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Biosynthesis of Porphyrins and Corrins [and Discussion]

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Biosynthesis of porphyrins and corrins

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Haem, chlorophyll and vitamin B_{12} are all derived ultimately from four molecules of the pyrrole porphobilinogen (PBG) and the initial enzyme catalysed condensation of PBG leads to the unsymmetrical type III isomer of uroporphyrinogen. On the basis of straightforward chemical considerations the type I isomer should be formed and so the porphyrinogen-forming enzymes of all living systems must catalyse a highly specific rearrangement process. The nature and chemical mechanism of this rearrangement poses one of the most fascinating problems in the porphyrin field and so it is not surprising that over 20 hypothetical schemes have been proposed to account for it.

Analysis of the problem suggested that the incorporation of doubly ^{13}C-labelled precursors into the rearranged macrocyclic rings would give valuable new information on the nature of the rearrangement process. In this approach the *meso*-bridge atoms are of crucial importance, and several unambiguous syntheses of ^{13}C-labelled pyrroles and porphyrins were developed to allow rigorous n.m.r. assignments to be made, and also to provide substrates for enzymic experiments. Studies carried out with enzymes from both avian blood and from *Euglena gracilis* have revealed the precise nature of the assembly of four PBG molecules into the type-III macrocycle: it is the same in both systems despite their vastly different evolutionary development.

Complementary studies are in progress in order to determine the *intermediates* involved in the conversion of PBG into uroporphyrinogen III. The synthesis of amino methyl pyrromethanes and their interaction in the presence of PBG with the appropriate enzyme systems are described. It is important for the work to be able to separate not only isomeric pyrromethanes but also the four isomeric coproporphyrins. Powerful methods are described which make use of high pressure liquid chromatography for both types of separation process.

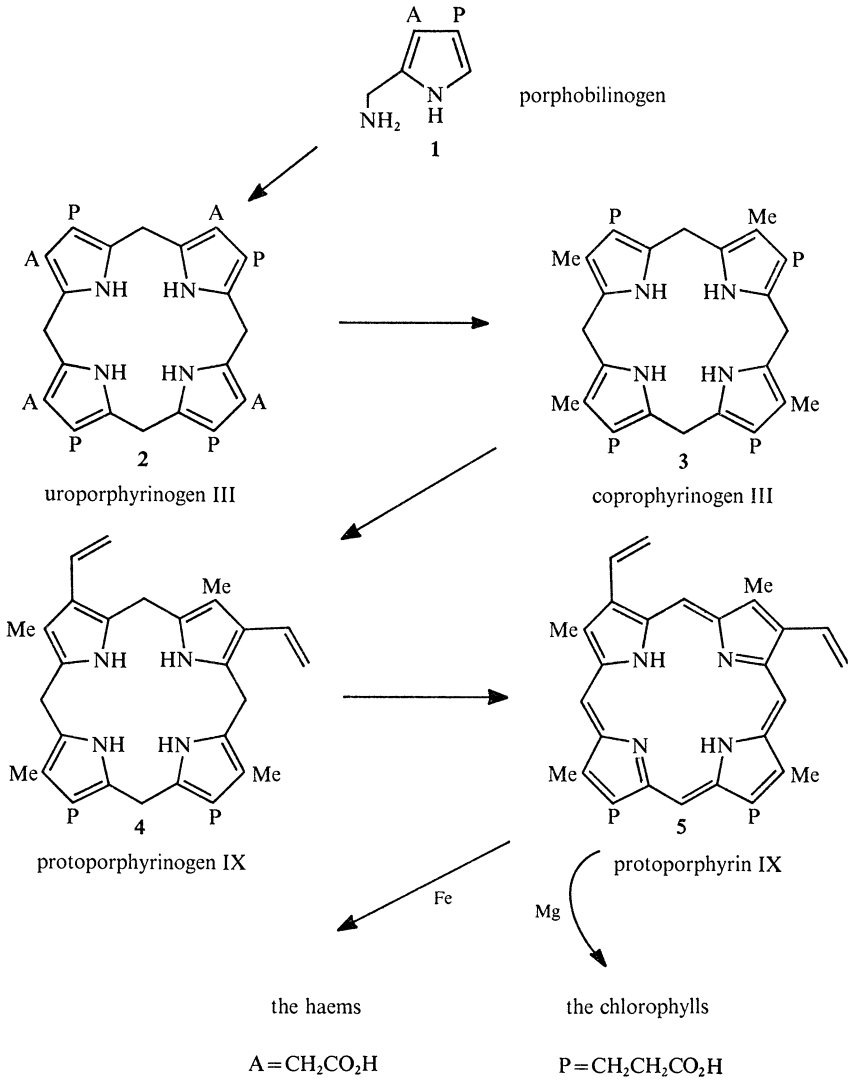
Once uroporhyrinogen III has been built enzymically, there is a stepwise enzymic decarboxylation of the four acetic acid residues. A heptacarboxylic porphyrin shown to be a type-III porphyrin is isolated from the action of avian blood enzymes on porphobilinogen. Spectroscopic studies with ^{13}C-labelling limit the possible structures to two and total synthesis of these substances shows that the natural product carries its methyl group on ring D.

An isomeric heptacarboxylic porphyrin having its methyl group on ring C is of particular interest in relation to the biosynthesis of vitamin B_{12}. This substance is synthesized together with uroporphyrin III, ^{14}C-labelled specifically in ring C. This latter product is used to settle one of the key questions concerning nature's route to vitamin B_{12} – that is, does the corrin macrocycle arise from uroporphyrinogen III? Incorporation studies and specific degradations prove specific incorporation of uroporphyrinogen III into cobyrinic acid, which is the known precursor of vitamin B_{12}.

The earlier contributions to this Meeting have covered those biosynthetic stages on the pathway to the porphyrins which build porphobilinogen (**1**). This is the stage at which construction of the macrocycle begins and it is fascinating that over evolutionary time, nature has chosen to build the type-III isomer, e.g. uroporphyrinogen III (**2**), for all its vitally important tetrapyrrolic substances.

Uroporphyrinogen III (**2**) is converted enzymically by decarboxylation into coproporphyrinogen III (**3**) and then by oxidative decarboxylation into protoporphyrinogen IX (**4**).

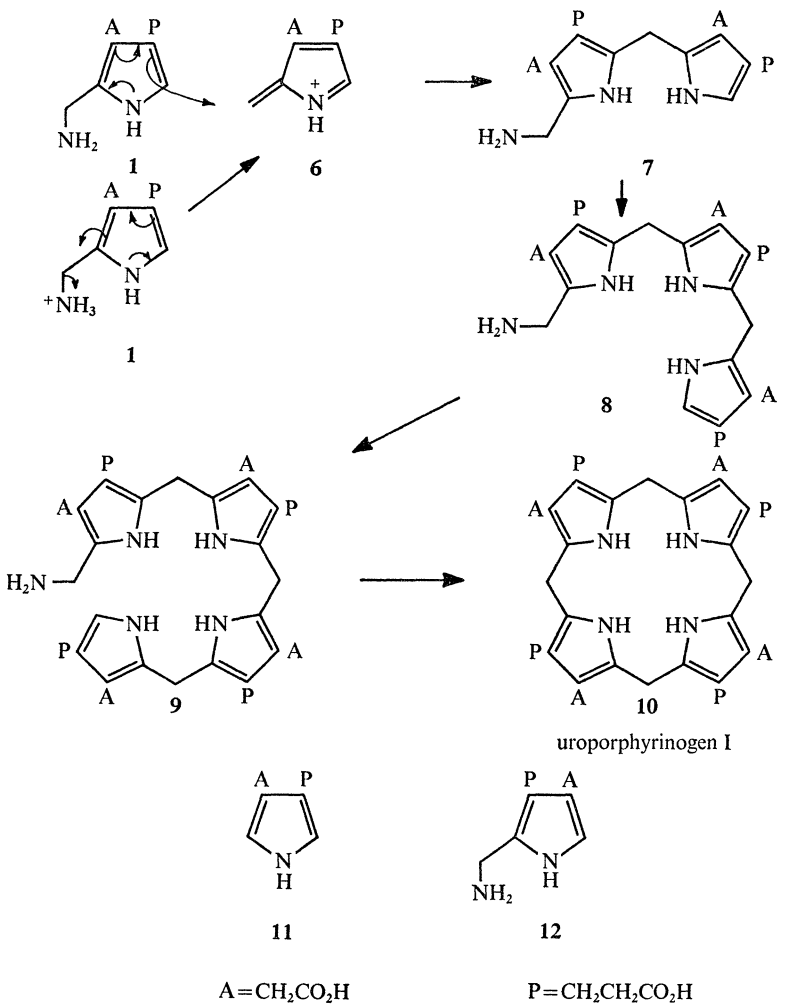
Finally, aromatization takes place to generate protoporphyrin IX, now as the fully conjugated macrocycle **5**; see scheme 1 for an outline of these steps. One cannot over-emphasize the importance of the superb work of Shemin, Neuberger, Granick, Bogorad and Rimington and the contributions from many others to the pathway just outlined. It is on this solid base that the more recent researches have been built.



