THE UNIVERSITY OF
SYDNEY

A STUDY OF SOME

PORPHYRINS OF VARIEGATE PORPHYRIA

A thesis submitted for admission to the

Degree of Master of Science in

The University of Sydney

by

VICTOR POULOS

ACKNOWLEDGEMENTS

The author wishes to thank his supervisor, Mr. W.H. Lockwood for guidance and discussion and Mrs. K.P. Carson and Miss P. Howe for their secretarial assistance.

The author also wishes to acknowledge the patient (J.M.) for his cooperation in this work.

The assistance of Professor A.V. Robertson and his staff of the Organic Chemistry Department, University of Sydney in carrying out mass spectrometry is also gratefully acknowledged.

TABLE OF CONTENTS

SUMMARY

1. The faecal porphyrins of a patient with variegate porphyria were investigated.
2. The ether-soluble porphyrins were purified and isolated by ether-mineral acid partition and thin layer chromatography of the esters.
3. With the assistance of mass spectrometry, structures have been proposed for nine of the ether-soluble porphyrins. These are:-

   ethyl propionic acid deuteroporphyrin methyl ester

   propionic acid deuteroporphyrin methyl ester

   methoxyethyl propionic acid deuteroporphyrin methyl ester

   bis (methoxyethyl) deuteroporphyrin methyl ester (tetramethyl haemato porphyrin)

   methoxyethyl hydroxyethyl deuteroporphyrin methyl ester

   vinyl hydroxyethyl deuteroporphyrin methyl ester

   ethyl hydroxyethyl deuteroporphyrin methyl ester

   hydroxyethyl deuteroporphyrin methyl ester

   bis (hydroxyethyl) deuteroporphyrin methyl ester (haemato porphyrin methyl ester)

4. A method of purifying water-soluble porphyrins on cellulose columns and thin layer chromatography of the esters is presented.
5. Most of the water-soluble porphyrin fraction was labile and gave ether-soluble porphyrins on manipulation but part

was stable and contained at least two water-soluble porphyrins.

6. The two stable water-soluble porphyrins were isolated and partially purified. They both appeared to contain a strong acidic group in addition to carboxylic acid groups.

7. The nature is discussed and an hypothesis is proposed that the two stable water-soluble porphyrins each contain a sulphonic acid group attached to an ethyl side chain.

8. From these findings, both on ether- and water-soluble porphyrins, it is suggested that there is a fault in the coproporphyrinogenase enzyme system of the liver or there is overloading of this enzyme system due to a fault elsewhere in the haem biosynthetic pathway.

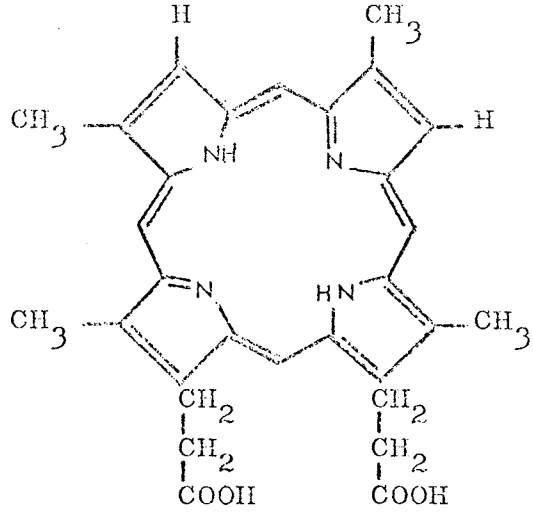
ABBREVIATIONS

ALA	δ-aminolaevulinic acid
ATP	adenosine triphosphate
CoA	coenzyme A
DNP	dinitrophenol
EDTA	ethylene diamine tetra-acetic acid
FDNB	fluorodinitrobenzene
NAD(P)	nicotinamide adenine dinucleotide(phosphate)
PBG	porphobilinogen
TLC	thin layer chromatography
copro	coproporphyrin
coprogen	coproporphyrinogen
	(- ogen transforms porphyrin to the porphyrinogen)
coprogenase	coproporphyrinogenase
dehydroisocopro	dehydroisocoproporphyrin
deutero	deuteroporphyrin
haemato	haematoporphyrin
hardero	harderoporphyrin
hepta	heptacarboxylic acid porphyrin
hexa	hexacarboxylic acid porphyrin
iso-hardero	iso-harderoporphyrin
meso	mesoporphyrin
penta	pentacarboxylic acid porphyrin
proto	protoporphyrin
uro	uroporphyrin

NOMENCLATURE

All porphyrins mentioned in this thesis are based on the IX configuration unless otherwise indicated.

The naming of porphyrins is based on substitution at the 2 and 4 positions of the basic deuteroporphyrin IX structure (below).



SECTION A

INTRODUCTION

In humans, porphyrins occur naturally in the urine, faeces and serum as by-products arising from the biosynthesis of haem and from dietary sources. The normal biosynthetic pathway for haem has been well established. Reviews by Heilmeyer (1964), Gajdos and Gajdos-Török (1969) and Marks (1969) are excellent. The pathway is shown diagrammatically in Figure 1.

Succinyl coenzyme A (succinyl-CoA) and glycine are condensed by α-aminolaevulinic acid synthetase (ALA synthetase) to form α-amino β-keto adipic acid which subsequently loses carbon dioxide to yield ALA. ALA dehydratase condenses two molecules of ALA to form the monopyrrole porphobilinogen (PBG). Four molecules of PBG are cyclized by the concerted action of two enzymes, PBG deaminase and uroporphyrinogen III cosynthetase (urogen III cosynthetase) to form urogen III.

Coproporphyrinogen III (coprogen III) is formed by the action of the decarboxylase enzyme (s) on urogen III. It is then converted by oxidative decarboxylation to protoporphyrinogen IX (protogen IX) by the enzyme coproporphyrinogenase (coprogenase). Protogen IX is oxidized to protoporphyrin IX (proto IX) and the enzyme ferrochelatase catalyzes the insertion of Fe^{2+} to form haem.

Proto synthesis leading to haem, cytochromes or

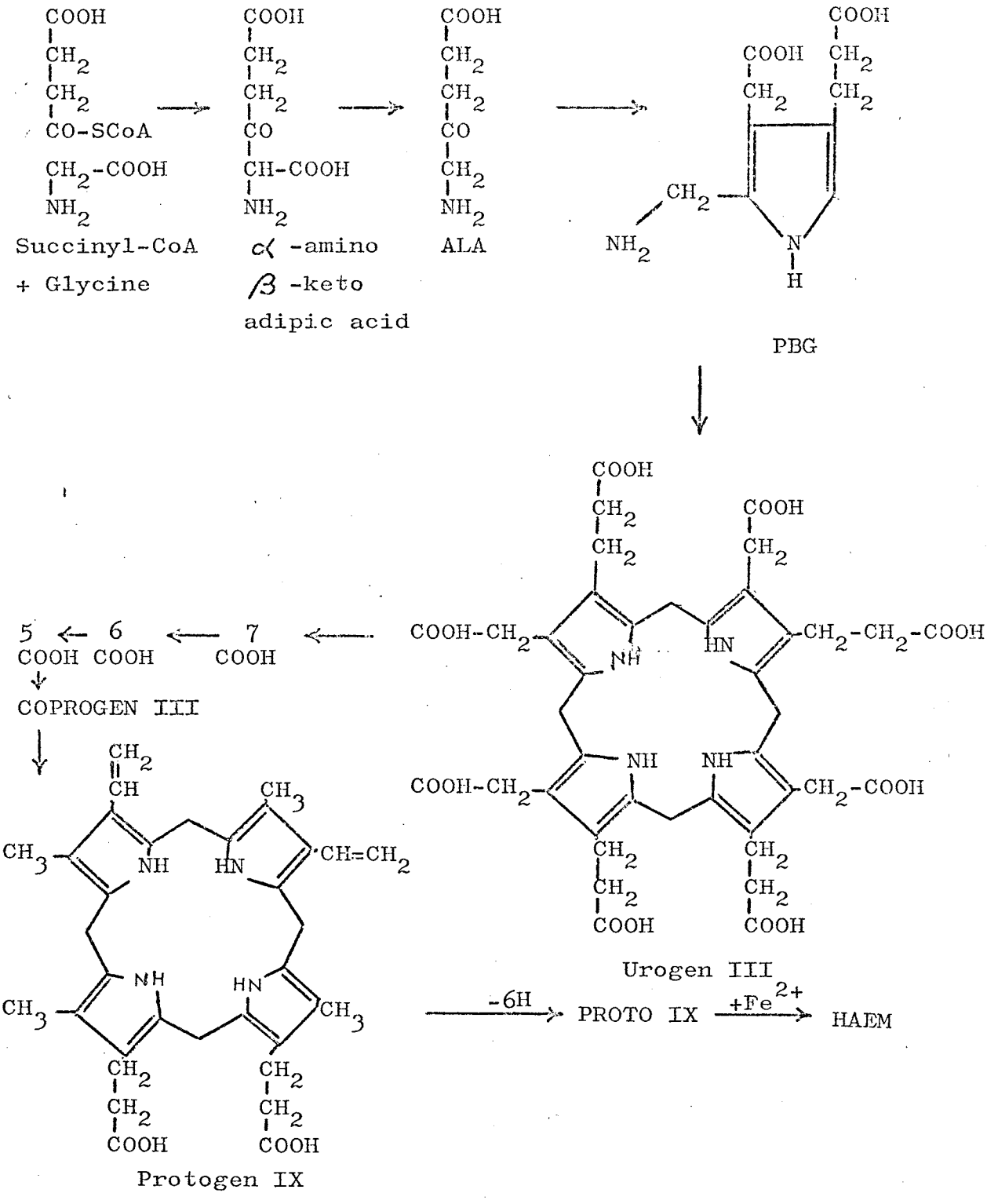


Figure 1. Biosynthesis of haem

chlorophyll occurs in almost all cells of all organisms. There has been no report of major differences in the pathway but minor differences in the properties of the individual enzymes have been reported. For example, coprogenase from mammalian liver mitochondria (Sano and Granick, 1961; Batlle et al., 1965), bacteria (Jacobs et al., 1971) and algae (Jacobs et al., 1971) has an absolute requirement for oxygen but anaerobic coprogenase activity has been reported in Rhodopseudomonas spheroides and Chromatium strain D under certain conditions (Tait, 1972). Because of the uniformity of the porphyrin biosynthetic pathway, workers have found it is convenient to choose a different source for each step. Bovine liver (Sano and Granick, 1961) and Euglena gracilis (Carell and Kahn, 1964) have been sources of the coprogenase enzyme system. Spinach leaf has been used as a rich source of PBG deaminase (Bogorad, 1958 a, b) while Kikuchi et al. (1958) have studied ALA synthetase prepared from R. spheroides.

(1) δ-AMINOLAEVULINIC ACID SYNTHESIS

ALA synthetase condenses glycine with succinyl-CoA to form α-amino β-keto adipic acid which loses carbon dioxide to yield ALA (Figure 2). This reaction can be thought of as the start of the haem biosynthetic pathway, since it condenses glycine and succinyl CoA which are common to many other reactions in the cell.

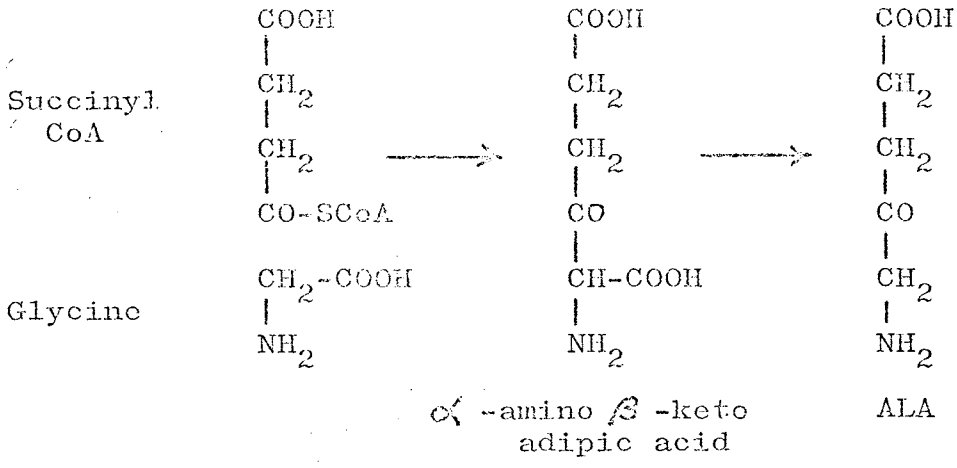


Figure 2. Synthesis of δ-aminolaevulinic acid

ALA synthetase is a particulate enzyme requiring pyridoxal phosphate as cofactor (Lascelles, 1957; Gibson et al., 1958). It has been studied in bacteria (Lascelles, 1957; Kikuchi et al., 1958), chicken erythrocytes (Brown, 1958).

Haem appears to be involved in the feedback regulation of ALA synthetase both by inhibiting the activity of the enzyme (Burnham and Lascelles, 1963) and by repression of its synthesis (Granick, 1966).

(2) PORPHOBILINOGEN SYNTHESIS

The enzyme ALA dehydratase catalyzes the condensation of two molecules of ALA to give PBG with a removal of one molecule of water (Figure 3). It is a soluble enzyme and requires free sulphydryl groups for activity (Gibson et al., 1958).

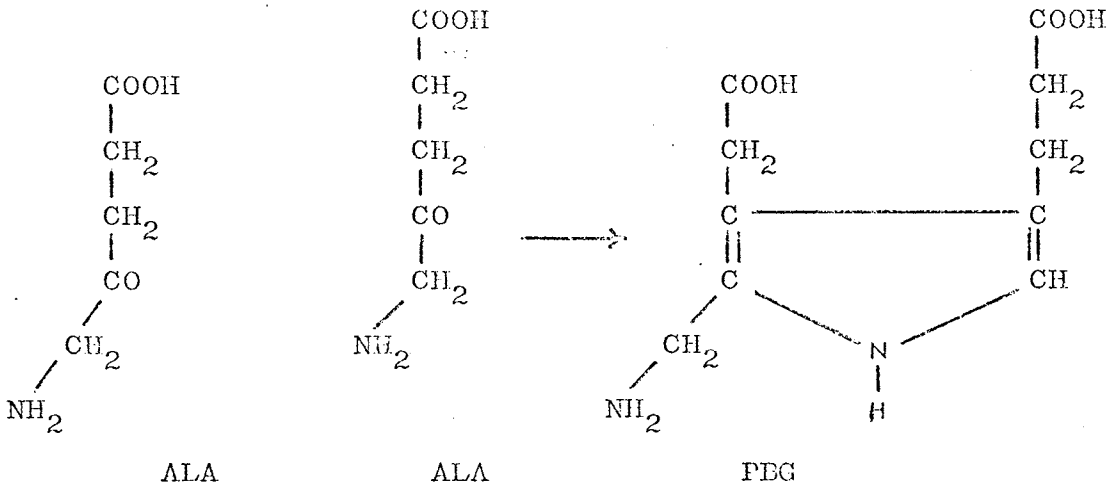


Figure 3. Formation of porphobilinogen from δ-aminolaevulinic acid.

The enzyme has been purified from ox liver (Gibson et al., 1958) and Rhodopseudomonas spheroides (Burnham and Lascelles, 1963) among other sources.

(3) UROPORPHYRINOGEN III SYNTHESIS

The condensation of four molecules of PBG gives rise to urogen III (Figure 4). The enzyme system for this reaction is composed of two factors, PBG deaminase (also called urogen I synthetase) and urogen III cosynthetase (also called urogen isomerase) which are present in the cytoplasm.

