hydrophilic porphyrins resembled albumin in any way. On the other hand electrophoresis of plasma taken from the patient as

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excreted large amounts of porphyrin "X" showed the major section of the porphyrin fluorescence to be travelling with the albumin fraction and only a minor part with β globulin. The porphyrin was shown to be predominantly tetracarboxylic and could only be extracted by vigorous means such as treatment with a heavy metal or as Rimington and Lockwood (1966) noted in a similar case, by trypsin digestion. The conditions under which we attempted to simulate the binding of albumin and coproporphyrin may not have been optimal and possibly a chemical bond is formed in vivo which may be broken by the kidney or liver and only the porphyrin bound to the β globulin is excreted in the conjugated form both from the plasma via the kidney into the urine, and directly from the liver into the bile. Vanotti (1952) reported a case of nephrosis with serious proteinuria. Protein bound porphyrins were noted in the urine. Large quantities of albumin were present but the greater part of the porphyrin was linked to the globulins.

It is interesting to note that hematoporphyrin and uroporphyrin were readily bound by the basic amino acids lysine and arginine which were present in all the hydrophilic porphyrin fractions; also Stenhagen and Rideal (1939) were able to demonstrate specific interaction between porphyrins and amine monolayers. Cysteine was found to bind not only with the reduced forms of protoporphyrinogen, coproporphyrinogen and hematoporphyrin, but also with the oxidised form of hematoporphyrin. To account for the three separate phases in which hematoporphyrin was released from the porphyrin-peptide complexes one could postulate that the hematoporphyrin present was not always linked to the peptide chain via the cysteine molecule but in some fractions via a diamino monocarboxylic amino acid such as lysine or arginine.

TABLE 33 (a)

PORPHYRIN BINDING STUDIESPresent Studies

Porphyrin	Protein	+ denotes presence of binding - denotes absence of binding	Confirmation by other authors
Uroporphyrin	cysteine	-	
"	histidine	-	
"	phenylalamine	-	
Coproporphyrin	cysteine	-	
"	histidine	-	
"	phenylalamine	-	
Protoporphyrin	cysteine	-	
"	histidine	-	
"	phenylalamine	-	
Protoporphyrinogen	cysteine	+	Theorell (1939); Popper + Tuppy (1963); Sano et al. (1964)
Coproporphyrinogen	cysteine	+	
Uroporphyrinogen	cysteine	-	
Hematoporphyrin	cysteine	+	Theorell (1939)
"	lysine	+	
"	arginine	+	Heathcote et al. (1968)
"	phenylalamine	-	
"	methionine	-	
"	alamine	-	
"	aspartic acid	-	
Hematoporphyrin	albumin	+	
Coproporphyrin	"	+	Bennhold (1938); Koskelo et al. (1970)
Uroporphyrin	"	+ -	VIII+ VI - } Koskelo et al. ('70); - Bennhold ('70) Popper & Tuppy (1963); Koskelo et al. (1970); Maehly (1961)
Protoporphyrin	"	+	
Coproporphyrin	cationic protein (lysosomes)	+	
Uroporphyrin	platelets	+	
Protoporphyrin	"	+	

TABLE 33 (b)

RELEVANT WORK OF OTHER AUTHORS

Uroporphyrin	Lysine	+	Davis et al. (1970)
	Arginine	+	"
	Nucleohistone	-	"
	Adenosine	+	"
	Cytosine	-	"
	Thymine	-	"
	Uracil	-	"
	Guanine	-	"
Coproporphyrin III	globulin	+	Koskelo et al. (1971)
Hematoporphyrin	Tryptophan	+	Heathcote et al. (1968)
	Globin	+	Hill and Holden (1926)
Hematoporphyrinogen	Methionine	-	Sano et al. (1964)
Protoporphyrin	Globin	+	Hill and Holden (1926); Holden (1941)
Protoporphyrinogen	Thioglycollic	+	Sano et al. (1964)
	Glutathione (reduced)	+	Sano et al. (1964)
Heme octapeptide	Histidine	+	Nanzo and Sano (1968)
	Methionine	+	" " "
	Tryptophan	+	" " "

Fig. 71.

THE BINDING OF COPROPORPHYRIN AND ALBUMIN AS DEPICTED BY A CHANGE IN ABSORPTION AND WAVELENGTH SHIFT

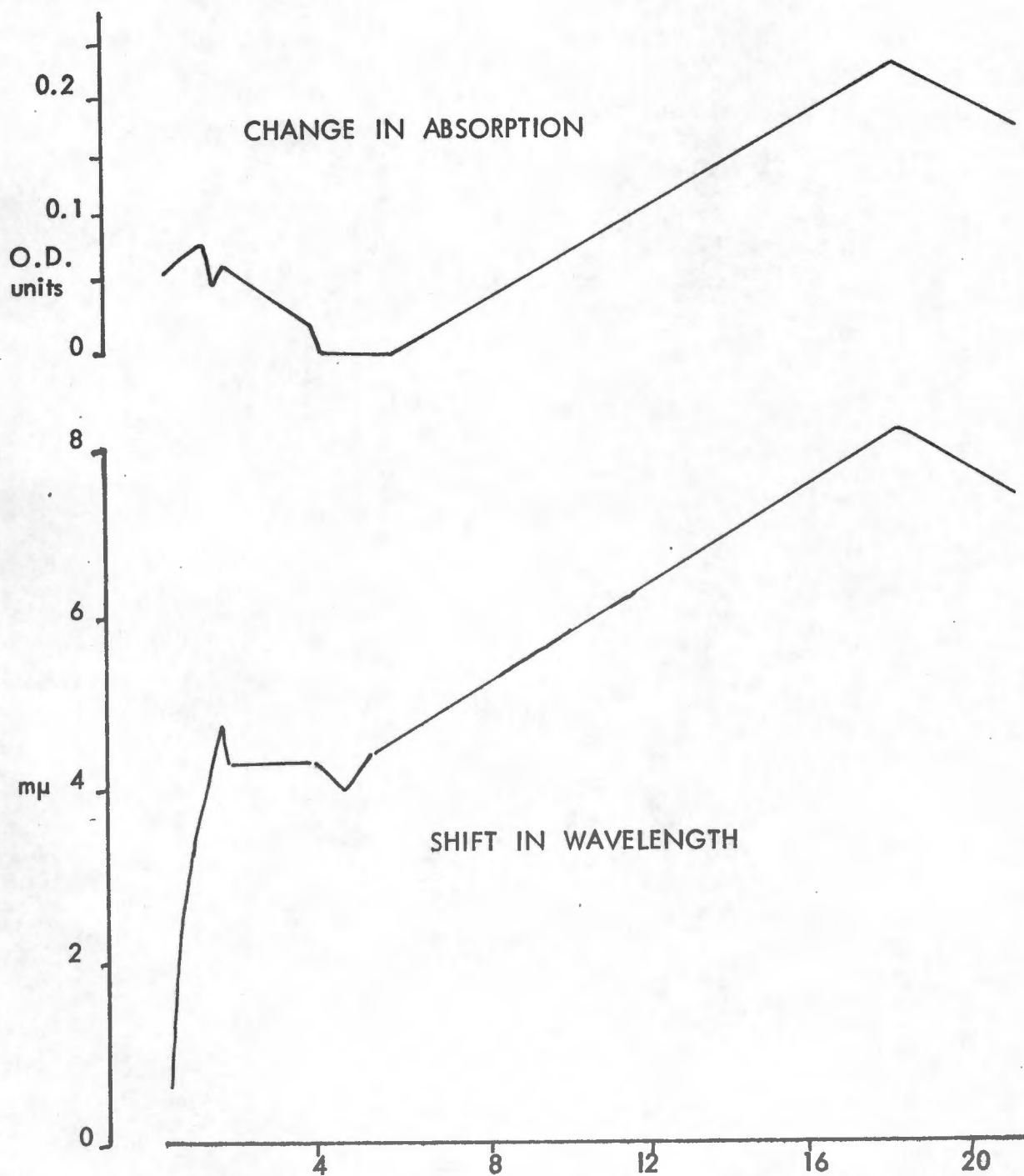


Fig. 72.

THE ABSORPTION SPECTRA OF PORPHYRIN + ALBUMIN
(0.1M Tris Buffer pH 7.4) AT VARIOUS TIME INTERVALS

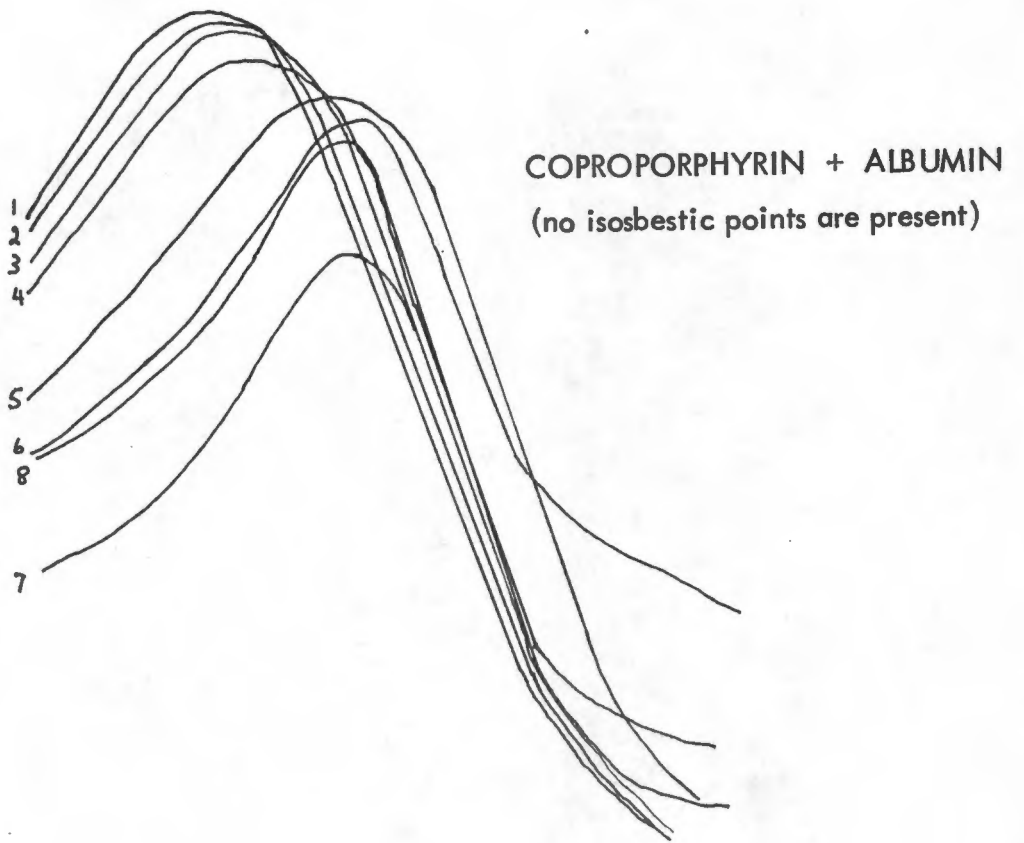


Fig. 73.

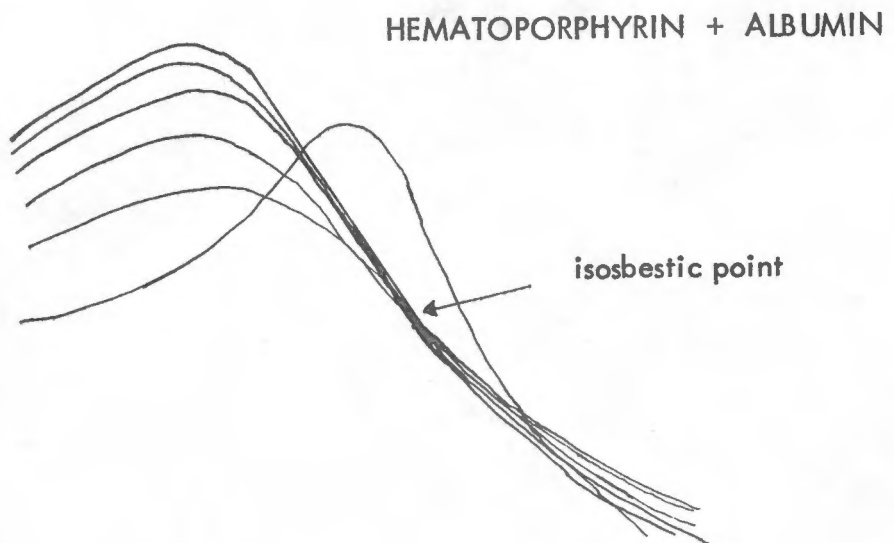
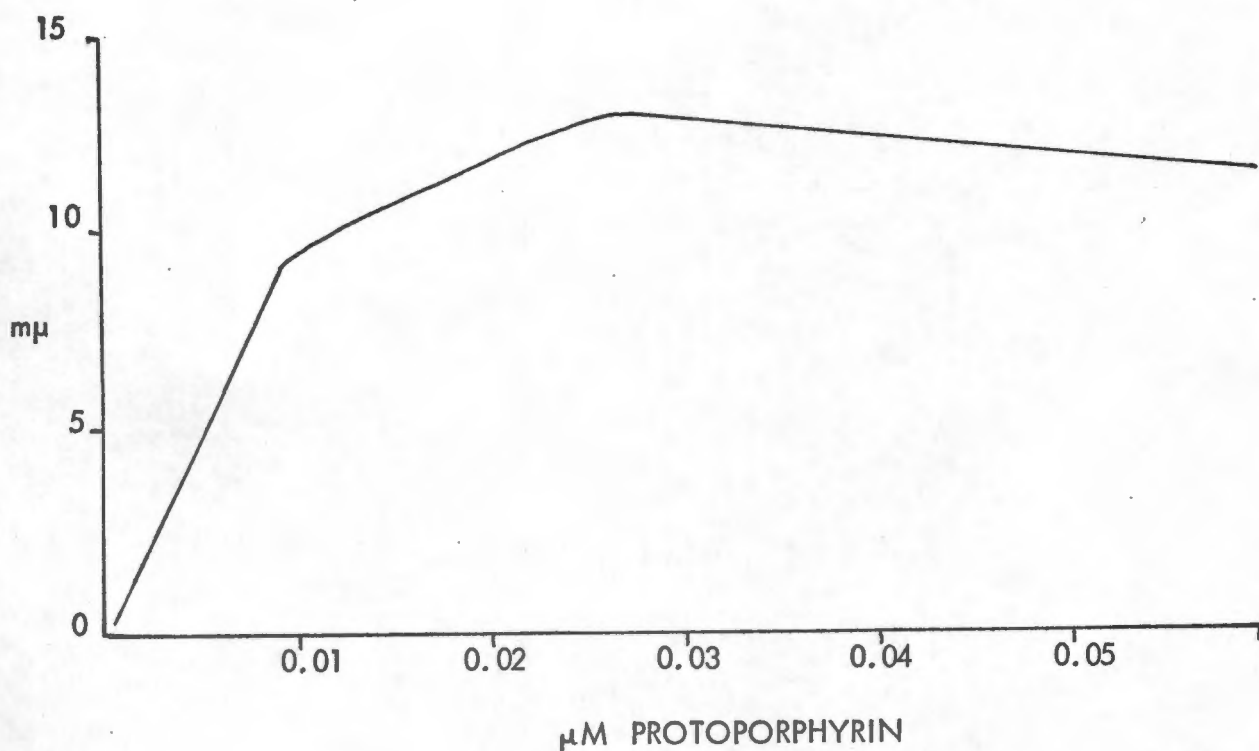