SCHEME 1

To recognize the central role played by the tetrapyrrolic macrocycles, especially by protoporphyrin IX (**5**), in living systems one need only consider the following three points: (*a*) that haem for haemoglobin is formed by enzymic insertion of an iron ion into protoporphyrin IX, (*b*) that chlorophyll *a* was shown also to be derived from protoporphyrin IX (Granick 1951) and this has recently been confirmed by radiotracer methods (Cox, Howarth, Jackson & Kenner 1969; Shien, Miller & Psenak 1974), (*c*) that there were pointers about which we will hear more later, that the corrin macrocycle of vitamin B_{12} is derived from uroporphyrinogen III (**2**).

Returning for a moment to the *enzymic* formation of uroporphyrinogen III (**2**), we should emphasize that it is the unexpected product. If we think only about the *simplest chemical pathway* by which four units of porphobilinogen (**1**) could be converted into the porphyrinogen macrocycle (ignoring enzymes at present), one derives scheme 2. The final product is the centrosymmetric uroporphyrinogen I (**10**). So living things have developed a way, unerringly in healthy systems, to form what is on chemical grounds the surprising rearranged isomer, type III (**2**). This part of the biosynthetic pathway has attracted intense interest and equally intense speculation leading to more than 20 mechanistic schemes being published. We will give here a brief historical survey of work on this problem, together with a more detailed account of recent researches.



SCHEME 2

An important early observation was that the biochemical formation of uroporphyrinogen III (**2**) from porphobilinogen (**1**) involves the cooperation of two proteins, one of which was much less stable to heat than the other (Bogorad & Granick 1953; Bogorad 1955, 1958). The relatively stable one is porphobilinogen deaminase (sometimes also called uroporphyrinogen I synthetase but the former name better describes its function; we will abbreviate the name to *deaminase*).

This enzyme converts porphobilinogen solely into uroporphyrinogen I (**10**). The less stable protein we will refer to as *cosynthetase* (full name uroporphyrinogen III cosynthetase). Treatment of porphobilinogen with deaminase in the presence of an excess of cosynthetase generates uroporphyrinogen III (**2**).

During the period following the above enzymic experiments and running up to about 1970 many studies were made to gain information about the specific biological formation of the type-III isomer. A complete survey of all this work is given elsewhere (Battersby & McDonald 1975). The most important findings are collected here.

It was found (Bogorad 1955, 1958, confirmed by many other workers) that four molecules of porphobilinogen are converted by deaminase-cosynthetase into uroporphyrinogen III and that no pyrrolic substances detectable by Ehrlich's reagent were present in the final solution. Free formaldehyde could not be found in the medium when porphobilinogen was converted enzymically into uroporphyrinogen III and neither [^{14}C]formaldehyde (Bogorad & Marks 1960*a*; Lockwood & Benson 1960) nor [^{14}C]opsopyrroledicarboxylic acid (**11**, scheme 2) (Bogorad 1960) were incorporated into the final macrocycle. The diacid (**11**) was an important intermediate in some mechanistic speculations about the formation of the type-III macrocycle. It was further found that cosynthetase did not transform porphobilinogen into a substrate for deaminase (e.g. into isoporphobilinogen (**12**)) nor did it join porphobilinogen units together (Bogorad 1955, 1958). Isoporphobilinogen (**12**) was examined as a substrate but it was not incorporated by deaminase-cosynthetase into uroporphyrinogen III (Carpenter & Scott 1961; Battersby, McDonald & Markwell 1974*e*); indeed, it acted as an inhibitor of the enzyme system (Frydman & Feinstein 1974). Importantly, uroporphyrinogen I (**10**) was proved not to be converted into its type-III isomer (**2**) by cosynthetase nor by the deaminase-cosynthetase combination (Bogorad 1955, 1958; Granick & Mauzerall 1958; Bogorad & Marks 1960*b*).

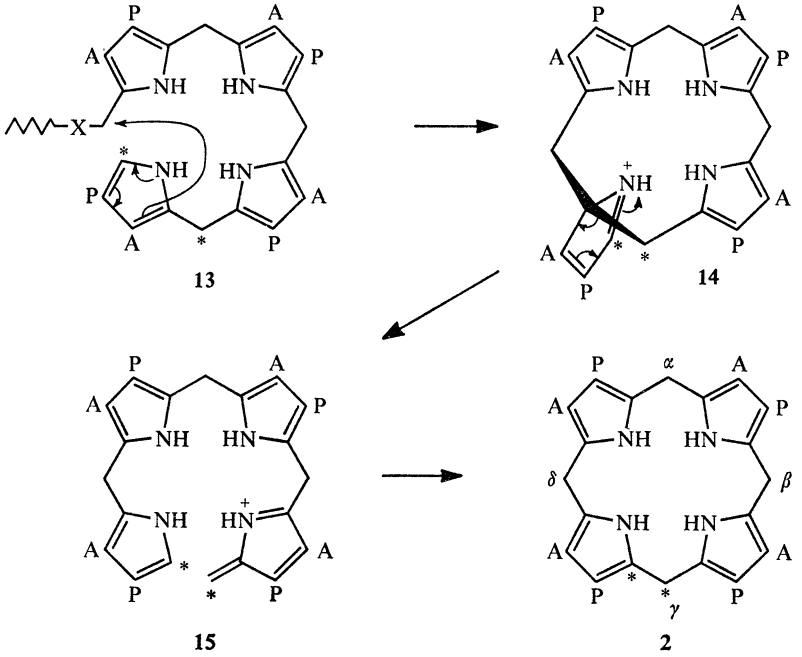
Recent studies of formation of the type-III isomer

Over the last five years or so, a renewed attack has been made on the so-called type-III problem and the effort has been directed along two complementary lines. One has made use of labelling with carbon-13 and by this approach it has been possible to study carbon–carbon bond breaking and bond making steps in the biosynthetic process. The second attack, which has been steadily progressing alongside the work with carbon-13, involves the study of intermediates lying between porphobilinogen and the uroporphyrinogen III macrocycle.

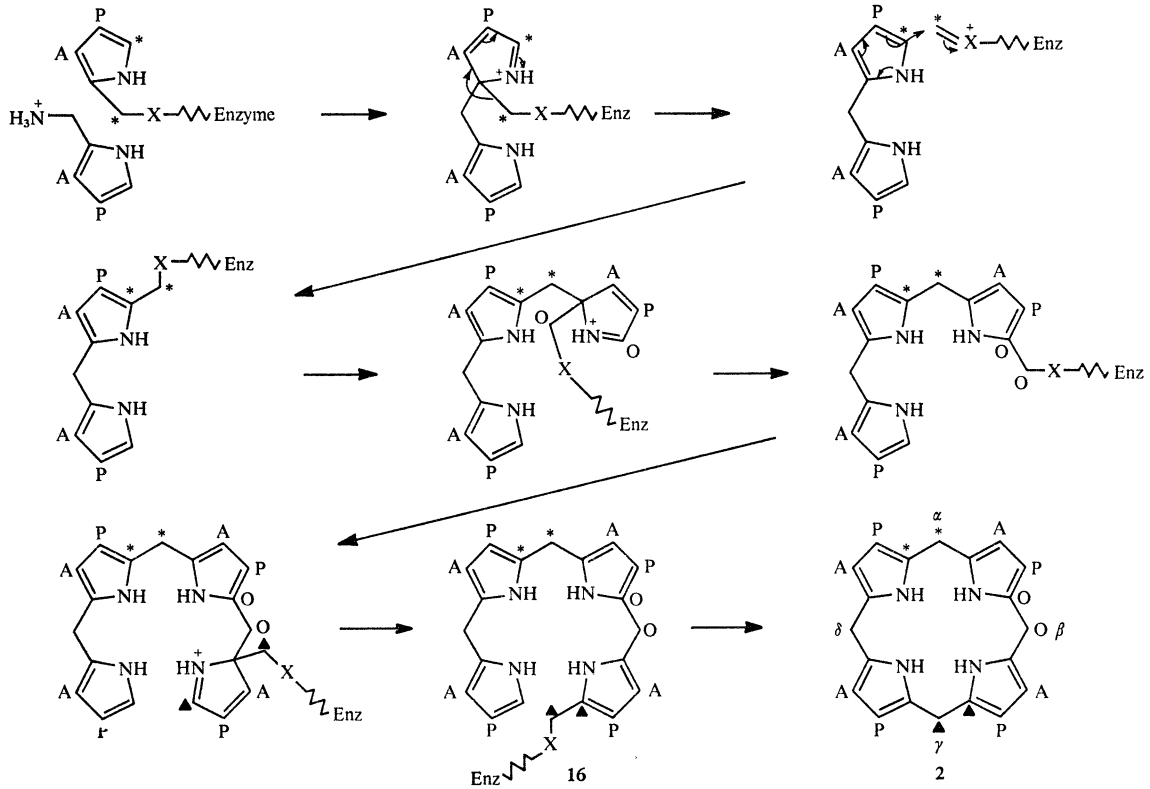
(*a*) *The ^{13}C-approach*

The work with carbon-13 can best be appreciated by considering two of the mechanistic proposals in the literature which aim to explain how the type-III system is formed.

