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# RECENT RESEARCHES ON THE BIOSYNTHESIS OF NATURAL PRODUCTS

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Abstract - The first part of the lecture covers research on the mechanism and stereochemistry of formation of the vinyl groups of protoporphyrin-IX and so of haem. This section leads into a survey of current biosynthetic investigations on vitamin $B_{12}$. Illustrations are given of the use of broken-cell enzyme preparations, carbon-13, carbon-14 and true double labelling for studying a molecule of the complexity of this vitamin.

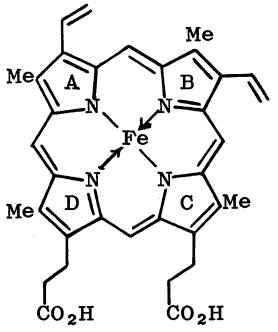
This is a particularly exciting time to be studying the way living systems synthesise the often complex natural products they produce. The reasons for this being so can perhaps be brought out by thinking back to the 4th IUPAC Symposium on Natural Products held in Stockholm in 1966. My aim at that time was to illustrate the way in which structure determination, chemical intuition and hypothesis, tracer experiments and isolation work were combining together in a remarkably powerful way for research on elucidation of biosynthetic pathways (Ref. 1). These mutually reinforcing approaches are as important ten years later as they were then and, thank goodness, nothing can replace what Hans Fischer would have called "phantasie" - the imagination of the researcher. In fact, the recent changes have mainly been in the new tools and techniques which have come available over this ten year period. One of these is Fourier transform n.m.r. spectroscopy, especially for carbon-13 but also for protons; important too have been the development of high pressure liquid chromatography and of the sophisticated mass spectrometric methods for work with unstable or involatile substances. Finally there have been major advances in methods for separating and handling enzymes.

It was clear to us, as these techniques gradually improved, that here was a golden opportunity. By combining these methods with the earlier approach and building in the power of organic chemistry, one could attack problems which were far beyond our reach in 1966. And one could anticipate deeper and more adventurous penetration into the problems which held our interest.

We can illustrate all these aspects by concentrating first on haem (1) whose function in the oxygen-binding sites of haemoglobin and myoglobin is of course well known. Later I want to turn to vitamin $B_{12}$ (3), a complex cobalt-containing corrin which is one of Nature's most beautiful gifts to the organic chemist. These substances and their relatives including the chlorophylls (*e.g.* 5) and the cytochromes (*e.g.* 4) act as pillars for the various forms of life. Clearly a full knowledge of the biosynthesis of this family of related macrocycles and of the relationship of their structures to function are of fundamental importance for our understanding of living systems.

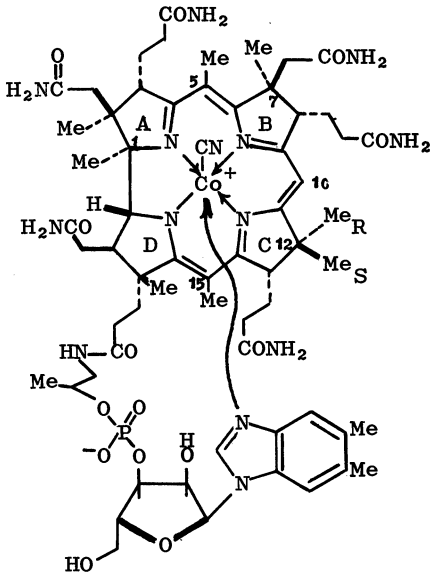
Looking again at haem (1), we can see three different types of groups around the periphery of the macrocycle, *viz.* methyl, vinyl and propionic acid. Only the last type is present as such from the early stages of the biosynthetic process; the others are fashioned step by step in a beautiful series of enzymic reactions. A set of intermediates were pin-pointed as the main markers on this biosynthetic pathway by the pioneering work of Shemin, Granick, Neuberger, Bogorad and Rimington with others too. It would be out of place to attempt here a detailed survey of these researches from the 1950's and 1960's but it is important to emphasise what marvellous progress was made at that time (see Ref. 2). Thus the primary pyrrolic building block for haem (1) was found to be porphobilinogen (6), [shortened to PBG in the sequel]. This is converted enzymically into uro'gen-III (7,

Note a) by an amazing process involving two enzymes which mediate a ring formation with a single intramolecular rearrangement; this rearrangement has been and is of great interest to us, but that is another story. Then decarboxylation of the acetic acid side-chains occurs to produce the four C-methyl groups of copro'gen-III (8) before the vinyl groups are generated.

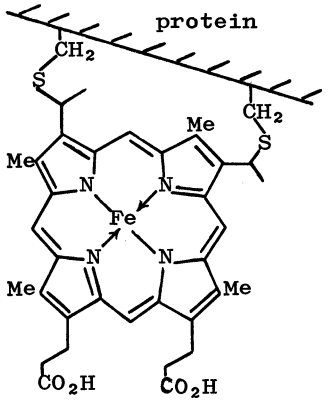


(1) Haem

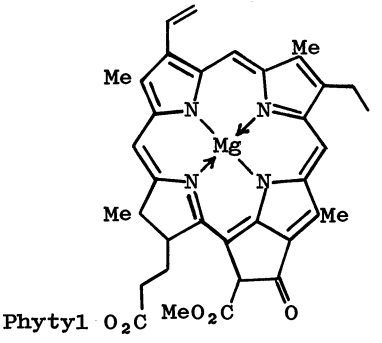
(2) Corresponding metal-free porphyrin = Protoporphyrin-IX



(3) Vitamin $B_{12}$



(4) Cytochrome c



(5) Chlorophyll a

The resultant proto'gen-IX (9) is then aromatised enzymically to yield protoporphyrin-IX (2) ready for iron insertion; this step too is catalysed by an enzyme and the final product is haem (1); (Ref. 2 also surveys recent work on the foregoing conversions).

Nature does not invariably call a halt when the vinyl groups of protoporphyrin-IX (2) have been built. These groups are the anchors by which haem is covalently attached to the protein of cytochrome c (4) and the vinyl residue on ring-B of (2) is reduced at some stage during the biosynthesis of the chlorophylls (e.g. 5) (Refs. 2, 3). Obviously, the redox and other properties of the macrocyclic complex will be affected by the presence or absence of vinyl groups and by the greater or lesser conjugation of vinyl

