"THE PORPHYRIAS IN SOUTH AFRICA"

A Study of the Excreted Porphyrins.

by

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This thesis is dedicated

to my wife Pat

who is responsible for many

of the illustrations.

INDEX.

PREFACE.

Study of the diseases of porphyrin metabolism has formed a major part of the work of the C.S.I.R.-U.C.T. Renal Metabolic Research Group since 1959. The scope of the activities of this Group has been wide; this Thesis concerns but one aspect, the excreted porphyrins. This is essentially a laboratory study but it is a study that would have lost much of its significance had it not been closely linked to the clinical studies of other members of the group, in particular Prof. L. Eales.

The unit has formed a stimulating environement in which to pursue these investigations and it is a pleasure to acknowledge the contributions to this thesis of all members of the group.

Acknowledgements.

It is a pleasure to acknowledge the helpful criticism and advice received from Prof. L. Eales during the course of the studies recorded here.

I am indebted to the Director of Veterinary Services, Onderstepoort, for access to the sole remaining member of the herd of cattle with congenital erythropoietic porphyria originally studied by Rimington and Fourie. Dr. Anderson, of the Rietfontein Laboratories, S.A.I.M.R. kindly supplied large volumes of ultrafiltrate from C.diphtheriae cultures.

I am indebted to Dr. S. F. MacDonald of Toronto for a generous gift of URO's II and IV and COPRO's I and III from his collection of synthetic porphyrins.

The first counter-current analyses were performed at the National Chemical Research Laboratories of the C.S.I.R. in the Department of Dr. P. Enslin, whose help in this regard is acknowledged; I am also grateful to Dr. Enslin for the loan of 220 Craig counter-current cells. Dr. C. Garbers of the Dept. of Organic Chemistry, University of Stellenbosch, kindly allowed me to borrow a 35 tube counter-current apparatus for some months.

Prof. Holliman, lately of the Dept. of Organic Chemistry (UCT) kindly allowed me to prepare KCl discs for infra-red spectroscopy and permitted these spectra to be recorded in his Department.

Mrs. M. Levey was responsible for the quantitative estimations of faecal and urine porphyrins.

Mr. Todt of the Dept. of Clinical Photography kindly helped with the many photographs.

I am grateful to Mrs. M. Birkenstock who coped so admirably with the task of typing this thesis.

The following abbreviations have been used:

AIP	Acute Intermittent Porphyria.
AIA	Allyl-isopropyl acetamide.
ALA	Delta-amino levulinic acid.
CCD	Counter-current Distribution.
COPRO	Coproporphyrin.
COPROGEN	Coproporphyrinogen
DDC	Dihydro-dicarbethoxy-collidine.
DEUTERO	Deuteroporphyrin.
d.w.	Dry weight.
DPN/DPNH	the oxidised and reduced forms of Co I or nicotine adenine diphosphonucleotide.
IR	Infra-red.
HAEMATO	Haematoporphyrin.
HCB	Hexachlorobenzene.
MESO	Mesoporphyrin.
PBG	Porphobilinogen.
PROTO	Protoporphyrin.
PSEUDOURO	Pseudouroporphyrin
PCT	Porphyria cutanea tarda.
URO	Uroporphyrin
UROGEN	Uroporphyrinogen.
UV	Ultra-violet.

CHAPTER I.

1. PORPHYRIA IN SOUTH AFRICA - an historical review.

In 1939 the first case of porphyria was described in South Africa (1, 2); by 1961 it had come to be realised that South Africa has probably the highest incidence in the world of the porphyrin diseases. Little progress was made over the war years, but over the short space of the last 15 years the status of these diseases has altered. They are no longer rare, never-diagnosed metabolic disorders hardly accorded space in text books, but constitute almost a national problem.

The increased use of all manner of drugs over the past 20 years may be responsible in part for a rise in the frequency of expression of the inherited form of the disorder, but this form is commonest in descendants of the trek-boers and their migration into urban areas where medical diagnostic facilities exist is a fairly recent phenomenon. The increase of porphyria in Africans can be attributed to various factors associated with the growth of industry in the towns. Despite the foregoing, the increase in apparent incidence of the porphyrias in South Africa is probably due mostly to increased awareness on the part of the medical profession. This follows largely the published works of two men, Dr's Dean and Barnes.

Barnes (3) published his first paper concerning porphyria in South Africa in 1945. His work, later performed in association with Dean, is responsible for much of our present knowledge of the porphyrin diseases in South Africa. In this early paper eleven cases are reported; the possibility of a familial trait is hinted at, as is the danger associated with the administration of barbiturates.

In his second paper Barnes (4) noted porphyrinuria in both European and African patients. Attempts were made to study the porphyrin chromatographically, but for reasons which are not clear, this did not seem very helpful. No striking differences were found between the porphyrinuria seen in Europeans and in Africans.

In 1953 Dean (5) commented upon the high incidence of porphyria in South Africa, and stressed the familial nature of the disorder. Cases of acute porphyria were described. Dean makes early reference in this paper to his renowned studies on the inheritance of porphyria in South Africa. As is well known, it was the frequency with which he had encountered acute porphyria in this country which prompted these studies.

Chemical studies at this stage were confined to the examination of urines for PBG (Watson-Schwartz test) and porphyrins. Urines were studied spectroscopically by Barnes. It was realised that, except during an acute attack, the urine could be normal spectroscopically and by the Watson-Schwartz test. It was realised also, that even when the urine is normal, the faeces should be examined.

Dean had observed the varied manifestation of porphyria in families he had studied. He concluded that attempts to classify porphyria on clinical grounds into cutanea tarda, acute and latent forms were artificial, not realising that he was dealing with an entity poorly documented in the world literature.

In 1955, the family studies to which Dean had referred briefly in his previous paper, were published. This paper, "The Inheritance of Porphyria" (6) represents the most extensive genetic survey of any condition, and is a milestone in the researches into porphyria in this country.

The biochemical investigations were extremely simple and need not be stressed. Urine was examined spectroscopically after acidification, to detect abnormal concentrations of porphyrin. The faeces were

examined if urine was negative on 2 occasions. The study showed clearly (1) that a very large number of porphyric patients in this country were descended from a single ancestor (2) that in a single family acute porphyria, chronic cutaneous porphyria and latent porphyria could co-exist.

In the same year Barnes (7) indicated that a form of porphyria was also common in Africans on the Witwatersrand. Porphyrin in excess of normal (i.e. detectable in acidified urine using a hand spectroscope) was found in 300 urines. Skin lesions were found in one third of the patients where urines had contained excess porphyrin. (Excess.) Urobilinogen was found in 49 of the 300 urines. Some of the patients had hepatomegaly. A possible aetiological role was ascribed to alcohol.

Barnes (8) published in 1958 results of a further study of porphyrin and porphyrin precursor excretion in 15 African patients using quantitative techniques. He noted that in all instances faecal porphyrin was less than 150 µg/g d.w. (Holti method) and the urine uroporphyrin excretion was markedly elevated in most cases. He noted that ALA excretion exceeded 10 mg/L

in four of the cases studied. In this paper, Barnes recognised Swedish and South African inherited porphyria, as well as what he termed Bantu porphyria, as separate entities. He rejected the idea that these conditions could be caused by the same metabolic lesion.

At this time it was realised that the South African and Swedish forms of inherited porphyria were similar in some respects but differed in others, notably in the occurrence of skin lesions in the South African form. To decide whether the differences were environemental or due to different inherited genes, Dean travelled to Sweden and Holland, studying cases of porphyria and sending specimens to Barnes for analysis. Dean and Barnes (9) compared clinical and biochemical findings in cases of acute and cutaneous porphyria encountered in Holland and Sweden, and showed that examples of porphyria in every way comparable with South African cases, occurred in Holland where cases identical to the Swedish ones could also be found.

Eales (10) showed that what had previously been referred to as Bantu porphyria did, in fact, occur in all three racial groups seen in Cape Town, Whites, Africans and people of mixed descent. He showed that

the nature of the cutaneous involvement was no clue to biochemical differences in the cases studied, but that two distinct patterns of porphyrin excretion were associated with cutaneous porphyria.

Barnes (11) had similarly observed that faecal porphyrin, in particular the ratio of COPRO to PROTO in the faeces, is a valuable index to distinguish inherited porphyria from the African variety. In this publication he compared faecal porphyrin excretion by 27 White and 19 African porphyric patients. While PROTO concentration in the White cases ranged from 103 - 1280 µg (mean 487), in the African cases the range was 11 - 134 (mean 45). COPRO concentrations in white cases ranged from 23 - 800 (mean 242), but in African cases from 6 - 161 (mean 58).

The foregoing paragraphs indicate that two forms of porphyria are prevalent in South Africa;

(a) a form in which dominant inheritance can be demonstrated, which is most common in white people but occurs also in persons of mixed descent, and

(b) a form seen in all racial groups, but more commonly in Africans and persons of mixed descent.

Other essential differences between these conditions may be summarised in Table 1-1.

Table 1 - 1.

	Inherited.	Acquired.*
Clinical Presentation	None. or Cutaneous Porphyria or Acute Porphyria. or Combinations.	None. or Cutaneous Porphyria.
Urine Porphyrin.	Variable.	Always raised.
Faecal Porphyrin.	Always raised.	Variable
ALA, PBG in the urine.	Raised in acute attacks.	Normal or only slightly raised.
Associated disease.	None.	Often evidence of impaired liver function.

* This appeared to be the most suitable term to use in this table, although it was not in general use in 1960.

There are reports in the South African literature of varieties of porphyria which clearly differ from those detailed above. Congenital erythropoietic porphyria has been described, (12) and there is an interesting case report of a white child of 5 years who had cutaneous porphyria from the age of 6 months. Urine

URO and COPRO was increased but faecal COPRO and PROTO were very high. Fluorocytes were not found in the bone marrow, but unfortunately free erthrocyte porphyrin was not estimated. The case resembles in some respects, that described by Porter and Lowe (14). Lest the problem appear to have been oversimplified, mention should be made of reports (10, 46) of acute porphyria occurring in apparently pure blooded Africans. Such instances are, however, very rare and open to various interpretations.

2. STATEMENT OF THE PROBLEM.

Analyses of excreted porphyrins referred to above have been by simple techniques. Initially urines were examined with a hand spectroscope, and stools and urines screened for excessive porphyrin content by observing fluorescence in UV light. These techniques throw no light on the nature of the porphyrins excreted in increased amounts. The introduction in 1956 of a method for the routine estimation of uroporphyrin in urine (Dresel et.al.-15) as well as coproporphyrin , and the introduction in 1958 by Holti et.al(16) of a simple method for faecal porphyrin analysis, represented major advances in porphyrin technique. The routine

estimation of URO and COPRO fractions in urine, and COPRO and PROTO fractions in faeces, became feasible in any laboratory equipped with a spectrophotometer operating in the range 380 - 430 mu. Eales, in particular, has made a systematic study of porphyrin excretion in health and disease using these methods. Upper limits of normal excretion have been defined and routine diagnostic problems can be dealt with.

Useful though these techniques may be, they provide only crude separations of the principal porphyrins. The urine porphyrin method only distinguishes ether soluble and ether insoluble/cyclohexanone soluble fractions; the faecal porphyrin method of Holti separates ether soluble porphyrin into a fraction which is extracted from ether by 0.1 N HCl and a fraction extracted by 1.5 N HCl.

On the basis of known physical properties of the porphyrins, it may be reasoned that these methods provide adequate separation of artificial mixtures of URO + COPRO and of COPRO + PROTO. The purity of the fractions obtained from pathological urines and faeces and labelled "URO," "COPRO" and "PROTO" is, however, more dubious.

By 1960 the broad outline of the steps leading to the biosynthesis of protoporphyrin from glycine and succinate had been defined. Although there was (and still is) much to learn regarding the enzymes catalysing the individual steps and of the integration of porphyrin synthesis into cellular metabolism, it had become possible to think of the porphyrin diseases in terms of defects at various stages in haem synthesis. For this reason a detailed study of porphyrin excretion was undertaken.

3. THE AIMS OF WORK TO BE DESCRIBED IN THIS THESIS.

It was considered necessary to identify precisely the principal porphyrins excreted by patients with the various porphyrin disorders encountered. Such a study would include both urine and faecal porphyrins. It was intended that isomer types of isolated porphyrins would be established.

Prior to the work described in this thesis, it had been established that the Swedish and South African forms of inherited porphyria, as well as acquired porphyria (variously referred to in the literature, see Chapter V), differed in the excretion patterns of porphyrins and porphyrin precursors. It was hoped that more precise analytical methods would clarify

these differences, and perhaps throw light on the nature of these disorders at an enzyme level.

During the course of these studies two other problems arose:- The first concerned the excretion of uroporphyrin in the faeces and the second the composition of dicarboxylic porphyrin mixtures in faeces.

There was no fundamental reason for investigating faecal uroporphyrin excretion, but until a method was available for extracting and measuring faecal URO, it was impossible to know whether or not significance should be attached to its occurrence. Watson, (whose technique of analysing faecal uroporphyrin is reviewed in Chapter VI), has stressed the importance of this measurement. (17).

Initial studies of faecal porphyrin showed that all samples contained a variable amount of "non-PROTO" dicarboxylic porphyrin. In patients (Chapter VII) with acquired porphyria, in one of the patients with S.A. genetic porphyria, and in the normal subject GS, these fractions were found to exceed PROTO. Until precisely identified, their significance remained uncertain. The problem was met by the development

of countercurrent distribution (CCD) techniques.

In the introduction to the studies which follow, one final point must be made. This work formed part of the research programme of the Renal Metabolic Research group. It was necessary that the group have at its disposal adequate technique for handling porphyrins, in any given situation. A systematic study of the physical and chemical properties of porphyrins, and of methods used in handling them, was therefore commenced.

CHAPTER II.

PHYSICO-CHEMICAL PROPERTIES OF PORPHYRINS.

1. **Introduction.** The role of the porphyrins in animal biochemistry is to modify the redox-potential of the reaction $Fe^{III} + \epsilon \rightleftharpoons Fe^{II}$ and couple this reaction to protein systems. This role is dependent upon the interesting physico-chemical properties of the porphyrin macrocycle. Discussion of these properties is essential for an understanding of many practical aspects of porphyrin behaviour including solubility, light absorption, fluorescence and acid-base properties.

Were it not for the striking physical characteristics of the porphyrins, their intense colour, vivid fluorescence and photosensitising action, the excessive excretion by humans of a few milligrams daily could have remained long undetected.

Important publications concerning porphyrin chemistry include Fischer's classical work "Die Chemie des Pyrroles" (18), a review by Granick and Gilder (19), the comprehensive treatise on biological aspects of porphyrins by Lemberg and Legge (20), the review by Granick and Mauzerall in 1961 (21), and most recent

of all, reviews by Falk (22) and Phillips (23) in 1963.

2. Nomenclature. Systematic nomenclature regards the porphyrins as derivatives of porphin, the structure of which is shown in Figure (II - 1).

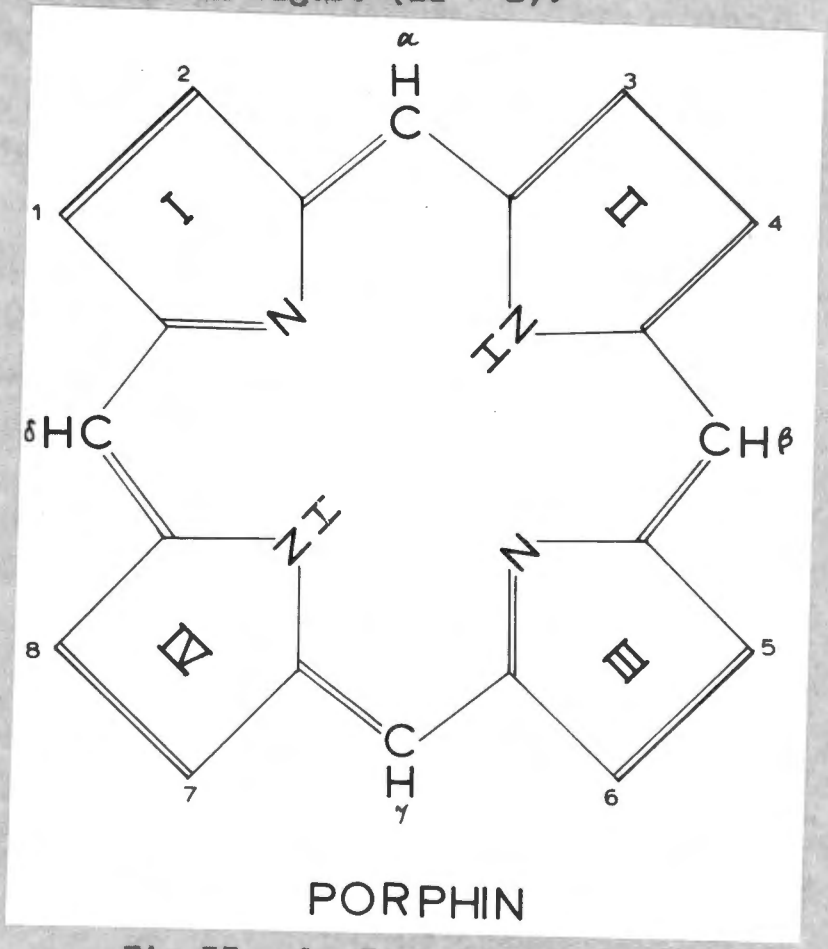


Fig.II - 1. Structure and nomenclature of Porphin. Numbering of the pyrrole rings, the methene carbons, and the eight unsubstituted beta positions is as shown in the figure. The beta positions being unsubstituted in porphin, no isomerisation can occur.

It was suggested at one time that the attachment of two hydrogens to two different ring nitrogens could lead to isomerisation, but this was refuted by Erdman and Corwin (24) who showed that methylation of one pyrrole N produced little change in the absorption spectrum.

Table II - 1.

	1	2	3	4	5	6	7	8
Porphin.	H	H	H	H	H	H	H	H
Aetio I	Me	Et	Me	Et	Me	Et	Me	Et
II	Me	Et	Et	Me	Me	Et	Et	Me
III	Me	Et	Me	Et	Me	Et	Et	Me
IV	Me	Et	Me	Et	Et	Me	Et	Me
Proto IX	Me	Vi	Me	Vi	Me	Pro	Pro	Me
Copro III	Me	Pro	Me	Pro	Me	Pro	Pro	Me
Uro III	Ac	Pro	Ac	Pro	Ac	Pro	Pro	Ac

Me = $-CH_3$ Et = $-CH_2CH_3$ Vi = $-CH{=}CH_2$

Ac = $-CH_2COOH$ Pro = $-CH_2\ CH_2COOH$

For other derivatives of protoporphyrin see III - 1.

Substituting each of the pyrrole rings I - IV with an ethyl and a methyl group leads to four isomeric aetioporphyrins. The four aetioporphyrins (see Table II - 1) were synthesised by Fischer who showed that

protoporphyrin derived from blood yielded aetioporphyrin III on reduction and decarboxylation. He later synthesised the fifteen possible mesoporphyrins and showed that protoporphyrin from haem could be reduced to mesoporphyrin IX in his series with vinyl groups in positions 2 and 4.

3. The electronic configuration of Pi electron molecules.

Current concepts regarding the disposition of the bonding (valency) electrons in the porphyrin molecules provides a theoretical basis for observed physical properties, in particular:-

1. Chemical stability.
2. Dependence of basicity of ring N's on beta substituents.
3. Spectral properties.
4. Fluorescence.
5. Biological function.

Electronic configuration will thus be considered in detail.

Benzene, C_6H_6, is conventionally represented as an hexagonal arrangement of 6 carbon atoms linked by alternating single and double bonds (conjugated double bonds.) This is an unsatisfactory representation because the molecule lacks the reactivity of the ethylenic bond, and in fact, X-ray diffraction studies

show each C to C bond in benzene to be of equal length, whereas true double bonds areshorter than single bonds. The C - C bonds of benzene are of intermediate length.

Two theories attempt to explain these facts. The valency bond method associated particularly with the name of L. Pauling, envisages benzene as an equilibrium mixture of various resonating forms, differing only in the disposition of the 18 electrons involved in C - C bonds, see Figure II - 2(a).

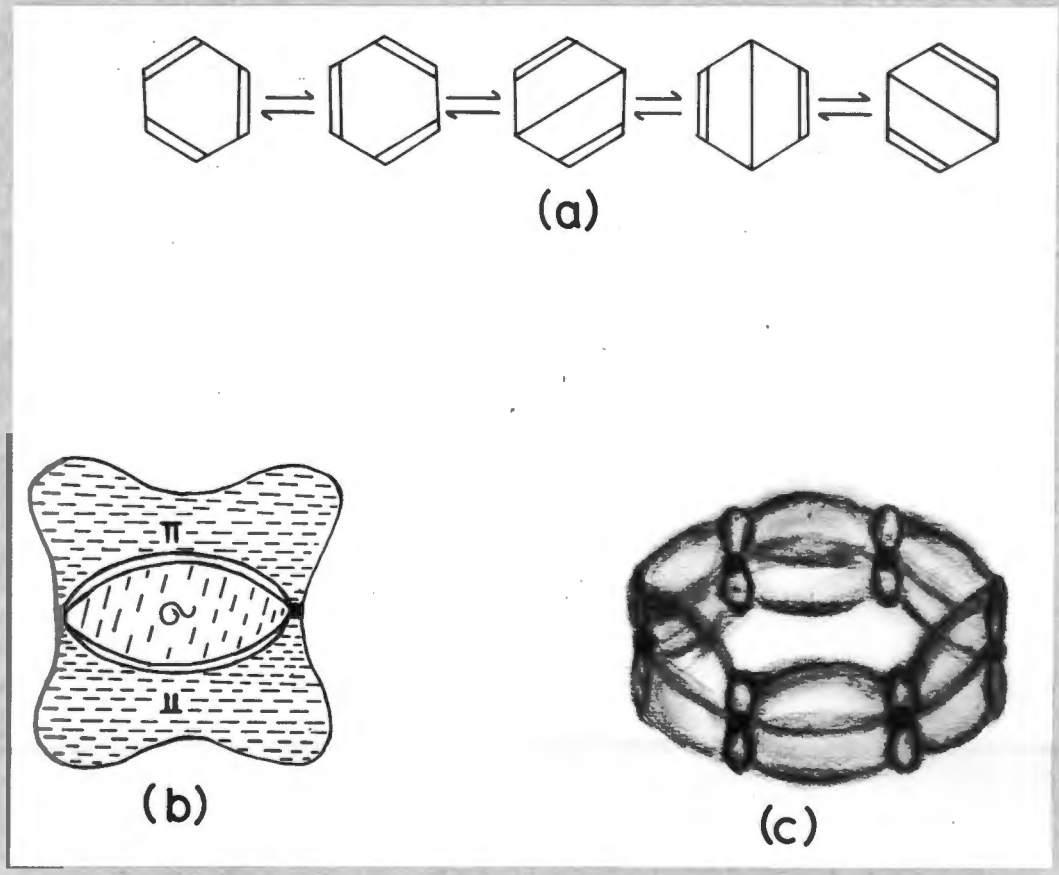


Fig.II - 2(a). See text.

Increased freedom of electron disposition allowed by resonance leads to a lowering of the free energy of the molecule with increased stability (resonance energy).

The Molecular Orbital method of Hund and others (25) is somewhat complex but provides a more dynamic picture. Where two carbon atoms are joined by a single bond, the two bonding electrons occupy an orbital symmetrical about the line joining the two carbon nuclei (sigma bond). A second bond between the two carbon atoms involves a Pi bond formed by the overlap of dumbbell-shaped orbits, normal to the plane of the sigma bond. Figure II - 2(b). Unlike the sigma bond electrons, Pi electrons are not strictly localised to their parent C atoms and in a molecule such as benzene where "single" and "double" bonds alternate, the Pi electron orbitals become confluent, (Figure II - 2(c). The increase in freedom of movement of the Pi electrons is associated with lowered free energy; this "delocalisation energy" is equivalent to resonance energy. A further consequence of Pi bonding is a planar (linear) molecule as this entails greatest overlap of the atomic Pi electron orbitals. By this treatment, (linear

combination of atomic orbitals with maximum overlap or L.C.A.O.) it is possible to deal mathematically with the Pi electrons as if they belonged to the molecule as a whole and not to its constituent atoms (molecular orbital or M.O. method.)

Energy levels of the Pi electron cloud can be quantised and, to some extent, absorption spectra due to electronic transitions justified mathematically. (26) The Pi electrons are free to move about the molecule and can be induced to do so by, for example, a magnetic field, rather like an electric current in a wire. Their relative density at one part of the molecule can be influenced by electron attracting or repelling groups at other parts. The porphyrin macrocycle is an example par excellence, of a Pi electron molecule and although conventionally drawn with 16 conjugated double bonds, it should be visualised as a planar skeleton of C and N atoms joined by single bonds, with clouds of mobile Pi electrons above and below the plane of the C and N atoms. The value of this concept will become clear in subsequent sections.

4. SPECTRAL PROPERTIES.

A theoretical analysis of porphyrin spectra is given by Platt (26). Certain practical aspects are dealt with in review articles already cited. A comprehensive review is that of Phillips (23).

The subject is a complex one, but for the purpose of this thesis only spectra of the "aetio" type and the modifications that result from solution in acid or from metal complex formation need be considered. In neutral and alkaline solvents aetioporphyrins possess four absorption bands in the visible region, and a sharp and very intense band around 400 mμ in the near ultra violet. This last band is named the Sorét band after its discoverer.

The porphyrins derived from haem precursors, and also certain derivatives of protoporphyrin have "aetio" type spectra in neutral solvents. A typical example is the absorption of COPRO ester in chloroform shown in Figure II - 3. The alpha, beta, gamma and delta bands have regularly increasing molar extinction co-efficients. (See appendix A for extinction co-efficients.)

The sharpness of the bands depends upon the dielectric constant of the solvent and in ether the extreme sharpness, particularly of the alpha band, allows rather accurate measurements to be made. Differences in alpha band position, though not large, have practical significance. (See Chapt. VI.)

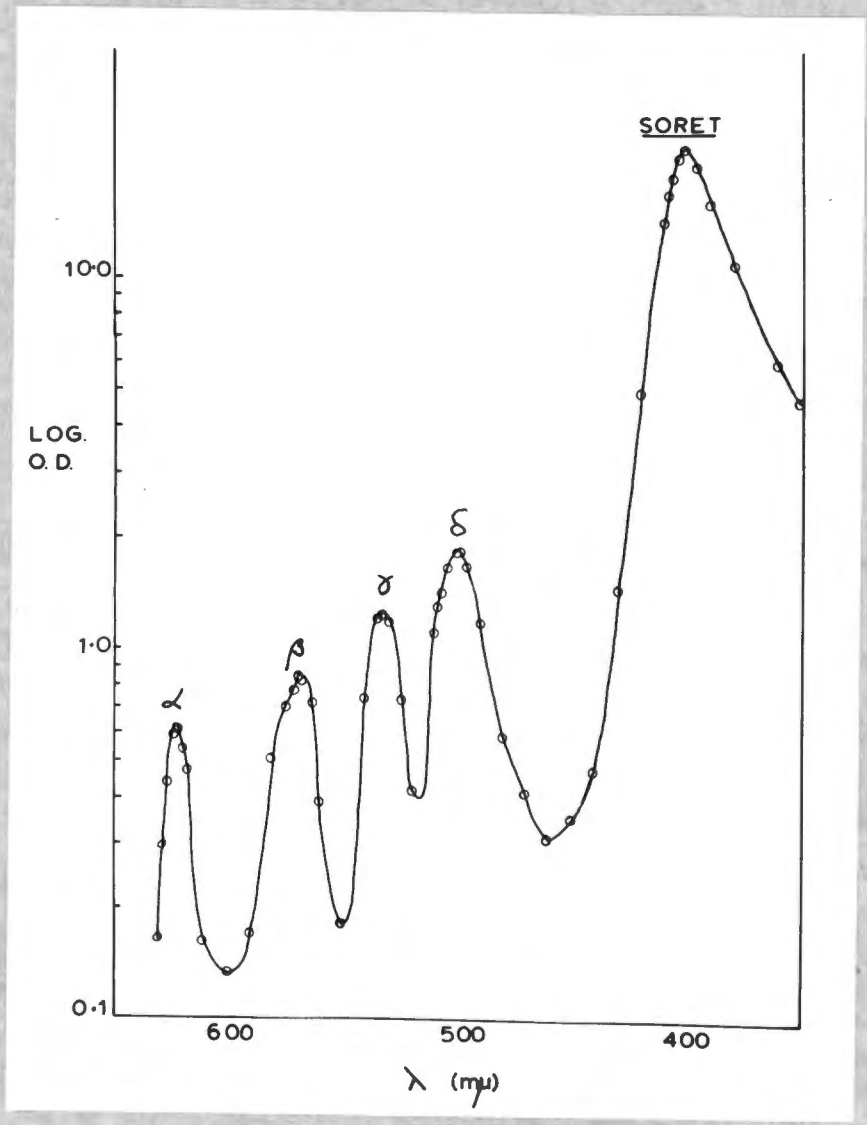


Fig.II - 3. The absorption spectrum of COPRO ester in chloroform.

Beta* substituents alter the position and shape of the porphyrin absorption bands. Aliphatic groups have little effect, and replacement of two H's at positions 2 and 4 in deuteroporphyrin by ethyl groups giving mesoporphyrin causes only a 2 mμ shift in the position of the alpha band. In the case of uroporphyrin, the carboxyl groups of the acetic residues are one methane C removed from the porphyrin macrocycle and each causes only about 1 mμ shift of the alpha band to longer wave-lengths; propionic carboxyl groups have about half this effect (compare mesoporphyrin and coproporphyrin.)

The vinyl groups of protoporphyrin, in contrast, each cause about 4 mμ alpha band shift to the red, and at the same time exert a "rhodofying" effect, increasing the height of the gamma band. The vinyl groups of protoporphyrin are, of course, conjugated to the nucleus.

It would seem that addition of vinyl groups increases the size of the Pi electron cloud, increases the delocalisation energy and lowers the quantum energy

* refers to the α and β carbon atoms of the pyrrole rings. If methene bridge carbons are referred to this will be specifically indicated.

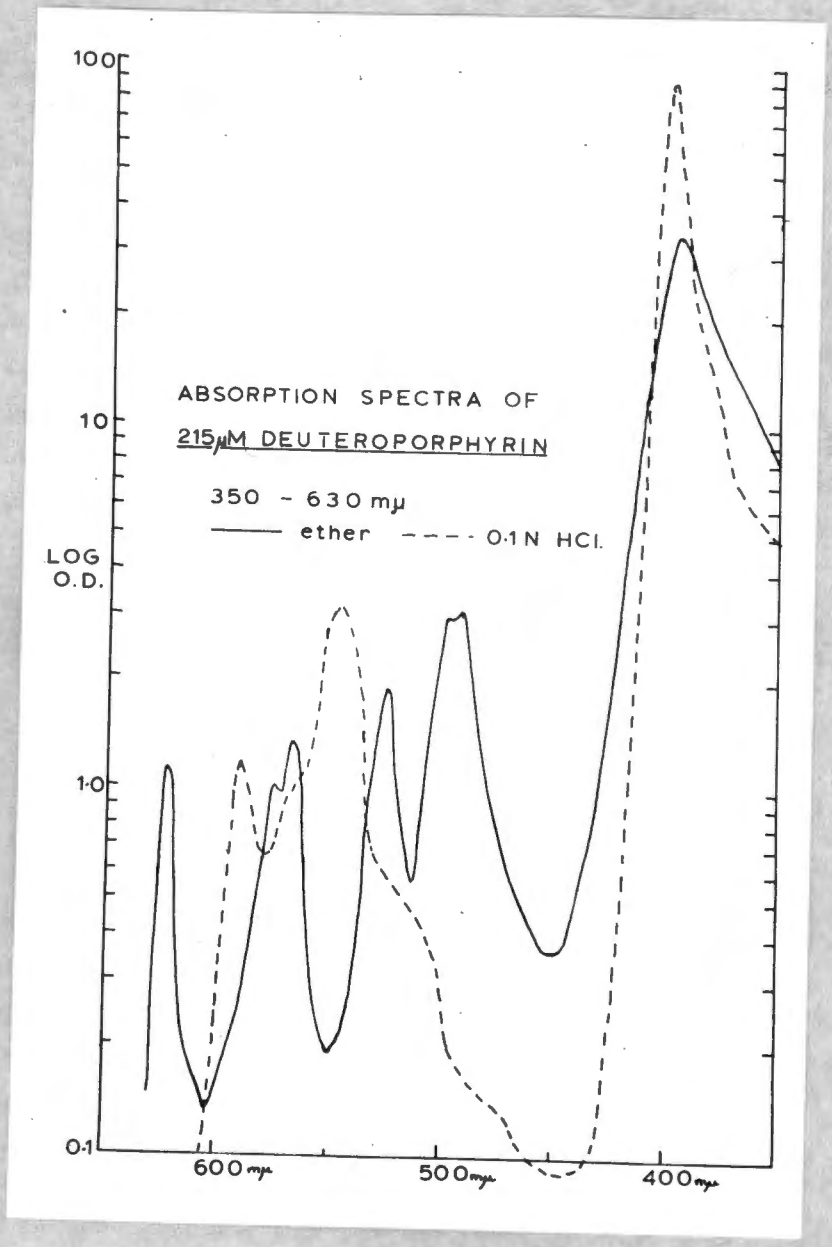


Fig.II - 4.

The absorption spectra of DEUTERO in ether and 0.2 N HCl.

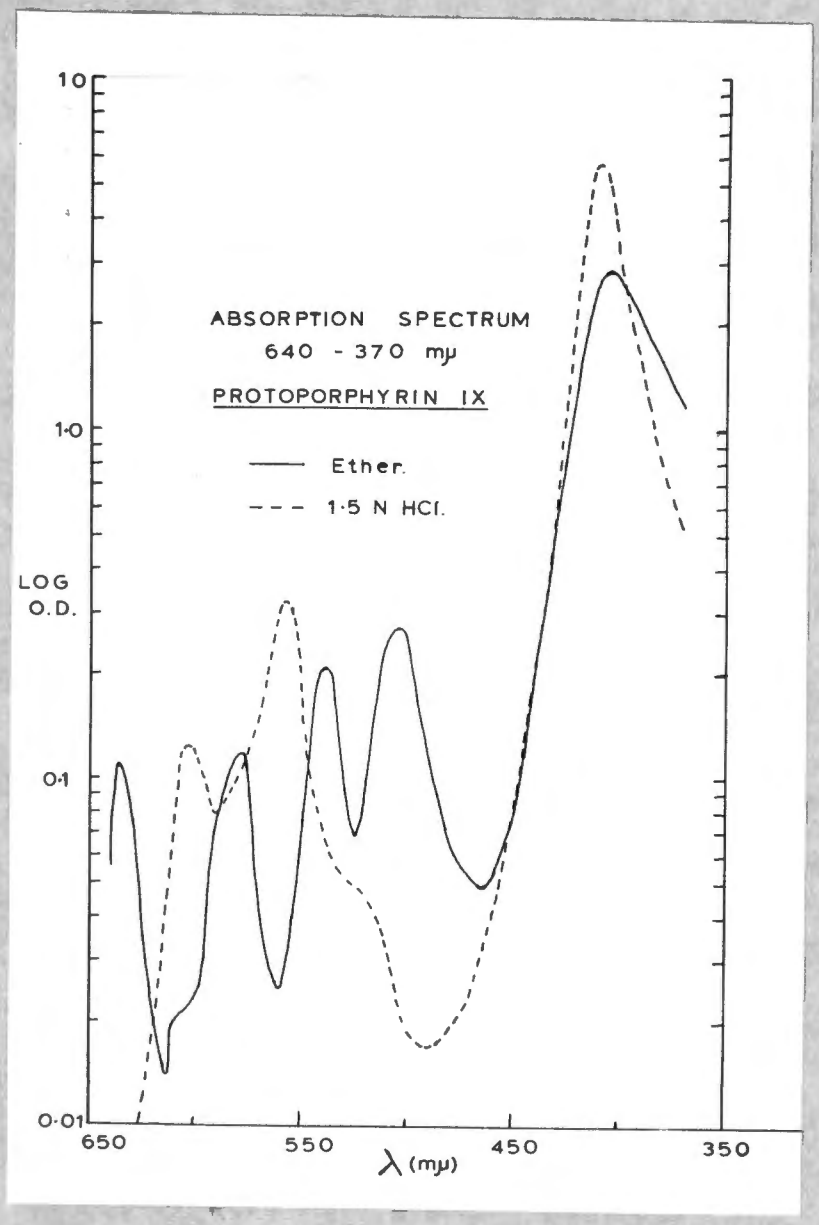


Fig. II - 5.

The absorption spectra of PROTO in ether and 1.5 N HCl.

of electronic transitions, thus causing a spectral shift towards the red. At the same time there is a relative decrease in electron density at the central ring nitrogens (rhodofying side chain are electron-attracting.). A decreased tendency to acquire protons is reflected in an increased HCl number.

In acid solution the four bands of the aetio-type spectrum undergo alteration and the Sorét band is shifted towards the red. Two bands are seen in the visible region with the shorter wave length band having the greater extinction co-efficient. The neutral to acid spectrum shifts in deuteroporphyrin and protoporphyrin are illustrated in Figures II -4,5.

Metal complex formation involves a similar change although the relative heights of the alpha and beta bands in the visible varies with the stability of the particular metal complex.

Oxidation of a methene carbon atom destroys the aetio-type spectrum. Before ring opening occurs an absorption band appears in the region 640 - 660 mu . Opening of the ring leaves a linear tetrapyrrole with a single broad band around 500 mu. Because porphyrin derivatives and degradation products have absorption

spectra different from their parent porphyrins, the ratio of E_{max} : E_{min} and the relative heights of two bands constitute valuable criteria of purity.

5. ACIDIC AND BASIC PROPERTIES.

Transfer of free porphyrin between acid solutions and organic solvents is an indispensable procedure in purification. For this and other reasons, it is essential to consider theoretical aspects of acidic and basic properties.

Porphyrins derived from haem precursors possess variable numbers of carboxyl groups and two basic pyrrolenene nitrogens. The carboxyl groups are hydrophylic at all pH's, though more so when ionised in alkaline solution. The nitrogens are hydrophobic unless quarternary; the pH at which transition to the quarternary state occurs is influenced by β substituents. As mentioned above, electron attracting groups in the beta positions of the pyrrole rings, e.g. vinyl, discourage acquisition of protons by the central ring N's.

All porphyrins are soluble in sufficiently strong HCl by virtue of their pyrrolenene nitrogens. Solubility in this and other acid solutions is influenced

by the number of carboxyl groups. All species possess an iso-electric point or pH of minimum solubility in the range 1.5 - 4.

It was appreciated by early workers that porphyrins could be partially separated by extracting ethereal solutions of a mixture with increasing concentrations of HCl. This led to the definition of a specific property of porphyrins, the HCl number:-

"That concentration in percent of HCl which will extract $^{2}/_{3}$ of the porphyrin from an equal volume of its solution in ether."

A table of HCl numbers of some porphyrins is given in appendix B.

The reaction between porphyrin and acid may be represented as below if it is assumed that solutions are sufficiently dilute for activity co-efficients to approximate to unity :

$$P + 2H^+ \rightleftharpoons PH_2^{2+}$$

By the law of mass action:

$$\frac{(P).(H^+)^2}{(PH_2^{2+})} = k$$

$$\frac{(P)}{(PH_2^{2+})} = \frac{k}{(H^+)^2}$$

and $$\frac{(PH_2^{2+})}{(P)} = k'(H^+)^2$$

Further, if it is assumed that PH_2^{2+} is insoluble in ether and P insoluble in acid, the partition of a given porphyrin species between ether and HCl may be written:

$$\frac{\text{Porphyrin in acid}}{\text{Porphyrin in ether}} = k'(H^+)^2$$

These assumptions may be tested by showing that a plot of

$$\log (PH_2^{2+}) - \log (P) = k'' + 2 \log (H^+)$$

is linear. This is found to be approximately so.

The distribution coefficient of porphyrin between aqueous HCl and ether is thus related to the square of the hydrogen ion concentrations; it is around

unity only over a small range of acid concentration and it is this range which should be selected for purification of a porphyrin by CCD. Further, if an ethereal solution of porphyrin is extracted with HCl, the concentration of which is increased stepwise, the change from "inefficient" to "efficient" extraction will be abrupt.

The effect of the hydrophilic carboxyl groups on solubility in aqueous media can be largely cancelled by esterification. For example, free coproporphyrin has an HCl number of 0.08, but the HCl number of the tetra methyl ester is 1.5. Haematoporphyrin free acid has an HCl number of 0.1, but the dimethyl ester, 0.8. Lesser change in HCl number results when haematoporphyrin is esterified because the lower HCl number is determined in part by the hydroxyethyl side chains at positions 2 and 4, which are only masked if the dimethyl ether derivative is formed.

6. FLUORESCENCE.

Energy is absorbed by molecules in discrete "packets" or quanta; the energy of a quantum is a

function of its wavelength. For energy to be absorbed by a molecule, some change must be induced requiring exactly the amount of energy supplied by the quantum. This may be an alteration in vibration or rotation of components of molecular structure, or excitation of electrons, or both.

The absorption bands of porphyrins in the regions 350 - 650 mu represent those regions where the probability of energy being absorbed is high. When absorption occurs in these regions, transitions occur in the energy levels of the Pi electrons resulting in an excited state of the molecule. The fate of absorbed radiant energy is dealt with by Lumry and Eyring (47). The topic is a complex one but it seems that the following may occur:-

1. The energy may be dissipated in small steps into vibration and rotation movements and appear as heat.
2. By direct interaction with the electron clouds of neighbouring molecules, the energy absorbed by one molecule may be transferred to another.
3. The molecule may decay from its excited state to ground state or near ground state with emission of a quantum of radiant energy. This is the physical basis of fluorescence.

Fluorescence is a phenomenon typically associated with Pi electron molecules because in the loosely held electron cloud of these molecules it is possible for an excited state to persist long enough for fluorescence to occur. The persistence of this excited state is interfered with by factors which facilitate (1) and (2) above. Thus neighbouring molecules cause quenching of fluorescence by interaction with the excited molecule at an electronic level before fluorescence can occur; heat also decreases fluorescence by increasing random rotation and vibration movements.

The fluorescence of porphyrins is a striking and beautiful attribute, and red fluorescence being relatively uncommon, allows minute quantities to be detected in any situation where porphyrin is in solution. Solid porphyrin does not fluoresce. 0.1 ug/cc is easily detected by the naked eye when fluorescence is activated by filtered UV light from a low pressure Hg arc. A fluorimeter equipped with a photomultiplier tube can detect 10^{-4} ug/cc.

Bands of fluorescence emission correspond to those of light absorption but the intensities are inversely related, thus the most intense fluorescent

band in neutral solution corresponds to the weakest absorption band.

7. SOLUBILITY.

Knowledge of the solubility properties is required for handling porphyrin in the laboratory. Certain aspects of solubility could be dealt with from a theoretical view-point, but only generalisations based on empirical observations will be recorded here.

A. Comparison of the solubilities of esters and free acids. Esterification results in the loss of ionisable side chains. Decreased basicity of the N's is reflected in increased HCl numbers. The esters are insoluble in strong bases which do dissolve free porphyrin acids, but have much greater solubility in relatively non-polar organic solvents, e.g. chloroform, carbon tetrachloride, carbon disulphide and ethyl acetate.

B. Solubility of Free Porphyrin Acids. Free porphyrins are very soluble in strong acids with the solubility in HCl > HCOOH > CH_3COOH. This property reflects the basicity of the ring nitrogens and proto-porphyrin with fairly high HCl number of 1.8, is relatively insoluble in glacial acetic acid. The

normality of HCl required to dissolve free porphyrin in the dry state is a function of the HCl number.

C. Solubility in Alkaline Solutions and Organic Bases.

Aqueous sodium and potassium hydroxide dissolve free porphyrin from the dry state and extract porphyrin from solution in ether. Salts form with the carboxylic acid group and these are relatively insoluble, although readily dissociated at neutral and acid pH. Free porphyrins are soluble in aqueous ammonia (25% W/W - about 13N) and the solubility appears to be a function of the number of carboxyl groups with the solubility of URO > COPRO > PROTO. This order is reversed for pyridine which is an excellent solvent for porphyrins with 2, 3 and 4 carboxyl groups.

Tetrahydrofuran (THF) is a weaker base than pyridine. Uroporphyrin is scarcely soluble in THF, coproporphyrin intermediately so, but dicarboxylic porphyrin very soluble. THF is a useful solvent, being a weak base and yet able to bring free porphyrin into solution from the dry state (see Countercurrent Distribution Chapt.VI-13) Free porphyrins and their metalloporphyrin complexes have similar solubilities in basic solvents.

D. Solubility in Neutral Organic Solvents. Free porphyrins are insoluble in non-polar organic solvents, and slightly soluble in ether, ethyl acetate and acetone. These solvents do not dissolve porphyrin in the dry state unless acidified, e.g. ether/acetic acid, ethyl acetate/acetic acid and acetone/HCl mixtures.

Certain alcohols are fairly good solvents. The water miscible alcohols are little used but n-butanol will extract any free porphyrin from aqueous phase at its iso-electric point.

Of the free porphyrins, uroporphyrin I is least soluble in organic solvents. Insoluble in ether, it is only sparingly soluble in ethyl acetate, but fairly soluble in ethyl acetate and n-butanol mixed in equal parts and also in cyclohexanone.

E. Solubility of Esters. The solubility of porphyrin esters decreases with increasing numbers of carboxyl groups and with decreasing polarity of the solvent, thus PROTO > COPRO > URO and, if solvents are listed in terms of their polarity, hexane, benzene, ether, ethyl acetate chloroform, methanol, acetone, water, it is found that the solubility of esters is maximal in chloroform but decreases with both increasing

and decreasing polarity. Substantial differences exist between the solubilities of the various isomers. Isomers I and II are less soluble and II and IV more soluble. These differences are used in purification by fractional crystallisation.

CHAPTER III.

CHEMISTRY OF THE PORPHYRINS.

The organic chemistry of the porphyrins is dealt with in Fischer's classic work "Die Chemie des Pyrroles" (18) for which there is no substitute in English. Falk's review (22) covers briefly certain theoretical and practical aspects. A valuable guide to laboratory technique is that of Schwartz et.al. (27). Only those chemical reactions relevant to analytical technique will be discussed here. Practical details are given in Chapt. VI.

1. REACTIONS OF THE SIDE CHAINS.

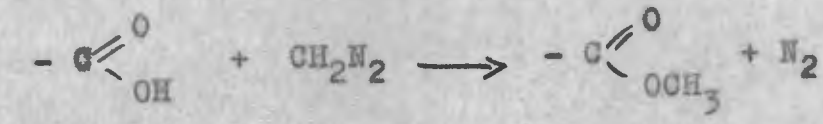
(A) Esterification.

Esterification of the carboxyl side chains is often made use of. The esters are more stable, they can be purified by column chromatography, and are very soluble in $CHCl_3$ and ethyl acetate. When pure, the esters form crystalline compounds with sharp melting points. Methyl esters are usually made, but ethyl esters of uroporphyrins have been prepared on account of their lower m.p. (28). Equilibrium in the reaction

$$-C(=O)OH + CH_3OH \rightleftharpoons -C(=O)O.CH_3 + H_2O$$

is driven to the right by strong mineral acid.

Diazomethane is also used:



(B) Hydrolysis and Saponification.

Porphyrin esters hydrolyse in aqueous mineral acid at room temperature. Saponification takes place if the ester is treated with methanolic KOH; potassium salts of the carboxyl groups form and may precipitate although they are more soluble than the sodium salts. Treatment of the salt with dilute acid will liberate free porphyrin. Saponification may be preferable to hydrolysis with strong acid if it is desireable to protect vinyl groups from hydroxy-ethylation.

(C) Decarboxylation.

If porphyrin with acetic side chains is heated at 180°C in 1 M HCl for 4 hours, decarboxylation to methyl groups occurs (29). Yields approach 100% if oxygen is excluded. The kinetics of the reaction, the effect of varying the normality of the acid, the temperature and the reaction time, have been investigated by Chu and Chu (30). Increasing acid concentration enhances stability of the COOH groups. Heating at lower

temperatures and/or for shorter times, increases the yield of porphyrins with 7, 6 and 5 (COOH) groups.

(D) Reactions of the Vinyl groups of Protoporphyrin.

(i) Hydroxyethylation.

In strong mineral acid the vinyl group reacts thus:

$$-CH=CH_2 + HOH \qquad -\overset{*}{C}H(OH)CH_3$$

the possibility of optical isomerisation should be noted; the asymmetric C atom is marked with an asterisk. The reaction is presumably a random one and a solution of protoporphyrin in acid will become an equilibrium mixture of divinyl, monovinyl-monohydroxy ethyl and dihydroxyethyl deuteroporphyrins.

The vinyl group will similarly add HBr and the adduct may be hydrolysed to haematoporphyrin.

$$-CH=CH_2 \xrightarrow[HBr/HAc]{} -CHBrCH_3 \xrightarrow{H_2O/NaOH} -CH(OH)CH_3 + HBr.$$

Haematoporphyrin produced in this way is optically inactive; chromatography of the ester on cellulose (31), has yielded 4 fractions which represent the expected number of optical isomers.† It is interesting that haematoporphyrin formed by cleaving the thio-ether

† The assumption has been made that the addition of H_2O or HBr to the vinyl groups obeys Markownikoff's Rule. This point does not seem to have been investigated but it is very likely that this assumption is valid.

bonds of cysteine to the porphyrin moiety of cytochrome c using silver salts, yield optically active haematoporphyrin (32).

Haematoporphyrin will lose 2 molecules of water to give protoporphyrin if heated in vacuo.

The hydroxyl groups of haematoporphyrin may be replaced by methoxy groups by refluxing with methanol-sulphuric acid. The propionic groups are simultaneously esterified and the dimethyl ester-dimethyl ether results.

(ii) Resorcinol Fusion.

Haemin fused with resorcinol at 200°C loses two vinyl groups to form deuterohaemin. Removal of Fe^{III} after reduction to Fe^{II} gives deuteroporphyrin. Haemin is used, rather than protoporphyrin, for resorcinol fusion because the side chains have greater reactivity in the ferriporphyrin complex than in the free porphyrin.

(iii) Reduction.

The vinyl groups of protoporphyrin may be reduced with hydriodic acid (33), or with hydrogen and a palladium catalyst (34) to yield mesoporphyrin. Granick reported an overall yield of 70% for the latter reaction conducted on a micro scale. Muir and Neuberger (35) on the other hand, suggest that conventional Pd catalysts are ineffective and recommend using Pd suspended in methyl methacrylate.

2. REACTIONS OF THE RING-NITROGENS.

(A) Formation of metal complexes.