Scheme 3 shows the proposal which involves a 'spiro' intermediate and it is set in a form slightly modified from the original one (Mathewson & Corwin 1961). The straightforwardly constructed bilane (**13**) is considered to undergo ring-closure not to the α-free position of the terminal pyrrole unit (which would yield the type-I isomer) but to the substituted α-position of that unit. The group X in scheme 3 is either the original amino residue or is a nucleophilic group in the enzyme's active site which has displaced the amino group of the first porphobilinogen unit to have been bound. Fragmentation of the spiro intermediate and fresh ring-closure of the methylenepyrrolenine (**15**) so formed brings about reversal of ring-D; the final



SCHEME 3. The 'spiro' hypothesis



SCHEME 4

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product is uroporphyrinogen III (**2**). This scheme involves no rearrangement of three porphobilinogen units and intramolecular rearrangement of the fourth one (see asterisks on scheme 3).

An alternative idea (Robinson 1955) is presented with minor modifications in scheme 4. In this proposal there is head-to-head joining of the first three porphobilinogen units and the methylene group of the original aminomethyl residue is transferred in each case on an 'enzyme arm' so as to make the process in effect intramolecular. When the fourth porphobilinogen unit has been inserted by head-to-tail joining, the resultant bilane (**16**) is ready to ring-close with formation of uroporphyrinogen III (**2**). In contrast to scheme 3 there are three intramolecular rearrangements involved in scheme 4 and, as illustrated, they affect the α, β and γ *meso* carbons. Similar analysis of all the available proposals (and others that had not been published) showed that most differed decisively in the number and type of the rearrangements involved (e.g. intramolecular with respect to a given porphobilinogen unit or not). Invariably these proposed rearrangement processes involved one or more of the *meso*-bridge carbon atoms (α, β, γ and δ in the schemes 3 and 4).

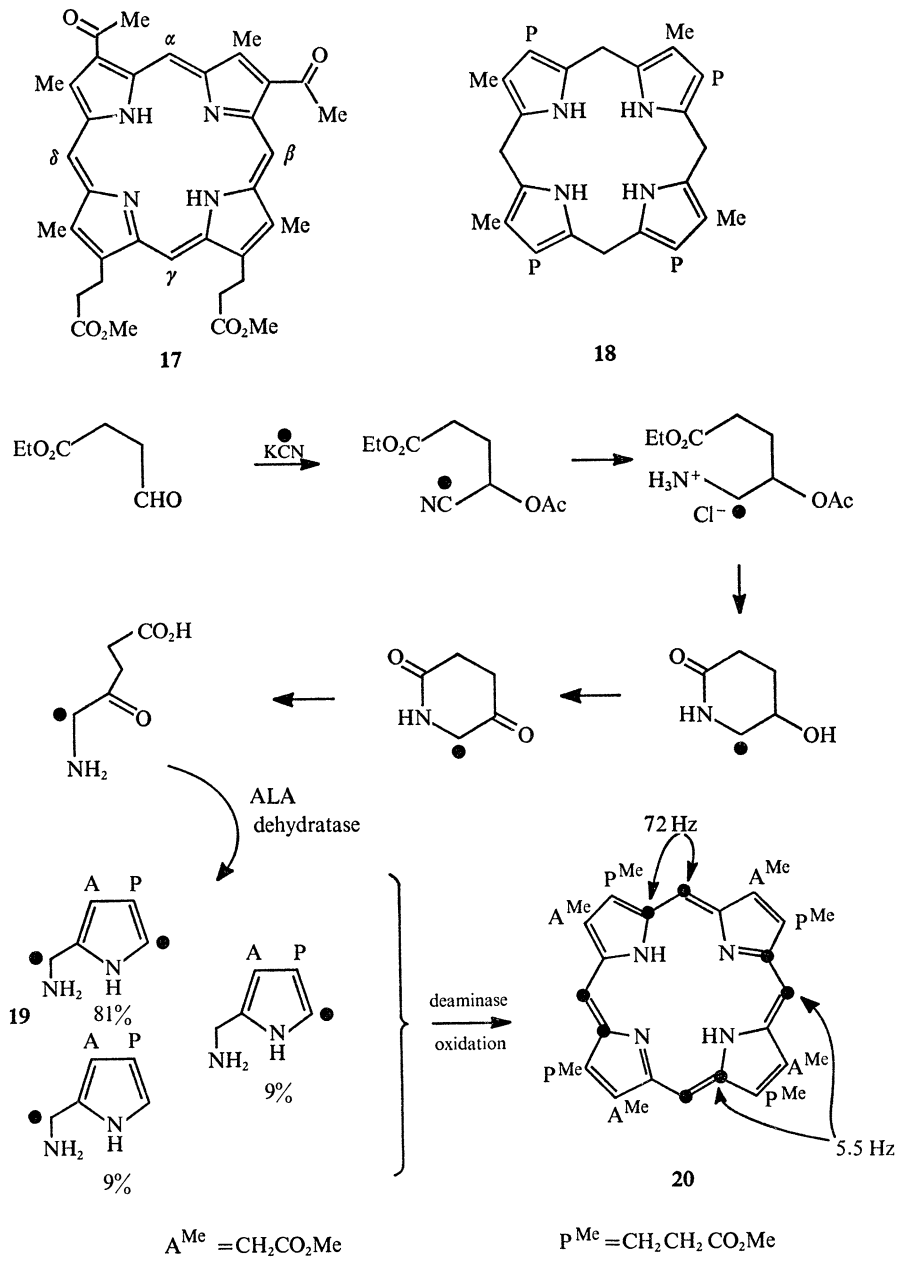
The above considerations led to our plan to study the type III problem by working out the origin and history of each *meso*-carbon atom; e.g. is the α-*meso* carbon still attached unchanged to its original pyrrole unit or not, and if it has rearranged, is the process an intramolecular one or not? Success in this plan depends first on choosing a porphyrin for study in which carbon-13 atoms at these crucially important *meso*-bridges give four *separate* ^{13}C-n.m.r. signals. This was immediately shown to hold good for the dimethyl ester of protoporphyrin IX (**5**), and the four signals from the *meso* positions appeared in the δ_c 96–98 parts/10^6 region (Battersby, Moron, McDonald & Feeney 1972; Battersby, Hunt, McDonald & Moron 1973*e*).

These four signals were assigned to individual *meso*-carbon atoms by unambiguous synthesis of specifically ^{13}C-labelled samples of protoporphyrin IX dimethyl ester (ester of **5**). Three such labelled samples were prepared, one enriched with carbon-13 at the δ-*meso* bridge, another ^{13}C-labelled at the γ-*meso* and the third at the β-*meso* position (Battersby *et al.* 1973*c*). The sequence of signals reading from low to high field was found in this way to be α, β, δ and γ.

The separation of the signals from the α and γ *meso*-carbons of protoporphyrin IX dimethyl ester (ester of **5**) was just under 2 parts/10^6 and by converting this porphyrin chemically into the diacetylporphyrin ester (**17**), the chemical shift difference between the signals at lowest and highest field increased to 6.9 parts/10^6 (Battersby *et al.* 1973*c*; for spectrum at natural abundance see Doddrell & Caughey 1972). The greater this separation, the easier it is to assign the splitting patterns with complete confidence. Chemical conversion of each of the foregoing ^{13}C-labelled samples of protoporphyrin IX dimethyl ester into the corresponding diacetylporphyrin (**17**) showed that for this latter product also, the signals from the *meso*-carbons appeared from low to high field in the same order, namely α, β, δ and γ.