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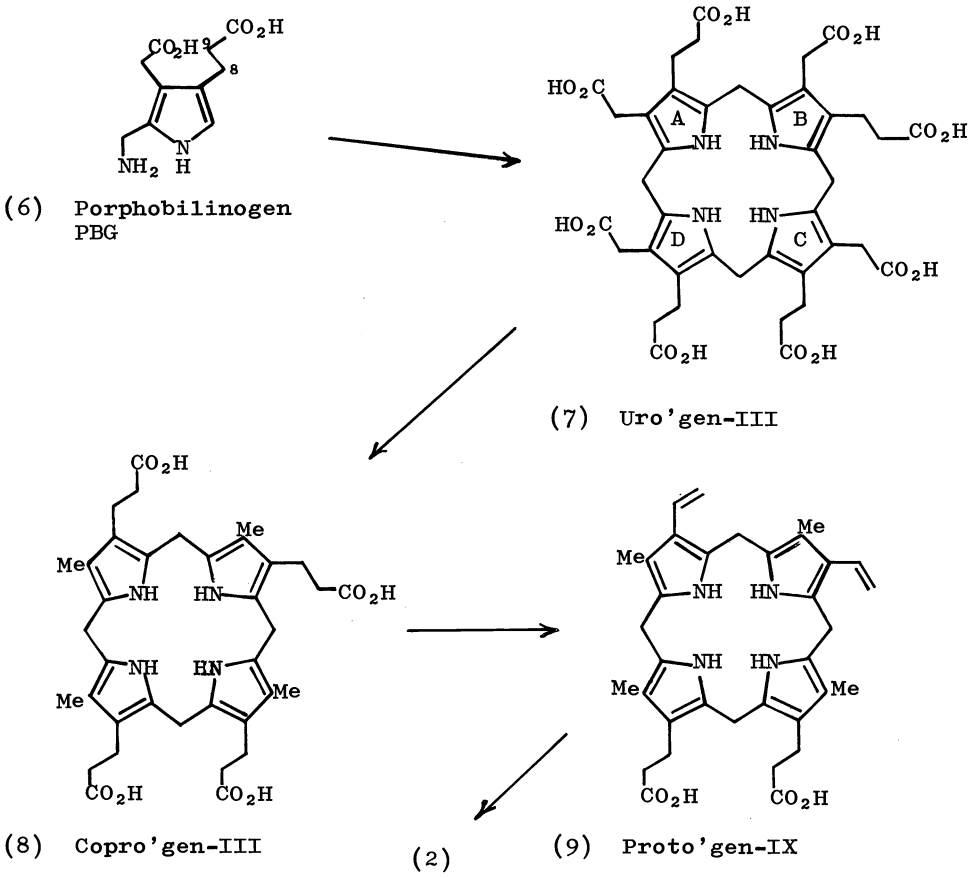
Note a. The full name for this product is uroporphyrinogen-III but shortened forms will be used for this and the other porphyrinogens throughout the lecture.




Scheme 1

with the main macrocycle depending on the relative angle at which they are held. These could be among Nature's methods for "fine tuning". At any rate, the main point to emphasise is that the vinyl groups are both important and chemically interesting. Our aim in the work I want to cover next is to discover, in as complete detail as possible, how the vinyl groups of protoporphyrin-IX (2) are formed.

There are several possible ways in which the conversion of a propionate group into vinyl could be achieved and three are shown in Scheme 2. The illustrated routes A, B and C are attractive in being both mechanistically and biochemically sound.

Our early work to decide among these routes is published (Ref. 4) and parallel studies were carried out by Professor Akhtar (Ref. 5), so it is only necessary to summarise briefly the main findings. We used an enzyme system from Euglena gracilis which converts PBG (6) on a preparative scale into a mixture containing coproporphyrin-III (the aromatised form of 8) and protoporphyrin-IX (2) (Note b). The enzyme in this enzymic cocktail which is responsible for transforming the propionate group into vinyl is called coproporphyrinogenase. Experiments with synthetic PBG (6) $^2H_2$-labelled in one sample at C-8 and in the other at C-9 (see 6) allowed proof that all eight deuterium atoms were retained as expected at the copro'gen-III stage (8, Scheme 1) in both experiments. However, the

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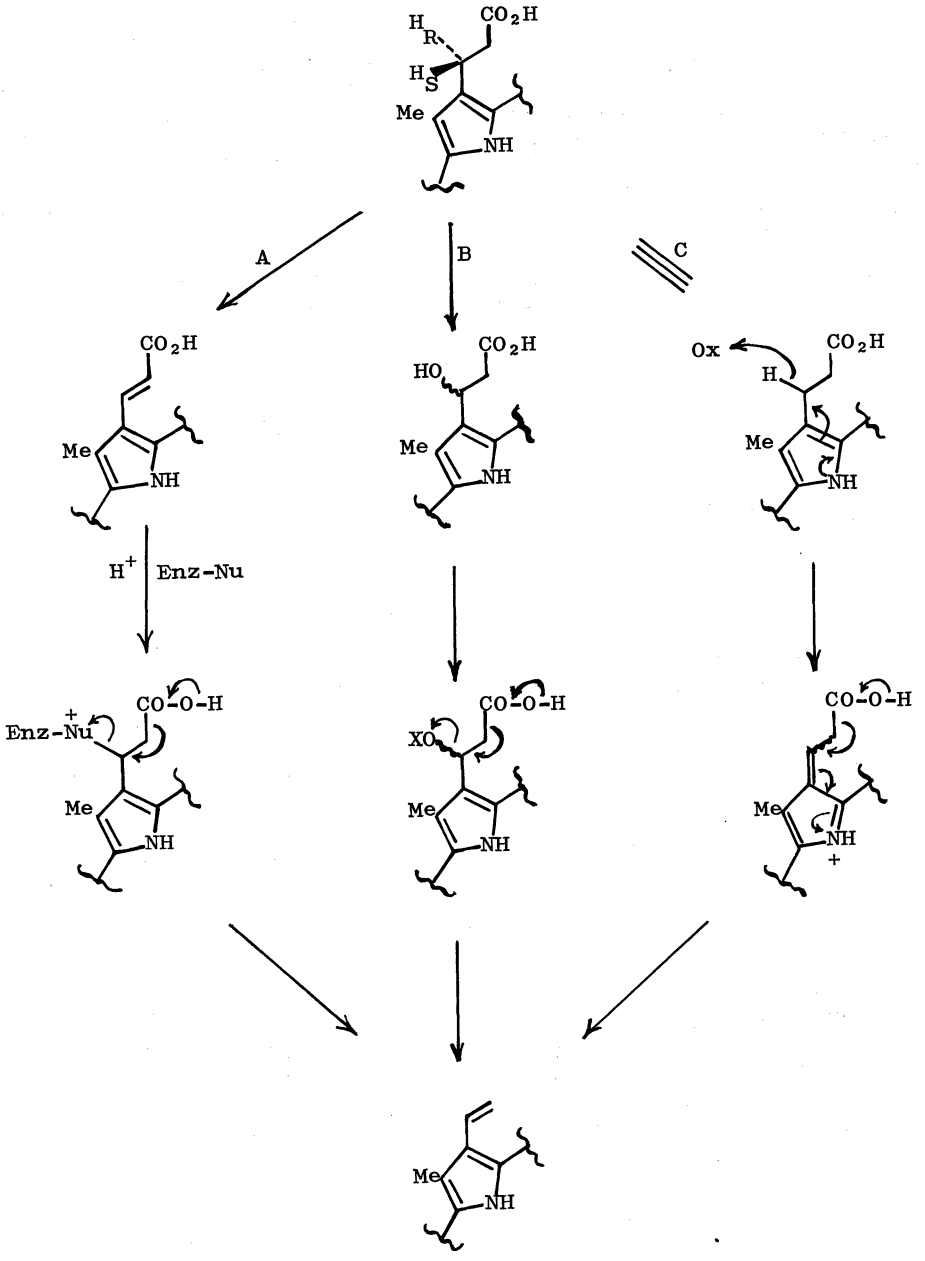
Note b. The mixture initially contains copro'gen-III (8) and proto'gen-IX (9) which are converted chemically into the corresponding porphyrins for isolation.




