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The discovery of nature's biosynthetic pathways*

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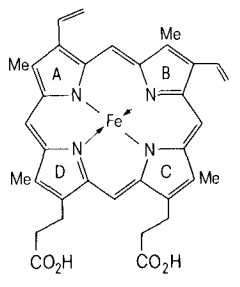
Summary. Uro'gen-III is a key intermediate on the biosynthetic pathways to the vitally important natural pigments haem, chlorophyll and the cytochromes. How the unexpected structure of uro'gen-III is synthesized by living things has long been a major puzzle. Studies based on ^{13}C-labelling are described which show a) that a single intramolecular rearrangement occurs and b) that this step occurs after the open-chain linear tetrapyrrole system has been built.

A second study involves stereospecific labelling with deuterium and tritium to elucidate the absolute stereochemistry of the enzymic reaction sequence which produces the vinyl groups of haem.

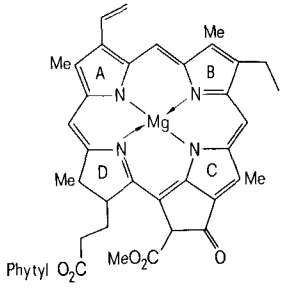
The third and last section of the lecture is focussed on the biosynthetic intermediates lying between uro'gen-III and cobyrinic acid on the pathway to vitamin B_{12}. An octacarboxylic isobacteriochlorin is isolated from a vitamin B_{12}-producing organism and this is shown to be identical with sirohydrochlorin, previously obtained by Kamin and Siegel as the metal-free prosthetic group of certain sulphite-reducing bacteria. The structure and absolute stereochemistry of sirohydrochlorin are studied and comment is made on the evolutionary interest of these findings.

There has been spectacular progress during the last two and a half decades in uncovering the sequences of chemical reactions which living systems use to synthesize the simple and the complex organic molecules they contain. This progress has required the full use of a wide range of approaches for each problem. In addition to showing the joy of studies in this area, my further aim is to illustrate how complex problems can be successfully solved by using synthesis, isotopic labelling and spectroscopy together with the latest techniques for separation of proteins and of closely related smaller molecules. This full array was certainly necessary to solve several fascinating problems in the field of porphyrins and corrins and the results have proved to be quite exciting. But the work has not been without aesthetic pleasure because the substances involved could hardly be more beautiful; they have magnificent colours and they have particularly beautiful structures and chemistry.

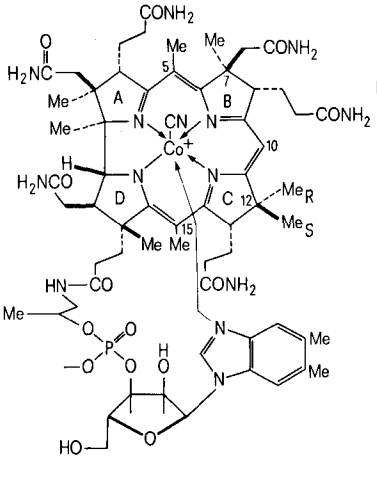
We shall be considering vitamin B_{12} which has a rich red-purple colour, the deep green chlorophyll *a* and protoporphyrin-IX. This last pigment has a lovely violet colour in acidic solution and it forms the organic part of haem; the function of haem at the oxygen binding site of haemoglobin is familiar to you all. We will also meet a deep blue pigment, but let us keep this as a surprise until its nature and relationship to the other pigments becomes evident later in the lecture.



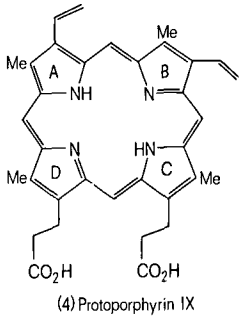
(1) Haem



(2) Chlorophyll a



(3) Vitamin B_{12}

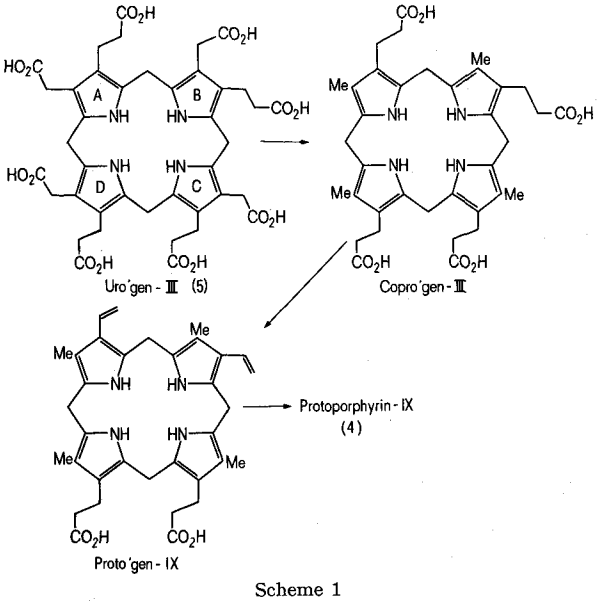


(4) Protoporphyrin IX

*This account is a slightly extended version of the Paul Karrer Lecture given on July 5th, 1977 at the University of Zürich.

When we think of haem (**1**), chlorophyll *a* (**2**) and vitamin B_{12} (**3**), it is clear that all these substances are of vital importance in living systems and this gives them a special attraction. The structures **1**, **2**, **3**, also have several common features. Haem (**1**) has Fe^{II} held at the centre of a macrocycle composed of 4 five-membered pyrrole residues connected by single carbon bridges, the so-called *meso*-bridges. Structure **1** has a variety of groups around the periphery of the macrocycle but notice that the 2 propionate groups on rings C and D occupy adjacent positions on the periphery.

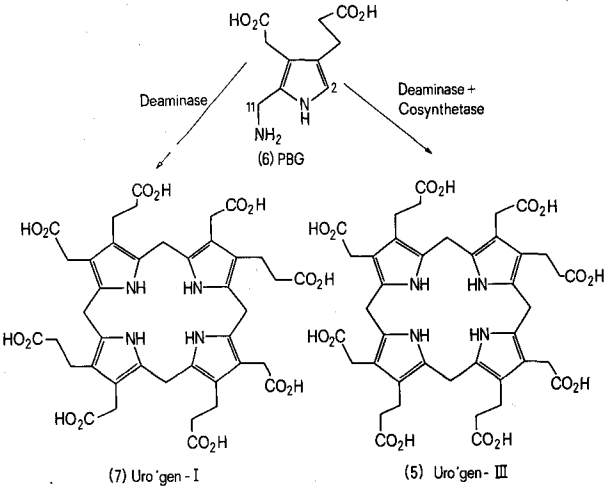
Chlorophyll *a* (**2**) is related structurally to haem. In this case Mg^{II} is the coordinated metal ion and the propionate group on ring-C has undergone ring-closure onto 1 of the meso-bridges. But this does not obscure the fact that the 2 propionate residues on rings C and D once again lie side by side. This characteristic structural feature can be traced back along the biosynthetic pathway until one reaches uroporphyrinogen-III (abbreviated to uro'gen-III) having structure **5**. Uro'gen-III is the 1st macrocycle to be built in living things, and both haem (**1**) and chlorophyll *a* (**2**) are biosynthesized from it by a marvellous sequence of reactions[1]; notice that the juxtaposition of the 2 propionic acid groups on rings C and D has been established by the biosynthetic process which produces uro'gen-III (**5**). The main steps[1] beyond uro'gen-III on the pathway to protoporphyrin-IX (**4**) and so to haem and chlorophyll *a* are illustrated in scheme 1.



Scheme 1

So the stage is set and this lecture will explore the biosynthesis of these macrocycles. In the available time, we can only look together at some of the high-lights. Most of the supporting work on synthesis, basic ^{13}C-NMR, structure determinations, enzyme isolations and separation techniques cannot be described today even though all of this represents about 90% of the total effort*. However, I am sure you will realize as we go forward that this solid foundation of supporting work is always there.