The fundamental base was thus laid down for further ^{13}C-n.m.r. study of the biosynthesis of protoporphyrin IX (**5**). However, it was brought out in the introduction that the rearrangement process which sets up the type-III macrocycle occurs on the pathway after porphobilinogen (**1**) but before uroporphyrinogen III (**2**). Work on protoporphyrin IX can only give information about the nature of the rearrangement if it can be proved that the type-III macrocycle does not change between uroporphyrinogen III (**2**) and protoporphyrin IX (**5**). Two separate studies (Franck, Gantz, Montforts & Schmidtchen 1972; Battersby, Staunton & Wightman 1972) showed this to be so by enzymic conversion of specifically ^{14}C-labelled uroporphyrinogen III

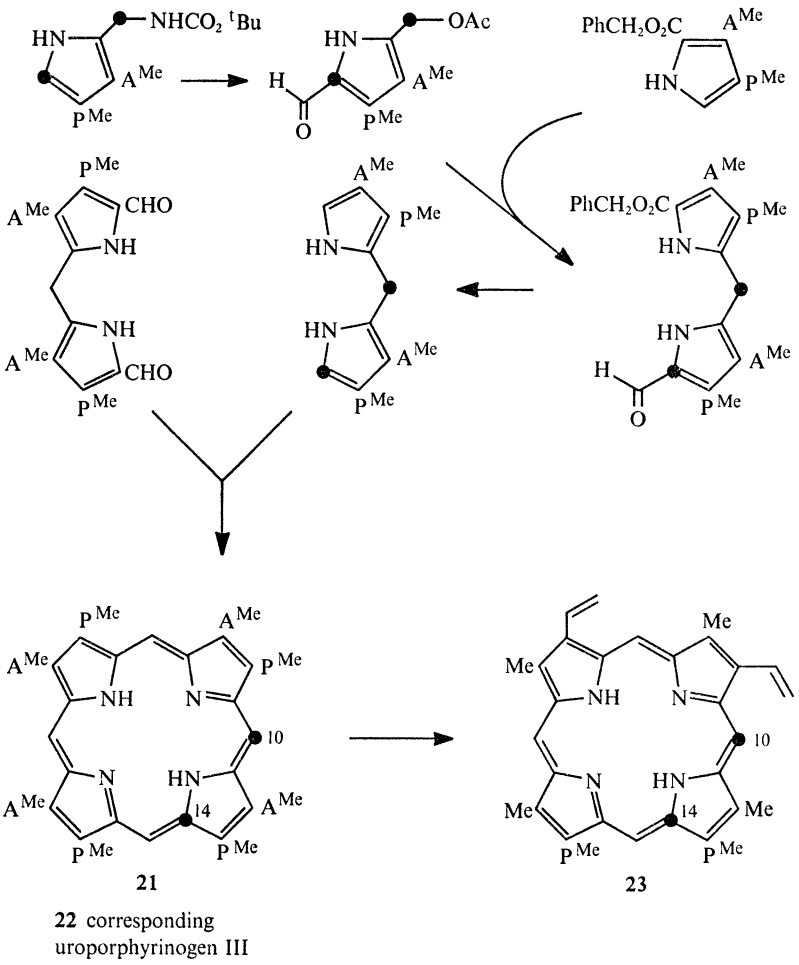
(**2**) and coproporphyrinogen III (**18**) into protoporphyrin IX (**5**). It was found by suitable degradative sequences that the latter porphyrin was in each case labelled as at the outset. Accordingly, what is discovered about the labelling patterns and ^{13}C–^{13}C couplings for protoporphyrin IX (**5**) produced enzymically also holds true for uroporphyrinogen III (**2**) and hence the findings are directly and reliably informative about the rearrangement process.



SCHEME 5

The first need was to prepare porphobilinogen (**19**) doubly labelled with carbon-13 at positions 2 and 11. This was achieved by enzymic condensation of specifically labelled [5-^{13}C]aminolaevulinic acid which had been prepared by the new synthesis illustrated in

scheme 5 (Battersby *et al.* 1973*e*). Since the [5-^{13}C]aminolaevulinic acid carried 90 atom % ^{13}C at C5, it follows that the *ca.* 81 % of the resultant porphobilinogen molecules *contain two* ^{13}*C atoms*. This product was converted by deaminase into uroporphyrinogen I and the ^{13}C-n.m.r. spectrum of the derived porphyrin ester (**20**, scheme 5) showed that the signals from the directly bonded ^{13}C-atoms are split by a 72 Hz coupling (Battersby, Hunt & McDonald 1973*d*). A further 5.5 Hz coupling was revealed by this spectrum and it was assigned to a coupling through three bonds as indicated on structure (**20**). It was recognized that this assignment, if correct, is just as important as the 72 Hz coupling because we will show later that *intact incorporation of a porphobilinogen unit* into a porphyrin can be established by the appearance of the 5.5 Hz coupling in the product's spectrum.

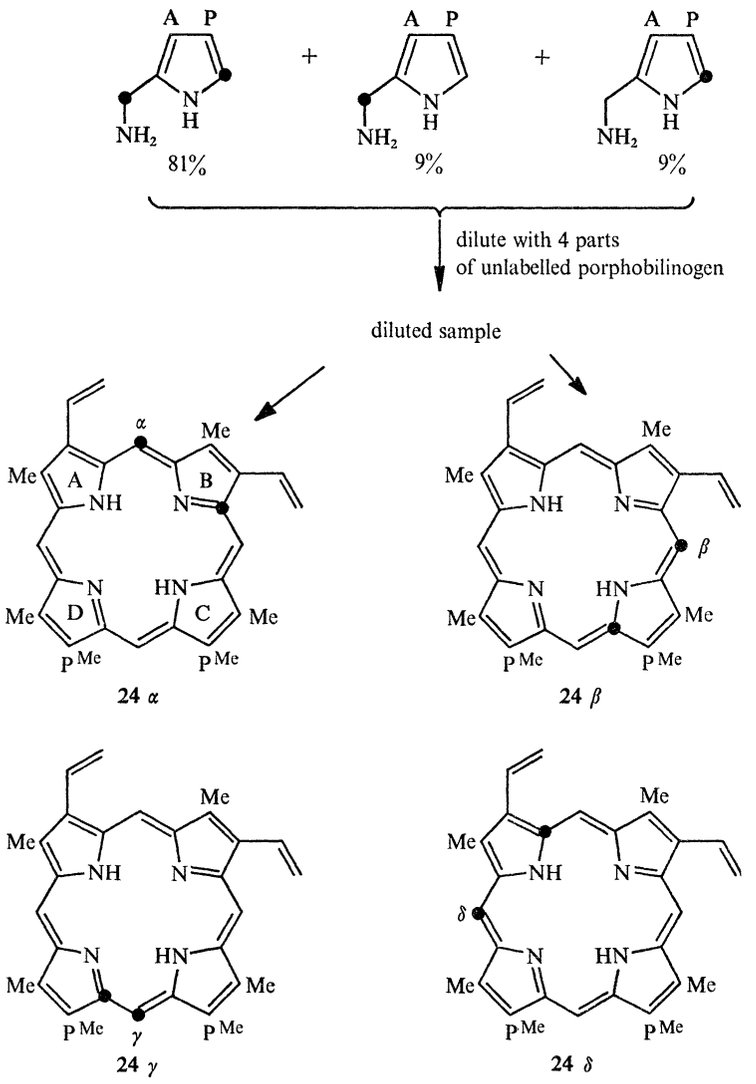


22 corresponding uroporphyrinogen III

SCHEME 6

An assignment of such importance had clearly to be given full experimental backing. For this purpose, a synthesis of protoporphyrin IX dimethyl ester (**23**) was carried out which yielded a product having 81 % of its molecules carrying two carbon-13 atoms at positions 10 and 14 as shown in scheme 6. The only readily available doubly labelled pyrrolic starting material was porphobilinogen (see earlier) and accordingly the first synthetic target became the ester of