Details of this reaction are considered by Phillips (23). The classical view was that two dissociable hydrogen atoms attached to the ring nitrogens were replaced by metal forming ionic bonds; the two remaining nitrogens contributed electron pairs to co-ordinate bonds. In fact, the bonding of the metal is the same to each nitrogen.

The nitrogen electron pairs are part of the π - electron cloud in the free porphyrin. Co-ordination of a metal therefore, alters the disposition of electrons and causes profound changes in absorption spectra.

Metals may be introduced into the porphyrin ring by heating the porphyrin and the acetate of the metal in its correct valency state in a suitable solvent, usually glacial acetic acid.

(B) The Removal of Metals from Metalloporphyrins.

The available methods are

(i) treatment with mineral acid

(ii) reduction with sodium amalgam and reoxidation

(iii) addition of $LiAlH_4$ to a solution of porphyrin in ethylene diamine. (36).

(i) above is the method most commonly used; the strength of acid required will depend upon the nature of the complex. Uroporphyrin Zn complex is dissociated in 1.5 N HCl. Coproporphyrin Cu complex requires concentrated sulphuric acid. The same applies to the Cu complex of uroporphyrin, "turacin." The stability of various metal complexes is considered by Oliver and Rawlinson (1951).

A special problem is the de-ironing of haems. Fe^{III} forms an exceptionally stable complex and before the free porphyrin can be obtained by treatment with acid, iron must be reduced to the ferrous form. The most convenient method is that of Grinstein (38). Haemin is dissolved in methanolic oxalic acid containing ferrous sulphate. Dry HCl gas is passed. The haem Fe^{III} is reduced and Fe^{II} replaced by protons to give proto-porphyrin which is esterified.

3. REACTIONS OF THE MACROCYCLE.

With the exception of deuteroporphyrin, the porphyrins dealt with in this thesis are fully substituted in the beta positions. The only reaction which need be considered here is reduction. The aromatic nature of the porphyrin nucleus makes it resistant to reduction by most common reducing agents, e.g. dithionite, H_2/Pd.

Reduction is possible with H_2/Pt but Na/Hg is most commonly used. (27). A third method is the acidification of an alkaline solution of porphyrin and sodium borohydride. (68). The addition of 6 hydrogen atoms results in complete reduction. All aromatic character is lost, the porphyrinogens are colourless and have no absorption bands in the visible or near UV. Metals are not complexed.

In acid solution porphyrinogens are unstable and dissociate reversibly into dipyrryl-methanes. Reassociation of different dipyrryl-methanes leads to alterations in isomer type. Fischer (loc.cit.) originally showed that mesoporphyrin reduced in hot acetic acid with zinc dust gave a mixture of one, two and three carboxyl porphyrins an re-oxidation. Recently Mauzerall (69) has shown that in hot acid solution UROGEN and COPROGEN rearrange to random mixtures of isomers, $\frac{1}{2}$ III, $\frac{1}{4}$ IV and $\frac{1}{8}$ each of I and II.

Porphyrinogens are unstable in solution but fairly stable in the solid state. The stepwise reduction of porphyrin was investigated by Granick (34). Greatest energy is required for addition of the first 2 hydrogens to produce a chlorin-type compound; this may be further

reduced to the hexahydroporphyrin under milder reducing conditions, e.g. with dithionite.

Porphyrinogens may be quantitatively oxidised to porphyrins with iodine. Other oxidants lead to variable losses. (70).

4. THE BIOSYNTHESIS OF PORPHYRINS.

In 1955 Shemin (39) summarised the state of knowledge at that time describing in detail the experimental work which demonstrated the origin of each carbon and nitrogen atom of protoporphyrin. Our knowledge of the biosynthesis of porphyrins has been placed in historical perspective by Schmid (40); a comprehensive review of biosynthesis is given by Granick and Mauzerall (21). Neuberger (41) stressed certain special aspects of the role of glycine and serine in porphyrin synthesis and Goldberg and Rimington (42) review the subject with the problems of diseases of porphyrin metabolism in mind.

(A) The Synthesis of Delta-Amino Levulinic acid.(ALA)

The enzyme ALA synthetase which catalyses the condensation of glycine and succinyl-CoA is located in

the mitocondria; apart from this enzyme, ALA synthesis requires a functioning citric acid cycle associated with an intact electron transport chain and active oxidative phosphorylation. In vitro ALA is synthesised by ALA synthetase in the presence of glycine, succinyl-CoA, pyridoxyl phosphate and magnesium ions. The evidence indicating the requirement of pyridoxyl phosphate is interesting. Wintrobe (43) observed that Vitamin B6 deficient pigs developed hypochromic anemia with a low erythrocyte protoporphyrin, and Schulman and Richert (44) observed that pyridoxine deficiency led to inefficient haem synthesis from glycine-C^{14} in ducklings. Compounds such as l-cysteine, l-penicillamine, isonicotinic acid hydrazide, cyanide and parachloro-mercuribenzoate (PCMB), which inhibit reactions requiring pyridoxal phosphate as cofactor, also will inhibit ALA formation.

(B) <u>The Shemin Cycle</u>. (45)

ALA can undergo further reactions other than those which lead to haem synthesis. A transamination reaction has been documented (48), which leads to alphaketo-glutaraldehyde which may lose a 1-C fragment

to form succinate. This cycle provides a possible metabolic pathway for glycine, the carboxyl carbon of glycine forming CO_2, and the alpha carbon contributing a 1-C fragment possibly to a labile pool. While there is good evidence that these reactions occur in mammals, their quantitative significance is unknown. The ALA cycle may be one special case of a cycle in which acyl-CoA derivatives condense with glycine with resultant metabolism of the various atoms of glycine and reformation of the acyl moiety as carboxylic acid.

ALA is the first substance formed which is specifically a haem precursor. The ALA synthetase reaction is thermodynamically irreversible and ALA must either condense to PBG or be metabolised by reactions of the Shemin cycle. Until the quantitative significance of this cycle in intact organisms is known it will not be possible to consider the significance of the ALA synthetase reaction as a control step in regulating haem synthesis. The possible importance of ALA synthetase as a control step has been emphasised by Sano and Granick (52).

(C) ALA Dehydrase.

The enzyme which catalyses the Knorr type condensation of two molecules of ALA to one molecule of PBG is situated in the soluble fraction of cells. (54) No special co-factors are required for this reaction, but the enzyme itself has been shown to contain copper (55) and EDTA is strongly inhibitory as are substances which interfere with SH groups, e.g. iodoacetamide and PCMB. The need for copper (50) may account for the hypochromic anemia known to follow copper deficiency. The enzyme is unique in biochemistry in that it catalyses the condensation of 2 molecules of its substrate. Both molecules of ALA are bound to the enzyme although one is more firmly held that the other (56).

(D) Porphobilinogen. (PBG).

PBG will condense to uroporphyrinogen in vitro. The reaction proceeds best in solutions of high concentration; when oxygen is rigorously excluded, yields can approach 100% (57). Under oxidising conditions coloured porphobilins (probably dipyrryl methenes) and

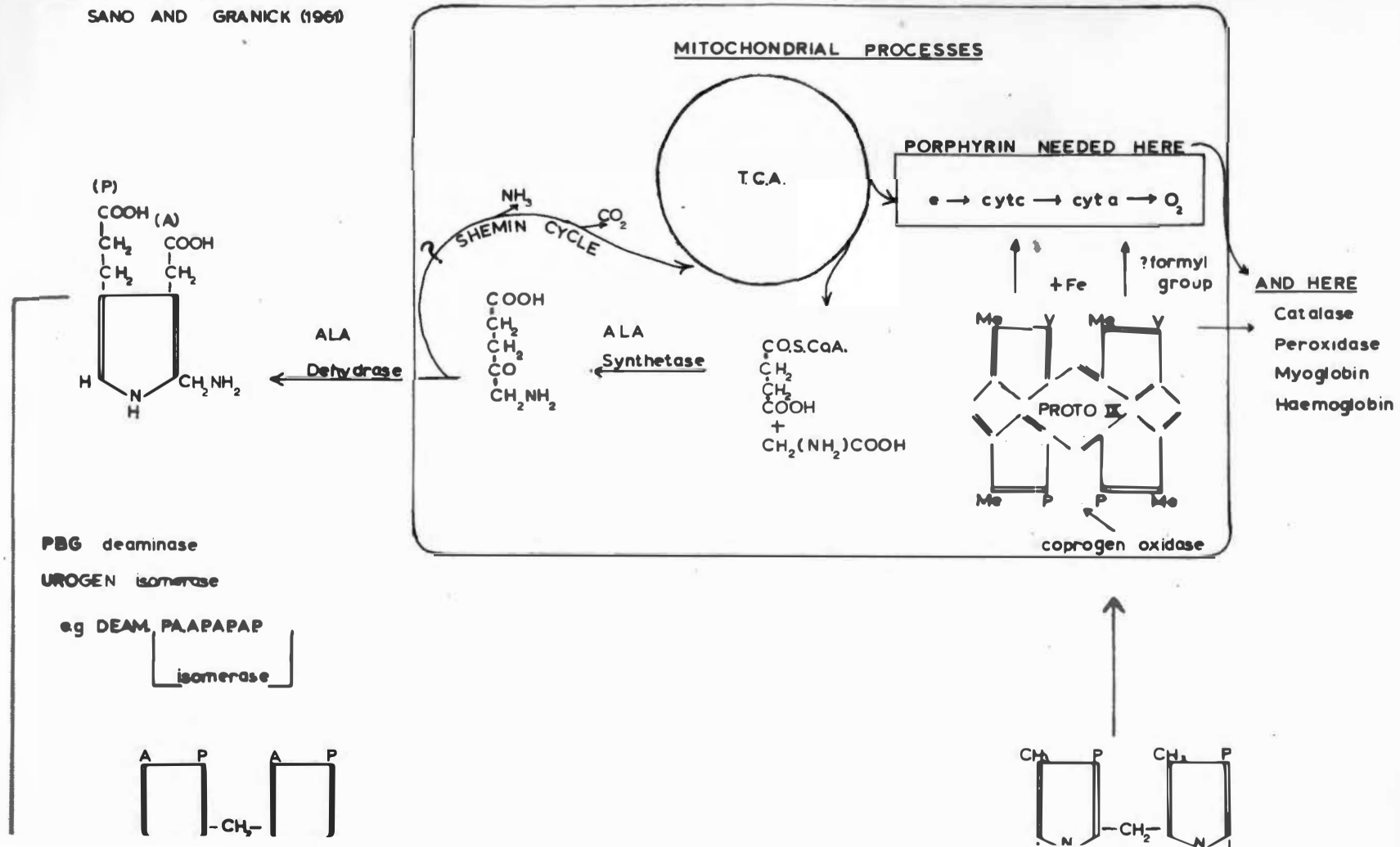
some porphyrins are formed. At physiological pH a large amount of isomer I UROGEN is formed although an exact figure has not been given. On the other hand, under acid conditions a mixture of isomers is formed, one half isomer III, one eighth of isomer II and isomer I, and one quarter of isomer IV, which mixture is that which would be expected from random condensation of PBG molecules to tetrapyrroles (57). In vitro conditions lead only to 8 carboxyl porphyrinogens.

Two enzymes have been described which participate in the condensation of PBG to UROGEN (58). The first, PBG deaminase, leads only to isomer I, the second, a heat labile "isomerase" does not act on PBG or UROGEN I, but if present with the deaminase, will cause PBG to condense to UROGEN III.

Various mechanisms have been advanced to explain these phenomena. Apart from the well documented existence of two enzyme fractions, the following facts must be accounted for.

1. Less than 4.5 moles of PBG form a mole of UROGEN. (No scheme involving "wastage" of PBG is acceptable.)

MODIFIED FROM
SANO AND GRANICK (1961)



2. The isomerising agent acts prior to ring-closure. (The isomerase has no action upon UROGEN.)

3. Free formaldehyde is not formed as postulated in some reaction mechanisms.

PBG shows a strong tendency to condense and form linear polypyrryl methanes. Cyclic tetrapyrryl methanes can form without strain. Tri- and penta-pyrryl methanes are improbable. The mechanism proposed by Bullock et. al. and supported by Rimington (59) appears probable; the deaminase attaches to PBG at the alpha-hydrogen activating the amino methyl side chain which undergoes de-amination. Successive molecules of PBG condense through their amino methyl side chains losing ammonia, forming the sequence ENZ; PA. AP.AP.AP. etc. In the absence of isomerase cyclisation only occurs when 5 PBG molecules have condensed in linear fashion and the last 4 cyclise to UROGEN I. The isomerase enzyme causes the pyrrole attached to the enzyme to be included in cyclisation when only 4 PBG molecules have condensed. Details of this hypothetical, though attractive, scheme are given by Rimington (loc.cit.)

(E) The Decarboxylation of UROGEN.

Our knowledge of the reactions which lead from UROGEN III to haem is incomplete. The demonstration by Neeve et.al. (67) that uroporphyrinogen rather than uroporphyrin was active in promoting haem synthesis in a red cell haemolysate represented a major advance in knowledge of porphyrin synthesis, but the subsequent steps are documented in rather few papers. Certain conclusions as regards anatomical localisation of enzyme action (52) may be premature. UROGEN III is decarboxylated in stepwise fashion to COPROGEN III. Evidence for this reaction has been obtained by Mauzerall and Granick (60) who achieved partial purification of a decarboxylase enzyme by zone electrophoresis on starch. The source was a haemolysate of rabbit reticulocytes. This enzyme had no special requirements and low specificity. UROGEN III was decarboxylated only twice as fast as UROGEN I. Even this degree of specificity is somewhat surprising as the enzyme must, in the course of stepwise decarboxylation, deal with some 34 possible substrates. Papers from Rimington's school concerning a decarboxylase

enzyme from human erythrocytes deny increased specificity for the series III isomer UROGEN (61). A heat stable ultrafilterable co-factor required for decarboxylase activity has been described by Hoare and Heath (67). The enzyme is inhibited by mercury, copper and manganese, also by oxygen. Yields of COPROGEN are improved by addition of GSH, cysteine and by lack of oxygen. This is not surprising as these are the factors which maintain porphyrinogens in their reduced state. The intermediately carboxylated porphyrinogens do not tend to accumulate because the enzyme has a low Michaelis constant and a high turnover number.

(F) COPROGEN Oxidase.

The conversion of coprogen to protoporphyrin is an interesting reaction. Decarboxylation of propionic side chains at positions 2 and 4 is coupled with oxidation of the remaining 2-carbon side chains to vinyl groups. Oxygen is required for the reaction but cyanide does not inhibit it, suggesting direct oxidation by molecular oxygen. Granick (63) cites unpublished evidence that oxidation and decarboxylation proceed at the same time,

and Sano and Granick (52) provide evidence that the enzyme is located chiefly in mitocondria. The specificity of this enzyme is apparently of a very high order. Only COPROGEN III can be converted to a di-vinyl porphyrin, and no evidence has ever been put forward that protoporphyrins of isomer type other than 9 occur in nature.

(G) The Incorporation of Iron into PROTO.

Various papers, (64, 65) cite evidence in favour of this reaction being mediated by an enzyme, the so-called "ferro-chelatase." One difficulty is that at physiological pH, if iron can be maintained in a ferrous state and PROTO maintained in solution at a reasonable concentration, haem will form in the absence of any enzyme. Protein, because of its solublising effect on PROTO, will, in a non-specific fashion, increase yields of haem. However, there is evidence that this reaction is enzyme catalysed. In Haemophilus influenzae, the vinyl groups of protoporphyrin are obligatory for the insertion of iron. Other haems (e.g. deuterohaem, mesohaem) support growth, but the organism will insert iron only into PROTO.

(H) Summary.

The main reactions, the enzymes involved, and their possible localisation within the cell as suggested by Sano and Granick, have been summarised in Figure III-1. The picture presented is a static one. The various isolated fragments of knowledge have been collected from study of plant enzyme systems, of red cell haemolysates, of liver homogenates, and purified cellular fractions. Virtually nothing is known of the manner in which these enzymes are integrated into the controlled formation of haems at a rate sufficient to meet the needs of cells. The daily synthesis of porphyrin in the human body is of the order of 300 mgm, yet the excretion of unused haeme precursors does not exceed 5 - 10 mgm, a state of affairs which suggests excellent control over the rate of haeme synthesis.

One mechanism of control has been suggested by Sano and Granick (52) who used the fact that the enzymes required to synthesise ALA as well as the enzyme(s) which finally convert COPROGEN to PROTO, are located within the mitocondria. The remaining enzymes are found in the soluble fraction of the cell. Granick suggests that the rate at which ALA leaves the mitocondria

serves to control the rate of haem synthesis. He does not suggest how egress of ALA may itself be controlled.

The task of unravelling the biosynthesis of protoporphyrin has been difficult enough, but the chain of events does not stop there. Iron protoporphyrin is found in catalase, peroxidease, myoglobin, and, with the vinyl groups involved in thioether linkages, in cytochrome C, but cytochrome oxidase has a formyl porphyrin with -CHO at position 8. (66). Nothing is known of the reactions which lead from protoporphyrin to porphyrin "a".

CHAPTER IV.

PORPHYRIN EXCRETION IN HEALTH AND DISEASE.

A Review of the Literature.

In the past, urine porphyrin excretion has been extensively studied, whereas faecal porphyrin has been rather neglected. This is unfortunate; the faeces represent the major pathway of porphyrin excretion in health and usually in disease. Further, in the urine, the occurrence of PBG which slowly converts to uroporphyrins has complicated the study of porphyrin excretion in diseased states. Much effort was directed towards elucidating the nature of uroporphyrins which could be recovered in crystalline form from pathological urines. This will be dealt with as a separate problem. Before considering pathological states, knowledge concerning normal porphyrin excretion must be reviewed.

1. NORMAL PORPHYRIN EXCRETION.

A. Urine Porphyrin. Comfort et.al. (71) collected 118 litres of "normal" human urine from persons known and unknown. Ether soluble porphyrins were extracted and the remaining porphyrin adsorbed onto talc. The

chief porphyrin present was Coproporphyrin which was mainly isomer I. Small amounts of porphyrins with from 5 to 7 carboxyl groups were identified chromatographically, and also a small amount of uroporphyrin which was not crystallised. Lockwood and Bloomfield (72) recovered crystalline uroporphyrin from normal human urine. They found the uroporphyrin to be chiefly isomer I, but coproporphyrin chiefly isomer III, although variations were encountered between the two subjects studied.

Watson (73) studied the daily excretion and isomer distribution of coproporphyrin in normal urine. He found that isomer I predominated although there was variation from person to person. Kehl (74) has reviewed these findings and studied the problem further. Using a more reliable technique than was available to earlier workers, he found normal urine coproporphyrin to be largely isomer III.

Eales and Saunders (75) have studied normal urine porphyrin excretion in the three racial groups seen in Cape Town. Coproporphyrin ranged from 1 - 204 ug/day and Uroporphyrin from 0 - 41 ug/day. Ranges and means for the different groups are cited.

B. Faecal Porphyrin. Various authors (8, 16, 75, 76, 77) have studied quantitative aspects of the excretion of ether soluble porphyrins in health using the Holti (16) method of porphyrin estimation. All authors have found faecal ether soluble porphyrin in health to be less than 100 ug/g dry weight, but whereas Haeger found coproporphyrin excretion to be 2 $\pm$ 2 ug/g dry weight and protoporphyrin 15 $\pm$ 12 ug/g dry weight, Eales found a range for coproporphyrin of 0 - 27 and protoporphyrin 2 - 99 ug/g dry weight. The difference may be due in part to variations in diet, for a diet rich in meat certainly increases faecal porphyrin (75) (78) All investigators have found the protoporphyrin fraction in the Holti method to exceed the coproporphyrin fraction by between 3:1 and 6:1. No thorough study has been reported of ether insoluble porphyrin in normal faeces. The only study appears to be that of Bashour, quoted by Watson (79) that a trace of uroporphyrin may be recovered from normal faeces. The important point is that it is only a trace, and Schwartz (27) gives a range of 10 - 40 ug/day for normal uroporphyrin excretion.

In summary, the excretion of porphyrin in health is low, despite the fact that the daily requirements of haem synthesis entail manufacture in the body of about 300 mg. of protoporphyrin; in the urine usually less than 0.1 mg. of haem precursors is excreted daily and in the faeces not more than 1-2 mg. daily. This excludes ALA and PBG of which a few milligrams may be found daily in the urine.

2. PORPHYRIN EXCRETION IN DISEASE.

(A) Congenital Erythropoietic Porphyria. (C.E.P.)

This condition originally tended to be confused with other forms of porphyria, but Schmid (80), following study of urine and tissue porphyrins, defined the condition and, reviewing the literature, accepted only 34 cases as being proven C.E.P. H. Fischer, in his original studies of the patient Petry, had shown that the urine contained large quantities of series I URO and COPRO. Post mortem studies of tissues from Petry showed the liver, spleen and bones likewise to contain large amounts of series I porphyrin.

(i) Findings in erythropoietic tissues.

Schmid studied the bone marrow in C.E.P. by fluo-

rescence microscopy. He found that some normoblasts fluoresced brilliantly while others were non-fluorescent. Bone marrow biopsy material contained large amounts of porphyrin and crystalline URO I was prepared.

(ii) Findings in the urine. Rimington and Miles (81) studied 15.7 litres of urine from a human case of C.E.P. 712 mg. of porphyrin was obtained of which 634 mg. was URO I; 11.5 mg. of COPRO I were also obtained. A few milligrams of 5 and 7 carboxyl porphyrins were present. 6 carboxyl material was not mentioned. Kench et.al. (82) studied the urine of a patient with C.E.P. chromatographically and found evidence of porphyrins with 7, 6, 5 and 4 carboxyl groups.

(iii) Findings in Faeces. Fischer showed the faeces to contain large amounts of COPRO I and lesser amounts of URO I. He noted that in the faeces some of the COPRO was present as a colourless precursor. The level of PROTO in the faeces is reported to be normal (11).

(iv) Metabolic Studies. An important observation was that of Gray (83) who gave N^{15}-glycine to a patient with C.E.P. and studied labelling of the

faecal stercobilin. He found a prominent early peak at 3-4 days which was much greater than in normal persons. This probably represents ineffective erythropoiesis and may be associated with the haemolytic element prominent in some cases of C.E.P.

Rimington and Booij (84) have shown that a haemolysate of red cells from a patient with C.E.P. is able to convert PBG to both URO I and URO III. Normal cells, however, form only URO III unless previously heated. There is thus only a partial inability to form the series III isomer. Presumably a total block would be incompatible with life.

(B) Swedish Genetic Porphyria. In common with C.E.P. this condition may be recognized as an entity in the literature although it is referred to by various names. (Chapt.V). One can only be certain of the diagnosis, however, when figures pertaining to faecal porphyrin excretion are cited.

(i) Findings in the Urine. The urine contains variable amounts of ALA and PBG in remission and large amounts in an acute attack. The quantity

of URO found in the urine will thus depend largely upon conditions of collection and storage. Freshly voided urine from patients with Swedish porphyria may contain little, if any, increase in uroporphyrin concentration. (85) On the other hand, urine from these patients, is often voided pinkish or dark and then contains increased amounts of porphyrin. The discolouration, of course, is due more to porphobilin than to porphyrin. It is not known how much of the porphyrin is formed while the urine is in the bladder. Unexplained as yet is the occurrence of porphyrin in the urine largely as a metal complex (probably zinc). This phenomenon requires further investigation. The composition of the urine uroporphyrin will be considered in a special section devoted to Uroporphyrin.

(ii) Findings in the Faeces. Kench (86) studied faecal porphyrin in acute porphyria in 1943. He found 18 ug/g of coproporphyrin during an acute attack, but no uroporphyrin. A similar report came from Gray in 1950. (87) From 350 gm. of faeces, 1.5 mg of Coproporphyrin I was obtained and an impure specimen of URO I. It was in this case that cytochrome

myoglobin and catalase were studied and found to be normal. Quantitative aspects of faecal porphyrin excretion in Swedish porphyria were studied by Barnes (9) and Haeger (76). Barnes showed that the faecal porphyrin levels could be relatively normal, but in 3 out of 10 cases studied total faecal porphyrin exceeded 100 ug/g d.w. Haeger, with a larger series, showed that faecal porphyrin was increased relative to normal but the ratio of PROTO to COPRO remained unchanged at about 6:1.

(iii) Findings in the Tissues. Schmid studied 11 cases of acute porphyria and showed no increase in the porphyrin in the bone marrow even after heating. In the liver, however, heating produced an increase in URO content. Faecal porphyrin excretion was not reported and some of these cases may have been of the S.A. Genetic variety.

(iv) Metabolic Studies. Scott (89) showed that persons with Swedish genetic porphyria excrete more of an administered dose of ALA as PBG than do normal persons. Richards and Scott (89) showed that a large oral dose of glycine (25 g)

caused increased urinary excretion of ALA in this type of porphyria. Dowdle et al (90) gave C^{14}-glycine to a patient with Swedish porphyria. They showed that there was no defect in the oxidation of glycine-2-C to CO_2 but that the specific activity of glycine which conjugated with benzoic acid to form hippuric acid was eight times the specific activity of the glycine which formed PBG.

(C) Reports from Rimington's School on patients with Porphyria Cutanea Tarda (PCT). A group of cases is described in the literature which has led to Rimington's concept of reciprocity affecting faecal and urine porphyrin excretion. The first such report was in 1948. Gray et al (91) described their concept of PCT : "porphyrinuria and sensitivity to light develops later in life and a scelerodermic tendency is common. The occurrence of abdominal colic, and excretion in the urine of large amounts of URO III, suggests a relationship to the acute type, but severe nervous symptoms are not present."

A 22 year old man was described in whom jaundice

was accompanied by a fall in faecal porphyrin excretion and an increase in urine porphyrin. In the urine both COPRO and an ether-insoluble porphyrin increased. This latter could not be decarboxylated to COPRO and had an alpha band ($CHCl_3$) at 623.5 mu. There was no evidence obtained that this ether-insoluble porphyrin was URO.

MacGregor (92) described a second instance of cutaneous porphyria with intermittent jaundice in a 14 year old boy. Again, the reciprocal relationship between urine and faecal porphyrin was well demonstrated, but insufficient data concerning the jaundice was given. In remission periods faecal porphyrin rose to as much as 45 mg. per day. Death eventually occurred in coma with a positive PBG reaction in the urine. The urine contained porphyrins with from 3 - 8 carboxyl groups; PROTO was isolated from the faeces and crystallised after purification by HCl number fractionation. A dicarboxylic porphyrin was obtained which melted at 165°C. It is interesting that at autopsy the nature of the porphyrin was not established. The skin of this patient was already affected at the age of 14, and at death scarring and deformity of the hands were severe.

A third example of jaundice associated with PCT was described by Rimington and Wells (93). The patient was a chronic beer drinker. Faecal porphyrin was described as greatly increased with the PROTO fraction exceeding COPRO. The serum fluoresced and was stated on spectroscopic grounds to contain protoporphyrin, but details of analytical methods were not given.

Holti et al (16) described a patient with PCT. The skin was involved, faeces contained a large excess of porphyrin. PROTO, as measured by the Holti method, exceeded COPRO. Urine porphyrin was sometimes increased and the Watson-Schwartz test was faintly positive if fresh urine was tested. Family studies indicated a dominant mode of inheritance. There is no reason to believe that this case differed from S.A. Genetic porphyria.

Comment. The reciprocity theory appears to have originated in the studies described in the case of Gray (90). It is important to note that although there was an increase in ether-insoluble porphyrin, this was never shown to be uroporphyrin and the nature remains obscure. In the second case, that of MacGregor, quantitative estimation of ALA and PBG were not performed. Although it was stated that testing the urine

for PBG gave a negative result, it was not stated whether the urine tested was fresh. From the published data it is not possible to assess the nature of the jaundice. Was this an event in the natural history of the porphyrin disease, or was it an unrelated obstructive jaundice which grossly altered the clinical presentation of the porphyria?

The terminal "acute attack" described in MacGregor's case was acute porphyria as the urine contained large amounts of PBG. Was this so in any of the other acute episodes? Data in the case of Rimington and Wells is again difficult to assess. The finding of protoporphyrin in the urine is interesting but inadequately documented. It is understandable that in a condition associated with the daily excretion of more than 40 mgm of porphyrin in the faeces, obstructive jaundice would lead to an increased urine excretion of porphyrin. The same porphyrin previously excreted in the faeces would be expected to appear in the urine.

(D) Other Reports. An interesting case is described by Calvy (94), with biochemical aspects of this case well documented in the studies of Lowry et al. (95). The patient was a 20 year old naval rating who

sufferred intermittent abdominal pain and excreted a urine which darkened on standing. His skin was severely involved. Vesicles and erosions occurred on the hands, but an acute photo-sensitive reaction had involved the face. A positive family history was confirmed by finding increased PBG in urines from other members of the family. The patient was admitted to hospital with an episode of jaundice with hepato-megaly and bile in the urine. PBG was absent at this time, urine COPRO was increased and PROTO exceeded COPRO in the faeces. Faecal porphyrins were type III isomers. Haem and porphyrin metabolism was studied following the administration of N^{15}-glycine. The label was rapidly incorporated into haemoglobin haem and into faecal COPRO, but there was a delay in the incorporation of N^{15} into faecal PROTO. The decay in activity of faecal PROTO was not exponential but could be accounted for by dilution in various pools.

An important paper concerning the classification of the porphyrias was that of Schmid et al (96). Amongst the patients described with hepatic porphyria a group with Swedish porphyria can easily be distinguished.

Tissue porphyrin was normal except after heating, recurrent abdominal pain was a prominent clinical feature, but liver function was variously disturbed. A further group may be distinguished with high urine URO, grossly fluorescent liver biopsy tissue and gross increase in the URO content of liver. These patients all had abnormal albumin-globulin ratios; frank cirrhosis was present in some, and in others a history of alcohol consumption.

Schmid et al make particular reference to case 86 in this article, described as Mixed Porphyria. The patient was seen on two different occasions. On the first occasion cutaneous involvement was prominent. On the second occasion abdominal pain was being experienced and the skin was described as normal. Liver function showed a reversed albumin-globulin ratio on the first occasion, but the content of porphyrin in the liver was not grossly abnormal, (URO 5.3 ug, COPRO 0.18 and PROTO 0.82 ug/gm liver.) Unfortunately no mention is made of faecal porphyrin in this article, but it seems likely that case 86 was similar to the familiar cases of S.A. Genetic porphyria.

Used in the context of this paper (Schmid et al) the term "mixed porphyria" could equally be applied to cases of S.A. genetic porphyria although, in this condition, it is questionable whether the skin, once abnormal, ever returns to normal.

(E) The Uroporphyrin Problem. In 1937 Waldenstrom (97) described the isolation from acute porphyria urine using ethyl acetate, of URO-methyl ester which melted in the range 258 - 260°C and which he considered to be largely isomer III. This finding was contrasted with Fischer's finding of uroporphyrin I in the urine of his patient with C.E.P.

In 1945 the first of a series of papers dealing with uroporphyrin came from the school of Watson (98). Uroporphyrin was recovered from the urines of patients with Swedish porphyria, C.E.P. and P.C.T. Some of the urines were heated prior to recovery of porphyrin. Crystalline uroporphyrin esters isolated from patients with Swedish porphyria and P.C.T. were described as Waldenstrom porphyrins. Crystal morphology was typical of URO, but melting points were somewhat broad, in the range 250 - 260°C, and decarboxylation

yielded a mixture of COPRO'S I AND III.

When URO from the case of P.C.T. was chromatographed on calcium carbonate, two fractions were obtained. The faster running (mp 284°C) yielded COPRO I on decarboxylation, the second fraction, mp 208°C COPRO III. The second fraction was absent from Waldenstrom's porphyrin derived from the urine of patients with Swedish genetic porphyria in the acute phase and also from uroporphyrin obtained from urine of patients with C.E.P. Elementary analysis showed the 208° porphyrin to be a heptacarboxylic porphyrin.

In 1949 Rimington (99) introduced lutidine chromatography for the analysis of porphyrin mixtures. In 1950 Nicholas and Rimington (100) systematically investigated the chromatographic behaviour of porphyrin esters on columns of alumina, magnesium oxide, magnesium carbonate and calcium carbonate. Lutidine chromatography showed that certain samples of uroporphyrin ran as two spots, that with a slightly higher Rf value be referred to as "pseudo-uroporphyrin." Column chromatography on magnesium oxide, magnesium carbonate and calcium carbonate did not appear to separate URO

and PSEUDOURO. In 1951 Chu and Chu (101) introduced their technique for the separation of COPRO ester isomers I and III, and Falk and Benson (102) extended this technique to URO ester isomers, using different solvents.

The uroporphyrin problem depended on whether Waldenstrom porphyrin from the urine of patients with P.C.T. contained a 7 carboxyl fraction which lowered the melting point of URO I with which it was mixed and whether this 7 carboxyl material was identical with PSEUDOURO observed chromatographically.

Despite the fact that Falk (22) still refers to PSEUDOURO as an unknown substance, there is now abundant evidence that it is the same as Watson's heptacarboxylic porphyrin. Chu and Chu (103) examined 17 litres of urine from a patient with P.C.T. using the excellent resolution of their "Hyflo" column chromatographic technique. Both URO'S I and III were isolated and in addition a 7 carboxyl porphyrin, melting point 224°C, which decarboxylated to COPRO III. In 1955 Watson's school published a very careful study of 11 mg. of porphyrin isolated by Professor Gray from the urine of a patient with acute porphyria. Although

this porphyrin behaved as URO III on Falk-Benson chromatography, it decarboxylated largely to isomer I COPRO. The melting point of the original specimen had been slightly raised by chromatography on calcium carbonate, but not on magnesium oxide.

A source of difficulty in establishing the nature of uroporphyrin samples has been the failure of investigators other than of the Watson school to duplicate the reported separations of 8 and 7 carboxyl porphyrins on calcium carbonate. Watson reported an investigation of this point in 1960 (104), and showed that only samples of calcium carbonate in which the correct proportions of calcite and aragonite (X-ray crystallography) were present, were effective in achieving this separation. Dresel and Falk (105), using a sample of calcium carbonate obtained from Dr. C. J. Watson, separated URO and PSEUDOURO methyl esters.

Various paper electrophoresis systems separate the 7 and 8 carboxyl porphyrins widely. This includes the system of Lockwood and Davies (106). These authors obtained sufficient PSEUDOURO for elementary analysis and showed it to be 7 carboxyl.

Various reports (85, 107), in addition to those already cited, indicate that the 7 carboxyl (or pseudouro-) porphyrin only occurs in significant quantity in uroporphyrin samples which have been formed by enzyme activity. Uroporphyrin formed by heating urine containing considerable amounts of PBG, does not contain the 7 carboxyl porphyrin and, more significantly, it is almost absent from the urine of C.E.P. (81) (Chapter VI, Section 15-A).

Summary. Uroporphyrin from congenital porphyria urine is almost pure uroporphyrin I and provides no problem. Urine porphyrin from the urine of acute porphyria may, or may not, contain significant amounts of 7 carboxyl porphyrin. If it does not, it will contain a mixture of URO isomers formed from PBG, the composition of the mixture depending upon the pH of urine. Such uroporphyrin will form a Waldenstrom-type A uroporphyrin, the melting point of which will not be altered by methods which separate off 7 carboxyl fractions. Uroporphyrin from patients with acquired porphyria usually referred to in literature as PCT, will yield a Waldenstrom-type B porphyrin, where 7

and 8 carboxyl porphyrins are admixed in varying proportions. The melting point of this material will be altered by techniques which separate 7 and 8 carboxyl porphyrins.

CHAPTER V.

CLASSIFICATION OF THE PORPHYRIAS.

Various classifications of the porphyrias have been suggested but none has encountered general approval. As emphasised by Eales (108), disagreement is inevitable until the various types of porphyria can be defined precisely in terms of a biochemical defect.

The original classification of Gunther (109) is now only of historical interest.

The classifications which are currently of interest are those of Waldenstrom (110), Watson (17), Eales (108) and Goldberg and Rimington (42). These are given below:

(i) Waldenstrom.

Porphyria congenita

Porphyria acuta (5 clinical subdivisions)

Porphyria cutanea tarda hereditaria

" " " symptomatica.

(ii) Watson.

I. Porphyria erythropoietica

II. Porphyria hepatica

A. Hereditary acute intermittent

1. Manifest
2. Latent.

B. Hereditary mixed or variegate

1. Cutaneous
2. Acute
3. Mixed (Various combinations)
4. Latent

C. Hereditary cutaneous.

D. Constitutional or Ideosyncratic (PCT)

1. Chemical
2. Ideopathic
3. With systemic disease.

E. Acquired.

1. Secondary to hepatoma
2. Secondary to fungicide.

(iii) Eales.

Eales added to Waldenstrom's classification names suggesting the known biochemical differences between the conditions.

1. Porphyria congenita
2. Porphyria acuta - pyrrolo_porphyria
3. PCT hereditaria - protocoproporphyria
4. PCT symptomatica - urocoproporphyria.

(iv) Goldberg and Rimington.

1. Congenital (erythropoietic) porphyria
2. Acute Intermittent Porphyria
3. Cutaneous Hepatic Porphyria
   (a) Hereditary forms
      i. Porphyria Cutanea Tarda Hereditaria or protocoproporphyria (Waldenstrom,1957)
      ii. Mixed Porphyria (Watson et al 1951)
      iii. Porphyria variegata (Dean and Barnes, 1959)
   (b) Acquired forms
      i. Porphyria Cutanea Tarda Symptomatica
      ii. Bantu Porphyria (Barnes,1959)
      iii. Turkish porphyria (Cetingil & Ozen, 1960)
      iv. Porphyrin producing hepatic adenoma (Tio 1957)
4. Experimentally induced porphyrias.

Erythropoietic protoporphyria remains unclassified.

Waldenstrom's scheme is clearly allied to Gunther's original classification; it is merely a list of the four familiar clinical variations of porphyria. The recent description of erythropoietic protoporphyria renders this list incomplete and the toxic human porphyrias are not referred to. The classification is entirely clinical; Bales attempted

to link the established clinical entities with specific biochemical disturbances, but the validity of this is questionable. Were it to be shown that a variety of "urocoproporphyria" or "PCT symptomatica" had an hereditary basis as now claimed by Waldenstrom, the relationship would become confusing.

Having studied the porphyrin content of various tissues, Schmid et al (1954) proposed that the porphyrias could be broadly divided into erythropoietic and hepatic varieties. Although Rimington (1952) at first questioned the validity of such a distinction, the current classification of Goldberg and Rimington indicates partial acceptance of Watson's view.

There is no problem in segregating the erythropoietic porphyrias from those porphyrias in which free erythrocyte porphyrin is normal. Even if the liver is only the <u>main</u> site of abnormal porphyrin metabolism in the hepatic porphyrias, this distinction is very useful in classification and diagnosis.

It is over the classification of the hepatic porphyrias that disagreement is greatest. Waldenstrom (1963) makes a plea that the genetically determined

porphyrias be regarded as entities which cannot interconvert any more than can, for example, Haemophilias A and B. With his series of over 300 cases he has a vast experience of Swedish genetic porphyria, and has established this as an entity. The principal biochemical features are raised ALA and PBG excretion in remission, but normal or only slightly increased faecal porphyrin concentrations.

South African genetic porphyria has been, if anything, better documented and is characterised by relatively normal ALA and PBG in remission and a high faecal porphyrin. Both the Swedish and South African varieties are inherited as Mendelian dominants and sufferers from either are liable to attacks of acute porphyria.

These two conditions are in every sense biochemical entities, but there are published reports which are either inaccurate or indicate that intermediate forms of genetically determined disease exist: Calvy et al (94) describe a patient with cutaneous porphyria whom Watson and his co-workers studied. (See Chapter IV). The faecal porphyrin was high but a positive family history was established by finding

PBG in the urine from two otherwise healthy relatives. This is typical of neither the Swedish nor the South African forms.

Watson's sub-classification of the hepatic porphyrias would place the "Swedish" and "South African" conditions into his categories (A) and (B) with further subdivision into clinical variants. His category of (C), hereditary cutaneous, remains to be established. Porphyria cutanea tarda symptomatica or urocoproporphyria which is seen as a complication of the excessive use of alcohol, will fall under Section D.

A major source of confusion, at present unresolved, is the relationship between liver function and the porphyrias. Watson (17) claims that liver function is frequently disturbed in "hepatic porphyria", but does not indicate that this takes the form of an obstructive lesion associated with jaundice. However, he maintains that impairment of BSP excretion is frequently encountered.

Waldenstrom (111) agrees with Brunsting (112) that in cutaneous porphyria seen in association with alcoholism there is usually evidence of liver disease but that frank cirrhosis is not often encountered.

In Swedish porphyria in remission liver function is normal, but Waldenstrom (110) has encountered jaundice in a number of patients with acute porphyria.

Reports from Rimington and his associate (see Chapter IV-2-C), suggest that episodes of obstructive jaundice characterise the natural history of inherited cutaneous porphyria in which the faecal porphyrin is increased. However, the data presented in the case reports do not indicate clearly that the liver disease which so grossly altered the clinical presentation of the porphyria was secondary to the porphyria.

The literature thus appears to provide two main points of difficulty: the first, found chiefly in American literature, is whether Swedish and South African genetic porphyria form clear cut entities as is so strongly emphasised by Waldenstrom, or whether intermediate forms occur. The second, apparent from case reports of British authors, is whether there is a type of porphyria characterised by a remission phase when the faecal porphyrin is high and skin lesions are absent or mild, and an acute phase characterised clinically by abdominal colic, jaundice, and an

exacerbation of the skin lesions, and biochemically by signs of obstructive jaundice, a fall in faecal porphyrin and a rise in urine porphyrin.

South African experience concerning the first point supports Waldenstrom's view with the reservation that moderate (not defined on purpose) increases in ALA and PBG may be encountered in remission in South African genetic porphyria.

However, South Africa, as recently emphasised by Dean (145) may represent a special case. In this country there is good evidence that all persons with variegate porphyria have inherited their affected chromosome from the same source. Perhaps a similar circumstances obtain in Sweden, but the existence of two disorders involving the haem synthetic pathway, similar in some respects but differing in others, suggests that other, perhaps intermediate forms could occur which would also prove to be genetically and biochemically distinct.

The second problem depends upon whether the jaundice of the English cases was part of the natural history of the porphyric disorder or an obstructive jaundice of unrelated cause occuring in patients with

the South African genetic type of porphyria. The latter view is favoured.

For the purpose of this thesis, a simple classification is given which covers only those varieties of porphyria encountered in this study and other types fully documented in the literature.

1. Erythropoietic porphyrias
   - (a) congenital erythropoietic porphyria
   - (b) erythropoietic protoporphyria
2. Hepatic porphyrias
   - (a) Swedish genetic porphyria (Acute Intermittent Porphyria)
   - (b) South African genetic porphyria (Variegate Porphyria)
   - (c) Acquired porphyria (Symptomatic Porphyria)
   - (d) Porphyria secondary to hepatoma.

The non-commital designations "Swedish" and "South African" are used as suggested by Dean and Barnes (113).

CHAPTER VI.

LABORATORY TECHNIQUE.

1. Purification of Solvents.

It is essential that metallic impurities are removed from solvents and from all reagents which are to be used with porphyrins. "Analar" solvents (British Drug Houses) have often been used as supplied but if time permits it is sound practice to distill all solvents.

Some solvents require special mention:

A. Water should come from an all-glass still or from a deionising column.

B. Diethyl ether must be purified before use. Peroxides may be removed from technical grade ether (114), the ether dried with sodium sulphate or calcium chloride, and distilled. Storage, when unavoidable, should be in a light-proof bottle. Ether supplied for anaesthesia is free of peroxides but contains a preservative (propyl gallate) which leaves a residue if the bulk of the solvent is evaporated.

C. Chloroform available commercially usually contains a small amount of ethanol as a preservative. This renders it unsuitable for use in chromatographic

procedures as ethanol greatly increases the eluting power of the solvent. The ethanol may be removed by washing commercial chloroform four or five times with one fifth its volume of water, drying the washed chloroform with calcium chloride (powdered) and distilling it. So prepared, chloroform is unstable. It must be stored in a dark bottle and prepared freshly every 3 - 4 weeks.

D. Methanol. Commercial reagent grade methanol contains less than 1% of water and this is dry enough for use in esterification reactions.

E. Methylated Spirit. This solvent is extremely cheap and is useful for rinsing glassware during experimental work. A supply of distilled "white" methylated spirit should be kept available.

F. Ethyl acetate (Analar) has been used as supplied. It is expensive and should be recovered after large volumes have been used for porphyrin extractions. The recovery is described by Schwartz et al (27).

G. Hexane. The commercial product is a fraction obtained during petroleum refining. It should be redistilled to remove high-boiling contaminants.

H. Pyridine. "Analar" pyridine has been used as supplied.

I. Lutidine. Tends to become discoloured. It can be purified and at the same time freed of metallic impurities by distillation under reduced pressure (about 75 millimetres of mercury).

J. Tetrahydrofuran is unstable and forms peroxides. It is usually preserved with hydroquinone. Practice has been to purify it before use by distillation over solid KOH pellets. Discoloured samples should be discarded.

2. Recovery of Porphyrins from Biological Material.

A. Urine.

Rimington and Sveinson (115) and Dresel and Falk (105) have described solvent extraction procedures which will recover both COPRO and URO from urine. COPRO is recovered by the classical procedure of shaking the urine with ether and acetic acid. URO is extracted with cyclohexanone at pH 1.5. It should be realised that some urines, contain appreciable concentrations of porphyrins with 7, 6 and 5 carboxyl groups similar to, or greater than the concentration of COPRO (Chapter VIII). These porphyrins are partially soluble in ether and the fractionation into COPRO and URO is not clear cut.

Both fractions will be contaminated by porphyrins more polar than COPRO but not insoluble in ether, as is URO. The cyclohexanone extract of urine also contains a considerable amount of brownish impurity which seems unavoidable if an organic solvent is used to extract the URO fraction.

Various adsorbents appear to be suitable for the recovery of porphyrin from urine. Talc has, however, been used routinely because it is easy to work with and appeared to have no particular disadvantages. The pH of the urine is corrected to the range pH 3 - 4 by the addition of glacial acetic acid. About 50 grams of talc is added per mg of porphyrin. After thorough mixing an aliquot is removed and centrifuged. 5cc of the supernatant is then shaken with 2 cc of a mixture of equal parts of ether, acetic acid and amyl alcohol and examined in filtered UV light, to ensure that all porphyrin has been adsorbed to the talc. If not, more talc is added. A large Buchner funnel is prepared with two layers of filter paper covered with "Celite" filter aid and the talc is filtered off. After washing with distilled water containing about 1% acetic acid, the talc and "Celite" filter cake is removed and dried

in a vacuum dessicator over calcium chloride. The cake is then broken up and left in methanol plus 5% sulphuric acid for 24 hours at room temperature to esterify adsorbed porphyrin. The talc is filtered off from the esterifying mixture and washed with fresh methanol containing 2% concentrated sulphuric acid. Porphyrin ester is transferred from the methanol-sulphuric acid mixture to chloroform as described below. (Section 3).

One possible advantage of using talc as an adsorbent which was discovered during the course of studies on porphyrinogens, is that talc, when added to a solution of porphyrinogen, causes the immediate oxidation of the porphyrinogen to porphyrin. Atmospheric oxygen is presumably required.

B. Bile (or Duodenal Aspirate).

When attempts were made to extract porphyrin from bile by shaking with various organic solvents, copious and stable emulsions formed. It was found possible to improve matters somewhat by diluting the bile ten times with water before attempting to extract the porphyrin. This, however, meant working with very large volumes

and extractions were probably rather inefficient. Later, it was found that if the bile was mixed with an equal volume of saturated ammonium sulphate, extractions could easily be performed with ether or ethyl acetate. This is the procedure which has been adopted for all studies reported here. Although fairly "clean" extracts of porphyrin can be prepared from bile in this fashion, it should be noted that there is still a tendency for the recovered porphyrin to form emulsions which make it tedious to analyse bile-porphyrin extracts in countercurrent distribution apparatus.

C. Faeces.

The recovery of porphyrin from faeces using ethyl acetate and acetic acid has been described (116). This method was used for much of the early work described in this thesis. However, the extraction of uroporphyrin from faeces is incomplete. This difficulty can be overcome by extracting the faecal residue remaining after extraction with an ethyl acetate/acetic acid mixture. Before extraction, uroporphyrin must first be esterified.

The residue is left soaking in methanol plus 5%

sulphuric acid for 24 hours. The acidified methanol containing porphyrin ester is filtered from the faecal residue which is then washed with further quantities of acidified methanol and the porphyrin ester then transferred from the pooled methanol sulphuric acid solutions to chloroform. Unfortunately, during transfer of the ester to chloroform, emulsions sometimes form which must be separated by centrifugation.

D. Tissue.

A method has been developed for the estimation of porphyrin present in liver, which depends upon the homogenization of the tissue in acetone and elution of porphyrin by addition to the acetone of 1-2% concentrated (36%w/v)hydrochloric acid. The method was developed because it was found that the technique of Schwartz et al (117) did not extract uroporphyrin present in the livers of rats poisoned with hexachlorobenzene. The acetone extraction on the other hand, is extremely efficient. At the same time, of course, haems are extracted. The amount of porphyrin recovered from some livers was so great that it was suspected that some of the haems were being de-ironed to leave free

porphyrin, but the amount of porphyrin obtained from normal liver was less than one microgram per gram, showing this not to be the case.

Method. A weighed sample of tissue is homogenised in acetone (AR). The homogenate is transferred to a small conical flask and 2% (v/v) conc. HCl added to the acetone. After violent shaking, the tissue residue is filtered off using a small Buchner or Hirsch funnel. The tissue residue remaining on the funnel is once more extracted by shaking with acid acetone and the two filtrates pooled. The extract of porphyrin and haems in acid acetone may be either evaporated to near dryness under vacuum in a rotary evaporator and esterified for subsequent analysis or analysed by solvent extraction procedures. (It is only necessary to evaporate the acetone if a fairly large volume of methanol + 5% sulphuric acid is added.)

Details of a Solvent Extraction Procedure. The acid acetone is added to an equal volume of water and the solution brought to pH 3.5 by the addition of aqueous sodium acetate. Ether soluble porphyrin is extracted by shaking the solution twice with equal volumes of ether. The ether extracts are pooled and washed twice

with half their combined volume of 3% sodium acetate, the first wash containing .005% iodine. The two sodium acetate washes are added to the original aqueous solution for the subsequent extraction of URO. COPRO is extracted from the washed ether using 0.1 N HCl, and PROTO with 1.5 N HCl. To extract URO from the combined aqueous solutions the pH is corrected to be in the range 1.5 to 2 by adding 3.0 N HCl, and URO is extracted to completion into a mixture of equal parts of ethyl acetate and n-butanol. Extraction is complete when the organic solvent no longer shows pink fluorescence in UV light after shaking with the aqueous phase. URO is extracted into 1.5 N HCl after petrol ether (any fraction) has been added to the ethyl acetate - n-butanol solution. The volume of petrol ether should approximately equal that of n-butanol. The porphyrin content of the extracts is quantitated as described in the appropriate section. An experiment was conducted in which liver from a patient with acquired porphyria was analysed by 3 methods:

(a) acetone)HCl extraction as described above followed by esterification, electrophoresis and quantitation of the electrophoresed porphyrin fractions.