Fig. 74. A SPECTROPHOTOMETRIC TITRATION OF ALBUMIN WITH PROTOPORPHYRIN

The albumin $0.02 \mu\text{M}$ in $0.2 \text{M } \text{Na}_2\text{HPO}_4$ buffer pH 7.6 was titrated with 0.01 to $0.06 \mu\text{M}$ protoporphyrin. The measurements were made 10 mins. after mixing.



Chapter 9.THE ISOLATION OF THE BLOOD PORPHYRINS.

The red blood cell porphyrins and plasma porphyrins were determined on the blood from the case of variegate porphyria, whose faeces and urine were studied for the hydrophilic porphyrins. As may be seen from fig. 75 the plasma showed a vivid red fluorescence.

FIG. 75.

Red Blood Cell Porphyrins :

Coproporphyrin 5.2 µg % rbc

Protoporphyrin 36.2 µg % rbc

Plasma Porphyrins :

Coproporphyrin 21.1 µg % plasma

Protoporphyrin 13.3 µg % plasma

As may be seen from these values very little of the plasma porphyrins could be extracted by normal procedures and considerable amounts were lost in the aqueous washes. The porphyrin could not be extracted from the aqueous phase into cyclohexanone at pH 1.5 nor at 2.9 nor at 4.5 (the pH at which the hydrophilic porphyrins pass into cyclohexanone). It was also noted that the porphyrins could not be adsorbed onto talc, and that precipitation of the plasma proteins with acid-acetone showed the porphyrins to be adsorbed or bound to the precipitated protein. The porphyrins could however be separated by treating the plasma with Hg as for splitting thio-ether bonds. After Hg treatment the protein was precipitated, and removed from the porphyrin which remained in solution. The latter was esterified and the porphyrin esters run on TLC. These showed 90% of the porphyrin to be 4 carboxyl with traces of 6 and 7 carboxyl porphyrins and hydrophilic porphyrins. Wells and Rimington (1953) investigated the plasma porphyrins in a case of porphyria cutanea tarda and, in that instance, found the main component to be protoporphyrin. This shows a reciprocal behaviour between the excreted porphyrins and the plasma porphyrins, namely in the case of variegate porphyria where large amounts of protoporphyrin were excreted in the faeces, none was found in the plasma, but in porphyria cutanea tarda where faecal protoporphyrin excretion was low, the plasma contained protoporphyrin.

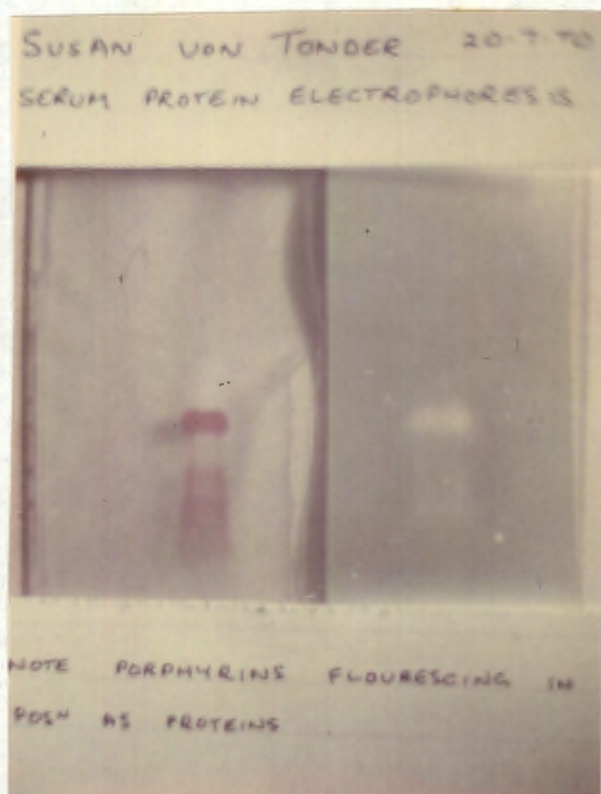
The serum proteins from the variegate porphyric were electrophoresed. An unstained strip was run alongside and viewed under UV light (fig. 76).

Fluorescence was seen corresponding with the albumin fraction mainly and smaller amounts of fluorescence with the α_2 and β globulins.

The serum protein quantitative values are as follows

Albumin		2.4	
Globulin	1	0.3	
	2	0.9	3.1
		0.9	
		1.0	

FIG. 76



It would be interesting to know whether the porphyrin is in fact binding with a β microglobulin and that it is by virtue of its size that the hematoporphyrin - β globulin complex is excreted by the kidney, whilst the porphyrin-albumin complex is retained in the plasma.



Chapter 10

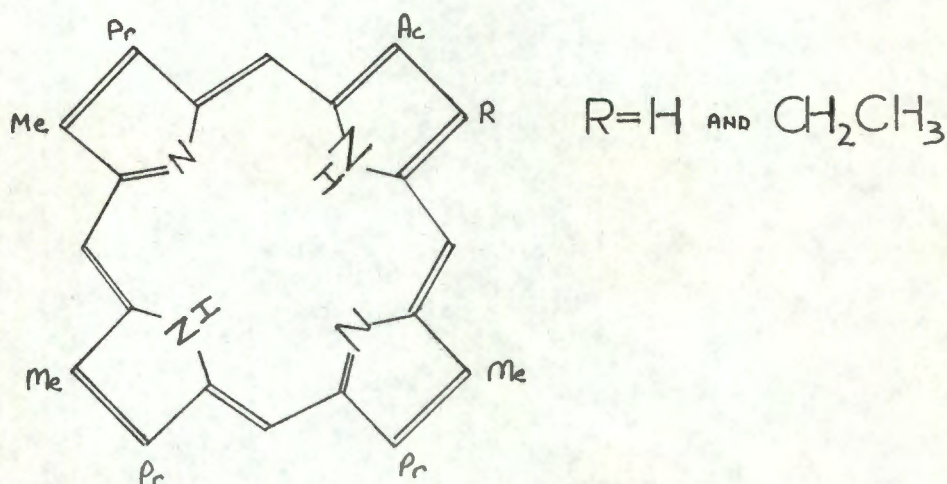
THE ISOLATION OF PORPHYRINS FROM
THE BLISTER FLUID

The patient with variegate porphyria and associated jaundice showed extensive skin lesions and blisters (fig. 77). Approximately 50 ml. of fluid was drawn from two of these blisters, and the porphyrin content was investigated.

Fig. 77



The proteins present in the fluid were precipitated with trichloroacetic acid but unlike the serum very little porphyrin fluorescence was seen in the precipitate. The supernatant fluoresced red under ultraviolet light. All the porphyrin was ether soluble and could be extracted in 0.1N HCl. A total porphyrin of 55 $\mu\text{g/L}$ was found. Paper chromatography and TLC showed the porphyrin to run mainly in the 4 carboxyl porphyrin position with a small amount in the 5 carboxyl position. The porphyrin therefore appears to be predominantly tetracarboxylic and shows an absorption spectrum with a Soret maximum at 403 $m\mu$ which suggests a possibility of it being similar to Elders porphyrin (1) 1972 with the following structure



Certain reservations are held concerning this since insufficient material prevented me from recrystallising the porphyrin and therefore the absorption spectra may not be 100% reliable but nevertheless a 3 $m\mu$ shift from 400 $m\mu$ (the Soret maximum for Coproporphyrin) is more than one would expect from impurities, but the possibilities of the blister fluid porphyrin being Coproporphyrin must also be considered. It was at one stage thought that the porphyrin-peptides might have been responsible for the extreme photosensitivity seen in this patient, but the

presence of free porphyrins in the blister fluid itself dispels this idea, and possibly the increased photosensitivity is merely due to the recirculation of the porphyrins with increased amounts present in the skin. It has been conclusively shown that the combination of porphyrin and ultraviolet rays cause an increase in photosensitivity (Magnus et al 1959, 1961, Gordon 1963, Rimington et al 1967). Allison et al (1966) showed that in the uptake of fluorescent substance by living cells the photosensitising agents are concentrated in the lysosomes, and it appears that the porphyrins damage the lysosomes by the production of free radicals which cause lipid peroxidation with the subsequent release of hydrolytic enzymes (Lenere and Kappas 1969). Zieve and Harvey (1965) were able to show the release of serotonin from platelets in a manner that was a linear function of the log dose of the porphyrin.

There appears to be little agreement on the relative photosensitizing action of different porphyrins. Schmid (1960) lists the porphyrins in decreasing photosensitising action as uroporphyrin coproporphyrin ALA protoporphyrin. Watson (1960) likewise found uroporphyrin to be the most sensitising porphyrin, protoporphyrin perhaps not at all sensitizing and coproporphyrin questionable so. Meyer Betz (1913) found extreme sensitivity to hemotoporphyrin. Gordon (1964) found coproporphyrin to have the maximum effect which is particularly interesting in view of this finding of a free tetracarboxylic porphyrin in the blister fluid itself.

It would therefore seem that the clinical lesions of porphyrin photosensitivity are related to many variables such as the intensity of light exposure, concentration and type of porphyrin photosensitizer and the cellular level at which porphyrins are deposited and although from experimental data it would seem that proteins in particular serum albumin, are able to afford protection against the photosensitizing action of UV rays on porphyrins (Rask and Howell 1928) they appear to be bound to the porphyrins only in the serum and not in the extracellular fluid of the epidermal blisters.

CONCLUSIONS

It may be concluded from the evidence shown here that porphyrin 'X' represents a group of porphyrin-peptide complexes, having primarily a porphyrin moiety that behaves chromatographically as hematoporphyrin but varying in their peptide components. The amino acid composition of all the fractions studied differed from that of cytochrome c therefore excluding the possibility of the latter being the parent substance of porphyrin 'X'.

It would appear that hematoporphyrin binds preferentially with acidic peptides in the liver. These peptides are relatively small and porphyrin 'X' appears to have a molecular weight of approximately 2000. The amino acid compositions of two of the porphyrin-peptide complexes isolated could be likened to those of hemopexin and β microglobulin. The presence of hemopexin in the excreta of variegate porphyrics has already been reported by Meiers and Ippen (1968). Its presence possibly acts as a protection mechanism by binding free hematoporphyrin thus preventing the circulation of a highly photosensitizing agent.

Coproporphyrin, the dominant porphyrin of the serum in this particular case studied, appeared to be similarly bound but preferentially to albumin and to a lesser extent to the β globulins. In vivo studies by Koskelo et al (1971) showed coproporphyrins to have a greater affinity for the β zone plasma proteins when Sephadex G200 was used as the differentiating method, but iso-electric focusing showed an affinity of coproporphyrin for albumin. They found no binding between coproporphyrin and pure hemopexin except in the presence of albumin. These latter findings are more in agreement with our own electrophoretic studies. Rask (1928) found serum albumin to have a protective mechanism which decreased the effect of hematoporphyrin in the presence of light. The porphyrin isolated from the blister

fluid of this same patient was found to be tetracarboxylic. This was not protein bound, although albumin and chemopexin are present in the interstitial fluid of the dermis (Zimmer and Dub, 1960). The question poses whether this tetracarboxylic porphyrin is coproporphyrin and if so whether the dissociation between the porphyrin and protein is due to photo-oxidation by sunlight. It is interesting to note that Gordon (1964) found coproporphyrin injected intracutaneously to be the most potent photosensitiser.

The study of amino acids obtained from urinary peptide hydrolysates showed a depletion of tyrosine and phenylalanine in the 6 cases of porphyria studied relative to the normals. This is interesting in so far as phenylalanine may be converted to tyrosine which under the influence of sunlight may give rise to melanin. This points to the idea of an increased melanin formation in porphyria with subsequent decreased excretion of tyrosine containing compounds. In support of this theory is the incidence of increased pigmentation so often observed in porphyria.