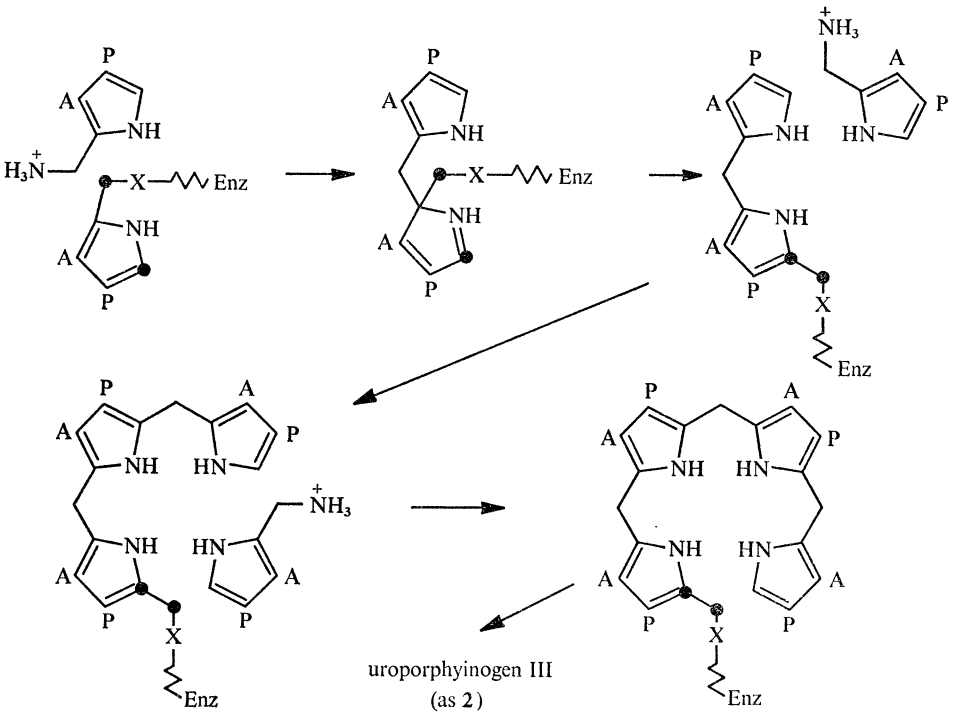
uroporphyrin III (**21**). The final steps through to protoporphyrin IX dimethyl ester (**23**) were carried out enzymically on [10,14-$^{13}C_2$]uroporphyrinogen (**22**) derived by hydrolysis and reduction from the synthetic porphyrin (**21**); the absence of rearrangements during these late enzymic steps has already been emphasized. The ^{13}C-n.m.r. spectrum of the resultant protoporphyrin IX ester (**23**) showed the 5.5 Hz coupling as did the octamethyl ester of the synthetic uroporphyrin III (**21**). This synthetic work set the n.m.r. assignment on a firm basis (Battersby *et al.* 1976*c*).



SCHEME 7

A crucially important step in the further study was dilution of the [2,11-$^{13}C_2$]porphobilinogen (**19**, scheme 5) with four parts of unlabelled porphobilinogen before it was converted into uroporphyrinogen III by the enzyme system isolated from avian erythrocytes (see scheme 7). Such a dilution has the result that the majority of single molecules in the biosynthesized uroporphyrinogen III and in the protoporphyrin IX (isolated as its dimethyl ester) enzymically derived from it, contain only one doubly labelled porphobilinogen unit and so carry only two

^{13}C atoms. If we ignore the small secondary isotope effects, then ring-A will be derived from a labelled porphobilinogen unit in one quarter of the protoporphyrin IX molecules, another quarter will have ring B labelled and similarly for rings C and D. This dilution allows each bridge and pyrrole ring of the final porphyrin to be studied separately; without the dilution, no information would have been gained about the nature of the rearrangement process. The labelled protoporphyrin IX so formed, and the 3,8-diacetylporphyrin (as **17**) which was prepared from it, both gave proton decoupled ^{13}C-n.m.r. spectra in which the signals from the α-, β- and δ-*meso* carbons were all 5.5 Hz doublets whereas that from the γ-*meso* carbon was a 72 Hz doublet. Bearing in mind the rigorous signal assignments and couplings which have already been described, it follows that the labelledprotoporphyrin IX dimethyl ester is mainly composed of the $^{13}C_2$ species (**24**α), (**24**β), (**24**δ), and (**24**γ), scheme 7. The observation that the two 'arms' of the 72 Hz doublet from the γ-*meso* carbon appeared as sharp single lines is in full agreement with these deductions since (**24**γ) is the only type of $^{13}C_2$ molecule present in the mixture which lacks a ^{13}C atom three bonds away from the bridge ^{13}C atom.



SCHEME 8

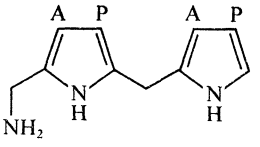
The results outlined in the previous paragraph (Battersby *et al.* 1973*d*; Battersby, Hodgson, Hunt & McDonald 1976*a*) establish the nature of the rearrangement process by which type-III porphyrins are biosynthesized and it is characterized by the following features:

(i) The three PBG units which form ring A and its attached δ-*meso* bridge, ring B and the α-*meso* bridge, and ring C with its β-*meso* bridge are all incorporated *intact without rearrangement.*

(ii) The PBG unit forming ring D is built in *with rearrangement which is intramolecular with respect to that PBG unit.*

(iii) The rearranged carbon atom becomes the γ-*meso* bridge.

These findings put strict limitations on the mechanisms which can be considered for the biosynthesis of type-III porphyrins and most of the 25 or so speculative mechanisms in the literature are eliminated. Several reasonable pathways satisfy the above requirements and schemes 3 and 8 illustrate two; for scheme 8, the intramolecular nature of the rearrangement step is preserved by the migrating methylene residue being carried by some flexible group on the enzyme. *The stage at which rearrangment is proposed to occur* differs in these schemes and so our studies of the intermediates between porphobilinogen and uroporphyrinogen III are important and complementary mechanistic probes.



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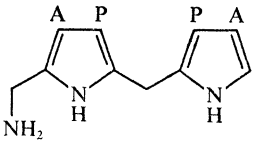

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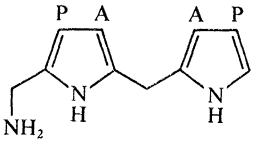

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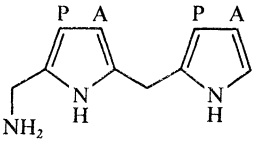

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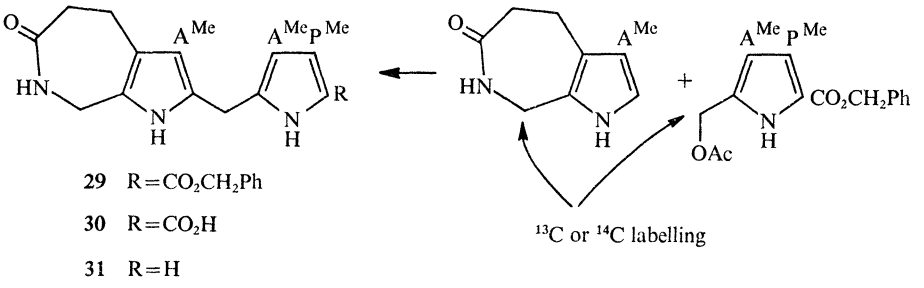

29 R = CO_2CH_2Ph

30 R = CO_2H

31 R = H

SCHEME 9

(*b*) *Enzymic studies with pyrromethanes*

The researches with carbon-13 have established the nature of the process by which the type-III porphyrins are biosynthesized; that is, our work showed exactly *what happens*. The aim of parallel studies with pyrromethanes has been and is to find out *when and how it happens*.

Professor Frydman has already described the important studies he and his colleagues have carried out in this area and so the following survey will deal largely with the researches in Cambridge.

There are four possible aminomethylpyrromethanes in which each pyrrole unit carries one acetic and one propionic residue; and four are AP.AP (**25**), AP.PA (**26**), PA.AP (**27**) and PA.PA (**28**). In this 'shorthand', the sequence of side chains is read from the aminomethyl end. Some of these are more likely to be derived enzymically from porphobilinogen than others but it seemed best at the outset (long before the ^{13}C studies were complete) to study all four

possibilities. The synthetic route used by the three groups involved has followed the same strategy of internal protection of the aminomethyl residue (Frydman *et al.* 1971; Battersby 1971; Ogersby *et al.* 1972; Battersby *et al.* 1973*a*; Battersby, Beck & McDonald 1974*a*; Valasinas, Levy & Frydman 1974). As an example, our synthesis of PA.AP (**27**) is illustrated in scheme 9; the other three isomers were prepared similarly.

