PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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Macrocyclic intermediates in the biosynthesis of porphyrins

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The hepta-, hexa- and penta-carboxylic porphyrins found in the faeces of rats poisoned with hexachlorobenzene have been separated by high-pressure liquid chromatography and characterized largely by spectroscopic methods. Their structures were confirmed by total synthesis, as part of a programme in which eleven of the fourteen hepta-, hexa- and penta-carboxylic porphyrins derived from uroporphyrin III have now been synthesized as their methyl esters. The four isomeric heptacarboxylic and three of the pentacarboxylic porphyrinogens have been incubated with haemolysates of chicken erythrocytes, and they are all converted into protoporphyrin IX but at different rates. On the basis of this and other evidence we conclude that the decarboxylation of uroporphyrinogen III to coproporphyrinogen III is a stepwise process taking place by a preferred pathway (both in normal and abnormal metabolism); the acetic acid groups are decarboxylated in a sequential clockwise fashion starting with that on the D ring and followed by those on the A, B and C rings. In the poisoned rats the uroporphyrinogen decarboxylase enzyme (or group of enzymes) is probably partially inhibited and the pentacarboxylic porphyrinogen with an acetic acid group on ring C accumulates. The latter is then transformed by a side pathway into dehydroisocoproporphyrinogen and thence into dehydroisocoproporphyrin and its congeners.

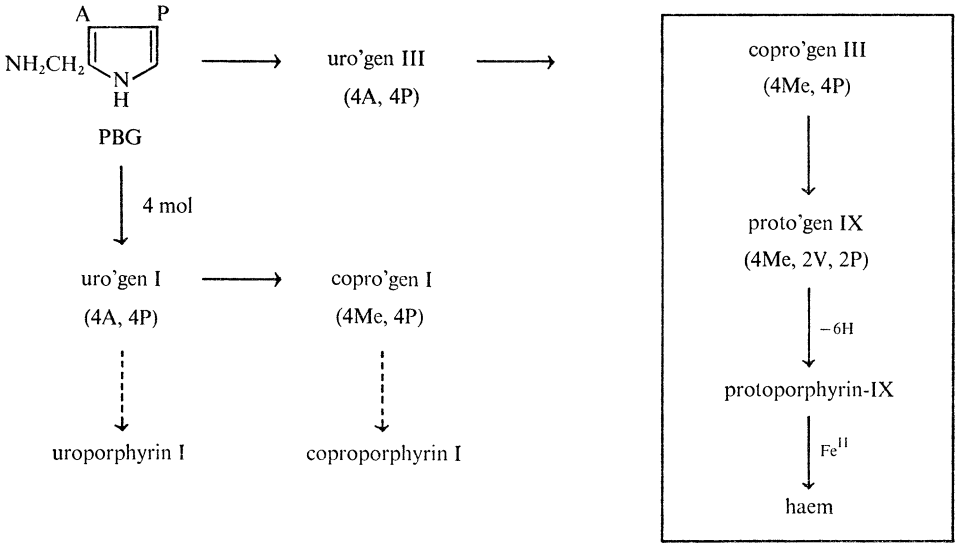
Introduction

The general outlines of haem and chlorophyll biosynthesis from succinate and glycine via porphobilinogen, various macrocyclic porphyrinogens, and protoporphyrin IX have now been known for some twenty years (see, for example, Mauzerall & Granick 1958; Bogorad 1966). Vitamin B_{12} (cobalamin), which is closely related structurally to the porphyrins, is also derived from succinate and glycine via porphobilinogen (PBG) (Corcoran & Shemin 1957; Bray & Shemin 1963) but the biosynthetic pathway probably diverges from the porphyrin pathway at the uroporphyrinogen-III stage (Burnham 1969; Scott, Townsend, Okada & Kajiwara 1973; Scott *et al.* 1974).

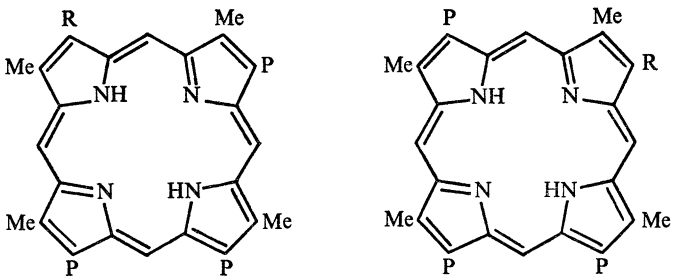
The overall pathway of haem biosynthesis (shown in scheme 1) may be divided into several stages (i) the formation of porphobilinogen PBG, (ii) the polymerization of PBG to uroporphyrinogen III, (iii) the decarboxylation of the four side chains of uroporphyrinogen III to coproporphyrinogen III, (iv) the conversion of two of the propionic acid side chains of coproporphyrinogen III to vinyl groups and dehydrogenation of the nucleus to form protoporphyrin IX and (v) the insertion of iron to form haem. Other papers in this symposium are concerned with the first two stages including the intriguing question of the mechanism of reversal of the D ring of uroporphyrinogen III, but our interests during the last few years have been largely concerned with stages (iii) and (iv). We were stimulated in part by structural studies of various newly

discovered porphyrins which had been isolated from mammalian sources, e.g. pemptoporphyrin, harderoporphyrin and the so-called 'S411' porphyrin (see scheme 2).

In normal metabolism only small amounts of free porphyrins are found in body tissues, body fluids or excreta and intermediate porphyrinogens are very rapidly transformed through protoporphyrin IX to haem; for example, the uroporphyrinogen decarboxylase appears to have a low Michaelis constant and high turnover number so that the concentration of intermediate



Scheme 1. The biosynthesis of haem.



harderoporphyrin R=CH=CH$_2$ isoharderoporphyrin
'S 411' porphyrin R=CH=CHCO$_2$H 'iso S411' porphyrin
pemptoporphyrin R=H

Scheme 2. Structures of harderoporphyrin, S411 porphyrin and pemptoporphyrin and their isomers.

porphyrinogens is very low at any one time and coproporphyrinogen III is rapidly formed (Mauzerall & Granick 1958). Notable exceptions to this general rule include the well-known occurrence of protoporphyrin IX as the brown colouring material of egg shells, and of protoporphyrin IX, harderoporphyrin and coproporphyrin III in the Harderian gland of the rat (situated at the rear of the eye) (Kennedy 1970; Kennedy, Jackson, Kenner & Suckling 1971). However, in various genetic and acquired disorders of haem metabolism (*porphyrias*) (see Marver & Schmid 1974) relatively large amounts of free porphyrins may be excreted in the urine or faeces, or deposited in the skin (leading in the latter case to photosensitivity and skin

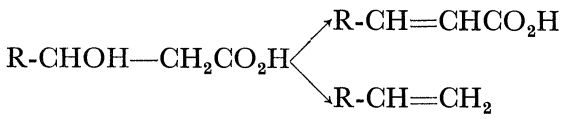
lesions). In adults about 300 mg per day is synthesized in the bone marrow, but the rate of excretion of free porphyrins in urine and faeces is only of the order of 0.1 mg/day or less in normal metabolism, whereas in some of the porphyrias excretion rates of several milligrams per day have been observed, and the urine is red and fluoresces brilliant orange under ultraviolet light.