(b) acetone HCl extraction procedure (as above) followed by the solvent extraction procedure, also described above.

(c) homogenisation of the tissue in methanol, elution of the porphyrin by acidification of the methanol with 5% ($^{v}/v$) of concentrated sulphuric acid, followed 24 hours later by transfer to chloroform of the methyl esters of porphyrin, and analysis by electrophoresis after hydrolysis.

(a) gave a result 117 micrograms total porphyrin per gram wet weight of liver, (b) 128 micrograms, and (c) 127 micrograms per gram wet weight of liver. Homogenisation in methanol and subsequent esterification of the porphyrin before separation from the tissue residue is not recommended as the emulsions formed were extremely troublesome.

3. The Esterification of Free Porphyrin Acids.

Techniques described above make use of 5% solutions of sulphuric acid in methanol for the esterification of porphyrins. This is probably the simplest procedure to use. Equally effective is a solution of dry hydrogen

chloride (118) in methanol. Falk et al (102), state that up to 24 hours is required for complete esterification using methanol-sulphuric acid at room temperature. However, if dry hydrogen chloride is passed into a solution of porphyrin in methanol, esterification is virtually immediate.

Enough HCl gas need not be passed to saturate the methanol (this is about 35% $^{w}/_{w}$). If the gas is bubbled fairly fast, the methanol-HCl solution will suddenly become warm and esterification proceeds rapidly. (38).

The technique for transferring porphyrin ester from acid methanol to chloroform is described in the reference already cited (116). It is most important that acid present be neutralised with <u>saturated</u> aqueous sodium acetate before water is added to separate the chloroform and aqueous phases. Unless this sequence is followed, some hydrolysis of porphyrin is inevitable.

Diazomethane has been somewhat neglected in recent years for use in the esterification of porphyrins, particularly since there is now a convenient method for its preparation. It is true that the reagent has a bad reputation on account of its poisonous nature and

explosive properties, but the amounts required for the esterification of porphyrins recovered from biological material are usually small and the dangers are thus slight.

Although it is normally stated that esterification is carried out in anhydrous ethereal solution, ether is a very poor solvent of free porphyrin in the dry state. However, experience has shown that diazomethane can be used in ethereal solutions containing tetrahydroguran (THF) or pyridine.

Diazomethane is prepared from N-methyl:N-nitroso:p-tolylsulphonic acid by the method cited by Vogel (loc.cit. p 971.) The method of synthesising this compound is also given. An ethereal solution of diazomethane is distilled into a solution of porphyrin in ether , with or without THF or pyridine, and cooled in ice. It is convenient to prepare at least 0.15 of a gram of diazomethane at a time although this usually represents a gross excess of that required for methylation. The presence of an excess of diazomethane is tested for by dipping a glass rod moistened with glacial acetic acid into the solution; bubbles of nitrogen are liberated

in the presence of diazomethane. The diazomethane and solvents are distilled off under vacuum or removed with a stream of dry nitrogen. A residue of the porphyrin ester remains which may be taken up in chloroform.

4. Separation of Porphyrin Esters by Column Chromatography.

A. The Use of Alumina Columns.

It is of great importance that the adsorbtive capacity of alumina be carefully standardised prior to use. For this reason exact details of the procedure found to be satisfactory by trial and error will be given. The alumina used was "Aluminium oxide, standardised for chromatographic analysis according to Brockmann, Merck." This was activated by heating at 220°C for three to four hours and cooling in a dessicator. Deactivation to between Grades IV and V (Brockmann and Schodder) was achieved by adding 10% (v/w) of distilled water to the alumina contained in a wide-necked flask, stoppering this firmly and shaking violently until the water had been dispersed and the alumina was again a fine, free-flowing powder.

A mixture of 2.5 milligrams each of uroporphyrin, coproporphyrin and protoporphyrin methyl esters can be separated on a column containing about 50 grams of alumina as prepared above. 50 grams of alumina is slurried with chloroform (alcohol free) and poured into a column 1½ inches across, closed at the bottom with a sintered glass disc of medium porosity with a tap below to control the rate of efflux. The tap is closed and the alumina allowed to settle. The layer of chloroform above the alumina is sucked off, or run off, and, taking great care not to disturb the surface of the adsorbent, the porphyrin mixture is added in solution in a minimum volume of alcohol free chlroform. The tap is opened to allow the ester to be adsorbed at the top of the column, but closed again just before the column runs dry. Again with great care, chloroform is added and the rate of efflux adjusted to 120 drops per minute. Protoporphyrin and then coproporphyrin will be collected as separate fractions, but the elution of uroporphyrin requires addition to the chloroform of 1% by volume of methanol. Recovery of protoporphyrin and coproporphyrin from such a column is virtually quantitative but, in an experiment performed, while 97% of protoporphyrin and

100% of coproporphyrin applied, was recovered, only 72% of uroporphyrin eluted. The remainder stayed at the top of the column and could not be displaced even by pure methanol. The ease of elution of porphyrins from alumina is MESO = DEUTERO = PROTO > COPRO > URO = HAEMATO.

B. Chromatography on Magnesium Oxide.

This technique has been largely abandomed for analytical work. Although batches of magnesium oxide initially supplied appeared to give good recoveries, recent batches have tended to adsorb irreversibly considerable amounts of the applied porphyrin esters. Magnesium oxide used to be used for separating the porphyrins of urine, but this is done far more efficiently by electrophoresis. There was previously a use for magnesium oxide column chromatography in the separation of protoporphyrin and other dicarboxylic porphyrins of faeces e.g. meso- or deuteroporphyrins. However, the separation was never clear cut, and losses of protoporphyrin were sometimes large. Countercurrent distribution (CCD) is now used for separating these porphyrins.

Although chromatography on MgO has been largely supplanted by CCD and electrophoresis in analytical studies it is still useful for preparative work. MgO is prepared in similar fashion to alumina but only 3% v/w of water is added to deactivate. A slurry is prepared in chloroform plus 1% (v/v) methanol and elution is accomplished with increasing concentrations of methanol. The ease of elution is URO > COPRO > DEUTERO = MESO > PROTO > HAEMATO.

5. <u>The Quantitation of Porphyrin in Solution.</u>

The concentration of porphyrin in solutions is measured either spectrophotometrically or fluorometrically. Spectrophotometry makes use of the sharp absorption bands in the visible region, or of the extremely sharp and intense absorption of the Soret band around 400 mu. in the near UV. Fluorometric measurements are made by activating fluorescence with light at the wave length of the Soret band of the porphyrin being measured and recording emission at the alpha band maximum which is around 600 mu. in acid solutions, and 620 - 630 mu. in neutral solutions.

A novel method of quantitating porphyrin in solution which will also be described, is the copper titration method of Oliver and Rawlinson (37) which is useful for determining molar concentrations of porphyrins whether or not the molecular weight is known.

A. Spectrophotometry.

Theoretical aspects concerning the behaviour of light passing through solutions are considered by Glasstone (120). Accurate measurements by absortiometry require (1) absolutely clear solutions, (2) that the molecular species being measured is not in any way associated and (3) that the concentration is such that deviations from Beer's Law can be neglected.

Molar extinctions of porphyrins at the Soret band maximum range from about 2.8×10^5 for protoporphyrin in acid solution to 5.2×10^5 for uroporphyrin in acid solution, and measurements may be made on solutions which contain less than about 1.5 to 2 micrograms of porphyrin per ml. at higher concentrations Beer's law is no longer followed. In organic solvents the extinctions are lower, ranging from 1.5 to 2×10^5 and more concentrated solutions can be measured using Soret band absorption.

For stronger solutions, either acid or neutral, the absorption bands in the visible region must be used or the solutions diluted. For accurate work, it need hardly be stated that these measurements are only valid if physical constants relevant to the particular porphyrin being measured are known. It often happens, though, that in solutions obtained from biological extracts, unknown porphyrin mixtures are present as well as impurities, and under these circumstances measurements can only be regarded as approximate.

Rimington and Sveinsson (115) have described a method for correcting absorption in the Soret band for the presence of impurities. Measurements are made at the peak of the Soret band absorption and at points approximately 25 mu. above and below the peak, i.e. at 430 mu. and 380 mu. The assumption is made that absorption due to the background impurities changes in a linear fashion from the point 25 mu. above the Soret band to that 25 mu. below. The sum of absorptions at these two points is subtracted from twice the Soret band maximum and the difference, divided by a factor which is unique for each porphyrin, represents the corrected optical density. Rimington

has published a list of correction factors (128). This method cannot profitably be applied to measurements made on the Soret band of neutral porphyrin solutions because in these solutions the absorption on the ultra violet side of the band does not fall off sharply.

B. Extinction Coefficients.

Extinction coefficients have been determined for the various reference porphyrins prepared. These are indicated in Appendix B. Weighing have been made on a "Sartorius Selecta" balance which allows the weight to be estimated to the nearest 0.1 mg. For extinction coefficients to be reasonably accurate at least 10 mg. of porphyrin should therefore be weighed out. This amount of pure porphyrin has not always been available, so that it is not claimed that extinctions quoted in this work are more accurate than other published figures. Those extinction coefficients which have been used in calculations are separately indicated in Appendix B.

The generally used extinction coefficients are those of Rimington (128). These are given to three significant figures implying a very high degree of

accuracy. Rimington's figure for Emol, COPRO (0.1 N HCl) is 4.89×10^5; Jope and O'Brien (121) after a painstaking purification of COPRO, arrived at the figure 5.2×10^5. However, it is not possible to assess the relative merits of published figures when details of technique, including the weighings, are not given.

A generally accepted extinction coefficient which should be viewed critically is that of PROTO in 1.5 N HCl. Rimington gives E_{mol} PROTO (1.5 N HCl) as 2.75×10^5 and the Soret peak as 408 mu. It has been found that both figures are probably rather low.

PROTO ester was hydrolysed and purified by CCD. (Ether/0.5 N HCl.) The molar concentration of this purified PROTO in solution in glacial acetic acid was determined by the Cu titration method (below) and the extinction in 1.5 N HCl was found to be 3.15×10^5 and 3.20×10^5 in two separate experiments. The Soret band maximum was at 409.5 mu on each occasion.

In a different experiment PROTO ester was purified by chromatography and hydrolysed with methanolic KOH. The molar extinction in 1.5 N HCl was 3.02×10^5 based on a weighed sample of 8.5 mgms.

The Soret band maximum was at 409 mu.

Acid hydrolysis of PROTO leads to an impure product and cannot be used if it is desired to measure extinction coefficients accurately. Despite the experiments reported here and these remarks, Rimington's figure of 2.75×10^5 has been used in most calculations. However, attempts to obtain correct extinction coefficients have highlighted difficulties in preparing pure samples of porphyrin.

C. Fluorimetry.

Fluorimetry was the generally accepted method of measuring the concentrations of porphyrin in very dilute solution before spectrophotometers able to operate in the near UV became generally available. The physical basis of fluorescence was considered briefly in Chapt.II. The monograph of Udenfriend (129) "Fluorescence Assay in Biology and Medicine" contains useful practical data and some theoretical discussion but makes only brief reference to porphyrins.

(i) Advantages of Fluorimetry. The overriding advantage is sensitivity. The Aminco-Bowman spectrofluorophotometer uses a Xenon light source, a photomultiplier tube detector and separate diffraction-grating monochromators for activating and emitted

light. This instrument will give a full scale deflection with as little as 10^{-4} ug. of porphyrin per ml. in acid solution. Only 1 ml. of solution is required.

A further advantage could be selectivity. Activation of fluorescence at 400 mu with emission at 600 - 630 mu is a fairly unique property; far more unique to porphyrins than is absorption at 400 mu. Fluorimeter readings are thus due mainly to porphyrin fluorescence. Unfortunately, enhancement and quenching of fluorescence by impurities detract from the precision of measurement and can generally not be adequately corrected for.

(ii) Standards. Measurements must be made by comparison with a known source. If possible this should be a standardised solution of the substance being measured. If this is not possible, conversion factors have to be used which vary with the following:

(a) slit widths of the monochromators
(b) photomultiplier tube response characteristics
(c) variations in spectral energy distribution of the activating source.

(It is assumed that the wavelength settings of the instrument are adjusted to give the maximum deflection from a sample before each reading is made.)

Only coproporphyrin is sufficiently stable for use as a standard in fluorimetry. In practice, solutions are used which contain 0.10 ug/mL in 0.10 N and 1.5 N HCl. Using the Aminco-Bowman instrument, slit-system No. 3, an IP 28 photomultiplier tube and expressing concentrations on a w/v basis in 1.5 N HCl, PROTO will give fluorimeter readings 70% of those of an equal concentration of COPRO and URO 75% of COPRO. (The concentrations of URO, COPRO and PROTO used in determining these conversion factors were derived from molar extinctions of 5.2, 4.8 and 2.8 x 10^5 respectively.) Because of (a) (b) and (c) above, these conversion factors are only applicable under the instrument conditions defined. Standard solutions of coproporphyrin, stored in the dark at room temperature, have proved quite stable over several months.

The relationship between fluorimeter readings and concentration is linear up to 0.2 ug/mL. Schwartz (27) reports that saturation of an acid solution of porphyrin with ethyl acetate causes an

8 - 12% enhancement of the fluorimeter reading. This was not found to be so when ether was the organic solvent used.

In summary, fluorimetric assay of porphyrins is useful when extreme sensitivity is required. It has also been useful and accurate in determining coproporphyrin isomer composition as absolute assays were not called for.

D. After Analysis by Countercurrent Distribution.

A special problem encountered was the need for rapid quantitation of porphyrin in the individual cells of countercurrent apparatus. After a countercurrent analysis, some 30 to 100 cells, each containing an ethereal and aqueous phase, must be analysed. Various porphyrins, some known and some unknown will be distributed throughout the system. The concentrations vary from fairly deeply coloured to only fairly fluorescent solutions. Granick and Bogorad (123), describing the countercurrent separations of haemato-deutero-meso- and protoporphyrins measured both phases simultaneously in a modified Beckman cell-holder, making the measurement at isobestic points of these particular porphyrins. They stress

the advantage of this method in speeding the analysis.

Inspection of a plot of porphyrin absorption spectra in acid and ether solutions e.g. Figs. IV-4,5 shows that at the isobestic points, the slope of the lines is extremely steep so that small errors in choosing the wavelengths of isobestics will lead to large differences in molar absorption.

Add to this the fact that the nature of the porphyrin being measured is unknown until after the countercurrent analysis and it will be apparent how unsuitable this technique is for unknown solutions. In practice, it has been found convenient to determine the extinctions, both in ether and in acid solution, of prominent bands in the visible as well as the Soret region of the porphyrins commonly encountered. After CCD the two phases have been separated, measured separately and all readings have been finally corrected to Soret Band absorption in acid. Inaccuracies are inevitable when "new" porphyrins are encountered but they are likely to be considerably less using this method than if measurements are made on supposed isobestic points. Absorption peaks may be rapidly determined but not isobestics.

E. The Copper Titration method of Oliver & Rawlinson. (37).

This method for measuring molar concentrations of porphyrin makes use of the fact that the absorption spectrum of the copper complex differs from that of the free porphyrin. To determine, for example, the molar concentration of a solution of protoporphyrin plots are made of the spectrum of the free porphyrin in glacial acetic acid and of its copper complex formed by heating the porphyrin with cupric acetate and glacial acetic acid in a water bath at 100° C for ten minutes. A point (or points) is chosen from the plotted spectrum where formation of the metal complex results in a large change in extinction.

A series of tubes is prepared each containing a known amount of porphyrin and graded amounts of cupric acetate and glacial acetic acid so that there will be some tubes in which conversion of the porphyrin to its copper complex is incomplete and two or three tubes where conversion is complete. The tubes are then heated at 100° C for 10 minutes and the optical density of each read at the point previously chosen. A plot is obtained as shown in Fig. VI - 1.

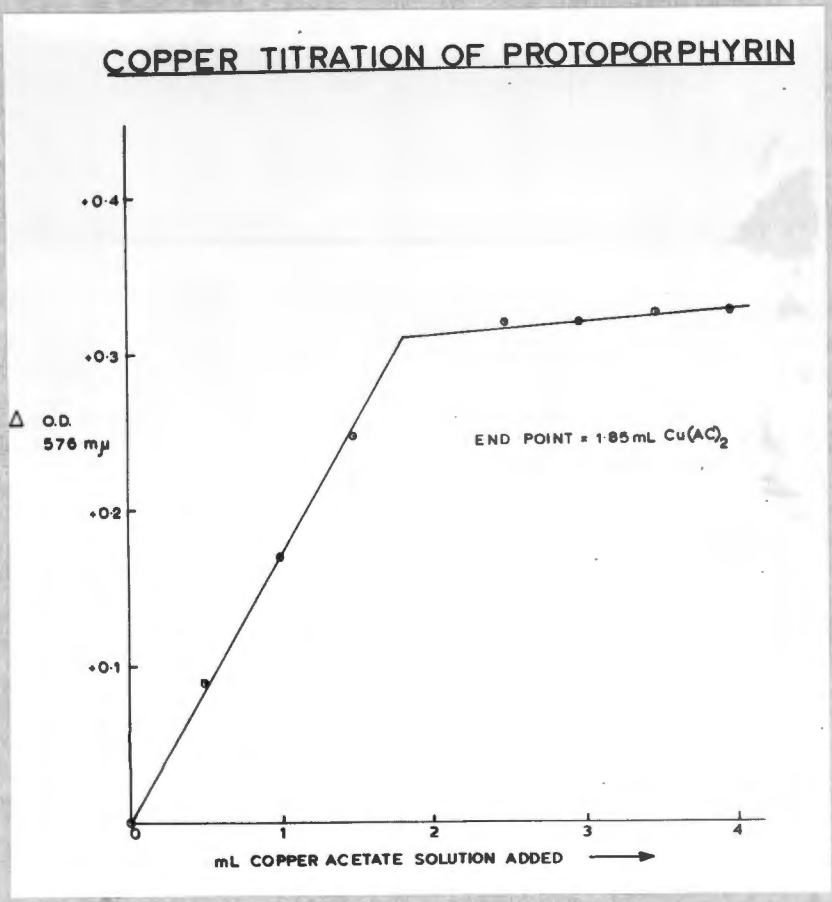


Fig. VI - 1. The estimation of the molar concentration of PROTO in glacial acetic acid by titration with copper.

From the intersection of the two lines is obtained that amount of copper exactly converting all the protoporphyrin present to the copper complex. The molar concentration of protoporphyrin in the original solution can then be calculated.

6. Crystallisation of Porphyrin Methyl Esters.

Concentrated solutions of porphyrin methyl esters are prepared in chloroform. Crystallisation is induced by adding a solvent in which the ester is relatively insoluble. (Referred to as precipitating solvents.) Crystal growth should be as slow as possible.

Optimum crystal morphology has been obtained by adding precipitating solvent to a chloroform solution of ester slowly, and with constant agitation; both solvents are warmed before mixing. The mixture is then stoppered and allowed to cool slowly in a beaker of tepid water. If no crystals form, cooling is continued in the refrigerator. If crystallisation still fails to occur, the total volume is reduced by evaporation in a stream of nitrogen while warming the tube in a water bath, further precipitating solvent can then be added.

Crystallisation should be carried out in a ground-glass stoppered test tube of as small a volume as possible. Filtration should precede crystallisation. All solvents must be distilled before use.

Uroporphyrins crystallise from chloroform on the addition of benzene, hexane, acetone, ether etc.

COPRO I crystallises well from chloroform methanol. This is a useful system because the chloroform can be evaporated on a water bath faster than the methanol so that if crystals do not form after adding methanol and cooling as described above, the concentration of methanol and porphyrin may be simultaneously increased by evaporation.

COPRO III tends to crystallise very slowly. Hexane should be added to a very concentrated solution of the ester in chloroform. Crystallisation may take days or weeks.

Dicarboxylic porphyrins crystallise well from chloroform-methanol as described for COPRO I.

Crystals should be separated from their mother liquors by centrifugation. The mother liquor is removed using a Pasteur pipette and the crystals washed in fresh methanol or hexane, whichever solvent has been used to induce crystal growth.

It has been stated (121) that it is extremely difficult to remove traces of chloroform from crystalline porphyrin methyl esters. It is possible that clathrates form. If crystalline material is to be accurately weighed, it should be kept for a few days in a vacuum dessicator over paraffin wax, or,

preferably, dried in an Abderhalden pistol.

7. Melting Point Determinations.

A sharp melting point is one of the best single criteria of purity in an organic chemical compound. The porphyrins are no exception and the melting points of porphyrin methyl esters have been used since the early days of porphyrin chemistry as criteria of the purity of preparations and as a guide to their nature. Melting points described in this thesis have been obtained using a Reichert Microhotstage of the Koffler type which enables the crystals of porphyrin to be observed under a magnification of up to 100 times. Ordinary or polarised light may be used. The rate of heating is controlled by a Variac type transformer.

There is perhaps some disadvantage in such minute inspection of porphyrin crystals in the process of their melting. Instead of observing gross melting in a group of crystals, each individual crystal is observed. Sintering may be confused with melting although of course there should be no loss of birefringence. Crystals tend to reform in the melt and melting cannot be regarded as complete until all crystals in the melt have disappeared. Under these stringent

circumstances a sharp melting point, when the temperature is raised sufficiently slowly, for example, 2° per minute, is an excellent indication of purity, but it is encountered fairly uncommonly in porphyrins prepared from biological extracts. The porphyrin literature contains frequent reference to melting points given as a single figure. It is not easy to accept these because the most highly purified materials melt over a range of one or two degrees, when the melting point is as high as 200° C. The melting points of various reference porphyrins are given in Appendix B.

B. Spectroscopy.

A. The Visible Region. Absorption maxima and minima of porphyrins may be determined by plotting complete spectra using a spectrophotometer; the spectra are complex and this is an extremely time-consuming operation. Further, few spectrophotometers are equipped with gratings and prisms provide rather low dispersion in the red region where the important alpha band is situated.

The Hartridge Reversion Spectroscope was specially designed for investigating the spectra of

porphyrin compounds. It is able to measure the centre (not the peak unless the band is symmetrical) of absorption bands in the region 500 - 680 mu with quite remarkable accuracy and the instrument is extremely rapid to use.

Very accurate measurements can be made on the alpha band of neutral porphyrin spectra, especially in ether or dioxane solutions, and small alterations in the positions of this band provide useful information in analytical work. Tables of absorption maxima of various porphyrins as measured with the Reversion Spectroscope are given in Appendix B.

B. Infra-red Spectra. Different isomers of different porphyrins possess unique patterns of IR absorption in the finger-printing region of from 750 - 2250 cm^{-1}. In the case of URO's I and III these differences are not adequate to detect mixtures of isomers (27) but isomers I and III of COPRO differ quite considerably in the region 800 - 1300 cm^{-1}. It is not known whether there are significant differences in the spectra of isomers of PROTO although the marked asymmetries which should follow moving vinyl groups about suggest that there would be.

IR spectra can be recorded in solution or in the solid state. Only the latter appears to have been used in published works. (27, 141). Fig. VI-2 shows IR spectra obtained using KCl discs and a Unicam SP-100 double beam recording spectrophotometer. These spectra are in good agreement with those of Falk and Willis (141 which were obtained with porphyrins dispersed in Nujol. Only the record of absorption in the "finger-printing" region has been shown as it was found that differences between these porphyrins studied were negligible in other regions. The absorption maxima at 1740 mu are due to carbonyl oxygen. It is not possible to assign definite significance to any other peaks.

9. Paper Chromatography of Porphyrins.

A. Free Porphyrins. In 1949 Nicholas and Rimington (131) introduced the first solvent system suitable for the chromatography of free porphyrins on paper. The solvent consisted of water and a commercial mixture of 2:3 and 2:5 dimethyl pyridines and the chromatograms were run in an ammonia atmosphere. The Rf values of free porphyrins in this system depended chiefly upon the number of carboxyl

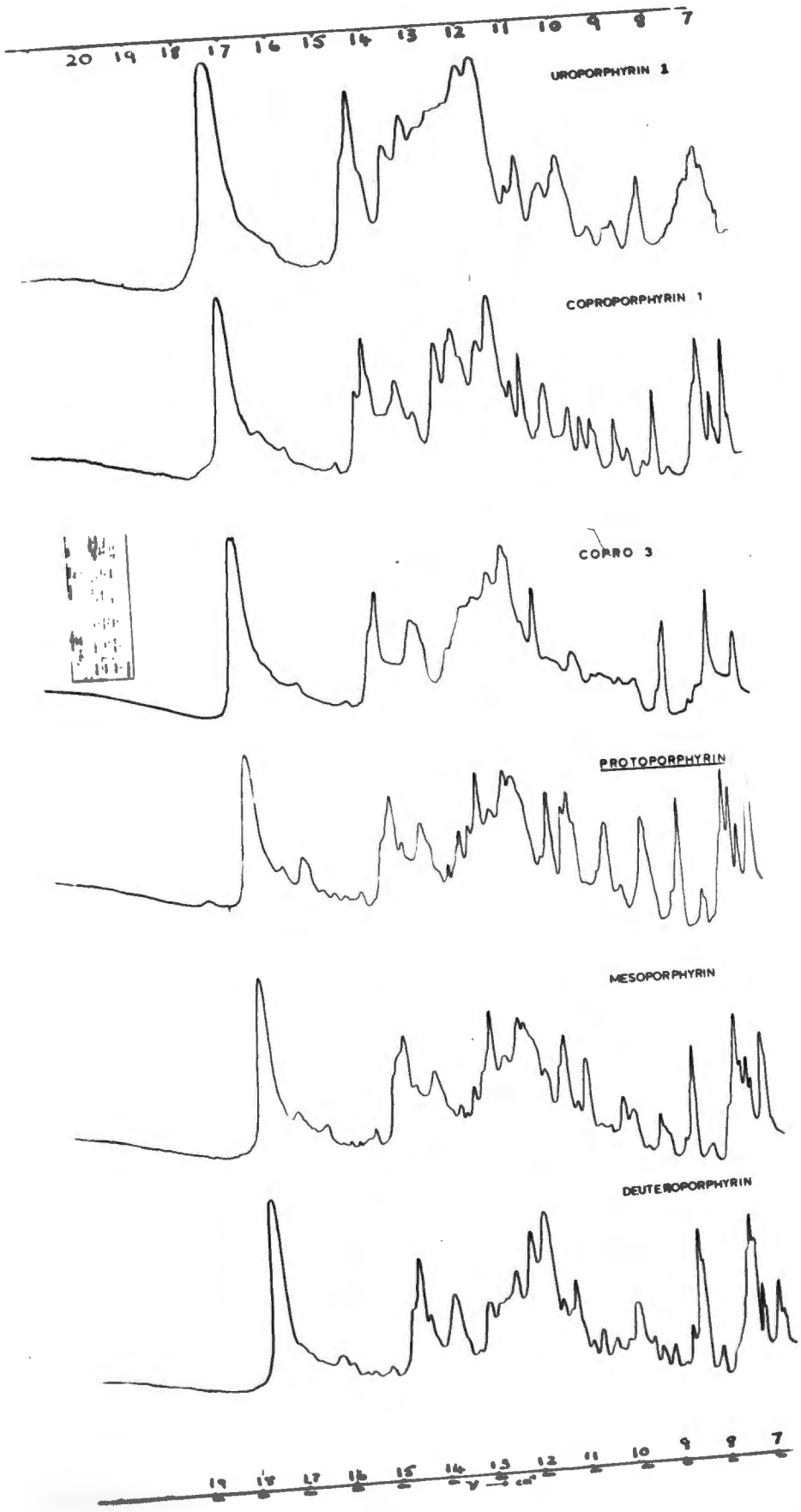

20 19 18 17 16 15 14 13 12 11 10 9 8 7
UROPORPHYRIN 1
COPROPORPHYRIN 1
COPRO 3
PROTOPORPHYRIN
MESOPORPHYRIN
DEUTEROPORPHYRIN
19 18 17 16 15 14 13 12 11 10 9 8 7

Fig IV-2

groups. The method was improved by Eriksen (132) who introduced 2:6 dimethyl pyridine in place of the previous isomers which were partly immiscible with water. Eriksen employed the convenient "ascending" technique. In 1958 the versatility of the method was extended when Eriksen showed that if the ratio of water to lutidine was changed from 3:5 to 2:5 that the method caused good separation of copro-porphyrin isomers, and that isomers I, II and III could be separated. Isomers III and IV did not separate. In 1961 Rimington showed that meso-porphyrins I and IX could be separated using water to lutidine in the ratio 1:5.

A method for the paper chromatography of free porphyrin acids has been described (133) using neutral salt solutions, but this method does not compare with the lutidine method as the quantity of porphyrin which can be applied to paper is small and streaking tends to be annoying.

B. Esterified Porphyrins. A technique has been described for the separation by paper chromatography of porphyrin esters on the basis of the number of carbmethoxy groups. (101). Two methods are documented which separate the I and III isomers of URO

and COPRO esters respectively (102,103). Care must be taken in using any one of these 3 systems that the capacity of the paper is not overloaded and the two techniques for isomer separation lead to anomalous results unless great care is taken in this respect. Both methods for isomer separations appear to depend upon the relative insolubility of the series I isomer esters in organic solvents, but this property must be exploited at exactly the right concentration. Further, the Falk-Benson technique for separating URO isomers requires the use of dioxane; this requires considerable purification prior to use and has very limited stability. Neither of these techniques has been used in this study. The Chu and Chu technique for coproporphyrin isomer separations was found to be inferior to that of Eriksen. The Falk-Benson technique leads to considerable difficulty when 7 carboxyl porphyrin (pseudo-uroporphyrin) is present, and the procedure followed in isomer analyses of URO and "PSEUDOURO" has been to decarboxylate and estimate the isomer content of the resulting copro-porphyrins.

C. Details of Technique.

(i) Lutidine Chromatography, System 1.

(To separate free porphyrins on the basis of the number carboxyl groups.)

Chromatography is carried out at room temperature in closed, light-proof glass tanks. The solvent used is 2:6-lutidine 5 parts, water 3.5 parts. The atmosphere is derived from a 100 cc beaker containing 5N ammonia. Pieces of Whatman's No. 1 filter paper (see below for details of preliminary washing required) 9" x 11" are spotted with porphyrin containing solution, 1 inch from the 9 inch border. The paper is dried in a current of warm air and stapled so as to form a cylinder which stands in a petri dish on the bottom of the glass cylinder. No time need by allowed for equilibration of the atmosphere in the tank. Solvent is placed in the petri dish and after 12 hours the paper is taken out, the solvent front marked, the paper dried in warm air, and examined in ultra violet light. The capacity of the paper is extremely high. Porphyrin may be applied in solutions containing 1 microgram each of uroporphyrin, coproporphyrin and protoporphyrin per microlitre of solvent. Concentrated (25%) ammonia solution is a suitable solvent for porphyrin mixtures containing predominantly uro-

porphyrin but pyridine must be used if the porphyrin present is predominantly dicarboxylic. For mixtures containing equal quantities of uro- copro- and dicarboxylic porphyrin, a 1:1 mixture of concentrated ammonia solution and pyridine usually works well. Dilute HCl is a good solvent for free porphyrin but if porphyrin is applied as the hydrochloride, anomalous Rf values are obtained and coproporphyrin runs as a double spot.

Quantitative Aspects. Initial attempts to recover porphyrin quantitatively from paper after lutidine chromatography were very unsatisfactory. Dicarboxylic porphyrin in particular was recovered in yields of from 5 to 10%. It was found that this could be overcome by washing the paper thoroughly before use in 1.5 N HCl, rinsing the paper thoroughly in distilled water until the washings were neutral, and drying it in a warm air oven at 100°C for one to two hours. After this treatment, which presumably removes metallic impurities, recoveries have been, for a chromatographic procedure, fairly good, on condition enough porphyrin is applied at the starting point.

(ii) Experiment: To determine the recovery from paper following separation of porphyrins by lutidine chromatography.

Solutions of methyl esters in chloroform were prepared of uroporphyrin I, coproporphyrin III and protoporphyrin IX. 0.5 cc of each was dried and hydrolysed for 24 hours in 1 cc of 7.5 N HCl. 4 cc of water was added and the solution diluted ten times with 1.5 N HCl. The optical densities and concentrations were:-

	O.D.	ug/cc
uroporphyrin	0.875	1.57
coproporphyrin	0.770	1.15
protoporphyrin	0.435	0.923

Into 6 B-14 glass stoppered test-tubes were pipetted in turn 0.5, 0.4, 0.3, 0.2, 0.1 and 0.05 cc of each ester solution. The ester mixtures were hydrolysed with 7.5 N HCl for 24 hours and the acid dried off under vacuum over KOH. 100 uL of a 1:1 mixture of NH_4OH (25%) and pyridine was added to each test tube and the free porphyrin acid-hydrochlorides dissolved with vigorous swirling. ("Rotomix.") 10 uL of each of the 6 concentrations was applied to a 2.5 cm line on Whatman's No. 1 paper (acid washed) and the 3 components of the mixture separated by lutidine chromatography (system 1) as described.

The chromatogram was dried at 40°C, the fluorescent spots marked in pencil and the porphyrin eluted into 5.0cc of 1.5N HCl. (The paper was macerated by shaking vigorously in B-14 glass stoppered test tubes with small glass balls.) The optical density readings and the % recoveries are tabulated (Table VI-1). Optical densities have been corrected for background absorption. (Sect. 5-A). No claim is made that this is a precise technique. It does, however, allow information of limited quantitative significance to be obtained from chromatographic analyses where previously visual inspection was the only means of assessing the relative concentrations of fractions. Time has not yet permitted a more rigorous evaluation of either quantitative lutidine chromatography or electrophoresis.

Table VI - 1.

Vol.of Ester Solution (cc)	0.5	0.4	0.3	0.2	0.1	0.05
URO O.D.	0.86	0.66	0.48	0.30	0.16	0.054
% Recovery	98	96	92	86	94	62
COPRO O.D.	0.59	0.48	0.34	0.21	0.12	.079
% Recovery	77	77	73	68	76	100 (?)
PROTO O.D.	0.17	0.19	0.11	.087	0.10	0.035
% Recovery	38	55	41	50	115(?)	77

Lutidine Chromatography System II - The Separation of Coproporphyrin Isomers.

Technique is similar to the above. The same apparatus is used and coproporphyrin dissolved in ammonia or ammonia-pyridine is applied in the same fashion to Whatman's I paper. The atmosphere in the tank however, is derived from a small beaker containing 25% (approximately 13.5 N) ammonium hydroxide. The solvent is 2:6 lutidine 5 parts, water 2 parts. After the paper has been placed in the tank together with the beaker of ammonia, 20 minutes is allowed for equilibration of the atmosphere in the tank before solvent is run into the Petri dish. Again a run of approximately 12 hours is satisfactory. The mobilities of the coproporphyrin depends to some extent on temperature, and in cold weather the separation of isomers is less good than on warmer days.

Quantitative Aspects. Whatman's No. 1 paper need not be washed prior to use for coproporphyrin isomer separations, and the technique of isomer analyses is readily quantitated by a fluorometric method. After chromatographic separation is complete, the paper is dried at 60° for about 20 minutes. The

spots are then marked in pencil, cut out, and placed in B-14 ground glass stoppered test tubes. To each test tube is added 5 ccs of 1.5 N HCl and some half dozen glass beads. (BDH). The tubes are stoppered and shaken violently until the paper has been entirely disrupted. After centrifugation the intensity of fluorescence in the supernatant fluid is measured. Fluorescence due to porphyrin eluted from the different spots is summated and the percentage fluorescence due to each calculated. Figure VI-3 shows the precision of this method.

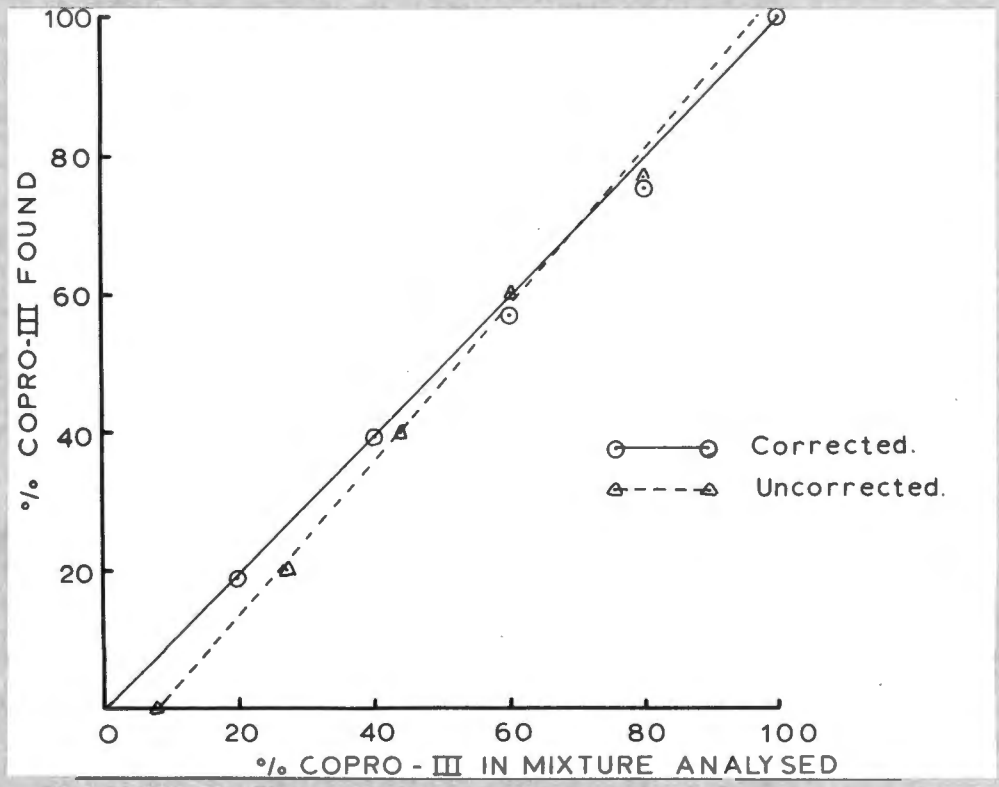


Fig. VI-3. The analysis of COPRO isomers.
In the corrected plot allowance has been made for 7% of COPRO III which contaminated the "pure" COPRO I.

This figure shows two plots, corrected and uncorrected. In the corrected plot allowance has been made for the fact that the analysis showed the standard coproporphyrin I solution to contain 7% of Series III isomer.

Note. Rf values have not been mentioned in connection with lutidine chromatography. It has been found in the case of System I where porphyrins separate according to the number of carboxyl groups, that Rf values depend greatly upon the concentration of ammonia in the tank which is not easily controlled. It is far preferable, with each run, to analyse a known mixture of uro- , copro- and protoporphyrins. Similarly in the technique for separation of coproporphyrin isomers (system II) known coproporphyrins I, II and III should always be chromatographed simultaneously with unknown mixtures.

B. Chromatography of Esterified Porphyrins.

Chu and Chu (101) have described a solvent system consisting of kerosene 35 parts, chloroform (alcohol free) 30 parts and n-propanol 2 parts (KER-CHLOR-PROP) which separates uro-, copro- and protoporphyrin fairly well on Whatman's I paper, in an atmosphere derived from

the same solvent mixture. The time required for separation is about 3 hours. Similar apparatus to that described above may be used, namely a closed glass jar, a petri dish on the bottom and ascending technique. It is of great importance that the ground glass cover of the jar should fit well. Although separation of porphyrins is largely dependent upon the number of carboxyl groups, there are certain anomalies. Firstly, haematoporphyrin moves at approximately the same rate as uroporphyrin. As mentioned elsewhere, mixtures of haematoporphyrin prepared by treating haemin with hydrogen bromide and acetic acid are not homogenous, and separate on this system into at least 4 components, the fastest moving at the same rate as uroporphyrin, which has an Rf of about .25. Also, protoporphyrin runs at a slightly different rate from meso- and deuteroporphyrins. The differences in Rf values are not sufficient to use this system for the separation of protoporphyrin from meso- and/or deuteroporphyrin, but if a porphyrin mixture is suspected to contain deuteroporphyrin contaminating protoporphyrin, this can be detected by inspecting the shape of the proto spot. No attempts have been made to quantitate this technique. Care must be taken not to overload the paper, and the capacity is approximately a tenth that of the

lutidine system. Free porphyrins remain at the point of application.

10. The Electrophoresis of Free Porphyrin Acids.

Electrophoretic separation of free porphyrin acids was first described by Papastamatis and Kench (135). Uroporphyrin, coproporphyrin and protoporphyrin were separated using agar gel and a phosphate buffer at pH 8.0. Subsequent papers (136) have described similar separations using various buffers and paper as the supporting medium. Lockwood and Davis (106) have recently described the semi-quantitative analysis of porphyrin containing mixtures using Whatman 3MM paper and 0.04 M Na_2CO_3 with 0.0001 M EDTA as electrolyte.

Electrophoresis is chiefly useful for separating porphyrins with more than four carboxyl groups on the basis of charge alone, i.e. there is no difference in the mobility of isomers as occurs in 2:6-lutidine chromatography. In particular, electrophoresis separates clearly 7- and 8- carboxyl porphyrins. (See Fig.VII-7)

Experiments with agar and agarose as the diffusion barrier for column electrophoresis have given separations of 7- and 8- carboxyl porphyrins inferior to those obtained

on Whatman's 3MM paper. The mobility of both fractions was extremely high and some separation appeared to occur but subsequent analysis of the "separated" fractions using paper electrophoresis did not confirm this.

A. Technique. The method of Lockwood and Davis (loc.cit.) is used but porphyrin is applied to the paper before wetting it with electrolyte. The paper on either side of the line of application of the sample is wet with electrolyte to within ½" of the sample. The electrolyte gap closes by capillarity. The voltage gradient is 5-6 v cm^{-1} and 2-3 hours is required for separation. The electrophoretogram may be inspected with UV light during "running" but such exposures should be brief and the electrophoresis box should be protected from light as far as possible. Further information on technique is given in Section 11.

B. Interpretation. In general, the mobility of porphyrins during electrophoresis is only a function of the number of carboxyl groups, but certain anomalies occur and are important. The movement of porphyrins during electrophoresis on paper is probably a fortunate combination of various factors including (i) electrophoresis, (ii) electro-endosmosis and (iii) adsorption

to the supporting material (cellulose.) If increasing amounts of COPRO are applied to paper and electrophoresed it will be found that there is a minimum mobility but not a maximum mobility; the COPRO behaves as though it were being adsorbed to the paper until this becomes saturated. This point is illustrated in Fig. VI-4.

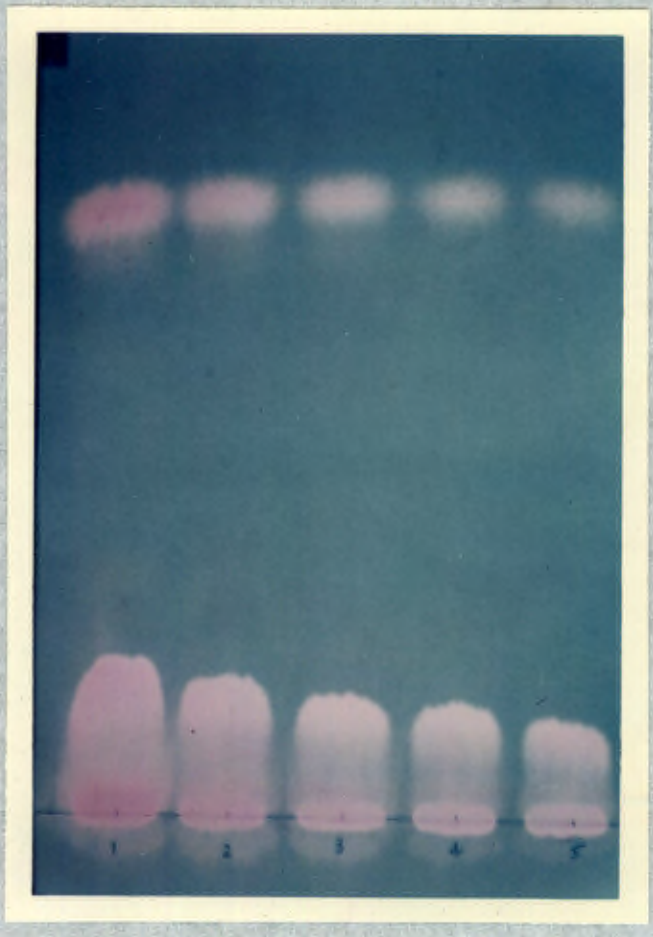


Fig. VI - 4.

Electrophoresis of increasing amounts of URO, COPRO, and PROTO.

Fig. VII - 7 shows that if there is tricarboxylic porphyrin present it will displace the slowest moving COPRO away from the starting line probably through the same mechanism of blocking adsorption sites on the paper. These difficulties do not arise in lutidine chromatography so that all analyses should be by both methods. Fig. VI-5 illustrates analysis of a mixture of porphyrins with 4, 5, 6 and 7 COOH groups performed by chromatography and electrophoresis.

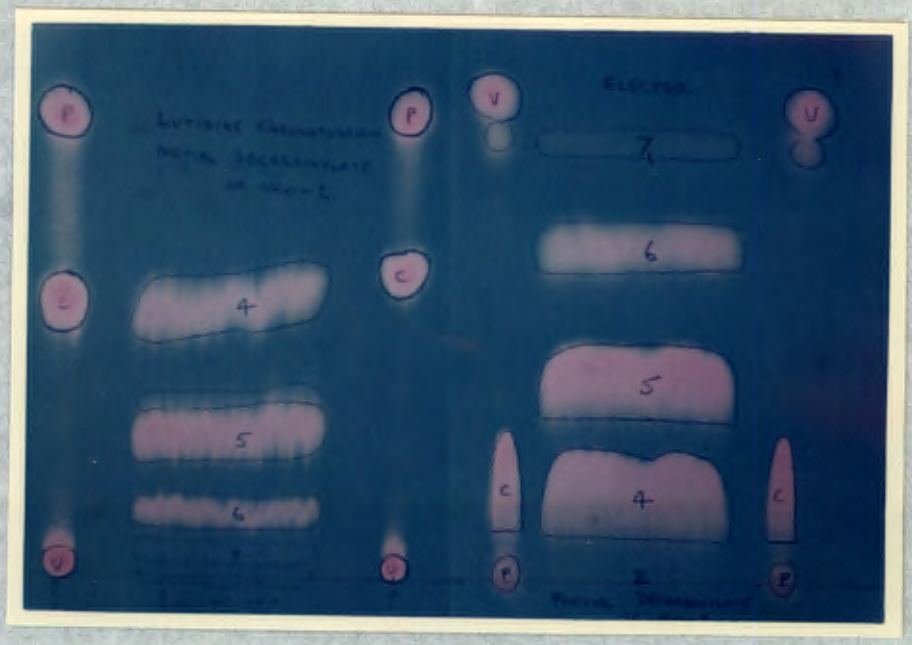


Fig. VI - 5.

Analysis of a mixture of 7, 6, 5 and 4 carboxyl porphyrins by electrophoresis and lutidine chromatography to show the differences in behaviour of porphyrins in these two systems.

Excellent correspondence between the number of carboxyl groups and mobility is observed in both systems. These porphyrins were all of isomer series I;* analysis by lutidine chromatography can be complicated by the fact that some separation of isomer types occurs, affecting in particular the mobilities of the 5 and 6 carboxyl porphyrins.

C. Quantitative Aspects. Section 5-C (ii) gave details of recoveries of porphyrin from paper after separations achieved with lutidine chromatography. The same recovery experiment was performed separating the same mixtures of URO, COPRO and PROTO by electrophoresis and the recoveries obtained are set out in Table VI - 2.

* obtained by decarboxylating URO I for ¾ hr. at 180°C in 0.5 M HCl.

Table VI - 2.

Vol. of ester solution (c.c.)	0.5	0.4	0.3	0.2	0.1	0.05
URO O.D.	.784	.568	.450	.223	.103	.023
% recovery	89	81	86	64	59	25
COPRO O.D.	.695	.530	.436	.227	.123	.047
% recovery	89	81	86	74	80	61
PROTO O.D.	.400	.300	.231	.145	.073	.012
% recovery	92	87	89	84	84	28

In Table VI - 2, "0.1 cc of ester solution" corresponds to roughly a concentration of 0.05 ug/mL porphyrin in the solution applied to the paper. Below this concentration recoveries are obviously very poor.

11. The Measurement of UroPorphyrin in Faeces.

Schwartz et al (27) and Rimington (137) have published techniques for the estimation of URO in faeces. Watson (17) has stated that such estimates may be "decisive" in diagnosing "porphyria cutanea tarda." Despite the significance attached to this fraction, Watson has not published the evidence that it is uroporphyrin which he measures. In later publications the fraction is referred to as "uro-type

porphyrin", indicating presumably mixtures of porphyrins with more than 5 or 6 (COOH) groups.

Watson extracts faecal porphyrin exhaustively with ethyl acetate and glacial acetic acid; uroporphyrin is washed from this with 3% sodium acetate. The faecal residue is then further extracted with 10% NH_4OH until extracts are no longer fluorescent in UV light. These extracts are combined with the sodium acetate washes and uroporphyrin recovered by chromatography on alumina.

Rimington's technique is similar but the initial faecal extraction is performed with ether rather than ethyl acetate and the recovery of uroporphyrin from the aqueous phases is with cyclohexanone at pH 1.5.

Neither method proved satisfactory. The ammonia extracts of faeces are difficult to work with, extraction of fluorescent material is relatively inefficient and the volume of extract is large. Much non-porphyrin pigment is extracted and some of this tends to be colloidal. Chromatography on alumina as described in Watson's method, largely eliminates this brown pigment but not the cyclohexanone extraction of Rimington. From a faecal specimen of a patient with S.A. genetic

porphyria in remission the equivalent of 98 ug/gm dry weight of uroporphyrin was obtained using Watson's method; this proved on lutidine chromatography to be almost entirely coproporphyrin with some dicarboxylic porphyria.

It was therefore concluded that this technique does not ensure that porphyrin measured as URO is in fact uro- or even "uro-type" porphyrin. It is not denied, however, that when uro- (or "uro-type")porphyrin is present in the faeces that this will appear in the eluate off alumina.

Reasons for Difficulty in Measuring Uroporphyrin in Faeces.

(1) In quantitative extraction techniques the chief physical property of the porphyrins made use of in purification is transfer from dilute HCl to an organic solvent and the reverse. In the case of uroporphyrin the organic solvents suitable are cyclohexanone, an n-butanol - ethyl acetate mixture and amyl alcohol; petrol ether must then be added before porphyrin can be extracted with acid. These solvents dissolve so much other organic material that the aqueous to organic transfer results in little purification.