Scheme 2

copro'gen-III (8) derived from [8-$^2H_2$]PBG lost two deuterium atoms as it was converted into proto'gen-IX whereas the copro'gen-III (8) formed from [9-$^2H_2$]PBG lost none. Clearly the three hydrogen atoms on each of the vinyl groups of protoporphyrin-IX (2) are three of the four originally present in the propionate side chain. Route A and a related scheme involving cis-acrylic acids as intermediates are thus eliminated (Note c). So we are left with routes B and C.

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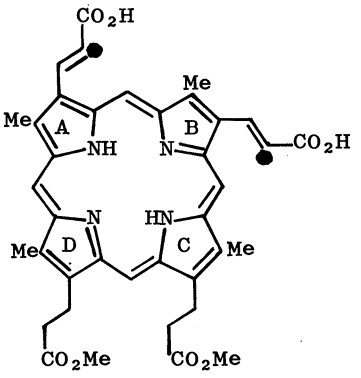
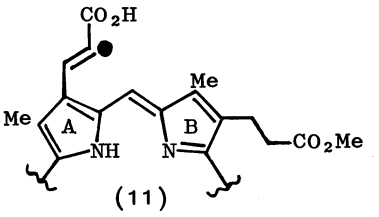
Note c. Ketonic intermediates were also considered in some schemes and obviously these too are eliminated by our findings.

It is evident that we must synthesise the hydroxy intermediates of route B but to give all the details would be outside the scope of this lecture. It is sufficient to say that the three $^{14}C$-labelled porphyrins (10), (11) and (12) were unambiguously synthesised by Richard Wightman and hydration then afforded the diol (13) and the mono-alcohols (14) and (15) in labelled form; the latter two products are racemic and the former undoubtedly a mixture of two RS-diastereoisomers. The mono-alcohols (14) and (15) have also been synthesised in Sydney by Professor Clezy and his colleagues (Ref. 6); comparison of the antipodean samples with ours settled their identity. Finally, the three porphyrin alcohols (13), (14) and (15) were hydrolysed and the resultant acids were then reduced to the corresponding porphyrinogens. This is necessary, you will recall, because all the transformations of these macrocycles prior to proto'gen-IX (9) occur in the living system at the porphyrinogen level (Scheme 1).



(10)



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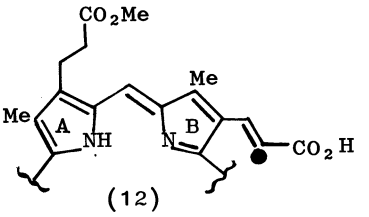

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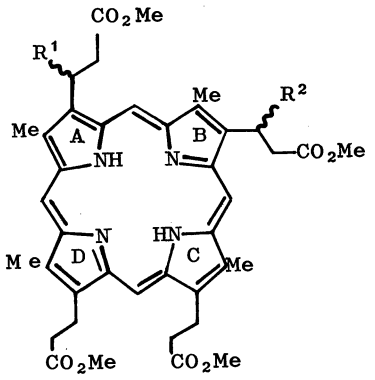

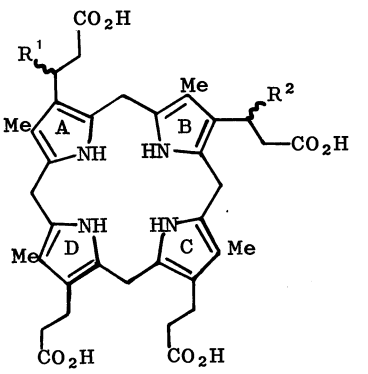

(13) $R^1 = R^2 = OH$

(14) $R^1 = OH$, $R^2 = H$

(15) $R^1 = H$, $R^2 = OH$

(16) $R^1 = R^2 = OH$

(17) $R^1 = OH$, $R^2 = H$

(18) $R^1 = H$, $R^2 = OH$

This then in outline is how we obtained the labelled porphyrinogen alcohols (16), (17) and (18) for test in the Euglena enzyme system. It turned out that the diol (16) was not significantly incorporated (Note d) into protoporphyrin-IX (2). However, the ring-A alcohol (17) was incorporated

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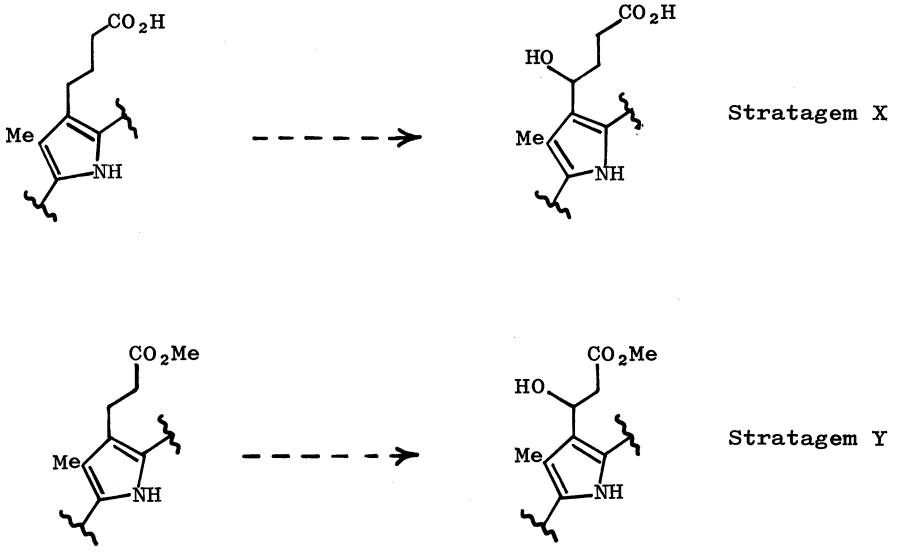
Note d. All the necessary controls and blanks were carried out. Also, the same result was obtained with coproporphyrinogenase from beef liver mitochondria.

into (2) and the conversion (2.8%) was somewhat higher than from the ring-B alcohol (2.2%). Synthetic $^{14}$C-copro'gen-III (8) was incorporated into protoporphyrin-IX to the extent of 8.8% in a strictly parallel experiment; when comparing this figure with the earlier ones, we should bear in mind that the hydroxy porphyrins used above were racemic.

We can conclude from these results that the diol (16) is not on the pathway to protoporphyrin-IX (2); see Ref. 7. This conclusion is in agreement with Kenner's finding (Ref. 8) that the propionate group on ring-A is converted into vinyl before that on ring-B and results to be described later also bear on this point. The incorporation of the ring-A alcohol (17) into protoporphyrin-IX (2) supports route B, Scheme 2 though the appreciable biological conversion of the ring-B alcohol (18) adds unwelcome ambiguity.