In Cardiff we have been concerned with the nature of the porphyrins excreted in both normal and abnormal metabolism, and whether they are the same or different. In the present paper we provide an answer to this question, and the subsequent discussion is divided into three parts, the first reviewing work on the nature of the intermediates between corproporphyrinogen III and protoporphyrin IX, the second with structural studies of abnormal metabolites related to intermediates between uroporphyrinogen III, and the third with some preliminary biosynthetic studies of uroporphyrinogen decarboxylase.

Intermediates between coproporphyrinogen III and protoporphyrin IX

Our earlier studies of the structure and synthesis of harderoporphyrin (scheme 2) led to the hypothesis that the related porphyrinogen was a normal intermediate between coproporphyrinogen III and protoporphyrinogen IX (Kennedy *et al.* 1971). This has since been confirmed by studying the incorporation of tritium-labelled harderoporphyrinogen and isoharderoporphyrinogen in both *Euglena gracilis* (Cavaleiro, Kenner & Smith 1973) and a haemolysate of chicken erythrocytes (Games *et al.* 1975). Harderoporphyrinogen was converted over ten times more efficiently (35 %) into protoporphyrin IX, than its isomer, thus suggesting the existence of a preferred pathway).

In this connexion the existence of the S411 porphyrin (scheme 2) (so-called because of the position of its Soret band in the near u.v.) in meconium (French, Nicholson & Rimington 1967) is of considerable interest because it is a dehydrocoproporphyrin III. Its structure, originally deduced from analytical and spectroscopic evidence together with biosynthetic reasoning has now been proved by synthesis and the isomer was also prepared (Couch 1973; see Jackson & Games 1975 and Jackson *et al.* 1974). Isotopic labelling experiments in *Euglena gracilis* showed that both of the hydrogen atoms in each of the methylene groups neighbouring the carboxyl groups of the 2- and 4-propionic acid groups of coproporphyrinogen III are retained in the resulting protoporphyrin IX (Battersby *et al.* 1972) and thus the S411 porphyrinogen cannot be an intermediate in normal metabolism. A possible explanation is that an intermediate α-hydroxypropionate porphyrinogen either eliminates water to form the acrylate porphyrinogen, or fragments with loss of water and carbon dioxide to form the vinyl porphyrinogen; the first pathway is sterically more favourable, but the latter may be preferred under enzymic control via a less-stable conformer (see Jackson *et al.* 1974; Jackson & Games 1975).

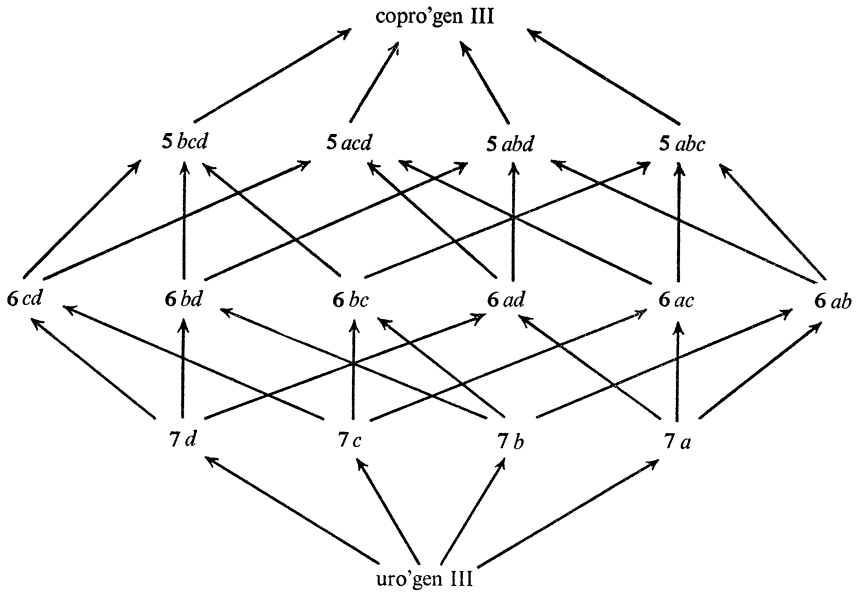


We have also recently shown in Cardiff that the final oxidation of protoporphyrinogen IX to protoporphyrin IX is essentially a sterospecific process in haemolysates of chicken erythrocytes (Jackson *et al.* 1974). Doubly labelled coproporphyrinogen III labelled with ^{14}C in the

2-propionic acid side-chain and with ^3H at the *meso*-positions was incorporated into protoporphyrin IX with 50 % loss of tritium label. Presumably stereospecific removal of hydrogen occurs from one side of the molecule only either directly by the enzyme itself, or alternatively from the side of the molecule remote from enzyme through the action of molecular oxygen, or other oxidizing agent. It was originally thought that the enzyme which carries out the conversion of the propionic acid side chains to vinyl groups ('coproporphyrinogen oxidative decarboxylase') also carried out the dehydrogenation to porphyrin. However, evidence for the existence of two separate enzymes has now been presented, and it has been suggested that repression of haem biosynthesis observed in aerobic cultures of the yeast *Saccharomyces cerevisiae* by high concentrations of glucose is due to inhibition of the second enzyme (Poulson & Polglase 1974).

Intermediates between uroporphyrinogen III and coproporphyrinogen III

In theory four heptacarboxylic, six hexacarboxylic and four pentacarboxylic porphyrins might be formed by successive decarboxylations of the acetic acid side-chains (scheme 3). There are thus 24 (factorial 4) possible routes between uroporphyrinogen III and coproporphyrinogen III, and a major aim of our work was to discover whether or not there was a specific (or preferred) route and whether this was the same in both normal and abnormal metabolism.

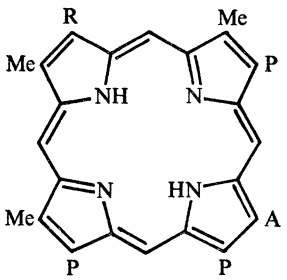


Scheme 3. Possible routes from uroporphyrinogen III and coproporphyrinogen III via hepta-, hexa- and pentacarboxylic porphyrinogens. The numbers refer to the number of carboxylic acid groups in the porphyrinogens, and the letters indicate the position of the methyl groups (i.e. which acetic acid groups have been decarboxylated) in relation to the four pyrrole rings of uroporphyrinogen III.