It will be seen later that the deaminase-cosynthetase enzyme system which actively forms uroporphyrinogen III from porphobilinogen is reluctant to accept any pyrromethane system. Considerable quantities of the pyrromethanes have therefore to be used in the incubation mixtures even to compete inefficiently with porphobilinogen. In these circumstances the presence of small amounts of impurities or other isomers in the synthetic pyrromethanes could be disastrous and considerable effort has been put into the task of eliminating any doubt on this score. Firstly, all four benzyl esters (**29** and isomers) and all four α-free lactams (**31** and isomers) have been obtained crystalline and have been rigorously purified. Further, conditions have been developed using high-pressure liquid chromatography which allow complete separation of the four benzyl esters (**29** and isomers) each from the others and in a separate run, all four α-free lactams (**31** and isomers) have been cleanly separated. All our crystalline pyrromethanes both labelled and unlabelled were shown by this powerful method to be free from isomeric pyrromethanes and from other impurities. The labelled pyrromethanes carried carbon-14 or carbon-13 at the inter-pyrrolic methylene group or at the methylene of the aminomethyl residue (Battersby *et al.* 1973*a*; Battersby *et al.* 1974*a*; Battersby, McDonald & Markwell 1974*e*).

Alkaline hydrolysis of the four α-free lactams (**31** and isomers) released the aminomethyl group to yield the pyrromethanes **25**, **26**, **27** and **28**. It was found that the ^{1}H-n.m.r. spectra of these substances could be grouped into two very similar pairs one comprising AP.AP (**25**) and PA.AP (**27**) and the other being from AP.PA (**26**) and PA.PA (**28**); the two pairs differed appreciably. However, when the spectra were of high quality, the partners in the similar pairs could also be clearly distinguished. These findings are of importance for current and future work on the isolation and positive identification of biosynthetic intermediates.

With our base thus secure, we can now consider the results of incorporation experiments with the four aminomethylpyrromethanes. However, the reader has to appreciate some of the problems which confront workers handling aminomethylpyrromethanes and he also needs to be familiar with various experimental methods before he can sensibly assess the results which have been obtained.

A real difficulty for incorporation experiments with aminomethylpyrromethanes is their *non-enzymic conversion* into porphyrinogens which produces a troublesome blank. Enzymic incorporation has thus to be recognized as an increase above this often large blank value. Despite these severe problems, the picture is steadily becoming clearer.

All the incorporation experiments so far by our group and by others (see Frydman *et al.*, this volume, and Battersby & McDonald 1975) have been by one or other of two methods. The first involves incubation with the enzyme of excess *radioinactive* pyrromethane with [^{14}C]porphobilinogen; this will be called the *dilution method*. If the resultant uroporphyrin III (**38**) has a lower molar radioactivity than that isolated from the blank experiment, then the interpretation is that enzymic incorporation has occurred; and conversely, if no lowering is found, then this is interpreted as showing no incorporation.

The dilution method requires great accuracy in determinations of molar activities (since the blank is usually large) and also high chemical purities of starting materials and products.

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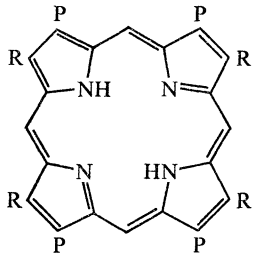
The second method is the *direct one* of incubating radioactive pyrromethane and radioinactive porphobilinogen with the deaminase-cosynthetase enzyme system. Clearly this method requires complete radiochemical and chemical purity of the isolated porphyrin.

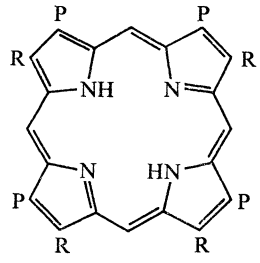
We can now consider the results we have obtained so far. The *AP.PA pyrromethane* (**26**) did not act as a substrate alone or in the presence of porphobilinogen for the deaminase-cosynthetase enzyme system from duck erythrocytes or *Euglena gracilis* as judged by the essentially complete lack of radioactivity in the isolated protoporphyrin IX. In the absence of enzyme, this pyrromethane (**26**) underwent chemical (non-enzymic) conversion into uroporphyrin II (**32**) which was pure as far as could be judged by the chromatographic methods used at the time (t.l.c. on cellulose); this material has not so far been studied by high-pressure liquid chromatography (h.p.l.c., see later), (Battersby *et al.* 1973 *b*). The foregoing results and those of Professor Frydman's group are in agreement.

Our further studies with the ^{14}C-labelled *PA.PA pyrromethane* (**28**) showed that this also fails to act as a precursor of the type-III macrocycle when incubated with porphobilinogen and the enzyme system from *Euglena gracilis* (Battersby *et al.* 1974*e*). Indeed, it would have been surprising if the PA.PA pyrromethane (**28**) had been incorporated because there is no way we can envisage for the formation of (**28**) which does not run counter to the strict requirements of the ^{13}C-studies on page 170; the two approaches thus reinforce each other.



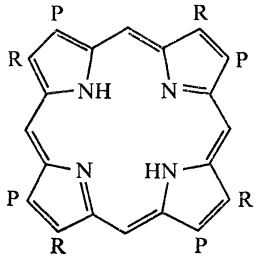
32 R=A, uroporphyrin II

33 R=Me, coproporphyrin II



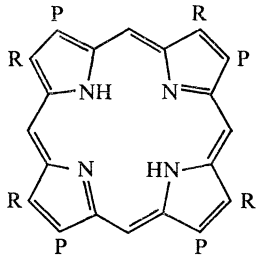
34 R=A, uroporphyrin IV

35 R=Me, coproporphyrin IV



36 R=A, uroporphyrin I

37 R=Me, coproporphyrin I



38 R=A, uroporphyrin III

39 R=Me, coproporphyrin III

A=CH_2CO_2H P=$CH_2CH_2CO_2H$

SCHEME 10

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The foregoing experiments made use of enzyme systems which carry the biosynthesis through to protoporphyrin IX (**5**) and this product was assayed for radioactivity. Other experiments involved isolated deaminase-cosynthetase preparations and these led to mixtures of uroporphyrin isomers (see scheme 10) produced partly by enzymic synthesis and partly by chemical conversion. One immediately faces the problem of determining which of the four uroporphyrins is (or are) labelled, and to what level, for both the enzymic runs and for the control runs with boiled enzyme. Since uroporphyrins are almost quantitatively converted by hot acid into the corresponding coproporphyrins (Falk 1964), an extensive study of h.p.l.c. for the separation of the four coproporphyrin isomers has been carried out. As a result, conditions have been established for complete separation of the tetraethyl ester of coproporphyrin I (**37**) from that of coproporphyrin II (**33**) and both quantitatively from the mixture of coproporphyrins III (**39**) and IV (**35**) on reverse-phase columns with 10 μm packings. A micro flow-cell monitor set on the Soret band and coupled to a recorder and integrator provide a direct picture of the separation. The collected mixture of tetraethyl coproporphyrins III (**39**) and IV (**35**) is converted by transesterification into the tetramethyl esters which are then separated on a column of 10 μm silica particles. These methods have the advantage of allowing accurate collection of pure products or of fractions across a particular peak.

Our incorporation experiments on biosynthesis of the type-III macrocycle with the pyrromethanes AP.AP (**25**) and PA.AP (**27**) in the presence of porphobilinogen were carried out mainly before the foregoing h.p.l.c. work and partly after that development. When the intact enzyme systems from *Euglena gracilis* or avian etythrocytes were used, radioactivity was found in the isolated protoporphyrin IX (**5**) from both ^{14}C-labelled AP.AP (**25**) (range 1.6–9.0 % with duck erythrocytes and 0.2–0.5 % with *Euglena*) and from ^{14}C-labelled PA.AP (**27**) (range 0.06–0.12 % with duck erythrocytes and 0.01–0.015 % for *Euglena*). It will be clear, however, from the points made above that *some or all* of the [^{14}C]protoporphyrin IX may arise from [^{14}C]uroporphyrinogen III (as **2**) which has been produced chemically with rearrangement from the pyrromethanes.

The h.p.l.c. separations having been developed, the way is open to determine accurately the radioactivity in the various uroporphyrin isomers from control runs and enzymic experiments with AP.AP amd PA.AP with purified deaminase-cosynthetase preparations.