S U M M A R Y

Methods for quantitative porphyrin determinations have been reviewed and a survey of faecal porphyrins performed on the 3 types of porphyria presenting in South Africa. A normal upper limit of total faecal porphyrin was found to be 107 $\mu\text{g}/\text{G}$. The presence of uroporphyrin in faeces has been demonstrated in 89% of the cases studied. The normals showed an upper limit of 30 $\mu\text{g}/\text{G}$, the symptomatic porphyrics 80 $\mu\text{g}/\text{G}$ and variegate porphyrics 270 $\mu\text{g}/\text{G}$. The pattern of excretion of faecal porphyrins in symptomatic porphyria has been shown to be very similar to that of the urine with the exception of the 8 carboxyl porphyrins excretion generally exceeding that of the 7 carboxyl porphyrin.

Mesoporphyrin was found to be the dominant dicarboxylic porphyrin present in the faeces from the 2 variegate porphyria patients studied.

3 carboxyl porphyrins were present in 75% of the cases of variegate porphyria.

The isolation and identification of porphyrin 'X', a hydrophilic porphyrin-peptide complex was attempted from urine and faeces. A quantitative survey of porphyrin 'X' showed an upper limit for normals to be 10 $\mu\text{g}/\text{G}$. Raised levels in both Variegate Porphyria and symptomatic porphyria were found. Children from families with a history of porphyria showed a statistically significant variation to a 2% level in their protoporphyrin and porphyrin 'X' excretion from that of normal children.

Porphyrin 'X' was found to represent a group of porphyrin-peptide complexes. The main porphyrin component being hematoporphyrin linked via a thio-ether bond to a small acidic peptide. Two of these peptides were similar to hemopexin and β microglobulin. The molecular weight of the complexes were all in the vicinity of 2000. Coproporphyrin of the serum was likewise found to be protein bound. Electrophoretic studies showed the protein to be predominantly albumin. Blister

fluid isolated from a variegate porphyric and also from a congenital erythropoietic porphyric showed the presence of a tetracarboxylic porphyrin.

The amino acid composition of peptide hydrolysates from the urine of porphyrics showed a depletion of phenylalanine and tyrosine relative to the normals studied. The significance of these findings have been discussed and the role of porphyrin 'X' postulated as a protective device for the elimination of the potentially harmful photosensitising agent hematoporphyrin.

APPENDIX A

REAGENTS.

All solvents used were of "analar" grade and several of these required additional purification. Care should be taken to exclude metal contamination since porphyrin chelates are readily formed.

Water. - glass distilled water was used. The presence of metals is undesirable due to metalloporphyrin complexes forming.

Chloroform. - Chloroform contains ethanol as a preservative; this must be removed when it is used for chromatographic purposes since the ethanol increases the eluting power of the solvent. Traces of phosgene, if present, may be removed simultaneously by washing the chloroform with distilled water several times, and then drying it with calcium chloride and finally distilling it. The pure chloroform is unstable and may not be kept for more than a few days. It must be stored in a dark bottle.

Glacial Acetic Acid. - "Analar" grade acetic acid frequently contains peroxides which must be completely removed. This is best achieved by freezing and thawing incompletely several times and finally distilling the liquid portion from metal-free glassware.

Diethyl Ether. - Peroxides are removed by washing with a fresh solution of ferrous sulphate in very dilute sulphuric acid; the ether is then washed three times with water, dried over calcium chloride, filtered and distilled.

Methanol. - Absolute dry methanol must be used for esterifications, chromatography and crystallizations.

10 g of dry Mg plus 1 g iodine is placed in a 3 L flask, fitted with a double surface reflux condenser. 100 ml methanol is added and the flask gently heated until all the Mg is converted to magnesium methoxide. 2 L of methanol is now added and the mixture refluxed for 30 min; the methanol is then distilled over, collecting the 65-66° fraction.

Kerosene. - is fractionally distilled and the fraction distilling in the range 120 - 200°C collected.

Lutidine. - Generally it is not necessary to distil Lutidine, but if yellow in colour it may be distilled under reduced pressure (75 mm Hg).

Methyl Cellosolve. - A check must be made for the presence of peroxides. (3 ml methyl cellosolve + 3 ml of 4% KI). Liberation of iodine indicates the presence of peroxides.

ALA - ^{14}C . - as the acid hydrochloride is purchased from New England Nuclear Corp., Boston Mass. (15.1 $\mu\text{c}/\mu\text{M}$).

Scintillants. - Primary scintillant - "PPO"
Secondary scintillant - "POPOP"
Solvents - Toluene
- Toluene plus Triton - X 100.

SPECTROSCOPY OF PORPHYRINS.

Porphyrins may be measured either by absorption spectroscopy or fluorescent spectroscopy. The absorption characteristics of the porphyrins are related primarily to the intense resonance of the molecule; changes in the side chains, result in minor shifts in wavelength, whereas metal complexes which affect the resonance structure by bonding to the nitrogen atoms have a dramatic effect on the absorption spectra. Most of the porphyrins discussed here have an aetio type spectrum with an intense "Soret" band around 400 m μ . Porphyrins in alkaline solutions or organic solvents show four absorption bands in the visible region plus the Soret band and are used for identification of the porphyrins.

In acid solution, 2 absorption bands and the Soret band are present. Extinction values in diluted HCl at the Soret band maxima have been worked out (See Appendix 2).

The use of fluorescence spectroscopy allows the detection and quantitation of porphyrins at very low concentrations. One of the unique properties of porphyrins is their absorption of light around 400 m μ and the emittance of red fluorescence at 600 m μ .

An Amin ϕ Bowman Fluorometer has been used in these studies; after standardisation of the instrument linearity of fluorometer readings and porphyrin concentrations could be achieved for concentrations lying between 10^{-4} - 0.5 mM/L.

PORPHYRIN ESTIMATIONS.

Solvent-partitioning provides a convenient method for the separation of faecal and urinary porphyrins. The initial partitioning is between ether and aqueous phases. Of the natural porphyrins those with the higher number of carboxyl groups favour the aqueous phase, while the dicarboxylic and tetracarboxylic porphyrins are readily soluble in ether.

The ether soluble porphyrins are then separated according to their HCl number. (The HCl number is defined as the concentration of HCl (expressed as %) which will extract 2/3 of the porphyrin from an equal volume of ether). The HCl number depends upon the ether-water partition coefficient and on the dissociation of the porphyrin as a base. The HCl numbers are recorded in Appendix 2.

The hydrophilic porphyrins, namely uroporphyrin and porphyrin "X", are separated according to the solubility of the porphyrins in organic solvents at varying pH's.

The resulting fractions are not pure as shown in section 1, but nevertheless give an approximate value of the amount of porphyrin present.

DETERMINATION OF FAECAL PORPHYRIN.

1. Protoporphyrin and Coproporphyrin (Holti et al., 1958)

Reagents : Glacial acetic acid

Ether

1% Iodine in Ethanol. Dil. 1 : 200 with water before use.

Saturated Sodium Acetate.

0.1 N HCl 8.6 ml conc. 1000 ml H₂O

1.5 N HCl 129 ml conc. 1000 ml H₂O.

Procedure :

The wet/dry weight ratio of the faeces was determined by weighing approximately 0.8 g faeces into a crucible, drying it at 110° for 4 hours and then reweighing.

To determine the amount of faeces needed for the porphyrin estimation, approximately 0.5 gm stool and 3 ml Dean's solution (amyl alcohol : glacial acetic acid : ether 1 : 1 : 1) was taken and the fluorescence viewed under a UV light. Depending upon the result of the screening test between 0.1 to 1.0 g stool was taken for analysis; this was weighed into a 50 ml ground glass stoppered test tube. 3 ml glacial acetic acid and 20 ml ether were added. It was stirred well with a glass rod and the clear supernatant (centrifuge if necessary) decanted. This was repeated until no more fluorescence was seen in the ethereal layer. The ether extracts were combined and washed with (a) 20 ml saturated sodium acetate; (b) 20 ml 0.005% aqueous iodine; (c) 20 ml water. The washes were discarded.

Coproporphyrin from the ethereal solution was extracted with 0.1 N HCl 2 ml fractions until no more fluorescence was seen. The acid volume was recorded and then the procedure repeated, using 1.5 N HCl to extract the protoporphyrin.

The optical density of the HCl extracts was read at 430 m μ , 380 m μ

Calculation :

$$\text{Coproporphyrin } \mu\text{g/G} = \frac{0.D \times 730 \times \text{Vol (acid)} \times \text{wt (wet)}}{\text{wt for analysis} \times \text{wt (dry)}}$$

$$\text{Protoporphyrin } \mu\text{g/G} = \frac{0.D \times 1080 \times \text{vol (acid)} \times \text{wt (wet)}}{\text{wt of analysis} \times \text{wt (dry)}}$$

where $0.D = 2 D_{\text{max}} - (D_{430} + D_{380})$.

II. The Faecal Hydrophilic Porphyrins (for Routine Investigation) (Rimington et al. 1968)

Reagents : Ether

Glacial Acetic Acid

45% (w/v) urea/triton 4%

Butanol

1.4 N HCl 121 ml conc. \longrightarrow 1000 ml H₂O.

Procedure :

The faecal residue remaining after the extraction of protoporphyrin and coproporphyrin by acidified ether was further extracted with 45% (w/v) urea/triton 4%. The hydrophilic porphyrins were then extracted from the urea/triton into butanol, which was washed several times with distilled water, and then finally extracted into 1.4 N HCl after the addition of an equal volume of ether to separate the phases.

Calculation :

$$\text{Hydrophilic Porphyrins (ug/G)} = \frac{\text{O.D.} \times 0.886 \times \text{vol (acid)}}{\text{wt of analysis} \times \text{wt (wet) / wt (dry)}}$$

where O.D. = $2 D_{\text{max}} - (D_{430} + D_{380})$.

This method has been adhered to for comparative purposes, but as Rimington himself describes, it only measures the major portion of the hydrophilic porphyrins.

The urea/triton does not always extract all the porphyrin from the faeces; neither are all the hydrophilic porphyrins butanol-soluble.

Rimington has laid out a scheme which ramifies into many fractions. We were able to reproduce these results, but due to the limitations of the solvent extraction procedures these fractions are of a heterogeneous nature. More exacting chromatographic methods were preferred for the structure analysis (see text).

THE DETERMINATION OF URINARY PORPHYRINS.

I. Coproporphyrin and Uroporphyrin (Rimington and Sveinsson 1950).

Reagents : Glacial acetic acid.

Ether

0.5% (w/v) Sodium Acetate

Iodine stock solution 1% in ethanol. Dilute 1 : 200 with distilled water before use.

10% HCl 200 ml conc. 720 ml H₂O

1.5 N HCl 129 ml conc. 1000 ml H₂O

2% HCl 40 ml conc. 720 ml H₂O

Cyclohexanone

Petroleum ether.

Procedure :

The urine was screened with Dean's solution and, depending upon the intensity of red fluorescence, between 1 to 50 ml urine taken. This was acidified with 1/10th the volume with glacial acetic acid, 20 ml of ether was added and shaken gently. Continued extractions with ether were performed until the ether phase was negative; the ether extracts were pooled and the residual aqueous phase reserved for uroporphyrin determinations. The ether extracts were washed with (a) 20 ml 0.5% sodium acetate; (b) 20 ml iodine solution; (c) 10 ml water. The aqueous washes were combined and added to the residual urine for uroporphyrin determination.

The ether solution was extracted with 1.5 N HCl until negative and the acid volume recorded - coproporphyrin.

The pH of the residual urine and combined aqueous washes was adjusted to pH 1.5 using sodium acetate and 10% HCl (approximately 2.5 ml Na Ac and 8 ml 10% HCl). This solution was then extracted twice with 40 ml volumes

of cyclohexanone. (Centrifuged if necessary). 80 ml petroleum ether was added to the cyclohexanone and the uroporphyrin extracted into 2% HCl using 2 ml portions, until no further red fluorescence could be removed.

The volume of the acid extracts was recorded. The O.D. of the two HCl extracts were read at 380 $m\mu$, 430 $m\mu$ and at the solet peak.

Calculation :

$$\text{Coproporphyrin (} \mu\text{g/L)} = \frac{\text{O.D.} \times 728 \times \text{vol. (acid)}}{\text{vol. aliquot}}$$

$$\text{Uroporphyrin (} \mu\text{g/L)} = \frac{\text{O.D.} \times 855 \times \text{vol. (acid)}}{\text{vol. aliquot}}$$

$$\text{where O.D.} = 2 D_{\text{max}} - (D_{380} + D_{430}).$$

II. The Urinary Hydrophilic Porphyrins

(for Routine Investigation) (Rimington et al. 1968)

Reagents : Glacial Acetic Acid

Ether

0.5% Sodium Acetate

Iodine 1% in ethanol. Dilute 1 : 200 before use

Cyclohexanone

1.4 N HCl 121 ml conc. 1000 ml H₂O.

Procedure :

The procedure was identical to that for copro and uroporphyrin determinations to the point where uroporphyrin was extracted into the cyclohexanone at pH 1.5; subsequently the pH of the aqueous phase was raised to 2.9 and then extracted again with cyclohexanone. This extraction was repeated three times. 2.5 volumes of ether and 4 ml 1.4 HCl were added to the second beaker of cyclohexanone and the porphyrin peptide fraction extracted to completion with HCl. The acid volume was recorded and the optical density at 380 m μ , 430 m μ and the soret peak (406) read on a spectrophotometer.

Calculation :

The porphyrin peptide was calculated as for uroporphyrin, i.e.

$$\mu\text{g/L} = \frac{\text{O.D.} \times 855 \times \text{vol. (acid)}}{\text{vol. aliquot}}$$

$$\text{O.D.} = 2 D_{\text{max}} - (D_{380} + D_{430})$$

In another paper Rimington (1967) amended this method and suggested extracting the porphyrin "X" before the uroporphyrin. The coproporphyrin was extracted as per usual and then the pH of the aqueous adjusted to 5.0 and extracted with cyclohexanone. This removes the porphyrin "X" first; the pH is then lowered to 1.5 and the uroporphyrin finally extracted into cyclohexanone.