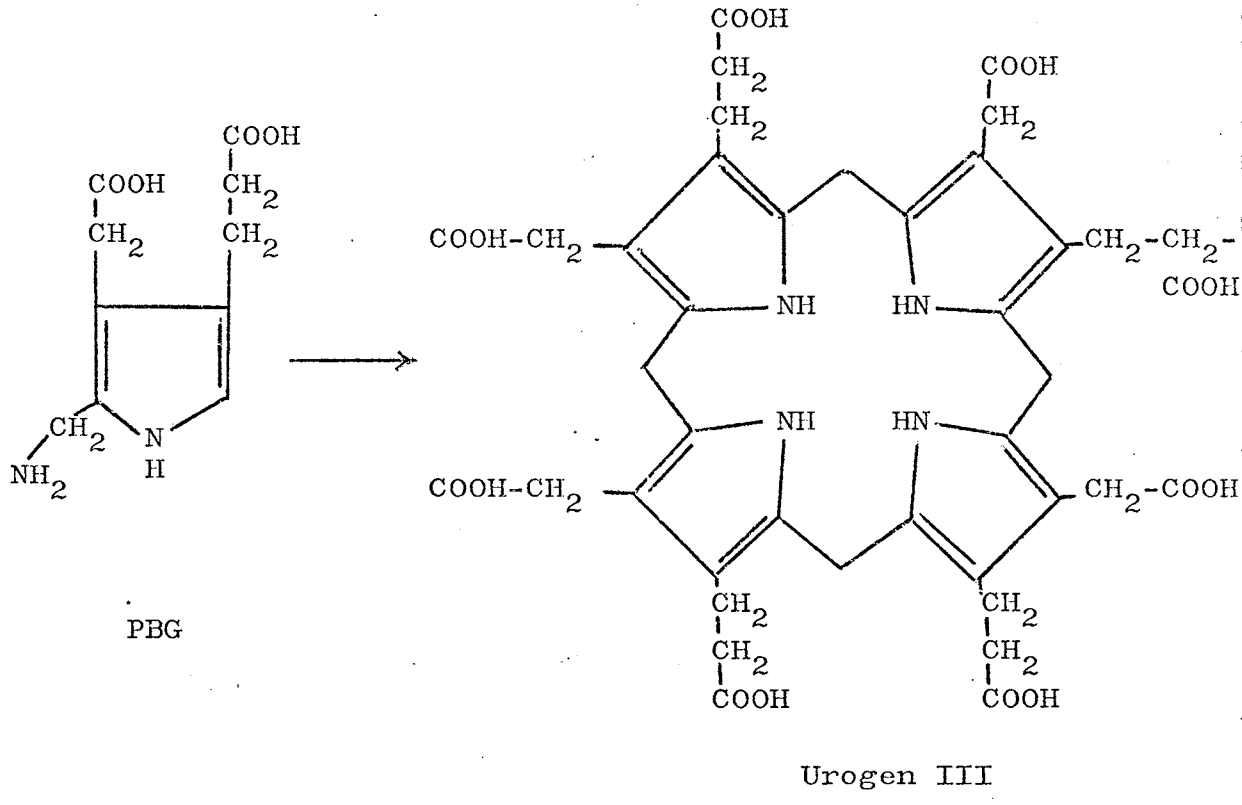


Figure 4. Formation of uroporphyrinogen III from porphobilinogen.

Bogorad and Granick (1953) with an enzyme preparation from Chlorella showed that PBG was converted to urogen III. After heating the preparation to 60°C, however, only urogen I was formed. This indicated that at least two enzymes, PBG deaminase and urogen III cosynthetase, were necessary for conversion of PBG to urogen III and that the enzymes differ in their susceptibility to heat.

PBG deaminase has been isolated from spinach leaf and in the absence of urogen III cosynthetase converts PBG to urogen I (Bogorad, 1958b). Urogen III cosynthetase has been prepared from wheat germ (Bogorad, 1958a) and has no effect on PBG when incubated alone. When incubated together with PBG deaminase, PBG was converted to urogen III. A simple cyclization of four PBG molecules would give urogen I (Figure 5). However, under the action of two enzymes, there is a migration of a methylene bridge carbon to give urogen III.

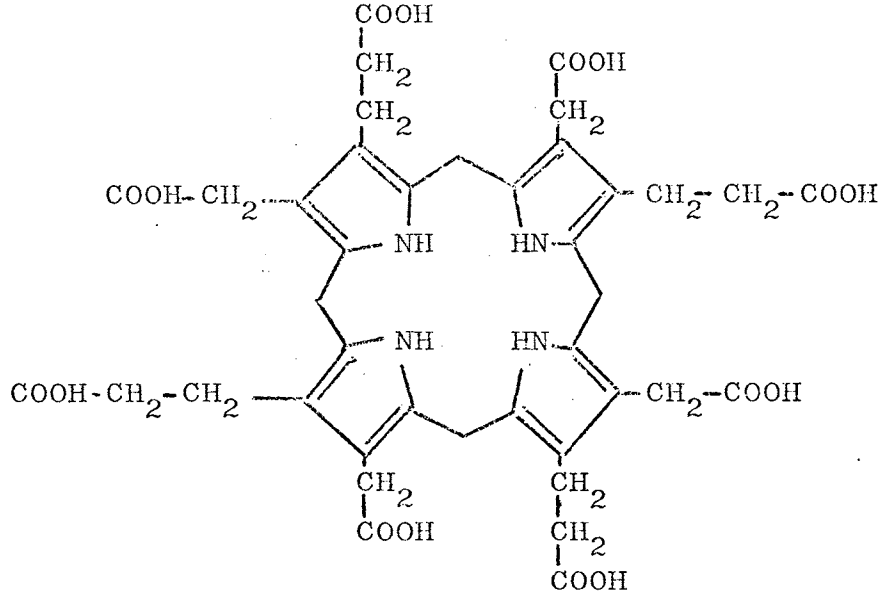


Figure 5. Uroporphyrinogen I.

PBG deaminase is strongly inhibited by p-chloromercuribenzoic acid but this inhibition is reversed by cysteine, suggesting the requirement of free sulphydryl groups for activity (Bogorad, 1958a).

(4) CONVERSION OF UROPORPHYRINOGEN III TO COPROPORPHYRINOGEN III

Urogen III is enzymically decarboxylated to coprogen III by the successive removal of the carboxylic acid groups from the acetic acid side-chains (Figure 6). The enzyme system for this conversion is not particulate and acts only on porphyrinogens, not porphyrins as substrates.

Sources from which the decarboxylase has been isolated include R. spheroides (Hoare and Heath, 1959), rabbit reticulocytes (Mauzerall and Granick, 1958) and chick reticulocytes (Tomio et al., 1970).

Hoare and Heath (1959) have shown the requirement of an ultra-filterable factor for the enzyme system from R. spheroides. However, no such requirement exists for the human erythrocyte system (Cornford, 1964).

Heptacarboxylic acid porphyrin (hepta; pseudouroporphyrin of Falk et al., 1955) and also porphyrins possessing six and five carboxyl groups, have been detected in incubations of urogen III with chicken red cell fractions (Batlle and Grinstein, 1962a, b, 1964 a,b). Porphyrinogens containing 7, 6 and 5 carboxyls have been established as the true intermediates in the transformation and not the corresponding

porphyrins (Garcia et al., 1973).

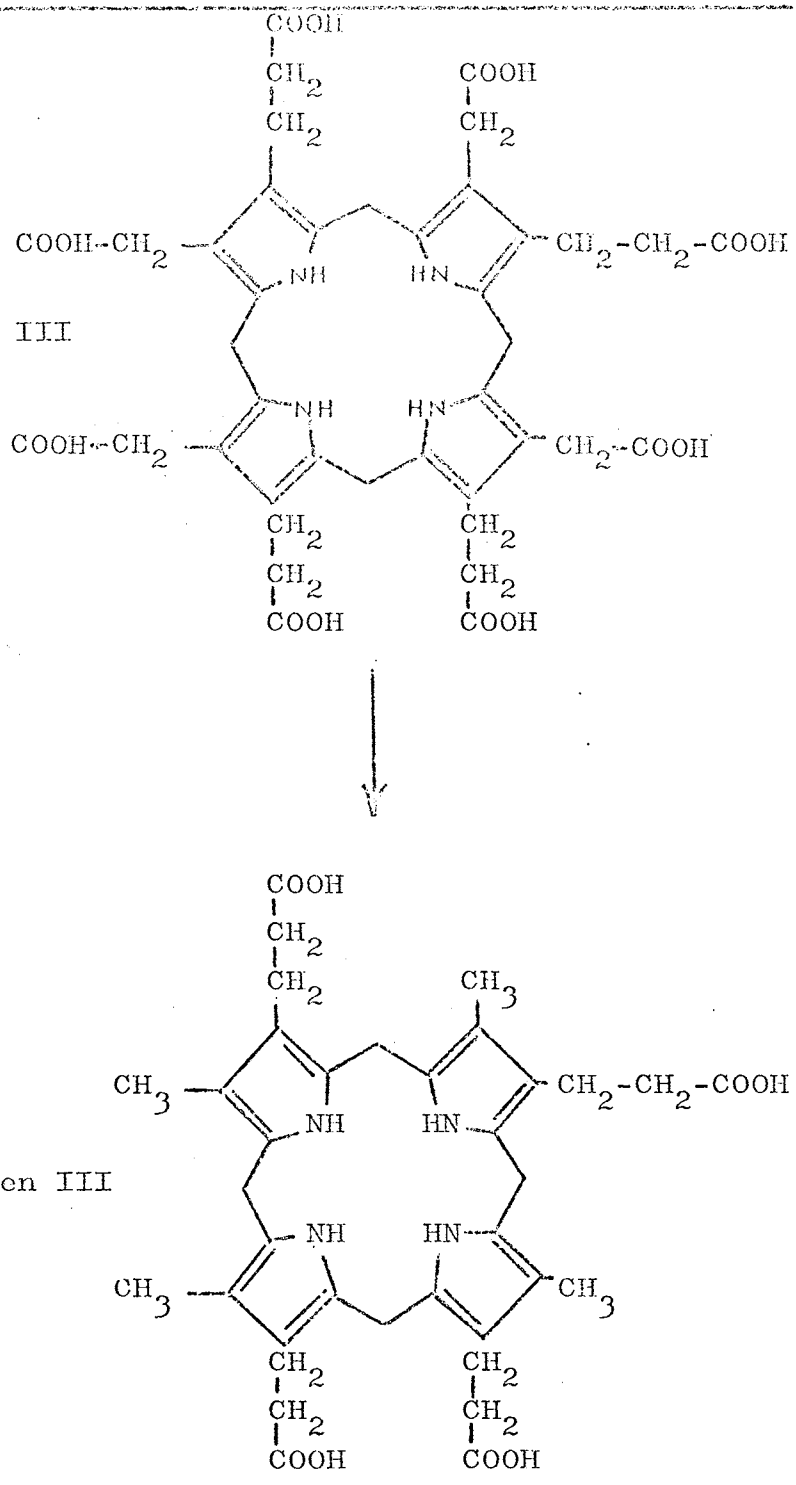
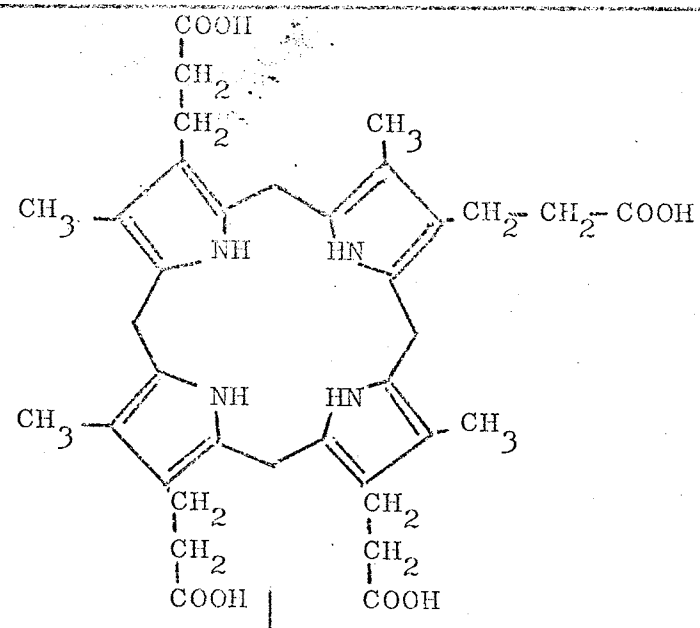


Figure 6. Conversion of uroporphyrinogen III to coproporphyrinogen III

(5) CONVERSION OF COPROPORPHYRINOGEN TO PROTOPORPHYRIN IX

Coprogenase is a particulate enzyme which converts coprogen III to protogen IX by the oxidative decarboxylation of two propionic acid side-chains (Figure 7).

Coprogen III



Protogen IX

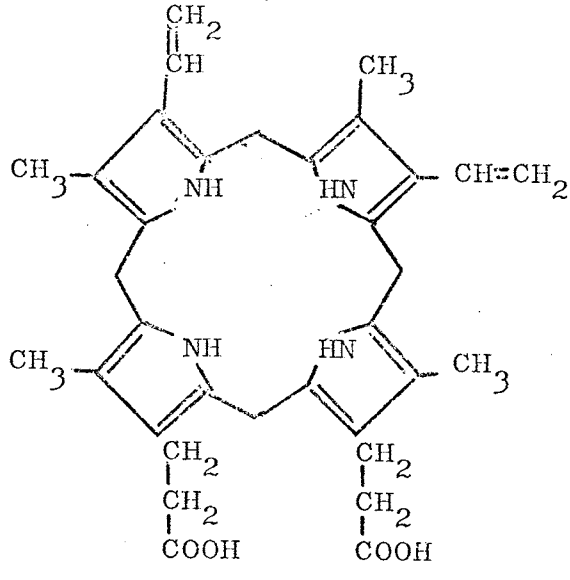


Figure 7. Conversion of coproporphyrinogen III to protoporphyrinogen IX

Protogen IX is subsequently oxidised to proto IX by the removal of six hydrogens, but it is not known whether this process is enzymic.

Coprogen IV has been shown to react with coprogenase but at one tenth the rate of coprogen III (Porra, 1962). Coprogen I and II, however, are unaffected by the enzyme (Granick et al., 1958, 1964; Sano et al., 1961; Porra 1962).

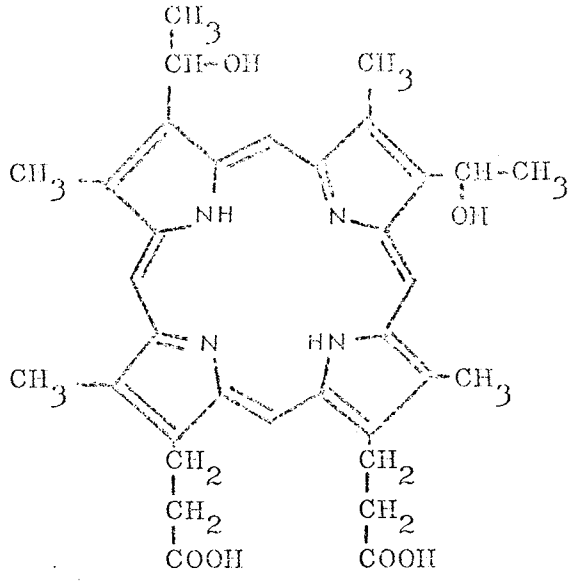
Coprogenase has been prepared from many sources including E. gracilis (Carell and Kahn, 1964), liver mitochondria (Sano and Granick, 1961; Porra and Falk, 1961; Rimington and Tooth, 1961; Batlle et al., 1965) and chick erythrocytes (Granick and Mauzerall, 1958).

Until recently no substitute has been found for molecular oxygen as the electron acceptor for the oxidative decarboxylation in a variety of organisms - mammalian liver mitochondria (Sano and Granick, 1961; Batlle et al., 1965), tobacco-leaf mitochondria (Hsu and Miller, 1970), bacteria (Jacobs et al., 1971) and even the anaerobe Chromatium strain D (Mori and Sano, 1968). Two examples of coprogenase activity in the absence of oxygen have now been reported: in an extract of R. spheroides possibly with NAD(P) as the final electron acceptor, and in a crude extract of Chromatium strain D in the presence of S-adenosylmethionine or ATP plus methionine (Tait, 1972).

The activity of coprogenase prepared from rat-liver mitochondria was not affected by prolonged dialysis (Batlle et al., 1965). However, the coprogenase preparations from bovine liver mitochondria of Sano and Granick (1961) were shown to contain traces of flavine. Batlle et al. (1965) in their work on rat liver mitochondria could neither isolate nor reveal flavine and concluded that, if present, the flavine must be very tightly bound to protein and that its spectrum must be masked by other components.

Inhibition of coprogenase was observed with Cd^{2+} and Pb^{2+} suggesting the presence of thiol groups on the enzyme (Batlle et al., 1965). These workers also demonstrated that the monothiols, cysteine and glutathione, inhibit the activity of the coprogenase system. They suggest that this inhibition may be due to competition for the substrate between the monothiol and an SH group of the enzyme or due to reaction of an intermediate with the monothiol.

Several porphyrinogens have been proposed as intermediates in the conversion of coprogen to protogen. Bogorad and Granick (1953) and Granick and Bogorad (1953) found haematoporphyrin IX (haemato IX) and mono hydroxyethyl monovinyl deuteroporphyrin IX (mono hydroxyethyl monovinyl deutero IX) in cultures of a Chlorella mutant (Figure 8).