Nonetheless, the sum of evidence at present favours the C-hydroxylation route and in accord with this, an absolute requirement for oxygen has been found for coproporphyrinogenase in almost every system studied and the enzyme behaves like a mixed function oxidase (Ref. 2, see also Ref. 9).

One naturally thinks about trapping intermediates between copro'gen-III (8) and proto'gen-IX (9), Scheme 1, and we hope to simplify the task by "cheating" the enzyme. Route B of Scheme 2 (and route C also) has strict requirements for the nature and relative location of the key groupings on propionate side-chain. Compare route B operating on a carboxyethyl side-chain with what may happen when the following slightly modified porphyrinogens are treated with coproporphyrinogenase. If normal hydroxylation of a carboxypropyl group occurs (as at X in Scheme 3) then the normal subsequent step of fragmentation is frustrated by the additional methylene unit. Conversely, the relative arrangement is left unchanged at Y, Scheme 3 but the block to fragmentation is introduced by esterification.

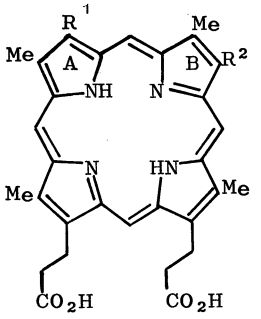



Scheme 3

The crucial contribution of synthesis to our researches will already be abundantly clear and now we need the porphyrins (19), (20), (21) and (22). This study, carried out by John Robinson, involved synthetic control by selective protecting groups and now very satisfactory sequences are available for these materials which were shown to be >98% homogeneous by high pressure liquid chromatography (H.p.l.c. in the sequel); see Ref. 10.

The position so far is that the porphyrinogen derived from the ring-B butyric acid analogue (19) is smoothly converted by coproporphyrinogenase into a porphyrinogen which after aromatisation is isolable as the ring-A

vinyl porphyrin (23). In contrast, the porphyrinogen prepared from the isomeric ring-A butyrate (20) is largely unaffected by the enzyme and these main findings interlock with what was said earlier about the sequence of operations at rings A and B. In addition, small quantities of other products are formed in both cases which are currently being examined. It will not have escaped your notice that the monohydroxy esters required for




(19) $R^1 = CH_2CH_2CO_2H$
$R^2 = CH_2CH_2CH_2CO_2H$

(20) $R^1 = CH_2CH_2CH_2CO_2H$
$R^2 = CH_2CH_2CO_2H$

(21) $R^1 = CH_2CH_2CO_2Me$
$R^2 = CH_2CH_2CO_2H$

(22) $R^1 = CH_2CH_2CO_2H$
$R^2 = CH_2CH_2CO_2Me$

(23) $R^1 = CH{=}CH_2$
$R^2 = CH_2CH_2CH_2CO_2H$

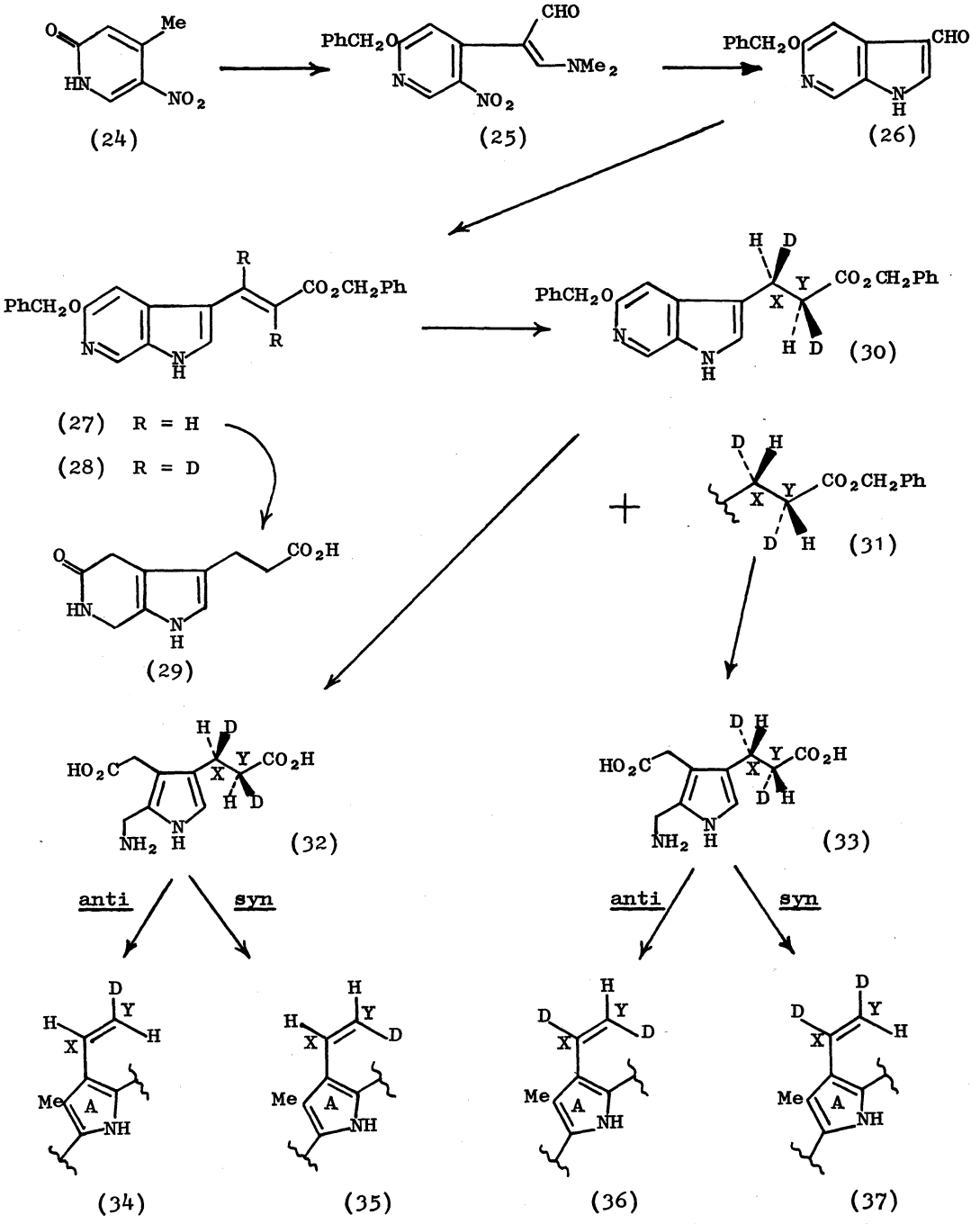
comparison with products from stratagem Y, Scheme 3 are already available. Also one of the two hydroxybutyrate porphyrin esters needed to check stratagem X has recently been synthesised.

So the net is tightening on the last few features of the process and this study has already been informative about enzymic specificity. In fact the net has already closed on one important prize, the stereochemistry of the olefin formation.