When our own studies started in this area some 10 years ago, we were building on the splendid contributions[1] made by Shemin, Granick, Neuberger, Rimington and Bogorad, and other groups too, during the nineteen fifties. Some of their findings have already been summarized and in addition, the exciting discovery was made that uro'gen-III (**5**) is biosynthesized from 4 molecules of porphobilinogen (**6**), usually shortened to PBG (scheme 2). The product



Scheme 2

from straightforward combination of 4 PBG units head-to-tail would be uro'gen-I (**7**) in which the acetic and propionic groups are set in sequence around the periphery of the macrocycle. Indeed, uro'gen-I is formed when PBG is treated with the enzyme deaminase alone. In living systems, however, deaminase works cooperatively with a second protein called cosynthetase to produce uro'gen-III (**5**) from PBG (scheme 2). It is important to emphasize that uro'gen-I (**7**) is not rearranged to uro'gen-III (**5**) by cosynthetase or by deaminase plus cosynthetase. So the rearrangement step or steps occur(s) somewhere along the right hand arrow in scheme 2. Notice that the first-formed macrocycle (**5**) is a reduced, colourless system – a porphyrinogen – and that the subsequent biochemical steps shown in scheme 1 also take place at this oxidation level; aromatisation[4,8] to form protoporphyrin-IX (**4**) is the last step.

You will see from the brief summary that haem and chlorophyll *a* are biosynthesized from the unexpected isomer (uro'gen-III). It is fascinating that living systems have developed over evolutionary time a highly specific way to catalyze a rearrangement process which produces this type-III isomer having adjacent propionate groups on rings C and D.

*For accounts of these aspects, see references 2–7 inclusive.

How can we discover the way living things carry out this subtle chemistry? Notice that 4 identical building blocks are used to form uro'gen-III (**5**) so something rather special will be needed to dig out the secret. The problem separates into 3 parts: What happens? When does it happen? How does it happen?

I shall describe our published work[5] rather briefly but the results form an essential foundation for understanding the latest experiments. Let us think first about *what happens;* for this, we have to answer the 3 questions collected in scheme 3. The plan was to use [2,11-$^{13}C_2$] labelled PBG* (as **6**) in a special way to be

Scheme 3

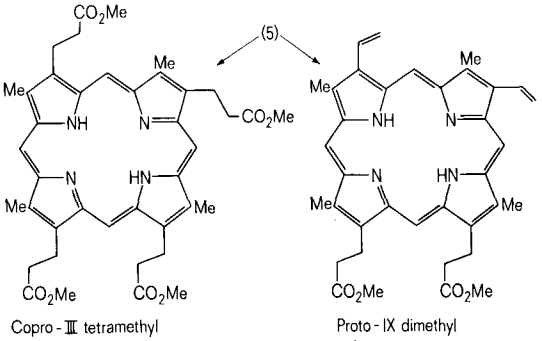
A How many rearrangements occur?

B Consider each rearrangement – is it int*ra*molecular or int*er*molecular?

C Where do the migrating carbon(s) appear in the final type-III macrocycle?

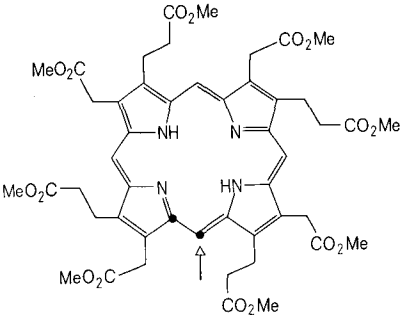
described later, for the enzymic formation of uro'gen-III (**5**). We hoped that by analysis of the product using NMR, it would be possible to detect carbon-carbon bond formation (or conversely, carbon-carbon bond cleavage). The simple principle is that nonequivalent ^{13}C-atoms which are directly bonded show strong spin-spin coupling whereas more remote separation of two ^{13}C atoms results in little or no coupling.

Such a plan requires there to be a rigorous way of proving where the ^{13}C-labels lie in the enzymically formed uro'gen-III. This substance is too unstable to handle satisfactorily and so for all experiments, uro'-gen-III (**5**) was converted, by methods which cause no rearrangement of the macrocycle, either into copro-porphyrin-III tetramethyl ester (**8**) [copro-III ester] or into protoporphyrin-IX dimethyl ester (**9**) [proto-IX ester]. These stable, crystalline esters were then assayed by NMR.

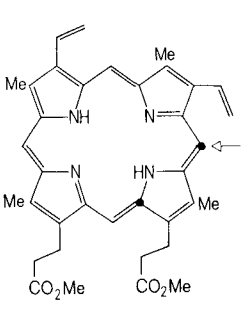


Block B

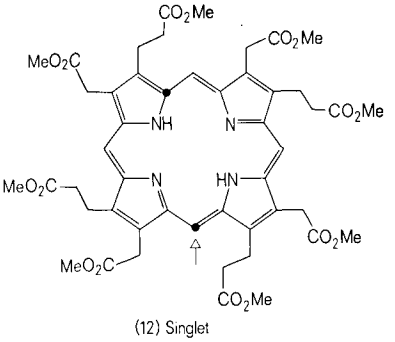
Finally, we must hold in our hands standard samples of porphyrin esters which have been synthesized[6] or prepared in some other unambiguous way[5], so as to place 2 ^{13}C-labels with at least 90 atom% enrichment in the various possible relative arrangements. Analysis of the problem shows that there are 3 possibilities and for clarity, a slightly simplified account is given. One possibility is to have the 2 ^{13}C atoms directly bonded (as **10**); for this case, the ^{13}C-signal from the arrowed meso-bridge was found to be a 72 Hz doublet. A 2nd possibility is to have the ^{13}C atoms, as in the original PBG (**6**), set 3 bonds apart (as **11**); the synthetic sample of this material showed a ^{13}C-signal for C-10 as a 5Hz doublet arizing from longer range coupling. The last possibility is that the 2 ^{13}C atoms are well separated (as **12**) and in this case we found no significant coupling between the two. All 3 possible arrangements can thus be clearly distinguished by this approach.



(10) 72 Hz doublet



(11) 5 Hz doublet



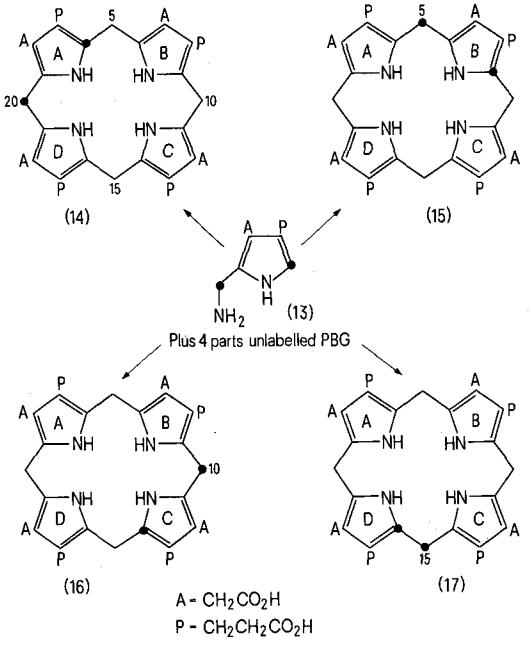
(12) Singlet

Block C

We are now ready for the first key experiment which involved dilution of the $^{13}C_2$-PBG (**13**), scheme 4, with 4 parts of unlabelled PBG. This dilution has the effect that the majority of uro'gen-III molecules which are then enzymically formed from PBG will contain only 1 doubly-labelled PBG residue. So approximately 1 quarter of the uro'gen-III molecules will have ring-A derived from a labelled PBG unit (and rings B, C and D will be unlabelled), another quarter will have ring-B so labelled and similarly for rings-C and D. This allows us to determine by NMR what has happened at each bridge and each ring individually.

* Note that the labelled atoms which each carry 90 atom % carbon-13 are the 2 involved in bond formation as the macrocycle is biosynthesized.