Haematoporphyrin

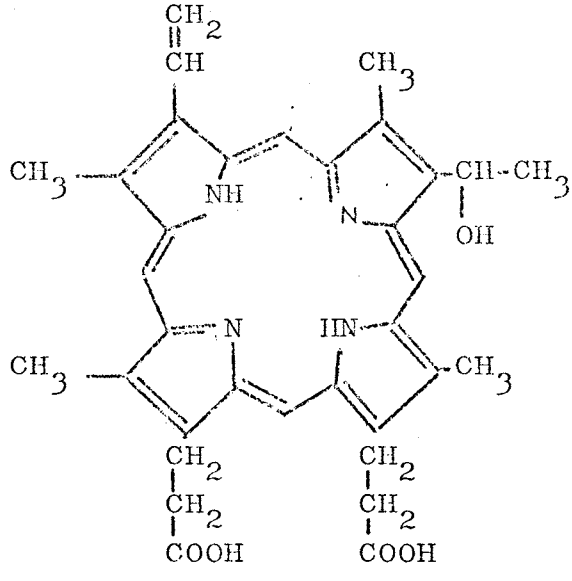


Figure 8. Mono hydroxyethyl mono vinyl deuteroporphyrin.

However, Granick et al. (1960) and Marks and Bogorad (1958) showed neither haemato IX nor haematogen IX to be utilized as substrates by frozen and thawed Chlorella mutants. Also semicarbazide and hydroxylamine added to incubations of the enzyme system had no effect, evidence against a carbonyl group in any intermediate.

Harderoporphyrin (hardero) (Figure 9) was isolated by Kennedy et al. (1970) from rat Harderian glands and identified as 2-vinyl 4-propionic deutero by mass spectroscopy and by comparison with authentic samples, prepared synthetically, of 2-vinyl 4-propionic acid deutero and 4-vinyl 2-propionic acid deutero (iso-hardero).

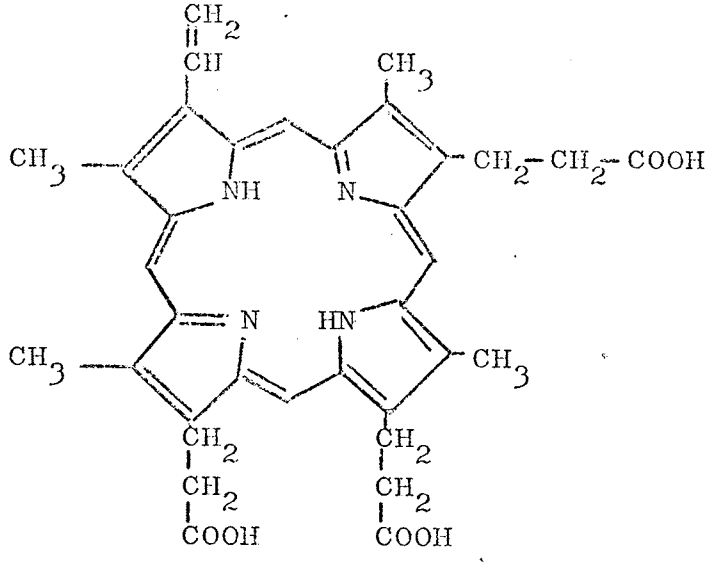


Figure 9. Harderoporphyrin (2-vinyl 4-propionic acid deuteroporphyrin).

The porphyrinogen from hardero is converted by a coprogenase system from E. gracilis to protogen, but iso-harderoporphyrinogen (iso-harderogen), the 4-vinyl 2-propionic acid isomer is not (Cavaleiro et al., 1973).

A tetracarboxylic acid porphyrin with a rhodo-type spectrum has been isolated from foal and calf meconium (French et al., 1970) and from human bile (Smith et al., 1968, 1969a, b, 1971). The porphyrin from both sources, on reduction with one mole of hydrogen, yields a porphyrin convincingly identified as copro III. This reasonably establishes the structure as acrylic acid propionic acid deutero IX (Figure 10).

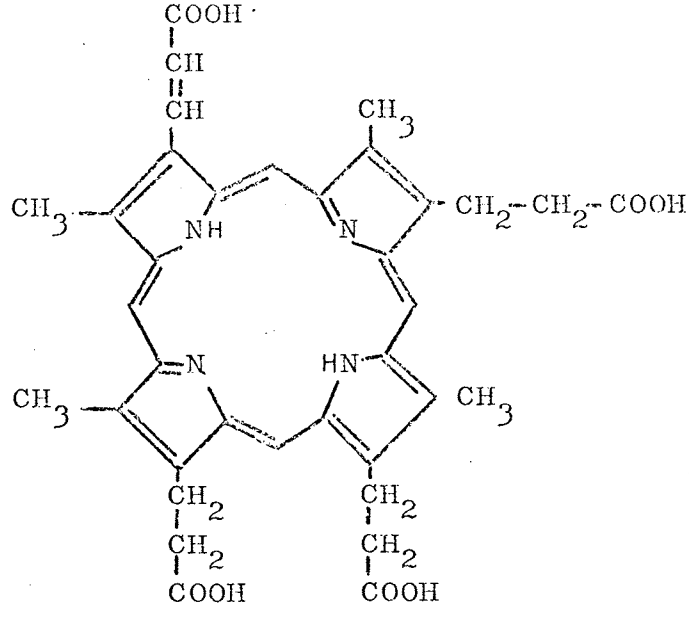


Figure 10. Acrylic acid propionic acid deuteroporphyrin IX.

French et al. (1970) were able to show that the major part was the cis isomer. Neither the porphyrin nor the corresponding porphyrinogen has been tested as an intermediate with the coprogenase system. On the other hand, Sano and Granick (1961) have shown that 2,4-trans-diacrylate deuteroporphyrinogen (2,4-trans-diacrylate deuterogen) was not utilized by coprogenase prepared from beef liver mitochondria.

Sano (1966) has synthesized 2,4-bis (β-hydroxypropionic acid) deuterogen IX, Figure 11. This compound gave a 20-22% yield of proto IX when incubated with a coprogenase preparation from beef liver mitochondria.

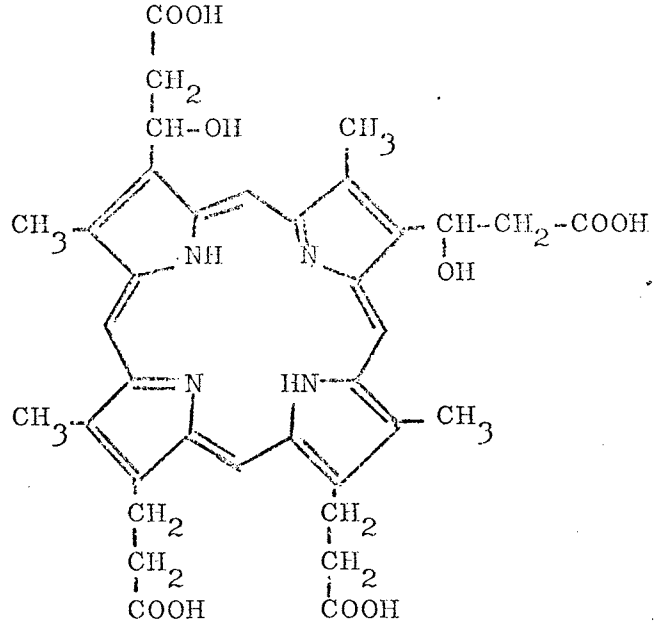


Figure 11. 2,4-bis (β-hydroxypropionic acid) deuteroporphyrin IX.

Zaman et al. (1972) incubated a chicken haemolysate preparation with radioactive ALA chemically labelled on the β-carbon with tritium and on the γ-carbon with ^{14}C as an internal standard.

$$NH_2 - CH_2 - {}^{14}CO - CHT - CH_2 - COOH$$

(β - T, γ - ^{14}C) ALA

The haems formed were recovered, converted to meso and oxidized with chromic acid to give two maleimides; methyl ethyl maleimide arising from rings A and B of the porphyrin ring and carboxyethyl methyl maleimide arising from rings C and D. The ratio of tritium to ^{14}C in the carboxyethyl methyl maleimide was twice that in the methyl ethyl maleimide. From this it follows that rings A and B had lost half their tritium (Figure 12).

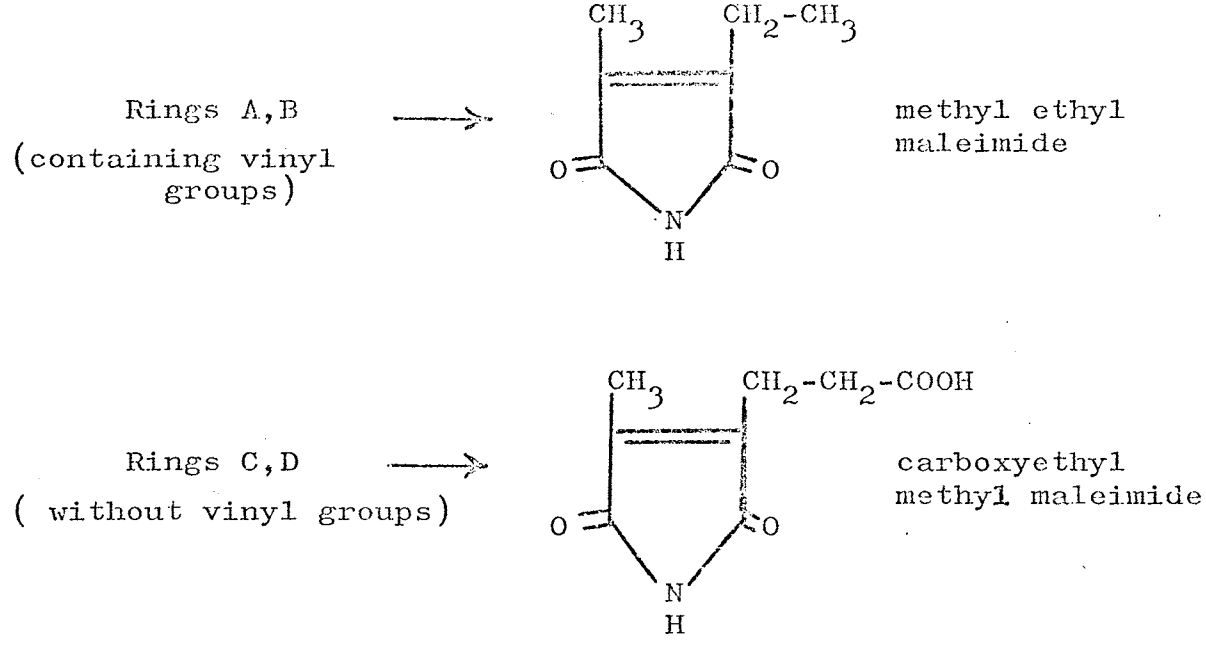


Figure 12.

They then incubated the haemolysate with α-ketoglutarate labelled asymmetrically with tritium in position 3R (Figure 13). In this case no loss of labelling was noted after recovering the haems.

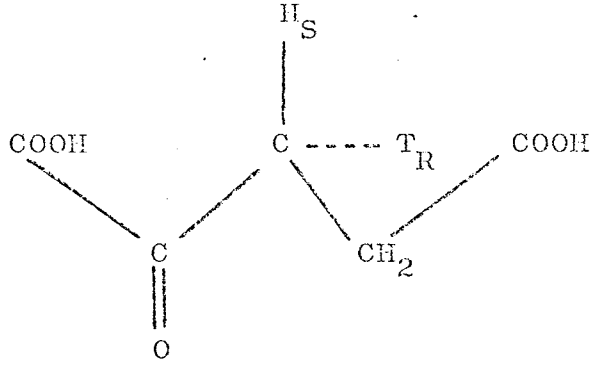
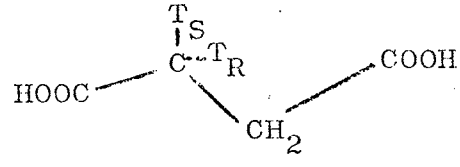


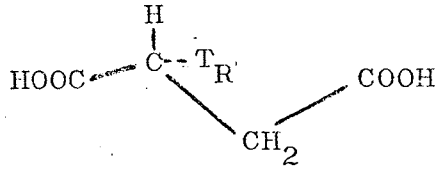
Figure 13. α-keto- (3R - ^{3}H) glutarate

Thirdly, they prepared three succinates labelled stereospecifically with tritium (symmetrically labelled RS and asymmetrically labelled R and S - Figure 14). With these compounds, incorporation of tritium into haem was 5/6, 6/6 and 4/6 respectively.

(2RS - T_2) - succinate



(2R - T) - succinate



(2S - T) - succinate

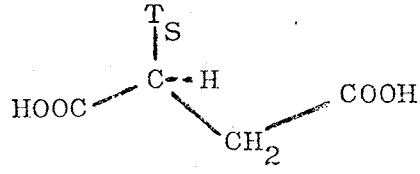


Figure 14.

Battersby et al. (1972) performed labelling experiments using Euglena preparations. From PBG labelled with two deuterium atoms in the β-position of the propionic acid side chain (below) they recovered copro containing eight deuterium atoms and proto with six deuterium atoms. From PBG labelled with two deuterium atoms in the α-position (Figure 15) both the copro and proto recovered had eight deuterium atoms incorporated.

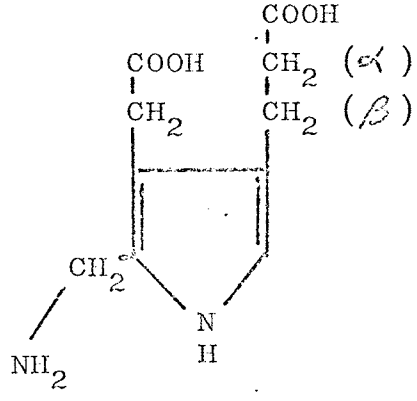


Figure 15 α- and β-positions of PBG for labelling experiments.

These two papers are convincing evidence that both the α-hydrogen atoms of the propionic acid side chains are retained in the formation of the vinyl group of proto. However, at the β-position one hydrogen is stereospecifically removed while the other hydrogen remains attached throughout the reaction.

On the assumption that the intermediates would be the same in other systems, it would appear that haemato and monohydroxyethylmonovinyl deutero (and the corresponding porphyrinogens) found by Bogorad and Granick (1953) and Granick and Bogorad (1953) were by-products and not intermediates since they would be expected to lose one third of the hydrogens from the terminal methyl group. This is supported by their failure to act as intermediates.

Further the acrylic acid derivatives of French et al. (1970) and Smith et al. (1971) seem unlikely as intermediates since they have already lost one hydrogen from the α-position. Sano's (1961) failure to get conversion of the diacrylate deuterogen is not conclusive since this was the trans isomer and the only evidence for the natural compound is that it is the cis isomer.

Sano's success in achieving 20-22% yield of proto from 2,4-bis (β-hydroxypropionic acid) deuterogen IX is important since the idea of this compound as an intermediate is consistent with the loss of one hydrogen from the β-position and no loss from the α-position on the propionic acid side chain. The compound used by Sano was chemically prepared and should contain all of the four possible isomers expected from the two asymmetric carbon atoms. In the light of the evidence of Zaman and colleagues (1972) it is reasonable to assume that only one of these four isomers would be an intermediate.

The utilization of harderogen and the failure to utilize iso-harderogen establishes that position two of the porphyrin ring is attacked before position four, at least in Euglena, but adds nothing to our understanding of the mechanisms of the reactions of the propionic acid side chains.

Porra and Falk (1961) have made an interesting observation on the coprogenase enzyme system. On incubating with coprogen III aerobically, up to 40% of the porphyrin was bound to the protein. The protein-bound porphyrin was stable in glacial acetic acid, ethyl acetate-acetic acid, acetone-HCl and several other solvents which would be expected to remove loosely bound porphyrin. Methods which did split this complex and gave ether-soluble porphyrins were hydrolysed at 40°C for 60 hours with 5N HCl or 1N NaOH, the silver salt method of Paul (1950) and treatment overnight at room temperature with 50% HBr in glacial acetic acid (Hill and Keilin, 1930). The porphyrin hence appeared to be covalently bound. Copro III, copro I, coprogen I aerobically and coprogen III anaerobically were not bound. Protogen aerobically was bound but only loosely. Porra and Falk suggested that the stable protein-bound porphyrin compound was a protein-porphyrinogen intermediate in the reaction.

Sano (1966) has proposed the following reaction mechanism for the conversion of coprogen to protogen (Figure 16):

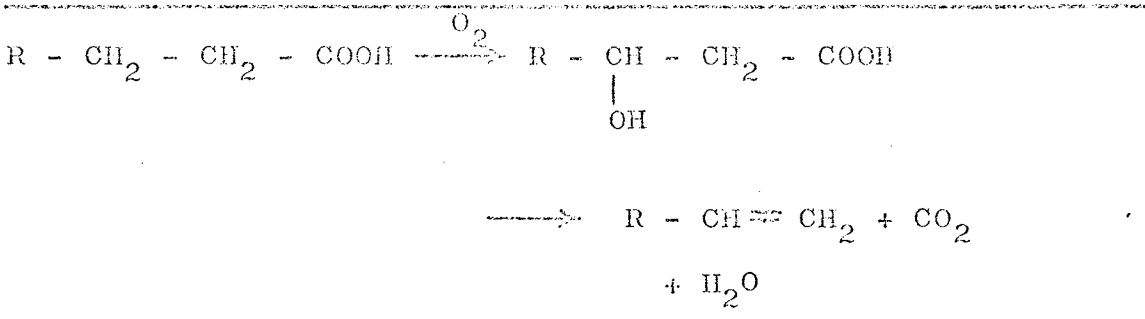


Figure 16. One postulated reaction mechanism for vinyl side chain formation.