We had previously gained evidence from tritium labelling which indicated that, as each vinyl group of protoporphyrin-IX is formed, a stereospecific loss of the single hydrogen atom occurs from the β-carbon of the propionate chain (Ref. 4) and Professor Akhtar concluded (Ref. 5) that it is the forward hydrogen atom (pro-S) which is removed (see Scheme 2).

So it remains to uncover the stereochemical relationship between the carboxyl group and the eliminated hydrogen atom in the olefin forming step. To study this problem, we need a sample of PBG (6) stereospecifically labelled with deuterium at the centres X and Y (see Scheme 4). The shortened PBG synthesis which was developed for this purpose is outlined in Scheme 4 (Ref. 11 and 12). This synthesis and all the labelling and spectroscopic work was carried out by Hanns Wurziger.

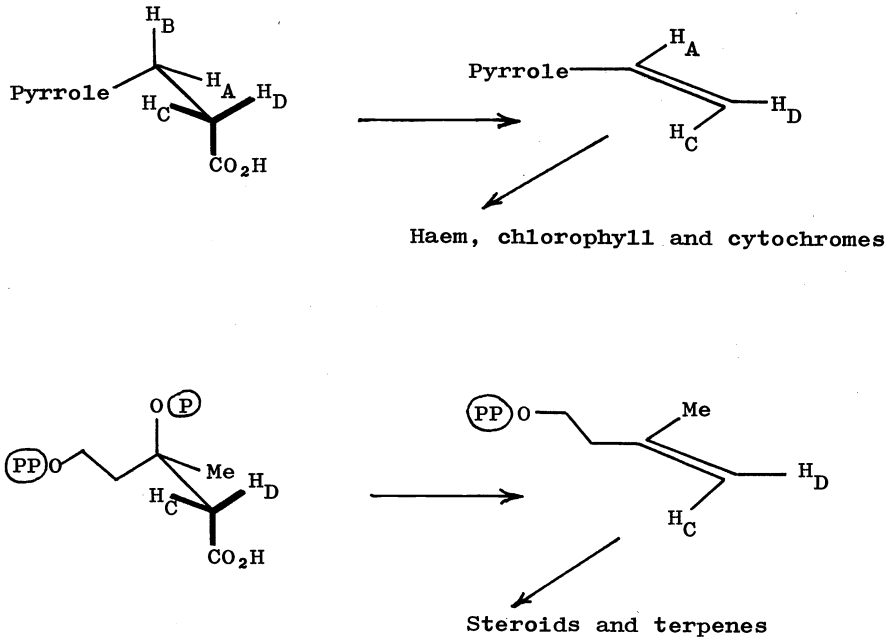
A double Vilsmeier reaction on (24) followed by benzyl ether formation and hydrolysis yielded (25). This was reductively ring-closed to the aldehyde (26) and reaction with monobenzyl malonate then gave the acrylic ester (27) ready for a multi-purpose hydrogenation step leading directly to PBG lactam (29). This is a thoroughly practical route to large quantities of PBG and it has a further important advantage. By reducing the dideuterio analogue (28) with diimide, a racemic product (30) + (31) is obtained in which the relative configuration at centres X and Y has been fixed by the <u>syn</u>-stereospecificity of diimide. This arrangement is not affected by the subsequent reactions which produces PBG as a racemate (32) + (33).




Scheme 4

Consider now the outcome of the two possible ways for formation of the vinyl group, viz. anti-, or syn-elimination, from the molecules (32) and (33); the resultant labelling patterns for the vinyl groups are shown in Scheme 4 at (34)-(37). So by focussing the $^1$H-n.m.r. analysis on the hydrogen at centre X, neither product (36) nor (37) from enantiomer (33) will register and a clear $^1$H-$^1$H coupling pattern should be seen in the product (34) or (35) from (32). Fortunately, at the right concentration of protoporphyrin-IX dimethyl ester, one can see separate signals from each $H_X$ on the two vinyl groups. So the sample of protoporphyrin-IX ester derived from the labelled PBG (32) + (33) using the Euglena enzyme cocktail was examined at this concentration and two doublets with trans-coupling (18Hz) were observed from the two $H_X$.

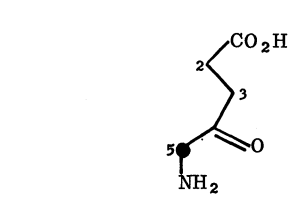
The two vinyl groups of protoporphyrin-IX (2) and so of haem (1) are thus formed by an overall anti-elimination as in Scheme 5. Also illustrated there is the stereochemical course of the process which generates another vitally important vinyl group in isopentenyl pyrophosphate from the illustrated mevalonate derivative (Ref. 13). So there is stereochemical unity and in each case, Nature is following what organic chemists would regard as the best path.



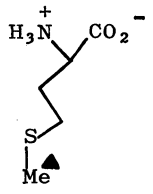

Scheme 5

Now let us turn from the biosynthesis of haem (1) to another challenging problem, that of vitamin $B_{12}$ (3). Again the aim is to find out how Nature builds such a marvellous structure and I will cover the progress which has been made so far, starting briefly with published work up to the 1972-73 period (see also Ref. 2). Bernhauer's group (Ref. 14) made the important observation that cobyrinic acid (40) is a biosynthetic precursor of vitamin $B_{12}$; so the various amides and attachments around the periphery of the vitamin (3) are added late in the sequence. The major problem thus becomes that of understanding the biosynthesis of cobyrinic acid. Because of the obvious structural relation of cobyrinic acid (40) to the porphyrins (Note e), Bray and Shemin had tested $^{14}$C-labelled forms of δ-aminolaevulinic acid, ALA (38) as a precursor of vitamin $B_{12}$ (Ref. 15); ALA was well known by that time as the immediate precursor of PBG (6).

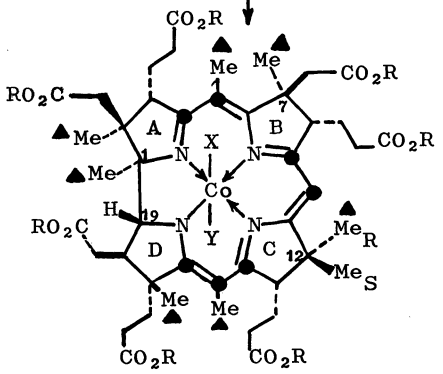
Note e. It is particularly striking to compare the nature and arrangement of the side-chains for cobyrinic acid (40) with uro'gen-III (7).