The outcome of this experiment was to establish that the 4 major species produced by deaminase-cosynthetase from [2,11-$^{13}C_2$] PBG (**13**) are labelled* as shown for **14**, **15**, **16** and **17** in scheme 4. These patterns allow one to set down in scheme 5 precisely what has happened in the formation of uro'gen-III. These findings strictly limit the possible mechanisms by which uro'gen-III can be formed from PBG (around 30 different



Scheme 4

Scheme 5

Nature of biochemical rearrangement

a The 3 PBG units which form ring A and its attached C-20 bridge, ring B and the C-5 bridge and ring C with its C-10 bridge are all incorparated *intact without rearrangement.*

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

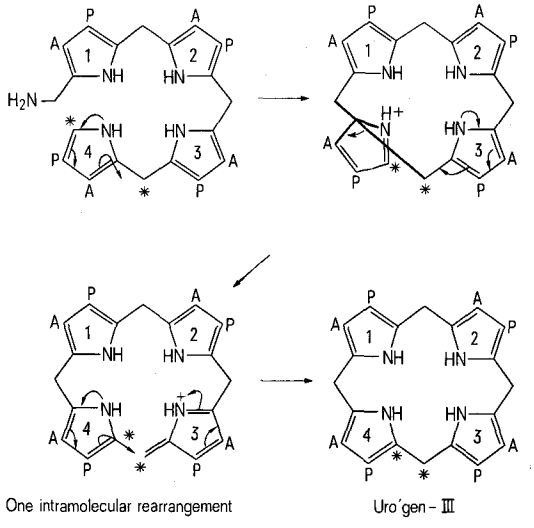
c The rearranged carbon forms the *C-15 bridge.*

ones had been proposed), and 2 which fit the evidence so far are shown in schemes 6 and 7. These differ in mechanism and in the timing of the rearrangement step; in scheme 6, the rearrangement comes after straightforward head-to-tail assembly of 4 PBG units whereas rearrangement occurs at the outset in scheme 7. So we must now answer the question '*When* does the single intramolecular rearrangement (established above) take place?'

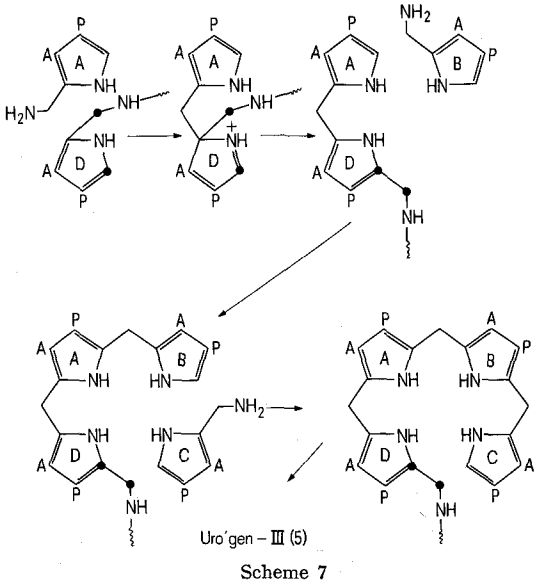
*It is essential to be absolutely clear that single molecules are illustrated in scheme 4, that is, both carbon-13 atoms are in the same molecule. The point is made to avoid confusion with ^{14}C-labelling which is normally at tracer level. When a substance is ^{14}C-labelled at 2 or more sites, then illustrated labelling patterns represent an average pattern derived from labelled molecules each of which carries only 1 ^{14}C-atom.

Before dealing with that problem, however, let us be sure about the enzymic formation of uro'gen-I (**7**). Is our tacit assumption justified that uro'gen-I is the product of uncomplicated head-to-tail joining of 4 PBG units? We were happy to find, by exactly the same approach based on [2,11-$^{13}C_2$] PBG, that deaminase builds uro'gen-I from 4 PBG units, joined head-to-tail, which remain intact throughout. So we now know exactly the difference between enzymic formation of the type-I and type-III macrocycles.

Turning next to the problem of *when* the single rearrangement occurs which produces uro'gen-III, a promising approach is to study intermediates between PBG and the uro'gen-III macrocycle. However, intermediates are not normally released from deaminase-cosynthetase[1] nor is it known whether partly built di-, tri- or tetrapyrrolic substances are covalently or physically bound to the enzyme. Such di-, tri-, and tetrapyrrolic materials are known to be highly reactive



Scheme 6

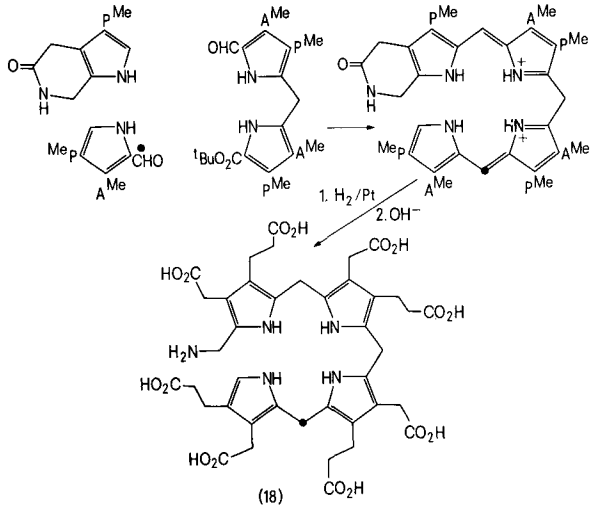


Scheme 7

so it must be expected that synthetic materials of this type which we treat with deaminase-cosynthetase will also undergo nonenzymic (chemical) cyclisation to porphyrinogens. The combination of competing chemical and enzymic processes will produce mixtures of isomeric porphyrinogens.

Without going into all the details, 2 crucial steps had to be taken: a) deaminase-cosynthetase was isolated in purified and concentrated form to encourage maximum movement of our added intermediates along the enzymic pathway; b) quantitative methods were devised, based on high pressure liquid chromatography (HPLC), for the separation of isomeric porphyrins[7]. As for the earlier NMR-studies, the HPLC separations were carried out on the mixture of coproporphyrin esters derived from the initially-formed uro'gens. Incidentally, there has been considerable medical interest in these HPLC methods for research on various porphyrias.

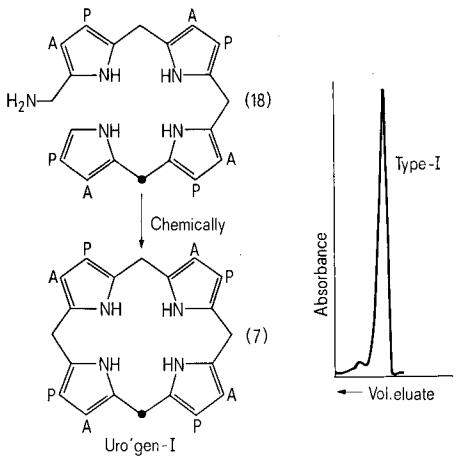
All the tools were thus available to handle the 2nd set of key experiments. The plan was to synthesize the aminomethylbilane (**18**), corresponding to straightforward head-to-tail combination of 4 PBG units; a single ^{13}C-label was introduced at the illustrated site. The route to this highly reactive substance (scheme 8)



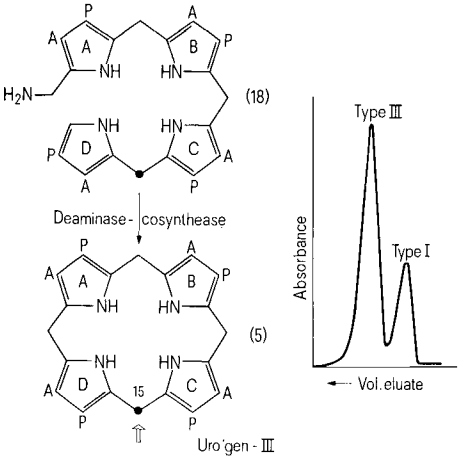
Scheme 8

made use of protected, stable intermediates. This bilane (**18**) ring-closed chemically at pH 7.2, under exactly the conditions used for the subsequent enzymic experiment, and the product was shown to be > 95% pure uro'gen-I, see scheme 9. This was marvellous because it showed that no significant rearrangement occurred at pH 7.2 as the aminomethylbilane (**18**) cyclized chemically to the uro'gen macrocycle[9].