Another possibility, which incorporates the suggestion that a porphyrinogen is bound by a thio-ether linkage during the conversion, is given in Figure 17.

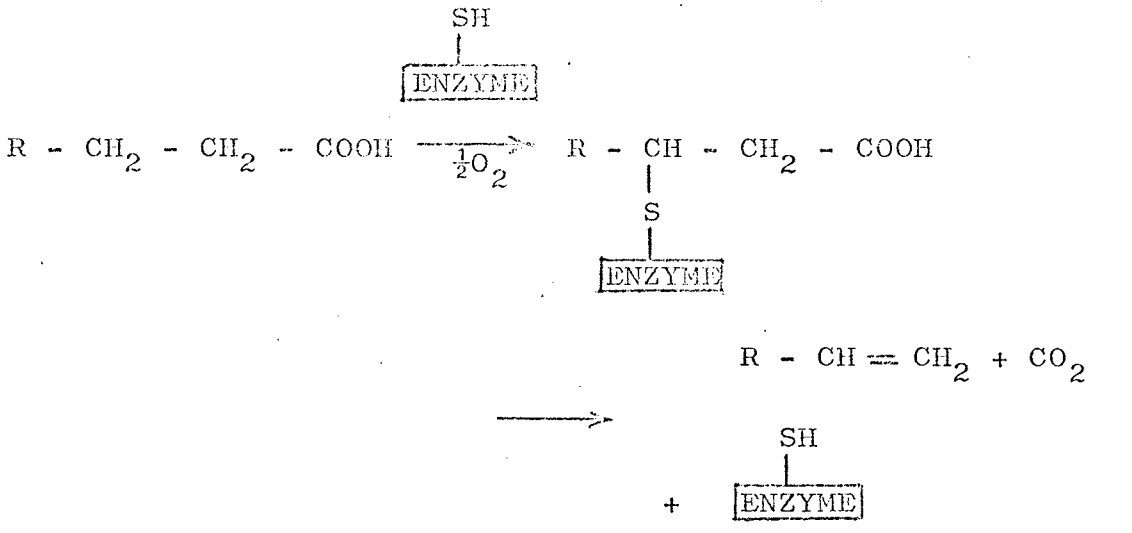


Figure 17. One postulated reaction mechanism for vinyl side chain formation.

These two suggestions for the conversion are consistent with the findings of Zaman *et al.* (1972) and Battersby *et al.* (1972).

(6) FORMATION OF HAEM

Ferrochelatase catalyzes the insertion of Fe^{2+} into proto to form haem.

The enzyme is particulate and has been studied from sources including rat liver (Labbe and Hubbard, 1961) pig liver (Porra and Jones, 1963), duck erythrocytes (Oyama et al, 1961) and bacteria (Lascelles, 1964).

Ferrochelatase will not react with porphyrinogens. It is specific neither for porphyrin nor for metal. Meso, for example, reacts much faster than proto and Co^{2+} is incorporated at about the same rate as Fe^{2+} (Jones and Jones, 1969).

(7) THE PORPHYRIAS

The porphyrias are a group of metabolic diseases having in common some defect in the haem biosynthetic pathway. As well as occurring in man, porphyria has also been reported in several domestic animals including bulls and pigs. The term has even been used in describing some yeast and bacterial cultures with a high porphyrin excretion into the medium.

There are two types of symptoms which may be expressed in the porphyrias in man. There are the acute symptoms which include nausea, abdominal pain and other neurological disturbances and there is photosensitivity in which blisters may form on exposure to sunlight.

The porphyrias can be conveniently divided into two

major groups, depending on whether the fault is manifest in the bone marrow or in the liver. Further classification of the porphyrias relies on the symptoms expressed and the metabolites excreted. The porphyrias are summarized in Table 1.

TABLE 1

The Porphyrias

Erythropoietic	Hepatic
Erythropoietic porphyria	Acute intermittent porphyria
Erythropoietic protoporphyria	Toxic porphyria
	Variegate porphyria
	Coproporphyria
	Porphyria associated with hepatic adenoma

In erythropoietic porphyria a large amount of urogen I is formed apparently due to insufficient activity of the urogen III cosynthetase. Urogen I is partly decarboxylated to coprogen and intermediates and is excreted in the urine and faeces. The presence of large amounts of urogen I in the peripheral circulation gives rise to severe photosensitivity in the patient.

In erythropoietic protoporphyria there is a 10 to 50 fold increase in the amount of free protoporphyrin in

the red blood cells. The presence of excess porphyrin that is not converted by the enzyme ferrochelatase to haem gives rise to mild photosensitivity.

There are several hepatic porphyrias. In acute intermittent porphyria, the urine contains raised levels of ALA and PBG. These precursors also are raised in almost all latent carriers and are important for diagnosis.

Porphyrin excretion is generally but not always raised. The raised porphyrin excretion in the urine is due partly to non-enzymic condensation of PBG to uro (that is known to occur on standing) but hepta is also often raised above normal; this is evidence of enzymic origin of the porphyrin.

Tschudy et al. (1965) found that the activity of ALA synthetase in the liver is greatly increased in acute intermittent porphyria. Strand et al. (1971) have shown that, as well as the increase in ALA synthetase activity, there is a marked decrease in the activity of urogen I synthetase. These workers suggest that the basic fault is due to lowered activity of urogen I synthetase and that increased ALA synthetase production results from feedback control due to lowered haem synthesis.

In toxic porphyria there is an increase in the excretion of uro and of partially decarboxylated porphyrins which cause the photosensitivity. Also dehydroisocopro-porphyrin (dehydroisocopro) shown in Figure 18 and two

related porphyrins (possibly formed from bacterial action on dehydroisocopro) have been isolated and characterized by Elder (1972) from faeces of patients with toxic porphyria, where they occur in large amounts (50-100 µg/g. dry wt., W.H. Lockwood, personal communication).

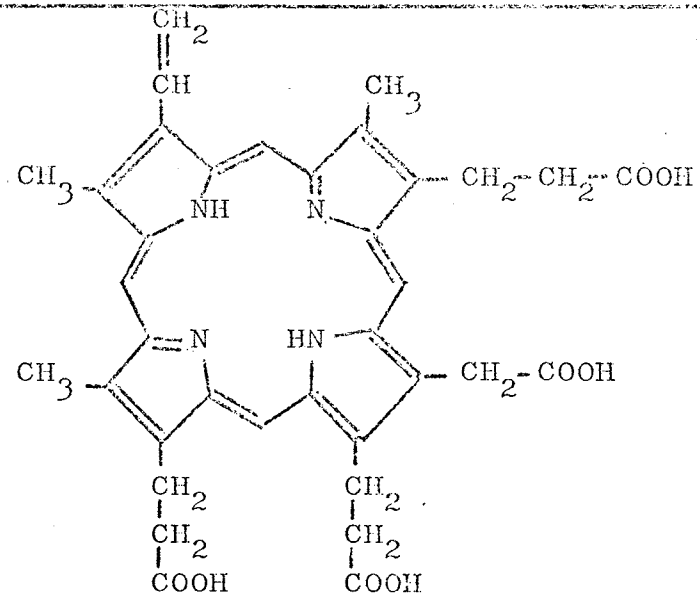


Figure 18. Structure of dehydroisocopro proposed by Elder (1972).

The basic fault in toxic porphyria seems to be a breakdown in the decarboxylation. Increased levels of partially decarboxylated porphyrins accumulate. If coprogenase acts on pentacarboxylic acid porphyrinogen this would explain the increased amounts of dehydroisocopro present.

Hepatic hereditary coproporphyria is an uncommon form of porphyria characterized biochemically by the excretion of large amounts of copro III mainly in the faeces but also in the urine. Excretion of ALA and PBG is generally elevated even during remission, but a more marked elevation occurs during attacks (Lomholt and With, 1969). Many people carrying the gene for coproporphyria are asymptomatic and therefore difficult to recognise.

There are several other rare forms of porphyria including a porphyrin-producing hepatic adenoma found in a patient of Tio et al. (1957).

Lastly, variegate porphyria is one of the inherited hepatic porphyrias. It is called variegate since both acute and photosensitive symptoms may occur, some patients suffering from both forms, others from only one. Acute attacks, particularly the first acute attack, are often precipitated by barbiturates and other drugs.

Variegate porphyria is inherited as a Mendelian dominant gene which is not sex-linked. In some carriers of the genetic defect, no clinical symptoms occur. This form of porphyria is particularly common in the white population of South Africa. Dean (1963) estimates the gene is present to the extent of 3 in 1000 and has traced most cases to the arrival of a Dutch settler in 1685.

Rimington et al. (1968) have reported two families of sufferers. There is a large family of Polish-Jewish origin distributed throughout the world; sixteen were identified as carrying the porphyria gene and another four were possible carriers. In another large family of British origin four members were shown unambiguously to be carrying a similar porphyria gene.

Hammström et al. (1967) report three Swedish families in which the variegate gene is present. A total of seventy members were investigated; five manifest suffers were found and nineteen latent carriers.

At the Institute of Medical Research, Royal North Shore Hospital of Sydney, three families of sufferers have been investigated. One family from Sydney comprises a mother who has clinical symptoms and a son who has been tested biochemically and clinically to be positive. It is his porphyrin excretion which forms the basis of this thesis. Seven members of another family in Sydney have been investigated; one has clinical symptoms, three are definite carriers and one a possible carrier of the variegate gene. A Canberra family of variegate sufferers has one member with manifest porphyria and is known to have relatives who are affected. However, no details or family tree are available.

Watson (1960) in a review on porphyria mentions several cases of mixed porphyria, but it is difficult to

judge either from family history of from analytical data whether these are variegate porphyria.

Clinically the cutaneous symptoms are the most common, Dean (1963) and Rimington et al. (1968) both stressing skin fragility and light sensitivity. Acute attacks appear sometimes to be spontaneous but more often there is a history of a precipitating factor particularly barbiturates. No reliable study of the incidence of the various manifestations has been found. However, it appears from some families discussed in detail by Dean (1963) that most carriers exhibit some skin abnormality and about one third have suffered from an attack of acute porphyria. Only a few appear to be truly latent. Dean makes the point that the acute form is a disease of this century and is an expression of the development and wide use of new drugs.

Biochemically, variegate porphyria frequently shows elevated urinary porphyrins, particularly copro, but uro may also be raised. During an attack of acute porphyria both porphyrins and the precursors ALA and PBG in the urine are raised. At other times PBG and ALA may be normal or slightly raised.

Faecal porphyrin excretion in variegate porphyria is permanently raised, although Gray et al. (1948) and Rimington et al. (1968) observe that, during acute attacks, urinary porphyrin excretion rises, whereas faecal excretion

declines. The raised faecal porphyrin excretion is due mainly to the ether-soluble porphyrins copro and proto which are often more than ten times their normal values. As well as ether-soluble porphyrins, there is an abnormal excretion of faecal water-soluble porphyrin. The elevated ether-soluble porphyrins, particularly proto (Dean, 1963) and more recently the excretion of faecal water-soluble porphyrin (Rimington et al., 1968) form the most reliable biochemical diagnostic criteria for variegate porphyria.

Comparison of published values for porphyrin and precursor excretion in normal individuals shows wide variation, making diagnosis difficult in certain cases (Goreczky et al., 1968). For example, Prato et al. (1967) obtained normal PBG excretion of 671 ± 414 µg/day, while Heilmeyer (1964) obtained values of 1513 ± 220 µg/day. Similar contradictory values have been recorded for urinary copro and uro determinations. Possible explanations for these variations may be in the choice of normal patients and also in the methods of determination.

(8) WATER-SOLUBLE PORPHYRINS

In 1948 Gray and coworkers isolated water-soluble porphyrins from the urine of a porphyria patient with recurrent jaundice. These porphyrins were not identified but were not uro; the methyl ester could not be crystallized and, unlike uro, did not give copro by decarboxylation.

In 1963, Sweeney and Eales reported the presence of water-soluble porphyrins unlike uro and hepta in the urine of a porphyric patient during an episode of intrahepatic cholestatic jaundice. In an undescribed chromatography system, these porphyrins migrated as a mixture containing two to four carboxyl groups.

Beginning in 1966, Rimington and co-workers (Rimington and Lockwood, 1966; Rimington et al, 1968) investigated the urine of a patient who had developed jaundice while recovering from an attack of acute porphyria. As well as copro and uro, the urine contained a water-soluble porphyrin. Unlike uro, the porphyrin was not extracted from aqueous solution at pH 1.5 by cyclohexanone but was extracted readily at pH 2.9, behaviour resembling that of porphyrin c (dicysteinyl haematoporphyrin).

For isolation total porphyrins were adsorbed onto talc from urine at pH 3.5. The porphyrins were eluted with formic acid and a portion of this eluate was evaporated. Attempts at esterification of the dry residue with ethereal diazo-methane were unsuccessful but were successful with 5% (v/v) sulphuric acid in methanol. On paper chromatography (Chu et al., 1951) most of the porphyrin remained at the origin but distinct spots were also seen in the position for copro I and III and haemato. Re-esterification did not alter the position of the base-line spot.

The remainder of the ester was hydrolyzed and then treated with silver sulphate as a test for thio-ether linkages (Paul, 1951). An ether-soluble porphyrin was obtained, which ran as a dicarboxylic on lutidine chromatography and, after esterification migrated as haemato ester on the chromatography of Chu and Chu (1954).

Another portion of the formic acid eluate of the talc was added to water and porphyrins were extracted into ethyl acetate. Porphyrins not extracted into ethyl acetate were extracted with butanol. These two fractions were purified from amino acid impurities on Amberlite CG-50 columns. Each fraction was treated with (U - ^{14}C) FDNB and ether-soluble porphyrins obtained were examined by lutidine chromatography and autoradiography. For both fractions, the radioactive area coincided with the red fluorescent streak from the tricarboxylic to the dicarboxylic positions. Reaction of porphyrin c with (U-^{14}C) FDNB followed by similar chromatographic procedures gave an ether-soluble porphyrin which migrated with the radioactivity.

Identification of the amino acid components was attempted on bulked DNP-porphyrin material from the urine. The material was heated with 20% (w/v) HCl in a sealed, evacuated tube for 24 hours at 105°C. Terminal amino acid DNP derivatives were removed from the acid solution by extraction with ether. The aqueous phase was treated with (U - ^{14}C) FDNB; ether extraction after acidification removed

any DNP derivatives of amino acids which had been part of a peptide chain. The aqueous phase after neutralization to pH 3.5 was extracted with ether to remove any cysteinyl porphyrin DNP derivative. Paper chromatography using suitable markers suggested that: (i) the N-terminal amino acids were mainly phenylalanine with a trace of glutamic acid; (ii) of the remaining amino acids present, glutamic and aspartic acids, serine and phenylalanine were part of a peptide chain; (iii) there was evidence for the presence of cysteinyl porphyrin DNP.

Grosser et al. (1971) examined the urine of a patient with infective hepatitis and found similar water-soluble porphyrins. The total urinary water-soluble porphyrins were adsorbed onto talc and eluted with acetone-HCl. Ether-soluble porphyrins were removed and the water-soluble fraction was passed through an Amberlite CG-50 column. The major porphyrin band obtained from this column gave a strong ninhydrin reaction. This band was further purified on a Dowex 50 column and found to contain free peptides similar in amounts to that obtained by washing the talc with water prior to elution with acetone/HCl. The major porphyrin fraction on the Dowex 50 column was eluted with lutidine-water and at this stage 20% of the porphyrin was found to be haemato. After silver salt treatment, a further small fraction (less than one fifth of the total porphyrin present) appeared as haemato while

60% of the porphyrin remained unchanged. Removal of both free haemato and that released by silver salt treatment was accompanied by a change in the acid Soret maximum from 400 nm to 410 nm. The shape of the Soret peak at 400 nm suggested a mixture of substances with wavelengths of 400 and 410 nm while the Soret band at 410 nm of the residual fraction seemed to be uniform. Comparison of the chromatography of the water-soluble porphyrin and porphyrin octapeptide on acetone/HCl and lutidine/water systems showed dissimilar migration.

Water-soluble porphyrins have also been reported in liver, plasma and bile (Rimington and Lockwood, 1966; Rimington et al., 1968). Belcher et al. (1969) prepared water-soluble porphyrins from variegate bile by removing ether-soluble porphyrins and extracting the aqueous fraction with butanol. The porphyrins were extracted from the butanol with 1.5N HCl and prepared for treatment on a Sephadex G25 column. Four peaks containing porphyrin were obtained on developing the column. The material in peaks 2, 3 and 4 was reacted with (^{14}C) FDNB and lutidine chromatography and autoradiography showed the radioactivity corresponding with the porphyrin fluorescence at the tricarboxylic acid position. The porphyrin in peak 2 gave haemato on treatment with silver salt. These workers give evidence that the porphyrin from bile has a higher molecular weight than that obtained from faeces by Rimington et al. (1968).