(38) ALA



(39) Methionine

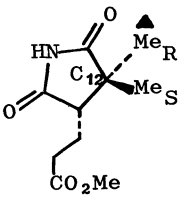


(40) R = H Cobyrinic acid

(41) R = Me, X = Y = CN
Cobester

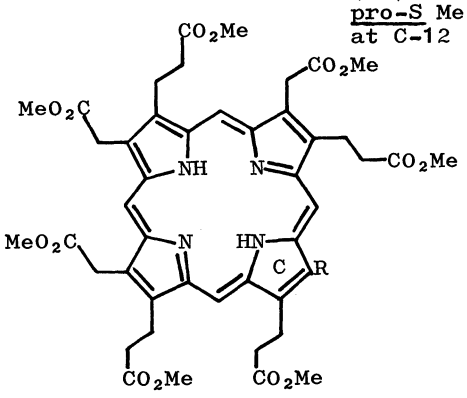
Vitamin $B_{12}$
(3)

$O_3$



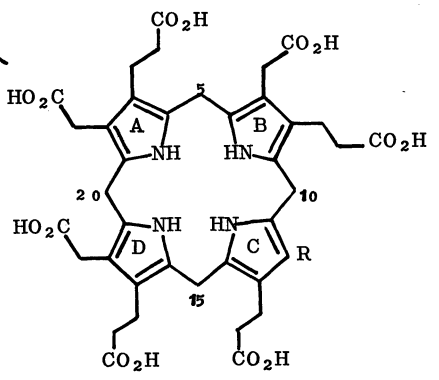
(42)

(45) labels
pro-S Me group
at C-12



(43) R = $\overset{*}{C}H_2CO_2Me$

(44) R = $\overset{*}{Me}$



(45) R = $\overset{*}{C}H_2CO_2H$

(46) R = $\overset{*}{Me}$

Scheme 6

These studies and others with labelled PBG (Ref. 16) were important in establishing that the natural porphyrins and corrins are constructed from the same early building blocks. Most of the C-methyl groups of vitamin $B_{12}$ were found to be derived from methionine (39) including one of the geminal methyls at C-12 of ring-C; the other is formed by decarboxylation of an acetic acid residue (see also later). However, degradative methods were not available, and are not available even now, to locate precisely all the labelled atoms in such radioactive samples of vitamin $B_{12}$. So we decided to base as many of our biosynthetic experiments as possible on $^{13}C$-labelled precursors; in principle, the sites of labelling could then be unambiguously determined by $^{13}C$-n.m.r. spectroscopy. As it turned out, Shemin's group and Scott's had independently decided on a similar approach though there were differences which resulted in their work and ours on these early stages being mutually reinforcing.

We decided to use Propionibacterium shermanii as our biological partner and to carry out most of the n.m.r. work on the beautifully crystalline heptamethyl dicyanocobyrinate (41), usually called cobester; this is available in $>90\%$ yield from vitamin $B_{12}$ by Professor Eschenmoser's procedure (Ref. 17 and Note f). The advantages are that cobester (41) lacks the 17-carbon nucleotide ligand and importantly has three fewer C-methyl groups than vitamin $B_{12}$ so its $^{13}C$-n.m.r. spectrum is simpler and more readily assigned. Indeed, fully resolved $^{13}C$-signals are observed from 43 of the 45 skeletal carbon atoms of cobester (41).

The American groups (Refs. 18, 19) used vitamin $B_{12}$ itself for their spectroscopic studies, or dicyanocobalamin (as 3, replace heterocyclic ligand by CN). In experiments with P. shermanii, they confirmed the earlier $^{14}C$-results and made the important finding that the C-1 methyl group of vitamin $B_{12}$ is derived from methionine rather than from C-5 of ALA (38). Our results with the same two precursors, viz. ALA and methionine, were also unambiguous and confirmatory; the vitamin $B_{12}$ derived biologically from [5-$^{13}C$]ALA (38) yielded cobester (41) showing seven (not eight) strongly enhanced signals in its $^{13}C$-n.m.r. spectrum. [See (41) for labelling pattern]. The vitamin $B_{12}$ similarly derived from [methyl-$^{13}C$]methionine (39) yielded cobester which displayed seven greatly enhanced signals from C-methyl groups in its $^{13}C$-n.m.r. spectrum (Ref. 20); the $^{13}C$-pattern is illustrated on (41).

The sum of the foregoing work from the three groups based on $^{14}C$- and $^{13}C$-labelling was to show that the corrin nucleus of vitamin $B_{12}$ (3) is built from eight molecules of ALA (38) with seven methyl groups being donated by methionine (39). During the construction process, one carbon atom (originally at C-5 of ALA) is eliminated and carbon dioxide is lost from a carboxyl group which was originally C-1 of another ALA molecule.

When confronted with a molecule as complex as that of cobyrinic acid (40), every line must be followed which conceivably could give clues about the biosynthetic pathway. We felt that knowledge of the stereochemistry of the C-methylation process at C-12 of ring-C (40) could possibly help and certainly it is information which must eventually be accommodated in the final biosynthetic scheme; this aspect is the last to be covered in our brief survey of published work.

Degradation by ozonolysis (Ref. 21) of the cobester (41) labelled by biological incorporation of [$^{13}C$-methyl]methionine gave the ring-C imide (42). Its beautiful $^{1}H$-n.m.r. spectrum showed two satellites, due to $^{1}H$-$^{13}C$ coupling, centred on the unsplit signal from the pro-R methyl group (see 42). Thus it is $Me_R$ (see 42) which carries carbon-13 and therefore is the one derived from methionine. So again we see a constancy in Nature with rings A, B and C of cobyrinic acid (40) all being methylated from the rear face of the molecule as it is drawn; ring-D differs in this respect but for all four rings, the biosynthetic process corresponds to the formal

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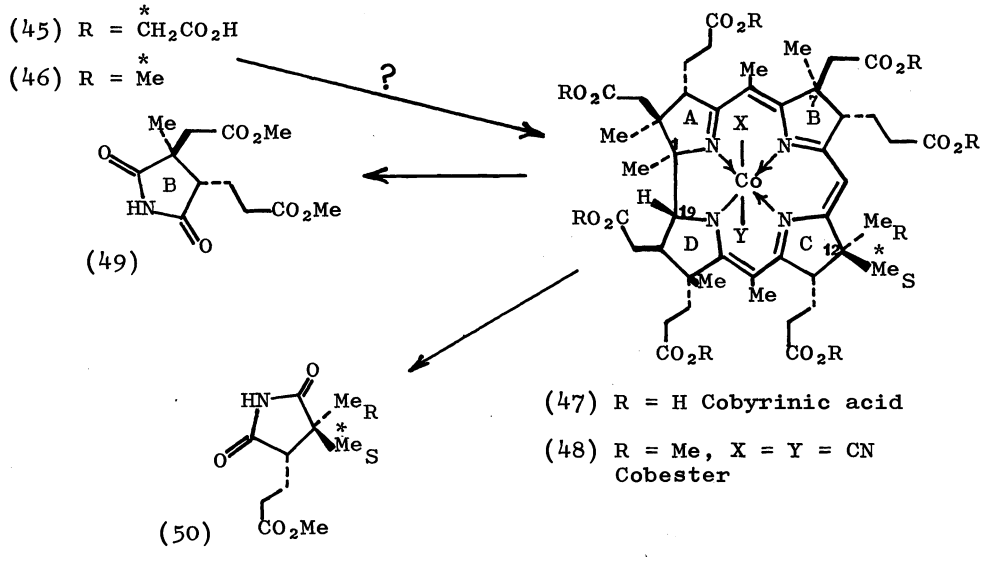
Note f. We are indebted to Professors A. Eschenmoser and R. B. Woodward for samples of cobester and unpublished information concerning it.

addition of Me and H in an overall trans manner (Ref. 20). It was possible to confirm this result by deuterium labelling (Ref. 22) and also by a different approach at Yale (Ref. 23). Additionally, our experiments with [methyl-$^{3}H_2$]methionine (as 39) demonstrated that the biological methyl transfer occurs intact from methionine to C-7 and C-12 of cobyrinic acid (40); we will return to the other five C-methylations later.