Normal human urine contains only small amounts of porphyrins between uroporphyrin and coproporphyrin, but no protoporphyrin, whereas faeces only contains traces of copro- and protoporphyrin. However, in the urine of patients with certain types of porphyria (see San Martin de Viale & Grinstein 1968, and references therein), and from porphyria induced by hexachlorobenzene (Chu & Chu 1965; De Matteis, Prior & Rimington 1961; San Martin de

Viale, Viale, Nacht & Grinstein 1970; Elder 1972) relatively large quantities of porphyrins with 8, 7, 6, 5, and 4 carboxylic acids are produced. Small amounts of hepta-, hexa-, penta- and tetracarboxylic porphyrins have been isolated previously (Batlle & Grinstein 1964; San Martin de Viale & Grinstein 1968), either from haemolysates of chicken red blood cells by incubation with uroporphyrinogen III or better from the urine of rats fed for several months with hexachlorobenzene. The porphyrins obtained in this way were all shown to be derived from uroporphyrinogen III and the tetracarboxylic porphyrin was shown to be coproporphyrin III.

More recently a group of four closely related tetracarboxylic porphyrins has been isolated from the faeces of patients with symptomatic cutaneous hepatic porphyria and from rats with porphyria due to hexachlorobenzene (Elder 1972). One of these porphyrins, *isocoproporphyrin*, has been identified by a combination of n.m.r. and mass spectrometric methods as an isomer of coproporphyrin III (Stoll *et al.* 1973). Confirmation of the structure of isocoproporphyrin has recently been provided by synthesis (de Almedia, Kenner, Smith & Sutton 1975). The other three porphyrins were tentatively identified as the analogous vinyl, hydroxyethyl and desethyl compounds (scheme 4). The corresponding vinyl porphyrinogen (*dehydroisocoproporphyrinogen*) was assumed to be the primary metabolite, while the ethyl, hydroxyethyl and desethyl porphyrins presumably arise by hydrogenation, hydration or extrusion of the vinyl group respectively (by bacterial action in the gut). A precedent for loss of the vinyl group is provided by pemptoporphyrin, a normal component of faeces, which is almost certainly derived from protoporphyrin IX in nature, whereas extrusion of vinyl groups from porphyrins can only be achieved in the laboratory under fairly severe conditions (i.e. heating in a resorcinol melt).



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(*a*) R=Et isocoproporphyrin

(*b*) R=V

(*c*) R=CHOH.CH_3

(*d*) R=H

Scheme 4. Isocoproporphyrin and its congeners.

The structure deduced for dehydroisocoproporphyrin was somewhat unexpected in view of the previously well-established normal pathway of porphyrin biosynthesis through coproporphyrinogen III and it led us to consider the possibility that dehydroisocoproporphyrinogen might arise by a minor alternate pathway from a pentacarboxylic precursor of coproporphyrinogen III (cf. scheme 1). In the light of these considerations it became all the more desirable to identify the penta-, hexa- and hepta-carboxylic porphyrins produced in rats poisoned with hexachlorobenzene, and to discover whether or not these compounds were identical with those produced in much smaller amounts in normal mammalian metabolism.

Isocoproporphyrin and its congeners were isolated as their methyl esters and separated by careful fractionation on thin-layer chromatograms (Elder 1972). For the isolation of the higher carboxylic fractions, however, we chose to use the newer technique of high-pressure liquid

chromatography (h.p.l.c.). During the last two years we have been developing the use of h.p.l.c. for the rapid quantitative analysis of mixtures of porphyrins in urine and faeces. The separation of porphyrin esters with different numbers of ester side-chains has proved to be relatively straightforward, those with fewer esters being eluted first (figure 1).

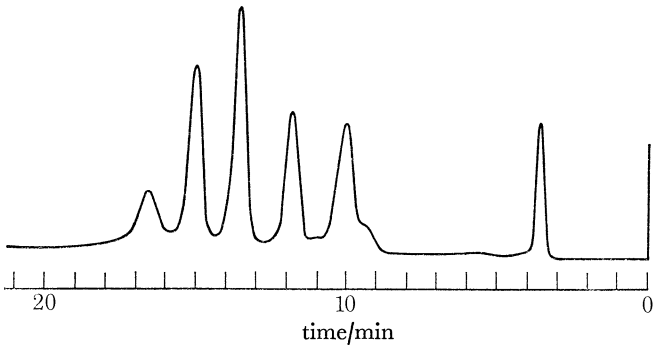

FIGURE 1. Analytical separation of the porphyrin esters obtained from hexachlorobenzene poisoned rats by high-pressure liquid chromatography. (Column: 0.6 m × 3 mm Merckosorb S160, 10 μm silica. Mobile phase: gradient of hexane to ethyl acetate at a flow rate of 2 ml/min.)

In some instances isomeric compounds can be separated, e.g. hardero- and isohardero-porphyrin trimethyl esters (Cavaleiro, Kenner & Smith 1974; Evans, Games, Jackson & Matlin 1975) coproporphyrin and isocoproporphyrin tetramethyl esters (Evans *et al.* 1975); proto-porphyrin I and IX methyl esters, and some of the coproporphyrin esters have also been separated (G. W. Kenner & K. M. Smith, private communication). On the other hand we have not so far succeeded in separating the S411 and iso-S411 porphyrin tetramethyl esters by h.p.l.c., although the free acids could be separated by countercurrent distribution (Couch 1973).

To speed up the elution of the higher esters gradient elution methods have now also been developed and these can be applied to both analytical and preparative separations. Quantitative analysis (figure 1) of the eluates was facilitated by monitoring the intense Soret band exhibited by all porphyrins in the near u.v. (*ca.* 400 nm), and the nature of the individual porphyrins was confirmed by their retention times and by mass spectrometry. *Field desorption* mass spectrometry has proved to be a particularly valuable technique for this purpose because the emitter wire can be dipped directly into the fractions eluted from the h.p.l.c. columns. Furthermore as both porphyrin free acids and their esters give essentially only molecular ions (with very little fragmentation in most cases) preliminary qualitative analyses can be carried out directly on the mixtures prior to h.p.l.c. (Evans *et al.* 1975).

With this experience in hand we have now also carried out preparative scale separations of the porphyrins excreted in the faeces of rats which had been poisoned with hexachlorobenzene. These proved to be mixtures of porphyrins mainly with four to eight carboxylic acid side-chains, and their methyl esters were separated on preparative h.p.l.c. column using gradient elution. In this way 10–20 mg amounts of each of the octa-, hepta-, hexa- and pentacarboxylic fractions were obtained and their characterization is described below. Confirmation that the various porphyrins were of the 'type-III' series (i.e. derived from uroporphyrinogen III rather than uroporphyrinogen I) was obtained by hot alkaline decarboxylation to coproporphyrin III which was shown chromatographically to contain no more than a trace of the type-I isomer. This result is in agreement with earlier results described above (San Martin de Viale & Grinstein 1968) and the octacarboxylic porphyrin was uroporphyrin-III.