For consistency of results the first method was adhered to.

DETERMINATION OF δ - AMINOLEVULINIC ACID (ALA)

AND PORPHOBILINOGEN (PBG) (Mauzerall and Granick 1956).

Reagents : Anion exchange resin - Dowex 2 x 8 (50 - 100 mesh) or amberlite CG 400 type 1 (100 - 200 mesh).

The superfines were removed with distilled water and the resin then prepared in the acetate form by running through 1 M Sodium Acetate until the elute was chloride free. It was then washed with distilled water until the elute was neutral and stored in distilled water.

Cation exchange resin - Dowex 50 x 8 (200 - 400 mesh) or amberlite CG 120 type 1.

The superfines were removed with distilled water and 2 vols 2N NaOH added. This was stirred for 30 hours, then washed until neutral. 1 vol. 4 N HCl was added and stirred for 20 hours.

The acid was removed and the resin washed once with distilled water. Finally 1 vol. 1 N HCl was added and the resin stored as such.

0.5 M Sodium Acetate

1.0 M Acetate buffer pH 4.6

1 N Acetic Acid

0.2 N Acetic Acid

Acetyl Acetone

Ehrlich's aldehyde reagent : 1 g paradimethylaminobenzaldehyde +
20 ml glacial acetic acid + 8 ml 70%
perchloric acid 50 ml with glacial
acetic acid. Prepare freshly before use.

ALA - HCl Std. (10.25 μ g ALA.HCl/ml).

Procedure :

The pH of the urine was adjusted to exactly 6.6. 1 ml urine was applied to a 2 x 1 cm column of anion exchange resin and the column washed through with 5 ml water. PBG was adsorbed, the eluate containing ALA was transferred to a column of cation exchange resin which was washed with 30 ml water and 3 ml 0.5 M Na acetate.

The PBG was eluted with 2 ml 1 N acetic acid followed by 2 ml 0.2 N acetic acid. The eluate was made up to 10 ml with distilled water. 2 ml was taken and 2 ml Ehrlich's reagent added. The absorption was read at 555 m μ against a reagent blank.

The ALA was eluted with 7 ml 0.5 M Na acetate and made up to 10 ml with acetate buffer pH 4.6. 0.2 ml acetyl acetone was added. The tubes were stoppered and placed in a boiling water bath for 10 min. After cooling 2 ml was taken and added to 2 ml Ehrlich's reagent. The absorption was read at 553 m μ after 10 min.

Calculation :

$$\text{PBG mg\%} = \text{O.D.} \times 12.6 \quad (\text{Mol. wt} = 226.2) \\ (\text{E mol} = 6.1 \times 10^4)$$

$$\text{ALA mg\%} = \frac{\text{O.D. of unknown}}{\text{O.D. of standard}} \times 0.8$$

$$(\text{Mol. wt ALA} = 131.1 ; \text{ALA} - \text{HCl} = 167.6)$$

CHROMATOGRAPHY OF PORPHYRINS

(a) Column Chromatography.

The chromatographic processes involved for the separation of porphyrins make use of both adsorption and partition chromatography. The most commonly used adsorbants for column chromatography of the porphyrin esters are aluminium oxide, magnesium oxide (Falk 1964; Nicholas 1951; Waldenström 1935; Mc Swiney et al. 1950) and hyflo Supercel (Chu and Chu 1957, 1959). Talc and cellulose have been used for the separation of free porphyrins (Comfort 1949; Eriksen 1957). The separation of the porphyrins on the column is achieved by gradually increasing the concentrations of an eluting solvent which is diluted by a relatively inert solvent such as petroleum ether. Separation of porphyrins by molecular sieving has been discussed in a separate section. This method has limited application for porphyrin work due to adsorption of the porphyrins by the sephadex.

Column chromatography has been used for the preparation of reference porphyrins and the method of choice is as follows.

Procedure :

Alumina, standardised for chromatographic analysis according to Brockmann Merck, has been used. This was activated by heating at 220° for 3 to 4 hours and cooled in a desiccator. Deactivation was achieved by adding 10% (v/w) distilled water. The flask was shaken vigorously until the water was completely dispersed and the alumina appeared a free-flowing powder. A 20 cm column with a 2.5 cm diameter and a sintered glass disc of medium porosity was used. The Al_2O_3 was slurried in alcohol-free chloroform and poured onto the column; the Al_2O_3 was allowed to settle and the excess chloroform was sucked off the top of the column. The sample, dissolved in alcohol-free chloroform was applied carefully to the top of the column and the column developed at

120 drops per minute initially with chloroform to separate the proto and coproporphyrin and then with 1% methanol in chloroform to elute the higher carboxyl porphyrins. The ease of elution is meso, deuterio, proto copro uro. Some of the more hydrophilic porphyrins may be removed with 2% methanol in chloroform, others are more tightly adsorbed and may be removed with hot glacial acetic acid (peroxide free) which also elutes the dipyrroles.

(b) The Paper Chromatography of Porphyrins.

Paper chromatography is a form of partition chromatography where the paper serves as a carrier for the stationary phase and for the mobile solvent, which is driven forward by capillary forces.

(i) The separation of free porphyrins by paper chromatography was first attempted by Nicholas and Rimington (1949) since which time Eriksen (1958) has introduced several modifications.

The separation of free porphyrins by the use of neutral salt solutions (With 1957) does not produce such good resolution. Eriksen's method has been the one of choice.

Procedure :

The sample was dissolved in a 1 : 1 mixture of pyridine : ammonia and spotted on Whatman No. 1 chromatography paper (25 cm x 40 cm). The paper was folded into a cylinder, held by means of staples and placed in a petri dish in a light-proof chromatography tank. No time need be allowed for the equilibration of the atmosphere in the tank. The solvent (2 : 6 - Lutidine 5 parts, water 3.5 parts) is placed in the petri dish and a 100 ml beaker containing 11 N NH_4OH placed in the centre of the paper cylinder. The chromatogram was developed for 12 hours, after which time the paper was removed and dried for 20 min in an oven at 60°C . The paper could then be

viewed under UV light and the red fluorescent porphyrin spots marked with a pencil. If a quantitative result was required the spots were cut out and put into a B₁₂ ground glass stoppered test tube together with some small glass beads and 3 ml of 1.5 HCl. This was then shaken vigorously for $\frac{1}{2}$ hour and finally the paper centrifuged down so that the clear acid containing the porphyrin could be taken off and the absorption or fluorescence read.

(ii) Several methods for the chromatography of the porphyrin methyl esters on paper have been developed (Falk and Benson 1953; Cornford and Benson 1963; Chu, Green and Chu 1951). The paper chromatography of the porphyrin esters has generally been superseded by thin layer chromatography which gives better resolution. During the earlier part of this work the method of Chu, Green and Chu (1951) was used.

Procedure :

The porphyrin esters were dissolved in chloroform and spotted on Whatman No. 1 chromatography paper. The paper was folded into a cylinder and placed in a petri dish in a light-proof tank. The chromatogram was developed for 3 hours in a solvent system consisting of kerosene : chloroform : n-propanol 35 : 30 : 2, in an atmosphere derived from the same solvent mixture. This method was only used for qualitative work.

(c) Electrophoresis of Free Porphyrins.

The first method for the electrophoretic separation of porphyrins was described by Papastamatis and Kench (1952) who used agar gel as a supporting medium. More recent methods are described using paper as the supporting medium (With 1956, 1957; Verghese 1958; Eriksen 1958; Lockwood and Davies 1962). The method of Lockwood and Davies (1962) was used. The electrolyte used was slightly alkali and therefore the carboxyl groups of the porphyrin

nucleus were ionised; therefore the porphyrins with the higher number of carboxyl groups travelled the furthest. Other factors determining travel are electro-endo-osmosis and adsorption. Sweeney (1963) showed that there was a minimum mobility but not a maximum mobility and that the paper appeared to adsorb the porphyrin; once saturation was reached an increase in mobility was noted. Care must therefore be taken in interpreting the electrophoretogram.

Procedure :

The porphyrins were applied in buffer to Whatman 3 MM paper and the complete paper except for the application line made wet with the electrolyte consisting of 0.04 M Na_2CO_3 + .001 M EDTA. The paper was then laid in position and a voltage of 5 - 6 v per cm applied across it for 2 - 3 hours. The electrophoretogram could be examined by UV light while running.

(d) Thin layer chromatography.

This section has been described fully in the text.

CRYSTALLISATION OF REFERENCE PORPHYRINS.

The porphyrin methyl ester was dissolved in chloroform and a solvent added in which it is relatively insoluble. The tube is agitated constantly and cooled. Crystal formation was allowed to take place slowly. If crystallisation failed to occur the volume of the solvents could be reduced by evaporation in a stream of nitrogen.

Dicarboxylic porphyrins and coproporphyrin III crystallised readily upon the addition of methanol to the chloroform.

Uroporphyrin crystallised from chloroform on the addition of benzene, ether or acetone.

DETERMINATION OF FREE ERYTHROCYTE AND PLASMA PORPHYRINS.

(Schwartz and Wikoff, 1952).

Reagents :

1. Ethyl acetate : glacial acetic acid (4 : 1) solvent mixture.
2. 3% Anhydrous sodium acetate in water.
3. 1% Iodine in ethanol. Diluted 1 : 200 before use.
4. 3 N HCl.
5. 0.1 N HCl.
6. 1.5 N HCl.
7. Congo red paper.
8. Saturated sodium acetate.

Procedure :

1. The red cells from 10 - 15 ml (record volume) of anti-coagulated blood (or 10 ml plasma) were added to approximately 100 ml of ethyl acetate : acetic acid solvent mixture in a medium porosity sintered glass funnel on a suction flask with stirring.
2. The cell mush (or plasma) was ground well with a pestle against the sintered glass end.
3. Repeated extractions (4 or 5 times or until clear) were performed with 40 ml volumes of the same solvent mixture.
4. Combined extracts were washed with :
 - (a) 100 ml 0.005% I₂.
 - (b) 50 ml 3% Na acetate (twice).
 - (c) 50 ml water.The washes were discarded.
5. The porphyrins from the organic phase were extracted with repeated 5 ml volumes of 3 N HCl until no further red fluorescence was seen.

6. The combined HCl extracts were neutralised to congo red with saturated Na acetate and extracted 3 times with 50 ml portions of ethyl acetate.
7. The combined ethyl acetate extracts were washed 3 times with water (discarded) and then extracted exhaustively with repeated 3 ml volumes of 0.1 N HCl to remove the coproporphyrin. The ethyl acetate layer contained protoporphyrin and was treated as in Step 11 below.
8. The 0.1 N HCl solution was washed with chloroform (2 x 5 ml portions) and the chloroform added to the ethyl acetate residue.
9. The volume of 0.1 N HCl extract was recorded. 1/15th volume of 3 N HCl was added to adjust finally to 0.3 N and to clear of opalescence.
10. The coproporphyrin was determined fluorimetrically with a coproporphyrin standard in 0.3 N HCl.
11. The protoporphyrin from the ethyl acetate residue was extracted (Step 7 above) repeatedly with 3 ml volumes of 1.5 N HCl. The volume of the combined 1.5 N HCl extracts was recorded and the protoporphyrin determined fluorimetrically.

DETECTION OF DIPYRROLES (Gilbertson et al., 1959)

50 ml of urine was acidified to pH 5 with HOAc and extracted with petroleum ether (B.P. 30° - 60°) to remove urobilinogen. The aqueous solution was then heated to 80° on a boiling water bath overnight, after which it was extracted 3 x with butyl alcohol containing 2% HOAc. The butyl alcohol was washed 3 x with water and filtered through moistened paper. The volume was concentrated to 5 ml and 30 ml MeOH and 10 ml sat. alcoholic zinc acetate added and allowed to stand overnight or until a Zn complex of dipyrroles precipitated. The precipitate was separated and washed with MeOH and then esterified with 5% $H_2SO_4/MeOH$. The ester was extracted into $CHCl_3$ which was washed 2 x with H_2O , 1 x with 3% NH_4OH , 1 x with 7% NaCl. The solution was then evaporated and put on a Al_2O_3 column that had been saturated with ethyl ether.

Elution was performed in the following order :

- (1) $CHCl_3$: ethyl ether (1 : 10)
- (2) $CHCl_3$: ethyl ether (1 : 3)
- (3) $CHCl_3$ alone.

The brown band of mesobilifuscin was finally eluted with hot HOAc. This was mixed with several volumes of $CHCl_3$, the $CHCl_3$ washed with H_2O and the solution concentrated. The mesobilifuscin was precipitated by the addition of 10 volumes of pet ether.

An absorption scan should show diffuse absorption below 500 mp.

MEASUREMENT OF PORPHYRIN - PROTEIN BINDING.

Several methods were investigated for detecting and measuring the presence of porphyrin bound by protein.

(a) Sephadex columns (Davis et al. 1970; Koskelo et al. 1970).

The use of Sephadex columns is a common way of determining protein binding, but there are certain disadvantages when the other component is porphyrin. The column should be equilibrated with porphyrin and then the protein introduced onto the column. A record of the optical density of the eluate should theoretically show a peak where the protein plus protein complex emerges and then a trough immediately following this representing a decrease in porphyrin concentration equivalent to the amount removed to form the complex.

The difficulty with this method is that a Sephadex should be used that completely excludes the protein used, such as a G 25 Sephadex, but free porphyrins tend to be strongly adsorbed onto this and equilibrium can only be reached after several days (Bradlid 1970). Sephadex G 200 may be used without prior equilibration with porphyrin if only separation of the components is required and not quantitation.