The striking relationship in structure of cobyrinic acid (40) to uro'gen-III (45) had long suggested a biosynthetic link; the characteristic sequence of acetic and propionic side-chains around both macrocycles is particularly indicative. Indeed, there had been hints from isolation studies which supported such a biochemical connection (Ref. 24).




The experimental strategy for testing this relationship was based in Cambridge on two decisions (a) that an isolated enzyme system should be employed and (b) that it was essential to use specifically labelled uro'gen-III (45). Of course, all the "small" precursors used so far (such as ALA and methionine) readily penetrated the cell walls of P. shermanii but for a molecule such as uro'gen-III (45) one's chances of success would be improved if the cell-wall barrier were removed. There is no need here to cover the extensive experimentation by which a broken cell enzyme system was developed from P. shermanii. It is enough to know that under the right conditions and with the appropriate cofactors, the system worked well giving 7-12% incorporations of PBG (6) into the isolated cobester (41), (Ref. 25). Further, an unambiguous synthesis of the [C-12-methylene-$^{14}$C]-uroporphyrin-III ester (43) was devised (Ref. 26) ready for reduction to labelled uro'gen-III (45); there were good reasons for choosing this site for $^{14}$C-labelling as will become clear in the sequel.

During these developments, conflicting reports were published about uro'gen-III as a precursor of cobyrinic acid (Ref. 27, 28). The positive incorporation of activity found at Yale (Ref. 28) into vitamin $B_{12}$ was from uro'gen-III (7) plus isomer(s). These materials were labelled equivalently at each propionic acid side-chain and this valuable finding spurred efforts to study non-equivalently labelled, pure uro'gen-III (7) as precursor of the corrin nucleus to establish intact incorporation of the porphyrinogen macrocycle. Intact is used here in the sense of excluding the possibility of breakdown and re-incorporation of monopyrrolic or dipyrrolic fragments.

Our study was carried out by Masataka Ihara, Fumio Satoh and Clive Williams (Ref. 29) and they incubated [12-methylene-$^{14}$C]uro'gen-III (45), prepared from the ester (43), with the above enzyme system from P. shermanii; the resultant cobyrinic acid (47) was then isolated as cobester (48). It was highly radioactive and its radiochemical purity was rigorously established by multiple crystallisation and by high pressure liquid chromatography.

Several such experiments gave incorporation values in the range 5-8% which is very satisfactory for an isolated mixture of enzymes catalysing many steps.

The reason for choosing the illustrated labelling site for (45) is now obvious in that it allows the $^{14}C$-pattern in the resultant cobester (48) to be assayed by ozonolysis as earlier. If the incorporation process is perfect, it would yield cobester with carbon-14 only at the pro-S methyl group at C-12 (see 48). The result from the actual degradation of the labelled cobester was to yield ring-C imide (50) which carried, on a molar basis, 90% of the radio-activity of the original cobester (as 48). The activity of the analogous imide (49) from ring-B was confirmatory and corresponded to less than 3.5% of the activity originally present in (48). Scott's parallel work (Ref. 30) showed that [5,15-$^{13}C$]uro'gen-III (cf. 45) was converted by whole cells of P. shermanii into vitamin $B_{12}$ (3) enriched with $^{13}C$ at C-5 and C-15. The combined results firmly establish the intermediacy of uro'gen-III (45) on the pathway to cobyrinic acid (47) and so also to vitamin $B_{12}$ itself (3).

It is cautionary that the $^{14}C$-labelling found at ring-B of cobyrinic acid (47) in our foregoing experiment demonstrates that some breakdown of uro'gen-III (45) and scatter of activity does indeed occur in the incubation step; but now we know it is a minor side process. More recently, Dauner and Müller have developed a cell-free system from Clostridium tetanomorphum (Ref. 31) which on a small scale gives high incorporations of precursors into cobyrinic acid. They have used this system to convert [12-methylene-$^{14}C$]uro'gen-III (45), prepared from our synthetic sample (43), into cobyrinic acid with ca. 34% incorporation; I am very grateful for this information from Professor Müller (Ref. 32).

Direct comparison of structures (45) and (47) shows that the biochemical transformation of the former into the latter requires the following structural changes:

(a) introduction of seven methyl groups from methionine (39) at carbons C-1, C-2, C-5, C-7, C-12 (pro-R), C-15 and C-17

(b) decarboxylation of the acetic acid side-chain at C-12

(c) extrusion of C-20 from the skeleton of uro'gen-III

(d) possible adjustment of the oxidation level

(e) insertion of cobalt

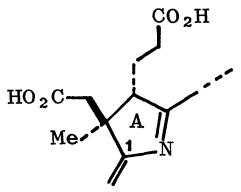
These eleven steps can in principle be carried out in "factorial eleven" ways, which my arithmetic works out to be 39,916,800 possibilities. How can we reduce this enormous number? One way is to gain further clues from the biological system by mutations, by trapping experiments and in other ways; all these lines are being actively followed at present. But we can also speculate, shrewdly one hopes, about the first step beyond uro'gen-III (45). We reasoned that the most probable candidates are (a) C-methylation or (b) decarboxylation of the C-12 acetic acid side chain to yield a hepta-carboxylic porphyrinogen (46) and both possibilities are being studied in Cambridge. The latter possibility is simpler to test and results are now available from these experiments.

The necessary [12-methyl-$^{14}C$]porphyrin ester (44) was synthesised essentially by standard methods and it is important to recognise the logic behind labelling this material at the C-12 methyl group. Firstly, if incorporation of the corresponding porphyrinogen (46) does occur into cobyrinic acid (47) then the labelling can be assayed as before by ozonolysis to afford the ring-C imide (50). Equally important though, this labelling site allowed us to be certain that any traces of uroporphyrin-III ester (as 43) which could be formed during the synthesis would necessarily be radio-inactive. Without this safeguard, a small incorporation of activity from the corresponding porphyrinogen carboxylic acid into cobyrinic acid could arise from uro'gen-III and so be spurious.

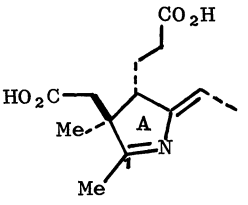
At this stage, everything was available for a meaningful comparison of the efficiencies of the $^{14}C$-labelled ring-C methyl derivative (46) and uro'gen-III (45) as precursors of cobyrinic acid. The broken cell enzyme

preparation from P. shermanii described earlier was used and half of one batch was incubated with the ring-C methyl system (46) and the other half with uro'gen-III (45). The outcome from three such pairs of strictly parallel experiments was that uro'gen-III (45) is 30-50 times more effective as precursor of cobyrinic acid (47) than is the ring-C methyl porphyrinogen (46). In fact, this activity of the cobester (48) isolated from the experiments with (46) was too low to allow the labelling pattern to be pin-pointed by degradation. We have tested and eliminated several unlikely (but conceivable) explanations of this large difference in efficiency and these necessary control experiments are described in our full paper. Of course, there must always be caution with interpretation, but it seems probable that the ring-C methyl derivative (46) is not a normal intermediate in corrin biosynthesis. It is possible though that this hepta-acid (46) is inefficiently metabolised in one way or another towards the corrin system by enzymes which normally transform molecules with an acetic acid side-chain at C-12 (Note g).