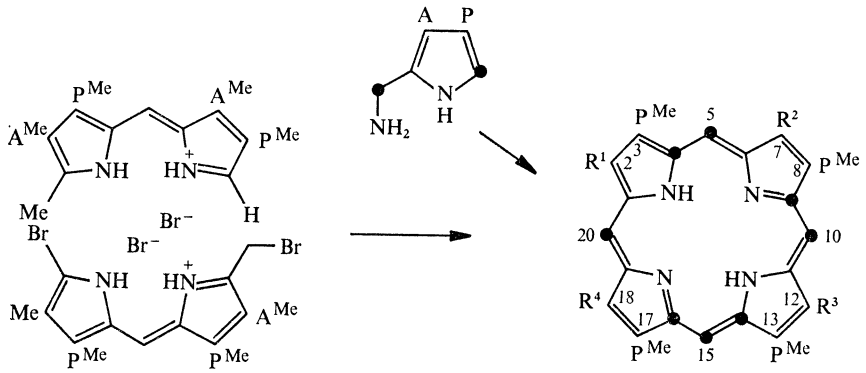
Our conclusion at present is that AP.PA (**26**) and PA.PA (**28**) need be considered no further but that neither AP.AP (**25**) nor PA.AP (**27**) can yet be rigorously excluded as being the intermediate (or related to the bound intermediate) on the pathway from porphobilinogen (**1**) to uroporphyrinogen III (**2**). (Battersby *et al.* 1973*b*; Battersby, Hodgson, McDonald & Markwell 1974*b*).

To look beyond the present stage, it must be shown that the proven enzymic incorporation is a specific one (i.e. without randomization) and the site(s) of labelling must be determined. These are very difficult requirements and they have not been met so far for any porphyrin proved to have had a pyrromethane enzymically incorporated into its macrocycle.

Enzymic decarboxylation of uroporphyrinogen III

We can turn now to those biosynthetic stages immediately following uroporphyrinogen III (**2**) and which precede coproporphyrinogen III (**3**). Earlier studies had clearly indicated that enzymic decarboxylation of the four acetic acid side chains is a stepwise process (Bogorad &

Granick 1953; Dresel & Falk 1956; Mauzerall & Granick 1958; Frydman, Tomaro, Wanschelbaum & Frydman 1972). So it was of considerable interest that we consistently isolated a porphyrin from incubation of porphobilinogen with the enzyme system from duck erythrocytes which was found by mass spectrometry to be a heptacarboxylic acid, $C_{46}H_{52}N_4O_{14}$ as its heptamethyl ester. It gave coproporphyrin III (**39**) on decarboxylation showing it to be a type III isomer and its properties agreed with those of phyriaporphyrin III (Batlle & Grinstein 1964). This substance has also been called porphyrin-208 and pseudo-uroporphyrin (Grinstein, Schwartz & Watson 1945; Falk, Dresel, Benson & Knight 1956). Four structures (**40**, **41**, **42** and **43**) must therefore be considered for the heptamethyl ester of this porphyrin and the possibilities were further reduced by enzymic preparation of the porphyrin from undiluted [2,11-$^{13}C_2$]porphobilinogen which generated the porphyrin with 90 atom % ^{13}C at each labelled site (see scheme 11). The ^{13}C-n.m.r. signals from positions 15 and 20 could be unambiguously assigned by their unique patterns of 72 Hz triplet and 5 Hz triplet, respectively (see Battersby *et al.* 1973*d*) whereas those



44

40 R^1=Me, R^2=R^3=R^4=A^{Me}
41 R^2=Me, R^1=R^3=R^4=A^{Me}
42 R^3=Me, R^1=R^2=R^4=A^{Me}
43 R^4=Me, R^1=R^2=R^3=A^{Me}

SCHEME 11

from positions 5 and 10 were both double doublets (J = 5 and 72 Hz) and so will be considered together. When the praseodymium shift reagent Pr([$^2H_9$]fod)$_3$ was added stepwise to the labelled heptamethyl ester, it was the signal from position 20 which moved upfield *least* for a given addition of shift reagent. For ^1H spectroscopy, lanthanide shift reagents affect most strongly the signals from protons at the *meso*-carbons flanked by two ester groups (Stoll *et al.* 1973). So our results strongly indicated that the least affected *meso*-carbon (C 20) is flanked by the C-methyl group; this leads to structures **40** and **43**, referred to as the C2 methyl and C18 methyl porphyrins. The former possibility was eliminated by synthesis of **40** by the MacDonald method and the product differed from the ester of the natural sample. The C7 and C12 methyl porphyrins **41** and **42** were also synthesized in a similar way and were readily distinguishable from the natural material so the deductions from the ^{13}C-n.m.r. study were confirmed. With structures **40**, **41** and **42** shown not to correspond with the ester of the natural sample, it follows that the correct structure is the C18 methyl porphyrin (**43**) (Battersby *et al.* 1974*c*). This structure has been confirmed during the preparation of this manuscript by unambiguous synthesis by

Dr J. B. Paine III using the pyrromethene method (Harris, Johnson & Kay 1966) as in scheme 11. A sample of this porphyrin (**43**) was previously synthesized by Professor Clezy and his co-workers (University of New South Wales) and kindly sent to us and this too has been proved to be identical with the ester of our natural product; we wish to thank Professor Clezy for his material.

The above isolation of the heptacarboxylic porphyrin corresponding to structure **43** *probably* means that enzymic decarboxylation of uroporphyrinogen III (**2**) is initiated at the acetic acid residue on ring D. This is of particular interest in relation to the next topic, that of the biosynthesis of corrins and vitamin B_{12}, which arises logically from the foregoing studies on uroporphyrinogen III (**2**) and the heptacarboxylic porphyrins **40**, **41**, **42** and **43**. A bridge is thus built to subsequent lectures in this Discussion.

Biosynthesis of corrins and vitamin B_{12}

The historical aspects and developments up to the early 1970s with leading references are covered in Professor Scott's paper and it suffices to say here that the corrin macrocycle of vitamin B_{12} (**48**) had been shown to be biosynthesized from porphobilinogen (**1**), derived from 5-aminolaevulinic acid (**45**), with methionine acting as the source of methyl groups. The important observation had also been made that the methyl group at C1 of corrins is inserted from methionine and is *not* a modified residue from C11 of porphobilinogen (equivalent to C5 of aminolaevulinic acid). It was further known that vitamin B_{12} itself (**48**) and the corresponding coenzyme are constructed *in vivo* from cobyrinic acid (**46**) (Bernhauer *et al.* 1968).

The similarity of corrins to natural porphyrinogens and especially the 'type-III' pattern of acetic and propionic side chains in corrins (see **46**) had long caused speculation (Porra 1965; Burnham & Plane 1966), as to whether corrins are biochemically derived from uroporphyrinogen III (**50**). This question thus became a key one on which future progress depends and there have been differing results from different groups. Incorporation experiments with *Propionibacterium shermanii* involving unsymmetrically labelled [5,15-^{14}C]uroporphyrinogen III (as **50**) gave negligible incorporations into vitamin B_{12} (**48**) (Franck *et al.* 1972). However, positive results were obtained when uroporphyrinogen III and isomer(s) were used carrying labels at each of the propionic side chains (Scott *et al.* 1972, 1974; Scott, this volume). It was generally recognized that with such symmetrical labelling, either intact incorporation or incorporation following breakdown would give the same result.

A single label at the methylene of the acetic acid residue on ring C of uroporphyrinogen III (**50**) was chosen for the following studies. If intact incorporation occurs, it will lead to labelling only at the *pro-S* methyl group at C12 of the corrin system (Battersby *et al.* 1973*f*, 1974*d*; Scott, Townsend & Cushley 1973) whereas incorporation as a result of breakdown will cause scatter of activity.