A second sample of the same bilane (**18**) was then treated at pH 7.2 with the purified deaminase-cosynthetase enzyme system. The striking result is shown in scheme 10; approximately 80% of the product was



Scheme 9



Scheme 10

type-III*, the remainder being type-I. Not only does this demonstrate a major enzymic rearrangement of the unrearranged bilane but the proportion of type-III product shows an enzymic rate enhancement relative to the competitive chemical process which produces the 20% of type-I isomer[9].

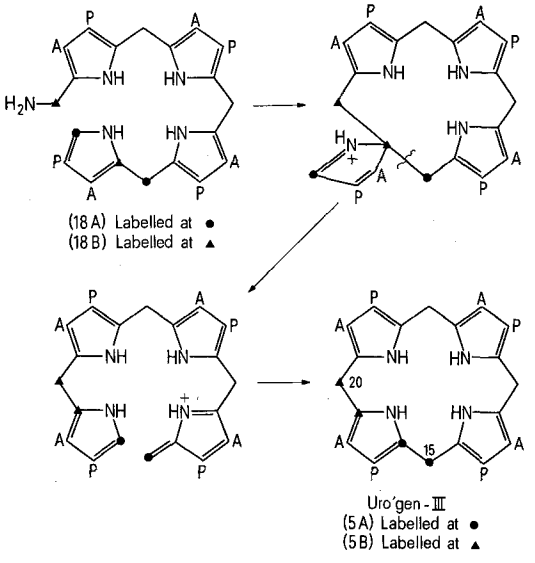
By now it will be evident that the labelling pattern of this enzymically formed uro'gen-III can be determined by NMR and it was found to be specifically labelled at C-15. This finding not only proves that uro'gen-III is formed without significant breakdown of the bilane but it also registers the former with respect to the latter and the corresponding rings are lettered (scheme 10). The clear conclusion from these experiments[9] is that the single intramolecular rearrangement involved in the formation of uro'gen-III occurs after head-to-tail assembly of 4 PBG units.

In parallel experiments, Professor Müller's group[10] found that deaminase-cosynthetase converted the unlabelled form of bilane (**18**) (prepared in a different

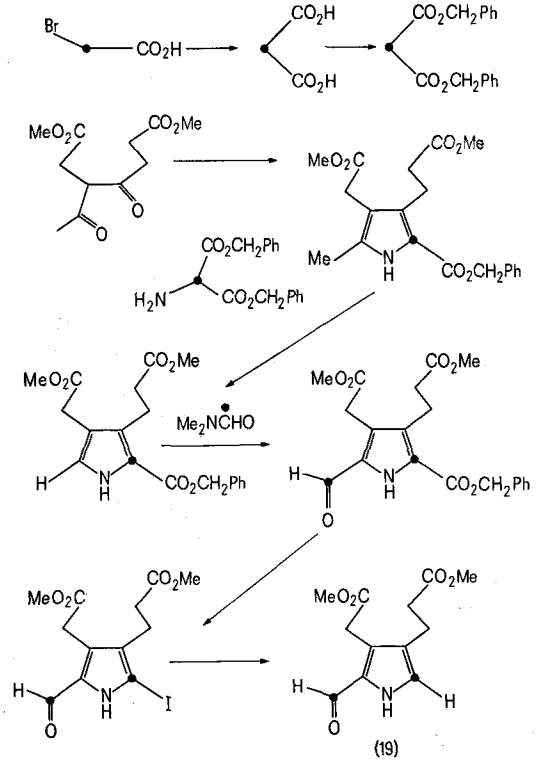
*Since copro-IV ester does not separate from copro-III ester under the HPLC conditions used for scheme 9, the major peak was isolated preparatively and was shown in a different HPLC system to contain negligible quantities of the type-IV isomer (0–<5%).

way from ours) into 14–18% of uro'gen-III (**5**) and 82–80% of uro'gen-I (**7**). It is true that these conversions into the type-III system are much lower, probably because the amounts of enzyme used were less, but nevertheless, these results add further strength.

We felt it was essential (see later) to gain overwhelming evidence for the enzymic conversion of the bilane (**18**) into uro'gen-III (**5**) with intramolecular rearrangement as the final stage. If one could synthesize 2 samples of the bilane (**18**), 1 labelled at 90 atom% with ^{13}C at the full circles and a 2nd with ^{13}C at the triangles, then such a proof should be possible, (see scheme 11). The aim is to demonstrate that during enzymic conversion of the bilane into uro'gen-III the 2 circled carbon atoms become directly bonded and, in a separate experiment, that the carbons marked with triangles bond with each other. If this is proved to happen in each case by an intramolecular process, then so to speak, the mouse hole will have been sealed at each end.



Scheme 11



Scheme 12

Both experiments are important but I suspect an advertizing agent would describe the one marked with triangles (**18B**) as 'super-important'. The reason is that in this case the labels are at the 2 ends of the bilane; so if the carbons marked with triangles become bonded together intramolecularly, then it is proven that the entire bilane (**18B**) has been converted intact into uro'gen-III (**5B**).

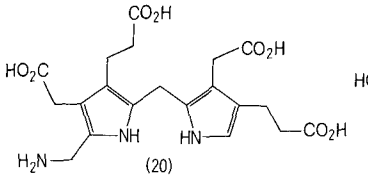
The synthetic problem involved in constructing these doubly-labelled materials is not a trivial one but we cannot savour it in detail. It is enough to see the approach used to put together one of the required building blocks and this is outlined in scheme 12. The doubly-labelled aldehyde (**19**) carrying 90 atom% ^{13}C at each labelled site was then put to work for synthesis of one of the required labelled forms of the bilane following the route you have already seen.

We can now consider the 3rd key set of enzymic experiments, firstly with the bilane (**18A**) labelled at the circled sites. This substance was diluted with 3 parts of unlabelled bilane (to allow the test for intramolecular rearrangement) and incubated at pH 7.2 with purified deaminase-cosynthetase. Uro'gen-III (**5A**) again formed 80% of the product, the remainder being uro'-gen-I. NMR-analysis of the uro'gen-III (as copro-III ester as earlier) showed that the ^{13}C-signal, which was proved to be from C-15, was a 72 Hz doublet corresponding to direct bonding with the 2nd carbon-13 atom; the uro'gen-III was thus labelled as in **5A**. Knowing the original ^{13}C-enrichment and the dilution with unlabelled material, one can calculate what the relative intensities of the split and unsplit signals should be. The experimental findings corresponded, within experimental error, to a fully intramolecular rearrangement process[11].

Similarly, the bilane (**18B**) with ^{13}C at the triangled sites was diluted with unlabelled bilane and enzymically converted in the same way into uro'gen-III (**5B**); the NMR-signal from C-20* of the derived copro-III ester was a 70 Hz doublet, so establishing the labelling pattern illustrated in structure **5B**. Here too, the NMR-spectrum corresponded accurately to rearrangement by an intramolecular sequence[12].

* Rigorous proof of labelling at C-20 was obtained by total synthesis of [20-^{13}C] uroporphyrin-III octamethyl ester followed by appropriate NMR-comparisons.

These results were a joy to see and taken with the earlier findings for PBG, they establish beyond question that the biosynthesis of the natural porphyrins, chlorins (and also, we shall see, of vitamin B_{12}) involves the following steps. Deaminase-cosynthetase joins 4 PBG units head-to-tail and the resultant bilane, bound covalently or by physical forces to the enzyme, is then converted into uro'gen-III by an intramolecular rearrangement process which directly affects only ring-D and the 2 carbons which become C-15 and C-20. The most attractive intermediate between the bilane (**18**) and uro'gen-III is the spiro-system illustrated in scheme 11*; this scheme is the same in principle as Mathewson and Corwin envisaged in 1961[13]. Naturally, the prospect of preparing such a spiro-molecule is an exciting one (even if we cannot grasp it) and work is in progress.