Water-soluble porphyrins have been investigated in variegate faeces by Rimington et al. (1968) and results indicate similarity with the urinary compounds. The residue after exhaustive acetic/ether extracts of the faeces (or the precipitate obtained by extracting this residue with acetic and adding ether) was hydrolyzed with boiling 6N HCl and then esterified with methanol-sulphuric. An ether-soluble porphyrin fraction was obtained which behaved similarly to porphyrin c ester of Zeile and Meyer (1939) in being extracted from ether by citrate buffer pH 2.9, in being returned to ether at pH 4.9, and also in losing its high basicity after standing in ether. This porphyrin was applied to an Amberlite CG-50 column and washed well with pyridine-acetate buffer pH 5. The porphyrin was stripped from the column with 30% aqueous pyridine, freeze-dried and treated with silver sulphate. The ether-soluble porphyrin obtained behaved after esterification as haemato ester. The aqueous fraction was oxidised with performic acid and subjected to two-dimensional paper chromatography. A faint and slowly developing ninhydrin-positive spot appeared in the cysteic acid position.

The precipitate obtained by adding ether to the acetic acid-ether extract of the residue (after acetic-ether extraction) was hydrolyzed in 20% H_2SO_4 for 40 hours at 120-130°C. The porphyrins present in the supernatant were extracted with butanol, applied to an Amberlite CG-50

column and washed with pyridine-acetate buffer as before. The porphyrin eluted from the column with aqueous pyridine was treated with (U-^{14}C) FDNB and extracted into ether. The ether was extracted with 10% HCl and lutidine chromatography and autoradiography showed a "strong porphyrin fluorescence in ultraviolet light and coincident with prominent radioactivity" at the dicarboxylic position and a smaller fluorescent spot with radioactivity at the tricarboxylic position, A trace of copro III without associated radioactivity was also present.

The faecal residue after exhaustive ether-acetic extraction was extracted with Triton-urea (4% v/v, 45% w/v) and prepared for paper electrophoresis in alkali. As well as a fast moving brown band there was a slower moving red fluorescing band. Elution of the slower band, and treatment with (U-^{14}C) FDNB followed by lutidine chromatography and autoradiography showed a copro moving spot devoid of radioactivity and a tricarboxylic fraction in which radioactivity was coincident with the porphyrin fluorescence.

These results on water-soluble porphyrins have been interpreted as establishing the presence of a series of porphyrin-peptides, in which the porphyrin is bound to the peptide portion through a thio-ether bond. It is suggested that these compounds are formed in the liver and result from a faulty biosynthesis of proto. They normally pass with the bile into the duodenum and thence through the gut, but

occasionally the bile is interrupted and they then appear, temporarily, in the urine.

Several important questions remain on the nature of water-soluble porphyrin. There are difficulties in interpreting the formation of haemato; either it is formed too readily or not readily enough. Secondly, no pure component of water-soluble porphyrin has been prepared for investigation. It is also difficult to imagine a porphyrin-peptide remaining unaltered after action of digestive enzymes and intestinal bacteria during passage through the gut. It might be expected therefore that the faecal compounds differ from the urinary and bile compounds.

(9) AIM OF THE INVESTIGATION

The present work was undertaken in order to identify both ether-and water-soluble porphyrins in the faeces of variegate porphyria and to interpret these compounds in terms of porphyrins emptied into the duodenum with the bile. These findings may then add to a better understanding of the metabolic fault in variegate porphyria.

SECTION B

MATERIALS AND METHODS

(1) REAGENTS AND MATERIALS

Reagents were of analytical grade.

Technical ether was obtained from Drug Houses of Australia and was stored in the dark to avoid peroxide formation.

Chloroform containing 1% absolute alcohol was supplied by Riedel de Haën.

Cellulose powder (standard grade) was supplied by Whatman.

Paper for electrophoresis was Whatman 3MM, 7 cm wide, in roll form.

TLC aluminium backed sheets without fluorescent indicator were supplied by Merck. The sheets were pre-coated with silica gel 0.25 mm in thickness.

All references to percentages in relation to concentrations of HCl were in weight per volume (w/v).

Tetramethyl haemato was kindly supplied by Dr. M.R. Lemberg.

Crystalline proto ester was supplied by Mr. W.H. Lockwood.

Haemato was supplied by Calbiochem. After esterification, it was purified on TLC.

(2) DIAGNOSIS AND CLINICAL HISTORY OF VARIEGATE PORPHYRIA PATIENT

The patient (JM) is a male, 29 years old and has complained for many years of skin fragility and photosensitivity on exposed areas. Bullous eruptions on the backs of fingers and hands sometimes occur and after rupture tend to leave small scars. The patient also has been troubled by periods of nausea and nervousness, symptoms which have often been noted in cases of variegate porphyria (Dean, 1963).

The patient has been diagnosed as suffering from variegate porphyria by several criteria. Analysis of the urine showed raised levels of ALA (132 µg/l), PBG (7.4 µg/l) and porphyrins (1900 µg/l). The elevation in porphyrin excretion was due mainly to copro, but uro, hepta, hexa and penta are also present in increased amounts.

Porphyrin excretion in the faeces was also elevated (1000 µg/g dry wt) being mainly proto and copro but raised amounts of uro, hepta, hexa and penta were also present. Large amounts of water-soluble porphyrins were found in the faeces which Rimington *et al.* (1968) have shown to be diagnostic of variegate porphyria.

The mother of the patient has not suffered noticeably from skin fragility or photosensitivity, but is troubled by neurological symptoms. The grandfather of the patient on his mother's side possessed both photosensitivity and neurological

symptoms. No biochemical investigations of the mother and grandfather have been undertaken.

The patients paternal relatives have no symptoms of the disease.

(3) SPECIMEN COLLECTION

Faecal specimens were collected, covered with methanol and stored at $4^{o}C$. Within seven days of collection, batched samples were homogenized in methanol to a thick slurry. Porphyrins were extracted from the faeces within two weeks of collection.

(4) INSTRUMENTS

A Cary Recording Spectrophotometer Model 14R manufactured by the Applied Physics Corporation was used for recording the visible spectra of porphyrin solutions. Visible porphyrin bands were also measured with a Hartridge Reversion Spectroscope.

Soret bands of porphyrins in acid solution were measured with a Hilger and Watts manual spectrophotometer Model H700.

(5) DETECTION OF PORPHYRINS

The visible absorption spectra and fluorescence emission spectra of porphyrins in acid and neutral solvents were viewed with a hand spectroscope.

Porphyrins on TLC plates were detected by their red fluorescence under ultraviolet light.

(6) PORPHYRIN ESTIMATIONS

Porphyrin solutions were measured spectrophotometrically in a 1 cm cell according to the method of Rimington and Sveinsson (1950) with the modification that readings were taken in 10% HCl at 390 and 425 nm. The porphyrin was calculated according to the formulae:

(a) $$\Delta = E^{\lambda max} - E^{\lambda 390} \times \frac{425 - \lambda max}{35} - E^{\lambda 425} \times \frac{\lambda max - 390}{35}$$

(b) Amount of porphyrin (ug) in volume V (ml) $= \Delta \times 2 \times V$.

The factor of 2 in formula (b) is not a precise value. For example, for solutions of uro the correct factor is 2.05, for copro 2.01 and for proto 2.8 (W.H. Lockwood, personal communication). However, the factor 2.0 is a useful approximation for mixtures of porphyrins and is self-consistent in estimating, for example, porphyrin recoveries.

Porphyrin samples that were not soluble in 10% HCl were measured in 10% HCl:glacial acetic acid (1:1 v/v). A copro sample, diluted in this solution gave a porphyrin estimation 2% higher than one diluted with 10% HCl.

In measuring porphyrin in neutral solvents such as ether or methanol a factor of 4.0 is used since the Soret in neutral solvents is approximately half that of acid solutions.

(7) CONTAMINATION COEFFICIENT

The contamination coefficient E $\frac{\lambda 425}{\lambda \text{max}}$ which has been used throughout this thesis is a measure of the coloured impurities present in the preparations. The absorption of the porphyrin at 425 nm is very low compared to that of the impurities.

(8) ETHER EXTRACTION OF PORPHYRINS AND PORPHYRIN ESTERS

Porphyrins and their esters were extracted with ether from neutralized aqueous solutions (pH 3-4) by the "cascade" method. In this method two separating funnels are used and both contain ether. The aqueous solution is shaken with the ether in the first separating funnel and then transferred to the second separating funnel, shaken and finally discarded. The washings, with water once, with 0.5 M K_2CO_3 once and with water twice, are all performed in the same order so that the second separating funnel effectively recovers most of the small collective losses from the first separating funnel.

(9) ESTERIFICATION OF PORPHYRINS WITH METHANOL-SULPHURIC ACID

Free porphyrins were esterified in methanol-sulphuric (90:5 v/v) at room temperature in the dark for at least 15 hours. Reagents were not dried since With (1971) has shown that up to 20% (v/v) water in the esterification mixture has little effect on the completeness of the reaction for uro.

(10) HYDROLYSIS OF PORPHYRIN ESTERS

Dried porphyrin esters were hydrolyzed by wetting with two drops of glacial acetic acid and adding two drops of concentrated hydrochloric acid. The acid solution (approx. 6 M HCl) was left to stand at room temperature in the dark for at least 15 hours.

After hydrolysis, the mixture was evaporated in a vacuum desiccator. The free porphyrins could be dissolved in electrophoresis buffer (see Electrophoresis section, Materials and Methods) for transfer to electrophoresis paper.

(11) ACETYLATION

The following modification of Barrett's method (1959) was used for detection of porphyrins containing hydroxyl or amino groups.

A solution of pyridine:acetic anhydride (10:1) was added to a dried porphyrin ester sample and the reaction mixture allowed to stand for 15 hours at room temperature in the dark. Three volumes of water were added and ether-soluble porphyrins that had been treated were extracted into ether while water-soluble porphyrins that had been treated were extracted into chloroform. Ether extracts were washed with water and evaporated while the chloroform extracts were evaporated without washing.

The porphyrins after treatment were spotted onto

TLC plates alongside porphyrin controls that had not been treated. After chromatography in a suitable solvent system, an increase in R_F of the acetylated porphyrin indicated the presence of a free hydroxyl or amino group.

(12) THIN LAYER CHROMATOGRAPHY

Blue fluorescing impurities were pushed to the top of the TLC plates by standing in a small volume of methanol for one day. The sheets were activated prior to use by heating for 45 minutes in an oven at 105°C.

Solvents used for developing chromatograms were the following:

(a) Benzene:ethyl acetate:methanol (85:13.5:1.5 v/v) abbreviated BEM throughout this thesis (Doss, 1969).

(b) Benzene:methanol (99:1 v/v) abbreviated BM.

(c) Chloroform:methanol (85:15 v/v) abbreviated CM.

(d) Chloroform.

It must be noted that variable separations occurred with the above solvent mixtures. A test run was normally carried out, to see whether more or less methanol should be added to ensure good separation.

(13) ELECTROPHORESIS

Free porphyrins were electrophoresed in alkali on suspended paper according to the method of Lockwood and

Davies (1962).

Electrophoresis buffer was 0.04 M with respect to K_2CO_3 and 0.004 M with respect to Na_2EDTA.

Whatman 3 MM roll paper, 7 or 10 cm wide, was dipped in the electrophoresis buffer and drained before use. Dried free porphyrins were dissolved in the electrophoresis buffer and transferred to paper with a capillary tube.

The marker used was a mixture of faecal porphyrins containing from 2 to 8 carboxylic acid groups.

The current was kept below 5 milliamps per centimetre width to prevent excessive heating.

(14) CELLULOSE COLUMN PREPARATION

Cellulose powder in water was packed in a glass column and washed in succession with 1 vol of 10% HCl; 0.2 vol. of water; 0.2 vol of saturated sodium acetate and thoroughly with water. The column was then ready for adsorption of porphyrins from aqueous solutions at pH 3-4.

(15) STANDARD WATER-SOLUBLE PORPHYRIN PREPARATION

A thick slurry of faeces in methanol was prepared by homogenization. The slurry was shaken in sealed centrifuge tubes with three volumes of glacial acetic acid and the suspension centrifuged. The supernatant was collected and the extraction repeated with two volumes of glacial acetic acid.

The combined acetic acid extracts were added to four

volumes of ether in a separating funnel. Enough water was added to form two layers on shaking. The aqueous layer containing a dark brown precipitate was collected and three further water extractions were carried out. The ether was kept for extraction of ether-soluble porphyrins as described later.

The combined aqueous extracts were extracted three times with three volumes of ether (total of nine volumes) to remove loosely bound ether-soluble porphyrins.

The aqueous was heated gently with shaking to evaporate dissolved ether and centrifuged. The supernatant and the precipitate both contained a rich supply of water-soluble porphyrins and the purification procedure for these two fractions is given separately.

(a) Treatment of the precipitate

The precipitate was washed once with water then extracted twice with 5 volumes of 10% HCl. The combined extracts were neutralized rapidly with an equal volume of saturated sodium acetate solution.

Five volumes of neutralized extracts were passed through one bed volume of a washed cellulose column (see Cellulose Column Preparation, Materials and Methods). The column was washed well with water to remove most of the brown material and blue fluorescing compounds; the adsorbed porphyrin was eluted with 10% HCl as a compact pink band.

Porphyrin of higher optical purity could be obtained by neutralizing the 10% HCl eluate, applying to another cellulose column and repeating the procedure.

(b) Treatment of the supernatant

To one volume of the supernatant was added one third of a volume of concentrated HCl. The solution was immediately neutralized with an equal volume of saturated sodium acetate.

Water-soluble porphyrin was then prepared from this solution by treatment on a cellulose column in the manner described for the precipitate.

(16) ESTERIFICATION AND CONCENTRATION OF WATER-SOLUBLE PORPHYRIN

One volume of the 10% HCl eluate from the cellulose column containing purified water-soluble porphyrin was added to nine volumes of an esterifying solution of methanol-sulphuric (95:5 v/v).

After standing for at least 15 hours at room temperature in the dark, the solution was neutralized by adding three volumes of dilute sodium acetate in a separating funnel and porphyrin esters were extracted exhaustively into chloroform.

The chloroform and the trace of acetic acid present were removed on a rotary evaporator.

After drying, the water-soluble porphyrin esters were dissolved in small amounts of clean chloroform.

(17) THIN LAYER CHROMATOGRAPHY OF WATER-SOLUBLE PORPHYRIN ESTERS

Water-soluble porphyrin esters were spotted with chloroform onto washed TLC plates (see Thin Layer Chromatography, Materials and Methods). The plates were developed in the CM (85:15 v/v) solvent system alongside markers of ether-soluble porphyrin esters.

Porphyrins were recovered by scraping off the plates and extracting the silica gel three times with methanol. The methanol extracts were passed through filter paper to remove silica gel and the methanol removed with a rotary evaporator.

(18) STANDARD PREPARATION OF ETHER-SOLUBLE PORPHYRINS

The ether-acetic solution remaining after removal of water-soluble porphyrins was extracted four times with a total of one fifth volume of 10% HCl. The extracts were immediately neutralized with saturated sodium acetate and the porphyrins extracted into ether twice in cascade (see Ether Extraction of Porphyrins and Porphyrin Esters, Materials and Methods).

The porphyrins were concentrated by re-extraction into a minimum volume of 10% HCl. After neutralization the porphyrins were returned to a small volume of ether. The ether

was evaporated and the porphyrins esterified with methanol-sulphuric (95:5 v/v). The porphyrin esters were taken into ether and the ether removed by evaporation.

(19) THIN LAYER CHROMATOGRAPHY OF ETHER-SOLUBLE PORPHYRIN ESTERS

The dried ether-soluble porphyrin esters were dissolved in a minimum amount of chloroform and transferred to washed TLC plates alongside standard porphyrin esters. The amount applied was 0.2 - 0.5 mg per cm. The plates were developed in the BEM (85:13.5:1.5 v/v) solvent system until the solvent front had travelled about 17 centimetres.

Three regions of the plates were scraped off and eluted separately. These regions were between proto and copro (P-C); between copro and a brown band at R_F 0.3 including the brown band (C-B) and between the brown band and origin (B-O). Each region was extracted separately by shaking three times with methanol in an Ehrlenmeyer flask. The extracts were filtered through paper and the porphyrin esters extracted by partitioning between water and ether. The ether was washed well with water and evaporated.

The (P-C) region porphyrin esters were separated by TLC against proto and copro ester standards in the BM (99:1 v/v) solvent system. The plates were chromatographed three times, drying the plate between each run. Porphyrin bands were extracted from the plate as before.