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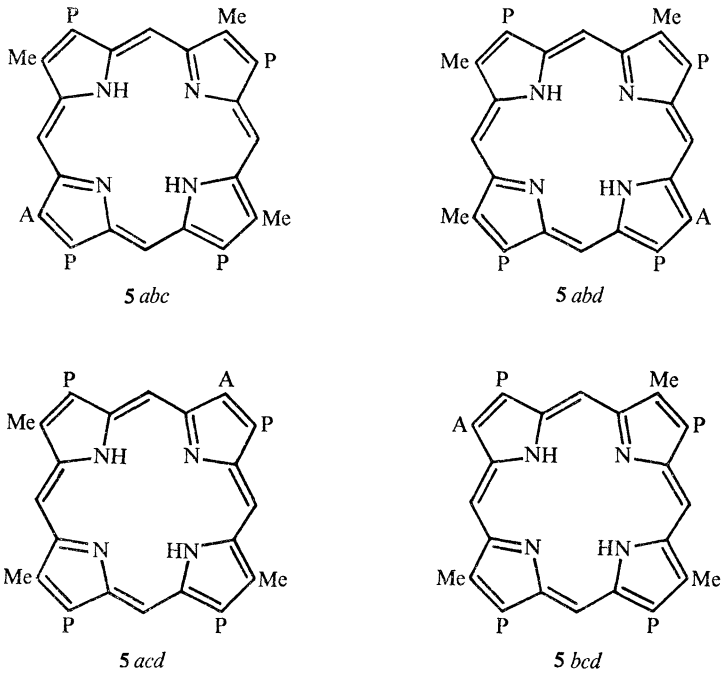
Structure of the pentacarboxylic porphyrin

Four possible pentacarboxylic porphyrins could be derived from uroporphyrinogen III by decarboxylation of three of the acetic acid side chains (scheme 5). *A priori*, however, we thought it very likely that the compound isolated from the rat faeces was the isomer (**5***abd*) with the remaining acetic acid residue on ring C, because the corresponding porphyrinogen is presumably the precursor of both dehydroisocoproporphyrinogen (see scheme 4) and of coproporphyrinogen III.



Scheme 5. Structures of the four pentacarboxylic porphyrins derived from uroporphyrin III.

In our earlier work on the structure of isocoproporphyrin we had shown the utility of n.m.r. spectral studies especially in combination with europium shift reagents (Stoll *et al.* 1973). These reagents appear to form bi-dentate complexes with neighbouring ester groups, whereas individual ester groups are only very weakly complexed; in consequence the resonances of *meso*-protons which are flanked by two ester side chains move rapidly to low field on titration with the shift reagent whereas those between two alkyl groups or between one alkyl and one ester group are very little affected. Inspection of the structures of the four pentacarboxylic porphyrin esters shows that they would be expected to fall into two groups on titration with shift reagent; in the isomers **5***acd* and **5***abd* two of the *meso*-proton resonances would be expected to move to low field, whereas in the other two isomers **5***abc* and **5***bcd* only one *meso*-proton resonance would be affected in this way. Titrations of the naturally derived ester showed that two *meso*-proton resonances moved to low field† (figure 2) and hence its structure must be either **5***acd* or **5***abd*. Confirmation that it had structure **5***abd* rather than **5***acd* was then provided by synthesis using the MacDonald route (cf. Jackson & Smith 1973) from a pyrromethane dialdehyde and a pyrromethane dicarboxylic acid under mildly acidic conditions (scheme 6). The resulting

† Similar titrations have also been carried out by Dr M. S. Stoll with naturally derived material separated (by G.H.E.) by t.l.c., and the results were in good agreement.

porphyrin ester was shown to be identical with the pentamethyl ester of natural product by mixed melting point, and by its n.m.r. spectral behaviour on titration with europium shift reagent. The pentacarboxylic esters (**5***acd*) and (**5***bcd*) have also been synthesized in a similar manner by the MacDonald route, and their n.m.r. spectral behaviour was in agreement with that expected. These three pentacarboxylic porphyrins have also been synthesized in Australia (Dr P. S. Clezy, private communication). The fourth pentacarboxylic porphyrin (**5***abc*) cannot be prepared by the MacDonald method because it lacks any element of symmetry, but its synthesis is in hand by a route involving preparation of an appropriate open-chain tetrapyrrole.

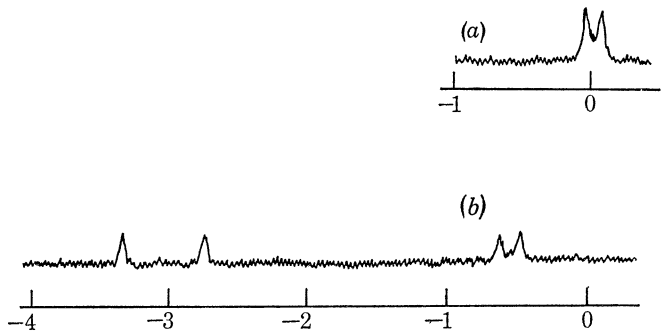
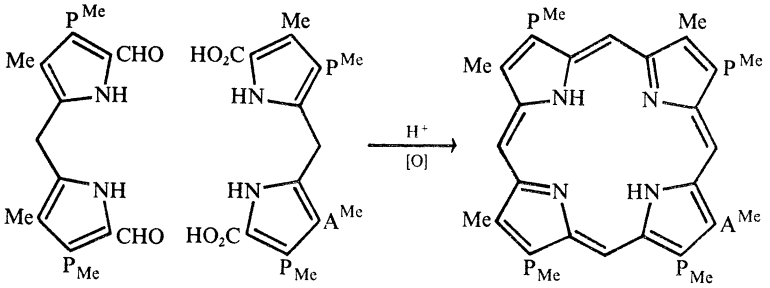


FIGURE 2. N.m.r. spectra of the *meso*-protons of the pentacarboxylic porphyrin pentamethyl ester obtained from poisoned rats (*a*) in $CDCl_3$, (*b*) in $CDCl_3$ containing *ca.* 1 mol of $Eu(fod\text{-}d_9)_3$.



5*dab*

SCHEME 6. Synthesis of the pentacarboxylic porphyrin ('**5***dab*') pentamethyl ester.

Structure of the hexacarboxylic porphyrin

Six possible isomeric porphyrins can theoretically be derived from uroporphyrinogen III by decarboxylation of two of the acetic acid side-chains (scheme 7). Determination of the effects of europium shift reagents on the n.m.r. spectrum of the naturally derived porphyrin hexamethyl ester revealed that three of the *meso*-proton resonances moved rapidly to low field on titration with the shift reagent whereas the other was little affected. This result uniquely defined the structure of the hexacarboxylic porphyrin from rat faeces as (**6***ad*) because only two of the *meso*-proton resonances in each of the other five isomeric hexacarboxylic porphyrin methyl esters would be expected to move markedly to low field on titration with shift reagent.