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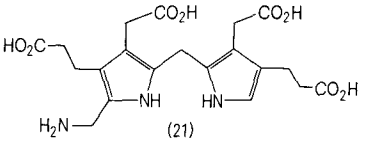


(21)

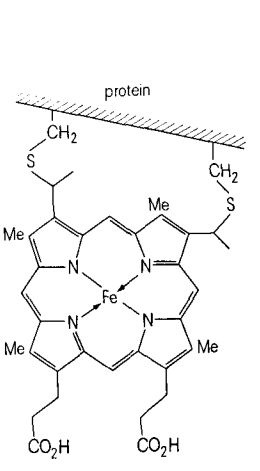
Block D

Finally, it should be added that the results from the many incorporation experiments in Cambridge based upon aminomethylpyrromethanes, e.g. **20** and **21**, agree with and give strong support to the conclusions summarized above. Time does not allow these researches to be described here but you will find them covered in references 14–18 inclusive.

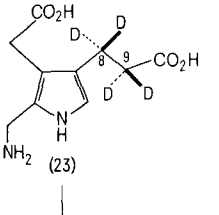
The reason it was important to provide such overwhelming evidence is because of experiments in other laboratories with pyrromethanes[19] and especially because it had been thought that small incorporations of a 'headless' pyrromethane had established[20] that the rearrangement occurs at the start of the building process when the first 2 PBG units are combined (as in scheme 7). It will be evident that such a view can no longer be seriously held.

I want now to consider 2 important biochemical modifications of uro'gen-III which, on the one hand, leads eventually to haem and on the other to vitamin B_{12}. Taking haem first, we have already seen in scheme 1 that uro'gen-III undergoes enzymic decarboxylation to copro'gen-III before the 2 propionic residues on rings A and B are converted into vinyl groups; the macrocycle is then aromatized to form protoporphyrin-IX (**4**) ready for insertion of Fe^{II}. So for haem, the vinyl groups remain as such but use is made of them, in various ways, to build other important natural systems. For example, in cytochrome C (**22**), the vinyl groups are used to allow covalent attachment of the haem system to the protein. Also the vinyl group on ring B is reduced at some stage during the biosynthesis of chlorophyll *a* (**2**). Obviously, the redox and other properties of the macrocyclic complex will be affected by whether the molecule carries vinyl groups or not. These properties will also be modified by the greater or lesser conjugation of any vinyl group(s) with the main macrocycle depending on the dihedral angle between vinyl and macrocycle. Perhaps these may be among Nature's methods for 'fine tuning' the properties of the macrocycle. Be that as it may, I am sure you will agree that the vinyl groups are structurally important and chemically interesting. Our aim in the work to be described now was to determine the complete stereochemistry of formation of the vinyl groups of protoporphyrin-IX (**4**).

Our early work is published[21,22] and parallel studies were carried out by Professor Akhtar[23], so the main findings can be summarized briefly in scheme 13. We used an enzyme 'cocktail' prepared from *Euglena gracilis* which converts PBG (**23**), through all the necessary steps, into protoporphyrin-IX (**24**). 1 sample of PBG was $^2H_2$-labelled at C-8 and a 2nd sample similarly $^2H_2$-labelled at C-9 and when these materials were enzymically converted into protoporphyrin-IX it was found that the 3 hydrogen atoms on each vinyl group are 3 of the 4 originally present in the propionate side-chain of PBG. The 2 sets of results are combined together in scheme 13 for brevity.



(22) Cytochrome C



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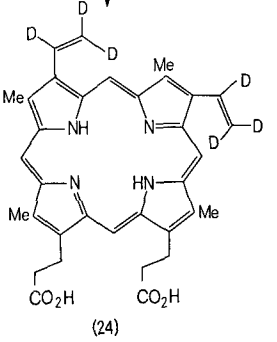


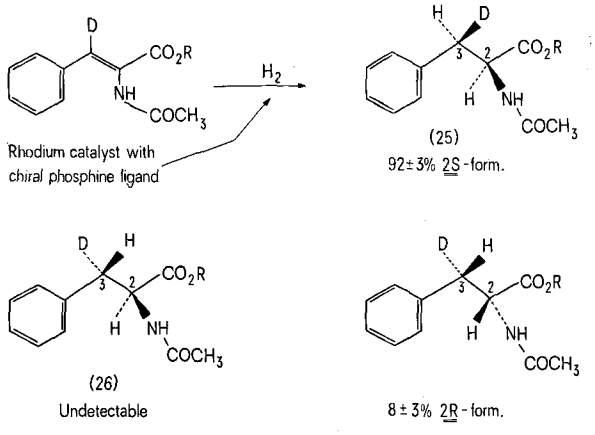
(24)

Scheme 13

* Our emotions are all against an alternative possibility, but the following process is also conceivable. Ring-D could be cleaved, following protonation of the enzyme-bound bilane, and this pyrrole unit (unsubstituted at both α-positions) could turn over without being allowed to escape from the catalytic site. Fresh bonding to the rest of the bound tripyrrole unit could then yield a rearranged bilane ready for ring-closure to uro'gen-III.

The stereochemical problem now stands out; which of the enantiotopic hydrogens at C-8 of PBG remains in each vinyl group and how are the enantiotopic hydrogens at C-9 of PBG finally arranged in each vinylic methylene residue.

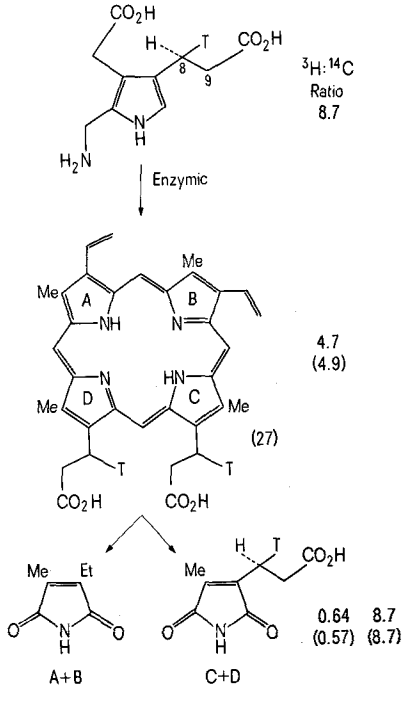
Our plan for C-8 was to use asymmetric hydrogenation by the chiral homogeneous catalyst developed by Knowles[24]. But first, we wished to confirm in this series that such a rhodium catalyst causes syn-addition of hydrogen. This was achieved by carrying out the hydrogenation shown in scheme 14. The resultant phenylalanine derivative contained ca. 92% of the (2S)-isomer, corresponding to high asymmetric induction. Moreover, NMR[25] showed this major product to be **25** and the product **26** which would have resulted from anti-addition was undetectable. So the foundation was a firm one.