(2) Uroporphyrin, because of its highly polar nature, is strongly adsorbed to various surfaces and it tends to remain adsorbed to faecal residues during extraction.

(3) There is a negligible amount of uroporphyrin in normal faeces; in symptomatic porphyria when a marked increase in uroporphyrin excretion occurs, this is chiefly into the urine and in genetic porphyria in remission it is doubtful whether the amount of uroporphyrin exceeds normal. Great sensitivity is thus required in a method measuring faecal uroporphyrin.

The Method Developed for Estimation of Faecal Uroporphyrin.

Eight and seven carboxyl porphyrins can only be separated from each other and from other porphyrins quantitatively on a micro-scale using chromatography or electrophoresis. The final quantitative step thus chosen was a modification of Lockwood's technique of quantitative electrophoresis of porphyrins.

Porphyrin was extracted in two stages; firstly, an ethyl acetate extract was made followed by recovery of uroporphyrin with other residual porphyrins after esterification.

Details. Between 5 and 10g. of wet faeces is weighed into a B-24 glass-stoppered test tube. 2 glass marbles are added and with violent shaking porphyrin is extracted into ethyl acetate and glacial acetic acid mixed in the ratio 6:1. After 20 minutes of shaking by hand or mechanically, the tube is centrifuged and the solvent is poured off. Fresh solvent is added. Extraction is repeated 6 times or until only a faint blue fluorescence remains. One extraction is now performed with ether which is added to the ethyl acetate and the faecal residue is allowed to dry.

5% H_2SO_4 in methanol is added to the test tube containing the dry faecal residue and this is left for 24 hours after vigorous agitation. The methylating mixture is filtered off and the residue repeatedly extracted with fresh 5% sulphuric acid in methanol until extracts are non-fluorescent.

Porphyrin is transferred from the ethyl acetate - acetic acid - ether mixture into 1.5 N HCl. The completeness of extraction must be judged in UV light. Fluorescence may remain in the organic phase while the acid layer is non-fluorescent if chlorophyll degradation products are present. The 1.5 N HCl extract is dried

under high vacuum in a rotary drier and the residue redissolved in 5% sulphuric acid and methanol. Methylation may be presumed complete after 24 hours.

Esterified porphyrin in the two fractions described is transferred to chloroform. Details of the transfer have already been given. Should emulsions form when the esterified faecal residue is transferred to chloroform, these must be separated by centrifugation. The chloroform solutions are dried and each ester fraction redissolved in 10 cc volumes of fresh chloroform with 1% (v/v) of methanol as preservative. These solutions must be stored so as to avoid evaporation.

Aliquots of the chloroform solutions of the extracted esters are transferred to B-14 glass-stoppered test tubes, dried, redissolved in 7.5 N HCl and left in the dark for 24 hours. The acid is evaporated in a vacuum desicator over solid KOH. A vacuum better than 1 mm of mercury is desirable.

A critical step in this quantitative method is securing complete re-solution of the free porphyrin acids. Dilute HCl is unsuitable. (See under Lutidine chromatography.) Dicarboxylic porphyrins are sparingly soluble in strong (25%, about 13.5 N) ammonia solution

and uroporphyrin is relatively insoluble in pyridine. In practice, most porphyrin mixtures will dissolve in one or other of these solvents or a mixture of equal parts of pyridine and ammonia. Pure ammonia should not be used for mixed faecal porphyrin residues, but if a significant fraction of uroporphyrin is anticipated, the pyridine-ammonia mixture should be used.

When solution of the hydrolysed porphyrins has been obtained in an accurately known volume of solvent (usually 0.1 cc) an aliquot of this is spotted onto dry Whatman's 3MM paper. 0.02 cc can be applied as a line 2.5 cm. long. Electrophoresis is by the method of Lockwood previously described.

After the electrophoretic strip has been dried in a warm-air oven at 45°C, the fluorescent areas are marked in pencil, cut out and placed in B-14 glass-stoppered test tubes each containing 5 cc of 1.5 N HCl. About six $\frac{1}{8}$" glass bals (B.D.H.) are added and the tubes shaken vigorously to fragment the paper. After centrifugation Soret band absorption in the centrifugate is measured spectrophotometrically. The corrections of Rimington and Sveinsson are applied. Fluorimetry may be used when concentrations of porphyrin are too low for

spectrophotometry, but at these levels low overall recoveries are obtained.

Lutidine chromatography may also be used to separate porphyrins prior to estimation. 3MM paper is less suitable than Whatman's No.1 or No.2, and to these papers only 0.01 cc of porphyrin solution can be applied per 2.5 cms.

A Recovery Experiment. The porphyrin content of a specimen of normal faeces was measured by the Holti method. The copro content was 26 ug. and proto 25 ug per gram dry weight.

Separate solutions of uro- (1.38 mg), copro- (2.25 mg) and proto- (2.24 mg) porphyrins were prepared in 1.5 N HCl. These were combined, the acid rapidly neutralised with saturated sodium acetate and this solution added to 32 g (dry weight) of the above-mentioned faecal specimen in a Waring blender. The stool and porphyrin were homogenised and the homogenate transferred to an evaporating dish. The excess fluid was dried off in vacuum over calcium chloride. (P_2O_5 should have been used, drying was very slow). Porphyrin was then extracted by the method just described.

If it is assumed that the Holti method provides an accurate estimate of total ether soluble porphyrin, and that only traces of uroporphyrin occur in normal faeces, then:

(1) the recovery of ether soluble porphyrin was 46%

(2) the recovery of uroporphyrin was 32%

Only 5% of the uroporphyrin (isomer I) was extracted in the ethyl acetate fraction.

Recoveries in this experiment were poor. The question remains as to which steps in the manipulations lead to losses and the extent to which these depend upon concentrations of porphyrin present. Because of the rather low level of porphyrin added quantitation by electrophoresis was required to operate at levels at which recoveries by this method are known to be poor. This was the chief reason for low recoveries obtained and would not apply to specimens containing more porphyrin. The concentrations of porphyrin added were:

uroporphyrin ..... 43)
coproporphyrin..... 70) ug/g dry weight faeces.
protoporphyrin..... 70)

12. Determination of HCl Numbers.

To determine HCl numbers a series of known concentrations of HCl saturated with ether are prepared. It is more convenient not, as was the custom, to express the concentration of acid as a percent, but to consider HCl numbers in terms of normalities, and the range of concentrations of HCl required is from 0.01 N to 2.5 N. A solution of porphyrin in ether is then prepared (about 1 ug/c.c.) and saturated with water. Equal volumes of the various strengths of acid and ether solution are pipetted into well stoppered test tubes, shaken, and the two phases allowed to equilibrate. The concentration of porphyrin remaining in the ethereal phase in each tube is now measured spectrophotometrically and a graph prepared plotting HCl concentration versus the amount of porphyrin extracted into the acid.

It is obviously impossible to determine the amount of porphyrin in acid directly as molar extinctions are a function of (H^+) concentration.

Published figures for HCl number of various well-known porphyrins do not agree precisely and the reason for this probably lay in the difficulty experienced by early workers in quantitating the

porphyrin content of the ether. This is easy with a spectrophotometer which can measure the intensity of the Soret band, but such instruments were not readily available before 1945.

The HCl number is a fairly crude estimation and can be supplanted in some cases by distribution coefficients in countercurrent apparatus.

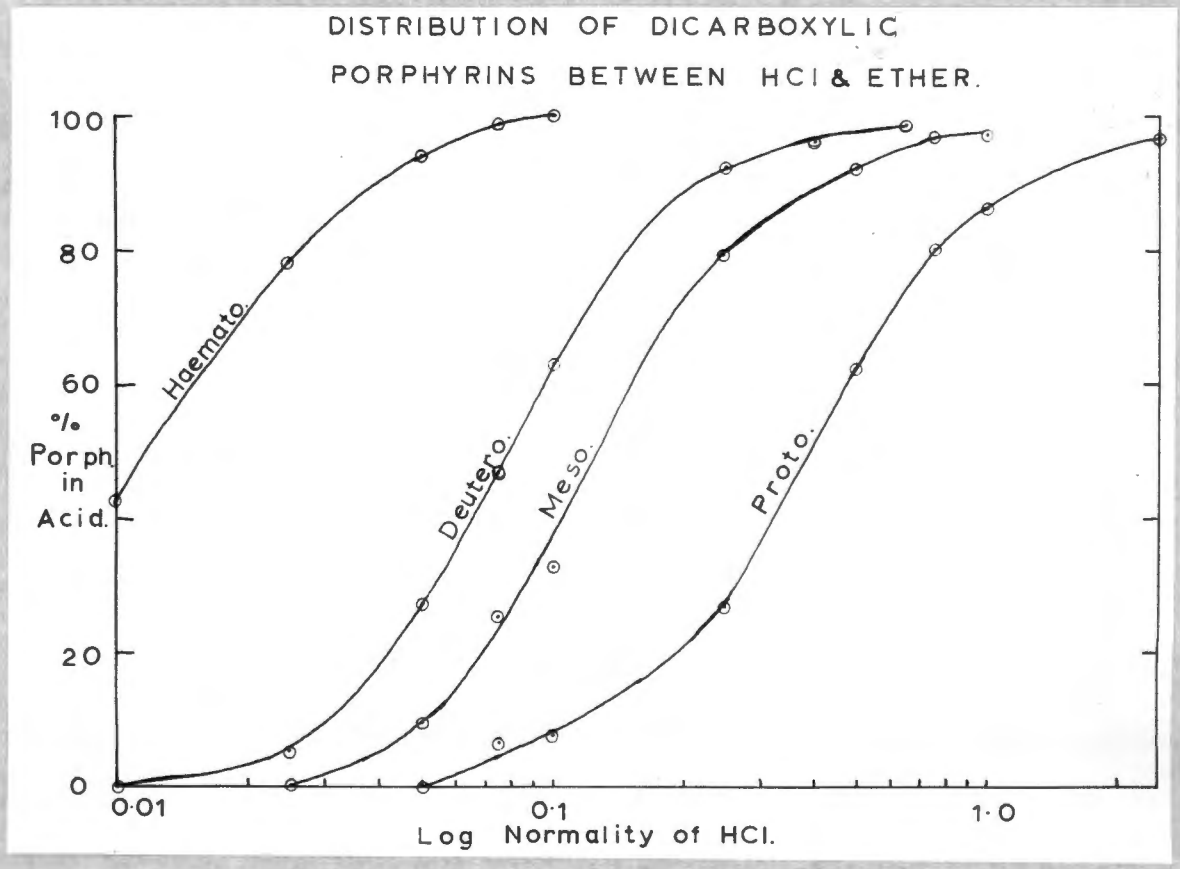


Fig. VI - 6.

Plots from which were derived HCl numbers of various dicarboxylic porphyrins.

Figure VI - 6 shows plots obtained by the above method which enabled the HCl numbers of haematoporphyrin, deuteroporphyrin, mesoporphyrin and protoporphyrin to be determined. These are set out in Table VI - 3 and compared with the results of earlier workers.

Table VI - 3.

Porphyrin.	This work.	Source of Data. Schwartz et al(27)	Zeile and Rau (146)
Proto-	1.85	2.0	1.90
Meso-	0.63	0.5	0.60
Deutero-	0.37	0.4	0.36
Haemato-	0.066	0.1	-

The accuracy of values obtained have been confirmed by the patterns of distribution of the same porphyrins in countercurrent apparatus. (Fig. VI - 7)

13. Counter-current Distribution Technique.

The HCl number has already been defined and the manner of its determination described. This single property, more than any other, has been used in the separation and purification of porphyrins extracted from biological materials.

Differing HCl numbers indicate differing partition coefficients between ether and dilute HCl and thus provide a property whereby porphyrins may be separated using counter-current distribution apparatus. Craig and Post (122) introduced CCD in 1946 and it is rather surprising that there are only four references in the literature to the use of this apparatus in porphyrin chemistry.

In 1953, Granick and Bogorad (123) used a large automatic apparatus to separate various porphyrins produced by Chlorella mutants. They also showed how efficiently a mixture of haemato-,deutero-,meso- and protoporphyrins could be separated. Paul (124) showed that a fifteen tube system could separate haemato-, meso- and protoporphyrins. In 1956, Falk et al (125) used a twenty tube CCD chain to show that when PROTO ester is hydrolysed in acid the hydrolysate contains impurities of a low HCl number. England et al (126) have published a brief account of an investigation into faecal porphyrin in steatorrhoea using CCD.

A. General Principles and Practical Details.

CCD apparatus consists of a train of many tubes which are numbered serially. With the tubes containing

upper and lower phases of immiscible (or partially miscible) solvents the inter-connections are such that transfer of the upper phases to overlie lower phase of the succeeding tubes is accomplished merely by correct rotation of the frame holding the tubes.

The sequence of operation is as follows:-

(i) Each tube is filled with the correct volume of lower phase (or an excess) previously saturated with upper phase.

(ii) The material to be analysed is introduced in solution in upper phase (saturated with lower phase) into the first tube.

(iii) The phases are equilibrated by rocking the apparatus to and fro about 6 - 10 times.

(iv) The phases are allowed to separate.

(v) By the correct manipulation of the handle, upper phase in tube 1 is transferred to tube 2.

(vi) Fresh upper phase (saturated with lower phase) is added to tube 1, and the whole cycle is repeated.

(vii) When the end of the counter-current train is reached, or when sufficient number of transfers has been made, the contents of the tubes is removed from the apparatus.

It is not necessary to determine the contents of each tube in order to plot a distribution curve; the differences between successive tubes are insufficient to warrant this. Practice has been to collect the contents of the odd-numbered tubes in numbered test tubes separately for spectrophotometry (See Section 5 - D.) The apparatus was specially designed with taps on alternate tubes to make this possible.

Three different varieties of CCD apparatus have been used. Initial studies were made using a large-volume apparatus housed in the National Chemical Research Laboratories of the C.S.I.R. in Pretoria. This was unsuitable for the type of work to be described because of its size. A small 35-tube apparatus was then borrowed from the Dept. of Organic Chemistry, University of Stellenbosch. This apparatus (Buhler) was unsuitable because each tube was open to the atmosphere leading to considerable losses of ether by evaporation. The consequent variations in volume of the upper phases made calculation of distribution coefficients inaccurate.

Most of the CCD analyses reported here were performed using 100 "Craig" 10 mL CCD tubes on a frame

made locally. The apparatus was entirely closed and there was no loss of upper phase due to evaporation during the course of distribution. Although the apparatus was entirely manually operated, it became possible, with practice, to perform a 60 transfer distribution, read the samples on the spectrophotometer, plot the distribution curve and wash the apparatus all in the space of 4-5 hours. Speed is of some importance in working with unstable material such as porphyrins.

It is of paramount importance that the solvents used in CCD be pure. In the 100 tube apparatus relatively small amounts of porphyrin become distributed in up to 2 Litres of ether and acid. The fractions are recovered by neutralising the acid, transferring the porphyrin to the ethereal phase and distilling off the ether (after washing it with water.) Impurities as well as the porphyrins become greatly concentrated. In practice, glass-distilled water and distilled peroxide-free ether have been used.

B. Choice of Solvents and Solvent Systems.

Technical difficulties were encountered when introducing material into the counter current apparatus.

It is essential that porphyrin be introduced in a small volume, viz. one, two or three upper phases in the first 1 - 3 tubes. Ideally, the apparatus should be charged by filling only the first tube with material to be separated, but this imposes too severe a limitation on the capacity. Porphyrin is prepared in solution in ether. The ether is washed well with water and then concentrated by evaporation in a rotary drier to the required volume of between 20 and 30 ccs. Without delay the concentrated solution is introduced into the first tubes. Granick and Bogorad suggest dissolving dicarboxylic porphyrin in tetrahydrofuran (THF) to increase the capacity of the first few tubes. They mention that a precipitate forms as the THF becomes diluted and maintain that this does not affect the quality of their separations. Experience has been otherwise; it was found that if precipitates formed they would slowly redissolve over the course of perhaps 10 or 20 transfers, entirely spoiling the separation of fractions.

THF is nevertheless useful as a solvent for introducing porphyrin into the first tube, but care must be taken that precipitation does not occur. Experience has shown that the best technique is to

add 2 - 5 cc of THF-porphyrin solution to the acid phase in tube 1, equilibrate this mixture and then add enough ether to bring the total volume in tube 1 to 20 cc. Pyridine cannot be used because it will neutralise the acid.

Counter current technique has only been used for separating ether soluble porphyrins. It was therefore only necessary to choose a particular strength of HCl with which to partition the porphyrin with ether. It is useful to start by determining the HCl number of the porphyrin mixture to be analysed and to select the concentration of acid which will give a known distribution coefficient of about 1 for the bulk of porphyrin in the mixture to be analysed. It is always possible, after an initial run, to further fractionate concentration bands at one or other end of the counter current apparatus, using different strengths of acid. Fig. VI - 7 shows the distribution of haematoporphyrin, mesoporphyrin, deuteroporphyrin and protoporphyrin after a 60 tube transfer, using the system ether/0.095 N HCl. The separation obtained may be compared with the HCl numbers of the same porphyrins which were given above. (Fig. VI - 6.)

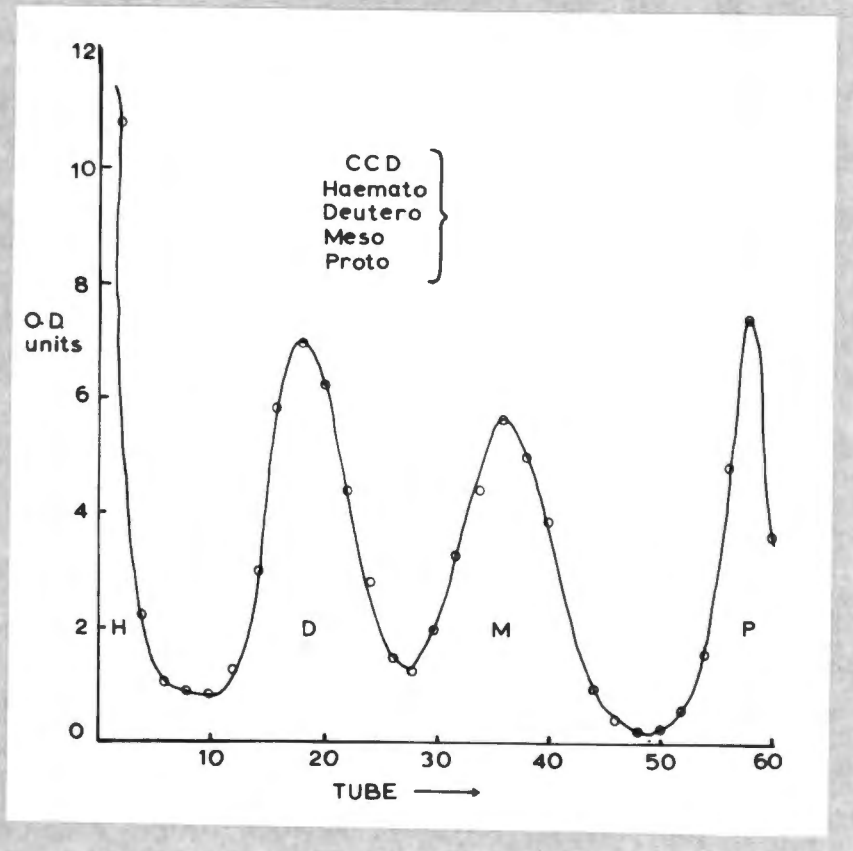


Fig. VI - 7.

Counter current distribution pattern of HAEMATO, DEUTERO, MESO and PROTO after 60 transfers between diethyl ether and 0.095 N HCl.

C. Manipulation of the Results. When a CCD analysis has been completed the quantity of porphyrin in each tube must be determined (that present in upper and lower phases is summated) and the distribution plotted graphically. The method of determining the

porphyrin content of each tube has been dealt with in section 5 above. It is convenient to plot the concentration in terms of optical density rather than amounts of porphyrin as the nature of fractions may be obscure when a graph is plotted.

The information available after plotting the distribution includes:-

(i) the concentration of porphyrin in any given tube

(ii) the tube number (n) of maximum concentration

(iii) the total number of transfer(r).

The partition coefficient, $K = \frac{n}{r - n}$, assuming that the volumes of upper and lower planes are equal. Recalling the relationship given in Chapt.II-5, the HCl number of a given porphyrin can be calculated from its <u>distribution</u> coefficient:

$$\text{HCl number} = 3.65 \times \sqrt{(N_{HCl})^2 \frac{K}{0.5}}$$

Where N_{HCl} is the normality of the HCl used in the lower phase.

In practice it is unnecessary to measure the contents of each tube following CCD. This stage is rather time consuming and it is adequate, after a run of 30 - 90 transfers to read alternate tubes.

The detailed mathematical treatment of results has been dealt with by King and Craig (127). If p and q are the fractional amounts of solute in upper and lower phases so that (p + q) = 1 "p" may be regarded as the probability that a given solute molecule will be transferred. With this statistical basis, the distribution of solute about its maximum (n) follows a Gaussian curve.

The fractional amount of solute present in the n^{th} tube after r transfers is

$$T_{r,n} = \frac{r!}{n!\,(r-n)!} \cdot p^{n}\, q^{(r-n)}$$

which expression, or its simplified form:

$$T_{r,n} = \frac{1}{\left(\frac{2rK}{(K+1)^2}\right)^{\frac{1}{2}}} \; e^{-\left(\frac{x^2}{\frac{2rK}{(K+1)^2}}\right)}$$

(where x denotes displacement from n_{max})

may be used to calculate a theoretical distribution curve and assess any deviations therefrom due to impurities, or non-ideal behaviour of the solute.

14. The Decarboxylation of Uroporphyrins.

The acetic side chains of URO are less stable than the propionic side chains and will decarboxylate if heated to 180° C. in dilute HCl. (The propionic side chains can be decarboxylated by heating with soda-lime.) Recoveries can approach 100% if oxygen is rigorously excluded from the reaction tube. This reaction allows COPRO to be prepared from any porphyrins with more than 4 carboxyl groups and the isomer composition of the original porphyrin can be investigated by using lutidine chromatography (system 2) to analyse the COPRO.

A mixture of equal amounts of URO's I and III was prepared and decarboxylated by the method to be given below. The isomer composition of the resulting COPRO was 52% isomer I and 48% isomer III. Decarboxylation does not therefore appear to alter the isomer composition of mixtures.

Method: The reaction is carried out in a Carius tube of suitable capacity. A 15 ml tube will enable about 3 mg of porphyrin to be decarboxylated, the limiting factor is the solubility of the porphyrin in 7.5 N HCl.

The porphyrin is introduced into the tube as the ester in solution in chloroform or in solution

in a suitable volume of HCl. The ester is dried onto the walls of the tube and dissolved in 7.5 N HCl using 1/25 the final volume of solution to be heated. (Decarboxylation is carried out in 1% HCl) It is not necessary to wait for ester to hydrolyse. The acid is diluted to 1% (0.35 N) and the tube attached to the apparatus shown in Fig. VI - 8.

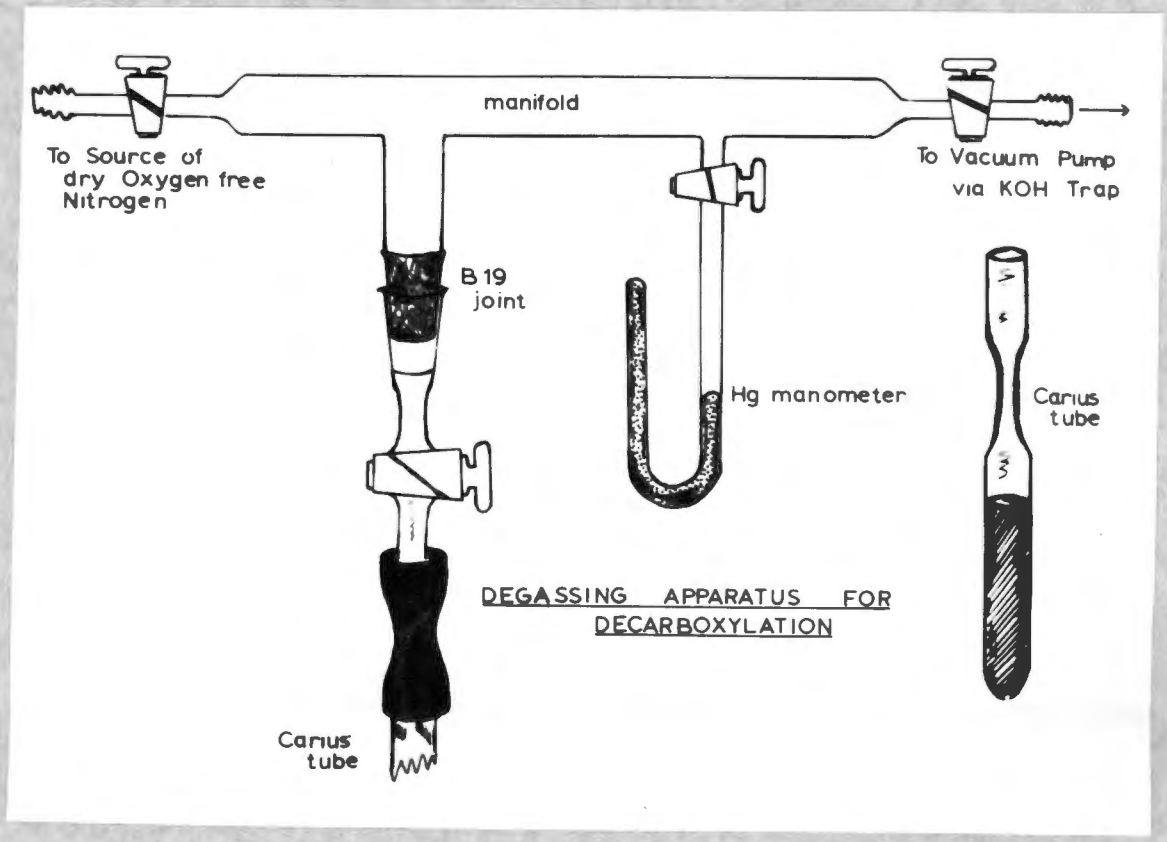


Fig. VI - 8.

After freezing the contents, the tube is evacuated to a pressure of 0.1 mm Hg. After a few minutes the tap leading to the pump is closed and the tube thawed. Purified nitrogen is allowed to fill the system. This cycle is repeated twice; on the third introduction of nitrogen the pressure is only allowed to reach 10 mm Hg. The tap isolating the Carius tube from the manifold is closed and the constriction in the Carius tube sealed in an oxygen flame. The tube is placed in an oven at 180° C. for three hours and allowed to cool. COPRO is transferred to ether after neutralisation of the HCl, the ether is washed and taken to dryness. The porphyrin residue may be taken up in ammonia for chromatography, or esterified, as required.

If due care is exercised, as little as 5 ug. of porphyrin can be decarboxylated to COPRO with recovery of enough material for chromatography.

15. Preparation of Reference Porphyrins.

In the following pages practical details of methods used in the preparation of reference porphyrins are given. Only outlines are provided as much of the information is to be found in the foregoing sections of this chapter.

Physical constants of the reference porphyrins are to be found in Appendix B.

A. Uroporphyrin I. Uroporphyrin I has been prepared for reference purposes on two occasions. The first preparation was from the urine of a cow with congenital erythropoietic porphyria. This animal is the sole surviving member of the herd originally studied by Rimington and Fourie (138). The techniques used were those described by With(139).

URO I was crystallised as the methyl ester from chloroform - benzene. The m.p. was 289 - 291° C and this porphyrin ran as a single spot when electrophoresed, thus indicating freedom from seven carboxyl porphyrin. The second preparation of URO I was from the urine of a patient with acquired porphyria who had received chloroquine. (140) About 200 mgms of mixed seven and eight carboxyl porphyrin containing about 60% isomer I was available. This was crystallised repeatedly from chloroform - acetone and, after seven recrystallisations, a fraction was obtained which melted over the range 289 - 291° C., which decarboxylated only to COPRO I, and which moved as a single spot on electrophoresis. The yield was 35 mgms.

When an attempt was made to determine the molar extinction of this specimen of URO I, it was found to be slightly contaminated with metalloporphyrin. Purification was achieved by precipitation from, and resolution in, dilute HCl on two occasions. The E_{mol} was found to be 5.6×10^5 in 1.5 N HCl. This figure is 7.6% too great.

B. Uroporphyrin III. The conventional source of Uroporphyrin III is Turacin, a copper complex of URO III found in the feathers of birds of the family Musophagidae. Preparation of pure URO III from this source has been described by Nicholas and Rimington (134).

Feathers of this bird were kindly supplied by Dr. Winterbotham, and an attempt made to prepare the free porphyrin. Following the method of Rimington, the feathers were extracted with concentrated sulphuric acid. What was obtained, however, was not pure uroporphyrin III but a mixture of porphyrins with 8, 7, 6 and 5 carboxyl groups, but all of the III isomer series. Small quantities of 8 carboxyl series III porphyrin could be separated by electrophoresis, but attempts to prepare the pure material by column chromatography failed. There are two

possible explanations for this unsatisfactory state of affairs. Firstly, the feathers were old and had been lying about in a drawer for some years. Some decarboxylation may have occurred spontaneously. Secondly, there are species differences amongst the luries, whose feathers contain Turacin. The feathers studied came from Turacus Corythaix, whereas Rimington had obtained feathers from Turacus Indicus.

The URO III used was a gift from Prof. C.Rimington to Prof. L. Bales.

C. COPRO I. Coproporphyrin I contaminated by 7% of COPRO III was prepared from about 3 Kg of calf meconium. Meconium was dried in an oven at 100° C. and then extracted with ether after thorough mixing with glacial acetic acid. The organic phase was removed by decantation. The ether was distilled off and the remaining acetic acid solution of pigment mixed with 10% HCl. Fatty material which precipitated overnight, was filtered off, and the resultant solution was vacuum distilled to dryness. The residue was treated for 24 hours with 5% H_2SO_4 in methanol and porphyrin ester with considerable impurity was then transferred to chloroform.

Crystalline COPRO I.

After chromatography on MgO, crystallisation and recrystallisation from chloroform - methanol, 30 mgm of small birefringent needles were obtained. After sintering at 248° melting occurred in the range 251-4° C. (See opposite page.)

This material behaved in the expected manner when chromatographed on paper using lutidine/water (systems 1, 2) and the system of Chu and Chu (101) for the identification of COPRO isomers, but a small amount of COPRO III was still present. An I-R spectrum (Fig VI - 2) (KCL disc) agreed precisely with that of Falk and Willis (141) and Schwartz et al (27)

D. Coproporphyrin III. The usual source for this porphyrin is an ultrafiltrate of a culture of Corynebacterium Diphtheriae, which has been grown on a medium containing that amount of iron which is optimal for the production of toxin. A large volume of such ultrafiltrate was obtained from the Rietfontein Laboratories of the S.A.I.M.R. After arrival by rail it was found to contain 400 ug/L of ether soluble porphyrin. The porphyrin was adsorbed onto talc after correcting the pH to 3.5 After esterification the COPRO was found to be contaminated by a metal complex, probably copper. Separation

was achieved on a column of magnesium oxide which had to be at least 12 cm long. A sample of COPRO III was prepared with a molar extinction of 4.56×10^5 in 1.5 N HCl. The melting point was typical of this compound; melting occurred first at 125 - 130° C, solidification occurred with formation of birifringent crystals which remelted at 178 - 180° C. Crystallisation was achieved from chloroform-hexane. The crystals were extremely dense, appearing amorphous unless crushed onto a glass slide.

E. Protoporphyrin IX. Preparation by the method of Grinstein (38) is convenient; however, the product is impure. Chromatography on alumina will remove a contaminating green pigment (see below). Chromatography on MgO has been abandoned because of variations in batches of this adsorbent and high losses due to irreversible adsorption.

CCD is the method of choice for purification of crude PROTO ester. Hydrolysis with 25% HCl (24 hours) or saponification with 5% methanolic KOH is followed by CCD between ether and 0.45 N HCl. An experiment was performed in which 2 samples of PROTO ester were hydrolysed by the above methods and distri-

buted separately over 60 tubes. There was a band of porphyrin of low HCl number only in the specimen hydrolysed with acid.

Diazomethane is preferred to strong mineral acid/methanol for the esterification of protoporphyrin because it avoids any treatment with strong acid. Esterification with CH_2N_2 should be followed by chromatography on alumina.

Protoporphyrin is unstable in solution, particularly in chloroform, decomposing to a green pigment of high HCl-number which absorbs at about 660 mu and at 500 mu. (Falk (22) considers formation of this pigment to be enhanced by phosgene present in old chloroform.)

Crystallisation of the dimethyl ester occurs readily from chloroform methanol, the characteristic habit appears to be the "boomerang" shaped crystals shown rather poorly in Fig. VI - 9. The melting point of pure specimens is 231 - 233° C. Falk quotes the single figure 231°. The melting point of 224° given by Schwartz et al (27) though unfortunately accepted during earlier work in this thesis, is definitely too low. The molar extinction coefficient in dilute HCl is discussed in Section 5 above.

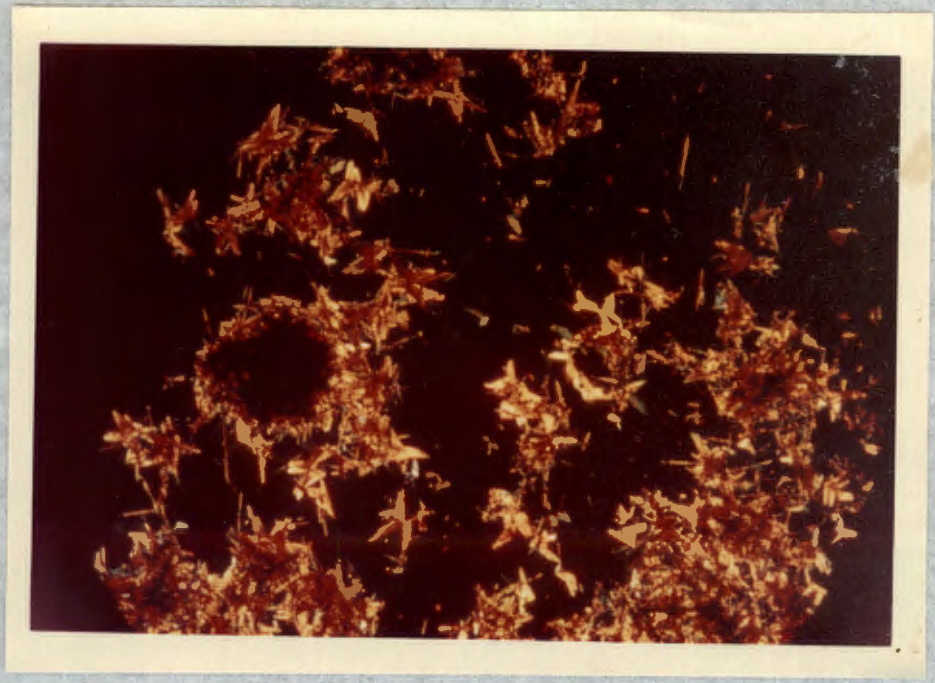


Fig. VI - 9.

Crystals of PROTO IX dimethyl ester viewed in polarised light. (X 100).

F. Deuteroporphyrin IX. Haemin chloride is prepared by the method of Fischer (142). This is converted to deuteroporphyrin by fusion with resorcinol followed by removal of the iron. The reaction to remove the vinyl side chain is conducted under extreme conditions and yields are rather low. The original

method described for removal of iron was that of Fischer who reduced Fe^{III} by adding iron dust to a solution of the porphyrin in acetic acid. Chu and Chu (143) recommended a reduction time of 15 minutes, but this was found to cause extensive loss of porphyrin. Much better yields of deuteroporphyrin were obtained if de-ironing was carried out according to the method of Grinstein, used for the preparation of protoporphyrin dimethyl ester.

The procedure adopted was as follows:

100 mgms. of recrystallised haemin chloride was intimately mixed with 500 mg of resorcinol. The mixture was maintained at 190 - 200° C. for 15 minutes in an oil bath in a lightly-stoppered flask, and then cooled. The reaction mixture was dissolved in 100 cc of absolute methanol and 4 g of ferrous sulphate added. Dry hydrogen chloride gas was bubbled through until the solution became warm, and, at the same time, turned purple. After standing aside on the bench to cool, the porphyrin ester was transferred to solution in chloroform and evaporated to dryness. The crude ester was taken up in chloroform and chromatographed on magnesium oxide. The yield was 30.9 mgms. A small quantity of deutero-haemin ran ahead of the porphyrin

on the magnesium oxide column. Physical properties of the porphyrin are given in the appendix. The melting point (226° C.) is higher than previously reported figures. It is suggested that this is due to a purer preparation resulting from the improved technique for removal of iron.

G. Mesoporphyrin IX. Reduction of the vinyl groups of PROTO to ethyl groups may be accomplished using either hydriodic acid (33) or H_2 with a palladium catalyst (34). Both methods have been used. Although losses appear to be much less when reduction is carried out catalystically a purer sample of mesoporphyrin was obtained from the HI reduction method.

(a) HI reduction 30 mg of PROTO ester was intimately mixed with 300 mg of ascorbic acid and dissolved in 6 ml of glacial acetic acid. 1.65 ml of HI (S.G. = 1.94) was added and the mixture boiled over a naked flame for ¾ minute. The reaction mixture was cooled in water and added to 150 ml of ether. The ether was washed twice with an aqueous solution of 10% acetate with 3% sodium sulphite and then three times with water. The porphyrin was extracted into 0.75 N

HCl, the acid neutralised and the porphyrin taken back into ether. The ether was evaporated off, 50 ml of methanol was added and dry HCl gas passed until the solution became warm. Mesoporphyrin ester was transferred to chloroform, chromatographed on MgO and crystallised from chloroform - methanol.
m.p. 212 - 214° C.

(b) H_2/Pd reduction: 21 mg of PROTO ester was dissolved in 2.0 ml of 99-100% formic acid (BDH) and 30 mg palladised charcoal (10%) added. The mixture was maintained at 60° C for 10 minutes while H_2 bubbled slowly through. Ether was added and the acid neutralised with saturated aqueous sodium acetate. Further purification was as described above.
m.p. 195 - 210° C.

CHAPTER VII.

STUDIES OF PORPHYRIN EXCRETION.

1. Introduction - The Evolution of Technique.

The studies described below evolved over the period January 1961 to September 1963. Initially the intention was to perform analyses of a type previously reported in the literature but not previously reported on the varieties of porphyria encountered in South Africa. To isolate and characterise a porphyrin appeared to be an end in itself. Only when this aim had been achieved was it realised how meaningless the information was divorced from quantitative aspects of porphyrin excretion.

Quantitative records were therefore kept of extraction and analytical procedures; measurements were made of porphyrin concentrations using physical constants which were most appropriate. However, a high degree of precision was not possible when dealing with "unknown" fractions. Much of the analysis was chromatographic. Fractions obtained from column chromatography could be measured and column chromatography on alumina is almost quantitative; at first, however, paper chromatography and electrophoresis were

only qualitative. Relative amounts of various porphyrins present in mixtures were judged by visual inspection of the fluorescent spots and a photographic record of paper strips was kept. At the time this procedure appeared the best available, but it is no longer defended.

It was easy for the experimenter to become convinced of certain findings after inspecting chromatograms but, even with photographic records, difficult to present such data to others.

Attempts were made to place lutidine chromatography and electrophoresis on a quantitative basis. This work was fruitful; recoveries were not complete but good enough to provide useful data, and the attempts to increase recoveries resulted in improvements in technique. The quantitative estimation of coproporphyrin isomer ratios proved to be particularly precise. (Fig. VI - 3.) Early impressions gained by inspecting chromatograms in UV light were confirmed and the information could be more acceptably presented.

Precision for the results of quantitative electrophoresis is not claimed. Reference to the relevant section of Chapt.VI makes it clear that recoveries are

a function of the amount of porphyrin applied; in analysing unknown mixtures this cannot ways be controlled. Conclusions have, however, been reached with a clear appreciation of the limitations of the methods employed.

In this chapter the records of analytical work on excreta from various patients with porphyria and from 3 normal subjects will be presented. With the exception of normal subject G.S. (the author) all patients studied were in-patients at Groote Schuur Hospital, receiving either the general ward diet or a diabetic step 6 diet. Urines were collected in amber Winchester bottles and porphyrin extracted as soon as possible after receipt. Faecal specimens were stored deep frozen if delay in analysis was unavoidable.

Quantitative measurement of urine ALA, PBG and porphyrins and faecal ether soluble porphyrins were made using standard methods already referred to. (151, 115, 16.) Dry weight of faeces were determined after drying an aliquot for 4 hours at 105° C.

The 24 subjects studied may be grouped as follows:-

Section.	Disease.	Number of patients studied.
2	Normal	3
3	Acquired Porphyria	6
4	S.A.Genetic (variegate) porphyria	10
	A. Remission - 5	
	B. Acute - 5	
5	Swedish Genetic (acute intermittent) porphyria	4
	A. Remission - 4	
	B. Acute - 0	
6	Erythropoietic protoporphyria	1

The laboratory techniques which have been employed are described or referred to in Chapter VI. Column chromatographic procedures are somewhat involved; these are set out diagrammatically in the sections on cases 4-A(v) and 4-B(i).

Brief case reports are given in Appendix B. It was not considered necessary to describe the clinical status of the patients at length; biochemical data sufficient to justify diagnosis of a particular type of porphyria has been given, and enough clinical data to indicate that there was no factor present which might modify the presentation of the porphyrin disease.

2. Normal Porphyrin Excretion.

A. Urine. Porphyrin excretion in normal urine has been adequately described by others (chapt.IV)

B. Faeces. Table VII - 1 lists quantitative aspects of the excretion of porphyrins and porphyrin precursors by three normal subjects.

Table VII - 1.

Case:	F.B.	J.K.	G.S.
Urine (mg/L)			
ALA	0	0.83	
PBG	1.5	0	normal
URO	0	0	
COPRO	.037	.094	
Faeces ug/g d.w.			
COPRO	.021	.028	.036
PROTO	.024	.047	.020

(i) (F.B.) European male, 23 years. Fractured femur reduced 6 weeks before study. Not a heavy drinker and had not taken alcohol since admission. The serum proteins and flocculation tests of liver function were normal.

100 gms (dry weight) of faeces was extracted and the crude ester mixture chromatographed on alumina to yield 3 fractions.

Table VII - 2.

Fraction.	mg.	($CHCl_3$)	(COOH)
1	3.0	627	2
2	.37	622	4
3	.92	620(diffuse)	4
	4.3		

Fraction 1. Chromatographed on MgO to yield a small component with alpha band at 621 mu (chloroform) and a major fraction with alpha band at 629 mu (chloroform).

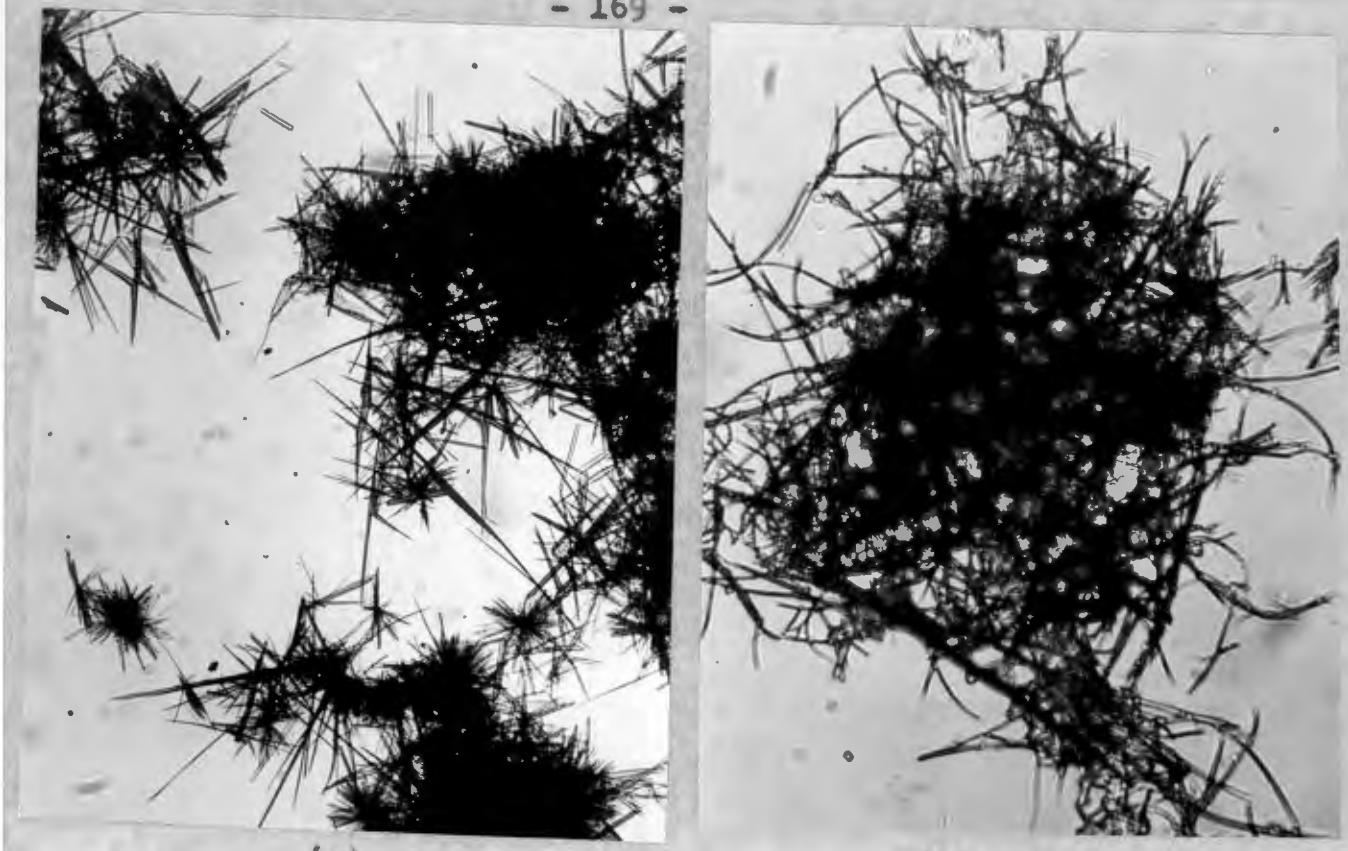
This was crystallised (m.p. 218 - 222° C) and recrystallised (m.p. 222 - 224° C.) Crystals of protoporphyrin (as judged by behaviour on MgO, absorption spectrum and m.p. of crystals) are shown in Fig.VII-1a.

Fractions 2 and 3. Both were $(COOH)_4$ but (3) was partly metalloporphyrin - hence the alpha band shift. This was dissociated with conc. H_2SO_4, re-esterified and examined by lutidine chromatography (system 2).

Found: about 70% isomer I

30% isomer III.

Crystallised, (Fig.VII-1b) mp.240-250°C.



(a) (b)

Fig. VII - 1.
Crystalline porphyrin from faeces of a normal subject.
(a) PROTO. (b) COPRO I.

(ii) (J.K.) African. Receiving treatment for cervical spondylosis. Liver function normal. No evidence of alcohol excesses.

110 g (dry weight) of faeces extracted and chromatographed on alumina. Three fractions obtained.

Table VII - 3.

Fraction.	mg.	($CHCl_3$)	(COOH)
1	about 2.5	624	2
2	0.63	622.4	4
3	small fraction not measured.	624	4

Fraction 1. Chromatographed on MgO. A minor fraction with alpha band (chloroform) at 619.4 mu was followed by 1.84 mg protoporphyrin (alpha band 628.8 mu.)

Fraction 2.and 3. Analysed by lutidine chromatography (system 2). Found: about 60% isomer I
40% isomer III

(iii) (G.S.) 29 years, white male. No diseases, liver function normal. The benzidine test on the faecal specimen was positive after 5 - 10 secs. but as the subject was consuming a meat-rich diet, the significance of this test was dubious. (144).

Urine - not examined quantitatively on this occasion but previously normal on many occasions.

Faeces - COPRO 36)
PROTO 20) ug/gm dry weight

38 gm dry weight of faeces was extracted. Chromatography on alumina yielded 2 fractions.

Table VII - 4.

Fraction.	mg.	$CHCl_3$ mu	COOH (Fig.VII-3)
1	0.97	620.4 (!)	2
2	0.27	621.7	4 + trace 3

Fraction 1. The position of the alpha band and behaviour of the ester when chromatographed using the KER-CHLORO-PROP system, suggested that the greater part was DEUTERO or MESO. Counter current distribution was performed (Fig. VII - 2)

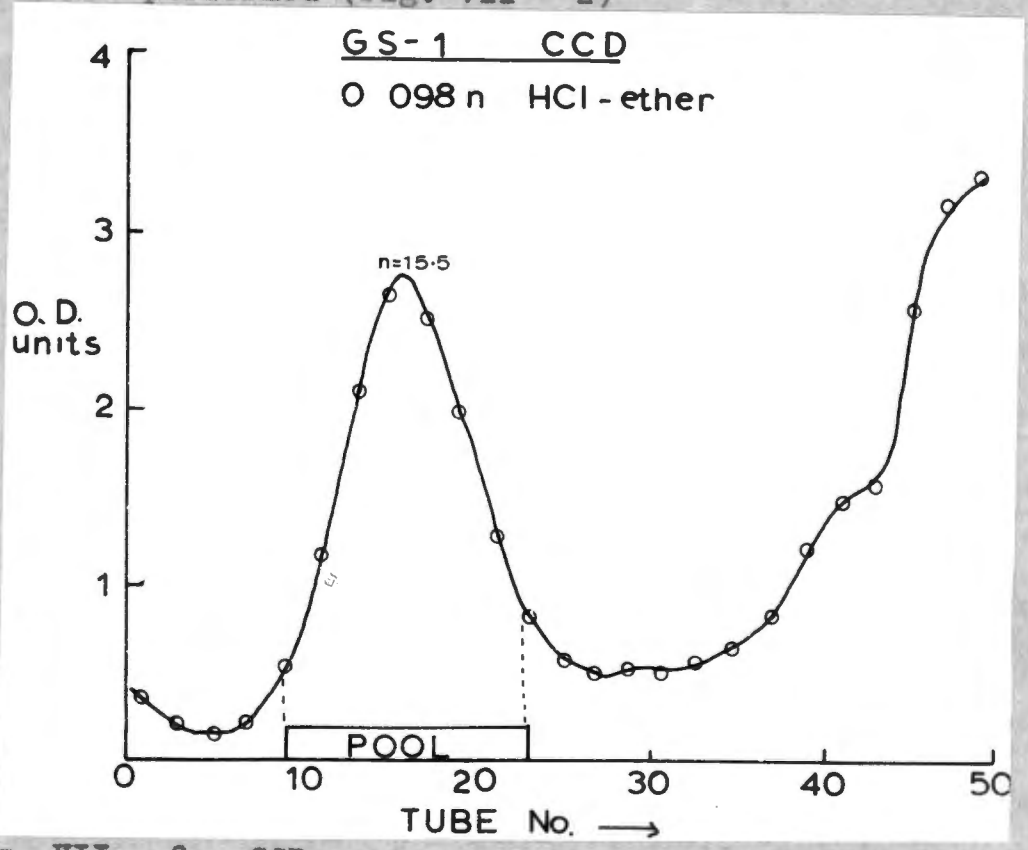


Fig. VII - 2. CCD pattern of dicarboxylic porphyrin from normal subject G.S. Note the prominent peak due to DEUTERO.

and indicated a prominent discrete peak due to DEUTERO and peaks due to two poorly separated components with higher HCl numbers, probably mono and divinyl deuteroporphyrin. DEUTERO was the largest single fraction.