(b) Equilibrium dialysis (Breckenridge and Rosen 1971; Davis et al. 1970; Sano and Tanaka 1964).

This method may likewise be used for separation of components but not for quantitation. The porphyrins are adsorbed by the cellulose membranes.

(c) Precipitation (Davis et al. 1970).

The separation of porphyrin, protein and porphyrin-protein complexes may be achieved by precipitating the complex with the protein and measuring the amount of complex formed by the decrease in free porphyrin concentration.

This method, although far removed from physiological conditions, proved to be most satisfactory.

- (d) Changes in spectral wavelength and intensity. (Rossi-Fanelli et al. 1959; Holden 1937, 1946; Hill and Holden 1926; Nanzyo and Sano 1968; Asakura and Yonetani 1969; Maehly 1961).

The presence of porphyrin-protein complex formation may also be measured by changes in wavelength of the solet peak and changes in O.D.

- (e) Electrophoresis and Chromatography. (Koskelo et al. 1969, 1970; Dossett and Bentley 1963).

The porphyrin-protein complexes may be separated from the free porphyrin and protein by paper chromatography, thin layer chromatography or electrophoresis.

- (f) Fluorescence quenching. (Velick et al. 1960).

When certain kinds of molecules become attached to proteins either as biochemically specific complexes or synthetic chemical conjugates, the quantum yield of the fluorescence of the tryptophane residues of the proteins is diminished. A condition for such an effect is the overlap of an absorption band of the ligand with the emission band of protein. Porphyrin absorption at 340 $m\mu$ is only minor, and at 400 $m\mu$ where the porphyrin absorbs strongly, the emission by the protein is only one tenth of that at 340 $m\mu$. Therefore this method is not entirely satisfactory.

URINARY CREATININE.

This method makes use of the Jaffe reaction, the production of a red colour with an alkaline picrate solution. The reaction is not specific for creatinine, but in urine only up to 5% of the chromogens may be non-creatinine substances.

Reagents : 5% Sodium Tungstate
0.3 N Sulphuric Acid
0.04 M Picric Acid
3% Sodium Hydroxide
0.1% Stock Creatinine Solution.

Procedure :

The urine was diluted ten times; 1 ml urine (dil.) + 5 ml H_2O + 2 ml Na Tungstate + 2 ml H_2SO_4 were added together to precipitate any protein. This was centrifuged and 5 ml of the supernatant put into a Klett tube and 2 ml picric acid and 2 ml NaOH added. The absorption was read at 520 m μ after 10 min. A blank and standard were run with the samples.

DETERMINATION OF RADIOACTIVITY.

Known quantities of the porphyrin methyl esters were placed into B₂₄ ground glass stoppered test tubes and taken to dryness. Combustion was then carried out according to the method of Van Slyke et al. (1951).

Reagents : (a) KIO₃ and K₂Cr₂O₇ mixed in the ratio 2 : 1.

Ground to mix thoroughly.

(b) Liquid reagent : 67 ml fuming H₂SO₄

33 ml H₃PO₄

1 gm KIO₃

Heated to 160 - 180°C till KIO₃ was dissolved.

(c) CO₂ free NaOH (IN)

(d) CO₂ free distilled H₂O

(e) 2 M NH₄Cl

(f) 25% BaCl₂

(g) Scintillator : 3 gms 2,5 diphenyloxazole

0.3 gms p - bis - 2 - (5 - phenyl-oxazolyl) -
benzene

1 L Toluene

4% Carbosil.

Procedure :

1 gm of reagent (a) and 5 ml reagent (b) were added to the B₂₄ test tube containing the sample. To a B₁₉ tube 5 ml of IN NaOH was added. The B₁₉ and B₂₄ tubes were joined with an adaptor which had a side piece for evacuation. Using a water pump the system was evacuated. The B₂₄ tube was warmed gently over a Bunsen flame and gradually the contents heated to boiling. When white fumes appeared the reaction was finished and the tubes were left overnight to allow diffusion of CO₂ to the NaOH to take place. The following morning

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the vacuum was released and 2 ml of 2M NH_4Cl and 3 ml 25% BaCl_2 was added to the NaOH . A white precipitate of BaCO_3 formed. This was collected on a previously weighed millipore filter and placed in a weighed counting vial. The BaCO_3 was then dried, removed from the filter paper, crushed and weighed into the counting vial and then suspended in 15 ml scintillator and counted on a Beckman Liquid Scintillation Counter.

ACETYLATION (BARRETT 1959)

Porphyrin methyl esters were dissolved in pyridine-acetic anhydride (2 : 1 v/v 0.3 ml), left overnight at room temperature in the dark and reagents removed under N_2 at 50° C.

THE ISOLATION OF CATIONIC PROTEIN FROM PLATELETS

(Nashman et al 1970, Chandler et al 1965, Marcus et al 1960.)

Alsevers solution

glucose 2.05 g friso ^d ium citrate 0.8 g 0.9 g $C_6H_5O_7Na_3 \cdot 2H_2O$ NaCl 0.42 g	}	→	H_2O 100 ml Adjust pH to 6.1 with 5% citric acid
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Veronal buffer

NaCl 20.9 g NaHCO ₃ 0.63 g Na5,5, diethyl barbital 0.75 g 5,5 diethyl barbituric acid 1.15 g	}	→	+ 300 ml H_2O Make up to 500 ml when cool dissolve in 100 ml boiling H_2O
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Gairter buffer pH 7.6[±] 0.1

KH ₂ PO ₄ 0.67 g NaH ₂ PO ₄ 5.529 6.24 (2H ₂ O) NaCl 6.09 10% NaOH - 16 ml glucose 1 g	}	→	H_2O 1000 ml
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0.44 Sucrose (0.001 M EDTA)

75.308 g sucrose 0.186 g EDTA	}	→	H_2O 500 ml
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0.25 M Sucrose

42.788 g → 500 ml

0.2 N H₂SO₄

1.4 ml → 250 ml

Buffered saline pH 7.4

0.15 M NaCl	0.0175 M PO ₄	
4.383 g NaCl	} → 500 ml	
1.242 g Na ₂ HPO ₄		
	or	1.0497 NaH ₂ PO ₄

30% Sucrose

30 g sucrose	} → 100 ml
.0372 g EDTA	

60% SucroseIsolation of platelets

1. Human citrated whole blood was used. It was centrifuged at 320 g (1253 rpm on large centrifuge) for 15 min.
2. The protein rich plasma (PRP) was again centrifuged at 185 g (930 rpm) for 5 min. The contaminating red cells were removed.
3. Platelets could be stabilized by incubation for 30 min. at 44°C.
4. The platelets were spun for 4 min. at 320 g and then at 2,000 g (2620 rpm on our centrifuge) for ½ hour.
5. The platelets were washed with Alsevers solution 4 x.
6. Then washed with Gainter buffer, 4 x.
7. The platelets were homogenised at 1700 rpm for 5 min. (Teflon pestle). The homogeniser was washed with 2 ml sucrose. All the sucrose was combined.
8. This was centrifuged at 2,000 g for 15 min. (± 10 ml).
Sediment - platelets, granules and cellular debris.
Supernatant - granules and membranes.

9. A sucrose gradient was prepared. 7 steps 40 - 55% were used, 0.5 ml per conc.
The supernatant solution was placed on top with a curved pipette.
10. The sucrose gradient was submitted to ultracentrifugation. Spinco model L 2 HV - 2 hours at 3°C (SW - 39 L head used at 39,400 rpm) (130,5769 at centre of tube).
A hole was pierced in the bottom of the tube with a 25 gauge needle, and fractions collected manually.
11. The fractions were washed with 0.25 M sucrose.
The membranes sedimented at 39,400 rpm for 1 hour
The granules sedimented at 20,000 rpm for 20 min.
12. The granules were washed twice with Gainter buffer to remove adsorbed plasma protein.
13. They were then dialysed extensively against water.
14. The granules were sonicated for 10 seconds in buffer at 20 KC under ice with a Branson sonifier at full amplitude.
15. Cationic proteins were extracted.
The solution was extracted 3 x with 2 ml volumes of ice cold 0.2 N H₂SO₄.
Combined acid extracts were cleared by centrifuging at 12,000 g (12,500 rpm).
16. The acid extracts were then dialysed for 4 hrs at 10°C against 3 changes of buffered saline pH 7.4 (0.15 M NaCl 0.0175 M Phosphate).

ESTIMATION OF FREE AND BOUND PEPTIDE.

Estimates of the total amount of peptide present in urine varies according to the procedure used, the most accepted method is the estimation of the individual amino acids before and after hydrolysis. Free amino acids are measured directly from an aliquot of urine, and then bound as follows : -

- 10 ml urine
- + 1 ml 0.67 NH_2SO_4
- + 1 ml 10% Na Tungstate.

The precipitate was removed and 1 or 2 ml of the protein free urine (0.2 - 0.5 mg total amino N) was hydrolysed in a sealed tube at 105°C for 18 hrs with an equal volume of concentrated HCl. The hydrolysate was filtered and washed through glass wool and dried over KOH in vacuo, and then put on an amino acid analyser.

Alternatively the peptides may be precipitated from a protein free urine by the use of phosphotungstate; the precipitate can then be dissolved in Folin phenol reagent and the concentration read on a spectrophotometer (Goiffon 1934).

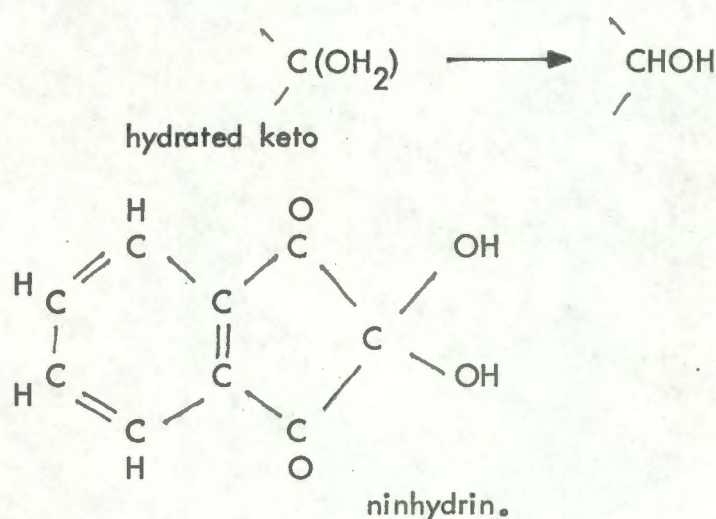
Measurement of UV absorption at $280\text{ m}\mu$ will give a rough indication of the amount of protein present, but the latter is due to tyrosine and tryptophan residues which vary in different fractions. Waddell (1956) recommends using the differences between 2 wavelengths, i.e. $215\text{ m}\mu$ and $225\text{ m}\mu$ to minimise the error from non protein constituents. This absorption is independent of pH in the range pH 4-8. If high absorption at $260\text{ m}\mu$ is seen there is the possibility that the peptides may contain pyrimidine bases or nucleotides.

More accurate measurements are made by sensitive colour reactions such as the ninhydrin reaction or the Folin reaction. The ninhydrin (Triketohydrindene hydrate) colour reaction is the one used in the Beckman automatic amino acid

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analyser and the method of choice performed throughout most of this work. Ninhydrin reacts with the carboxyl and α amino group of the same amino acid molecule at the same time. One is therefore able to distinguish between free amino acids and peptides. The reactant is a $-\text{NH}_2$ group or $\text{N}-\text{H}$ group; if both H atoms are substituted no reaction is possible. The $\text{N}-\text{H}$ - forming a peptide bond does not contribute to the ninhydrin colour produced by peptides and proteins. Therefore a peptide may be undetected when its α amino group gives a low colour yield e.g. in large peptides, also cyclization of an NH_2 terminal glutamine residue to form the pyrrolidine derivative may prevent the ninhydrin reaction, also if the NH_2 -terminals are acetylated, but apart from these few exceptions the approximate length of a peptide chain can be determined if the ninhydrin reactions are done before and after hydrolysis.

When the ninhydrin is heated with an aqueous solutions of the amino acid in the presence of a reducing agent, the amino acid is oxidised to CO_2 , NH_3 and an aldehyde which contains 1 carbon less than the amino acid. The ninhydrin itself is reduced to an alcohol.



The reduced form combines the excess of the hydrated Keto form and ammonia to give a purplish-blue colour due to diketohydrindylidene - diketohydrinamine (DYDA), whose absorption can be recorded at 440 μ for proline and 570 for the other amino acids. Other ninhydrin positive constituents in urine are urea, taurine

The colour development can be accelerated and increased by organic solvents especially phenol and pyridine (Troll, 1953).

The presence of phosphates in the urine precludes the use of Stannous chloride as a reducing agent and either ascorbic acid (Yemm, 1955) or cyanide (Moore and Stein, 1954) should preferentially be used.

The Folin reaction by Lowry yields colour values in rough proportion to the number of peptide bonds in the chain, but the amount of colour varies with different proteins, the ninhydrin reaction however has the added advantage of being able to detect free amino acids which are often present in proteolytic digests of protein chains and is therefore of greater general utility. The Folin reaction has the advantage that it is several fold more sensitive than the ninhydrin reaction.

For the detection of peptides on T.L.C. or paper chromatography the chlorine/tolidine reagent of Reindel and Hoppe (1954) as modified and shown by Stahl (TLC p.412, 1965. Academic Press N.Y.) is more sensitive than the ninhydrin reagent and can detect up to 0.1 μg .

PROCEDURE :

	<u>Before Hydrolysis</u>	<u>After Alkaline Hydrolysis</u>
Mix thoroughly	{ 0.2 ml sample 2.2 ml distilled water 2.0 ml ninhydrin reagent	pH 5 { 0.2 ml sample 1.0 ml NaOH (2.5 N). 1.2 ml AcOH (30%) 2.0 ml ninhydrin

Standards and Blanks were included in each run. The tubes are mixed and heated to 60° for approximately 45 minutes or until a blue colour develops. The optical density was then read at 570 mμ on a spectrophotometer.