[^{14}C]Uroporphyrin III (**52**) was synthesized in specifically labelled form as illustrated from the pyrrole (**54**) (Battersby *et al.* 1975*b*) and the macrocycle was reduced to the corresponding [^{14}C]uroporphyrinogen III (**50**). This was incubated with a broken cell preparation from *Propionibacterium shermanii* containing a range of cofactors and the cobyrinic acid (**46**) formed was isolated by dilution. The derived dicyano cobester (**47**) was rigorously purified and its constant specific activity corresponded to an absolute incorporation of 4.7 %. Ozonolysis of the labelled cobester (**47**) by the method of Eschenmoser (Dubs, Kesse, Werthemann & Eschenmoser 1968)

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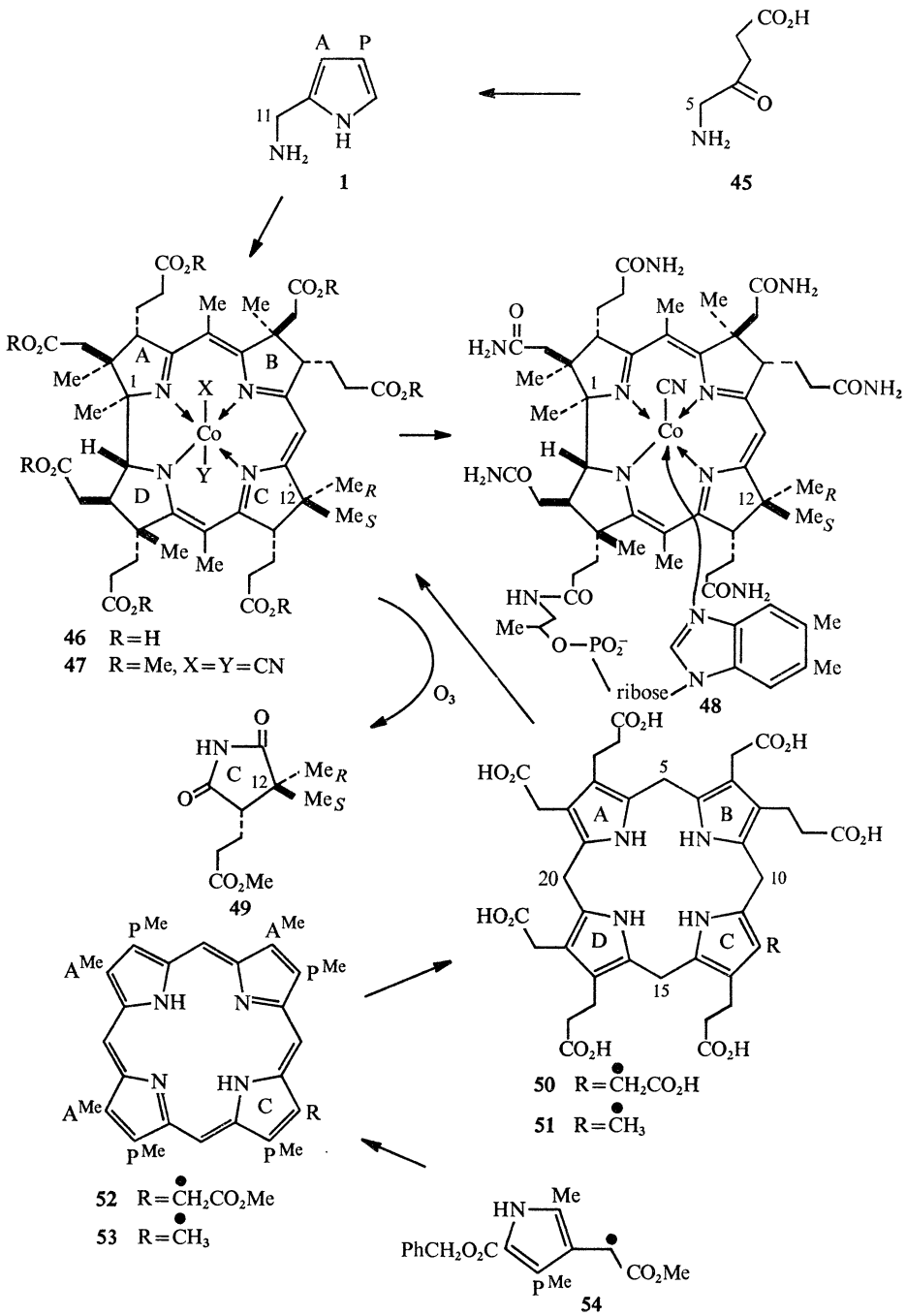
gave the ring-C imide (**49**) which in molar terms carried 90 % of the radioactivity of the original cobester (**47**). This work provides rigorous proof for the biological derivation of the corrin macrocycle (**46**) from uroporphyrinogen III (**50**).

The possibility that the C12 methyl heptacarboxylic acid (**51**) follows uroporphyrinogen III (**50**) on the biochemical pathway to cobyrinic acid (**46**) is now being tested with the illustrated labelled form (**51**) prepared from the totally synthetic porphyrin (**53**) (Battersby *et al.* 1974*c*).



SCHEME 12

Now that the pathway from porphobilinogen (**1**) to cobyrinic acid (**46**) is signposted by uroporphyrinogen III (**50**) as a macrocyclic marker, the problem of determining the mechanism by which uroporphyrinogen III is converted into corrin can be attacked. One could not wish for a more fascinating problem than this or those posed by the type-III macrocycle of the porphyrins. The next five years should be exciting.

The progress described in this lecture could not have been made without the invaluable work over the past five years of an outstanding group of colleagues and we wish to emphasize their contributions. Drs G. L. Hodgson, E. Hunt, M. Ihara and J. Saunders carried out the mechanistic researches using carbon-13. The studies on the synthesis and chemistry of pyrromethanes, h.p.l.c. separations and enzymic work with pyrromethanes were by Drs J. F. Beck, D. G. Buckley, D. A. Evans, K. H. Gibson, L. Mander, R. E. Markwell, J. Moron, L. N. Nixon and J. Saunders. Finally, Drs F. Satoh, E. Hunt, M. Ihara, J. B. Paine III, J. R. Redfern (née Stephenson) and D. C. Williams carried out the work on the heptacarboxylic porphyrins and on vitamin B_{12}.

We are grateful for this opportunity to record our warmest thanks to all of them and to the Nuffield Foundation and S.R.C. for financial support.

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Discussion

J. W. Cornforth (*Shell Research Ltd, Sittingbourne, Kent*). I wonder if the question of intermediates in the assembly of the porphyrinogen ring may be complicated by covalent binding of PBG (for example) to a group on the enzyme by displacement of ammonia. One has a similar situation with the fatty-acid synthases, which will accept acetate and malonate as thiolesters and turn out palmitic acid. Some reasonable 'intermediates' in this synthesis are not accepted as normal substrates when supplied as thiolesters; the growing chain in the normal synthesis is always bound to one of two enzymic sulphydryl groups.

A. R. Battersby. Displacement of ammonia from PBG by a nucleophilic group on the enzyme is undoubtedly a possibility and this reaction forms the basis of a speculative scheme we put forward recently (Battersby & McDonald 1975). Current work by Graham Morgan is designed to test the idea of such a displacement.

B. Frydman (*Facultad De Farmacia y Bioquimica, Universidad de Buenos Aires*). I would like Professor Battersby to comment on how he can distinguish between the chemical formation of a protoporphyrin resulting from the chemical dimerization of the dipyrrylmethane he calls AP.AP, and the enzymic formation of protoporphyrin IX at the expense of the former dipyrrylmethane. In our paper we presented evidence that dipyrrylmethane AP.AP is dimerized to uroporphyrinogen IV and the latter is enzymically transformed by the whole system into a protoporphyrin isomeric with protoporphyrin IX. Since in your system there is such a large endogenous pool of protoporphyrin IX, it is very likely that the latter will dilute the isomeric protoporphyrin formed

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at the expense of uroporphyrinogen IV and it will be very difficult to separate them. In this case the incorporation of label which you detect in protoporphyrin IX at the expense of the dipyrrylmethane AP.AP will be due only to a chemical artefact and will not reflect the negative enzymatic results. I would like Professor Battersby to comment on how he prepares his control experiments in order to exclude this possibility.

A. R. Battersby. There are two points to cover here. Taking first the possibility of carrying with the natural protoporphyrin-IX an isomer of it formed from coproporphyrinogen-IV. The formation of such an isomer was known from the earlier work of Porra & Falk (1964 *Biochem. J.* **90**, 69) and the extensive precautions we took in examining the radiochemical purity of our isolated protoporphyrin-IX have been outlined; you can see that we applied every method then available for purification including chemical modification and degradation with proof of constant specific activity at each stage. Current work is aimed at the preparation and synthesis of the pure porphyrin derived from coproporphyrinogen-IV and at its separation from protoporphyrin-IX by high-pressure liquid chromatography (h.p.l.c.).

The second point is that the distinction between a small enzymic formation of a porphyrinogen from PBG and a pyrromethane and the usually larger chemical formation is a problem we all face. Controls were done strictly in parallel with boiled enzyme (or equivalent buffer). It is precisely the common difficulty of the blank which leads us to keep an open mind at present about the status of AP.AP and PA.AP, and in addition, we feel the point made by Professor Cornforth is important. Our current work makes use of the h.p.l.c. separations outlined in our paper. A rigorous answer depends on showing not only which one is significantly incorporated but also on determining whether the resultant labelling of the porphyrin is specific or scattered. The latter result would not be a helpful one.

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