The porphyrin esters in the (C-B) and (B-O) regions were separated by TLC using chloroform as solvent. The plates were developed two and three times respectively, drying between each run and the porphyrin bands scraped off the plate and extracted as described previously.

SECTION C

RESULTS

The first stage of the investigation was to isolate and separate the ether- and water-soluble porphyrins from the faeces. The procedures finally adopted are presented in the Materials and Methods.

The preparation of ether-soluble porphyrins presented little difficulty. Porphyrins can be extracted from the faeces with glacial acetic acid and partitioned between water and ether. The ether-soluble porphyrins may then be purified by extracting into mineral acid and by returning to ether. The separation of individual porphyrins after esterification by chromatography has been well studied.

The isolation and separation of the water-soluble porphyrins is more difficult. There is a multiplicity of water-soluble compounds in faeces resulting from degradation of the gut contents by digestive enzymes and bacteria; there are partly degraded insoluble food and bacterial residues; there are also highly coloured compounds of known and unknown nature including bile pigments and the ill-defined bilifuscins. Also the water-soluble and ether-insoluble properties of the water-soluble porphyrins make purification difficult. The following two methods were tried but abandoned.

(1) Preliminary Esterification

Preliminary esterification of the porphyrins by suspension of the faeces in methanol-sulphuric for 15 hours brings most of the porphyrins into solution but leaves a residue containing some porphyrin. After neutralization of the esterification solution, the ether-soluble porphyrin esters were removed with ether; much of the ether-insoluble porphyrin ester remained partly in aqueous solution and partly bound to a brown precipitate.

Attempts to isolate a soluble fraction from the brown precipitate were unfruitful but about 80% of the porphyrin esters in aqueous solution could be extracted into butanol and thence, after addition of petroleum ether, into 10% HCl. The results of a typical experiment are given in Table 2.

TABLE 2

Isolation of ether- and water-soluble porphyrins by preliminary esterification

	Amount (μg/g dry wt)	Contamination Coefficient
Ether-soluble esters	747	0.22
Water-soluble esters	179	0.66
Purified through BuOH followed 10% HCl	142	0.56
Bound to brown ppt.	320	0.54

(2) Extraction with 10% HCl

Faecal suspension (1.3 g) was mixed with 10% HCl and centrifuged. The residue was suspended in a further 4 ml of 10% HCl. The combined extracts were neutralized to pH 3.5 and purified on cellulose as described in the Materials and Methods. The eluate from the cellulose column was neutralized to pH 3.5 and extracted twice with ether to leave the purified water-soluble porphyrin in the aqueous layer. The results are recorded in Table 3.

TABLE 3

Extraction of porphyrin from faecal suspension by extraction with 10% HCl

	Amount (µg/g dry wt)	Contamination Coefficient
Combined 10% HCl extracts	780	0.38
After purification on cellulose (contains ether- and water-soluble porphyrins)	635	0.14
Water-soluble porphyrin remaining in aqueous	152	0.25

(3) Yields and Losses during Water-Soluble Porphyrin Preparation

None of these methods was as successful as glacial acetic acid extraction as outlined in the Materials and Methods for obtaining good supplies of both ether-and water-soluble porphyrins. However, porphyrin is lost at every stage: in the faecal residue after acetic acid extraction; in the precipitate from the acetic acid extracts; during cellulose column purification and during esterification. The yields and losses of each stage were investigated. In the following tables, quantities are given for a typical preparation and alternative methods of purification (for example, different types of columns) are given.

Only the first two acetic acid extracts of the faeces were used. Table 4 gives results of six successive acetic acid extracts from 2.0 g (0.28 g dry wt) of methanol suspension of faeces.

The relative amounts of dicarboxylic, copro and total ether-soluble porphyrin esters of the first and third extracts were 1.0, 1.0 and 0.8 respectively showing little differential extraction of these porphyrins.

After six acetic acid extractions, a considerable amount of porphyrin remained in the residue. As shown by Table 5 further amounts of porphyrin can be extracted.

TABLE 4

Extraction of porphyrin from faeces with glacial acetic acid

Extract No.*	Porphyrin extracted** (µg/g dry wt)	% compared to total extracted
1	990	80.0%
2	180	14.5%
3	41	3.3%
4	15	1.2%
5	7.1	0.6%
6	5.6	0.5%

* 5 ml of glacial acetic acid are added to 2 gm of suspension to give the first extract. The residue after centrifugation is suspended in 7 ml of glacial acetic acid for the remaining 5 extractions.

** Contains a contribution from phylloerythrin.

TABLE 5

Extraction of porphyrin with various reagents from faecal residue after glacial acetic acid extraction

Reagent	First extract*		Second extract	
	Porphyrin (µg/g dry wt)	Contamination coefficient	Porphyrin (µg/g dry wt)	Contamination coefficient
Concentrated HCl	180	0.64	26.8	0.72
10% HCl	58	0.62	10	0.79
1% HCl	58	0.59	-	-
Triton-urea	81	0.59	17	0.62

* 5.5 ml of reagent were added to 2 g of suspension of faecal residue (equivalent to 0.28 g dry wt of original faeces) after six-fold extraction with acetic acid. The mixture was centrifuged and the residue extracted with a further 5 ml of reagent (second extract).

It must remain uncertain whether the value of porphyrin in the concentrated HCl extracts is in fact all due to porphyrin since the full spectrum is obscured with impurities. However, when these extracts are diluted with water to a concentration of 10% HCl, neutralized and purified on cellulose as described in the Materials and Methods, approximately 30% is recovered as purified porphyrin material with an unambiguous spectrum. None of these fractions was investigated further.

When the acetic acid extracts of the faeces were partitioned between ether and water a precipitate formed which collected at the interface. This precipitate contained porphyrin and about half of this amount of porphyrin could be extracted by 10% HCl. Table 6 shows the amount that may be extracted.

As shown in Table 7, further 10% HCl extraction of the precipitate yielded a small amount of porphyrin and the residue still contained considerable amounts of porphyrin.

Purification of the 10% HCl extract was attempted on several adsorbents. Columns of cellulose were prepared (10 cm), Dowex 50 in acetate form (3 cm), Dowex 2 in acid form (3 cm) and alumina (3 cm). Equal amounts of a 10% HCl extract of interface material were neutralized with saturated sodium acetate and placed on the columns. After washing with water, porphyrin was removed with a suitable eluant. The results are recorded in Table 8.

TABLE 6

Extraction of porphyrin from brown precipitate* with various concentrations of HCl

	1st Extract		2nd Extract		3rd Extract	
Reagent	Amount (µg/g dry wt)	Contam. coeff.	Amount (µg/g dry wt)	Contam. coeff.	Amount (µg/g dry wt)	Contam. Coeff.
0.1% HCl	16.0	0.31	10.5	0.29	-	-
1% ** HCl	50.4	0.26	11.5	0.33	-	-
10% HCl	53.3	0.33	11.0	0.37	5.5	0.42

* The precipitate to be extracted contained 138 µg/g dry wt of porphyrin.

** The 1% HCl extracts, although optically clearer than the 10% HCl extracts, were unsatisfactory in the following purification on cellulose columns. For the standard procedure 10% HCl was used and only two extracts.

TABLE 7

Extraction of porphyrin from brown precipitate with 10% HCl to exhaustion and then with methanol-sulphuric.

Operation	Amount (µg/g dry wt of porphyrin)	Contamination coefficient
1st and 2nd 10% HCl extract	64	0.33
3rd, 4th and 5th 10% HCl extract	11.1	0.57
6th 10% HCl extract	1.7	0.53
Residue in methanol-sulphuric (1,2)	75.7	0.60
Ether-soluble porphyrin from methanol-sulphuric	8.3	0.49

(1) Porphyrin was measured by diluting the methanol-sulphuric with acetic acid-HCl (50% acetic acid - 5% HCl)

(2) A small amount of material remained insoluble.

TABLE 8

Column purification of 10% HCl extract of brown precipitate.

Adsorbents	Amount Applied (µg)	Amount Not retained (µg)	Eluant	Eluted Porphyrin (µg)	% Recovery	Contam Coeff.
Cellulose	11.8	0.76	10% HCl	7.4	63%	0.20
Alumina	11.8	2.07	10% HCl	6.2	53%	0.26
Dowex 2	11.8	4.2	10% HCl	2.92	25%	0.20
Dowex 50	11.8	3.5	2N NaOH	trace	-	-

Cellulose column treatment of the 10% HCl extracts was chosen.

In the isolation and purification of the ether-soluble porphyrin esters as set out in the Materials and Methods, there was a loss of 60 µg/g dry wt; 30µg/g dry wt of this was bound to a brown precipitate which formed during the extraction procedure.

A summary of the more significant yields and losses in large scale preparation of the water-soluble fractions is shown in Table 9.

TABLE 9

Yields and losses in preparing the porphyrin fractions

	Yield (µg/g dry wt)	Contam. coeff.	Loss (µg/g dry wt)
Extraction from faeces			
2 x glacial acetic extraction:	1170	0.50	
Not extracted with 2 x glacial acetic extractions (extracts 3 to 6)			69
Remaining as residue			207
Ether-Water partition			
Water-soluble porphyrins in brown precipitate	138	0.45	
Water-soluble porphyrins in supernatant	54	0.58	
HCl extract from the ether layer	639	0.29	
Re-partition of ether-soluble porphyrins			
Porphyrin which would not return into ether			155
HCl extract of porphyrin which returned to ether	379	0.25	
Extraction of water-soluble porphyrins from brown precipitate			
2 x 10% HCl extracts	64	0.33	
Not extracted by 2 x 10% HCl extracts (3 to 6)			13
Porphyrin not extractable by 10% HCl			76
Cellulose column purification			
Water-soluble porphyrin in brown precipitate	56	0.20	8
Water-soluble porphyrin in supernatant	15	0.20	39
TOTAL PURIFIED WATER-SOLUBLE PORPHYRIN	71	0.20	47
Esterification	60		11
TLC	51		9

(4) ETHER-SOLUBLE PORPHYRINS

(4.1) Thin Layer Chromatography

The ether-soluble porphyrin esters were chromatographed on TLC in the BEM solvent system alongside markers of proto, copro and haemato esters. A pink band slowly changing to brown moved near the front followed by a pink band. From the origin up to these bands, the chromatogram was light brown in colour.

Under ultraviolet light, the chromatogram showed a continuous fluorescence throughout its length which was more intense towards the origin. Against this background of continuous fluorescence, at least ten red fluorescent bands could be distinguished; three of these corresponded to the three porphyrin markers. The R_F values for these red fluorescent bands when the porphyrin esters have been applied at 2 µg/cm are given in Table 10.

The ether-soluble porphyrin esters from J.M.'s faeces were compared with those from another patient with variegate porphyria (G.J.) and two different samples of normal faeces. The samples were applied in strips, approximately 2 µg of porphyrin per centimetre, and run in the BEM solvent system. All of the above bands could be seen in the sample from J.M.'s and G.J.'s faeces. The porphyrin esters from the normal faeces showed red fluorescent bands corresponding with proto and copro but little red fluorescence in other parts of the chromatogram.

TABLE 10

R_F values of ether-soluble prophyrin esters.

Red fluorescent Band	R_F value *
Proto marker	0.92
Copro marker	0.75
Haemato marker	0.11 (main band)
Band 1	0.92 (proto)
" 2 **	0.87
" 2' **	0.81
" 3	0.75 (copro)
" 4	0.65 (penta)
" 5	0.59
" 6	0.53
" 7	0.45
" 8	0.40
" 9	0.32
" 10	0.11 (haemato)
" 11	0.05
" 12	Origin

* R_F values vary with room temperature, humidity and loading

** On the preparative plates bands 2 and 2' cannot be seen as separate bands

The porphyrin from the region between proto and copro in the chromatogram of J.M.'s ether-soluble esters was eluted and rerun in the BM solvent system as described in TLC of Ether-Soluble Porphyrin Esters, Materials and Methods. Six major red fluorescent bands were observed under ultraviolet light. The first and sixth bands are proto and copro respectively and represent contaminations from bands 1 and 3 of the BEM chromatogram and have been named for identification 1 and 3. The intermediate porphyrins which arise apparently from bands 2 and 2' of the BEM chromatogram have been named 2a, 2b, 2c and 2d and the R_F values are given in Table 11.

Each of these porphyrin bands was eluted separately and rerun again in the BM solvent system as described in TLC of Ether-Soluble Porphyrin Esters, Materials and Methods. In the purification of each separate fraction no attempt was made to recover the small amounts of porphyrin from the neighbouring bands.

The porphyrin from the region between and including bands 4 and 7 in the original BEM chromatogram was eluted and run in chloroform as described in the Materials and Methods. The chromatogram showed four major red fluorescent bands with R_F values given in Table 12.

TABLE 11

R_F values on BM solvent system of ether-soluble porphyrin esters migrating between proto and copro on BEM solvent system.

Red Fluorescent Band	R_F* value
Band 1 (proto)	0.9
" 2a	0.7
" 2b	0.55
" 2c	0.45
" 2d	0.3
" 3 (copro)	0.2

* Approximate only since there is much variation

TABLE 12

R_F values in chloroform system of ether-soluble porphyrin esters migrating between copro and band 8 in BEM system.

Red Fluorescent Band	R_F* value
Band 4 (penta)	0.7
" 5	0.6
" 6	0.5
" 7	0.35

* Approximate only since there is much variation

Each band was eluted separately and run again in chloroform and this process repeated. On the first chloroform chromatogram, there was sufficient porphyrin in the neighbouring bands to justify their recovery.

The porphyrin from the region between band 7 and the origin in the original BEM chromatogram was eluted and rerun in chloroform as described in the Materials and Methods. The chromatograms showed four major red fluorescent bands with R_F values given in Table 13.

TABLE 13

R_F values in chloroform system of ether-soluble porphyrin esters migrating between band 7 and origin on BEM system.

Red Fluorescent Band	R_F^* value
Band 8	0.6
" 9	0.45
" 10	0.3
" 11	0.2

* Approximate only since there is much variation

Each band was eluted separately and rerun on TLC with chloroform. Small samples of all twelve fractions isolated were tested in the BEM and also the second solvent system (either BM or chloroform) to ensure that the fractions were not artefacts of chromatography. In all test runs, the purif-

ied fractions gave a main spot with traces of impurities and, as stated above for fractions 2a to 2d, the position of the spot was the same, allowing for differences of loading, as the original position of the band in the preparative plates.

It is difficult to estimate the amount of each fraction present in the original mixture of esters of ether-soluble porphyrins. Measurements of porphyrins eluted from the original BEM solvent plate show that approximately 190µg/g dry wt are present in the proto and copro bands combined. The combined porphyrins in the remaining area represented 130µg/g dry wt. The proto and copro fractions have not been further studied since they are well established constituents of variegate porphyria faeces. The proto band probably contains small amounts of at least fraction 2a and almost certainly small amounts of meso and deutero; meso and deutero are normal constituents of faeces. The spectrum of the proto band in chloroform (band I, 631 nm; authentic proto ester, 631 nm) and in 10% HCl (Soret 410 nm; authentic proto ester, 411 nm) show that it is mainly proto. Likewise the copro band almost certainly contains fractions 2d, 4 and probably others. The original BEM chromatogram showed continuous fluorescence and this is true of each of the three subpurifications. In all subpurifications, minor satellite bands were present and probably most of these, represent porphyrins different from any of the twelve isolated. Conservative judgement, however, would be that at least half of the 130 µg/g dry wt of the preparative BEM plate was due to the 12 porphyrin fractions. Examination by hand spectroscope

throughout the preparation suggested that the twelve fractions were each present in roughly the same amounts, namely approximately 6 µg/g dry wt for each one. Allowing for the uncertainties in this judgement, it is still reasonable to claim that there are from 4 to 20 µg/g dry wt for each fraction present in the original ester mixture. This is consistent, given the considerable losses during the preparation, with the approximate yields given in Table 14 of those fractions which were further investigated.

TABLE 14

Yields of individual fractions

Porphyrin	Amount (mg)	Approx. Yield (ug/g dry wt)
2 b	0.5	5
2 c	0.4	5
2 d	1.4	15
5*	0.1	-
6*	0.1	-
7*	0.4	-

* Fractions 5, 6 and 7 represented pooling from several experiments and no yield can be given.

(4.2) Acetylation

The 12 porphyrin fractions were submitted to acetylation (see Materials and Methods). They were then compared with the original porphyrins on the BEM solvent system. Acetylation of fractions 5, 6, 7, 8, 9, 10 and 11 increased their mobility on TLC. This is evidence that all of these seven fractions possess one or more hydroxyl groups. The mobility of fractions 2a, 2c, 2d and 3 was unchanged after the acetylation procedure.

Fraction 10 and authentic haemato ester moved to the same position on TLC in the BEM solvent system and their acetylated derivatives showed the same increase in mobility. Comparison by Chu and Chu chromatography (1954) of fraction 10 with authentic haemato ester confirmed its identification as haemato ester.