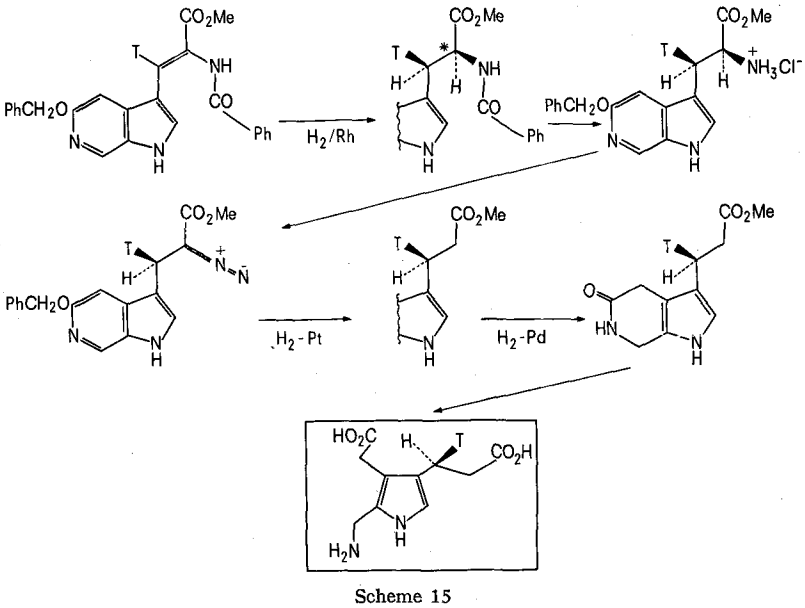
Scheme 14

The hydrogenation required for synthesis of PBG is shown at the start of scheme 15 and as before the 2S-configuration was generated at the starred chiral centre. The remaining steps leading to labelled PBG are self-explanatory. This sample was mixed with ^{14}C-labelled PBG to give a ^{3}H: ^{14}C ratio of 8.7 and on conversion enzymically into protoporphyrin-IX (**27**), the ratio fell almost to half (see scheme 16). Further degradation as illustrated (after hydrogenation of the vinyl groups) clearly showed that tritium had largely been lost during biosynthesis of the vinyl groups. The values found correspond closely to those expected (shown in brackets) for ca. 92% configurational purity from the initial hydrogenation step in scheme 15.

Thus it is the Si-hydrogen atom which is removed from C-8 of PBG during the biochemical formation of the



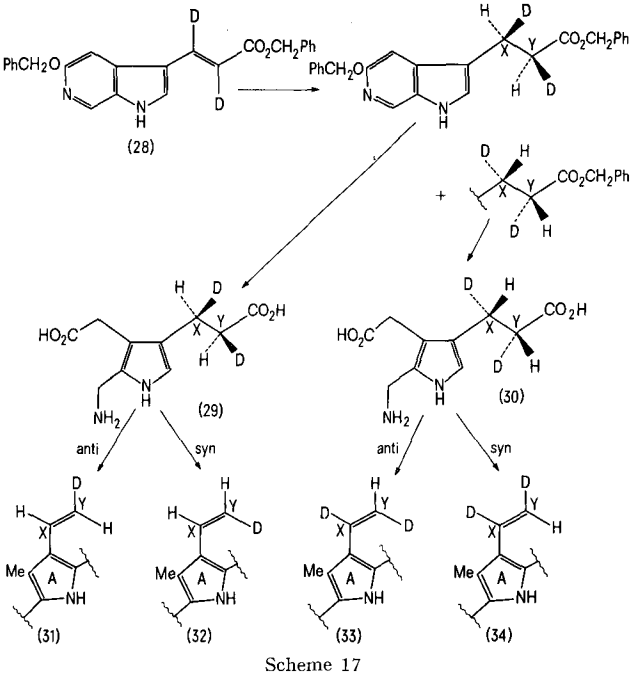
Scheme 16



Scheme 15

vinyl group on ring A and on ring-B of protoporphyrin-IX[26]. This result agrees with Professor Akhtar's earlier study[23] of this centre based upon labelling very early in the biosynthetic sequence, so there is interlocking strength from the 2 approaches.

We can now build on this stereochemical knowledge to discuss how Nature is working at the vinylic methylene groups. This study demands a sample of PBG

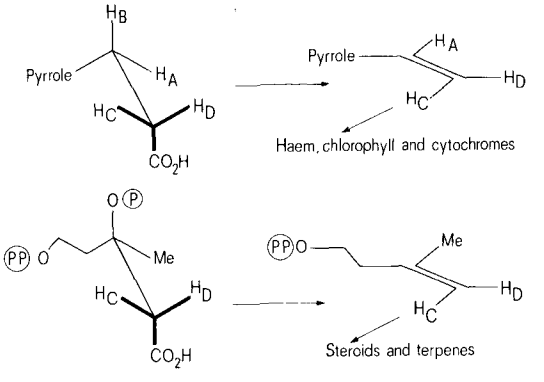


Scheme 17

labelled stereospecifically at centres X and Y and it was realized that the racemate (**29**) + (**30**) was sufficient.

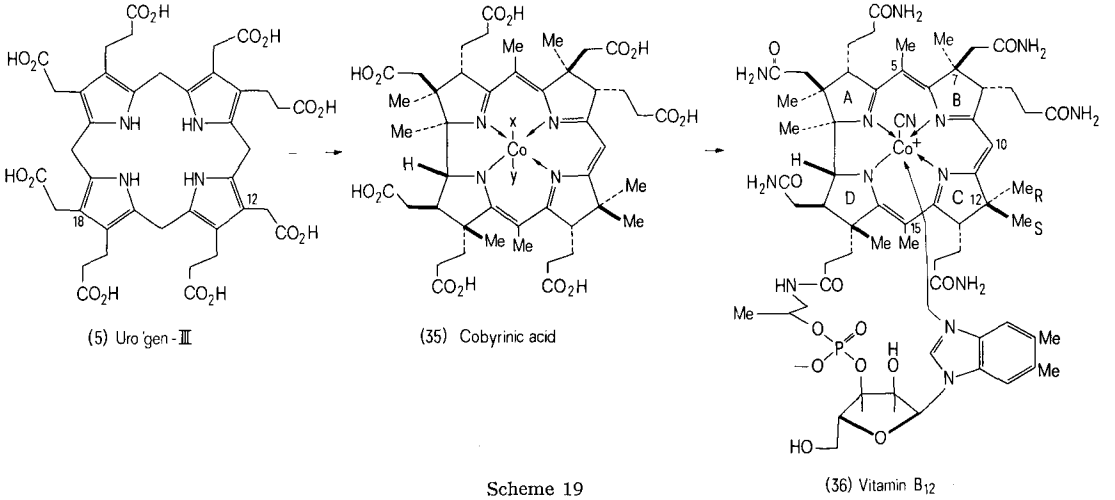
The preparation started (scheme 17) by reduction of the dideutero acrylic ester (**28**) with diimide which by its syn-stereospecificity fixes the relative configuration at centres X and Y. This arrangement is not affected by the subsequent reactions which produce PBG **29** and **30**.

Now consider the outcome of overall anti- or syn-elimination (irrespective of the precise mechanism) from the molecules **29** and **30**. The resultant labelling patterns for the vinyl groups are illustrated in scheme 17. So by focussing the ^{1}H-NMR-analysis on the hydrogen at centre X, neither product **33** nor **34** from enantiomer **30** will register and a clear ^{1}H-^{1}H coupling pattern should be seen from products **31** or **32**. Under suitable conditions, separate signals can be seen from H_X on each of the vinyl groups of protoporphyrin-IX ester. So this ester, derived enzymically from the labelled PBG **29** and **30**, was examined by NMR in this way and 2 doublets with trans-coupling (18 Hz) were observed from the 2 H_X (see **31**). Both vinyl groups of protoporphyrin-IX (**4**) and so of haem (**1**) are thus formed by an overall anti-elimination[27] and the absolute stereochemistry of this process is shown in scheme 18. Also illustrated there is the stereochemical course of the process which generates another vitally important vinyl group in isopentenyl pyrophosphate[28]. The correspondence is striking.



Scheme 18

The final part of this lecture concerns vitamin B_{12} and this takes us to the 2nd modification of uro'gen-III. But in addition, this section brings together, in a most fascinating way, 2 stories which at first sight seem unrelated.



Scheme 19

Look at the structure of vitamin B_{12} (**36**). The aim is to find out how living systems build such a marvellous molecule. Bernhauer's group[29] had made the important observation that vitamin B_{12} (**36**) is formed from cobyrinic acid (**35**); so the various amide residues and other attachments around the periphery of the vitamin are added late in the sequence. The major problem thus becomes that of understanding the biosynthesis of cobyrinic acid (**35**).

I want to leap over all the early work on this problem by several groups – those of Shemin, Scott, Arigoni, Müller and ours in Cambridge (see references 1 and 30–35 inclusive) – and start at the point that cobyrinic acid had been rigorously proved[31,33–35], to be biosynthesized from uro'gen-III (**5**); see scheme 19. Many steps are obviously needed for this conversion and the required operations are listed in scheme 20. We reasoned that the most probable enzymic step carried out next on uro'gen-III (**5**) is either C-methylation or decarboxylation of the acetic acid residue at C-12. The appropriate tracer experiments[35b] pointed clearly against decarboxylation and therefore our efforts were focussed on seeking C-methylated intermediates.