Ninhydrin reagent :-

- 8 g ninhydrin
- 0.16 g stannous chloride (or ascorbic acid or cyanide)
- 300 ml methyl cellosolve
- 100 ml sodium acetate buffer.

The methyl cellosolve and sodium acetate buffer were mixed and nitrogen bubbled through the solution for approximately half an hour; the reducing agent was then added and thoroughly dissolved before the addition of the ninhydrin. Nitrogen was again bubbled through and the solution allowed to stand stoppered in a dark bottle for several hours before use.

Sodium acetate buffer:-

136 g Na Acetate . 3 H₂O 100 ml dist. H₂O.

Heated to dissolve and then cooled. 25 ml glacial acetic acid was added and the solution made up to 250 ml with distilled water.

Amino Acid Standard:-

0.0131 g Leucine → 10 ml Glacial Acetic Acid (10 mM solution)

(i) dilute 1 → 10 (1.0 mM)

(ii) " 0.5 → 10 (0.5 mM)

(iii) dilute 0.1 \rightarrow 10 (0.1 mM)

0.2 ml of each treated as per sample.

METHODS OF SEPARATION OF PEPTIDES, AMINO ACIDS AND THE
HYDROPHILIC PORPHYRINS.

The isolation, separation and purification of peptides is sometimes hampered by their polymerisation, but several depolymerising methods may be used such as the use of histidine, urea, guanidine hydrochloride or extremes of pH, or merely dilution of the sample.

The separation of a mixture of peptides depends primarily upon variations of one or more of properties such as molecular weight, charge or solubility. The simplest procedure is by the use of dialysis membranes.

(a) Dialysis membranes.

The type of membrane used will depend upon the property being exploited, the most common in use are the collodion membranes. The pore size can be adjusted by varying the drying time during manufacture, they are weak acid cation exchanges. The ion exchange capacity is very low, but it can be increased by treatment with NaOH before or after the membrane formation. The NaOH causes partial degradation of the collodion which in turn is oxidised by the degradation products. Other types of membranes are homogeneous ones, such as linear polystyrene, or heterogeneous membranes where ion exchange particles are embedded into an inert binder. The ion exchange membranes have two unique properties, i.e. a permeability which is much higher for counter ions than for co-ions and high electric conductivity. The non-electrolytes diffuse at an intermediate rate and the rate of diffusion across the membrane is controlled by the slowest process, i.e. the diffusion of the co-ion.

Purification by means of dialysis had only a limited application in this instance due to the small size of the molecule and the method could only be used during isolation of the crude material in which the porphyrin peptide was probably polymerised; even the use of membranes at this stage of isolation was

abandoned in the latter studies in favour of more suitable methods, where a lower risk of loss of material would be encountered.

(b) Gel chromatography and electrophoresis. (Anderson and Stoddart 1967; Berman 1964; Blumer and Snyder 1967; Haurowitz 1963; Janson 1967; Kellett 1967; Male 1967; Morris 1964; Moschera et al. 1970; Neurath 1963; Porath 1960, 1962, 1967; Wieland and Deteman 1967; Whitaker 1963).

The different types of gels available may be grouped according to their hydrophilic or lipophilic properties.

The hydrophilic gels are the cross-linked Dextrans, polyacrylamides and agarose gels. A lipophilic gel is Styragel.

Sephadex is a cross-linked dextran gel whose polar properties are due to a high content of hydroxyl groups. The G types are unsubstituted sephadex and vary in degree of cross-linkage and hence in their porosity and fractional range. Sephadex LH 20 is an example of a lipophilic-hydrophilic gel. It is the Hydroxypropyl ether of Sephadex G 25. The numerical suffix of sephadex refers to the water regain of the gel whereas the numerical suffix of Biogel, a polyacrylamide, refers to the exclusion limit. The Styragels used with organic solvents are cross-linked polystyrene gels.

These gels were used either in columns or as thin layer plates, the former being the more common practice. The resolution obtained from a gel column is dependent upon the column length and sample size and the type of gel employed, the superfine giving the best resolutions. This molecular sieve chromatography is a form of partition chromatography which utilises the distribution behaviour of solutes between solvent phases in 2 physically distinguishable environments.

$$K_{AV} = \frac{V_e - V_o}{V_I}$$

K_{AV} = distribution coefficient

V_e = elution volume

V_o = void volume

V_I = int. volume that is accessible in the solute

= volume of column - V_o .

For large molecules $V_e = V_o$ ∴ $K_{AV} = 0$

For small molecules $V_e = V_o + V_I$ ∴ $K_{AV} = 1$.

A linear correlation exists between K_{AV} and log of the molecular weight of the sample.

One disadvantage of Sephadex is its adsorption properties due to electrostatic interaction or structure. A planar system and an extending system of conjugated bonds as found in porphyrins favour adsorption. Such aromatic adsorption can be depressed by the use of phenol : HOAc : H₂O as solvent or substances such as urea, pyridine, phenol and glacial acetic acid may be used to block the adsorption sites.

Adsorption due to ionisation can be affected by changes in pH which is either due to π electron rearrangement or to the fact that the highly charged molecule is surrounded by a larger ionic double layer preventing the matrix from entering the adsorption sites. Basic amino acids in HOAc are highly charged, their ionic double layers are very large and they are therefore almost excluded; on addition of salt the hydration layer will decrease and they will be slightly retarded. The adsorption due to the increase in salt concentration can also be due to an increase in sites available. An example of the effect of ionisation adsorption is the difference in elution between glutamic acid and glycine; the former being eluted first due to repulsion of the 2 negatively charged carboxylated groups by equally charged centres of the gel, preventing diffusion into the pores.

Early elution may be due to aggregation or complex formation. This may be avoided by the use of phenolate or alkaline urea solutions (Porath 1962). Urea tends to close the pores and meshes of Sephadex gel, probably because it is bound to the CHO network, since the swelling is in fact increased in strong urea solutions.

Due to these strong adsorption effects of porphyrins on the Sephadex a salt free solution was found to be most suitable. SG 25 superfine which has an exclusion limit of 5,000 was used as the last stage in purification and in order to obtain good resolution columns of 1.5 metres long were required, since the porphyrin-peptide fractions have very similar molecular weights. Rimington et al. (1968) recommended the use of a 0.05 M borate buffer pH 8.6 with Sephadex G 25.

Sephadex LH 20 used with a Benzene : MeOH solvent system provides an excellent method for the separation of porphyrin esters and was found useful for the separation of the crude porphyrin-peptide complexes.

It was thought that the undesirable adsorption effect seen when using a hydrophilic Sephadex gel might be eliminated by the use of Biogel (an inert matrix of polyacrylamide which contains no ionic group) but resolution of free porphyrins using Biogel P was not found to be so good as by use of Sephadex LH 20.

Gels of agar, starch or polyacrylamide are also used for electrophoresis. In gels with a molecular sieve effect the fractional retardation of the larger molecules is compensated by a greater net charge, so that many proteins of widely different molecular sizes have equal electrophoretic mobilities. When the gel pore size is very small the frictional retardation of the larger molecules outweighs the increased charge. Such gels act as molecular sieves, such as the acrylamide gels. The latter were used for isoelectric focusing, by the introduction

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of ampholytes into the gel. The pH range may be varied according to the range of the ampholytes, but the higher pH range was often difficult to achieve in the polyacrylamide gels.

PROCEDURES.

Sephadex G 25 (Superfine) (fractionation range 500 - 10,000 Mw).

The Sephadex gel was suspended in excess solvent and allowed to swell for 3 hours. It was then deaerated for approximately 30 mins by use of water suction.

Column : 1.3 metres long, 0.9 cm diam.

Solvent : 30% HAC or Phosphate/Borate buffer 0.05 M pH 8.4

Na H ₂ PO ₄ · 2 H ₂ O	2.34 g	} made up to 1 L with H ₂ O , pH 8.4
Na ₂ B ₄ O ₇ · 10H ₂ O	4.29 g	

Speed : 10 ml/hour maintained by constant pressure pump.

The spectral absorption at 280 and 400 mu was monitored with a Beckman automatic DB-G spectrophotometer.

Sephadex G 100 (Medium)

Column : 50 cm long, 1.9 cm diam.

Solvent : 0.05 M Barbitol or Borate buffer.

Speed : 20 ml/hour.

Sephadex LH 20 (Medium)

Column : 50 cm long, 1.9 cm diam.

Solvent : Benz. : MeOH (1 : 1).

Speed : ± 20 ml/hour.

Polyacrylamide electrophoresis and electrofocusing (Fawcett 1968; Dale and Latner 1968; Percival et al. 1970; Awdeh et al. 1968; Wrigley 1968).

Shandon Disc electrophoresis apparatus used.

Glass tubes 5 mm diameter and 8 cm long were stoppered at the bottom and filled to within 2 cm from the open end with a deaerated solution containing acrylamide (8%), ammonium persulfate (.03%), sucrose (40%) and dimethyl amino propronitrile (0.8%). Ampholine (1%) was added for electrofocusing. After polymerisation of the gel the stopper at the lower end was removed and the tubes ^{placed} in the supporting vessel. For electrophoresis the anode and cathode chambers were filled with a glycine/Tris buffer pH 9.5. For electrofocusing several combinations were tried for the cathode and anode electrolyte solutions in order to obtain a system in which a wide range of pH's could be obtained. The main difficulty was in finding a system which allowed for the higher pH values.

<u>System</u>	<u>Anode</u>	<u>Cathode</u>
A (Fawcett 1968)	ampholine (1%)	tetramethyl ethylene diamine (1%)
B (Dale and Latner 1968)	H ₃ PO ₄ (25 ml → 1750 ml)	ethanolamine (20%)
C (Wrigley 1968)	H ₂ SO ₄ (0.2%)	ethanolamine (0.4%)
D (Percival et al. 1970)	H ₂ SO ₄ (various strengths)	NaOH (1%)

A voltage of 300 V was applied across the tubes for approximately 6 hrs.

Immediately after disconnecting the current one gel was sliced into 8 sections. Each piece was placed in a separate tube, 1 ml of boiled distilled water was added and the tube immediately stoppered. The tubes stood overnight and the pH of each tube was read the following day in order to determine the pH gradient present. The other gels were fixed in 10% Trichloroacetic acid (TCA) which was changed several times to remove the ampholytes from the gel and then stained in 0.01% amido black (made up 0.01% TCA) overnight and destained with 40% EtOH.

Isoelectricfocusing with the use of LKB 8 121 equipment, consisting of a 110 ml column with built in electrodes, was achieved using ampholine carrier pH 3-10. The anode electrolyte was dilute sulphuric acid, and the cathode electrolyte was placed at the bottom of the column and consisted of a sodium hydroxide solution containing sucrose. Solutions were prepared according to the manual. The total time of running was 94 hours.

(c) Paper chromatography and Electrophoresis.

Many chromatographic methods exist for the separation of peptides and amino acids. The ionic dissociation of the amino groups requires an acid pH at which however the carboxyls cease to be charged. At an alkaline pH ionisation of basic groups retrocedes and carboxyls become dissociated. The nett charge of a protein therefore depends on the pH of the surrounding medium. Both the protein and the porphyrin are therefore amphoteric, the isoelectric point being the pH at which there is a nett zero charge.

The specific electrophoretic mobility is expressed by u ($\text{cm}^2 \text{v}^{-1} \text{sec}^{-1}$), where $u = \frac{S/t}{F}$

s = distance (cms) travelled by protein

t = duration of migration (secs)

F = field strength (v cm^{-1})

u depends on pH, qualitative composition and ionic strength of the buffer. The ionic strength is usually 0.05 - 0.1 since at higher concentrations the conductivity of the buffer would cause the development of an undesirable amount of heat and an inconveniently slow migration rate. Barbiturate ions are useful because they are univalent and therefore contribute little to the ionic strength and interact minimally with proteins.

The following chromatographic solvent systems were used

1. BuOH/HAC/H₂O 4 : 1 : 5 (Harris and Roos 1959)
2. BuOH/HAC/H₂O 3 : 1 : 1 (Leggett and Bailey 1967
Margoliash et al. 1962)
3. Pyr/isomylalcohol/H₂O 30 : 30 : 35 (Baglioni 1961)
4. Lut/H₂O 2 : 1 (Gross 1959).

They were generally used in conjunction with HV electrophoresis, in which event a Whatman No. 3 MM paper was used; alternatively the chromatography or electrophoresis was used as a second dimension after initial fractionation by column chromatography.

Electrophoretic separation of amino acids using aqueous 0.75 M HCOOH pH 2.0, 30 V/cm 40 min. gave an excellent resolution (Gross, 1959).

Other systems tried included

Pyridine : HAC : H₂O (10 : 0.4 : 90), pH 6.5, 35-40 V/cm 150 min (Laggett and Bailey, 1967) or

Pyridine : HAC : H₂O (1 : 10 : 189), pH 3.5, 35-40 V/cm, 2 hrs or the use of other pyridine or borate buffers.

Blackburn (1960) gives a review of combinations of HV Electrophoresis and chromatography.

(a) Electrophoresis in Pyr : HAC buffer pH 3-6 for 20 min at 1500 V, followed by chromatography in n propanol 0.05 M Na pyrophosphate 0.1 M NaCl buffer of pH 7 : 3 (70 : 30).

(b) Electrophoresis at pH 2 Formic acid : HAC buffer followed by chromatography in Lut/H₂O (2 : 1).

(c) Electrophoresis under Toluene using a Formic acid - Pyridine buffer, followed by chromatography in n-butanol - n-butylacetate - HAC - H₂O (19 : 1 : 5 : 25).