(4.3) Electrophoresis

Samples of all 12 fractions were hydrolyzed. They were compared on paper electrophoresis in alkali (see Materials and Methods) with a mixture of porphyrin markers including porphyrin dicarboxylic acids, copro and penta.

On electrophoresis, porphyrins with not more than three carboxyl groups show hardly any movement. Those with four or more carboxyl groups move towards the anode at a rate depending on the number of carboxyl groups. Fraction 3 behaved as copro and fraction 4 as penta confirming the identification of fractions 3 and 4 as copro and penta respectively.

All the other fractions and the dicarboxylic acid markers remained close to the origin. This is evidence that none (except fractions 3 and 4) has more than 3 carboxylic acid groups.

(4.4) Visible Spectra

Visible spectra on fractions 2b, 2c, 2d, 5, 6 and 7 in chloroform were measured and are given in Table 15 at the end of this section. Fractions 5 and 6 still contained considerable amounts of coloured impurities evident from the flattening of the curve. However, the spectra still appeared to be of aetio-type. The other porphyrin fractions were much cleaner from the visible spectra and possessed obvious aetio-type spectra.

(4.5) Mass Spectrometry

Porphyrin fractions 2b, 2c and 2d were submitted for mass spectrum analysis. Since porphyrins 5, 6 and 7 appeared to be hydroxy compounds, silyl derivatives were prepared for mass spectrum analysis. The silyl compounds were purified on TLC in the BEM solvent system; they all migrated with an R_F of 0.95 being faster than proto. The nature of the visible spectrum after silylation and purification remained essentially unchanged. The spectra of the silyl derivatives is given in Table 15 at the end of this section.

The mass spectrum of fraction 2b showed two major peaks at 652 and 624 attributed to porphyrins. This was

confirmed by the accompanying corresponding peaks of their copper complexes. In all the mass spectra discussed, peaks attributed to porphyrins were accompanied by peaks corresponding to the copper complexes. The peak at 624 is highly unlikely to be a daughter peak of that at 652 since the loss of ethylene occurs only in certain structures which could not be envisaged as part of these porphyrins. There was evidence for three propionic acid side chains in each of the parent compounds. The mass spectrum is interpreted as demonstration of the presence of the trimethyl esters of propionic acid deutero and ethyl propionic acid deutero. These two compounds would be unlikely to separate well on TLC since the analogues meso and deutero esters separate poorly in the same system.

The mass spectrum of fraction 2c showed two major peaks of similar heights with m/e values of 682 and 650 separated by the weight of a methanol molecule. The peak at 682 has been interpreted as the parent peak of 2c. On the basis of a full study of the results of the mass spectrum of fraction 2c, a constitution of methoxyethyl propionic acid deutero ester is proposed.

The mass spectrum of 2d showed three major peaks approximately equal in height with m/e values of 654, 622 and 590. The identification of the peak at 654 as the parent was confirmed by the presence of a metastable peak

corresponding to the transition of 654 to 622 and by comparison with a spectrum of authentic tetramethyl haemato. The loss of 32 between major peaks is attributed to the loss of methanol to give successively monomethoxyethyl monovinyl deutero ester, 622, and proto ester, 590.

The mass spectrum of the silyl derivative of fraction 6 showed peaks attributed to porphyrins at 712, 696, 682, 606 and 592. The spectrum was interpreted as that of a mixture of three compounds with parent peaks at 712, 696, and 682 and daughter peaks at 622, 606 and 592 formed by the loss of silyl groups. The parent peaks at 712 and 682 were attributed to the silyl derivatives of methoxyethyl hydroxyethyl deutero ester and ethyl hydroxyethyl deutero ester respectively. No convincing structure could be attributed to the compound with a parent peak at 696.

The mass spectrum of fraction 7 also appeared to be that of a mixture. It had parent peaks at 682 and 680 with daughter peaks at 592 and 590. The peak at 682 was attributed to the silyl derivative of ethyl hydroxyethyl deutero ester and the peak at 680 to the silyl derivative of vinyl hydroxyethyl deutero ester. The presence of ethyl hydroxyethyl deutero ester both in fraction 6 and fraction 7 is not unexpected. There was also present on the mass spectrum a smaller peak at 564 which is thought to be the daughter peak from the silyl derivative of hydroxyethyl deutero ester. The parent would have been at 654. This is in the centre of the cluster

attributed to the copper complexes with peaks at 651, 653, 655 corresponding to the porphyrins with peaks at 592 and 590.

The silyl derivative of fraction 5 was submitted for mass spectrometry but no satisfactory spectrum was obtained.

A full description of the mass spectral results for fractions 2b, 2c, 2d, 6 and 7 will be published separately in collaboration with Professor Robertson.

A summary of the ether-soluble porphyrin esters is given in Table 15 and their proposed structures are given in Table 16.

(5) WATER SOLUBLE PORPHYRINS

(5.1) Thin Layer Chromatography

The water-soluble porphyrin esters prepared as described in Materials and Methods were chromatographed on TLC in the CM solvent system and compared with the ether-soluble porphyrin esters on the same plate. Much of the water-soluble esters migrated with the front as did the ether-soluble markers; another significant fraction remained at the origin. There were, however, two distinct porphyrin bands with R_F values 0.2 and 0.3 (called fractions A and B respectively).

Eluting sections of the chromatogram and testing for ether-solubility, showed that the front was composed of ether-

TABLE 15

Summary of ether-soluble porphyrin esters

Porphyrin	Paper Electro-phoresis	Effect of acetylation	Visible maxima in $CHCl_3$				Parent Peak	Identification
			I	II	III	IV		
Authentic proto ester	No migration	-	631	577	542	508	590	
Authentic copro ester	Migrates to anode	-	621	568	532	499	710	
Authentic haemato ester	No migration	R_F increased	-	-	-	-	-	
Authentic tetramethyl haemato	No migration	R_F unchanged	623	570	535	500	654	
Authentic silyl derivative haemato ester	-	-	623	570	535	500	770	
Fraction 2a	No migration	R_F unchanged	-	-	-	-	-	
Fraction 2b	No migration	R_F unchanged	-	-	-	-	652	ethyl propionic acid deutero ester
							624	propionic acid deutero ester
Fraction 2c	No migration	R_F unchanged	622	571	537	503	682	methoxyethyl propionic acid deutero ester
Fraction 2d	No migration	R_F unchanged	623	570	535	500	654	tetramethyl haemato

CONTINUED OVERLEAF

TABLE 15 (Continued)

Fraction 3	Same as copro	R_F unchanged	-	-	-	-	-	copro ester
Fraction 4	same as penta	R_F unchanged	-	-	-	-	-	penta ester
Fraction 5	No migration	R_F increased	628	573	539	505	-	-
Fraction 5-silyl derivative	-	-	627	572	539	504	-	Not identified
Fraction 6	No migration	R_F increased	627	574	540	504	-	-
Fraction 6-silyl derivative	-	-	627	572	540	504	712	silyl derivative of methoxyethyl hydroxyethyl deutero ester
							696	not identified
							682	silyl derivative of ethyl hydroxyethyl deutero ester
Fraction 7	No migration	R_F increased	627	572	539	503	-	-
Fraction 7-silyl derivative	-	-	626	572	539	503	682	silyl derivative of ethyl hydroxyethyl deutero ester
							680	silyl derivative of vinyl hydroxyethyl deutero ester
							(654)*	silyl derivative of hydroxyethyl deutero ester
Fraction 8	No migration	R_F increased	-	-	-	-	-	-

CONTINUED OVERLEAF

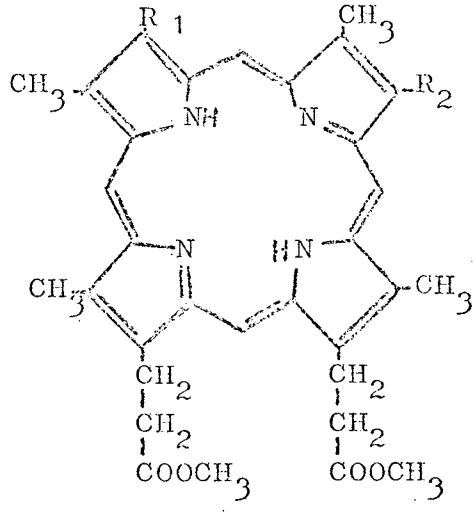
* Postulated from daughter peak at 564

TABLE 15 (Continued)

Fraction 9	No migration	R_F increased	-	-	-	-	-	-
Fraction 10	No migration	R_F increased	-	-	-	-	-	haemato ester
Fraction 11	No migration	R_F increased	-	-	-	-	-	-

TABLE 16

Proposed structures of ether-soluble porphyrins



The proposed structures can all be regarded as 2,4-substituted deutero IX methyl esters. No evidence has been presented to distinguish between substitution in positions 2 and 4.

CONTINUED OVERLEAF

TABLE 16 (continued)

FRACTION	R_1 (2)	R_2 (1)	NAME
2b	$-CH_2-CH_3$	$-CH_2-CH_2-COOH$	ethyl propionic acid deutero methyl ester
	H	$-CH_2-CH_2-COOH$	propionic acid deutero methyl ester
2c	$-CH(OCH_3)-CH_3$	$-CH_2-CH_2-COOH$	methoxyethyl propionic acid deutero methyl ester
2d	$-CH(OCH_3)-CH_3$	$-CH(OCH_3)-CH_3$	bis (methoxyethyl) deutero methyl ester (tetramethyl haemato)
6	$-CH(OCH_3)-CH_3$	$-CH(OH)-CH_3$	methoxyethyl hydroxyethyl deutero methyl ester
7	$-CH=CH_2$	$-CH(OH)-CH_3$	vinyl hydroxyethyl deutero methyl ester
	$-CH_2-CH_3$	$-CH(OH)-CH_3$	ethyl hydroxyethyl deutero methyl ester
	H	$-CH(OH)-CH_3$	hydroxyethyl deutero methyl ester
11	$-CH(OH)-CH_3$	$-CH(OH)-CH_3$	bis (hydroxyethyl) deutero methyl ester (haemato methyl ester)

soluble porphyrins while fractions A and B and the origin material were predominantly water-soluble. The amounts of porphyrin in each section of the chromatogram were measured and the results are shown in Table 17.

TABLE 17

CM chromatography of water-soluble porphyrin esters.

Fraction	% Porphyrin
Front	50
Band B	5
Band A	5
Origin	40

The material at the origin when eluted and run again, gave a substantially identical development, namely porphyrin at the front, bands A and B and origin material. It thus appears that bands A and B each comprise about 10% of the water-soluble ester preparation.

The porphyrins of fractions A and B were purified by re-running on TLC and about 150 µg of each fraction were prepared. When tested by TLC, fraction A still contained appreciable amounts of impurities while fraction B was seen to be much more homogeneous.

(5.2) Properties of Fractions A and B.

The dried esters of fraction A and fraction B dissolved with difficulty in water but were readily taken up in alkali. Esters of porphyrins such as copro and proto are not soluble in alkali.

On paper electrophoresis in alkali, fraction B, which is an ester, showed no significant movement but paper electrophoresis is not suitable for porphyrins with one, two or three carboxylic acid groups. On electrophoresis in polyacrylamide gel (Ornstein, L., 1964) in alkali (4% cross linked gel -pH 8.3 tris buffer; tanks-electrophoresis buffer, Materials and Methods) fraction B ester migrated at half the rate of free proto. Copro ester applied as a control failed to move.

Samples of fractions A and B were hydrolysed by the standard method. There was no movement of the hydrolysed porphyrins on TLC in the CM solvent system consistent with the presence of propionic acid groups unmasked by the hydrolysis. Hydrolysed fractions A and B were electrophoresed on paper in alkali with markers of proto and copro: fraction B migrated at the same rate as copro suggesting four acid functions in the molecule; fraction A showed a diffuse movement between proto and copro.

Fractions A and B were hydrolysed in 10% HCl in the dark both at 80°C and at room temperature. After drying, the

porphyrins were re-esterified in methanol-sulphuric and taken into chloroform. TLC compared with untreated samples showed no alteration by hydrolysis at room temperature. In the samples hydrolysed at 80°C some ether-soluble porphyrins had been liberated but the main fractions were unchanged.

Attempts at acetylation (Barrett, 1959) and silylation (Elder, 1972) of fractions A and B failed; the reacted products moved with the same mobility on TLC as the untreated porphyrins.

Visible spectra measured in chloroform of both fractions A and B were of aetio-type although the spectra showed appreciable amounts of coloured impurities in the preparations. The positions of the maxima were the same for each fraction being:-

Band I	634 nm
Band II	580 nm
Band III	548 nm
Band IV	514 nm

Samples of fractions A and B were treated with diazo-methane prepared according to Lipsky and Landowne (1963) and the major part of both fractions A and B became ether-soluble as witnessed by inspection under ultraviolet light. The action of diazo-methane on fraction B was studied quantitatively. Equal amounts of fraction B were evaporated in two

tubes. Methanol-glacial acetic acid (0.1 ml; 49:1 v/v) was added to each tube. To one tube an ether solution of diazo-methane was added until the yellow colour persisted (approximately 2 ml) and to the control tube the same volume of ether was added. Ether (6 ml) was added to each tube and the preparations washed with M acetic acid (8 ml) and water (8 ml). The ether layers were measured as such and the aqueous and water washings were measured after acidification with HCl. The results are given in Table 18.

TABLE 18

Effect of diazo-methane on fraction B of water-soluble porphyrin ester.

	Amount in ether (µg)	Amount in Aqueous and Washings (µg)
Not treated with diazo-methane	2	8
Treated with diazo-methane	10	2

About 75 µg of fractions A and B were then treated with diazo-methane. The esters were washed with M acetic acid, 0.5 M K_2CO_3 and water and purified by TLC on the BEM solvent system. Fraction B migrated close to tetramethyl haemato ester (R_F value 0.77) and fraction A close to hepa

ester (R_F value 0.3). About 30 µg of each of the diazomethane ester fractions were available for mass spectrometry.

The mass spectrum of fraction A treated with diazomethane showed peaks which could be attributed to the impurities from the TLC plates but no peaks which could be attributed to porphyrins. Fraction B treated with diazo-methane showed a peak with m/e value of 564 in addition to the other peaks attributed to impurities from the TLC plates. The peak at 564 could be due to vinyl deutero ester arising from a parent compound.

(5.3) Labile Water-Soluble Porphyrins

The ether-soluble porphyrin arising from the water-soluble porphyrin esters and migrating to the front in the CM solvent system were eluted and a sample run on TLC in the BEM solvent system. The chromatogram, except for the much reduced amounts of copro and proto, appeared the same as that given by the ether-soluble porphyrin esters.

Some further experiments were carried out on the water-soluble porphyrin (unesterified) from the initial cellulose column eluate. Treatment of this fraction with mineral acid releases substantial amounts of ether-soluble porphyrins. Trace amounts of ether-soluble porphyrins were first removed from a water-soluble preparation and the water-soluble porphyrins remaining were concentrated by adsorption

on cellulose and elution with 6N HCl. The acid eluate was heated at 80°C for 18 hours. The ether-soluble porphyrins liberated were removed and measured and the water-soluble porphyrin remaining was concentrated as described above. The heating was repeated for longer time intervals. Table 19 shows the amount of ether-soluble porphyrins released.

TABLE 19

Release of ether-soluble porphyrin by hydrolysis of water-soluble porphyrin in 6N HCl at 80°C.

Time	% ether-soluble porphyrins released
18 hours	30
36 hours	40
54 hours	43
120 hours	46

The amount of ether-soluble porphyrins released from the free water-soluble porphyrin preparation is about the same as the amount released on TLC from the total water-soluble porphyrin ester fraction. The ether-soluble porphyrins released under acid hydrolysis when esterified and run on TLC in the BEM solvent system appeared the same, except for the much reduced amount of copro and proto, as the ether-soluble porphyrin esters, but there was insufficient for further investigations.

(5.4) Acid Electrophoresis of Unesterified Material

The free water-soluble porphyrins prepared from the cellulose column were submitted to paper electrophoresis in 0.015 M H_2SO_4. Movement is slow in acid but they moved at the same rate as the marker (mixed porphyrins with from two to eight carboxyl groups), that is, towards the cathode as a compact band. This suggested the absence of a free amino group on porphyrins in this fraction since faster migration would be expected.

SECTION D

DISCUSSION

Comparison of the ether-soluble faecal porphyrins from two variegate porphyria patients with those of toxic porphyria and normal faeces showed in variegate porphyria faeces the presence in appreciable amounts of at least nine porphyrins which were absent from the other faeces.

It must be remembered that the faecal porphyrins are not necessarily those which would be found at the site of formation. They have been subjected to enzymic and putrefactive processes of passage through the gut. Also, all have been treated with mineral acid in the isolation procedure.

All naturally occurring dicarboxylic porphyrins that have been reported by other workers contain the deutero IX structure. In considering the porphyrins isolated in the present work, it seems reasonable to assume that these also are derivatives of deutero IX. Other position isomers, however, would not have been distinguished by the experiments performed in the present work.