Fraction 2. Coproporphyrin, isomer composition analysed by the quantitative method:

Found: 63% isomer I

37% isomer III.

(iv) Comment on normal faecal porphyrin. Recoveries of faecal porphyrin have been about one half those of the Holti method. This may be attributed to:

(a) Technical difficulty in exhaustively extracting large quantities of faeces with reasonable volumes of solvent.

(b) Sampling errors in the Holti method: faecal debris consists partly of large particles, e.g. beans, fruit, skins, pips. These are not usually taken for quantitative measurement.

(c) Loss on alumina chromatography. Pure esters of COPRO and PROTO are quantitatively recovered but in faecal analyses a fluorescent band is always present which elutes very slowly with 1-2% methanol in chloroform.

This is recovered heavily contaminated with bile pigment and has never been examined in detail in normal faeces. In faeces of S.A. Genetic porphyria in remission it has been found to be dicarboxylic porphyrin. It is either unesterified PROTO, or possibly esterified haematoporphyrin which does behave in this fashion on alumina columns.

In the three samples examined above, none contained porphyrin with more than 4 (COOH) groups. This was evident when crude mixed porphyrin was chromatographed (lutidine, system 1) before column chromatography.

In each instance dicarboxylic porphyrin was roughly 3 X coproporphyrin. In 2 cases the dicarboxylic porphyrin was mostly PROTO judging from the alpha band near 630 mu. In the third case there was an increase in DEUTERO and hardly any PROTO. This increase in DEUTERO seems to have been at the expense of PROTO but it may be associated with the positive benzidine test. (The concentration of porphyrin was normal.)

All three cases demonstrated a preponderance of isomer I COPRO.

3. Porphyrin Excretion in Acquired Porphyria.

The criteria for the diagnosis of acquired porphyria were:-

1. No family history of the disorder.
2. A gross increase in urine porphyrin concentration with "URO" greater than "COPRO", using the quantitative method already referred to.
3. Normal or only slightly increased excretion of ALA and PBG in the urine.
4. Faecal concentration normal or only moderately raised with "COPRO" exceeding "PROTO", using the quantitative method already referred to.
5. A history of excessive alcohol intake was suggestive of the diagnosis.

Table VII - 5 lists essential clinical data, Table VII - 6 the results of routine chemical investigations, and Table VII - 7 the histological findings (Dr. C.J.Uys) and porphyrin levels in liver biopsy specimens. Brief reports of typical cases are given in Appendix B. The excretion of porphyrins and porphyrin precursors is given in Table VII - 8.

Table VII - 5.

Case:	J.T.	G.S.	E.C.	J.F.	G.O.	F.M.
	All had lesions of cutaneous porphyria.					
Age (years)	41	43	46	41	47	41
Race*	C	C	C	C	C	A
Duration of alcohol excesses (years)	15		28	20	22	
Duration of porphyric symptoms		3 months	5 months	6 years	6 months	
Hepatomegaly	1f	3f	1f	none	3f	1f
Urobilinogenuria	-	+	-	-	-	+
Associated diseases	-	-	Decreased creat. clear.	-	**	-

* (C denotes mixed descent.
(A denotes pure blooded African (Bantu).

** (i) Diabetic GTC
(ii) abnormal pancreatic function.

Table VII - 7.

Case:	J.T.	G.S.	E.C.	J.F.	G.O.	F.M.
Fatty change	Mild	Marked	-	Present	-	Present
Regeneration	Present	Present	(No specimen)	Doubtful		Present
Prominent portal tracts	Present	Present		Absent		Present
Cirrhosis	Present	Absent		Absent		Absent
Siderosis	Moderate	Moderate		Moderate		Severe
Liver porphyrin ug/g wet weight.		175		216	197	470

Table VII - 6.

Case:	J.T.	G.S.	E.O.	J.F.	G.O.	F.M.
S.Albumin (g%)	2.6	4.0	3.6	3.3	4	3.8
S.Globulin(g%)	3.4	3.6	2.5	4.1	3.1	2.8
Bil.-total(mg%)	1.9	1.4	0.5	0.3	1.5	1.0
-conj.(mg%)	nil	trace	nil	0.1	0.4	0.4
Alk.Phos.(Bod. units)	8.0	3.7	3.5	11	4	8.5
Urea (mg %)	39	37	30	19		15
Creat.Clear(mL/min)	116	123	67			
Serum Iron(ug %)	260	85	176			140
Thymol.turb.(units)	4	1	2	1	1	2
Zinc.turb. (units)	12	12	16	8	8	6
SGOT (Karm.units)	-	26	33	24	35	23
P.I. %	78				97	
BSP retention (45 mins.)	nil	nil	nil	nil		nil

Table VII - 8.

Case:	J.T.	G.S.	E.O.	J.F.	G.O.	F.M.
Urine mg/L						
ALA	2.4	4.2	3.0	2.3	2.0	3.2
PBG	2.3	3.0	1.7	3.7	2.5	1.6
URO	2.9	4.3	2.5	1.3	1.39	2.87
COPRO	0.21	1.0	0.23	0.3	0.33	0.13
Faeces ug/g d.w.						
COPRO	0.18	0.73	0.091	0.057	0.049	0.14
PROTO	0.10	0.24	0.039	0.150	0.031	0.11

A. J.T. Coloured male, labourer, aged 31 years.

(i) Urine porphyrin. Porphyrin was extracted from a 2-day collection of urine which contained 6.6 mg of porphyrin (quantitative method.) Paper chromatography and electrophoresis showed porphyrins with from 8 to 4 carboxyl groups. 7 and 8 carboxyl porphyrins appeared to be the principal components. The other spots were of approximately equal intensity. Chromatography on MgO yielded 3 fractions:-

Table VII - 9.

Fraction.	($CHCl_3$)	(COOH)*
1	625	8=7>6
2	622	4
3	622 (faint)	4

* based on chromatography and electrophoresis.

Fraction 1. Crystallised to give crystals with typical morphology of URO (Fig. VII - 3) m.p. 255 - 257°C. Decarboxylated to COPRO. Found: about 60% isomer III

Fig. VII - 3. Crystalline URO from urine of J.T. m.p. 255 - 257° C.

Fraction 2 and 3. Largely COPRO on spectroscopic and chromatographic evidence; about 85% isomer III. The fraction was contaminated with porphyrin with more than 4 carboxyl groups.

(ii) Faecal Porphyrin. Porphyrin was extracted from 270 g (wet weight) of faeces and esterified. The crude ester was examined by lutidine chromatography (system 1). The most intense spot corresponded to a $(COOH)_4$ porphyrin. Faint spots corresponded to 2 and 8 carboxyl material, but a series of spots of intermediate intensity had relative mobilities between those of the URO and COPRO markers.

Chromatography on alumina yielded 4 fractions:

Table VII - 10.

Fraction.	mg.	(COOH)	($CHCl_3$)
1	0.4	2 and 3	620 - broad
2	-	3	-
3	-	4 > 5 > 6	-
4	-	contaminated with bile	-

Fraction 1. Further analysis on MgO yielded 0.12 mg of dicarboxylic and 0.21 mg of tricarboxylic porphyrin. The rather broad alpha band of the dicarboxylic

porphyrin was at 626 mu showing that, although protoporphyrin may have been present, it was certainly not pure.

Fraction 3. Because of contamination by more highly carboxylated porphyrin, pure COPRO was not obtained. Isomer analysis by lutidine chromatography (system 2) indicated that about 85% of the COPRO present was isomer III.

B. G.S. male, Coloured, Sewage worker, 44 years old.

(i) Urine Porphyrin. 4.7 L of urine was collected over 4 days. Porphyrin was extracted and esterified. After hydrolysis, the porphyrin was analysed by lutidine chromatography and electrophoresis.

Lutidine chromatography (system 1) indicated porphyrins with 3 to 8 carboxyl groups. This was the only evidence obtained for a tricarboxylic porphyrin which may have been partially hydrolysed material. This $(COOH)_3$ spot was fainter, and the $(COOH)_{7\ \&\ 8}$ spots stronger, than the rest which were of about equal intensity.

At a later date the relative amounts of each fraction present was determined by quantitative electrophoresis:

Table VII - 11.

COOH	8	7	6	5	4
%	32	35	9	13	11
Approximate % Isomer III	30	75			60

Crude porphyrin ester was chromatographed on a column of magnesium oxide. Four bands were eluted:

Table VII - 12.

	mg.	$CHCl_3$	(COOH)	Approx.% Isomer III.
1.	11.9	624.5	8=7>6	60
2.	0.9	623.1	5	60
3.	1.2	621.2	4	70
4.	0.4	622	4	0
	14.4			

Both electrophoresis and chromatography on MgO confirmed the preponderance of 7 and 8 carboxyl porphyrin. Significant amounts of porphyrins with 6 and 5 carboxyl groups were also indicated. Isomer analyses were performed on fractions separated both by chromatography on MgO and electrophoresis (Tables VII - 11, 12) and it will be noted that the results do not differ significantly. Some isomer separation

occurred on the MgO column which is unusual.

(ii) Faecal Porphyrin. Quantitative analysis of faecal porphyrin had indicated a "COPRO" fraction of 0.62 mg/g. This extremely high level made more precise analysis of this specimen of special interest.

Porphyrin was extracted from 204 g (wet weight) of faeces and esterified. Lutidine chromatography (Fig. VII - 4) showed components with from 8 to 2 carboxyl groups of which COPRO was most intense.

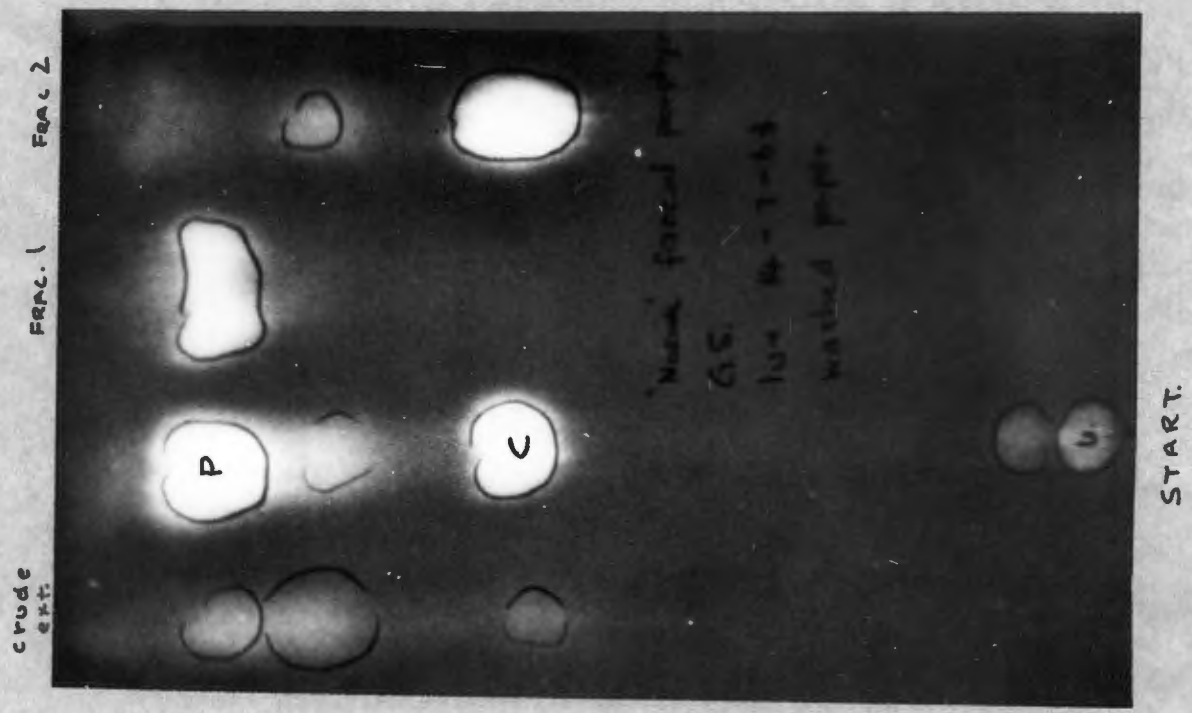


Fig. VII - 4.
Lutidine chromatography (system 1) on extract of faecal porphyrin from acquired porphyria. (G.S.)

Chromatography on alumina yielded 3 fractions.

Table VII - 13.

Table VII - 13.

Fraction.	mg.	($CHCl_3$)	(COOH.)
1	1.7	620.6	2 > 3
2	9.5	622	4 > 5
3	6	(contaminated with bile pigment)	2 - 8

Fraction 1 was largely dicarboxylic porphyrin from its behaviour on lutidine chromatography and when chromatographed as the ester on paper. (KER-CHLOR-PROP) The position of the alpha band indicated that proto-porphyrin was not the major fraction. This fraction was chromatographed on an MgO column and 3 sub-fractions obtained (Table VII - 14).

Table VII - 14.

Fraction.	mg.	($CHCl_3$)
1-1	0.1	622
1-2	0.8	620 - 621
1-3	0.5	627 (diffuse)

Fraction 1-3 represents the closest approach to PROTO from this faecal sample and this 0.5 mg fraction

equalled 10 ug/gm d.w., whereas the COPRO recovered represented 190 ug/gm d.w. and porphyrins with more than 4 carboxyl groups about 120 ug/gm d.w.

Fraction 2 was mostly COPRO and only 5 - 10% was isomer I. Crystalline COPRO III was prepared. The m.p. was very obviously dimorphic, mp 120 - 130° C, 155 - 165° C. Recrystallisation did not entirely free this specimen from contaminating 5-carboxyl porphyrin, though it elevated the mp's to 130-135° C and 165 - 168° C.

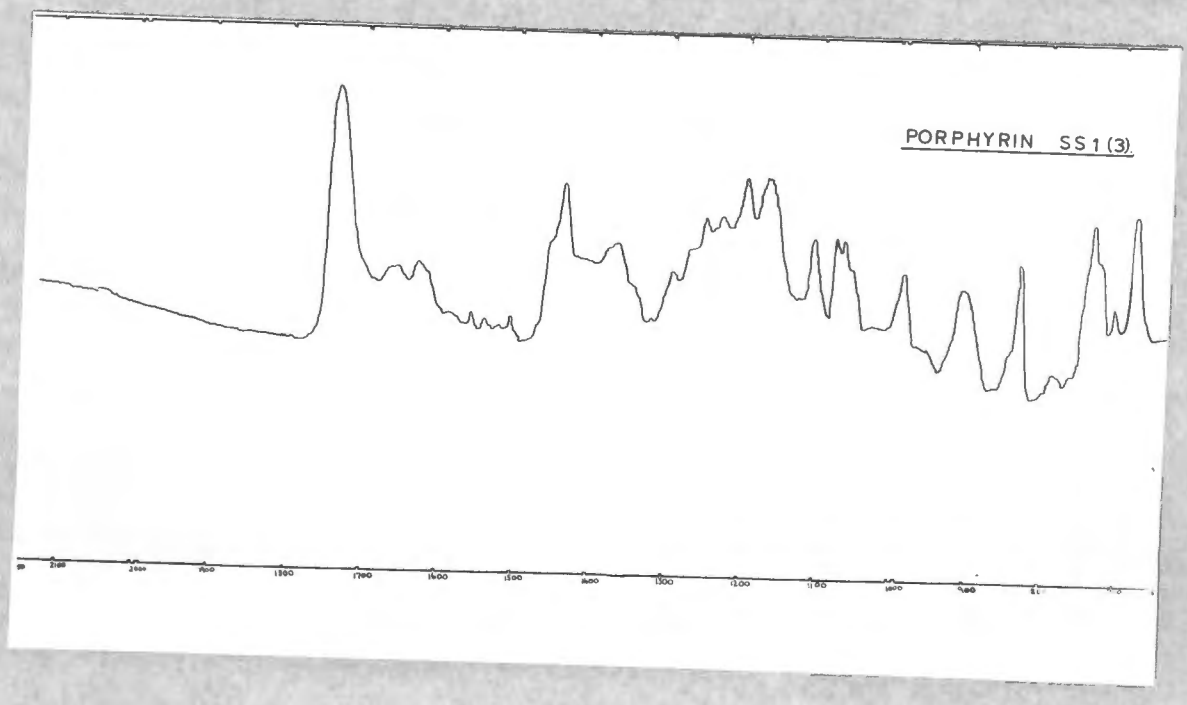


Fig. VII - 5.
IR Spedtrum of COPRO from faeces of G.S.

The IR spectrum (Fig. VII - 5) agreed fairly well with that of COPRO III, but a peak at 1125 cm^{-1} was missing in the "unknown" specimen. A Cu^{II} complex was prepared. (mp 211 - 212° C).

Fraction 3 was analysed by electrophoresis. Porphyrins with from 2 - 8 carboxyl groups were identified. The $(COOH)_2$ spot was faint, $(COOH)_3$ does not appear separately by this technique and the $(COOH)_8$ spot was barely detectable. $(COOH)_{4,5,6 \& 7}$ were approximately equal in intensity.

C. E.C. Coloured male, trawlerman, 46 years.

(i) Urine Porphyrin was extracted from 4.7 Litres of urine containing a total of 12.9 mgm of porphyrin. After esterification this porphyrin was chromatographed on MgO. Three fractions were obtained (Table VII - 15). These were quantitated and examined spectroscopically by chromatography and by electrophoresis.

Table VII - 15.

Fraction.	mg.	($CHCl_3$)	(COOH)
1	6.7	626	7, 8
2	0.17	-	5, 6
3	0.13	622	4

Fraction 1 was further separated into 7 and 8 carboxyl fractions by electrophoresis on Whatman's 3MM paper. The eight carboxyl porphyrin (URO) was 30% isomer III and the seven carboxyl (PSEUDOURO) 85% isomer III. Only isomers I and III were found. (The isomer analyses were performed by lutidine chromatography, (system 2) on COPRO's prepared by decarboxylation of the 7 and 8 carboxyl fractions separated electrophoretically.) (Fig VII-6).

Fraction 2 was not analysed further.

Fraction 3 COPRO 75% isomer III.

(ii) Faecal Porphyrin. Porphyrin was extracted from 62 gms (dry weight) of faeces. By the Holti method this contained 6.8 mg "COPRO" and 2.9 mg "PROTO". Chromatography (lutidine system 1) of the crude extract showed porphyrins with from 7 - 2 carboxyl groups. The strongest spot corresponded to the COPRO marker, all others were of approximately equal intensity.

5 fractions were obtained from a column of alumina; these were examined spectroscopically, quantitated and chromatographed on paper.

Table VII - 16.

Fraction.	mg.	($CHCl_3$)	(COOH)
1	0.56*	620 - 630 (diffuse)	2
2	0.57	-	3, 4
3	4.5	621.4	4
4	1.4	-	5, 4
5	±1.5	(contaminated with bile) pigment.)	7,6,5

* quantitated after chromatography on MgO.

Fraction 1 was chromatographed on MgO and two sub-fractions were obtained; 0.21 mg with alpha band in chloroform at 620 mu, followed by 0.35 mg with alpha band at 628.4 mu i.e. fairly pure protoporphyrin.

Fraction 2 - not analysed further.

Fraction 3 - isomer composition investigated chromatographically. Found: 75% isomer III.(Fig.VII-6).

Fractions 4 and 5 were heavily contaminated by bile pigment. Judging the electrophoresis strip visually they consisted of about equal amounts of porphyrin with 4, 5, 6 and 7 carboxyl groups. There was a trace of uroporphyrin.

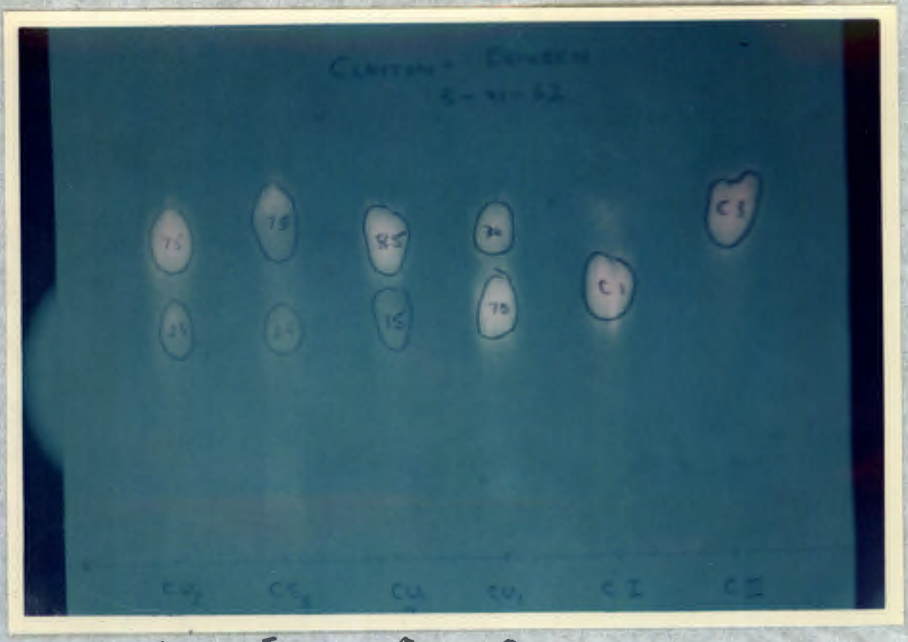


Fig. VII - 6.
Separation of COPRO isomers I and III by lutidine chromatography (system 2.)

Note: (i) As in the previous case, it was found that dicarboxylic porphyrin constituted only a small fraction of the total faecal porphyrin and of this small fraction, a substantial amount (40%) was definitely not PROTO. The pure COPRO fraction amounted to 73 µg/g dry weight; other porphyrin with more than 2 carboxyl groups to 56 ug/g dry weight, and dicarboxylic porphyrin to only 10 ug/gm dry weight of faeces.

(ii) It was apparent that considerable amounts of porphyrin with 7, 6 and 5 carboxyl groups were present. By the nature of the extraction procedure it was possible that URO was not being extracted. This was further investigated as follows:

2.44 g (dry weight) of faeces was extracted in two stages, first with ethyl acetate - acetic acid and then by esterification of the faecal residue. Neither fraction contained significant amounts of URO but the "residue" showed a prominent 7 carboxyl spot on electrophoresis, indicating incomplete extraction with ethyl acetate - acetic acid.

Ester from the "residue" fraction was chromatographed on MgO as on this adsorbent URO runs ahead of other porphyrins and may be recovered in good yield. Again, a mixture of 7 and 8 carboxyl porphyrin was obtained but the 8 carboxyl (URO) was only present in trace amounts.

In other patients quantitative data on this point will be presented. In this patient it was concluded that uroporphyrin was present, but in ~~much~~ lesser amounts than the 7 carboxyl porphyrin.

D. J.F. Coloured male, 41 years. Builder.

(i) Urine Porphyrin. No detailed study was made of urine porphyrin. The following attempt was made to determine the ratio of COPRO to COPROGEN.

Urine was voided by the patient directly into a dry dark bottle. This was worked upon immediately in the laboratory in dull red light. 5 cc of urine, 5 cc of glacial acetic acid, 25 cc of water and 50 cc of ether were shaken in a separating funnel and the aqueous phase discarded. The ether was washed 3 X with 10 cc aliquots of distilled water. The ether was extracted to completion with 0.1 N HCl (COPRO) and 0.1 cc of 1% iodine in alcohol added to the ether. Porphyrin was again extracted to completion with 0.1 N HCl (COPROGEN).

Found: $\frac{\text{COPROGEN}}{\text{COPRO}} = 1.96$

This analysis is imperfect. It does not ensure that porphyrinogens with 6 and 5 carboxyl groups are not measured as "COPRO", nor does it ensure quantitative recovery of COPRO. Both these factors, however, would tend to increase the COPROGEN/COPRO ratio.

(ii) Faecal Porphyrin was studied only by quantitative electrophoresis. Samples were analysed before and during the response of the patient to the chloroquine (140). Results are set out in Table VII - 16.

Table VII - 16.

(COOH) Groups.

Date.	2	3+4	5	6	7	8	
22/12	36	195	110		119	24	ug/gm d.w.
9/1	21	88		58	200	55	
10/1	21	210	154	160	440	164	

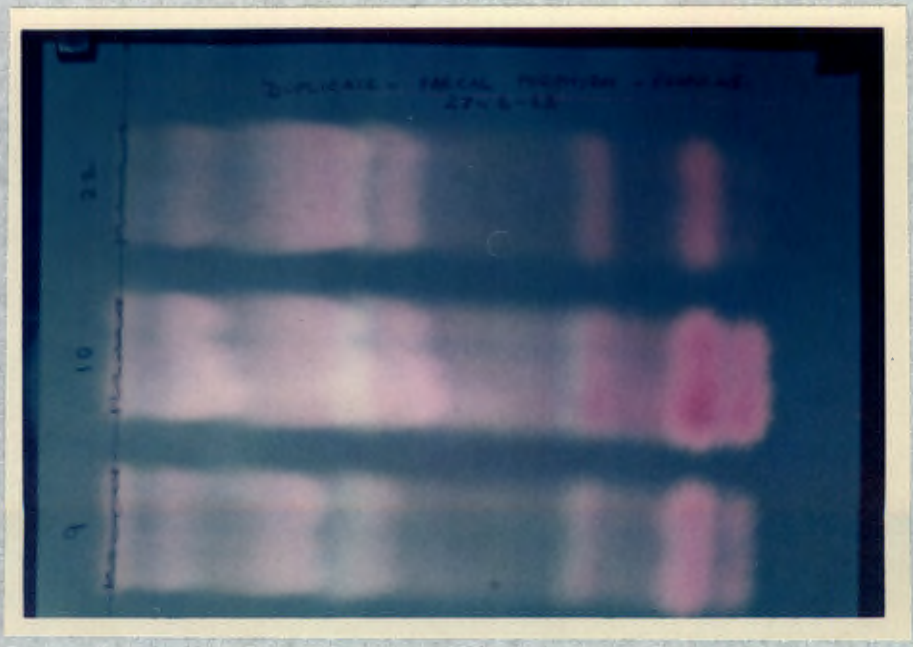


Fig. VII - 7. Electrophoresis analysis of faecal porphyrin on 3 days before (22nd) and after (9th,10th) the administration of chloroquine. Note:
(i) Very high concentration of URO on 10th.
(ii) Predominance of $(COOH)_7$ over URO.
(iii) Effect of $(COOH)_3$ fraction on the mobility of COPRO.

Figure VII - 7 illustrates the electrophoretic analysis which contributed the quantitative data given in Table VII - 16. Such high levels of faecal 7 and 8 carboxyl porphyrin have only been encountered in this single instance.

Case F.M. was also studied during the administration of chloroquine but no similar increase in faecal porphyrin occurred.

(iii) Liver Porphyrin. A liver biopsy specimen contained 216 ug/g wet weight of liver. Lutidine chromatography (system 1) and electrophoresis showed this to be only 7 and 8 carboxyl porphyrins.

E. G.O. Coloured male, electrician, aged 47.

(i) Urine Porphyrin. Urine collected during the first two days of hospitalisation was pooled. Porphyrin was extracted and esterified. By conventional quantitative analysis, the porphyrin content was "URO" 1.39 and "COPRO" 0.33 mg/L. Quantitative electrophoresis gave the following result:

Table VII -17.

(COOH)	8	7	6	5	4	Total.
ug/L	345	389	196	152	158	1,240.

(ii) Bile Porphyrin. Ether soluble porphyrin was extracted from 250 cc of duodenal aspirate. Preliminary purification involved two extractions into acid and back into ether to minimise emulsion formation in the CCD apparatus. CCD was then carried out through 24 transfers, using the phases diethyl ether and 0.105 N HCl, mutually saturated.

Major peaks are shown in Fig. VII - 8 and Table VII - 18.

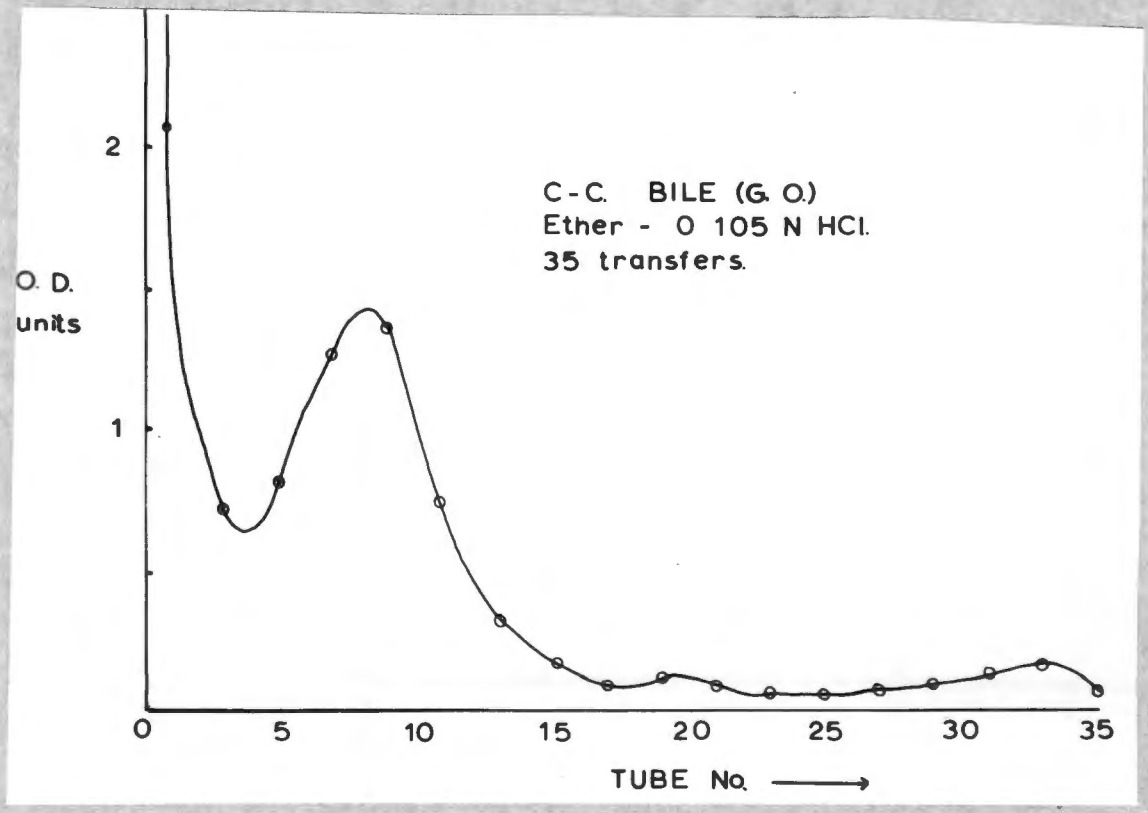


Fig. VII - 8.
CCD pattern of porphyrin from bile of G.O.

Table VII - 18.

n.	k	"HCl* no."	ug.	Probable nature.
0	0.03	-	97	COPRO
7	0.26	0.277	117	DEUTERO + $(COOH)_{3+4}$
19	1.26	.524	8	MESO
33	17	-	18	PROTO

(* Calculated from k)

Quantitative data given in Table VII - 18 is approximate. Separations were not clear-cut. The calculated HCl numbers are rather low because in this apparatus, which was not entirely closed, ether was lost by evaporation during transfer, with consequent deviations from unity of the ratio of the phase volumes.

(iii) Faecal Porphyrin. Porphyrin was extracted from 100 g. wet weight (51 g dry wt.) of faeces and esterified. As it was the intention to study only ether soluble porphyrin the conventional procedure was slightly modified. Porphyrin was extracted from the neutralised 3.0 N HCl solution into ether, not ethyl acetate. After esterification, the crude ester mixture was analysed by lutidine chromatography (system 1) and then fractionated on an alumina column. 3 fractions were obtained (Table VII - 19) and Fig. VII - 9.

Table VII - 19.

Fraction.	mg.	($CHCl_3$)	(COOH)
1	0.48	625.7	2
2	0.23	621.0	3
3	3.8	622.1	4 ≫ 5, > 6, > 7
	4.5 mg.		

Fig. VII - 9.

Lutidine chromatography indicates the relative purity of fractions obtained from an alumina column.

The porphyrin recovered after chromatography on alumina amounted to 88 ug/g d.w. By the Holti method this faecal sample contained 80 ug/g d.w. porphyrin.

Fraction 1. The position of the alpha band indicated that this decarboxylic fraction (Fig.VII-9) contained a substantial amount of PROTO. After saponification in methanolic KOH, the free porphyrin was analysed by CCD. The first CCD was between ether and 0.095 N HCl (30 transfers) shown in Fig.VII - 10a and tubes 20 - 30 from this CCD were then redistributed between the phases ether and 0.35 N HCl. (Fig. VII - 10b). 3 Symmetrical peaks were obtained, one in the first system (n = 11) and two in the second (n = 5.5, n = 18). Further details are given in Table VII - 20.

Table VII - 20.

Fraction.	n	k	Calculated HCl no.	ug.	ether.	Probable nature.
1	11	0.61	0.32	110	622	DEUTERO
2	5.5	0.224	0.87	120	627	MONO VINYL DEUTERO
3	18	1.64	1.98	180	632	PROTO

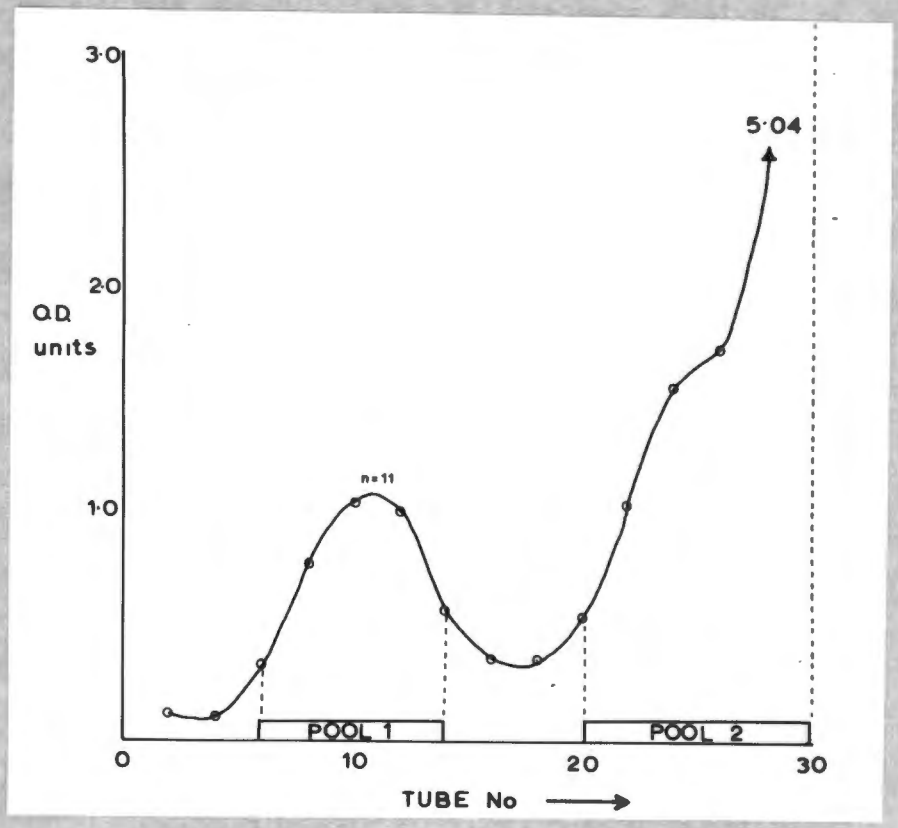


Fig. VII - 10 (a)

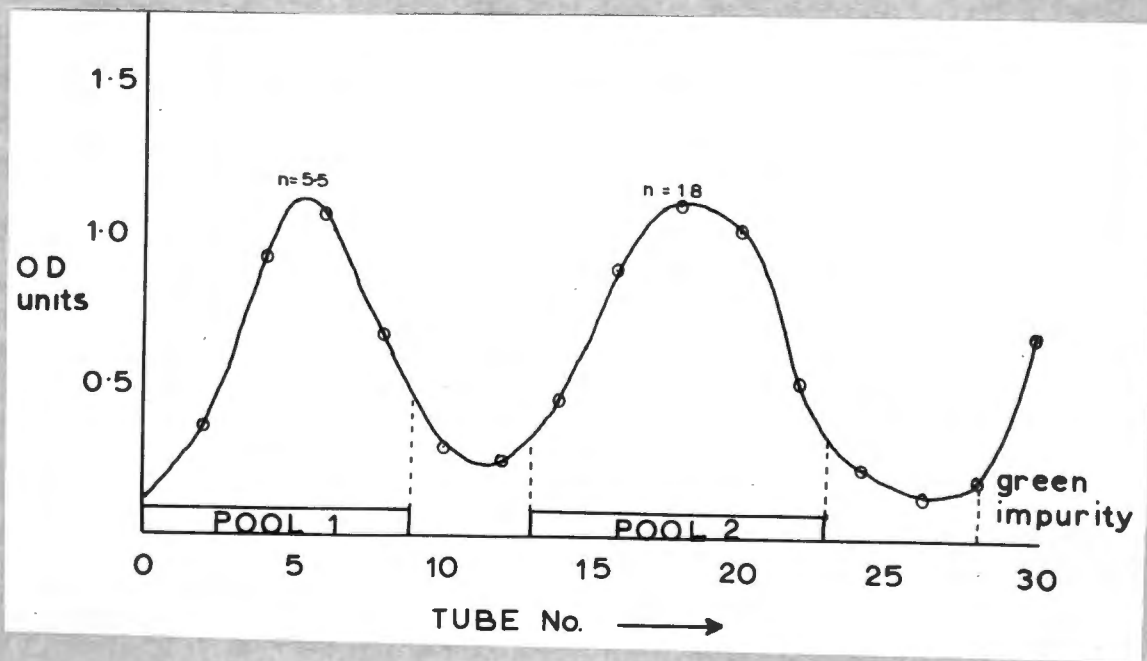


Fig. VII - 10 (b)

Fraction 2. Tricarboxylic porphyrin which was not studied further. The sharp alpha band at 621 mu. showed that no vinyl group was present at position 2 or 4.

Fraction 3. COPRO with small amounts of more highly carboxylated porphyrin. Further analysed by lutidine chromatography (system 2).

Found: isomer I 16%

isomer III 84%.

F. F.M. African male, labourer, aged 41 years.

(i) Urine Porphyrin. Urine was collected over a three day period and pooled. Porphyrin was extracted from an aliquot, esterified and analysed by quantitative electrophoresis. (Table VII - 21).

Table VII - 21.

COOH	2	4	5	6	7	8	
Urine		45	61	96	630	1140	ug/L
Faeces	86	141	27	18	48	46	ug/g d.w.

(ii) Faecal Porphyrin. Quantitative analysis of faecal porphyrin is shown in Table VII-21. For further analysis porphyrin was extracted from

135 g wet weight (26 g d.w.) of faeces. Only ether soluble porphyrin was esterified as described above (Case G.O.) Chromatography on alumina yielded 3 fractions (Table VII - 22).

Table VII - 22.

Fraction.	mg.	($CHCl_3$) mu.	(COOH)
1	1.45	622	2
2	0.10	-	3 > 4,=2
3	1.99	619.8	4
	3.5		

By the Holti method this faecal specimen contained 3.56 mg "COPRO" and 2.83 mg of "PROTO."

Fraction 1. This fraction was analysed by CCD and 3 fractions were obtained. All were dicarboxylic when examined by lutidine chromatography (system 1). 35 transfers were carried out between phases of ether and 0.1066 N HCl, mutually saturated. The distribution was not plotted graphically but 3 groups of tubes containing concentration bands were pooled. (Table VII - 23.)

Table VII - 23.

Fraction.	Tubes.	mg.	(ether)	Probable nature.
1-1	6-16	0.89	622	DEUTERO
1-2	23-29	0.47	627	MONOVINYL + MESO
1-3	30-34	0.30	631.6	PROTO + MONOVINYL.

These three fractions were esterified with diazomethane and chromatographed on alumina. Fraction 1-1 was inadvertently lost. Fraction 1-2 showed an alpha absorption band in chloroform at 625.6 mu, providing good evidence for one vinyl group at position 2 or 4. Fraction 1-3 showed alpha band absorption in chloroform at 629 mu; it was not pure PROTO but crystallised from chloroform/hexane to give the rosettes shown in Fig. VII - 11(a), mp 220-222° C.

Fraction 2. (from alumina) largely tricarboxylic porphyrin, was not analysed further.

Fraction 3. Coproporphyrin, analysed by lutidine chromatography (system 2).

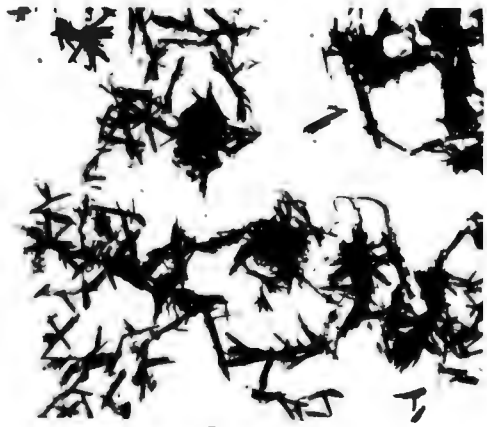
Found: isomer I 9%

isomer III 91%.

(iii) Liver Porphyrin. The total porphyrin content of a liver biopsy specimen was 470 ug/g wet wt. This consisted (lutidine chromatography, system 1) of seven and eight carboxyl porphyrin only.

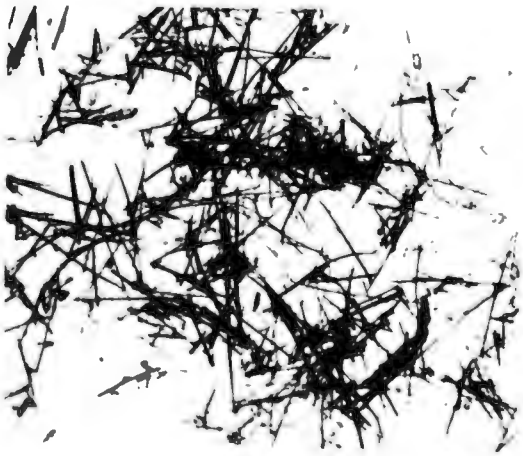
A

B

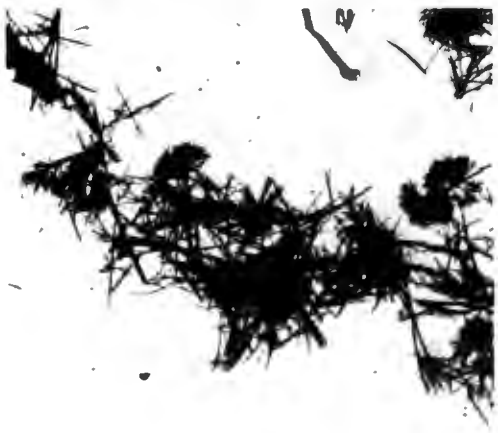


C

D



E



F

Fig VII-11

4. Porphyrin Excretion in S.A. Genetic Porphyria.

A. Remission Phase.

Criteria for the diagnosis of S.A. Genetic porphyria are as follows:

1. A family history of the disorder with the diagnosis in affected members confirmed biochemically.

2. Markedly elevated faecal copro- and protoporphyrin.

3. Urine porphyrin variably increased but with the "copro" fraction always exceeding the "uro" fraction, except when urine PBG is high during an acute attack.

4. Normal or only moderately increased excretion of ALA and PBG during the remission phase.

5. Clinically, patients may be asymptomatic, have cutaneous manifestations, or develop acute porphyria with or without coexistent cutaneous involvement.

There is no definite boundary which separates the acute attack from remission. If the excretion of ALA and PBG is normal the patient is in the remission phase; if these substances are present in the urine in high concentrations and the patient is suffering abdominal pains or showing signs of paresis or paralysis etc. there is no doubt that it is the acute phase of the illness. From the biochemical standpoint however, the distinction appears to be one of degree only.

Of the six patients studied during "remission" in only one (H.B.) was the urine ALA concentration below 6 mg/L and PBG below 2 mg/L, yet none had recently had an "acute attack" or shown symptoms of acute porphyria.

Table VII - 24.

Cases of S.A.Genetic porphyria in remission.

Case:	H.S.	A.V.	S.D.	P.V.	H.B.	J.C.
Race.	All were classed as "whites"					
Sex.	M.	M.	M.	F.	F.	M.
Age (yrs.)	30	21	37	23	50	57
Definite family history	+	+	+	+	+	-
Skin lesions	+	+	+	-	+	+
Previous acute attack	-	-	-	+	-	?
Associated disease	Mentally abnormal.	Nil.	Nil.	Nil.	*	-

* Angina pectoris
Hiatus hernia.

Table VII - 24 lists certain relevant clinical information; table VII - 25 gives data which enables the state of liver function in these patients to be assessed, and Table VII - 26 lists the results of quantitative determination of urine and faecal porphyrin

and urine ALA and PBG excretion at the time that the analyses to be described were conducted.

Table VII - 25.

Case:	H.S.	A.V.	S.D.	P.V.	H.B.	J.C.
Serum Albumin (g.%)	3.9		4.0	3.5	4.1	3.9
Serum Globulin (g.%)	1.9		2.3	2.2	2.8	3.0
Bilirubin						
Total (mg.%)	0.3		0.8	0.5	0.5	0.5
Conj. (mg.%)	neg.		0	0	0	nil
S.Alk.Phos. (Bod.units)	4.4		14.8	3.1	5.0	
Zinc turbidity (units)	10		0		0	1
Thymol turbidity (units)	2		5		4	11
B.S.P. (% at 45 min.)	nil	nil	24		nil	
SGOT (Karmen units)	17		14		26	

Table VII - 26.

Case:	H.S.	A.V.	S.D.	P.V.	H.B.	J.C.
Urine (mg/L)						
ALA	6.7	7.1	4.5		5.5	11.3
PBG	4.7	4.3	2.7		1.3	6.3
URO	0.19	0.067	0.35		.103	-
COPRO	0.48	0.68	1.25		1.11	-
Faeces (ug/g d.w.)						
COPRO	0.41	0.37	0.34	0.31	.53	.54
PROTO	0.38	0.34	0.48	0.44	.51	.51

(i) H.S. White male aged 30 years, unemployed.

(a) Urine Porphyrin. Porphyrin was extracted from 14 L of urine pooled during hospitalisation. Chromatographic and electrophoretic analysis of the crude porphyrin extract showed an intense COPRO spot and less intense spots corresponding to 7 and 8 carboxyl porphyrins.

Chromatography on MgO yielded 2 fractions. (Table VII - 27).

Table VII - 27.

Fraction.	($CHCl_3$)	(COOH)	% Isomer III.
1	624.6	7, 8	40
2	621.0	4	90

The isomer composition of Fraction 1 was determined after decarboxylation.

(b) Faecal Porphyrin. Porphyrin was extracted from pooled samples with a total wet weight of 300 g. The crude porphyrin extract was analysed chromatographically and electrophoretically. Spots corresponding to COPRO and PROTO markers were prominent; a faint spot indicating a tricarboxylic porphyrin and a trace of pentacarboxylic porphyrin was noted.

The crude ester was chromatographed on an alumina column. 3 fractions were obtained but records are incomplete.

Fraction 1. Dicarboxylic porphyrin. Further analysed on MgO to give three sub-fractions. The third and largest of these had the following properties:-

Absorption spectrum (mu) 629.6; 577.8; 540.6

m.p. 205 - 219° C.

I.R. absorption spectrum - peaks corresponded well with those of PROTO IX.

Fraction 2. 80% COPRO III. 20% COPRO I. Pure COPRO III (identified chromatographically) obtained by fractional crystallisation. m.p. not recorded.

Fraction 3. Heavily contaminated with bile. Not analysed further.

(ii) A.V. White male, 21 years. Farmer.

(a) Urine. Porphyrin was recovered from a pooled urine specimen. Chromatography (lutidine, system 1) showed COPRO to be the principal component. A definite spot corresponded to a six carboxyl porphyrin and a faint spot indicated URO. Seven carboxyl

porphyrin was not noted. COPRO, isolated by chromatography on MgO was 90% isomer III.

(b) **Faeces.** Porphyrin was extracted from 68 g (wet weight) of stool. By routine quantitative analysis this contained COPRO 0.41 and PROTO 0.43 mg/g dry weight. The crude extract was analysed by chromatography (lutidine, system 1). COPRO and PROTO spots were most prominent but weaker spots indicated porphyrins with 3 and 5 or 6 carboxyl groups.

The crude extract was chromatographed on alumina. Two fractions were obtained (Table VII - 28).

Table VII - 28.

Fraction.	mg.	($CHCl_3$)	(COOH)
1	2.06	626	2
2	1.36	621.4	4

Fraction 1: was chromatographed on MgO. Three fractions were obtained; the first contained a trace of COPRO. The second, dicarboxylic porphyrin, showed an alpha band at 620.5 mu and was thus of the DEUTERO-MESO type. The third, and largest, fraction had an alpha band at 629 mu. This fraction was crystallised from chloroform/hexane to give crystals

of poor morphology and m.p. 218 - 223°C. This fraction was regarded as PROTO IX.