We must therefore look in other directions. Naturally, intermediates beyond uro'gen-III (45) are being sought but it also seemed possible that some of the seven C-methyl groups introduced into the corrin macrocycle from methionine may be sources of hidden information. In particular, the methyl group at C-1 (see 47) might reveal something about the nature of the C-1 to C-19 bond formation in building the corrin nucleus. For example, the C-1 methyl group may temporarily become a methylidene residue with ring-A as (e.g. 51) in a seco-intermediate. Alternatively, the C-1 methyl may be unaffected throughout the process which bonds C-1 to C-19 and ring-A could be as (e.g. 52).




(51)




(52)

I cannot go into the reasons why the approach described earlier for proof of intact incorporation of the methyl groups at C-7 and C-12 would not serve for C-1. Let us take it as a fact and consider the new method which was developed; this approach should have wide applicability in similar situations. It is based on double labelling of methyl groups with carbon-13 and deuterium. The deuterium acts as the probe for checking hydrogen retention or loss and the carbon-13 provides the analytical capability by n.m.r. to follow the changes in deuterium content.

Roger Hollenstein synthesised [methyl-$^{13}CD_3$]methionine and used it for incorporation into vitamin $B_{12}$ by P. shermanii. The $^{13}$C-n.m.r. spectrum of the product was determined on dicyanocobalamin [as (3) with the dimethylbenzimidazole ligand replaced by CN] using $^{1}$H-lock and noise-decoupling of deuterium. Under these conditions, $^{13}CD_3$ groups will produce singlets, $^{13}CD_2H$ will give doublets (J ca. 130 Hz), $^{13}CDH_2$ triplets and $^{13}CH_3$ quartets.

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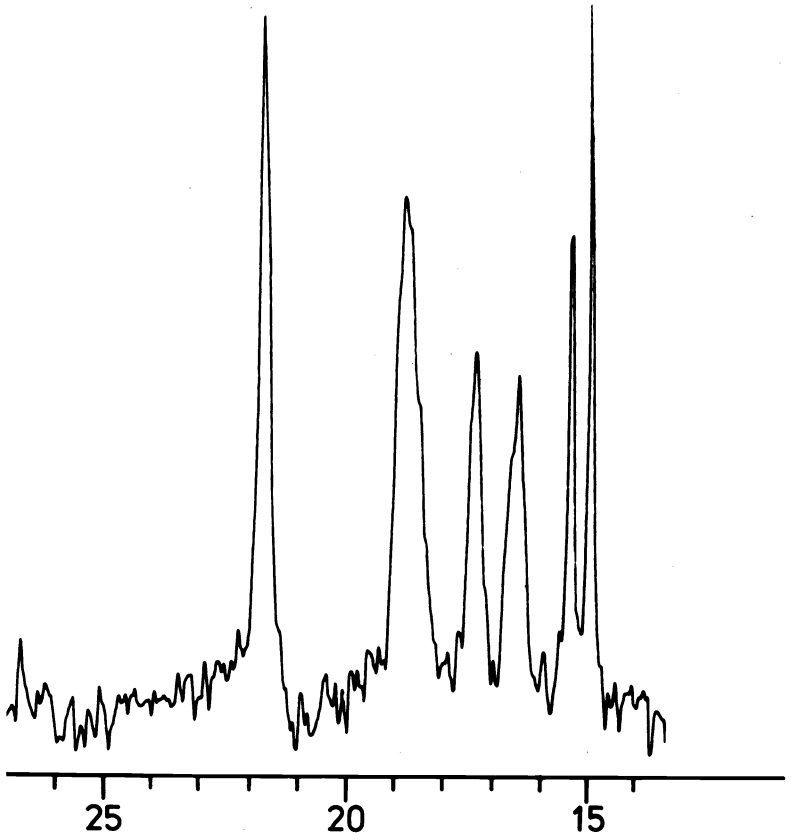
Note g. The possibility must also be borne in mind that the small incorporation of activity from (46) into (47) is simply by breakdown and reincorporation of fragments in the way observed earlier as a minor process with uro'gen-III (45).

It should also be mentioned that Scott et al (Ref. 30) have concluded on the basis of a 0.1% incorporation of $^{14}$C-(46) into cobyrinic acid of unknown labelling pattern that the methyl derivative (46) is an intermediate on the pathway to corrins and represents the point at which the haem and corrin paths separate.

The Figure shows the observed spectrum and all seven signals from the $^{13}C$-enriched methyl groups are singlets. Long range $^{1}H$-$^{13}C$ couplings broaden five of the signals and cause two of them to merge together at ca. 18.7 p.p.m. (Note h). These results show that all seven methyl groups of vitamin $B_{12}$ which are derived from methionine are transferred intact and none undergoes significant exchange of its hydrogens with the medium (Ref. 33). It is thus very unlikely that the biosynthesis of the corrin nucleus involves an intermediate in which the methyl group eventually appearing at C-1 has transiently been a methylidene residue (e.g. as 51). The same conclusions have been reached by the same double-labelling method in Zürich (Ref. 34) and by a different approach at Yale (Ref. 35).




Methyl region of $^{13}C$-FT n.m.r. spectrum of $^{13}C$-enriched dicyanocobalamin [as (3) with CN replacing dimethylbenzimidazole ligand] biosynthesised from [methyl-$^{13}CD_3$]methionine. Spectrum run on Varian XL-100 spectrometer with $^{1}H$-lock, noise decoupling of deuterium, in 0.1 M-KCN in $H_2O$, 84,785 transients. Chemical shifts are downfield from $(CH_3)_4Si$.

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Note h. Replacement of hydrogen by deuterium causes a small upfield shift of the signals from their normal positions.

It will be evident that an exciting stage has now been reached where the pathway to vitamin $B_{12}$ is taking form. At the moment, the feeling is rather like that of building a jig-saw puzzle after the pieces have been hidden around the room. Some pieces have been found and fit snugly into place; others are in our hands but we do not yet know exactly how they fit into the whole picture. The remaining pieces have still to be uncovered. However, one knows that eventually all the pieces will fit together and over the coming months or years, the collection of pieces will continue for this marvellous problem.

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I have been extremely fortunate in all these researches to work with enthusiastic colleagues whose experimental skills, ideas and vigour matched the scale of the problems. The names of the young men involved in the recent work have been given at the appropriate points in the lecture and I am very glad to have this opportunity to record my debt to them. I wish particularly to thank my senior colleague, Dr. Ted McDonald, whose many contributions to these researches have been invaluable.

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