This deduction has been confirmed by synthesis of the hexamethyl ester by the MacDonald route as shown in scheme 8, and comparisons with the naturally derived ester. The hexamethyl esters of three of the other hexacarboxylic porphyrins (**6***ab*), (**6***bc*) and (**6***cd*) have also been

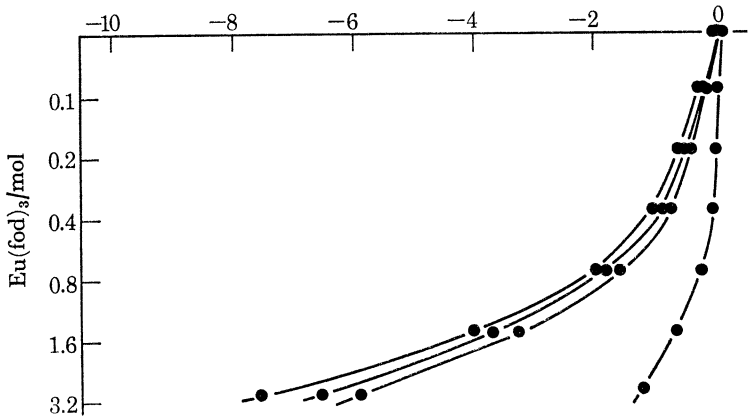
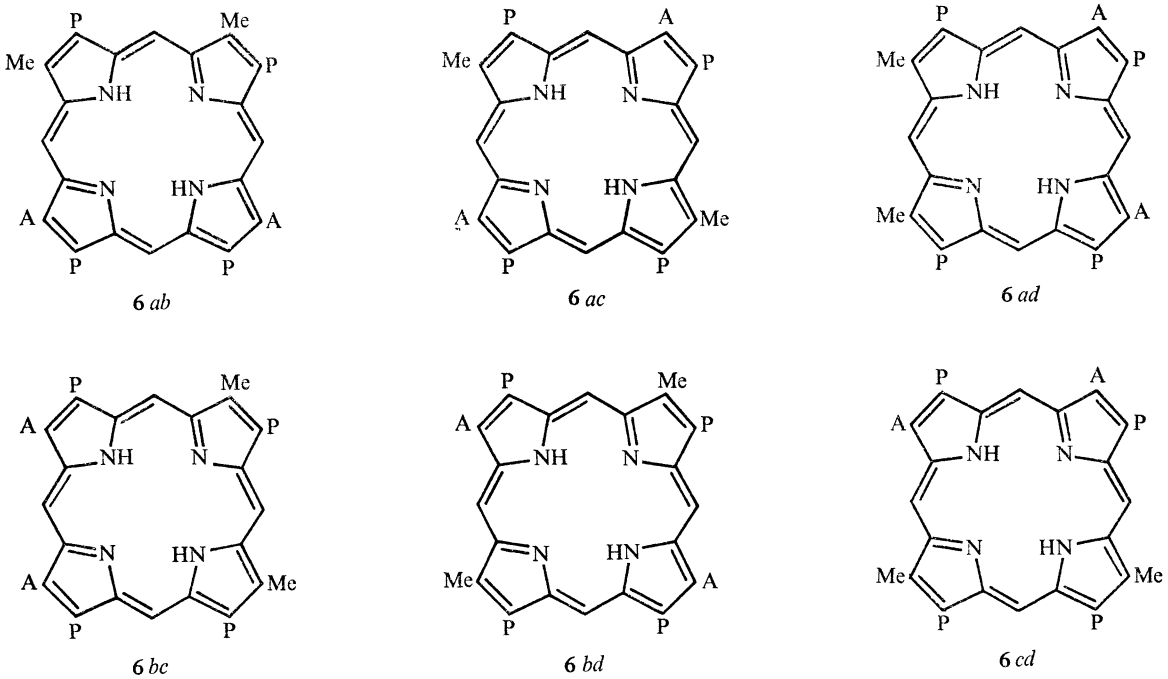
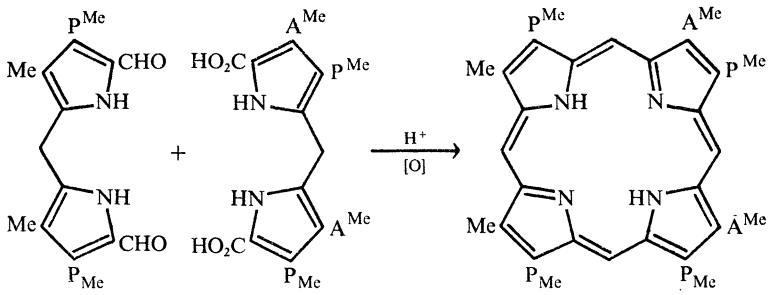


FIGURE 3. Variation of the n.m.r. chemical shifts of the *meso*-protons of the hexamethyl ester of the hexacarboxylic acid porphyrin obtained from poisoned rats on titration in $CDCl_3$ with the shift reagent $Eu(fod\text{-}d_9)_3$.

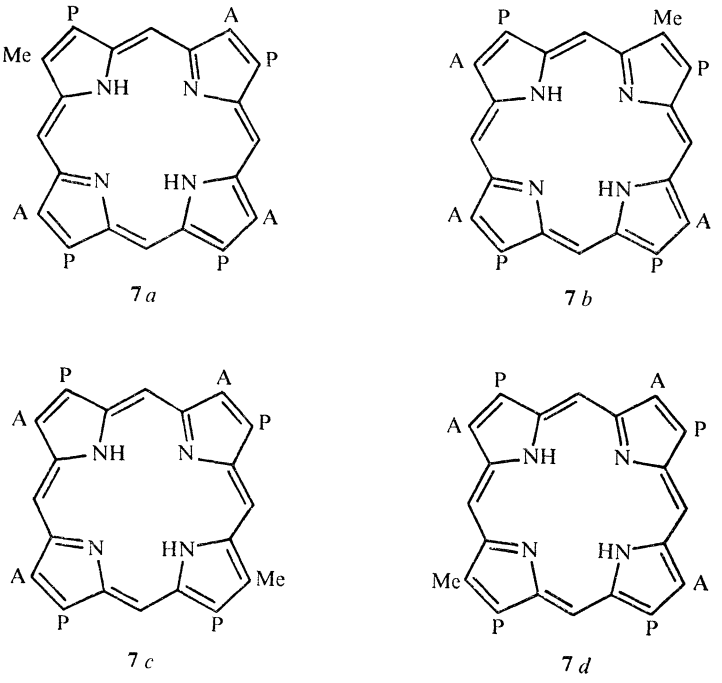


SCHEME 7. Structures of the six hexacarboxylic porphyrins derived from uroporphyrin III.