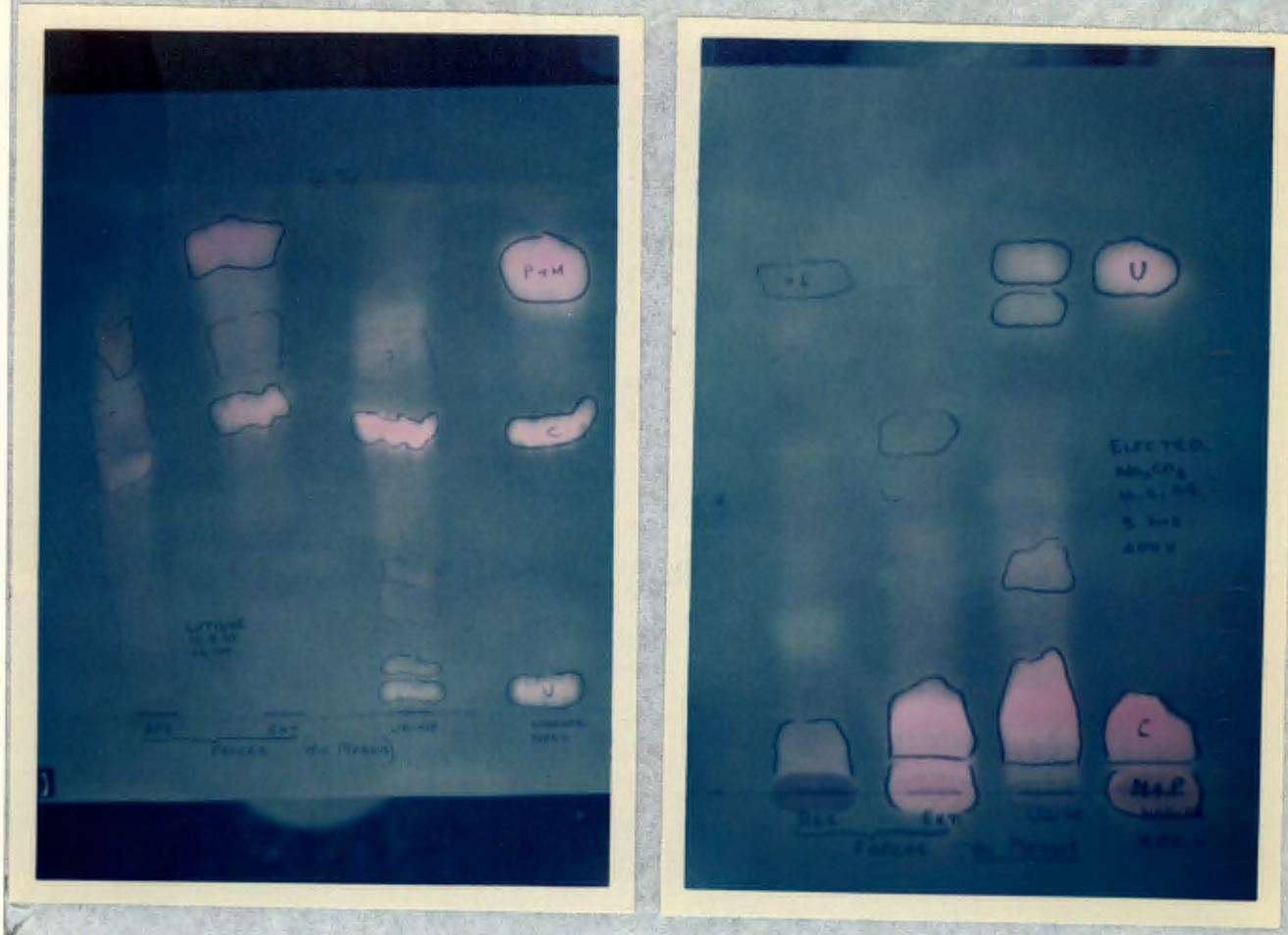
Fraction 2 was identified as COPRO chromatographically and spectroscopically. Lutidine chromatography (system 2) showed this fraction to be 90% isomer III.

(iii) S.D. White male, age 37 years. School teacher.

Both urine and faecal porphyrin were analysed by quantitative electrophoresis. The patterns are shown in Fig. VII - 12(a) and the results in Table VII - 29. Fig. VII - 12(b) enables lutidine chromatography (system 1) to be compared with the electrophoretic analysis.

Table VII - 29.

	COOH GROUPS						
	2	4	5	6	7	8	Total.
Faeces	280	244	-	-	-	trace	524 ug/g d.w.
Urine	-	625	62	25	14	19	745 ug/L



(a) (b)

Fig. VII - 12.

Analysis by lutidine chromatography and electrophoresis of faecal and urine porphyrin from Case (iii) (S.D.) URO, COPRO and PROTO + MESO markers appear on the right. Faecal porphyrin derives from two fractions, EXT (ethyl acetate extract) and RES (residue) (See Chapt.VI, Determination of URO in faeces.)

(iv)(P.v.d.L.) White female aged 23 yrs. Housewife.

This patient with S.A. Genetic Porphyria was admitted to hospital for two weeks for a metabolic study (90). She received an intravenous injection of glycine-2-C^{14} and the appearance of radioactivity in various metabolites of glycine-2-Carbon was studied. All stools were collected; crystalline COPRO and PROTO were prepared from each specimen by column chromatographic procedures. A very large quantity of faeces was processed and an opportunity thus provided to study in more detail (i) porphyrin from alumina columns eluting between dicarboxylic porphyrin and COPRO, possibly tricarboxylic, and (ii) porphyrin eluted from MgO columns ahead of PROTO, referred to previously as MESO/DEUTERO type.

Lutidine chromatography showed that the fraction referred to in (i) above was a mixture of approximately equal amounts of three and four carboxyl porphyrin. Alpha band absorption was fairly sharp and at 623 mu. It was thus unlikely that a large amount of this porphyrin was a tripropionic, monovinyl porphyrin which would have been of special interest. Analysis of this fraction by CCD has not yet been performed.

CCD of the fraction referred to in (ii) above has been completed. The porphyrin obtained by chromatography on MgO was saponified with 5% methanolic KOH, and analysed by CCD using as phases ether and 0.105 N HCl with 80 transfers. Three concentration bands were obtained which were maximal in tube 22, 34 and 45, but separations were incomplete. Tubes in which there was obvious overlap apparent, were discarded. These three fractions are described in Table VII - 30.

Table VII - 30.

n.	k.	Calc. HCl no.	Ether		Presumed nature.
			α	γ	
22	0.38	0.33	622	526.1	Deutero
34	0.74	0.47	622.6	-	Monoethyl-deutero
45	1.3	0.62	623.2	528.8	Meso

After esterification (CH_2N_2)

n	m.p.	Fig.VII - 11.
22	220 - 224° C	E
45	210 - 214° C	F

This study provided good evidence for the possible nature of "non-PROTO" dicarboxylic porphyrin

which has always been found in greater or lesser amounts in stools analysed. When, at a future date, the specific activity of these samples with respect to C^{14} is determined, it will be possible to decide whether they have arisen from PHOTO in the gut or from exogenous sources.

(v) H.B. White female aged 50 years.

(a) Urine Porphyrin. Urine passed during a 3-day period was pooled. By conventional analysis the concentration was URO 0.10 and COPRO 1.10 mg/L. Porphyrin was extracted by adsorption on talc, esterified, hydrolysed and analysed by quantitative electrophoresis.

Table VII - 31.

COOH Groups	4 5	6	7	8
Urine Porphyrin	490	8.6	5.4	8.9 ug/L

It was not possible to distinguish 4 and 5 carboxyl porphyrin because a large quantity of crude mixture was applied to the paper to minimise losses of these components present only in small amounts. Lutidine chromatography showed quite clearly that the main component was coproporphyrin.

(b) Bile Porphyrin. During study of pancreatic function, duodenal aspirate was collected. From 100 cc of this highly fluorescent fluid, ether soluble porphyrin was extracted quantitatively. There was no evidence of ether insoluble porphyrin. The porphyrin extract was analysed by CCD (ether, 0.1ON HCl.) Three concentration bands were obtained which were pooled and examined further: Table VII - 32.

Table VII - 32.

Tubes.	Fraction	Soret (ether) mu.	Amount mg.	(COOH)
0 - 5	"Copro"	400	1.48	4, 2
7 - 12	"Deutero"	398	1.68	2, 3, 4.
28 - 33	"Proto"	405	4.80	2, 1 (?)

Pinkish fluorescence in tubes 34 and 35 suggested the presence of a metal complex.

Tubes 0 - 5. Lutidine chromatography (system 1) showed the chief component present to be COPRO but, surprisingly enough, there was also a significant dicarboxylic porphyrin component. The COPRO was about 80% isomer III.

The dicarboxylic porphyrin present was either DEUTERO, inadequately separated, or porphyrin with a

lower HCl number such as HAEMATO. The pooled fraction was esterified with diazomethane, the ester dried and redissolved in fresh ether and extracted twice with half its volume of 1% HCl. (0.5% HCl should have been used.) The spectral properties of the porphyrin ester which was extracted by 1% HCl, and that remaining in the ether, were compared. The fraction with greater acid solubility showed an 0.6 mu shift to the red of the alpha bands in ether. Chromatography of the HCl extract showed a spot corresponding to a dicarboxylic porphyrin which was more intense than that corresponding to COPRO. Tenuous though this evidence is, it seems likely that the original bile sample contained between 0.2 and 0.5 mg/100 mL of mono- or dihydroxyethyl derivatives of PROTO. No other dicarboxylic porphyrin ester would have been efficiently extracted from ether under the conditions used.

(c) Faecal Porphyrin. A single specimen was analysed by the Holti quantitative technique, by quantitative electrophoresis and by column chromatographic procedures supplemented by CCD.(Table VII - 33).

Table VII - 33.

Holti method.

Copro 0.53
Proto 0.51
} mg/g d.w.

Quantitative Electrophoresis.

COOH	2	4	5	6	7	8	Total
	255	481	35	-	1.7	1.6	784 ug/g d.w.

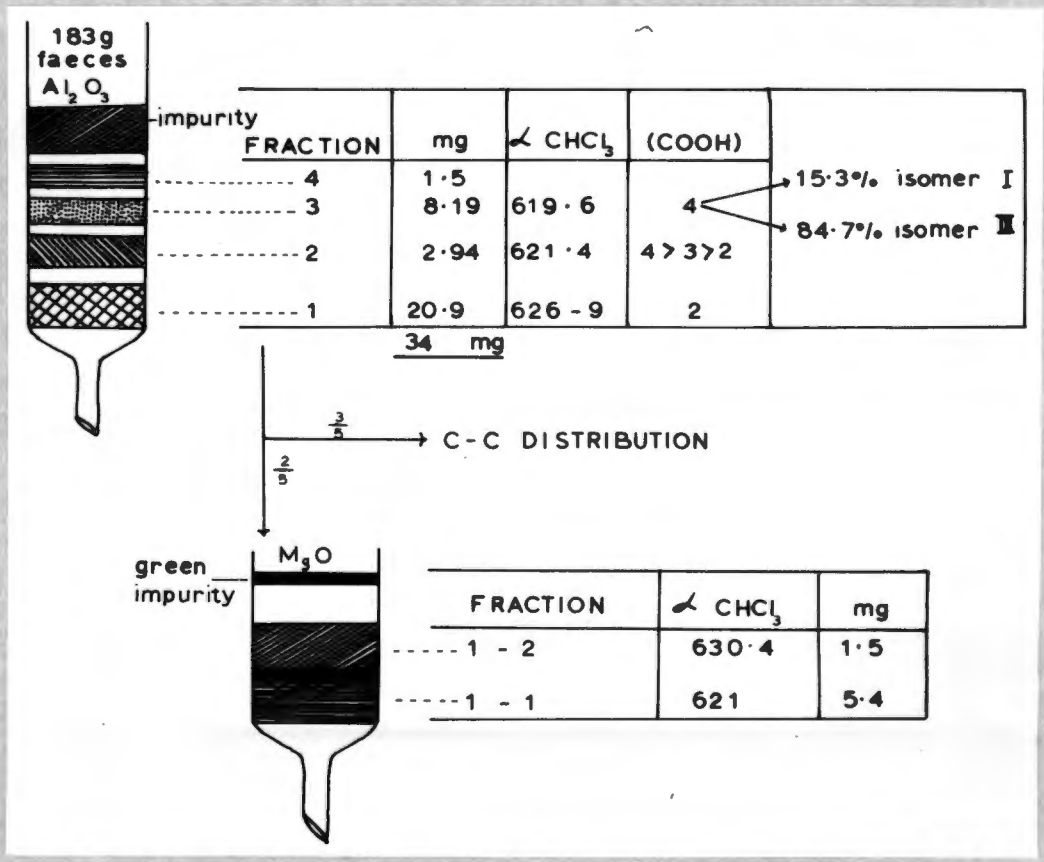


Fig. VII - 13.

Outline of analysis of faecal porphyrin of Case (v).H.B.

Column chromatography and CCD.

An outline of the procedure followed is set out in Fig. VII - 13. The crude extract separated into four fractions when chromatographed on alumina. The properties of these fractions are tabulated in the figure. Fraction 1, dicarboxylic porphyrin, was dried and redissolved in 5.0 cc $CHCl_3$. 2.0 cc was chromatographed on MgO and 3.0 cc was saponified. in 5% methanolic KOH prior to analysis by CCD. Fraction 2 was crystallised from chloroform-methanol and recrystallised twice from chloroform-hexane. Crystals (Fig.VII - 14) had typical COPRO III ester morphology. Careful heating revealed only a single sharp m.p. at 150-151° C.

Fig.VII - 14.
Crystalline COPRO III
m.p. 150 - 151°C.

Chromatography on MgO yielded 1.5 mg PROTO (alpha band, $CHCl_3$ = 630 mu. m.p. 218 - 220° C) (Fig. VII - 11C) and 5.4 mgms of dicarboxylic porphyrin having alpha band ($CHCl_3$) = 621 mu. Recovery of the PROTO fraction from the MgO column was incomplete because of irreversible adsorption. Despite this, MgO chromatography had indicated an excess of "non-PROTO" decarboxylic porphyrin over "PROTO" confirming the deduction based on behaviour of fraction 1 from the alumina column on paper chromatography. (KER- CHLORO- PROP).

Analysis by CCD now became of great interest. Two distributions were performed:

(i) ether - 0.096 N HCl - Fig. VII - 15a.

and (ii) ether - 0.45 N HCl on tubes 60-80 of (i) Fig. VII - 15b.

Figure VII - 15a, 0.096 N HCl, shows a distribution pattern with many peaks. The largest single fraction was concentrated in tubes 60 - 80 and this was again analysed by CCD using 0.45 N HCl. The peak maximal at tube 47 (k = 1.42, HCl no. = 0.51, α ether = 623 mu) was prominent; this was regarded as MESO. A definite peak was found maximal at tube 11; this behaved as tricarboxylic porphyrin on lutidine

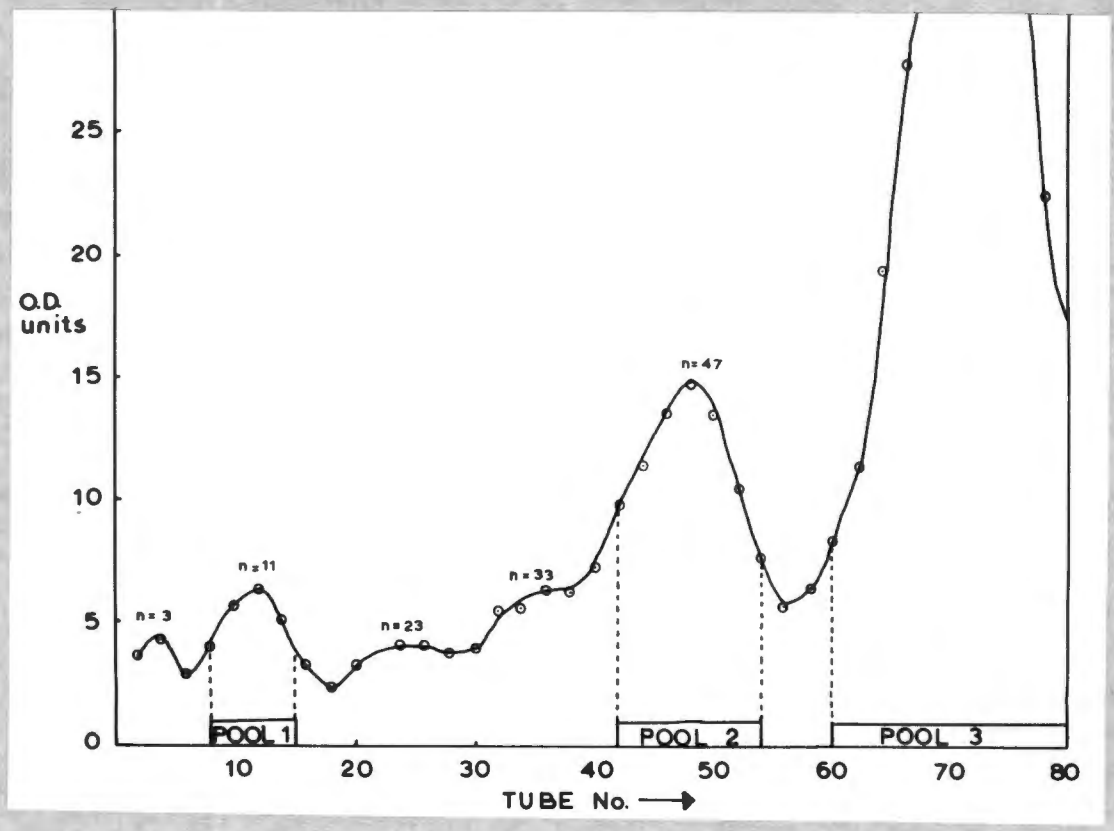


Fig. VII - 15a.

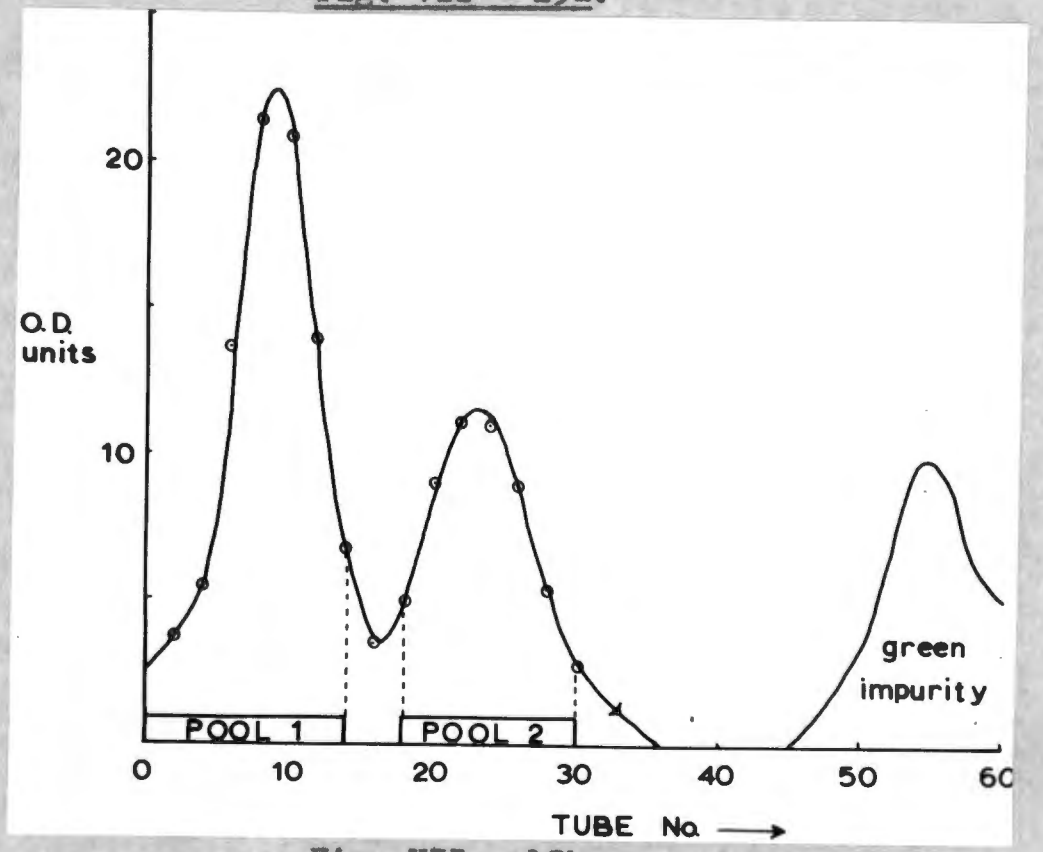


Fig. VII - 15b.

(system 1) chromatography. Further peaks were suggested at tubes 23 (DEUTERO) and 33 (DEUTERO - MESO hybrid). Tubes 60 - 80 were pooled and analysed by CCD using ether and 0.45 N HCl. Figure VII -15b illustrates the two clear cut concentration bands obtained. These were individually pooled and the properties are tabulated below. (Table VII - 34.)

Table VII - 34.

Pool	mg.	Calculated HCl no.	ether.	$CHCl_3$
1	2.95	0.89	628.6	-
2	2.1	1.87	633.0	630.6

It was thought likely that Pool 1 represented a monovinyl deuteroporphyrin. This fraction was therefore dried, dissolved in formic acid and reduced with hydrogen using a palladium charcoal catalyst. The alpha band in ether shifted from 628.6 to 623.0 mu which is approximately the shift which one vinyl group at position 2 or 4 would cause.

The green impurity shown to the right hand side of Fig. VII - 15 b absorbed at about 660 and 500 mu; it was non-fluorescent and could have been an oxidation

product of PROTO which had formed during storage in chloroform or during saponification. Although also noted during purification of PROTO prepared from blood, it had not previously been associated with the appearance of monovinyl porphyrin and there was no reason to regard this monovinyl porphyrin as an artifact.

Pool 2 was pure PROTO. After esterification with diazomethane and chromatography on alumina, crystallisation yielded beautiful dichroic crystals m.p. 231 - 233° C. (Fig. VII - 11d.

Comment. This analysis was particularly instructive. Although previously the column chromatographic procedures had yielded a fraction which would have been accepted as fairly pure, PROTO, CCD showed this to be impure judging from the m.p. To prepare pure PROTO it would be necessary to use a method capable of separating sharply monovinyl and divinyl deuteroporphyrins. MgO chromatography can barely separate PROTO from DEUTERO so that contamination of either fraction with monovinyl deuteroporphyrin would seem inevitable.

(vi) J.C. White male, 57 years old. Labourer.

Bile Porphyrin. 95 ml of duodenal aspirate was obtained from this patient and the porphyrin extracted. Analysis by CCD gave the pattern indicated in Fig.VII-16. Three fractions were obtained. Tubes 0-4 contained 122 ug of porphyrin and represents the "coproporphyrin" fraction. Tubes 8-16 contained 36 ug of porphyrin and was probably chiefly deuteroporphyrin. Protoporphyrin would be expected to be the major component in Tubes 25 - 30. This analysis has not yet been completed.

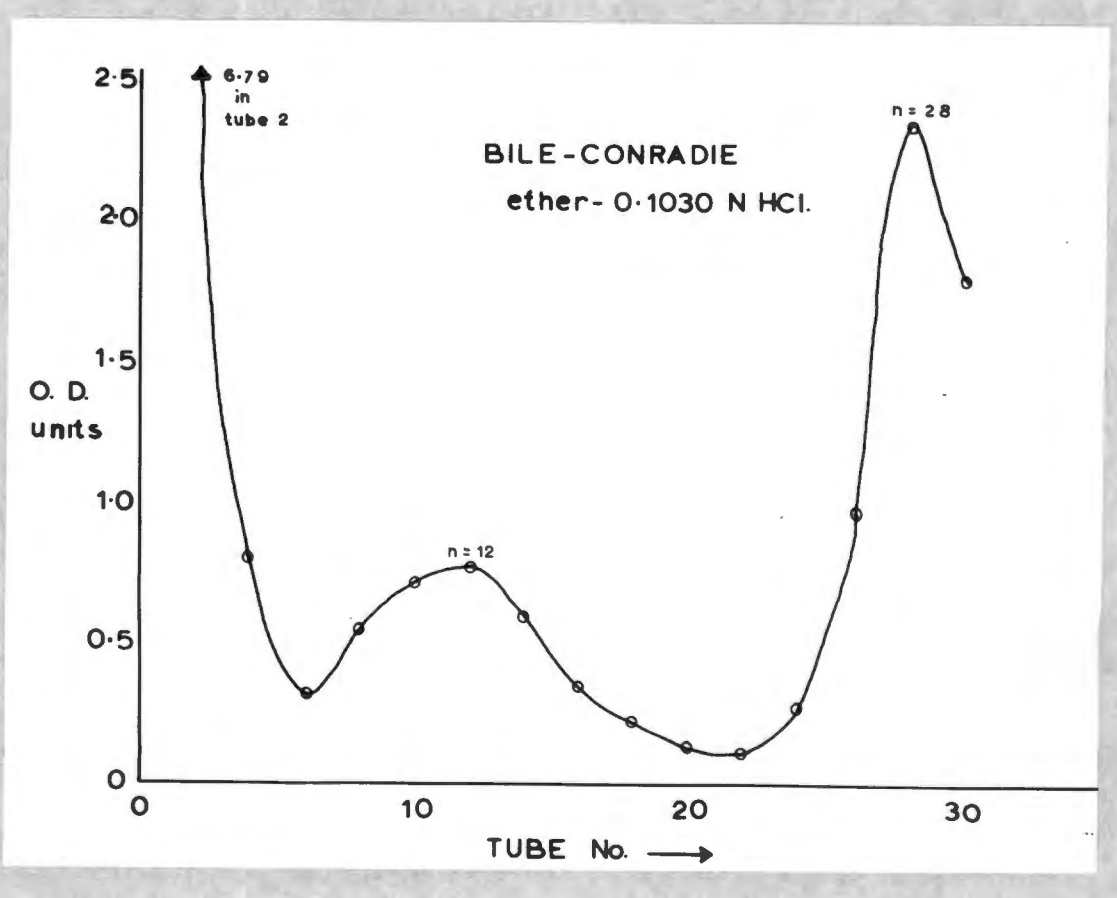


Fig. VII - 16.

CCD pattern of bile from J.C., Case (vi).

Table VII - 37*.

Case:	H.B.	A.S.	M.L.	M.N.	S.S.	
Urine mg/L					(i)	(ii)
ALA	22	26.7	23	17	5.0	20.5
PBG	68	58.2	47	35	24.3	35.7
URO	1.42	5.45	1.42	3.39	0.57	
COPRO	1.43	2.54	2.45	1.64	0.49	
Faeces ug/g d.w.						
COPRO	1.43	0.47	0.67	0.74	0.56	0.33
PROTO	0.83	0.42	0.47	1.01	1.00	0.28

* The excretion of porphyrins and porphyrin precursors on admission to hospital.

B. Cases Studied During Acute Attacks.

(i) H.B. White male aged 33 years. Farmer.

Relevant clinical and biochemical data is given in Tables VII - 35, 36, 37 and in Appendix B.

(a) Urine Porphyrin. Figure VII - 17 shows the result of serial estimations of urine PBG after admission to hospital. Porphyrin was recovered from urine collected over the three 2-day periods indicated. (A, B and C). Urine porphyrin fractions A-C were examined by paper chromatography and electrophoresis.

Tables VII - 35, 36 and 37 list relevant clinical and biochemical data referring to the five patients who were studied during attacks of acute porphyria.

Table VII - 35.

Case:	H.B.	A.S.	M.L.	M.N.	S.S.
Race	W	C	W	W	C
Sex	M	F	F	F	M
Age (yrs)	33	32	50	41	34
Family history of Porphyria	yes	no	?	yes	no
Skin lesions	yes	no	yes	yes	yes
Previous acute attack	no	no	no	no	yes
Associated disease	nil	nil	nil	nil	nil

Table VII - 36.

Case:	H.B.	A.S.	M.L.	M.N.	S.S.*
Serum albumin (g%)	3.2	4.1	3.3	3.3	
Serum globulin (g%)	2.0	3.3	2.1	2.2	
Ser.Bilirubin total (mg%)		0.9	0.6	0.9	0.4
conj. (mg%)		0	0.4	0.5	0.2
Serum Alk.Phos. (Bod. units)	3.2	3.8		-	5.0
Thymol turb. (units)	1		1	1	2
Zinc turb.(u)	6		5	6	11
BSP % retained at 45 mins.		nil			nil
S.G.O.T.	0	24			17
Blood urea	92	79	186	69	68
Creat.Clear.	78	60			

* Data refers to 2nd admission. Abnormalities of liver function including altered zinc turbidity and B.S.P. retention did appear after some weeks in hospital.

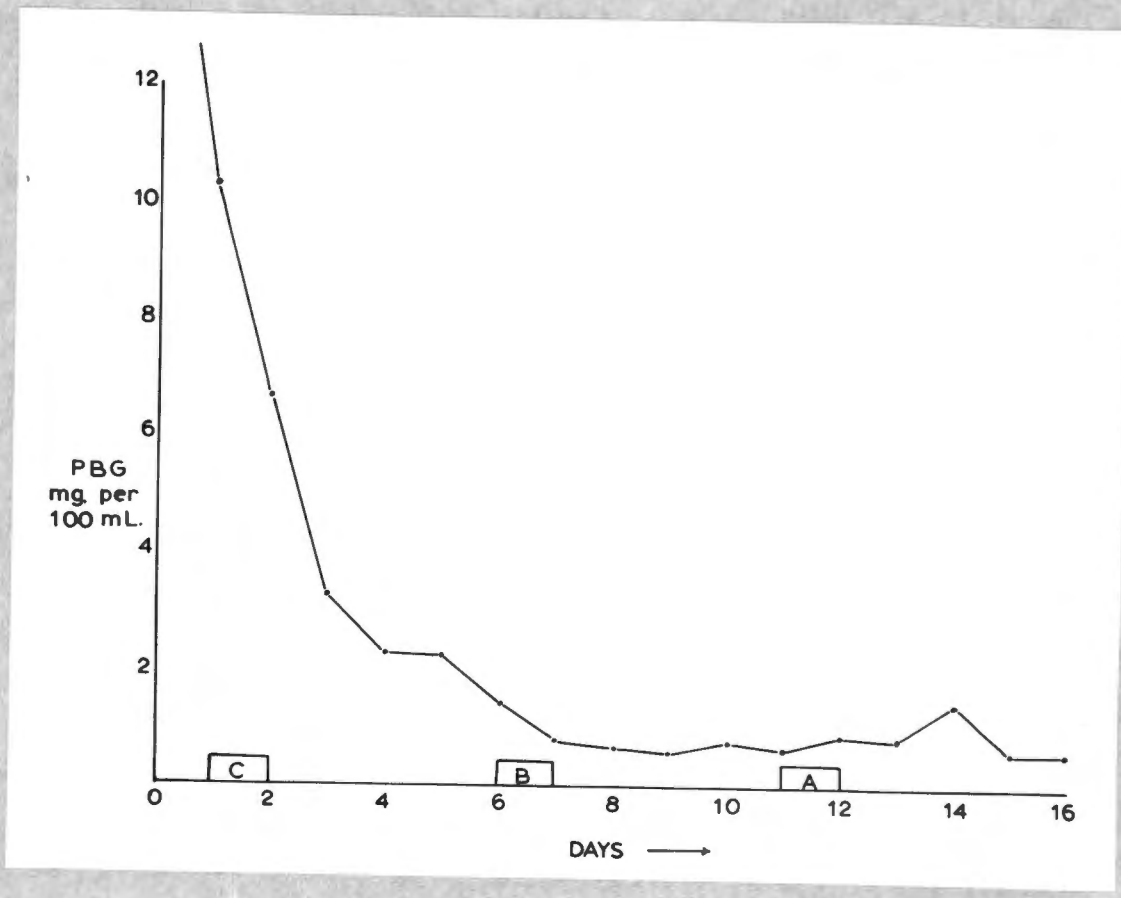


Fig. VII - 17.

The serial changes in the urine PBG concentration in Case (i) H.B. after admission to hospital.

Each fraction was chromatographed on MgO. The "URO" fractions, which were mixtures of 7 and 8 carboxyl porphyrin, decarboxylated and the isomer composition determined.

Urine specimens were never stored longer than 24 hours so that no special precautions were taken with regard to storage.

Electrophoresis of urine fractions A, B and C showed that with decreasing levels of PBG there was a relative decrease in the amounts of 8-carboxyl porphyrin with relative increase in the amount of 7 and 4 carboxyl porphyrin. (Fig. VII - 18).

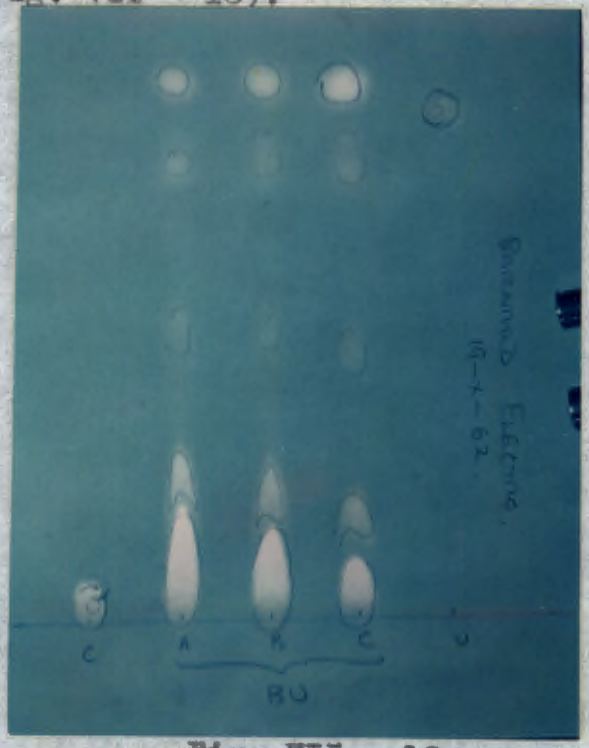


Fig. VII - 18.

Electrophoresis analysis of 3 urine specimens of Case (1) H.B. to show alterations in porphyrin content with decreasing PBG excretion.

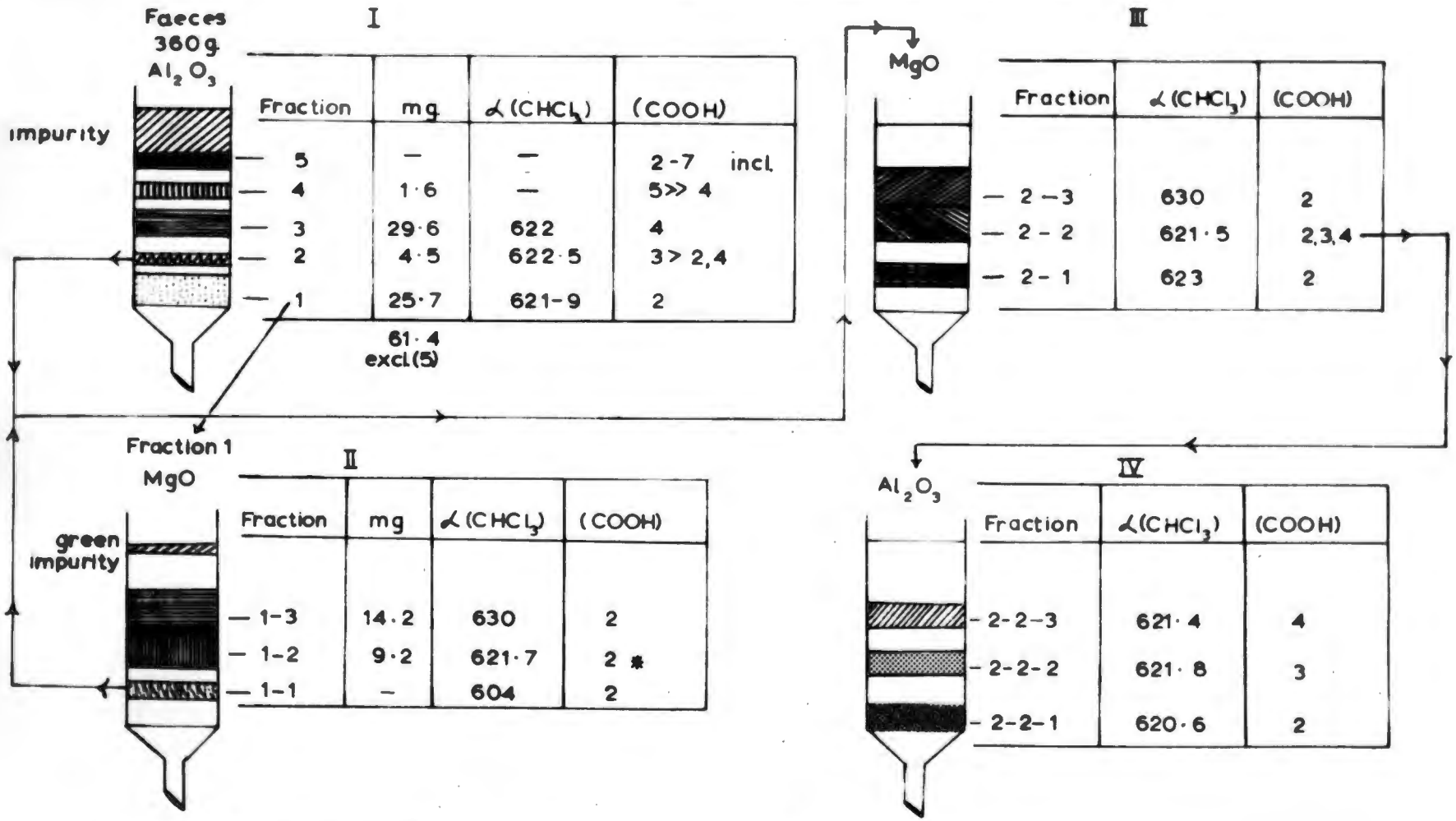
On MgO columns fractions A - C gave 3 subfractions each. (Table VII - 38).

Table VII - 38.

		Mg.	(COOH)	Isomers I	III/IV	II
A	1	1.1	4		90	
	2	0.3	5 > 6			
	3	0.8	8 > 7 > 6	60	40	0
B	1	1.49	4		90	
	2		4 > 5			
	3	1.24	8 > 7 > 6	50	45	5
C	1	1.1	4		90	
	2	0.6	5 > 4			
	3	4.7	8 > 7 > 6	45	45	10

It was of particular interest to find evidence of isomer II COPRO in the decarboxylated URO fractions B and C. The evidence for isomer II was a discrete spot on the chromatogram with an Rf just greater than that of isomer III. At the time reference isomer II COPRO was not available but later a specimen of synthetic URO II was obtained through the generosity of Dr. S. F. McDonald of Toronto. On decarboxylation this yielded COPRO II which behaved as described above. Because URO II was found, it was assumed that URO IV might also be present, but COPRO's II and IV are

CASE i FAECAL PORPHYRIN



*C-C distribution (Fig 7)

Fig VII-19

indistinguishable chromatographically.

Although the URO fractions consisted of mixtures of isomers, the urine COPRO was almost entirely isomer III.

(b) Faecal Porphyrin. After an initial period of constipation, stools were obtained on the 4th and 5th days after admission to G.S.H. (See Figure VII - 16). Table VII - 39 indicates the result of quantitative analysis.

Table VII - 39.

Date.	Wet.Wt. (g)	Dry Wt. (g)	Copro	Proto	Total	
13.8.62	100	38	1.83	1.24	3.07	ug/g dry wt.
14.8.62	183	69	0.62	0.42	1.04	

The analysis by column chromatography of the crude ester obtained from this pooled specimen was rather involved and has been shown diagrammatically in Fig.VII-19. Analysis was complicated by fractions with 4, 3 and 2 carboxyl groups which differed little in their physical properties.

The crude ester extract was fractionated on alumina (Column I) and 5 fractions obtained. These were quantitated except for (5) which was heavily contaminated with bile pigment. Spectroscopy and chromatography

gave the results shown in the figure.

Fraction 1. Analysed on MgO, colum II (Fig.VII-18) and 14.2 mg PROTO was obtained (Fraction 1-3). This was recrystallised 4 X; some of the crystals had the typical "boomerang" morphology of PROTO IX. The m.p. was 221 - 224° C. An I.R. spectrum agreed fairly well with that of reference PROTO IX.

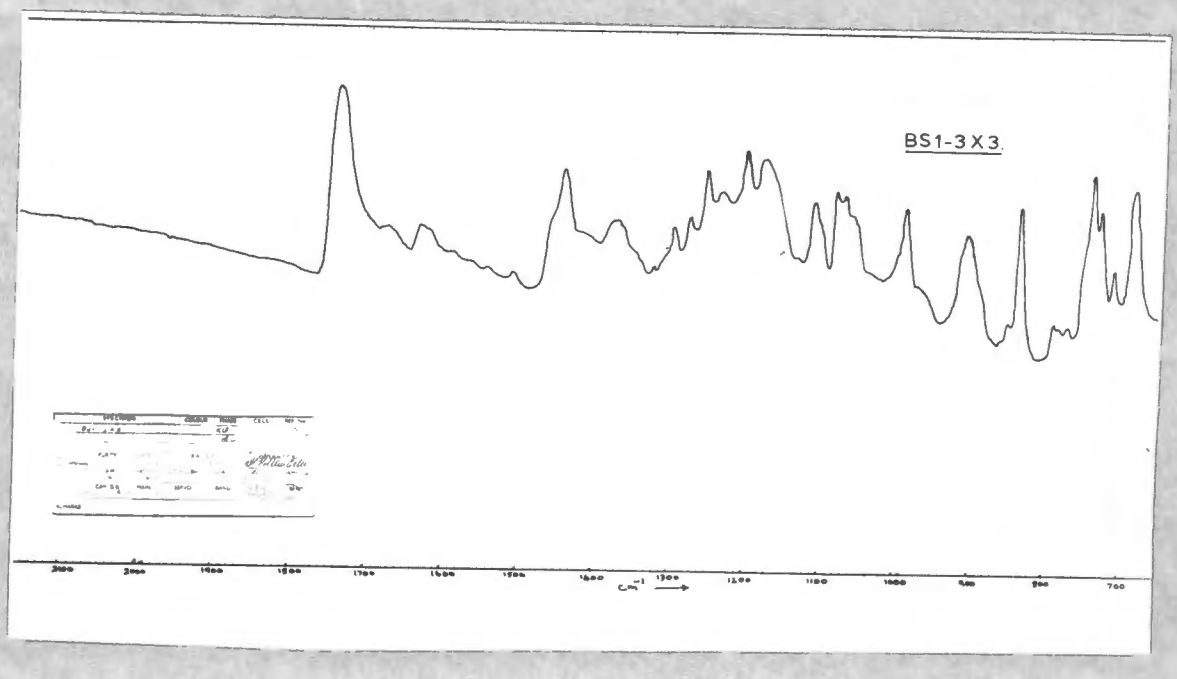


Fig. VII - 20.
IR spectrum of PROTO IX from faeces of Case (i) H.B.

On this evidence it was thought that the identity of the isolated porphyrin had been adequately established but later work with countercurrent distribution showed that the technique of chromatography on MgO used does not separate PROTO from monovinyl deuteroporphyrins. "Fairly good agreement" of the IR spectrum has thus been obtained with an impure specimen. This casts serious doubts on the value of IR spectra for proving identity of porphyrins unless the spectra are absolutely identical. Fraction 1-2 from the MgO column was analysed by CCD. Fig. VII - 21 shows the pattern obtained.

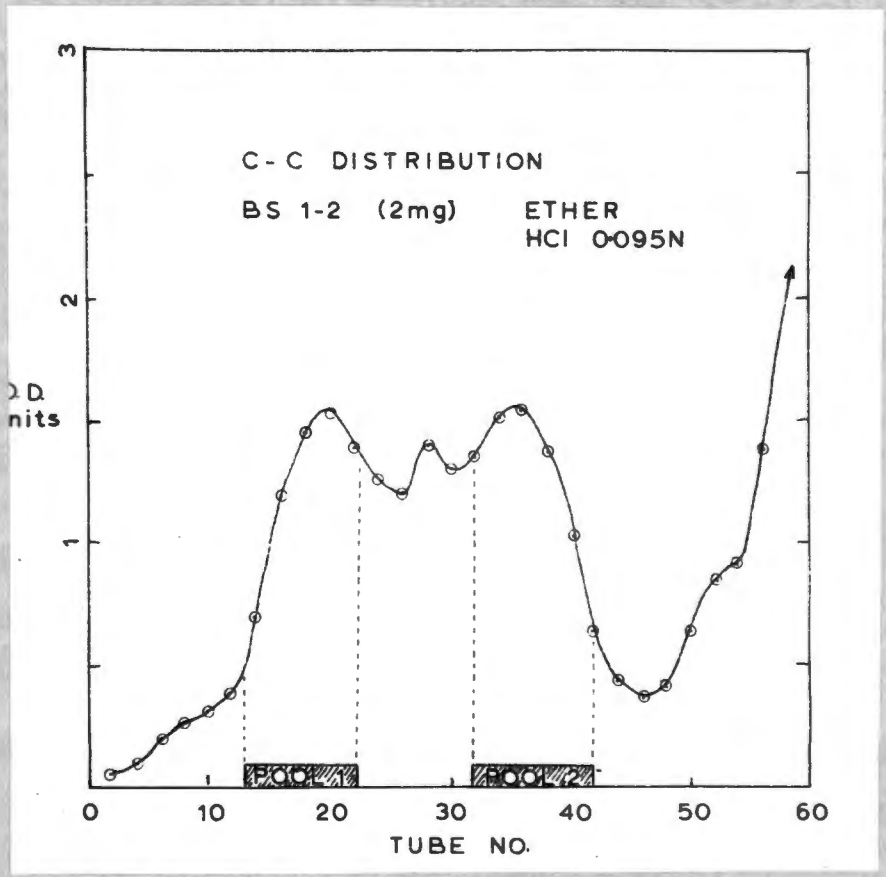


Fig. VII - 21.
CCD pattern of fraction 1-2, Case (i) H.B.

Two major components are indicated as Pools 1 and 2, which had the properties given in Table VII - 40. The fraction intermediate between pools 1 and 2 (n - 28) is probably (2 or 4) monoethyl deuteroporphyrin.

Table VII - 40.

Pool.	"n"	"k"	Calculated HCl no.	Absorption in ether.	Porphyrin.
1	20	0.50	0.345	622.0 573.3 526.2	DEUTERO
2	36	1.50	0.600	623.0 574.0 528.4	MESO

CCD analysis explained the failure to obtain crystalline material of good morphology and sharp m.p. from fraction 1-2. Crystals had been produced, the m.p. was 165 - 170° C, but morphology was not uniform. Fraction 2, from column I (Fig.VII - 18) was dealt with as shown in the diagram which is self-explanatory. Column IV (alumina) showed that it was possible to isolate 3 fractions differing little in alpha band absorption, but having 2, 3 and 4 carboxyl groups. These fractions probably had 4, 3 and 2 propionic carboxyl groups and could have been degradation products of COPROGEN.

Fraction 3. Lutidine chromatography (system 2) indicated 75% isomer III COPRO. Fractional crystallisation yielded 24 mg COPRO III (mp Cu^{II} complex 215-217°C, Fig. VII - 22.)

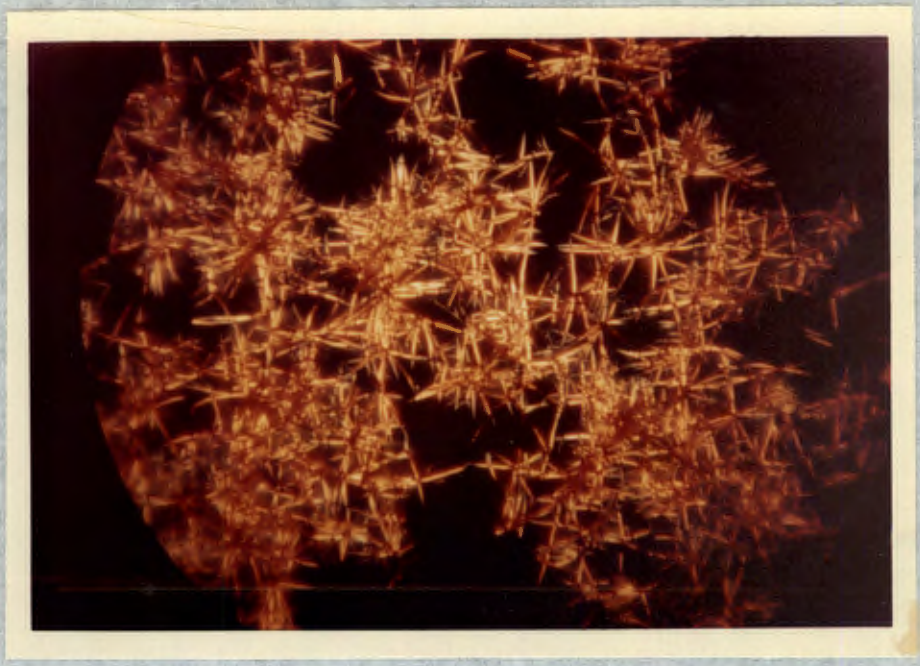


Fig. VII - 22.
Crystals of COPRO III Copper complex
m.p. 215-217°C.

and 7 mg COPRO I, mp 248 - 250°C. The IR spectra are shown in Fig. VII - 23.

Fraction 4. 1.6 pentacarboxyl porphyrin not investigated further.

Fraction 5 Lutidine chromatography (system 1) showed prominent 2 and 4 carboxyl porphyrin spots, but more highly carboxylated porphyrin was not conspicuous.

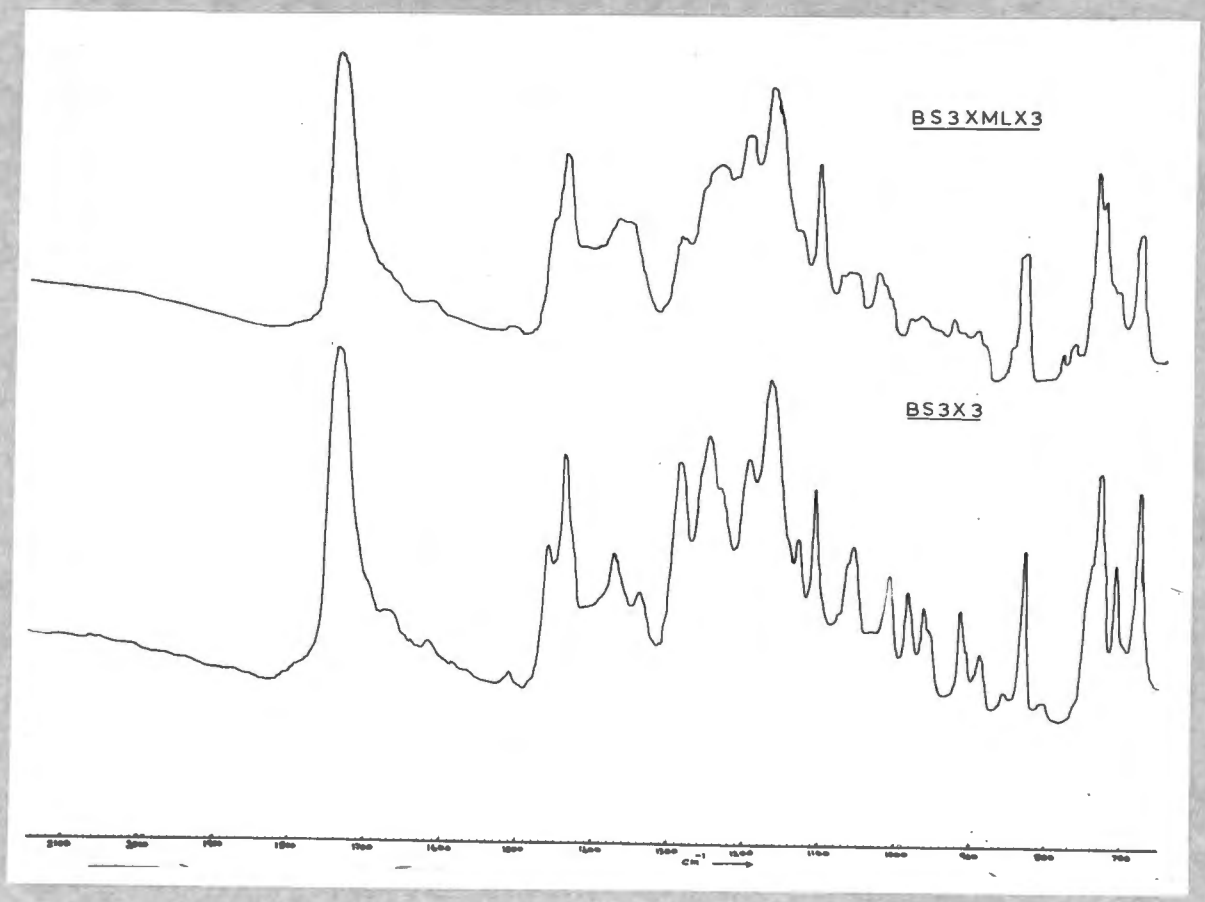


Fig. VII - 23.