(d) Electrophoresis in 2 N HAC - 0.6 N Formic acid (1 : 1) at 2800 V for 200 min followed by chromatography in Pyridine - HAC - H₂O (50 : 30 : 15).

System (b) was found to be the most suitable for the porphyrin-peptide complexes.

The peptides and amino acids were identified by spraying or dipping the paper in a 0.2% ninhydrin solution in acetone. The addition of CdCl₂ aided in identification. All the amino acids except pro and asp could be identified.

The advantages of using HV electrophoresis and chromatography were

1. the elimination of the need for desalting.
2. spots were rounder and more compact than those produced with 2-way chromatography.
3. very small quantities were detected which might not have been seen using 2 D chromatography.
4. there was also a better separation of certain amino acids.

2-Dimensional electrophoresis was also attempted. An example of such a system

A. 0.75 M Formic acid pH 2.0 30 V/cm for 40 min.; the paper is dried for 10 min at 90° and then for 1 hour in a stream of cold air and then sprayed with 0.05 M Na borate solution pH 9.2, turned through 90° and run again at 100 V/cm 20 min, and finally dried and sprayed with acidified ninhydrin.

Of all these systems the use of HV electrophoresis according to Gross (1959) i.e. 0.75 HCOOH pH 2.0 30 V/cm for 40 min, followed by ascending chromatography with lutidine/H₂O 2 : 1 as solvent proved the most satisfactory.

(d) Ion Exchange Chromatography.

The use of ion exchange chromatography for the separation of peptides and amino acids is probably the most widely used method of all. The type and strength of the exchanger is determined by the active groups which are part of the matrix. Cation exchangers employed phenolic hydroxyl, carboxyl and sulphonic groups and anion exchangers have aliphatic or aromatic amino groups, e.g. the trimethyl benzyl ammonium derivative of polyvinyl benzene. The sulphonated polystyrene resins which were used on this occasion have a cross-linking divinyl benzene agent which gives the material a 3-dimensional network of benzene rings. The SO_3^- ions are the active sites for cation exchange. The amino acids of peptides were separated by the ion-exchange resin by displacement development or by elution, the latter being the more common method. This method depends upon the binding of a protein to the cation exchanger at a pH below its isoelectric point (or vice versa with an anion exchanger), with subsequent release of the protein as the pH of the eluting buffer nears the isoelectric point of the protein.

Displacement development depends partially upon the suppression of the cationic form of the weaker bases by the stronger base and therefore displacement of the weaker base, but the system also depends on the relative affinities of the resin for the different cations.

The order of displacement is largely determined by the basic or acidic strengths of the solutes but other factors which contribute to the total adsorption energy are the valency and degree of hydration of the ion, molecular size and presence of non-ionic bonds, the latter being the reason for the retardation of aromatic amino acids such as Tyr, Phe, Try on cation exchange resins. This effect is minimised at higher temperatures, but generally the displacement is in the order of their pK values.

The highly cross linked resins form a matrix that is impermeable to all but the smallest molecules.

Analysis done by the elution technique gave high resolution. Factors governing the resolution of solutes apart from the relative affinities were the pH and ionic strength of the eluent, the temperature of the system and the characteristics of the resin.

Cellulose ion exchangers are a further improvement in the fractionation of proteins but are of limited value for peptide separation. The carboxyl methyl derivation of cellulose has an effective pK of approximately 4 and is therefore only partially ionised under the conditions favouring peptide binding; the phosphocellulose derivative does not have this drawback, but low yields suggest the presence of non-ionic bonding.

Preparation of Amberlite CG 50. (Margoliash and Smith 1962).

The resin was suspended in water and stirred, the fines were decanted and the process repeated 6 times. The resin was stirred with 2 N NaOH, washed with water and then stirred with 2 N HCl and again washed with water. This was repeated 4 times. It was then washed with acetone (after NaOH followed by water) and stirred with 2 N HCl again, then washed with water, then with 2 N NH_4OH . Washed 8 times with water. Finally it was suspended in 0.125 M ammonium acetate.

Preparation of Dowex 50 x 2. (Schroeder et al. 1962; Margoliash and Smith, 1962).

The resin was washed with 5 L water to remove fines. It was then washed successively with 5 L 1 N NaOH, 5 L water, 3 L 2 N HCl, 5 L water, 4 L 2 N pyridine (distilled) and finally with 3 L of pyridine buffer pH 3.1.

Preparation of Dowex 1 x 2 (Schroeder 1967)

The resin was washed with water to remove the fines and then washed successively with 1 L 0.3 N NaOH (carbonate free), 3 L water, 0.5 L 1N HCl, 3 L water, and finally with 500 ml pyridine buffer (pH 8.0)

Preparation of Dowex 50 x 8 (Schroeder 1967)

After removal of the fines, the resin was washed successively with 5L H₂O, 5 L of 1 N NaOH, 5 L H₂O, 3 L of 3 N HCl, 5 L H₂O, 4 L of 1 N NaOH, 3 L of pH 3.25 buffer

Preparation of Buffers.

(a) Pyridine Buffers:

pH	3.1	3.5	3.7	4.1	5.1
Pyridine (ml)	64.5	166	226	323	645
acetic acid(ml)	1114	1164	1130	1070	450
concentration	0.2M	0.5M	0.7M	1.0M	2.0M

Each one was made up to 4 L with distilled water.

(b) Sodium Citrate Buffers:

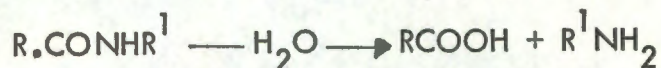
pH	2.2	3.25	4.25	5.28
Na conc.	0.2N	0.2N	0.2N	0.35N
Total volume (L)	1	4	4	4
Conc.HCl(ml)	16.5	50.3	33.5	26.2
Na Citrate.2H ₂ O	19.6	78.4	78.4	137.3
Pentachlorophenol(ml)0.1	0.4	0.4	0.4	0.4
Thiodiglycol(ml)	5	10	10	nil

PEPTIDE ANALYSIS

In order to determine the exact composition of the hydrophilic porphyrin and thereby obtain information which might lead to a better understanding of its function, we proceeded to determine the amino acid composition of the peptide part of the molecule. This involved determination of total amino acids present by acid or alkali hydrolysis, the determination of amino and carboxyl terminal end groups and the amino acid composition of enzymic digests.

a. Acid Hydrolysis.

Partial acid hydrolysis results in peptides of various lengths and consists of the cleavage of peptide and amide bonds according to



The conditions vary: for example the acid normality can lie between 0.02 N to 12 N, the temperature can vary from 37° 100° and the time of hydrolysis from 20 min to several days. Complete hydrolysis is generally attained by the use of 6 N constant boiling HCl which is sealed in an evacuated tube with the peptide and maintained at a temperature of 110° for 24 hours, after which the acid is removed under vacuum. The susceptibility of various peptide bonds to HCl is different; for example the peptide bonds formed by aspartyl residues or by serine or threonine are very easily hydrolysed. The -CO-NH-bond formed by amino acids of hydroxyacids is easily converted into an ester linkage -CO.O- in which the hydroxyl group of serine or threonine combines with the carboxyl group of an adjacent amino acid. Peptide bonds in which the carboxyl group is contributed by 1 isoleucine or valine are particularly stable; this is probably due to steric hindrance introduced by the side chain. Electrostatic effects explain the enhanced stability of dipeptides as compared to larger peptides. Therefore exposing the samples to varying times of acid

hydrolysis and extrapolating back to zero time gives an accurate estimation of the true value.

Acid hydrolysis causes destruction of tryptophan, glutamine and asparagine, the latter two are recovered as glutamic and aspartic acid. Serine, threonine, cystine and tyrosine also appear in slightly lower yields. The destruction of serine and threonine may be corrected for by the factors of 2.7% and 10% respectively (Moschera 1970). The fractions of glutamic and aspartic acid that exist in amide form may be roughly estimated from the amount of NH₃ produced, provided care to exclude atmospheric ammonia is observed. Destruction of serine and threonine also contributes to the ammonia. A more accurate determination of amide is via Conway's micro diffusion method or alternatively by digestion with leucine aminopeptidase and separation on a Dowex cation exchange column, where asparagine, glutamine and serine are normally eluted together at 50°, but a modification of the temperature causes serine to alter its position (Neurath 1963). Considerable amounts of the sulphur amino acids are converted to various products during acid hydrolysis, the modified amino acids being cysteic acid, S carboxymethylcysteine, methionine sulfoxide, methionine sulfone and carboxymethyl derivatives of lysine, histidine and methionine. Cysteine appears as cystine after hydrolysis.

Racemization at the α carbon of hydroxylysine occurs during acid hydrolysis and gives rise to appreciable amounts of allohydroxylysine which appears as a separate peak on effluent curves, also alloisoleucine emerges ahead of isoleucine and sometimes overlaps methionine.

Oxidation by cold performic acid prior to acid hydrolysis converts cystine and cysteine to cysteic acid, which is resistant in the action of boiling HCl.



Moore (1963) claims the recovery to be 94% efficient. In addition, the oxidation of phenolic groups and hydroxyl functions of serine and threonine occurs, and methionine is quantitatively converted to the sulphone. Tryptophan undergoes a multiplicity of transformations.

b. Alkaline Hydrolysis.

During alkali hydrolysis (2.5 N NaOH, temp. 250° press - 45 min.) there is more widespread destruction and racemization of amino acids (Neurath, 1967) but tryptophan is stable in the alkali and may be determined in such a digest, or alternatively by enzymic hydrolysis, e.g. by the use of leucine amino peptides (Margoliash, 1962). 4% thioglycolic acid in 6 N HCl may be used to prevent the oxidation of tryptophan, methionine, tyrosine and carboxymethylcysteine during hydrolysis. 95 - 100% recoveries were found. The use of thioglycolic acid also results in the minimum rearrangement of cystine peptides. The reagent affects the recovery of Pro. This may be overcome by exposing the hydrolysate to air before analysis (Moore and Stein, 1963). The presence of thioglycolic acid produces a peak between cysteic acid and aspartic acid and another at the carboxymethylcysteine valine position; it was therefore necessary to run the appropriate blanks.

c. Enzymic Hydrolysis.

Many native proteins are often resistant to attack by enzymes but are readily digested after denaturation or oxidation. All the peptides dealt with on this occasion had been denatured prior to digestion.

Enzymic hydrolysis can be divided into

- (i) endopeptidases - which split internal peptide bonds, e.g. trypsin, pepsin.
- (ii) exopeptidases - which are useful for the N terminal and C terminal amino acid determinations, e.g. amino peptidase, carboxypeptidase. For complete hydrolysis a combination of several enzymes is needed. Hill et al. (1962) recommend the use of papain, prolidase and leucine aminopeptidase, but on this occasion the complete hydrolysis was performed with acid.

Where the proteolytic enzyme is active under mild alkaline conditions the reaction was allowed to proceed in a "pH stat". Unfortunately the quantities of porphyrin peptide available were too small to obtain accurate estimations of the new COOH groups liberated.

Trypsin (mol. weight 23,000 - 24,000).

The action of trypsin is entirely restricted to bonds linking the carboxyl group of a basic amino acid to the amino group of another amino acid or to the hydroxyl group of an alcohol, and therefore the number of fragments is equivalent to the number of lysine and arginine residues plus the COOH terminal fragment which may contain neither. Regions with arginine and lysine adjacent to each other are often resistant, also bonds between proline and lysine (Harris 1958, Bell 1954) or bonds adjacent to negatively charged residues, e.g. glutamic acid, aspartic acid, cysteic acid or S carboxymethyl cysteine. Bonds involving histidine or ornithine are resistant. It is possible to mask specific basic groups or by introducing new ones the action of the enzyme may be limited or extended at will, e.g. lysine residues may be blocked by dinitro-phenylation (Redfield and Anfinsen, 1956) and in ribonuclease only the arginyl bonds are split. Sites may be created for example by the conversion of cysteine residues of a reduced protein to S - (β aminoethyl) residues by the action of

β -bromoethylamine (Lindley, 1956) or better still with ethylenimine (Rafferty and Cole, 1963). Arginine can be blocked by coupling with benzil ($C_6H_5COCOC_6H_5$) which does not react with lysine (Itano and Gottlieb, 1963). Once again our material was insufficient to do more than a simple trypsin digestion. We were merely able to note the number of fragments produced.

Any contamination of the trypsin with chymotrypsin may be reduced by partial inactivation of the trypsin with diisopropyl fluorophosphate or heat since chymotrypsin is preferentially more sensitive to both. Also short digestion times are preferable to minimise the effect of any contaminating chymotrypsin present; preferentially samples of the digest should be taken at intervals ranging from 5 min to 20 hrs., but unfortunately insufficient material prevented us from doing so. Shin-ichi Ishii (1967) found the 88 min digest confirmed the finding of a 20 hr digest and Harris (1959) found the reaction to be complete after 30 min as judged by means of a "pH stat".

Trypsin is most stable at pH 2-3 and most active in pH range 7-9; although most stable at low temperatures digestion is usually carried out at 20° - 40° which allows faster reaction rates. At a pH of 7.9 and 26°C a solution loses 50% of its activity in 30 min. It can be protected against autolysis by .01 M Ca^{2+} ions.

A high enzyme to substrate ratio ensures the splitting of difficult bonds, a ratio of 1 : 100 is usually used. The method of Thompson (1969) was used.

Approximately 1 μ mole of unknown was used and dissolved in 3 ml of H_2O and adjusted to pH 8.0 with 25 mM NaOH on a recording radiometer TTTIC autotitrator. The digestion was performed at 35°C by the addition of 1% (w/w enzyme/substrate) trypsin, made up in 10 μ l of 1 mM - HCl at zero time and a further 1% after 60 min. The digestion was followed on the auto-

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titrator and was determined after 100 min by boiling the solution to inactivate the trypsin. The digest was allowed to stand overnight at this pH at 4°C and any precipitate formed was centrifuged from the colourless solution containing the soluble peptide which was freeze dried.