Scheme 20

Eleven steps.

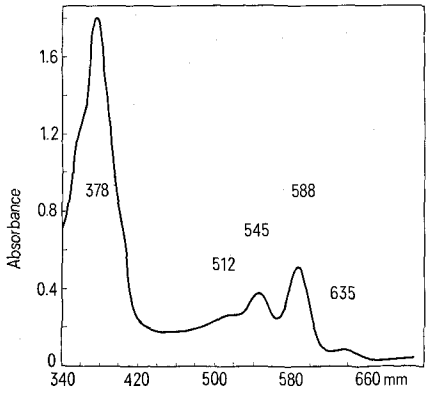
a Introduction of 7 methyl groups from methionine at carbons C-1, C-2, C-5, C-7, C-12 (pro-R), C-15 and C-17.

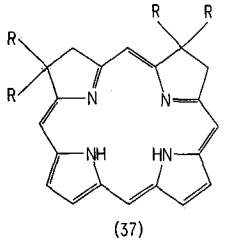
b Decarboxylation of the acetic acid side-chain at C-12.

c Extrusion of C-20 from the skeleton of uro'gen-III.

d Possible adjustment of the oxidation level.

e Insertion of cobalt.





(37)

Block E

We are fortunate at this stage to be able to collaborate with Professor Bykhovsky in Moscow who had obtained new pigments by modifying the growing conditions of *Propionibacterium shermanii* which biosynthesizes vitamin B_{12}. The modification involved inter alia strict exclusion of cobalt and a period of starvation. Professor Bykhovsky's group mainly carried out the biological work and we mainly handled the separation and structural problems. As a result of this excellent collaboration, many new pigments have been isolated but a simplified account is sufficient here; we will concentrate on 2 pigments, isolated as their methyl esters[36,36a]. Both showed the characteristic[37] isobacteriochlorin chromophore (**37**), which has 2 adjacent 'reduced' rings; 1 pigment was an octamethyl ester and the other had only 7 methyl ester groups. This important information came from field desorption mass spectrometry (carried out by Dr H. R. Morris, Imperial College) on the esters prepared firstly using CH_3OH and secondly using CD_3OH. The molecular weight of the octa-ester corresponded to the ester of a dimethylated derivative of uro'gen-III (**5**) minus 2 hydrogen atoms. When the IR-spectrum of the hepta-ester showed a strong band at 1775 cm^{-1}, corresponding to a 5-membered lactone, one could scent the probable relationship of the 2 pigments. It turned out that the lactone residue of the hepta-ester could be reductively cleaved and the product (after esterification) was the octa-ester as indicated on the left hand side of scheme 21. Thus, structural information derived from studies on the lactone hepta-ester also holds good for the octa-ester.

It is important to emphasize that the lactone is not a true natural product but is formed oxidatively during the handling of the acid corresponding to the octa-ester. Such oxidative ring closures are known in the corrin series[38] and its occurrence in this case was fortunate for us, as will be seen later.

I want to pause at this stage to consider the beautiful work of Kamin and Siegel et al.[39] on sulphite reductase enzymes. They isolated the metal-free prosthetic group from several such systems, and called it sirohydrochlorin. Their evidence was clear that this substance is an isobacteriochlorin and that it was a dimethylated derivative of uro'gen-III minus 2 hydrogen atoms. They tentatively proposed that the 2 methyl groups

Scheme 21

Vitamin B_{12} system (*Propionibacterium shermanii*)	Sulphite reducing system (*Desulphovibrio gigas*)
↓	↓
A Isobacteriochlorin octa methyl ester $C_{50}H_{62}N_4O_{16}$	*A* Sirohydrochlorin octa methyl ester $C_{50}H_{62}N_4O_{16}$
$Zn\text{-}CH_3CO_2H$ ↑ esterify	$Zn\text{-}CH_3CO_2H$ ↑ esterify
B Isobacteriochlorin hepta methyl ester (5-Lactone) $C_{49}H_{58}N_4O_{16}$	*B* A monolactone of sirohydrochlorin as hepta methyl ester $C_{49}H_{58}N_4O_{16}$

Identity by ^{1}H-NMR, UV-vis, IR, FD-MS, HPLC and CD.

had been substituted at C-12 and C-18 of uro'gen-III (**5**). They also made the inspired suggestion that sirohydrochlorin could represent an early intermediate on the pathway to vitamin B_{12} or at least share a proximate precursor.

This important lead was followed in Cambridge[40] in parallel with the work which has been outlined on the new pigments from *P. shermanii;* the metal-free prosthetic group was isolated from desulphoviridin isolated from *Desulphovibrio gigas*. We obtained, after esterification, a small quantity of sirohydrochlorin octamethyl ester and mainly a lactone hepta-ester*; data about these pigments from *D. gigas* are given on the right hand side of scheme 21. You can imagine the excitement when the octa-esters from *P. shermanii* and from *D. gigas* were found to be identical and identity was also established for the 2 samples of lactone hepta-ester[36a,40]. The methods used to prove identity are collected at the foot of scheme 21 and notice particularly that the circular dichroism curves were identical for the 2 octa-esters. They therefore match in absolute configuration.

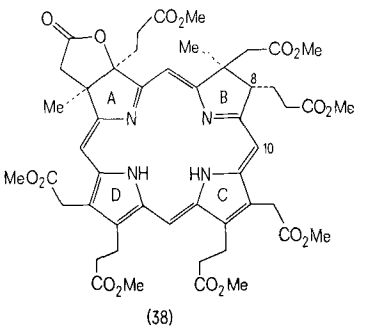
The NMR-spectrum of the lactone hepta-ester was highly informative. At low field appeared 4 characteristically well-spread signals[37] from the 4 CH's of the meso-bridges. The hydrogen atoms on 3 of the meso-bridges (between, and adjacent to the reduced rings) underwent exchange with CF_3CO_2D at different rates as was known from Bonnett's work[35] on simple isobacteriochlorins. Two 3H-singlets at high field corresponded to 2 C-methyl groups sited on quaternary carbon atoms.

A fascinating feature of the NMR-spectrum was that one of the signals from a meso-CH adjacent to a reduced ring was long-range coupled (0.9 Hz) to a single proton at δ 4.3 which we know from model studies corresponds to a proton on the periphery of a reduced ring. So we are observing a small allylic coupling from C-10 to C-8 (see structure **38**) or from an equivalent arrangement.

One must now use the foregoing data to analyse all possible isobacteriochlorin structures for the lactone ester bearing in mind that this substance, and its parent sirohydrochlorin, have been isolated by stressing a B_{12}-producing organism. The result is that 2 structures, **38** and **39**, fit all the information. Structures **40** and **41** then follow for sirohydrochlorin ester.

The argument which allows some choice between these 2 possibilities for sirohydrochlorin is as follows. There is presumptive evidence from the way it is produced by *P. shermanii* that sirohydrochlorin is on, or

* Further work is needed before this remarkably specific isolation of monolactone is fully understood.