Of the ether-soluble porphyrins identified with mass spectrometry there is a presumption of gut alteration in four: the ethyl groups of ethyl hydroxyethyl deutero and ethyl propionic acid deutero and the H groups of hydroxyethyl deutero

and propionic acid deutero can be presumed to arise from vinyl groups. The reduction of vinyl to ethyl is not known in animal biosynthetic pathways but is well established in bacteria. For example, the vinyl groups of bilirubin are known to be reduced by gut bacteria to the ethyl groups of stercobilin. Similarly, vinyl side chains are replaced by hydrogen in the formation of deutero by bacterial degredation (Fischer and Orth, 1937, p. 413).

The likely parent compound of ethyl propionic acid deutero is vinyl propionic acid deutero. Now harderogen is 2-vinyl 4-propionic acid deuterogen and acts as a substrate for the Euglena coprogenase system while the 4-vinyl 2-propionic acid deuterogen compound does not. Hence, the vinyl propionic acid deutero could arise from the intermediate harderogen or it could arise from the faulty action of coprogenase on the 4 position of coprogen giving a compound which cannot be utilized further and would thus accumulate.

The likely parent compound of both ethyl hydroxyethyl deutero and hydroxyethyl deutero is vinyl hydroxyethyl deutero.

The significance of all porphyrins isolated with hydroxyethyl and methoxyethyl side chains is obscure. Elder (1972) has isolated hydroxyisocopro from the faeces of a patient with toxic porphyria but otherwise porphyrins containing hydroxyethyl groups have only rarely been reported in faeces. The fact that haemato is not reported in normal faeces speaks

against the formation of hydroxyethyl groups by gut action on vinyl groups.

It is interesting here that in the porphyrins isolated with a potential hydroxyethyl group, several were present with methoxyethyl groups. The methoxyethyl group may be present on the porphyrins in the faeces although this seems unlikely. They could be formed from hydroxyethyl compounds during esterification with methanol-sulphuric but it has been shown that haemato treated similarly gives no significant formation of tetramethyl haemato. They could be formed by the action of methanol-sulphuric on proto although this has not been reported. Thus, it seems worth considering that they arise from a 1-substituted ethyl compound present in the faeces.

If the methoxyethyl groups arise from such a compound it suggests that hydroxyethyl groups could also arise in the same way. This postulate would agree with the findings of Rimington et al. (1968) who obtained release of haemato after treating faecal water-soluble porphyrin with silver salts. It would also agree with the work of Grosser and Eales (1971) who found release of haemato from a urinary water-soluble porphyrin after very mild manipulation. It is useless, however, to speculate on the type of binding or the nature of the conjugating group at this stage or whether these compounds are formed in the gut. Nor is there any evidence to decide whether free hydroxyethyl porphyrins are already present before the

isolation procedure.

Neither - $CHOHCH_3$ nor -$CHXCH_3$ side chains (where X is an unknown group) are present in intermediates in the conversion of coprogen to protogen by the coprogenase enzyme system according to the isotope studies but the presence of -$CHXCH_2COOH$ as a side chain of an intermediate is consistent with the isotope evidence. The hydroxyethyl compounds could arise from an intermediate with a -$CHXCH_2COOH$ group by a non-oxidative decarboxylation instead of the normal oxidative decarboxylation.

At least two types of porphyrins have been demonstrated in the water-soluble fraction; two porphyrins which are quite stable and another fraction which readily releases ether-soluble porphyrins during esterification and during chromatography of the esters.

Both the stable water-soluble porphyrins on treatment with methanol-sulphuric give esters which are water-soluble and still more readily soluble in alkali. One of the esters has been shown to migrate to the anode on polyacrylamide gel electrophoresis in alkali. The esters can be further esterified with diazo-methane to give ether-soluble compounds.

These properties suggest a strong acid function. In view of the evidence for thioether-linked porphyrin-peptides in the bile, the formation of sulphonic acid groups in the gut seems possible. A porphyrin with one or two sulphonic

acid groups and two carboxyl groups should have about the same electrophoretic mobility in alkali as copro. The propionic acid groups but not the sulphonic acid groups would be esterified with methanol-sulphuric. Diazo-methane would esterify the sulphonic acid groups; also a sulphonic acid group might well be stable tc acid hydrolysis.

The visible spectra in chloroform of the water-soluble porphyrins are both aetio-type and are consistent with a sulphonic acid group on a side chain of the porphyrins.

The porphyrin peak with m/e valuc of 564 recorded in the mass spectrum of one of the water-soluble porphyrins after reaction with diazo-methane, may be due to vinyl deutero ester. Vinyl deutero ester might be a daughter peak of a porphyrin with a sulphonic ester group on an ethyl side chain with the structure given in Figure 19.

The nature of the labile water-soluble porphyrin fraction is more difficult to explain. The fact that an ester fraction, which is quite water-soluble, gives large amounts of ether-soluble porphyrins after chromatography in an inert solvent system is strong evidence that the water-solubility is due to simple adsorption. From this it seems natural to assume that the water-solubility of the free porphyrins is also due to simple adsorption. This is consistent with the liberation of haemato from a urinary water-soluble porphyrin fraction on passing through a Dowex 50 column as

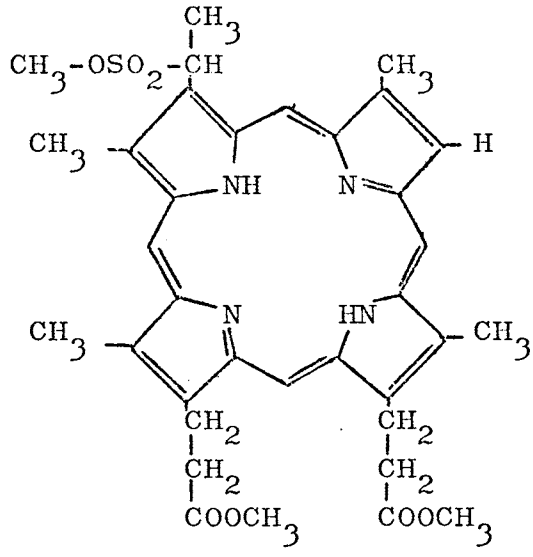


Figure 19. Proposed structure of stable water-soluble porphyrin ester

reported by Grosser and Eales (1971). However, the almost complete absence of copro and proto from the liberated ether-soluble porphyrins is puzzling. The suggestion should be considered that, while the esters of the labile fraction consist of ether-soluble porphyrins adsorbed to water-soluble material, the free porphyrins of the labile water-soluble fraction contain porphyrins conjugated to a water-soluble group by a weak covalent bond. This could be a 1-substituted ethyl compound as postulated in the discussion on the ether-soluble porphyrins.

Rimington et al. (1968) concluded that water-soluble porphyrin in faeces is a group of porphyrin-peptides containing

thioether linkages. This can no longer be regarded as a satisfactory description from present evidence.

Firstly, the present work has presented evidence for two stable water-soluble porphyrins which could not be thioethers, nor could they be released from thioethers during isolation. Secondly, the major part of the water-soluble porphyrin investigated is a labile porphyrin and there is good evidence that much of this is ether-soluble porphyrin adsorbed onto water-soluble compounds, although weak covalent bonds cannot be excluded.

Admittedly some half of the water-soluble fraction has not been studied in this investigation. The stable thioether peptides postulated by Rimington et al. (1968) may well occur in this fraction.

In conclusion, several ether-soluble porphyrins have been isolated and identified and shown to be peculiar to variegate porphyria. They are remarkable in having unusual substituents in the 2 and 4 positions of the porphyrin ring. Except for one unidentified porphyrin detected by mass spectrometry they can all be explained as derivatives of intermediates of the coprogenase system. The water-soluble porphyrins that have been studied have not been identified but what evidence there is suggests that they could also be derived from intermediates in the coprogenase system.

Two possibilities are suggested; that in variegate

porphyria the liver coprogenase system is faulty or that the fault lies elsewhere in the pathway and overloads the coprogenase system.

At least three avenues need further investigation. Firstly the isomeric structure of haemato and other ether-soluble porphyrins should be investigated. Secondly, the nature of the water-soluble porphyrin fraction requires elucidation and thirdly there is a need to study the isolated coprogenase system in relation to variegate porphyria.

REFERENCES

Barrett, J. (1959). Nature 183, 1185.

Batlle, A.M. del C. and Grinstein, M. (1962a). Biochim. Biophys. Acta 57, 191-194.

Batlle, A.M. del C. and Grinstein, M. (1962b). Biochim. Biophys. Acta 62, 197-200.

Batlle, A.M. del C. and Grinstein, M. (1964a). Biochim. Biophys. Acta 82, 1-12.

Batlle, A.M. del C. and Grinstein, M. (1964b). Biochim. Biophys. Acta 82, 13-20.

Batlle, A.M. del C., Benson, A. and Rimington, C. (1965). Biochem. J. 97, 731-740.

Battersby, A.R., Baldas, J., Collins, J., Grayson, D.H., James, K.J. and McDonald, E. (1972). J. Chem. Commun. 1265-1266.

Belcher, R.V., Smith, S.G. and Mahler, R. (1969). Clin. Chim. Acta 25, 45-52.

Bogorad, L. and Granick, S. (1953). J. Biol. Chem. 202, 793-800.

Bogorad, L. (1958a). J. Biol. Chem. 233, 501-515.

Bogorad, L. (1958b). J. Biol. Chem. 233, 516-519.

Brown, E.G. (1958). Biochem. J. 70, 313-321.

Burnham, B.F. and Lascelles, J. (1963). Biochem. J. 87, 462-472.

Carell, E.F. and Kahn, J.S. (1964). Arch. Biochem. Biophys. 108, 1-6.

Cavaleiro, J.A.S., Kenner, G.W. and Smith, K.M. (1973). J.C.S. Chem. Commun. 183-184.

Chu, T.C., Green, A.A. and Chu, E.J.-H. (1951). J. Biol. Chem. 190, 643-646.

Chu, T.C. and Chu, E.J.-H. (1954). J. Biol. Chem. 208, 537-541.

Cornford, P. (1964). Biochem. J. 91, 64-73.

Dean, G. (1963). The Porphyrias, London:Pitman.

Doss, M. (1969). Hoppe-Seyler's Z. Physiol. Chem. 350, 499-502.

Elder, G.H. (1972). Biochem. J. 126, 877-891.

Fischer, H. and Orth, H. Die Chemie des Pyrrols, Volume II, first half, p. 413, Akad. Verlagsgesellschaft, Leipzig (1937).

French, J., Nicholson, D.C. and Rimington, C. (1970). Biochem. J. 120, 393-397.

Gajdos, A. and Gajdos-Török, M. (1969). Porphyrines et Porphyries. Paris:Masson et Cie.

Garcia, R.C., San Martin de Viale, L.C., Tomio, J.M. and Grinstein, M. (1973). Biochim. Biophys. Acta 309, 203-210.

Gibson, K.D., Laver, W.G. and Neuberger, A. (1958). Biochem. J. 70, 71-81.

Goldberg, A., Rimington, C. and Lochhead, A.C. (1967). Lancet 1, 632-636.

Goreczky, V.L., Roth, I. and Breckner, M. (1968). Z. klin. u. klin. Biochem. 6, 489-492.

Granick, S. and Bogorad, L. (1953). J. Am. Chem. Soc. 75, 3610.

Granick, S. and Mauzerall, D. (1958). J. Biol. Chem. 232, 1119-1140.

Granick, S., Sparatore, F., Sano, S. and Mauzerall, D. (1960). Unpublished observations.

Granick, S. (1966). J. Biol. Chem. 241, (6), 1360-1375.

Gray, C.H., Rimington, C. and Thomson, S. (1948). Quart. J. Med. 17, 123-137.

Grosser, Y., Eales, L. and Sano, S. (1971). Sth. Afr. J. Lab. Clin. Med. 17, (2), 156-159.

Hamnström, B., Haeger-Aronsen, B., Waldenström, J., Hysing, B. and Molander, J. (1967). Brit. Med. J. 4, 449-453.

Heilmeyer, L. (1964). Die Störungen der Bluthämsynthese, Stuttgart: Georg Thieme Verlag.

Hill, R. and Keilin, D. (1930). Proc. Roy. Soc. B107, 286.

Hoare, D.S. and Heath, H. (1959). Biochem. J. 73, 679-690.

Hsu, W.P. and Miller, G.W. (1970). Biochem. J. 117, 215-220.

Jacobs, N.J., Jacobs, J.M. and Brent, P. (1971). J. Bact. 107 (1), 203-209.

Jones, M.S. and Jones, O.T.G. (1969). Biochem. J. 113, 507-514.

Kennedy, G.Y., Jackson, A.H., Kenner, G.W. and Suckling, C.J. (1970). FEBS Letters 6 (1), 9-12.

Kikuchi, G., Kumar, A., Talmage, P. and Shemin, D. (1958). J. Biol. Chem.233, 1214-1219.

Labbe, R.F. and Hubbard, N. (1961). Biochim. Biophys. Acta 52, 130-135.

Lascelles, J. (1957). Biochem. J. 66, 65-72.

Lipsky, S.R. and Landowne, R.A. (1963). in Methods in Enzymology VI, p. 515. (S.P. Colowick and N.O. Kaplan, Eds.) London and New York.

Lockwood, W.H. and Davies, J.L. (1962). Clin. Chim. Acta 7, 301-308.

Lomholt, J.C. and With, T.K. (1969). Acta Med. Scand. 186, 83-85.

Marks, G.S. (1969). Heme and Chlorophyll, London: D. van Nostrand.

Mauzerall, D. and Granick, S. (1958). J. Biol. Chem. 232, 1141.

Mori, M. and Sano, S. (1968). Biochem. Biophys. Res. Commun. 32, 611-615.

Ornstein, L. (1964). Ann. N.Y. Acad. Sci. 121, 321-349.

Oyama, H., Sugita, Y., Yoneyama, Y. and Yoshikaya, H. (1961). Biochim. Biophys. Acta 47, 314-414.

Paul, K.-G. (1950). Acta Chem. Scand. 4, 239-244.

Porra, R.J. and Falk, J.E. (1961). Biochem. Biophys. Res. Commun. 5, (3), 179-184.

Porra, R.J. (1962). Ph.D. thesis

Porra, R.J. and Jones, O.T.G. (1963). Biochem. J. 87, 181-185.

Prato, V., Mazza, U., Massaro, A.L. and Battistini, V. (1967). Arch. Immunol. Ther. Exp. 15, 765.

Rimington, C. and Sveinsson, S.L. (1950). Scand. J. Clin. Lab. Invest. 2, 209.

Rimington, C. and Tooth, B.E. (1961). J. Biochem. 39, 456.

Rimington, C., Lockwood, W.H. (1966). Panminerva Medica 8, 325-327.

Rimington, C., Lockwood, W.H. and Belcher, R.V. (1968). Clin. Sci. 35, 211-247.

Sano, S. and Granick, S. (1961). J. Biol. Chem. 236 (4), 1173-1180.

Sano, S. (1966). J. Biol. Chem. 241, (22), 5276-5283.

Smith, S.G., Belcher, R.V., Mahler, R. and Yudkin, J. (1968). Biochem. J. 110, 15P.

Smith, S.G., Belcher, R.V. and Mahler, R. (1969a). Biochem. J. 114, 88P.

Smith S.G., Belcher, R.V., Mahler, R. and Yudkin, J. (1969b). Clin. Chim. Acta 23, 241-243.

Smith, S.G., Belcher, R.V. and Mahler, R. (1971). Sth. Afr. J. Lab. Clin. Med. 17 (2), 150-156.

Strand, L.J., Manning, J. and Marver, H.S. (1971). S. Afr. J. Lab. Clin. Med. 17 (2), 108-111.

Sweeney, G.D. and Eales, L. (1963). S. Afr. J. Clin. Lab. Med. 9, 261 (Discussion).

Tait, G.H. (1972). Biochem. J. 128, 1159-1169.

Tio, (Tiong Hoo), Leijnse, B., Jarrett, A. and Rimington, C. (1957). Clin. Sci. 16, 517.

Tomio, J.M., Garcia, R.C., San Martin de Viale, L.C. and Grinstein, M. (1970). Biochim. Biophys. Acta 198, 353-363.

Tschudy, D.P., Perlroth, M.G., Marver, H.S., Collins, S., Hunter, G. and Rechcigl, M. (1965). Proc. Natl. Acad. Sci. 53, 841-846.

Watson, C.J. (1960). New Eng. J. Med. 263, (24), 1205-1215.

With, T.K. (1971). S. Afr. J. Lab. Clin. Med. 17 (2), 218-220.

Zaman, Z., Abboud, M.M. and Akhtar, M. (1972). J. Chem. Commun. 1263-1264.

Zeile, K. and Meyer, H. (1939). Hoppe-Seyler's Z. physiol. Chem. 261 (2), 178-198.