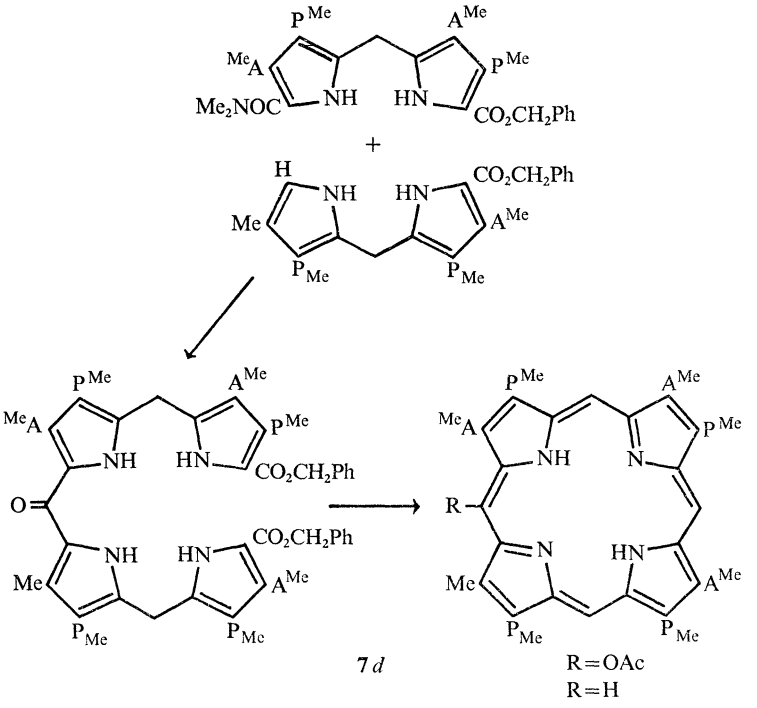


6 *da*

SCHEME 8. Synthesis of the hexamethyl ester of the hexacarboxylic porphyrin, '6 *da*.'



SCHEME 9. Structures of the four heptacarboxylic porphyrins derived from uroporphyrin III.



SCHEME 10. Synthesis of the heptamethyl ester of the heptacarboxylic porphyrin '7*d*' by the *b*-oxobilane route.

prepared in a similar manner, and their n.m.r. spectral behaviour was as expected. The synthesis of the remaining two hexacarboxylic porphyrins is in progress; like the fourth pentacarboxylic porphyrin they must also be prepared by cyclization of a rationally synthesized open-chain tetrapyrrole.

Structure of the heptacarboxylic porphyrin

The product isolated from rat faeces appeared to be identical with phyriaporphyrin (Batlle & Grinstein 1962, 1964*a*), 'porphyrin-208' (Grinstein, Schwartz & Watson 1945) and pseudouroporphyrin (Falk, Dresel, Benson & Knight 1956). To determine the structure it proved necessary to synthesize the heptamethyl esters of all four possible isomeric heptacarboxylic porphyrins which could be derived from uroporphyrinogen III (scheme 9). Three of the esters were synthesized by application of the MacDonald method but the fourth less symmetrical isomer with the methyl group on ring D necessitated the synthesis of an open-chain tetrapyrrole. Of the currently available syntheses of this type (Jackson & Smith 1973) we selected the *b*-oxobilane route (scheme 10). The pyrromethane amide (corresponding to the A and B rings of the final porphyrin) was synthesized by established methods and coupled as its phosphoryl complex with the α-free pyrromethane (corresponding to the C and D rings) (scheme 10).

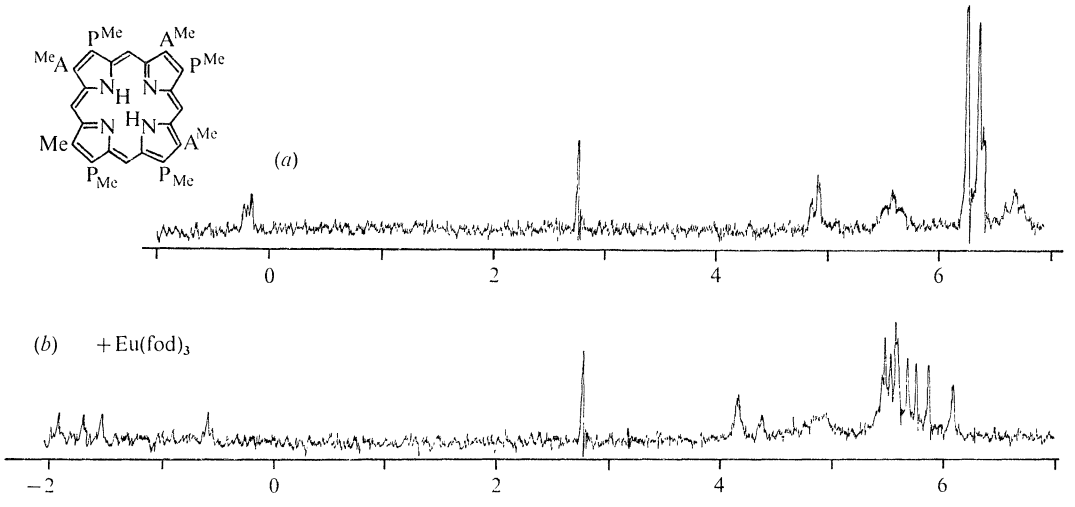


FIGURE 4. N.m.r. spectra of a mixture of natural and synthetic heptamethyl ester of the heptacarboxylic porphyrin obtained from poisoned rats (*a*) in $CDCl_3$, (*b*) in $CDCl_3$ containing *ca.* 1 mol of $Eu(fod\text{-}d_9)_3$.

After chromatographic purification the intermediate imine was hydrolysed to the *b*-oxobilane, dibenzyl ester before hydrogenolysis of the benzyl groups. The resulting di-acid was decarboxylated and cyclized by trichloroacetic acid and methyl orthoformate in methylene chloride and the oxophlorin. Acetylation afforded the *meso*-acetoxyporphyrin, hydrogenation of which followed by reoxidation, gave the desired heptacarboxylic porphyrin heptamethyl ester. Mixed m.p. determinations of all four isomers and of the natural product suggested that the latter was the isomer with the methyl group on ring D, but the results were not completely unequivocal.

Detailed studies of the effects of europium shift reagent on the synthetic and natural heptacarboxylic porphyrin methyl esters have also been carried out, and as expected three of the four *meso*-protons in each case moved markedly to low field on titration with the shift reagent (figure 4). Small second-order differences between the four isomers could be detected and in

accord with the m.p. determinations the most likely structure for the natural product appeared to be (**7***d*) (scheme 9). However, the individual titrations were not fully conclusive because the n.m.r. chemical shifts are somewhat susceptible to concentration effects. Consequently final confirmation of the structure of the natural material was obtained by mixing the heptamethyl ester with the synthetic compound and treating the mixture with shift reagent. The resulting 'mixed' n.m.r. spectrum (figure 4) clearly showed that only one compound was present as evidenced by the fact that only four *meso*-proton resonances and eight methyl resonances were observed. (A mixture of isomers may show up to eight *meso*-proton resonances and sixteen methyl resonances.)

It is interesting to note that in a parallel series of investigations, the Cambridge group (Battersby *et al.* 1974) have shown that a heptacarboxylic porphyrin isolated from a chicken red blood cell haemolysate also has the same structure as the porphyrin which we isolated from the faeces of the poisoned rats. Three of the four isomers were prepared by the MacDonald route in essentially the same manner as our syntheses, and these were shown by ^{13}C and ^{1}H n.m.r. spectral determinations, including mixed spectra, not to be identical with the natural product.