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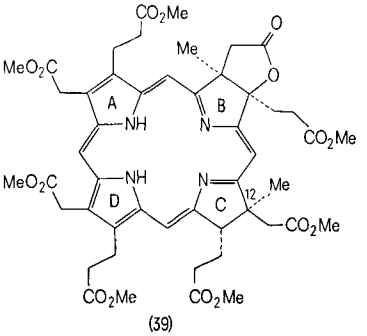

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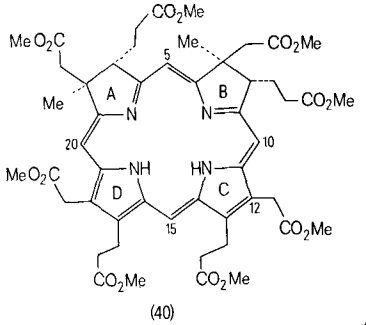

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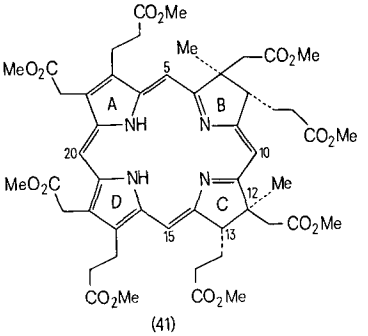

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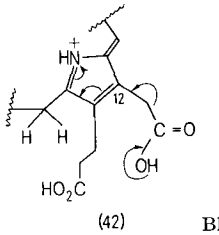


(42)

Block F

very close to, the biosynthetic pathway to vitamin B_{12} and incorporation experiments will be outlined later. On the basis of structure **40**, decarboxylation of the acetic acid side chain at C-12, necessary for the eventual production of cobyrinic acid (**35**), could occur by a straightforward acid-catalyzed process (see **42**)*. Such a sequence is blocked** in structure **41**. These are the grounds for preferring at present the A/B structure **40** for sirohydrochlorin octamethyl ester though it should be recognized that the argument against **41** is purely a biogenetic one. The necessary experiments finally to settle this point are in progress+.

As a final point, the C-methyl groups of sirohydrochlorin and the corresponding monolactone were shown to be derived as expected from [^{14}C-methyl] methionine by incorporation experiments using both *D. gigas*[40] and *P. shermanii*[41].

Sirohydrochlorin ester is visually very attractive. A solution in chloroform is purple-red, its fluorescence is an intense orange-red whilst in acidic solution, the colour is deep blue. This colour in acid is the basis of my reference at the outset to a new blue pigment and you can now see this pigment's relationship to haem, on the one hand, and to vitamin B_{12} on the other. Indeed, sirohydrochlorin (roughly speaking) is the combination of half of protoporphyrin-IX and half of cobyrinic acid. The future will undoubtedly yield much fascinating work in this area.

There is still a further theme to this story concerning the pigment, named Faktor II, isolated in Stuttgart[42] from *P. shermanii*. Importantly, singly and doubly labelled forms of Faktor II were shown[42] to be incorporated into cobyrinic acid (**35**) by an enzyme preparation from *Clostridium tetanomorphum*. It was clear to us from the reported properties of Faktor II that it must be sirohydrochlorin and this was rigorously established by comparing a sample, kindly sent by Professor Müller, with our material. Naturally, incorporation experiments are also in progress in Cambridge but already there is a firm interlock of the foregoing isolation, structural and labelling experiments on sirohydrochlorin (including the earlier evidence against C-12 decaboxylation being the next biosynthetic step beyond uro'gen-III). As a result, the pathway to cobyrinic acid (**35**) and vitamin B_{12} begins to take shape. This is outlined in scheme 22++. There is also preliminary evidence[40] that the third C-methylation occurs at C–5 and further work on this point is in hand. Indeed, we shall be seeking information by every helpful method about the pathway beyond sirohydrochlorin until we know, step by step, how the marvellous structure of vitamin B_{12} is built in life.

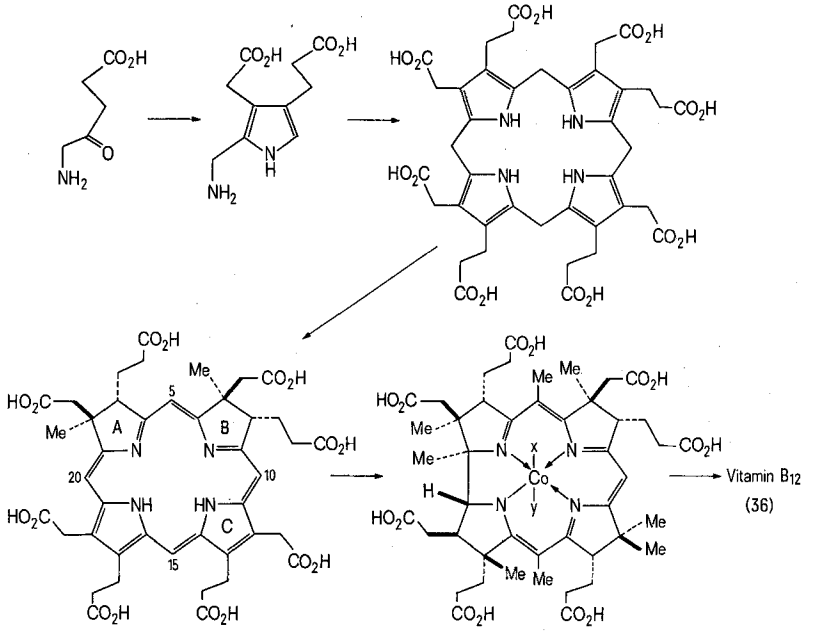
*This does not imply that decarboxylation is the next step on the biosynthetic pathway; the illustrated process, or very similar ones, could occur later provided C-12 has not undergone C-methylation.

**This blockage might be circumvented in a number of ways. Professor A. Eschenmoser (Zürich) urged us to keep these possibilities to the fore and he suggested 2 specific reaction sequences. I wish to thank him warmly for these valuable discussions during my visit to Zürich.

+ Sirohydrochlorin has also been isolated from *P. shermanii* by Professor A. I. Scott's group (Yale) and they too prefer the A/B structure **40**; we are grateful to Professor Scott for kindly sending this information (May 30th, 1977).

++ One should remember when viewing scheme 22 that 2 aspects require further study. Firstly, C-methylation twice of uro'gen-III (**5**) would produce a dihydroisobacteriochlorin. Therefor either a) the dihydro system is the true biosynthetic intermediate but the oxidized form is isolated; if this is so, the oxidized form must be reduced again by the enzyme preparation to the dihydro state to account for its incorporation into cobyrinic acid or b) the dihydro system first formed is dehydrogenated in vivo to the isobacteriochlorin which lies directly on the pathway and situable adjustment of oxidation level occurs at a later stage.

Secondly, rigorous evidence is needed to select finally between structures **40** and **41** for sirohydrochlorin octamethyl ester. Scheme 22 may need the change A/B to B/C, depending on the outcome.

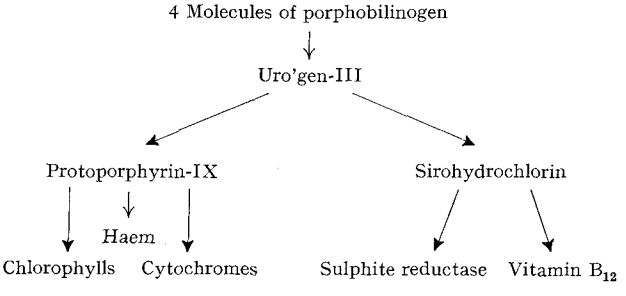


Scheme 22

The chemistry described in this lecture has given us enormous pleasure and I have tried to pass some of this on to you. But you will also enjoy thinking about the evolutionary aspects. Scheme 23 outlines the biosynthetic relations and it stands out that the 5 key structures at the bottom of the scheme are all moulded from uro'gen-III. The chemistry involved takes full advantage of the intrinsic reactivities of the various molecules though enzymic catalysis and control are operating throughout.

The beauty of Nature's chemistry fills one with admiration and the aim of this Paul Karrer Lecture has been to illuminate the biosynthetic pathways responsible for many of the conversions collected in scheme 23.

Scheme 23



Acknowledgments. I have been immensely fortunate over the last 10 years on these problems to have worked with groups of outstanding young colleagues. Their names are given on the published papers (see literature references) and the most recent unpublished work has been carried out by J. B. Campbell, C. J. R. Fookes, R. Hollenstein, K. Jones, M. J. Meegan, J. A. Robinson, M. Thompson and D. C. Williams. Today's account could not have been given without their enthusiastic efforts. I also wish to record my warmest thanks to my senior colleague, Dr Edward McDonald, whose contributions to the work have been invaluable throughout.

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