IR spectra of COPRO's I and III separated by fractional crystallisation from Fraction 3 (Fig.VII - 19.)

Comment. Analysis of the urine showed dissociation of the isomer composition of the COPRO and URO fractions. The appearance of a small amount (10%) of isomer II URO suggested that non-enzymic conversion of PBG might be the source of increased amounts of URO and this was corroborated by the excess of 8 over 7 carboxyl porphyrin. Urine COPRO in specimens A, B and C was virtually all

isomer III. (The possibility of isomer IV being present is not excluded by the chromatographic technique, but it is improbable.)

The faecal analysis was complex but the following conclusions appear valid: COPRO was the largest single fraction; 75 - 80% was isomer III and the m.p. of the Cu complex was determined. This excluded the possibility of isomer IV being present. The IR spectra of both COPRO isomers isolated agree well with spectra of reference material.

PROTO was not prepared in very pure form, but none of the properties of the four times recrystallised material suggested it was not largely PROTO IX.

MESO, DEUTERO and a hybrid of these porphyrins accounted for most of the 9.2 mg of fraction 1-2 (Fig. VII - 19)

$(COOH)_3$ porphyrin was isolated but it was not monovinyl and therefore not a biosynthetic intermediate.

(ii) A.S. Coloured female, 35 years. Housewife. Studied first during an acute attack and later in remission.

(a) Urine Porphyrin. Porphyrin was recovered from 2.1 L of urine(8 - 12/6/63). Chromatography on MgO and on paper provided the following fractions: (Table VII - 41).

Table VII - 41.

Fraction.	mg.	($CHCl_3$)	(COOH)	% Isomer III.
1	1.8	625.3	8 7	30
2	0.17	621.8	5 4	70
3	0.44	621.0	4	80

Chromatographic evidence for the presence of isomer II uroporphyrin was dubious; but the eight carboxyl porphyrin exceeded seven carboxyl porphyrin judging from the intensity of spots when the crude ester extract from the urine was analysed by electrophoresis and chromatography. This suggested that the eight carboxyl porphyrin had, in part, formed by non-enzymic condensation of PBG.

(b) Faecal Porphyrin. (a) The first specimen obtained after admission:-

The total weight was 340 gm (wet) or 75 gm dry weight. A crude extract of porphyrin ester was prepared and analysed; the chief components were porphyrins with two and four carboxyl groups, but the presence of

porphyrins with 5, 6, 7 and 8 carboxyl groups was clearly shown on electrophoresis and lutidine chromatography (system 1). Chromatography on alumina gave five fractions (Table VII - 42.)

Table VII - 42.

Fraction.	mg.	($CHCl_3$)	COOH.
1	10.8	625	2
2	4.5	621	3> 4
3	21.7	621.8	4
4	3.5	622.4	5>4
5	about 25	623	2 - 8
	66 mg.		

Fraction 1: Chromatographic behaviour and the position of the alpha band suggested the presence of a considerable amount of decarboxylic porphyrin which was not protoporphyrin; this was confirmed after chromatography on MgO (Table VII - 43.)

Table VII - 43.

Fraction.	mg.	($CHCl_3$)
1-1	0.53	621.7
1-2	5.32	620.0
1-3	2.55	629.5

Fraction 1-3 was crystallised and recrystallised from chloroform/hexane. Rhomboidal crystals of uniform size and morphology were obtained which melted in the range 218 - 222° C. Fraction 1-2 could not be crystallised; the position of the alpha band and behaviour on paper chromatography (KER-CHLOR-PROP) suggested that this fraction was a mixture of meso- and deuteroporphyrins.

This fraction was subsequently analysed by CCD. (Fig. VII - 24).

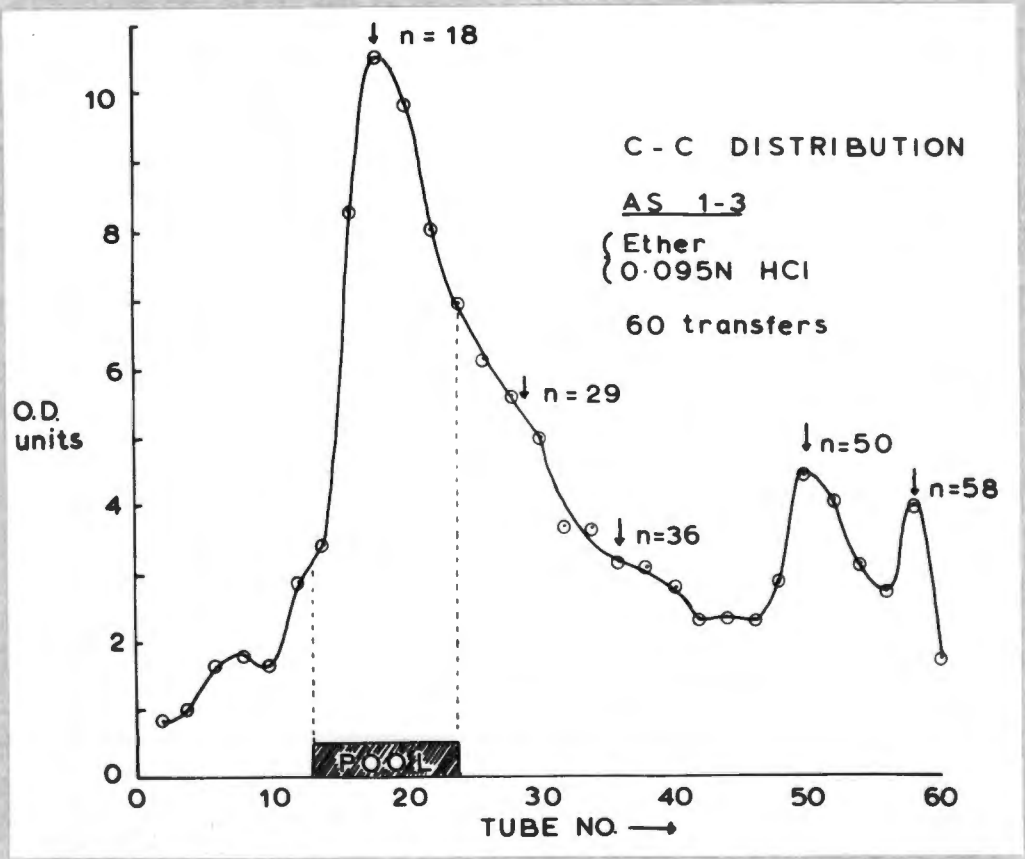


Fig. VII - 24.
CCD distribution pattern of fraction 1-2 from Case (ii) A.S.

The phases consisted of ether and 0.095 N HCl; 60 transfers were carried out. A complex pattern was obtained but the largest fraction was deutero-porphyrin (n = 18). The alpha and gamma bands in ether were at 621.8 and 527.0 mu respectively. After esterification with CH_2N_2 and chromatography on alumina, crystals were grown from chloroform/methanol. (Fig. VII - 11b.) The CCD pattern also suggested the presence of mono-ethyl-deutero- (n=29) and mesoporphyrins (n = 36). n=50, and n=58 indicate small amounts of monovinyl- and protoporphyrins in this fraction.

Fraction 2: not analysed further.

Fraction 3: Analysed by lutidine chromatography (system 2). Found: 50% isomer III

Fraction 4: Five carboxyl porphyrins, not investigated further.

Fraction 5: Electrophoresis showed the presence of approximately equal quantities of porphyrins with from 2 to 8 carboxyl groups.

Comment. It was noteworthy that the fraction 1-2, probably a mixture of MESO and DEUTERO, exceeded the PROTO fraction (1-3). Also interesting was the

presence of highly carboxylated porphyrin in the faeces, which as will be seen again later, is a feature distinguishing the acute attack from remission.

Faecal Porphyrin during remission. A faecal specimen was obtained four months after the acute attack. The urine at this time contained COPRO 0.21 and URO 0.06 mg/L. The result of quantitative analysis of faecal porphyrin was COPRO 0.29 and PROTO 0.45 mg/g d.w. Analysis was incomplete but paper chromatography (KER-CHLOR-PROP) of decarboxylic porphyrin separated by alumina chromatography showed this to be largely PROTO. This was confirmed by spectroscopy.

The COPRO in this specimen was analysed.

Found: 67% isomer III

33% isomer I.

(iii) P.N. White female aged 40 years.

(a) The first stool specimen obtained after admission with acute porphyria was analysed. The crude extract of porphyrin esters from 213 g of faeces was chromatographed on alumina. Five fractions were obtained (Table VII - 44.)

Table VII - 44.

Fraction.	mg.	($CHCl_3$)	(COOH)
1	8.6		2
2	2.35	no	4 > 3,2
3	12.0	record	4
4	3.16		5
5	heavily contaminated with bile		2 - 8

Fraction 1. Chromatography on MgO yielded 3.8 mg of PROTO judging from spectroscopic characteristics, behaviour when chromatographed on paper using the system (KER-CHLORO-PROP), crystal morphology and m.p. 222 - 224°C. (after three recrystallisations.) Only 0.6 mg of a mixture of MESO and DEUTERO was obtained.

Fractions 2 and 3. These were combined as fraction 2 was largely coproporphyrin. The isomer composition was analysed by lutidine chromatography (system 2).

Found: Isomer III 49%

Isomer I 51%

There was a small amount of porphyrin, perhaps 5%, which behaved like COPRO II in the chromatogram.

(b) The analysis described above had indicated the presence of porphyrin with more than 4 carboxyl groups. This problem was further investigated by analysing a specimen obtained on the following day by quantitative electrophoresis. (Table VII-45).

Table VII - 45.

COOH Groups.	2	4	5	6	7	8	Total.
Faecal Porphyrin	416	1177	277	74	41	140	2325 ug/g d.w.

This technique does not result in clear separation of 2, 3 and 4 carboxyl porphyrins but more highly carboxylated fractions are readily identified.

(iv) M.L. White female, age 50 years. Housewife.

(a) Faecal porphyrin was analysed during an attack of acute porphyria in order to investigate the excretion of highly carboxylated porphyrins. Table VII -46.

Table VII - 46.

COOH Groups	2	4	5	6	7	8	Total
Faecal Porphyrin	427	1125	379	174	150	176	2431 ug/g d.w.

(b) The urine porphyrin was not analysed in detail. After the excretion of PBG had returned to near normal levels, a sample of urine was collected

and the ratio of COPROGEN:COPRO determined by the same procedure described under Section 2, Case D,(J.F) The result was $\frac{\text{COPROGEN}}{\text{COPRO}} = 3.5$.

(v) S.S. Coloured male, unemployed.

This patient was studied during two different attacks of acute porphyria. On the first occasion faecal porphyrin was analysed by quantitative electrophoresis to investigate the excretion of highly carboxylated porphyrin. On the second occasion, a sample of bile was obtained and analysed by CCD.

(a) Faecal Porphyrin.

Table VII - 47.

COOH Groups.	2	4	5	6	7	8	Total	
Faecal Porphyrin	254	747	124	38	55	119	1337	ug/g d.w.

(b) Bile Porphyrin. The Ehrlich reaction on the bile was negative.

The CCD pattern after 40 transfers between the phases ether, 0.101 N HCl is shown in Fig. VII - 25. Three chief components are evident; "COPRO" to the left, and intermediate fraction and a "PROTO" fraction to the right. No ether insoluble porphyrin had been

noted when the bile was initially extracted.

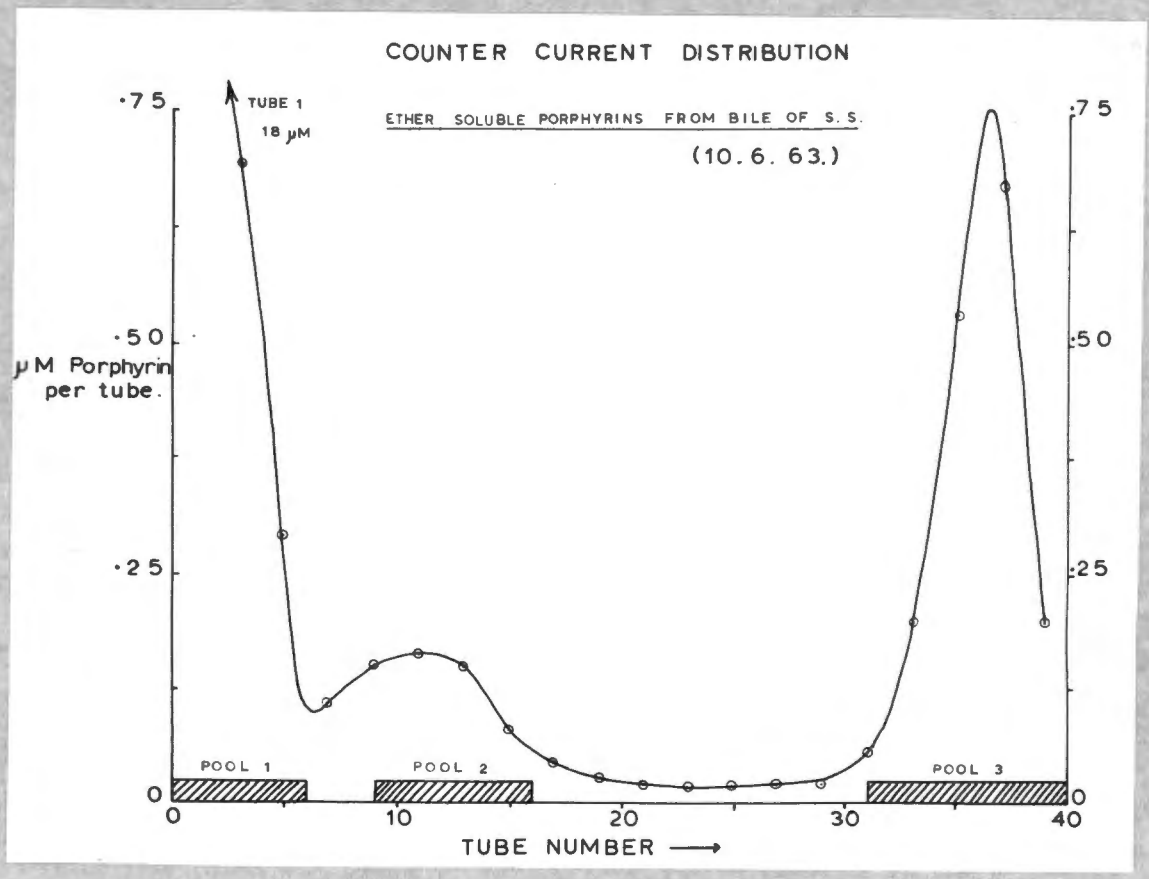


Fig. VII - 25.

CCD pattern of porphyrin from bile of Case (v) S.S.

Three pooled fractions were worked up further:

1. Tubes 1 - 7
2. Tubes 10 - 17
3. Tubes 32 - 41.

These fractions were esterified individually (diazomethane) and chromatographed on alumina. The fractions from alumina were hydrolysed and analysed by lutidine chromatography. (Fig. VII - 26)

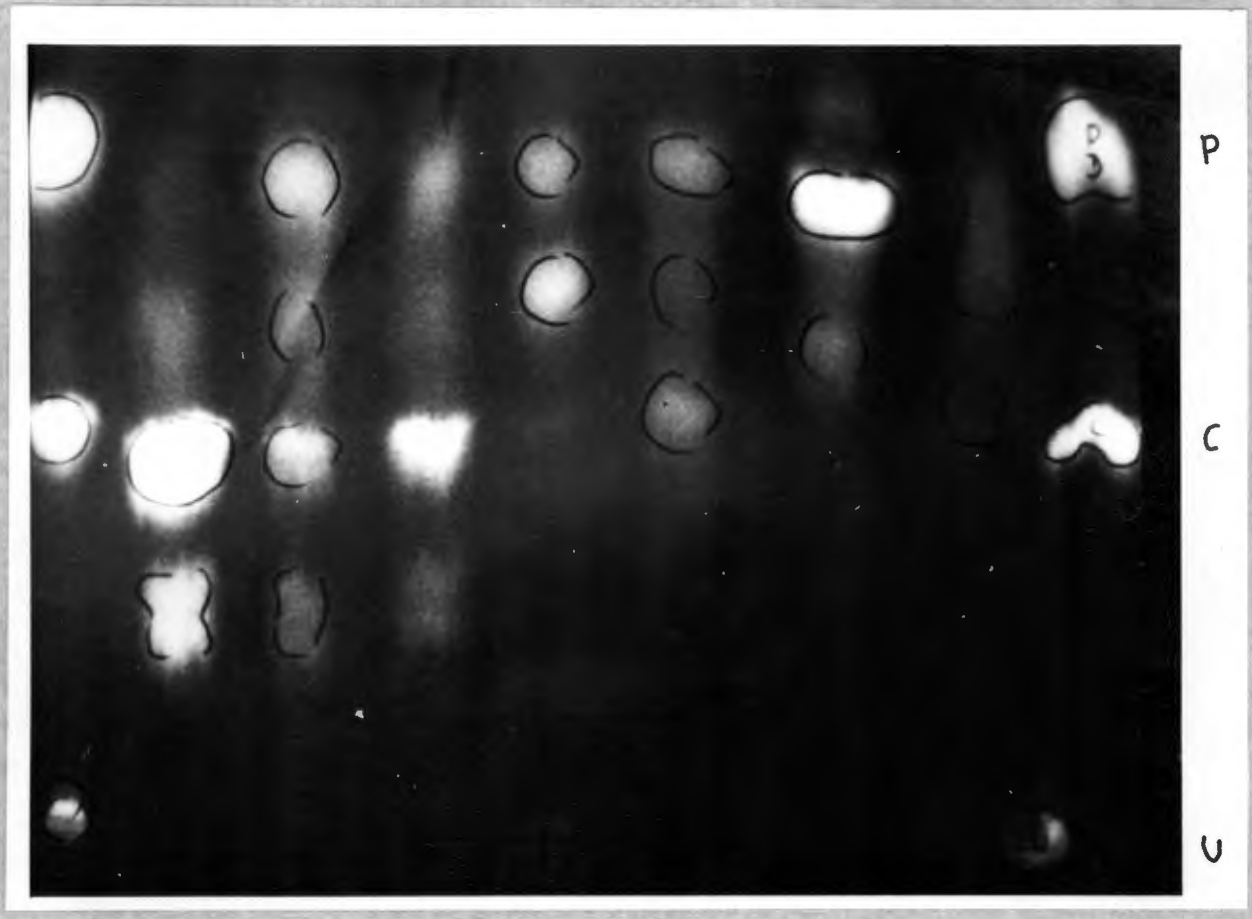


Fig. VII - 26.
Lutidine chromatogram of fractions of bile porphyrin (Case (v) separated by CCD.

It is quite clear from the chromatogram that these manipulations were not effective in isolating pure fractions. The principal component in tubes 1 - 7

was COPRO and in tubes 32 - 41 a dicarboxylic porphyrin. The intermediate fraction (Tubes 10-17) contained largely dicarboxylic porphyrin which was presumed to be of the meso - deutero type.

(c) Urine Porphyrin. The only study made was to measure the COPROGEN/COPRO ratio. The technique has already been referred to.

Found: $\frac{\text{COPROGEN}}{\text{COPRO}} = 3.75.$

5. Porphyrin Excretion in Swedish Genetic Porphyria.

Criteria for the diagnosis of Swedish Genetic porphyria are as follows:-

1. A family history of the disorder with the diagnosis in affected members confirmed biochemically.

2. Elevated urine ALA and PBG excretion at all times.

3. Normal, or only slightly increased, faecal porphyrin concentration.

4. The absence of all signs of cutaneous porphyria.

5. The occurrence of attacks of acute porphyria either in the patient or in members of his family.

The studies reported here have all been made on members of a single, rather small, family. The family

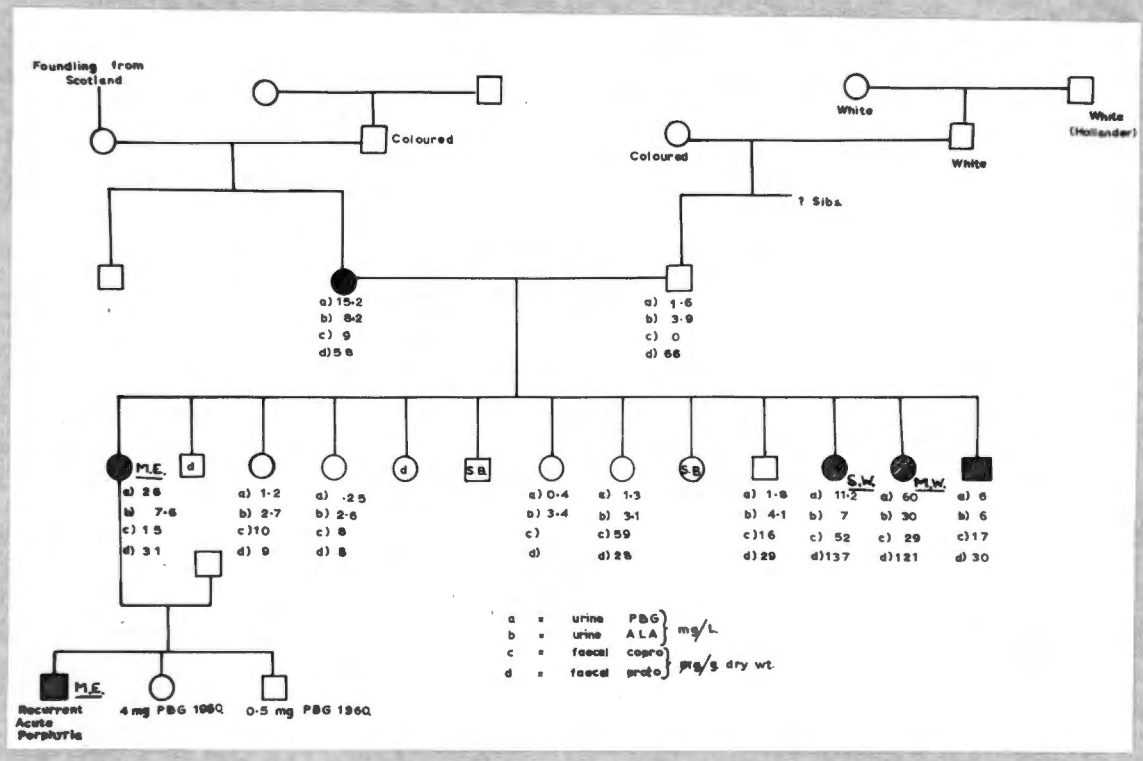


Fig. VII - 27.

Family studies by Miss A. Moodie on patients with Swedish genetic porphyria. Biochemical pictures are shaded.

tree is shown in Fig. VII - 27. The clinical course in Case 9 (A), M.E. has been dominated by severe rheumatic carditis. This patient has been admitted to Groote Schuur Hospital frequently and gross increases in urine ALA and PBG excretion while faecal porphyrin has been relatively normal, have been documented on many occasions. Case (B), M.W., is an aunt of M.E. and suffers periodic attacks of abdominal pain. She has been admitted to hospital on four occasions because of severe attacks of abdominal pain accompanied at the onset by grand mal seizures. Again, high urine levels of ALA and PBG have been accompanied by only moderately increased faecal porphyrin concentration.

Cases (C) and (D) have not been studied clinically. As far as is known they are asymptomatic but the figures given below show quite obviously that they are biochemically positive.

(A) M.E. Coloured male, 20 years, unemployed.

(i) Urine Porphyrin. Porphyrin was recovered daily from 24 hour specimens of urine collected during the course of a metabolic experiment. The delay between voiding the urine and recovering the porphyrin was thus minimised as was the formation

of uroporphyrin from PBG. A total of 7.3 L of urine was extracted. Chromatography of the crude ester showed porphyrin with 8, 5 and 4 carboxyl groups. This was chromatographed on MgO and the isomer content of the fractions obtained determined by lutidine chromatography (system 2). The findings are shown in Table VII - 48.

Table VII - 48.

Fraction.	mg.	Recovery ug/L	($CHCl_3$)	(COOH)	% Isomer III.
1	0.87	119	625.4	8 5	60
2	0.62	85	620.8	4	90

(ii) Faecal Porphyrin. It was anticipated that the yield of porphyrin from faeces of this patient would be low. For this reason a large specimen (850 g) was extracted. The crude ester extract obtained was chromatographed on alumina and three fractions were obtained (Table VII - 49.)

Table VII - 49.

Fraction.	mg.	($CHCl_3$)	(COOH)
1	22.0 (!)	629	2
2	2.1	622	4
3	largely bile pigment, not examined further.		

Fraction 1. Without further purification this fraction was crystallised (m.p. 217 - 219° C.) and recrystallised (m.p. 220 - 222° C.) Because of its spectroscopic and chromatographic behaviour and because of the mp, the fraction was regarded as PROTO IX. The amount recovered amounted to approximately 100 ug/g d.w. of faeces and must be regarded as increased.

Fraction 2. Spectroscopy and chromatography indicated this fraction to be COPRO. The isomer composition was analysed by lutidine chromatography (system 2).

Found: about 70% isomer I.

(B) M.W. Coloured female, aged 26. Housewife.

Faecal Porphyrin. A stool specimen weighing 125 gm (50 g d.w.) was analysed. This contained PROTO 81 and COPRO 74 ug/g d.w. (Holti method). The crude porphyrin ester extracted was chromatographed on alumina; the findings are recorded in Table VII - 50.

Table VII - 50.

Fraction.	mg.	($CHCl_3$)	(COOH)
1	0.75	metal complex	2
2	2.46	622.6	2
3	0.32	594.4 metal complex	3, 4
4	0.5	632.2	4

This analysis was unsatisfactory because a considerable amount of both 2 and 4 carboxyl porphyrin was recovered as metalloporphyrin. It is, however, significant that fraction 2 was not largely proto-porphyrin, judging from the alpha band absorption and behaviour of the ester when chromatographed on paper (KER-CHLORO-PROP). Further analysis of MgO resulted in loss of this fraction because of difficulty in preparing MgO with the correct degree of adsorbency. Fraction 4 was analysed by lutidine chromatography (system 2). Found: about 70% isomer I.

(C) S.W.
(D) M.E. Specimens of faeces were obtained from two further patients of the family to which cases (A) and (B) above belonged. Urine porphyrin precursors were estimated at the same time. The results of these estimations and of quantitative analyses of the stools is shown in Table VII - 51.

Table VII - 51.

	S.W.	M.E.
Urine (mg/L)		
PBG	11.2	26
ALA	7.0	7.6
Faeces (ug/g d.w.)		
Copro	52	15
Proto	137	31

(i) Quantitative electrophoresis of faecal porphyrin (Table VII - 52).

Table VII - 52.

COOH Groups.	2	4	5	6	7	8	Total.	
S.W.	98	23	-	trace	-	8.5	130)	ug/g d.w.
M.E.	62	16	-	trace	-	trace	78)	

Normal faeces have not been analysed by this technique so that it is uncertain whether these levels are abnormal. By the Holti method it was quite clear that faecal porphyrin of S.W. is increased. The electrophoretic technique did, however, show that in the patient who was excreting the greater quantity of PBG, a measureable amount of uroporphyrin could be detected in the faeces. Seven carboxyl porphyrin could not be measured.

(ii) Chromatography on alumina. Aliquots of the ethyl acetate extract of faeces which had been prepared for analysis by electrophoresis were analysed on alumina columns. The results are tabulated (Table VII - 53). The identity of the fractions is based on spectroscopic and chromatographic behaviour. Isomer analysis was by the quantitative lutidine (system 2) chromatographic technique.

Table VII - 53.

	S.W.	M.E.
Proto	.24	.19
Copro	.05	.05
% Isomer III	29	32

6. Porphyrin Excretion in Erythropoietic Protoporphyria.

Faecal porphyrin excretion has been studied in a single case of this condition. The patient was a fifteen year old white boy whose skin has been abnormal since the age of seven years. Unlike patients with S.A. Genetic porphyria who complain chiefly of the sequelae of increased cutaneous fragility, he has suffered, as the most prominent symptoms, oedema and erythema following prolonged exposure to sunlight. His parents are both quite normal. Following the report of Magnus et al (147), free erythrocyte porphyrin was measured and found to be grossly increased. Figure VII - 28 shows the appearance of a smear of peripheral blood in a fluorescence microscope.

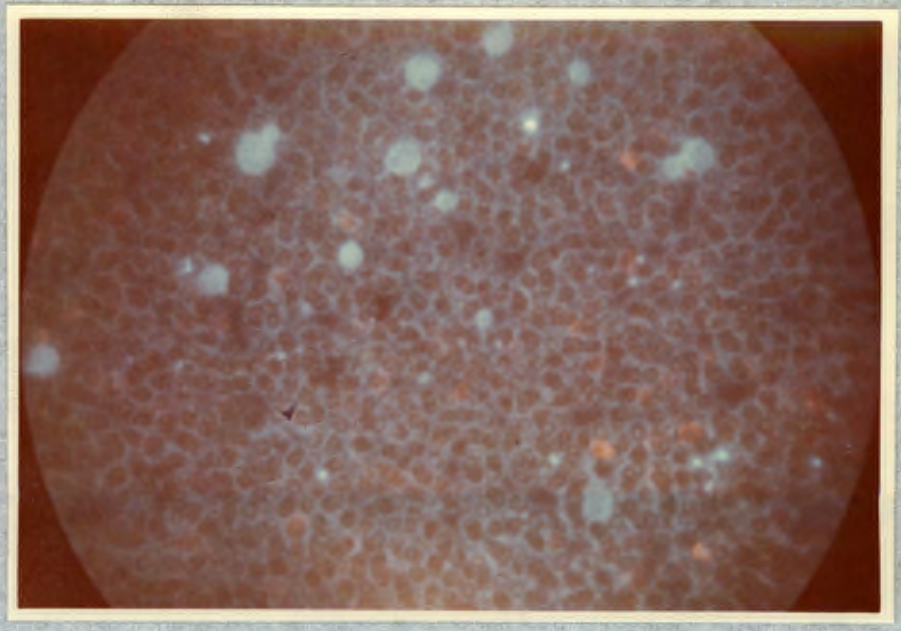


Fig. VII - 28.
Photomicrograph of the transient red fluorescence seen in erythrocytes from a patient with erythropoietic protoporphyria.

The levels of porphyrin and porphyrin precursors found in the urine were normal. Faecal porphyrin concentration was COPRO 0.04 and PROTO 0.52 mg/g d.w. Free erythrocyte porphyrin was COPRO 4.7 and PROTO 556 ug/100 c.c. blood. (Method of Schwartz and Wikoff,148).

Porphyrin was extracted from a stool weighing 113 g (wet wt.) Chromatography of the crude extract on paper (lutidine/water, system 1) showed the principal fraction to be dicarboxylic porphyrin. Chromatography on alumina yielded 4 fractions. Details are given in Table VII - 54.

Table VII - 54.

Fraction.	mg.	($CHCl_3$)mu	(COOH)
1	5.0	629.2	2
2	0.1	-	3
3	0.19	622	4
4	contaminated with bile		2

Fraction 1: PROTO judging from the spectroscopic and chromatographic behaviour; this fraction crystallised from chloroform-methanol to give crystals melting in the range 219 - 233° C.

Fraction 3: COPRO. Isomer analysis showed this to be 70% isomer I, 30% isomer III.

Comment: Significant points were the ratio of PROTO to COPRO evident from the quantitative analysis and after column chromatography. Column chromatography

showed this ratio to be more than 25:1, which sets this case apart from all others studied in this survey of porphyrin excretion. The COPRO isomer composition is that of a normal person and the COPRO is quantitatively not increased.

CHAPTER VIII.

COLLECTED ANALYTICAL DATA.

In the following sections the experimental results set out in Chapter VII will be summarised. Where applicable quantitative data obtained using standard laboratory methods will be included in an attempt to define the patterns of porphyrin excretion encountered in health and in the porphyrias studied.

1. Normal Porphyrin Excretion.

There is no normal level of porphyrin excretion; but only an upper limit arbitarily set because it exceeds the excretion of, for example, 95% of a healthy population. The upper limits of porphyrin excretion in health have been considered by Eales and Saunders (75).

A. Urine Porphyrin. 30 ug/L of "URO" and 130 ug/L of "COPRO" are accepted as the upper limits of normal. "URO" will consist chiefly of 8 carboxyl porphyrin but also some 7 carboxyl material. "COPRO" will be contaminated with traces of 5 and 6 carboxyl porphyrins. The COPRO of normal urine is mostly isomer III.

B. Faecal Porphyrin. The upper limits of normal are accepted as COPRO 27 ug/g dry weight and PROTO 75 ug/g dry weight. While the expression of faecal porphyrin as a concentration is useful in dealing with diagnostic problems, arguments will be developed below which require an approximate assessment of daily porphyrin excretion. It will be assumed that the average hospital patient consuming a full diet excretes about 25 g of faeces (dry weight) daily. (149). However, no reasoning will be advanced which relies heavily on the precision of this figure, which certainly does not apply to ill patients whose daily output of faeces may be very small.

The isomer content of faecal COPRO was investigated in 3 normal subjects (Chapt.VII -2). In each instance it was about 70% isomer I. No good evidence was obtained to prove that normal faecal PROTO is type IX. Crystalline material was only isolated in subject (A). The rather lame comment may be made that it is very unlikely to be any type other than IX.

C. Daily Excretion. Combining the various facts and assumptions given above, the upper limit of daily porphyrin excretion in health may be arrived at:-

	URO	COPRO I	COPRO III	PROTO	
Urine	30		130	-	)
Faeces	30-40	510	170	1900	) ug. daily.
Total	60-70	510	300	1900	)

2. Acquired Porphyria. (Chapt.VII Section 3.)

Urine.

The urine was studied in 5 cases. In 2 of these the results of electrophoretic analyses were expressed quantitatively. The inspection of chromatograms and electrophoresis strips gave similar results.

(1) There were large amounts of porphyrin with 7 and 8 carboxyl groups.

(2) All intermediates of UROGEN decarboxylation were represented.

(3) Coproporphyrin was not much more prominent than porphyrin with 5 or 6 (COOH) groups.

These results may be expressed quantitatively in Table VII - 1 using data from Tables VII - 11, 17 and 21. The results of quantitative analysis by conventional technique are given for comparison:-

Table VIII- 1.

							Conventional		
COOH group	8	7	6	5	4	Total	"URO"	"COPRO"	
Case E	345	389	196	152	158	1240.	1390	330	)
F	1140	630	96	61	45	1972	2870	130	)ug/L
B	1710	1850	470	690	580	*	4300	1000	)

* Table VII - 11 gives results only in terms of % of total. Figures for (COOH) 8 - 4 calculated from "conventional" total.

Isomer patterns of uroporphyrins were studied in cases A, B and C; the results are set out in Table VII-2. No evidence for isomer II was noted.

Table VIII - 2.

COOH	8	7	4
Case		% Iso.III	
A		60	85
B	30	75	60
C	30	85	75

It is concluded that the eight carboxyl porphyrin is largely isomer I but the 7 and 4 carboxyl porphyrins largely isomer III.

Faecal Porphyrin.

In only one case (D) did quantitative analysis by the Holti method indicate "PROTO" "COPRO". In only one instance (E) was the total faecal porphyrin normal but the "COPRO" fraction was definitely elevated.

Chromatography and electrophoresis of crude extracts of faecal porphyrin had indicated the presence of porphyrins with from 2 - 8 carboxyl groups. The quantities of highly carboxylated porphyrins were considerable in all cases.

Column chromatography enabled fairly pure fractions of di- and tetra- carboxyl porphyrin to be prepared. A significant finding which was quite different from the finding in normal persons, was the small amount of dicarboxylic porphyrin obtained relative to COPRO. Table VIII - 3 illustrates this point and includes, for interest, the $\frac{\text{COPRO}}{\text{PROTO}}$ ratio obtained by the Holti method.

Table VIII - 3.

Case.	$(COOH)_4$ mg.	$(COOH)_2$ mg.	$\frac{(COOH)_4}{(COOH)_2}$	Holti $\frac{\text{COPRO}}{\text{PROTO}}$.
B	9.5	1.7	5.6	3.23
C	4.5	0.6	6.8	2.3
E	3.8	0.5	7.6	1.6
F	2.0	1.5	1.34	1.3

The nature of the dicarboxylic porphyrin was further investigated by MgO chromatography in cases A, B and C and by CCD in cases E and F. Only from "F" was a fairly pure sample of "PROTO" obtained. (Fig.VII - 11a). In this instance recovered PROTO amounted to 12 ug/g d.w. but in none of the others did it exceed 10 ug/g d.w. The remainder of the dicarboxylic fractions obtained probably consisted of MESO, DEUTERO etc. but as these were never obtained in pure form, their nature could only be inferred.

Whereas the faecal COPRO is always increased, faecal $(COOH)_2$ is not increased above normal. (It would be meaningless to say that it is below normal because "normal" is being used here only to indicate that a value is below an upper limit.)

The isomer content of faecal COPRO was investigated in cases A, B, C, E and F. In A, B and C the measurement was only approximate; the results were 85%, 90% and 75% isomer III respectively. In E and F the technique was considered accurate and the results were 84% and 91% respectively of isomer III.

Highly carboxylated porphyrin which was noted in the faeces in all instances was measured by quantitative

electrophoresis in 2 cases. These porphyrins are usually regarded as being excreted largely into the urine, so, for the purpose of approximate comparison with amounts found in the urine, concentrations are expressed as "per g. d.w." and per 25 g. d.w." (Table VIII - 4.)

Table VIII - 4.

	COOH						
	8	7	6	5	4	2	
Case							
D (J.F.)	24	119		110	195	36	ug/g d.w.
	600	2980		2750	4850	972	ug/25g.dw
F (F.M.)	46	48	18	27	141	86	ug/g d.w.
	1140	1180	435	685	3530	2150	ug/25g.d.w.

If the assumption made regarding the normal daily stool weight is valid, then the faecal excretion of uroporphyrin and porphyrins with (7 - 5) carboxyl groups becomes highly significant, possibly even exceeding urinary excretion.

Duodenal aspirate from Case E (G.O.) was analysed by CCD. The pattern obtained was in agreement with the above comments on faecal porphyrin excretion in acquired porphyria.

Free porphyrin in the liver was measured in Cases B, D, E and F and found to be grossly increased. In Cases D and F this proved to be seven and eight carboxyl porphyrin only.

Summary of findings in 6 cases of acquired porphyria.

Urinary porphyrin was greatly increased; this consisted chiefly of 8 and 7 carboxyl porphyrins with lesser amounts of with 6, 5 and 4 carboxyl porphyrin groups. All except 8 carboxyl were chiefly isomer III; the eight carboxyl was about 70% isomer I. No evidence for other isomers was found.

Faecal porphyrin was usually increased but, more striking than the quantitative increase which was variable, was the reversed ratio of COPRO to dicarboxylic porphyrin and the significant amounts of porphyrins derived by oxidation from substrates of UROGEN decarboxylase.

Faecal COPRO ranged from 75 - 94% isomer III. Faecal PROTO was definitely not increased; more difficulty was experienced obtaining PROTO in pure form from patients with acquired porphyria than from normal persons.

3, S.A. Genetic Porphyria - Summary of Analytical Work.

(A) Patients studied during remission.

Analyses are reported on 6 cases (i - vi) H.S., A.V., S.D., P.V., H.B. and J.C.

(i) Urine Porphyrin. Urine porphyrin was studied in Cases (i), (ii), (iii) and (v). (i) and (ii) were studied by qualitative procedures and by chromatography on MgO. (iii) and (v) were studied by quantitative electrophoresis. In each instance the findings were similar. COPRO was the predominant fraction. In (i) and (ii) this was found to be almost entirely isomer III. URO formed a very much less significant fraction and was accompanied by seven carboxyl porphyrin. (e.g. Fig.VII - 12) Tables VII - 29 & 31 express these facts in quantitative terms. Only traces of porphyrin with five and six carboxyl groups have been encountered.

Much less attention has been paid to urine porphyrin excretion than to faecal porphyrin because, as previously emphasised, the urine is quantitatively a relatively unimportant route of porphyrin excretion unless the highly carboxylated porphyrins are being considered.

(ii) Faecal Porphyrin.

(a) Principal fractions. In each instance lutidine chromatography confirmed the presence of large amounts of COPRO and dicarboxylic porphyrin. These fractions were subsequently isolated by alumina chromatography. The findings in three cases are given in Table VII - 5 and contrasted with the COPRO/PROTO ratio derived from conventional analysis.

Table VIII - 5.

	COPRO mg.	$(COOH)_2$ mg.	Ratio $\frac{COPRO}{(COOH)_2}$	$\frac{C}{P}$ (Holti)
(ii) (A.V.)	1.36	2.06	0.66	1.09
(iii) (S.D.)	0.24	0.28	0.86	0.54
(iv)(H.B.)	8.2	20.9	0.39	1.04

No close correlation exists. These figures are given because they express in numerical terms a significant difference between faecal porphyrin in these patients and those with acquired porphyria. In S. A. genetic porphyria both COPRO and dicarboxylic porphyrin are considerably increased in the faeces.

(b) Faecal Uroporphyrin. In none of these cases were significant amounts of porphyrin with 6, 7 or 8 carboxyl groups obtained from the faeces.

Quantitative electrophoresis((iii) and (iv)) illustrates this point (Tables VII - 29 and 33.) This contrasts significantly with the finding in acquired porphyria and in S. A. genetic porphyria during the acute attack.

(c) Porphyrin from Duodenal Aspirate ("Bile").

Porphyrin from bile was studied by CCD in cases (v)(Table VII - 32) and (vi) (Fig.VII - 16). The amounts of porphyrin available were small but the distribution patterns obtained suggest that the mixtures of porphyrins encountered in bile are very similar to those encountered in faeces. Some evidence was obtained (Case v) that a porphyrin with the properties of HAEMATO was present in the bile.

(d) COPRO isomer composition. This was investigated in (i), (ii) and (v). In each instance COPRO was predominantly isomer III (80%, 90%, 85%) No evidence of isomer II was noted. Satisfactory crystalline COPRO III was only obtained from Case (v). The isomer composition differs strikingly from that seen in normals and in Swedish genetic porphyria.(Fig.VII - 1)

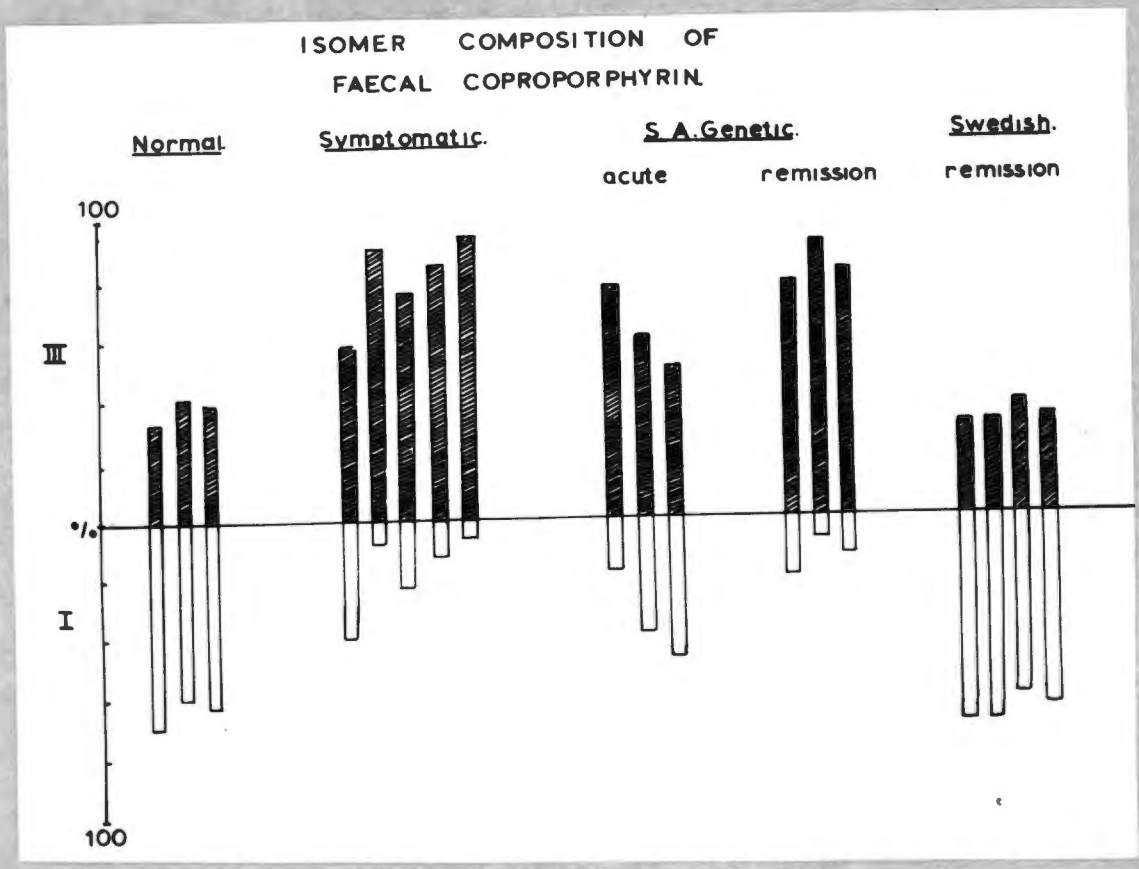


Fig. VIII - 1.
The isomer composition of COPRO isolated from faeces of the various cases described is summarised.

(e) Dicarboxylic porphyrin. During the earlier studies described (e.g. Cases i and ii) porphyrin which was dicarboxylic, which showed absorption bands in chloroform agreeing with reference PROTO and which formed crystals melting in the range 220 - 224°C was accepted as PROTO IX.

The CCD analysis of faecal porphyrin from Case v,

however, yielded a particularly pure PROTO preparation of m.p. 231 - 233°C. Reference PROTO was reviewed and reprepared confirming that the correct m.p. is close to this figure (232 - 233°C).

The evidence, therefore, for describing PROTO from the faeces of cases of S. A. genetic porphyria as identical to PROTO IX rests on this single m.p. determination. It does not seem justifiable to quote evidence based on I.R. spectral studies when m.p.'s have indicated clearly that the material studied has been impure. The best reason for referring to this PROTO as PROTO IX is actually the very great unlikelihood of it being anything else.

The CCD analysis on dicarboxylic porphyrin from Case V also revealed a monovinyl-deuteroporphyrin (Table VII - 34). Evidence for the presence of MESO has been found in Cases iv and v and also in i, studied during an acute attack. Evidence for DEUTERO was found in Cases iv and v during remission and in cases i and ii during acute attacks. CCD patterns in each of these instances provides evidence for a fraction with HCl number and spectroscopic properties intermediate between MESO and DEUTERO.

Porphyrins of the MESO-DEUTERO type have also been found in bile, probably as a result of an entero-hepatic circulation.

(B) Patients studied during acute attacks.

Various analyses were performed on urines and stools from 5 cases, (i - v)

(i) Urine Porphyrin. The striking biochemical abnormality in the urine during acute attacks is the excretion of large amounts of ALA and PBG. A variable amount of URO is formed by the non-enzymic condensation of PBG.

The urine porphyrins were only investigated in detail in Cases i and ii. The serial study in Case i indicated that the principal differences between the urine in remission and during the acute attack could be attributed to condensation of PBG to porphyrin. Evidence for this was the increase in eight, relative to seven, carboxyl porphyrin (see Chapt.IV - 3), the finding of COPRO II on decarboxylation of "URO" fractions obtained from chromatography on MgO columns and the rapid change to the remission pattern already described when PBG excretion decreased. Also, the isomer compo-

sition of urine COPRO remained constant at about 90% isomer III, while urine URO contained significant amounts of series I, II and probably IV URO's.

Porphyrins with 5, 6 and 7 carboxyl groups were never prominent on chromatograms of urine porphyrin from Cases i and ii. At no time was dicarboxylic porphyrin found in the urine during the acute attack.

(ii) Bile Porphyrin. Bile has been obtained only from Case v. CCD analysis and chromatography did not reveal any differences between excretion patterns in remission and in the acute attack. The Ehrlich reaction on this bile specimen was negative.

(iii) Faeces.

(a) Dicarboxylic porphyrin: there was no definite feature characterising the acute attack as opposed to the findings in remission already described. In Cases (i) and (ii) it seemed that an unusually large amount of "degraded" dicarboxylic porphyrin (MESO, DEUTERO etc.) was present, but in (iii) this was not so. There was no gross alteration in the ratio of COPRO to dicarboxylic porphyrin recovered.

(b) COPRO: A difference noted between remission and the acute attack was an increase

in the relative amount of COPRO I. (Fig. VIII - 1)

(c) Porphyrins with more than 4 carboxyl groups. In all cases studied there has been evidence of porphyrins with 5, 6 and 7 carboxyl groups, and even the ethyl acetate/acetic acid extracts sometimes contained URO. This point was investigated by quantitative electrophoresis in Cases (iii) (iv) and (v). (Table VIII - 6).

Table VIII - 6.

	(C O O H)						
	2	4	5	6	7	8	Total.
Case (iii)	416	1177	277	74	41	140	2325)
(iv)	427	1125	379	174	150	176	2431) ug/g d.w.
(v)	254	747	124	38	55	119	1337)

It is quite clear that in the acute attack increased amounts of highly carboxylated porphyrin appears in the faeces. This is significantly different from the findings in remission. It is perhaps surprising that more of porphyrins with 5 - 7 carboxyl groups was not found in the urine of Cases (i) and (ii).