It is clear from our work, and that in Cambridge, that not only are lanthanide n.m.r. spectral shift titrations of great value in porphyrin structure determination, but that the lanthanide shifted n.m.r. spectra of mixtures are perhaps the most valuable criteria for establishing the identity or otherwise of two specimens thought to be the same porphyrin. This criterion may also be useful in other fields, but in the case of porphyrins it is particularly helpful in view of the well-known tendency of many complex porphyrins to exhibit polymorphism, which renders mixed m.p. determinations and X-ray powder photographs less definitive.

Biosynthetic feeding experiments

In our previous work (Jackson *et al.* 1974) concerned with the various stages of the conversion of coproporphyrinogen III to protoporphyrin IX we employed a haemolysate (Dresel & Falk 1956) of chicken red blood cells and studied the incorporation of both singly (^{3}H) and doubly labelled (^{14}C and ^{3}H) intermediates. With this experience in hand we decided to continue with this system in studying the conversion of uroporphyrinogen III and the various possible hepta-, hexa- and pentacarboxylic porphyrinogen intermediates to coproporphyrinogen III and thence to protoporphyrin IX. In a series of preliminary experiments using porphyrinogen prepared from the synthetic porphyrins by sodium-amalgam reduction we established suitable conditions for the incorporations, and showed that it was possible to employ substrate concentrations some ten or twenty times greater than the level at which any of the porphyrinogens were normally present in the haemolysates without saturating the enzyme systems. In these circumstances there there was no need to use isotopically labelled porphyrinogens, and the products from the incubations were analysed both by thin-layer chromatography and by high pressure liquid chromatography.

In the preliminary experiments we confirmed that uroporphyrinogen III was rapidly converted by the haemolysates to protoporphyrin IX and that the maximum amount of the intermediate hepta- and hexa-carboxylic porphyrins formed at any one time was less than 10 % (figure 5). The four heptacarboxylic porphyrinogens have also been incubated with the haemolysates of chicken blood and the time-course of their conversion to protoporphyrin IX was

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followed by t.l.c. as shown in figure 6. It is clear that all four compounds can act as substrates for the decarboxylase enzyme, but the rate of disappearance of the isomer with a methyl group on ring B is appreciably slower than the other three isomers, and moreover there is a marked build-up of a pentacarboxylic intermediate.

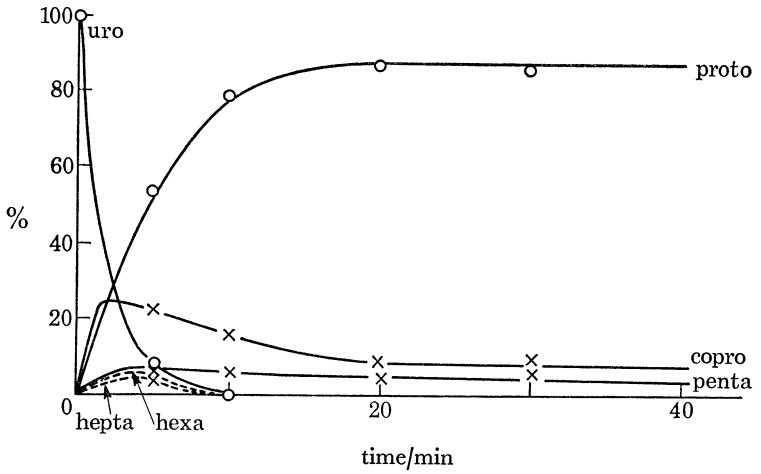

FIGURE 5. Rates of decarboxylation of uroporphyrinogen III to coproporphyrinogen III and protoporphyrinogen IX by incubation with chicken erythrocytes.

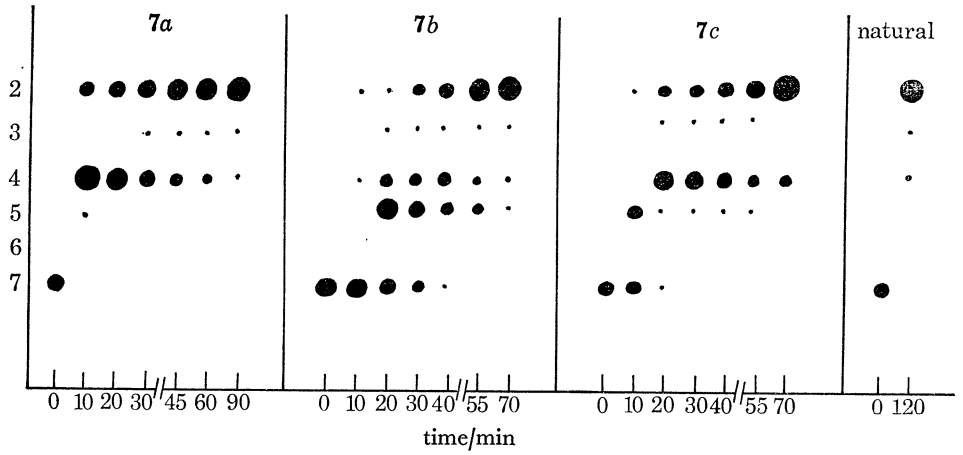

FIGURE 6. Thin-layer chromatograms showing the rates of decarboxylation of the four heptacarboxylic porphyrinogens related to uroporphyrinogen III. (The numbers in the left-hand margin refer to the numbers of carboxyl groups in the porphyrins isolated at various time intervals.)

A similar series of experiments has also been carried out with the three pentacarboxylic porphyrinogens. Again all three compounds can act as substrates for the decarboxylase and are eventually converted into protoporphyrin IX, but the isomer with the residual acetic acid group on ring B is decarboxylated to the intermediate coproporphyrinogen at a slower rate than the other two isomers (Smith *et al.* 1975).

Further experiments are in progress both with the hexacarboxylic porphyrinogens and to determine the nature of the intermediate pentacarboxylic porphyrinogen which accumulates during the decarboxylation of one of the heptacarboxylic porphyrinogens described above. It is conceivable that this pentacarboxylic porphyrinogen is the same as the slowest of the three

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synthetic compounds already tested; alternatively it could be the isomer which we have not yet synthetized, i.e. that with an acetic acid side-chain on ring D (cf. scheme 5).

Conclusions

The structural studies of the various porphyrins isolated from the faeces of rats poisoned with hexachlorobenzene present a fascinating picture and it is highly significant that the hepta-carboxylic porphyrin isolated from haemolysates by the Cambridge group is also identical with that from the rat faeces. The simplest interpretation of these results is that the corresponding porphyrinogens are the major intermediates in both the normal and abnormal metabolic processes, and that the effect of hexachlorobenzene is simply to slow up the overall rate of decarboxylation (by reduced activity of the decarboxylase enzyme). Alternative explanations are that (i) the porphyrins found in the faeces of the poisoned rats arise by a diversion from the normal metabolic pathway or (ii) that they accumulate because the corresponding porphyrinogens are metabolized at a slower rate than the isomeric compounds. However the second alternative cannot be correct because in our biosynthetic studies the naturally derived hepta- and pentacarboxylic porphyrins were metabolized at least as fast as any of the isomers which we have also synthesized. The first alternative also seems very unlikely because our n.m.r. studies indicated that each of the natural hepta-, hexa- and pentacarboxylic porphyrins was essentially a single isomer, and moreover the heptacarboxylic porphyrin was identical with that isolated from the chicken haemolysates.