Pepsin has a very wide specificity, but preferential attack is at bonds involving amino groups of aromatic amino acids, only peptide bonds are attacked, amides and esters are resistant. (Margoliash 1958).

0.5 ml sample (+ 2 μ M) was brought to a pH of 1.4 HCl and then made up to 1 ml with distilled water. To this was added 1 mg pepsin. This was incubated at 38° for 40 hours and then the solution boiled to destroy the pepsin.

Pronase hydrolyses 87% of total bonds and is the nearest approach to a universal proteolytic enzyme. (Nomoto 1959, 1960; Hiramatsu 1963).

1 ml enzyme solution (0.44 mg protein nitrogen per ml) + 1 ml 0.1 M substrate solution was adjusted to pH 7.0 and allowed to proceed at 40° for 24 hrs. The reaction mixture was acidified and the products separated chromatographically.

d. Terminal Group Analysis.

Determination of terminal residues may be accomplished by enzyme hydrolysis or chemical labelling. Proline amino peptidase cleaves exclusively N terminal L proline residues, N terminal hydroxy-L-proline residues are not split. Leucine aminopeptidase can hydrolyse the peptide bonds of all the common L amino acids when they occur in the NH₂ terminal position, but proline is very slow. In determining the amino groups it must be borne in mind that free amino groups occur in side chains of lysine. Carboxypeptidase cleaves C-terminal residues. These enzymes may be used in situ and sprayed onto the paper in which the peptide and amino acid are to be separated (Naughton and Hagopian, 1962).

The disadvantage of the use of enzymes is that the degradation depends on susceptibility of the peptide bonds and aliquots taken at different times show the release of some amino acids before others. When using chemical labelling to the terminal residues the main difficulty is to find a substituent which will not split off during the hydrolysis of the protein.

The end terminal may be reacted and then the whole peptide hydrolysed and the substituted amino acid determined or alternatively a stepwise degradation may be done. N terminal analysis may be performed by making a dinitro phenyl derivative. The Dansyl method is 100 x more sensitive and therefore more suitable for small quantities, but the DNP method may be adapted to the microscale by radioactive labelling of the reagent with C^{14} (F D N B - C^{14}) where as little as 0.1 μ mole may be detected by means of autoradiography of the chromatograms (Neurath 1967).

PROCEDURE

Samples taken for end group studies were dried thoroughly over H_2SO_4 , then redissolved in 10 - 15 μ l 0.2 M $NaHCO_3$. Evaporated to dryness and taken up again in 15 μ l deionised H_2O . The pH was checked and if below 9.5 more base was added. The pH should be such as to have most of the amino groups in the NH_2 form and low enough to have a limited hydrolysis (up to pH 9.5). An equal volume dansyl chloride was added and the tube covered with parafilm. This was left for 1 hour at 37° or 2-3 hours at room temperature.

(Excess reagent is hydrolysed to sulfonic acid and the solution becomes colourless).

This was dried in vacuo and 50 μ l 6 - 7 N HCl was added and the tube evacuated and sealed, and heated at 105° .

(Gross (1968) showed that the release of dansyl derivatives from the peptide chain is faster than that of free amino acid and destruction of the dansyl acids occurs rather rapidly; therefore a shortened time for hydrolysis of 4 hrs is

preferable unless valine, leucine and isoleucine are expected; then the longer period of 18 hrs. is necessary).

The tube was opened and the HCl evaporated in vacuo over NaOH pellets. (Peptide bonds of the form DNS-Val-X and DNS-Ileu-X are incompletely hydrolysed, in which case the end group may be deduced by comparison of electrophoretic mobilities at pH 1.9. DNS-Try cannot be identified after acid hydrolysis but may be released by incubating the labelled peptide with chymotrypsin).

The residue was dissolved in acetone/HOAc (IN) 3 : 2 and then spotted on Silica gel G thin layer plate, which was dried at 110° for ½ hour. The plate was run in Benz : Pyr : HOAc (16 : 4 : 1) Zdenek (1965) and then in CHCl₃ : benzylalcohol : HOAc (70 : 30 : 3) Morse (1966). The plate was evaporated at 110° for 5 min and viewed under UV. Yellow spots were seen. The plates were sometimes put in a desiccator with NH₃ fumes to enhance the fluorescence.

An alternative method to this is as follows :

The residue was dissolved in water at pH 3.5 and extracted with ether 3 x (Gross 1968).

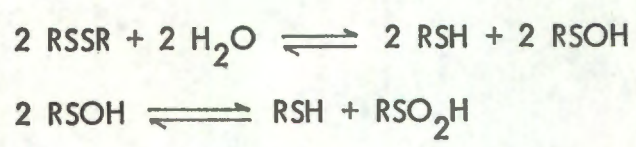
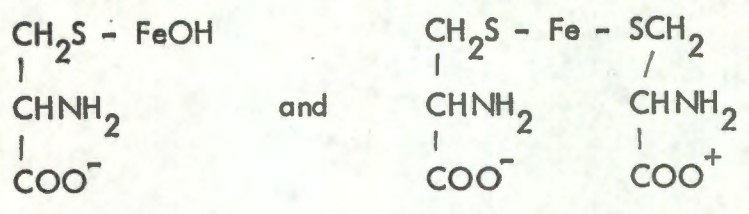
The extraction leaves the Dan OH in the waterphase. The ether and the water phases were brought to dryness and spotted on TLC.

The water phase contained D Arg., Dan-Hist., Dan-Lys., Dan-Tyr., Dan-CySO₃H, Dan-Cys. and Dan-OH and was chromatographed 2 dimensionally in the solvents toluene : monochloroethanol : 25% NH₄OH in ratio (6 : 10 : 4) and then in solvent toluene - monochloroethanol - NH₃ (6 : 10 : 0.5).

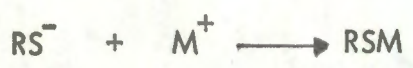
The organic phase contained the other dansyl amino acids and was chromatographed in solvents benzene - pyridine - HOAc (80 : 20 : 5) and then in solvents toluene - monochloroethanol - 25% NH₃ (6 : 10 : 4).

DETECTION OF SULFHYDRYL BONDS.

Disulphide bonds may be cleaved by oxidation of cystine with performic acid or 8 M urea or 4-6 M guanidine HCl or by the presence of Fe or OH ions.

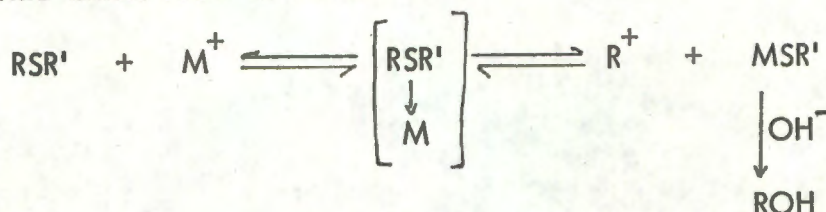


Heavy metals also promote hydrolysis. The presence of disulphide bridge is inferred if more half cystine residues (as cystine, S carboxymethylcysteine or cysteic acid) are found in an acid hydrolysate than can be accounted for by SH group estimation. The SH groups may be measured by mercaptide formation.



Silver or mercuric salts or organic Hg derivatives may be used. The affinity of the heavy metals for the RS⁻ ion results from their tendency to accept a pair of electrons combined with the willingness of RS⁻ to donate electrons with the resultant formation of slightly dissociated sulphides. Ag salts have the disadvantage that the Ag mercaptides of the simple thiols have a strong tendency to bind additional silver ions. Mercuric is more specific in its reactions with SH groups, but some ambiguities can arise from the valence of Hg. Either (RS)₂ Hg or Prot.S.HgX may be formed depending upon whether it is sterically possible for 2 sulphurs to react with the one Hg ion.

The Thio-ethers react as follows



The non-specificity of the reaction of heavy metal cations with proteins deserves stress. The metals Hg, Ag, Cu and Zn are known to bind other protein groups besides the -SH. Haarman (1943) found that Hg salts reacted stoichiometrically first with the -SH groups to form mercaptides and then combined with carboxyl and amino groups of the protein. At low concentrations the reaction with -SH does appear to be the most significant. Hughes (1954) comments that the use of methyl iodide increases the specificity for -SH since only the -SH groups have the affinity necessary to replace the iodide.

The presence of Thio-ether bonds was determined as follows (Boyer, '1959).

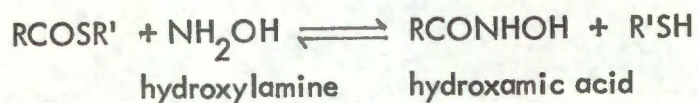
The sample was lyophilised and 2 ml 50% acetic acid added and 1 ml HgSO₄ (20 mg in 2.5 ml HAC + 2.5 ml H₂O). Nitrogen was passed through the solution and the tube then stoppered and incubated at 80° for 30 min in the dark. The solution was then brought to pH 3.5 and extracted with ether in order to detect any free porphyrin that would be liberated.

DETERMINATION OF THIO-ESTERS

The Thio esters are stable at pH 6 but labile at pH 9 and undergo non-enzymatic ester interchange reactions (Lipmann and Tuttle, 1945)



The rate of thiol ester hydrolysis may be measured by observing the fall in absorption at 231 μ . On this occasion measurements were made by allowing the thiol esters to react with hydroxylamine



The hydroxamic acid was treated with FeCl_3 and the absorption at 480 - 540 μ read.

APPENDIX B

TABLE (i)

The amino acid composition of the porphyrin-peptide isolation by isoelectric precipitation and then purified further by passage through an Amberlite CA 50 column with 0.125 M ammonium acid. The porphyrin-peptide was subjected to performic acid treatment before hydrolysis in order to preserve the cystine and cysteine residues as cysteic acid.

Asp	3.5
Thr	1.3
Ser	2.2
Pro	3.7
Glu	3.7
Gly	3.6
Ala	1.7
Val	1.3
Ileu	0.9
Leu	1.4
Phe	0.4
Lys	2.2
His	Trace
Arg	Trace

TABLE (ii)

ABSORPTION SPECTRA OF HYDROPHILIC PORPHYRINS
REPORTED BY OTHER AUTHORS.

<u>SOURCE</u>	<u>SORET</u>	IV	III	II	I	<u>AUTHOR</u>
Bile	407					Smith (1968)
	406					"
	405					"
	403					"
Urine of VP (ester in 0.05 HCl)			550	590		Rimington (1966)
(ester in CHCl ₃)	404	502.4	536.3	569.8	623.1	"
Faeces (SP) (ester in CHCl ₃)		498.7	531.0	565.3	619.9	MacGregor (1952)
		500.0	532.5	566.4	621.6	"
		508.1	551.5	576.0	621.0	640
Urine (VP) (ester in CHCl ₃)		503.0	536.6	573.2	623.5	Gray (1948)
Faeces (VP)(ester in CHCl ₃)	406	503.5	53.8	572.0	623.5	Rimington (1968)
	(free in Borate Buffer)	399	498.0		560.0	615.0

TABLE (iii)

SORET MAXIMA (λ) AND MILLIMOLAR EXTINCTION
COEFFICIENTS (E_{mM}) FOR PORPHYRIN METHYL ESTERS
IN CHLOROFORM (DOWDLE ET AL (1970)).

		E_{mM}
Uroporphyrin	406	216
Hepta-carboxylic porphyrin	405	206
Hexa- " "	404	203
Penta- " "	403	198
Coproporphyrin	400	181

TABLE (iv)

HCl NUMBERS OF PORPHYRINS (FISCHER & ORTH 1937)

	<u>Free Porphyrin</u>	<u>Porphyrim Methyl Ester</u>
Protoporphyrin	2.5	5.5
Deuteroporphyrin	0.3	2.0
Mesoporphyrin	0.5	2.5
Aetioporphyrin	3.0	
Haematoporphyrin	0.1	
Coproporphyrin	0.09	1.7
Uroporphyrin		5.0

STATISTICAL METHODS

A. Calculation of the mean (\bar{x})

$$\bar{x} = \frac{\sum x}{n}$$

n = number of samples

$\sum x$ = sum of all the observations

B. Calculation of the standard deviation (S.D.)

$$S.D. = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}}$$

68% of the population lie on either side of the mean within 1 standard deviation, and 95% lie within 2 S.D.

C. Calculation of the standard error (S.E.)

$$S.E. = \frac{S.D.}{\sqrt{n}}$$

D. Calculation of the Mann-Whitney U test, was used to test whether 2 independent groups had been drawn from the same population. This is a nonparametric test and was used as an alternative to the parametric 't' test.

The observations of the 2 groups have been combined and ranked in order of increasing size.

n_1 = number of cases in the smaller group

n_2 = number of cases in the larger group

The value of U is given by the number of times a value in the group with n_2 cases precedes a score in the group with n_1 cases in the ranking.

When $n_2 > 20$ the significance of U was determined by Z .

$$Z = \frac{U - \frac{n_1 n_2}{2}}{\sqrt{\frac{(n_1)(n_2)(n_1 + n_2 + 1)}{12}}}$$

or if ties exist between the 2 groups

$$Z = \frac{U - \frac{n_1 n_2}{2}}{\sqrt{\left(\frac{n_1 n_2}{N(N-1)}\right) \left(\frac{N^3 - N}{12} - \sum T\right)}}$$

where $T = \frac{t^3 - t}{12}$ and $N = n_1 + n_2$

t = the number of observations tied for a given rank

$\sum T$ = sum of T's over all groups of tied observations.

The p value (the probability that the 2 groups are drawn from the same population) was read from tables using the Z value.

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