(d) Quantitative Aspects of faecal porphyrin excretion during acute attacks:

The concentration of porphyrin in the faeces tends to be very high in specimens passed during attacks of acute porphyria. This usually coincides with a period of anorexia; constipation is usually a feature and the daily output of faeces may be very low indeed. The biochemical disturbance of the acute attack is usually short-lived and has resolved when daily faecal output returns to more normal levels. It is conceivable that by studying only concentrations of porphyrin, a marked decrease in faecal porphyrin excretion during the acute attack may have been overlooked.

The importance of this point cannot be over-emphasised, and will be considered again in Chapt. IX. An opportunity to investigate the relationship between daily faecal output and the urinary excretion of ALA and PBG arose on the second admission of S.S. (Case v) in an attack of acute porphyria. Shortly after admission the patient was transferred to a metabolic ward. All urines and stools were collected and Fig. VIII - 2 shows variations of weekly PBG and total faecal porphyrin excretion.

The inverse relationship was striking in the beginning but over the crucial first 2 weeks in a

general ward, faecal collections may have been incomplete. This single observation is however, strongly suggestive of an inverse relationship between faecal porphyrin excretion and precursor excretion, and further study is mandatory. Unfortunately, it is extremely unusual for precursor excretion to remain elevated for such long periods in S.A. genetic porphyria. In this instance, prescription of the sedative trichloralphenazone may have been partly responsible.

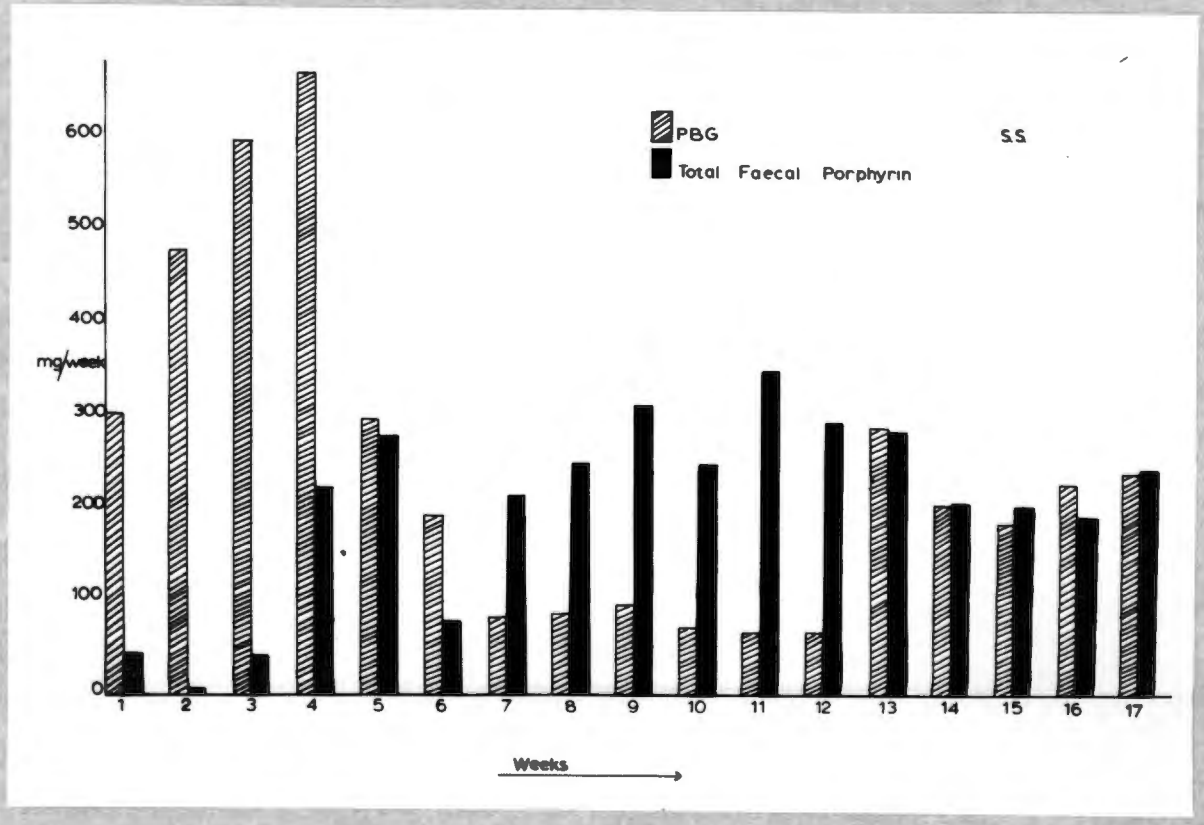


Fig. VIII - 2.
The relationship between weekly excretion rates of PBG and total faecal ether-soluble porphyrin in Case (v) over a 17 week period.

Summary of Findings in S.A. Genetic Porphyria.

Large amounts of COPRO and dicarboxylic porphyrin may be recovered from the faeces. The composition of the dicarboxylic porphyrin is variable, but PROTO IX is usually the chief fraction. MESO, DEUTERO, monovinyl (? monoethyl) DEUTERO and a MESO-DEUTERO hybrid have also been found. These are presumed to arise by bacterial activity.

COPRO in remission is largely (90%) isomer III but the amount of isomer III decreases relative to the amount of isomer I in the acute attack. The ratio of COPRO to dicarboxylic porphyrin both in remission and in the acute attack is in the region of unity.

Porphyrins with more than 5 carboxyl groups are largely absent from the faeces in remission, but are present in appreciable concentrations during the acute attack.

In remission the daily excretion of faecal porphyrin is probably about 25 - 40 mgms. (20 - 30 g dry weight of faeces daily.) This figure may well fall considerably during the acute attack but this point has not been adequately studied, nor is it readily amenable to study. There is, if anything,

a rise in urine coproporphyrin excretion during the acute attack, but quantitatively the change is not very large and could result from absorption of porphyrin from the bowel contents.

4. Swedish Genetic Porphyria.

Studies have been made on 4 cases, all from one fairly small family.

A. Urine Porphyrin. This was analysed in detail only in Case A (M.E.) Table VII - 48 shows that only 85 ug/L of COPRO was recovered; 90% of this was isomer III which may be significant. The faecal COPRO was only 30% isomer III.

B. Faecal Porphyrin. PROTO was recovered from the faeces of Case A in crystalline form. The dicarboxylic porphyrin from the faeces of Case B was mostly not PROTO, being of the MESO or DEUTERO type. Only small faecal specimens were analysed from Cases C and D but in each instance alpha band absorption indicated the dicarboxylic porphyrin to be largely PROTO.

In all four cases COPRO from the faeces was predominantly (70%) isomer I which is the same as has been found in normal subjects. (Fig.VIII - 1). Table VII - 52 shows that a small amount of URO was present in the faeces of both C and D. This point was not investigated in the other two. The higher figure for faecal URO (8.5 ug/g. d.w.) in (C) may correlate with the increased urinary excretion of PBG relative to D.

In 3 of these 4 cases (D excepted) there is evidence of an increased faecal porphyrin concentration and in each instance this is due to dicarboxylic porphyrin (to PROTO in 2 of these 3).
The ratios of COPRO:dicarboxylic porphyrin were:-

A	0.096
B	0.205
C	0.235
D	0.260

The average ratio in the three normal cases studied was 0.34 (0.25 - 0.43).

In summary, the pattern of porphyrin excretion is essentially normal but PROTO may be increased and URO is increased, probably when urinary PBG excretion is high.

CHAPTER IX.

THE SIGNIFICANCE OF DIFFERING PATTERNS OF PORPHYRIN EXCRETION.

1. Introduction.

In the preceding Chapter, I summarised results obtained using various analytical technique to investigate patterns of porphyrin excretion in normal and porphyric persons. Emphasis was placed on the low levels of porphyrin and porphyrin precursor excretion encountered in normal persons. In acquired porphyria it was shown that the urine and faeces contain excessive amounts of URO, porphyrins with 7, 6 and 5 carboxyl groups, and COPRO. The excretion of dicarboxylic porphyrin is normal and the urine contains, at most, a slight increase in ALA and PBG. Urine and faecal URO excretion is of the order of 1-10 mg daily, which may thus exceed 100 X the normal limit. Approximately the same amount of COPRO is excreted, but this represents a smaller increase relative to the normal of about 1 mg. daily.

The daily excretion of porphyrins and porphyrin precursors is greater in S.A. genetic and Swedish porphyria. COPRO and PROTO excreted in the former may exceed 30 mg daily, and in an acute attack as much

as 300 mg of ALA and PBG may be excreted daily.

In the recently recognised condition, erythropoietic protoporphyria (E.P.) only PROTO excretion appears to be abnormal. About 10-15 mg is excreted daily.

An attempt has been made to set out some of these features diagrammatically in Fig. IX - 1.

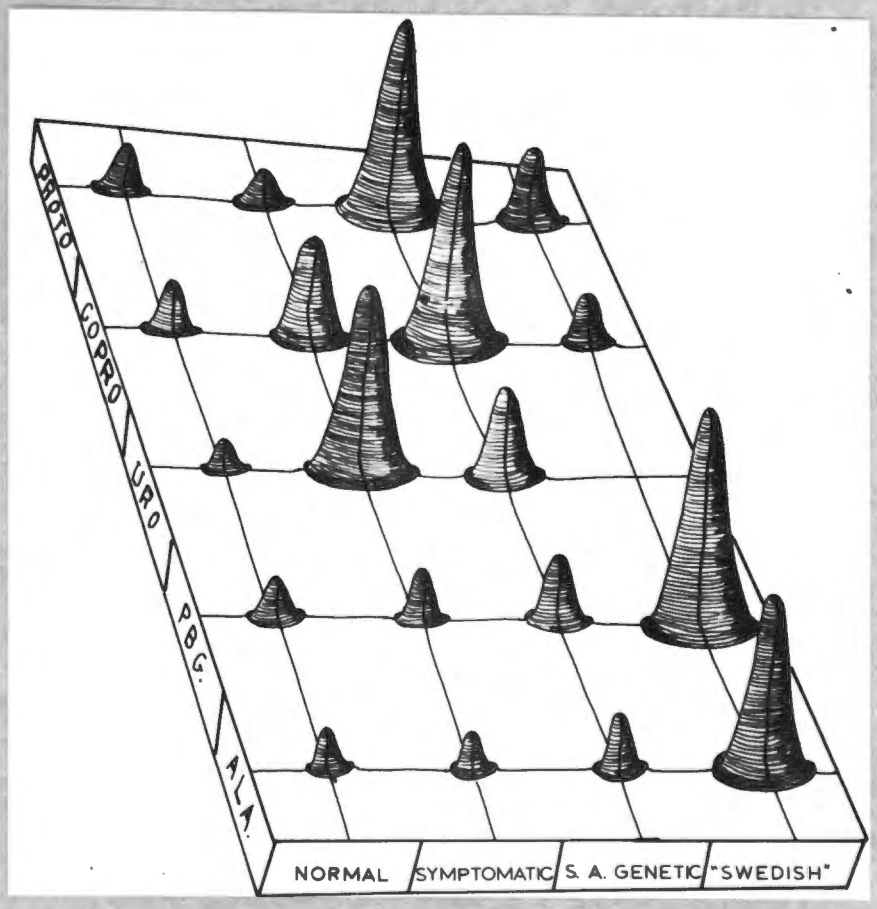


Fig. IX - 1.

The volumes of the solid cones in this figure represent increases in porphyrin excretion relative to the normal. Both faecal and urine porphyrin have been considered.

The different patterns of excretion illustrated in the figure suggest abnormal metabolic processes at different points on the haem synthetic pathway. In congenital erythropoietic porphyria there is good reason (Chapt.IV, Sect.2A) to attribute all the metabolic abnormalities to abnormal action by UROGEN isomerase. In none of the conditions which have been investigated in this study has a comparable state of affairs been found.

Before discussing the significance of different patterns of porphyrin excretion in relation to the metabolic lesions in the porphyrias, there are various comments to be made concerning the practical significance of these differences.

2. Practical Aspects.

A. Diagnostic Value. In Chapt. VII the criteria were stated which allow the porphyrias to be differentiated on biochemical grounds. Precise analytical data presented has confirmed the validity of these criteria and suggested how they may be augmented.

Electrophoretic and chromatographic analysis of urine from patients with cutaneous porphyria should

easily distinguish the acquired and S.A. genetic varieties. The excretion of considerable amounts of intermediately carboxylated porphyrins is typical of acquired porphyria. Similar information could be obtained by analysing faecal porphyrin, but the preparation of specimens is more involved.

It has been shown that the ratio of COPRO to dicarboxylic porphyrin in the faeces often differs in normal and in porphyric patients. Whether this will be reflected in the COPRO/PROTO ratio when these fractions are estimated by the Holti technique depends upon the relative amounts of PROTO and meso/deutero type porphyrin. Better use could be made of COPRO/dicarboxylic porphyrin ratios in the differential diagnosis of the porphyrias if separations were based on the number of carboxyl groups rather than on HCl numbers. This can be achieved by extracting dicarboxylic porphyrin from a solution of faecal porphyrin in 0.15 N HCl with chloroform, as recommended by Watson.

Much more isomer III COPRO was found in the faeces of patients with S.A. Genetic porphyria than in normal persons. This difference might prove to be of value in

differentiating between a porphyric person with only a slight increase in faecal porphyrin and a normal person whose faecal porphyrin concentration is towards the upper limit of normal. This problem is encountered in family studies, particularly when children are tested. However, it remains to be shown whether a knowledge of faecal COPRO isomer composition is of any value under these circumstances.

B. Inferences regarding Preferred Modes of Porphyrin Excretion. Rimington (59) and his co-workers have devoted much effort to determine the manner in which porphyrins and porphyrin precursors are excreted by the body. His views may be briefly summarised: URO and UROGEN are excreted largely by the kidney; dicarboxylic porphyrin (whether reduced or not) is excreted by the liver only. COPRO is excreted l rgely by the liver and COPROGEN partly by the kidney.

It was found that in acquired porphyria the urine and faeces contain roughly equal amounts of both eight and seven carboxyl porphyrin. In the urine the eight carboxyl, and in the faeces the seven carboxyl porphyrin were found to predominate. Much greater

amounts of COPRO were found in the faeces than in the urine. COPROGEN has not yet been measured in the bile, but in the urines of two cases of S. A. Genetic porphyria the COPROGEN/COPRO ratios were 3.5 and 3.75, which agrees with Rimington's predictions.

Dicarboxylic porphyrin has not been found in the urines of any of the patients discussed in this thesis. However, in a patient with S.A. Genetic porphyria studied recently with Prof. L. Eales, obstructive jaundice was accompanied by excretion in the urine of dicarboxylic porphyrin. It was of particular interest that the porphyrin was ether insoluble and an explanation for this is not yet available. An increase in urine porphyrin excretion following biliary obstruction is, of course, the sequence of events predicted by Rimington's reciprocity theory (see Chapt. IV). However, in the case referred to, there was reason to believe that the jaundice was not part of the natural history of the disease nor was the ether insoluble porphyrin excreted URO, but an unidentified dicarboxylic porphyrin. A possibility to be investigated is that under the abnormal circumstances of biliary obstruction,

dicarboxylic porphyrin can be conjugated (e.g. with glucuronic acid) prior to excretion in the urine.

C. The Fate of PROTO in the Gut. It has been shown that in addition to PROTO, other dicarboxylic porphyrins occur in the faeces. The spectroscopic properties and HCl numbers as indicated by behaviour in CCD apparatus indicate the occurrence of the porphyrins detailed in Table IX - 1. Confirmatory evidence for a monovinyl porphyrin was obtained by observing a 4-5 mu shift in the wavelength of alpha band absorption after catalytic reduction.

Table IX - 1.

Designation of porphyrin.	Substituents at positions 2 and 4.	Calculated HCl number.
PROTO	$-CH=CH_2$: $-CH=CH_2$	1.86
Monovinyl	$-CH=CH_2$: $-CH_2.CH_3$ or -H	0.96
Meso	$-CH_2.CH_3$: $-CH_2.CH_3$	0.60
Monoethyl deutero	$-CH_2.CH_3$: - H	0.46
Deutero	- H : - H	0.33

No physiological significance can be attached to any of the dicarboxylic porphyrins indicated in Table IX - 1, except PROTO. These fractions probably result from bacterial degradation of PROTO; porphyrins

with similarly low HCl numbers have also been encountered in bile and their presence there supports the concept of an enterohepatic circulation (161, 126).

The aromatic stability of the porphyrin nucleus protects it from reduction by bacteria but this does not prevent the vinyl groups from being reduced in a manner similar to the reduction of bilirubin in the gut.

3. Speculation regarding the Metabolic Lesions of the Porphyrias based on Observed Patterns of Porphyrin Excretion.

Basically, three possible explanations for the excessive excretion of haem precursors or their immediate derivatives have been advanced.

(i) For various reasons (not germane to the present arguments) there is excessive production of ALA which is metabolised to other haem precursors and thus excreted. (e.g. Granick (63), de Mattheis and Rimington (152) and Schmid (40).

(ii) The biosynthesis of a haem enzyme is blocked and the increased synthesis of haem precursors is an attempt to overcome such a block (153).

(iii) Porphyrinogens, the metabolic intermediates of haem synthesis, are irreversibly oxidised

to porphyrins at an abnormal rate. The porphyrins are excreted as the body has no other way of disposing of them.

In discussing these theories, acquired porphyria and erythropoietic protoporphyria will be considered separately; since the acute attack is common to the S.A. and Swedish forms of inherited porphyria, these will be considered together. Before considering the human diseases, brief mention will be made of experimental porphyrias in laboratory animals because the findings in these have often been compared with the findings in man.

A. Experimental Porphyria. Disturbances in porphyrin metabolism have been induced in laboratory animals (rats, guinea pigs and rabbits) by feeding allyl-isopropyl-thiocarbamide (Sedormid), allyl-isopropyl-acetamide (AIA), dihydrodicarbethoxycollidine (DDC) and hexachlorobenzene (HCB) (167, 168). Sedormid, AIA and DDC have, under the conditions used, produced similar disorders with excretion of increased amounts of proto- and coproporphyrins and porphyrin precursors. The onset of the disturbance of porphyrin metabolism has been rapid. HCB on the other hand, produces in the rat a condition similar to acquired

porphyria in man if fed as a 0.2% supplement to a standard laboratory diet. This substance came to be investigated following an outbreak of cutaneous porphyria in Turkey (170) which was traced to the injestion by peasants of HCB present in seed wheat as a fungicide.

Early work on experimental porphyria was dominated by the finding that the development of porphyria was accompanied by a profound fall in liver catalase activity. It was argued that the disturbance of porphyrin metabolism might represent either attempts to replace this haem-enzyme or failure of utilisation of haem precursors for catalase synthesis. Neither view has been supported by experimental work. Until recently, the induction of experimental porphyria with Sedormid or AIA or DDC remained an intriguing observation but could not be connected with any other specific alterations in intermediary metabolism. Ginsburg and Dowdle (171) have now shown that apart from alterations in porphyrin excretion, there is an increase in the activity of glucose-6-phosphate dehydrogenase in the liver of the rate, and a considerable increase in the excretion of ascorbic acid. At the same time there is a decrease in the activity of pyruvate kinase suggesting a deviation

of glucose-6-phosphate away from glycolytic to alternative pathways. There is as much reason, therefore, to refer to this intoxication as "experimental ascorbic-aciduria" as experimental porphyria!

The significance of the relationship between the disturbances of porphyrin metabolism and those of intermediary metabolism remains to be evaluated.

Studies of HCB porphyria have not yet provided any information on the pathogenesis of acquired porphyria except the observation that HCB produces both functional and anatomic evidence of liver disease.

In summary, experimental porphyria in animals can be expected to produce valuable information in the future regarding relationships between porphyrin metabolism and other pathways of intermediary metabolism, but at present the experimental porphyrias do not contribute to an understanding of the human diseases.

B. Acquired Porphyria. The metabolic abnormality responsible for this condition remains unknown, but the picture presented seems to be less confusing that that of the dominantly inherited porphyrias to be discussed in a later section.

Porphyrins derived from each porphyrinogen intermediate of PROTO synthesis are excreted in acquired porphyria and the amounts may range from normal (PROTO) to over 100 X normal (URO). The excretion of ALA and PBG is never more than slightly increased. This pattern does not suggest a metabolic block at any point. It could be argued that the pattern is due to increased loads of ALA on the biosynthetic pathway, but this does not account for the small increases in PBG excretion. Also, the pattern of excretion is very different from that found in a healthy person following the oral administration of a dose of ALA. (Fig. IX - 2)

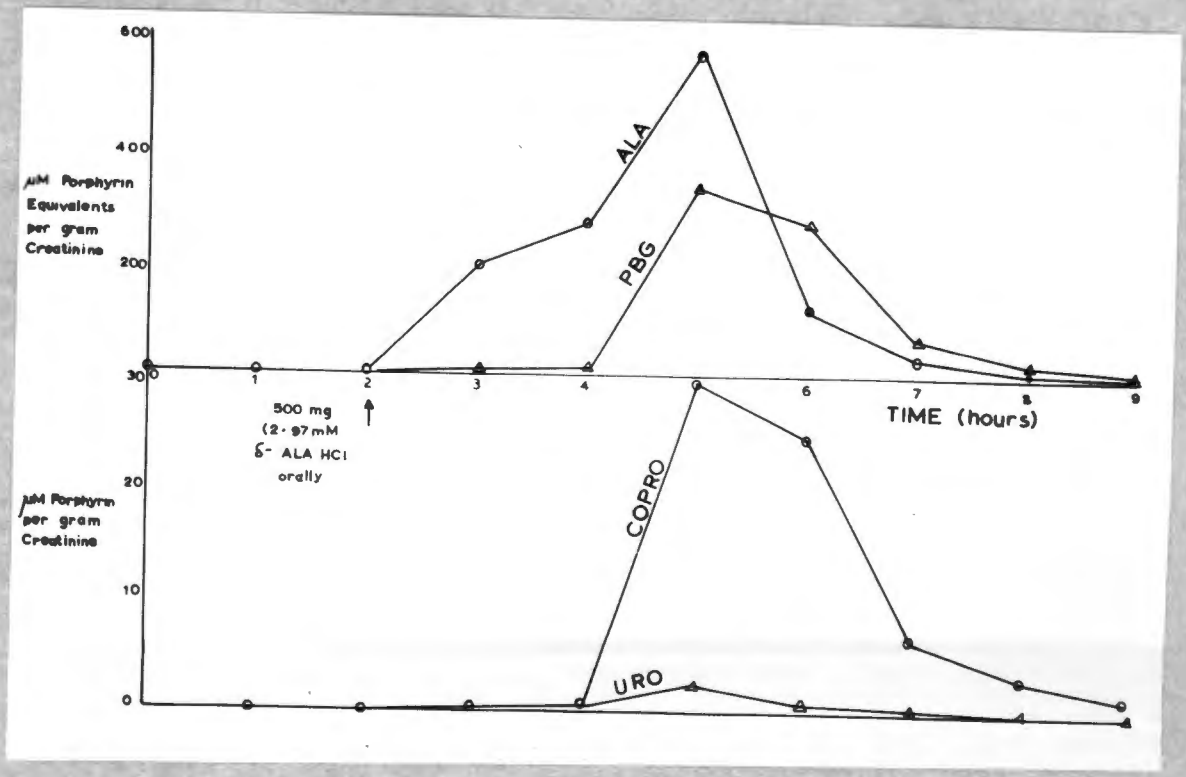


Fig. IX - 2.

The changes in excretion in the urine of ALA, PBG, URO and COPRO following an oral dose of 0.5g ALA.

Porphyrin excretion in acquired porphyria is adequately accounted for by the suggestion that the abnormal excretion of porphyrin follows an increased rate of porphyrinogen oxidation. Such a theory accounts for the excretion of about equal amounts of the various porphyrins with from 8 to 4 carboxyl groups, but normal amounts of dicarboxylic porphyrin. It also explains the relatively normal quantities of ALA and PBG excreted.

The validity of such an hypothesis could be checked by determining relative amounts of URO and copro which are excreted in the reduced form. It may even be possible to measure such ratios in serum. The technical difficulties are numerous and due consideration would have to be given to the different ways in which porphyrins and porphyrinogens are excreted by the kidney and liver.

While it is easy to discuss glibly the oxidation of porphyrinogen as a basic metabolic lesion in acquired porphyria, it must be remembered that such oxidation would require (a) atmospheric oxygen or some other electron acceptor and (b) a catalyst such as light, porphyrin or possibly Fe^{III}. There is a single account

in the literature of an enzyme isolated from spinach (172) which catalyses the oxidation of UROGEN but the significance of this is difficult to assess.

Perhaps the easiest explanation to accept is that porphyrinogen leaks from the cells in the reduced form and is oxidised elsewhere e.g. in the skin under the influence of light.

The observed pattern of porphyrin excretion is only one aspect of acquired porphyria which must be accounted for by any satisfactory hypothesis. Alcohol is almost a proven aetiological agent even though relatively few alcoholics develop porphyria. The Turkish epidemic due to HCB showed that other toxins can cause a similar disease. Recent work suggests a relationship between iron deposition in the liver and acquired porphyria. A further interesting fact to be explained is the reaction to chloroquine of patients with acquired porphyria. As far as this is known this reaction is specific to this disease.

The Possibility of an Inherited Factor.

The rarity of acquired porphyria in patients who consume excessive amounts of alcohol suggests the interaction of this toxin with a particular inherited constitution. A point of interest is whether this

inherited predisposition is multifactorial or dependent upon a single gene. Adequate studies of the families of patients may decide this. Waldenstrom and Aronsen (166) claim to have shown that acquired porphyria can be inherited.

Chloroquine induces a reaction in patients with acquired porphyria which is reminiscent of the reaction of patients with G-6-PD deficiency to primaquine (140). This again suggests an inherited factor and underlines the need for family studies.

The Role of Alcohol.

The metabolic fate of alcohol provides no support for the theory that acquired porphyria follows increased porphyrinogen oxidation as a result of an altered intracellular redox potential. The oxidation of alcohol (alcohol dehydrogenase) causes an increase in DPNH relative to DPN in acute experiments (163, 164, 165) but it is difficult to extrapolate from these observations to the possible effects of the long continued ingestion of alcohol. Alcohol may be responsible for the development of acquired porphyria purely through its effects on iron metabolism.

Disturbed Iron Metabolism.

Many alcoholic drinks contain increased amounts of iron. Increased iron absorption from the gut is a feature of chronic pancreatitis and hepatic cirrhosis, both of which conditions can follow intemperance. (155). It has also been suggested that alcohol per se causes an increase in iron absorption (173).

Uys (156) has consistently shown abnormal iron deposition in the liver in acquired porphyria, and Saunders (155) has shown that repeated venesection can lead to a marked decrease in urine porphyrin excretion. Theron et al (162) produced experimental siderosis in rats and found about a hundred fold increase in the activity of mitochondrial $DPNH$ oxidase. Such a change would support the concept of an altered intracellular redox potential favouring spontaneous oxidation of porphyrinogen.

C. South African and Swedish Genetic Porphyria.

To be acceptable, an hypothesis which attempts to explain these conditions must account for:-

i. Dominant inheritance.

ii. The different patterns of porphyrin and porphyrin precursor excretion encountered.

iii. The biochemical and clinical features of the acute attack.

iv. The ability of drugs to precipitate or aggravate the acute attack.

v. The complete normality of haemoglobin synthesis.

The significance of dominant inheritance will be dealt with first. Consideration of the other established facts listed above invites speculation but thoughts on the meaning of dominant inheritance emphasise the need for caution.

(i) Dominant Inheritance.

Before discussing the interpretation of the different patterns of excretion in these conditions the significance of dominant inheritance will be briefly considered.

When Garrod advanced his fertile concept of certain diseases being "Inborn Errors of Metabolism" he cited results of his studies on alcaptonuria, cystinuria, albinism, and pentosuria. It was no accident that these conditions are all inherited as Mendelian recessive characters. In these, and in other similarly inherited conditions, the abnormal

biochemical findings are adequately explained by postulating the absence of a single enzyme or species of protein. In some such diseases, in vitro studies have confirmed the absence of or gross decrease in activity of an enzyme or other protein.

It is accepted that in recessively inherited conditions two chromesomes are affected at the same locus with resultant deletion or modification of protein structurally dependent on that gene locus. Sex-linked dominant inheritance operates in a manner similar to the autosomal recessive because information is carried on only a single chromosome.

In diseases inherited as autosomal dominants there are no grounds for postulating that an enzyme is deleted. Evidence from two conditions (sickle cell trait and primaquine sensitivity in females) suggests that the inheritance of a single abnormal gene can cause a decrease in the amount of normal protein made in the body. In sickle cell trait the missing normal protein is replaced by abnormal protein. Neither carriers of sickle cell trait nor females heterozygous for G-6-PD deficiency show untoward effects unless exposed to the appropriate stress.

These two conditions are exceptional because quantitative deficiency of a particular protein leads to signs of disease. A number of other diseases are known which are inherited as Mendelian dominants, but in none is the nature of the metabolic lesion understood. Well documented conditions inherited as Mendelian dominants include:-

(i) S.A. and Swedish Genetic porphyria.

(ii) Periodic paralysis

(iii) Adynamia Episodica Hereditaria

(iv) Hereditary Spherocytosis

(v) Renal Glycosuria

(vi) Polycystic disease of the kidney.

In the last three conditions listed there is evidence of an anatomical abnormality at cellular level. Demonstrable abnormalities include an abnormal red cell envelope in hereditary spherocytosis, flattening of the tubular epithelium in renal glycosuria and in polycystic disease the failure of glomeruli and nephrons to connect in embryonic life. In each instance a particular tissue has been affected; the "metabolic defect" may lie in the chemistry of structural proteins rather than in a pathway of intermediary metabolism.

In South African and Swedish porphyria, in periodic paralysis and in adynamia episodica hereditaria a common feature is, again, involvement of a particular tissue. These three conditions are also similar in their phasic nature, although it is not suggested that the time relationships of attacks are the same.

There are other conditions probably inherited as Mendelian dominants, e.g. primary gout, certain familial hyperlipidaemias and hypercholesterolaemias and one form of localised albinism. In none of these has it been possible to attribute the diseases to an abnormal protein or enzyme.

In view of the foregoing remarks on the significance of autosomal dominant inheritance, it would be very surprising if the studies of porphyrin excretion recorded here enabled the metabolic defect in S.A. and Swedish porphyria to be exactly predicted. No such predictions will be made. The observed facts are confusing and it is probably correct to say that they will remain confusing until much more is known about the integration of isolated reactions and

reaction sequences within the cell. In particular, information is required on factors which control the permeability of cells to their contained metabolites.

(ii) The Different Patterns of Excretion.

The different patterns of porphyrin excretion in these conditions were summarised at the beginning of this chapter and set out diagrammatically in Fig.IX-1. In both conditions it is necessary to postulate excessive production of ALA; in Swedish porphyria this is excreted as ALA and PBG without being metabolised further, but in the South African form in remission the ALA is metabolised to, and excreted as, COPRO and PROTO.

When ALA was given to a normal individual (Fig.IX - 2) PBG and COPRO appeared in the urine, while COPRO and PROTO may have appeared in the faeces. This differs from the pattern of excretion in S.A.Genetic where PBG excretion remains low during remission. Low remission levels of PBG excretion could be explained by postulating adaptive changes in enzyme activities. However, in the acute attack of S.A. Genetic porphyria, faecal porphyrin does not increase pari passu with ALA and PBG excretion and it may even decrease. (Fig.VIII-2).

It is clear that in Swedish Porphyria some impediment exists to the further metabolism of PBG; in the South African variety a similar block would have to be postulated beyond PROTO. South African porphyria comes to resemble the Swedish variety more closely during the acute phase even to the extent that a partial block in the metabolism of PBG seems to develop.

The synthesis of haemoglobin in both these conditions appears to be quite normal; no defect in formation of any haemoprotein has been suspected, and, as far as is known, any defects are confined to the liver. These points make it unlikely that any enzyme defect exists comparable with the presumed absance of UROGEN isomerase in C.E.P.

The obvious differences between the haem synthetic pathways in the bone marrow and in the liver are a puzzling feature of Swedish and S.A. porphyria. The content of free porphyrin in the red cells is normal. It must be assumed that erythroid cells are impermeable to circulating proto-porphyrin precursors or these could be expected to diffuse inwards and be further metabolised to proto-porphyrin or haem.

In the first paragraph of this section, it was pointed out that excessive production of ALA had to be assumed. Whether this was due to events occurring outside the haem synthetic pathway or within this pathway (in the liver) was not discussed. In the two genetically distinct conditions being dealt with, the increased synthesis of ALA can manifest in divergent fashions (during the remission phase.) It is therefore more likely that any abnormalities of enzymes or cellular structures (a) involve the haem synthetic pathway in the liver and (b) involve it differently in the two conditions, than that the alterations in haem precursor synthesis are consequent on metabolic aberrations at some other site in intermediary metabolism.

The foregoing may appear contradictory. Firstly, it is suggested that there are metabolic abnormalities at various points in the haem synthetic pathway and that the excessive production of ALA is secondary. Secondly, it is pointed out that no evidence exists for an abnormal enzyme in the haem synthetic pathway.

However, this apparent contradiction should be considered together with the remarks regarding dominant

inheritance. Maybe nothing should be postulated until more facts become available. If a suggestion must be made, let it be that the "blocks" in metabolism of haem synthetic intermediates in the liver are due to anatomical derangements or abnormal permeabilities within the cells.

D. Erythropoietic Protoporphyria (E.P.)

In this condition erythrocytes are found to contain large amounts of free PROTO. Free PROTO (about 50 ug/100 cc blood) is found in the serum and 10-15 mg of PROTO is excreted daily into the faeces. The content of porphyrin in the liver is unknown. The liver in a single case was shown to contain large amounts of PROTO (14) but various features of this case were atypical.

Nothing more is known about the metabolic lesion in this condition. Redeker (157) made the significant observation that in lead poisoning and certain iron deficiency anaemias, PROTO is comparably increased in erythrocytes, but little escapes into the plasma, while in E.P. large amounts of PROTO are present in the plasma. Redeker suggested that the red cell envelope was abnormally

permeable to PROTO. This does not explain why erythrocyte porphyrin is increased rather than decreased.

No abnormalities of erythrokinetics or ferrokinetics have been shown in E.P. and it appears that the only disability suffered by affected persons is light sensitivity. Probably because of the increased serum protoporphyrin the clinical picture in E.P. is dominated by an acute reaction to light with erythema and oedema. Magnus (147) showed that variations in the amounts of light at varying wavelengths required to produce erythema in patients with E.P. corresponds to the absorption spectrum of protoporphyrin in the near UV. Abnormal fragility of the skin does develop in the sun-exposed areas.

A dominant mode of inheritance has been suggested (158) in erythropoietic protoporphyria although this is not yet proven. The concept of a defect in the red cell envelope is reminiscent of the defects suspected in other dominantly inherited conditions.

4. CONCLUSION.

The study of excreted porphyrins presented here has been useful in various ways. It has become possible

to define more accurately abnormalities of porphyrin excretion in the conditions studied. Additional diagnostic criteria have been defined. Information about the ways in which porphyrins are excreted has been gathered and in acquired porphyria the pattern of porphyrin excretion supports definite suggestions concerning pathogenesis. In the dominantly inherited porphyrias, while these studies have stimulated thought on possible mechanisms, it has not been possible to make any definite predictions regarding the fundamental metabolic lesions.

APPENDIX A.

PHYSICAL PROPERTIES OF PORPHYRINS.

If data of other authors is quoted the reference will be quoted.

1. SPECTROSCOPIC.

A. **Extinction coefficient.**

(i) Methyl esters in chloroform. (128)

Porphyrin.	mu.	$E_{mol} \times 10^{-5}$
URO	406	2.17
COPRO	399.5	1.80
DEUTERO	399	1.75
PROTO	407.5	1.71

Extinction coefficients do not appear to differ in the different isomers.

(ii) Free porphyrin in dilute HCl. (Soret band).

	Porphyrin.	(N) HCl.	mu.	Emol x 10^{-5}
(128)	URO	0.5	405.5	5.28
(128)	COPRO	0.1	399.5	4.89
(128)	DEUTERO	0.1	398	4.33
(22)	PROTO	1.37	408	2.75*

* See Chapt.VI, Sect.15.

(iii)(a) Free Porphyrins in Ether.

Porphyrin.	mu.	$E_{mol} \times 10^{-3}$
PROTO	405	154
	504	14.6
DEUTERO	395	170
	493	16.8

(b) Free porphyrins in dilute HCl.

Porphyrin.	(N)-HCl.	mu.	$E_{mol} \times 10^{-3}$
PROTO	1.5	410	310
		557	17.3
DEUTERO	0.2	400	424
		545	14.4

The constants given in this section were determined for use during CCD studies. Meso and Deutero have very similar extinction coefficients.

(iv) Free porphyrins in 1.5 N HCl following elution from chromatograms and electrophoresis strips.

Carboxyl groups.	Mol.Wt. (Me.esters)	$E_{mol} \times 10^{-5}$
2	590	4.0
3	650	4.0
4	711	4.2
5	769	4.5
6	827	4.7
7	885	5.0
8	943	5.2

Extinction coefficients are known accurately only for 4 and 8 carboxyl porphyrins. A correction factor of 1.8 is used in correcting for background absorption by the method of Rimington and Sveinsson(115)

B. Absorption maxima using the Hartridge Reversion Spectroscope.

(i) Porphyrin Methyl esters in chloroform.

Porphyrin.	α	β		γ
		middle	peak	
URO	625.0	573.0	568.6	535.3
COPRO	621.6	572.5	566.9	533.9
PROTO	630.6	579.6	575.0	541.5
MESO	620.8		570.9	532.9
DEUTERO	619.4		568.8	528.5

(ii) Free Porphyrin in ether.

Porphyrin	α	β		γ
		middle	peak	
PROTO	632.4	581.4	576.2	537.6
MESO	623.5	574.6	568.0	528.7
DEUTERO	621.8	572.4	565.6	526.1

(iii) Free Porphyrin in dilute HCl.

Porphyrin	(N)-HCl	α	β
PROTO	(0.5	599.4	553.8
	(1.5	601.3	555.5
MESO	0.13	589.0	545.9
DEUTERO	0.11	587.8	544.7

2. HCl NUMBERS.

Porphyrin	HCl number.
PROTO	1.85
MESO	0.63
DEUTERO	0.37
HAEMATO	0.066

3. MELTING POINTS OF METHYL ESTERS.

Porphyrin.	m.p. °C.	m.p. °C. Cu complex.
URO I	289-291	
URO III	254-261	
COPRO I	253-255	276 - 278
COPRO III	125-130 178-180	217 - 218
PROTO IX	232-234	
MESO IX	212-214	
DEUTERO IX	226-227	

Melting points were determined as described in Chapter VI. The figures are uncorrected for errors in thermometer calibration.

APPENDIX B - Brief Case Reports.

Tables included in Chapter VII provide data required for the assessment of liver function. The concentrations of porphyrins and porphyrin precursors in the urine and faeces is also tabulated in Chapter VII.

1. Patients with Acquired Porphyria.

A. (J.T.) A 41 year old Coloured labourer who was admitted to hospital because of lesions of cutaneous porphyria affecting his hands. The lesions had been present for some months prior to admission. No other member of the patient's family was similarly affected. For nearly 20 years he had drunk large amounts of wines and beer but mainly over week-ends.

The only abnormalities on physical examination were the skin lesions and a one finger, firm, non-tender hepatomegaly. The urine was normal. Haemoglobin was 12g/100 ml, ESR 5mm/hr and the blood pressure 130/70 mm Hg.

B. (G.J.) This patient was a 43 year old Coloured male employed in the municipal sewage department. Three months before admission blisters had appeared on the dorsa of his hands which broke down leaving ulcers which healed slowly and became infected. For years prior to admission the patient had consumed a number of bottles of cheap wine daily.

Physical examination showed typical lesions of cutaneous porphyria on the backs of the hands. The liver was three fingers enlarged and tender. During the first 7-10 days of hospitalisation the patient had a low grade pyrexia which was not adequately explained despite investigation. The haemoglobin was 14.5g/100 ml; the E.S.R. 14mm/hr (Westergren) and the B.P. was 150/105 mm Hg.

The patient suggested that his grandfather might have had some cutaneous disease; two living sisters were screened for porphyria but with negative results.

C. (E.C.) This 46 year old Coloured fisherman was admitted to hospital because of severely infected lesions of cutaneous porphyria. He had first noted that his skin was abnormally fragile 5 months prior to admission and the abraded areas were slow to heal. Since the age of 18 years he had indulged in frequent bouts of drinking. He would consume a number of bottles of cheap wine at a time. A month prior to admission he had noted that bouts of drinking were affecting his skin adversely and, of his own accord, desisted. Apart from obvious evidence of cutaneous porphyria, a palpable liver and mild hypertension, this

man was physically normal. His blood pressure was 155/85 mm Hg and sedimentation rate 10 mm/hr (Westergren). A reduced creatinine clearance (67 ml/min) was found but the reason for this was not discovered.

D. (J.F.) The patient was a 41 year old Coloured carpenter who had lesions of cutaneous porphyria for 6 years prior to admission. Apart from fragility of the skin and blister formation, scarring had been very severe and large areas of faecial skin had been replaced by scar tissue. Since about the age of 20 years the patient had consumed large amounts of alcohol, mostly as brandy.

The liver was slightly enlarged but apart from this finding and the cutaneous lesions the patient was physically healthy. The haemoglobin was 14.8 g/100 ml, ESR 40 mm/hr (Westergren) and the blood pressure 110/70 mm Hg.

E. (G.O.) This 47 year old Coloured man was employed by a firm making electrical goods. For six months prior to admission to hospital he had noted abnormal fragility of his skin with blisters following minor trauma. These had become infected and some

scarring had followed. For approximately the same period of time he had noticed parasthesiae of his feet and tenderness of his calf muscles.

Since his late teens this mad had drunk heavily, mostly cheap port. Over the past 8 years his consumption had increased to about 2 bottles of wine daily supplemented with other alcoholic drinks.

Physical examination showed a well-looking male with deeply pigmented scars on the darsae of his hands. Spider naevi were noted over the upper part of his trunk but there was no palmar erythema and the testes were normal. The liver was definitely enlarged being palpable three finger's breadths below the costal margin. There were no objective neurological signs of the peripheral neuritis suggested by his symptoms.

The B.P. was 120/70 mm Hg, haemoglobin 12.5 g/100 ml and the ESR 5 mm/hr (Westergren). Urobilinogen was present in the urine. A diabetic type of glucose tolerance curve was noted and pancreatic function was abnormal.

F. (F.M.) This 41 year old African male had suffered from lesions of cutaneous porphyria during the year preceding admission. He had also had colicky

abdominal pains and intermittent diarrhoea. For an unknown period of time he had been drinking large quantities of cheap wines and also illicitly brewed "Kaffir" beer.

Pigmentation of the sun exposed areas were prominent; scars and some recent and infected erosions were noted on the hands. The liver was palpable. Blood and mucus were present in the stool and proglottides of T.saginata were found. Amoebic dysentery was suspected. Treatment was with emetine followed by chloroquine and finally atebrine. Chloroquine produced a striking reaction such as has already been referred to (140) and atebrine lead to the expulsion of four tape worms.

The porphyrin studies described in Chapt. VII were performed prior to treatment with chloroquine.

2. Patients with S.A.Genetic Porphyria (Variegate Porphyria).

A. Those studied during the Remission Phase.

(i) (H.S.) This 30 year old White man was unemployed and supported by a state pension. Since the age of 7 years he has had an abnormal skin. When admitted to hospital in 1962 the skin of his hands and face showed numerous paper thin scars, milia, vesicles

and depigmented areas. The skin over the hands and forearms was abnormally fragile.

No other physical abnormalities were found on examination; the appendix had been removed and there was slight tenderness in the epigastrium. The patient was mentally dull.

One out of nine sibs has an abnormal skin similar to that of the patient and the father is also affected. The patient has never had a definite attack of acute porphyria, but the clinical record stated that PBG was present in the urine in 1959.

(ii) (A.V.) A 21 year old "white" male farmer from the Sutherland district. Since the age of 12 years he had noted that his skin was abnormally fragile. He had occasionally had mild attacks of abdominal pain when he noticed that his urine was dark. He denied taking any alcohol. Apart from minor skin lesions and definite cutaneous fragility, physical examination was negative.

The patient came from a family of five children two of whom have cutaneous porphyria. Two of his mother's sisters were also affected.

(iii) (S.D.) This patient was a 37 year old school-teacher. He was first seen as an out-patient at Groote Schuur Hospital in 1963 but he had been aware of having a "soft" skin for more than 10 years before that. This has become worse over the years and when seen at G.S.H. the backs of his hands and his forehead were extensively involved by erosions, ulcers and scars. No other physical abnormalities were noted. The patient denied excessive intake of alcohol. No definite family history could be obtained but his mother is thought to have had a soft skin.

The biochemical findings in the urine and faeces were typical of S.A. Genetic porphyria.

(iv) (P.V.) No clinical investigations were carried out on this patient during the hospital admission coinciding with the studies reported in this thesis. She had, however, been admitted to the hospital in 1959 with an attack of acute porphyria which followed removal of her appendix under pentathal anaesthesia. Porphobilinogen was found in the urine.

(v) (H.B.) This patient was diagnosed as suffering from S.A. Genetic porphyria in 1961 on the basis of:

(1) The characteristic skin lesions.

(2) A positive family history. (sister had had acute porphyria.)

(3) Elevated faecal porphyrin concentration. (Coproporphyrin 0.17 mg/g d.w. and protoporphyrin 0.78 mg/g d.w.)

The present admission was for the investigation of chest pain which proved to be due to angina pectoris. Typical cutaneous lesions were again notices. It is interesting that this patient had received a pentothal anaesthetic and taken butobarbitone sleeping tablets with no apparent ill-effect.

(vi) (J.C.) This patient was a 57 year old "white" casual labourer. He was referred to Groote Schuur Hospital in 1963 because of cutaneous porphyria. As a child he had suffered from "eczema" between the ages of 10 and 16 years. Signs of skin disease recurred between 25 and 30 years of age when he was engaged in heavy manual work. Blisters, which healed slowly leaving scars, appeared periodically on his hands.

In 1952 he had been treated conservatively in hospital for a suspected stomach ulcer. From the age of 30 onwards he had passed red urine periodically. There was no family history of porphyria and his six children were in apparently good health.

B. Those studied during Acute Attacks.

(i) (H.B.) This 33 year old "white" male was transferred from a country hospital in a semi-comatose condition. A week prior to transfer he had received tablets (? sulphonamides) as treatment for a flu-like illness. Profuse vomiting began soon after taking these and acute abdominal pain began a day later.

Since the age of 14 years the patient had had a fragile skin, trauma leading to blisters which burst, forming ulcers which healed slowly and with scarring. Two other members of the family have been diagnosed as having porphyria.

On examination the patient was confused and restless. He showed generalised pigmentation and healed scars on the dorsae of his hands and feet. The pulse was 108 and the blood pressure 145/90 mm Hg. The abdomen was tender in all areas. In addition to the

data given in Chapt. VII, the serum Na was 117 and K 4.9 mEq/L.

(ii) (A.S.) A week prior to admission this 35 year old coloured female was given a pentothal anaesthetic for the manipulation of her left ankle. Four days later she experienced the sudden onset of acute abdominal pain associated with vomiting and constipation. The urine became reddish in colour. The Ehrlich's test was strongly positive.

The backs of both hands and the forearms showed fine healed scars typical of cutaneous porphyria. No family history of porphyria could be obtained. Both parents had died years previously. A single living sister was located with the help of Miss A. Moodie, but was found to be normal.

While it is convenient to attribute the onset of acute porphyria in this patient to the pentothal anaesthetic, she had received four such anaesthetics in the previous 3 months without any apparent ill-effects.

(iii) (M.L.) This patient was a 50 year old "white" housewife, who was admitted to hospital 17 days after a hysterectomy operation performed under anaesthesia induced with pentothal. Recovery had apparently been

uneventful but 12 days after the surgery she became nauseous and felt pain over her whole body. Generalised motor weakness developed and the patient became confused.

When examined on admission there were no signs of porphyric skin lesions. The patient was confused, sensation was intact but there was a flaccid quadriplegia with absent reflexes. A tracheostomy was performed. Respiratory failure was treated with intermittent positive pressure respiration.

The urine had been found to contain large amounts of PBG.

Although the amounts of ALA and PBG in the urine decreased rapidly the patient's clinical course was stormy. IPPR had to be continued for over a month. Severe azotaemia marked the early phase of this acute attack.

(iv) (P.N.) The patient, a 40 year old "white" female was admitted to Groote Schuur Hospital with severe abdominal pain accompanied by vomiting. Shortly before the onset of these symptoms she had started a course of Antiphen treatment for a tapeworm. She had, however, been receiving phenobarbitone for an epileptic seizure two months previously.

When admitted the patient had been constipated since the onset of abdominal pain about a week before, and the urine had been a dark red. The Ehrlich's test was strongly positive for porphobilinogen.

(v) (S.S.) This 34 year old coloured male was admitted to Groote Schuur Hospital on two occasions while this study was in progress. On both occasions abdominal pain of considerable severity was accompanied by tachycardia and vomiting. No neurological lesions developed.

No family history has been obtained to substantiate the diagnosis of S.A. Genetic porphyria, but the patient has typical porphyric skin lesions and a grossly elevated faecal porphyrin.

Although Table VII-36 indicates a normal BSP clearance, this refers to his condition at the beginning of the second acute attack. In this rather unusual instance of acute porphyria in a patient with the S.A. Genetic type of porphyria the concentration of ALA and PBG in the urine remained increased for many weeks. The BSP clearance was impaired when repeated after 16 weeks in hospital.

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SUMMARY.

A brief review was given of the history of porphyria in South Africa and the aims of the study to be described were stated. Physical and chemical properties of those porphyrins of interest in a study of the intermediates of haem synthesis were described. Knowledge of the metabolic pathways leading to the synthesis of haem was reviewed.

Previous studies of porphyrin excretion in health and disease were discussed.

Considerable attention was given to laboratory techniques required for the analysis of porphyrins present in biological materials. It was hoped that this section of the thesis in particular would be of use to others.

Various analyses were conducted on urine, faeces and bile from 24 patients; 3 of these were normal persons 6 had acquired porphyria, 10 had S.A.Genetic porphyr of whom 5 were studied during acute attacks, 4 were ses of Swedish porphyria and there was an isola· case of erythropoietic protoporphyria.

as shown that the patterns of porphyrin excr encountered were unique to these various

conditions. This information could be helpful in elucidating diagnostic problems while it confirmed, in part, and extended previously expressed views on the modes of porphyrin excretion. Evidence was produced to show that protoporphyrin can be degraded in the gastro-intestinal tract and various products of degradation were described.

Emphasis was placed on the concept that the different patterns of porphyrin excretion in the porphyrias reflect different metabolic lesions in these diseases. The significance that could be attributed to various patterns was discussed. It was pointed out that in acquired porphyria our knowledge of the pathogenesis has made significant advances, but that in those conditions inherited by the Mendelian dominant mode the known facts are confusing and little progress has been made in understanding them.