Evidence in favour of a stepwise decarboxylation of uroporphyrinogen III has been obtained previously by Grinstein and his colleagues (Battle & Grinstein 1964; San Martin de Viale & Grinstein 1968) working with haemolysates of chicken erythrocytes. Our studies show that there is essentially only one preferred (and possibly specific) pathway (scheme 11) to coproporphyrinogen III but the decarboxylase enzyme is not specific for only the isomers on the preferred pathway. The fact that there is a preferred pathway is not surprising because the rates of decarboxylation of any one of the four acetic acid side-chains of uroporphyrinogen III for example are not likely to be quite identical even if the enzyme is non-specific; similar considerations apply to the subsequent decarboxylations and, moreover, the experimental evidence shows that isomeric compounds are metabolized at different rates. Small amounts of intermediates on other less-favoured routes may also have been formed, but these were not present in sufficient quantities to enable them to be detected by n.m.r.

Perhaps the most striking evidence for a preferred pathway of degradation, however, is that the structures of the compounds isolated show that decarboxylation occurs in a clockwise fasion starting with the acetic acid on the D ring of uroporphyrinogen III and proceeding via the successive decarboxylations of the acetic acid residues on rings A, B and C to form coproporphyrinogen III (scheme 11). It is as if the uroporphyrinogen in effect 'performs a cartwheel' on the enzyme surface as the acetic acid residues are succesively decarboxylated. However, this is largely speculative and it remains to be seen whether a single enzyme, or a group of enzymes is involved (cf. Garcia, San Martin de Viale, Tomio & Grinstein 1973). It is interesting to note that the oxidative decarboxylation of the two propionic acid side-chains of coproporphyrinogen III also appears to take place in a largely sequential clockwise fashion (Cavaleiro *et al.* 1973; Games *et al.* 1975; Jackson *et al.* 1974).

In contrast to our results, however, which show that the decarboxylation of the acetic acid

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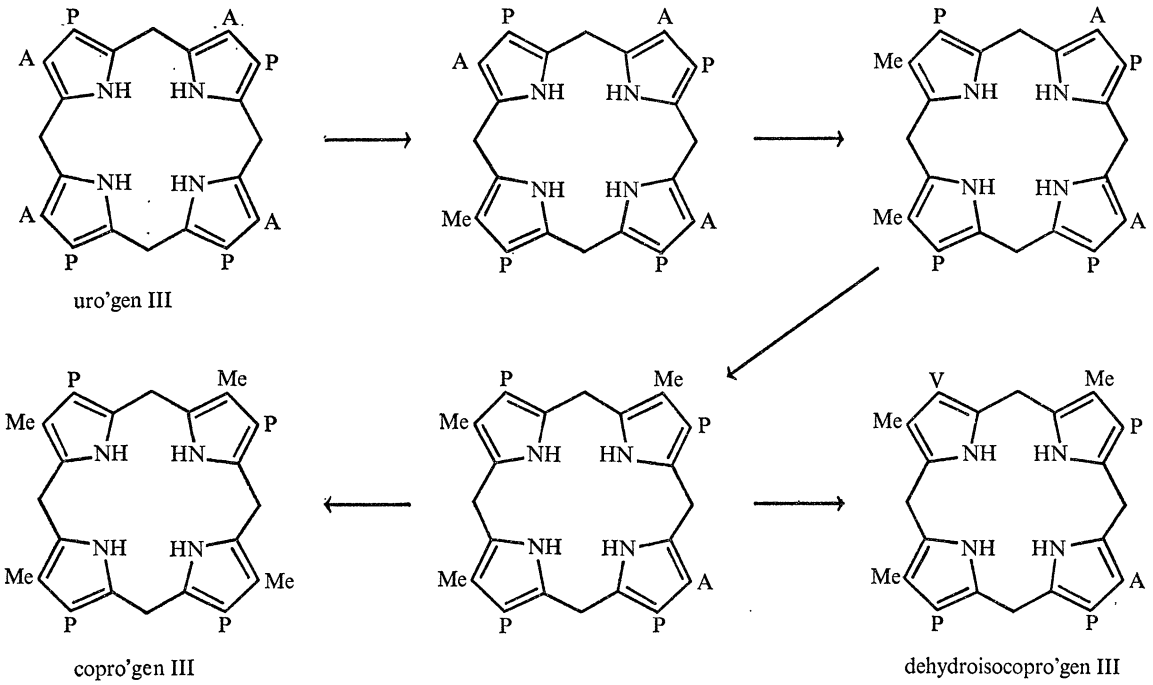
residues of uroporphyrinogen III starts with ring D and finishes with ring C, only the C ring acetic acid group has been decarboxylated in the formation of the corrin nucleus of vitamin B_{12}. Thus differentiation between the corrinoid and porphyrin pathways must presumably occur immediately after uroporphyrinogen III either as a result of the decarboxylation of the C-ring acetic acid group, or perhaps more likely as a result of C-methylation processes (cf. Scott *et al.* 1974).



SCHEME 11. Clockwise natural route for the decarboxylation of uroporphyrinogen III to coproporphyrinogen III and formation of dehydroisocoproporphyrinogen.

The mechanism of the decarboxylation of the acetic acid residues is unknown, but it may occur through protonation of the pyrrole nuclei in the porphyrinogens. The nearest analogy is the hot acid catalysed decarboxylation of the acetic acid side-chains of the uro-, hepta-, hexa- and pentacarboxylic porphyrins to give coproporphyrins.

The presence of dehydroisocoproporphyrinogen and its congeners in the faeces of poisoned rats as well as coproporphyrinogen III may well be due to impairment of the decarboxylation process so that the pentacarboxylic porphyrinogen precursor accumulates and transformation into dehydroisocoproporphyrinogen III could then occur by oxidative decarboxylation of the propionic acid side-chain in the same manner as the conversion of coproporphyrinogen III into protoporphyrinogen IX. However, one difficulty with this hypothesis is that uroporphyrinogen decarboxylase is a soluble enzyme, whereas it is commonly accepted that coproporphyrinogenase is a mitochondrial enzyme; a possible explanation for the formation of dehydroisocoproporphyrinogen is that the liver damage in both symptomatic hepatic cutaneous porphyria and hexachlorobenzene poisoning makes the mitochondrial enzymes accessible to the pentacarboxylate porphyrinogen as well as coproporphyrinogen III (cf. Elder 1972, 1974). It is a moot point whether dehydroisocoproporphyrinogen can be further transformed into harderoporphy-

rinogen and then into protoporphyrin IX; it may well be the final porphyrinogen in this abnormal pathway. The related ethyl and desvinyl compounds are formed by intestinal micro organisms after excretion into the bile (Elder 1974).

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