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Biosynthesis of Vitamin B_{12}: Isobacteriochlorins, the Link between Corrins and Sulphite Reductases[1]

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The beautiful structure of vitamin B_{12} has attracted great interest in its biosynthesis. This review briefly outlines the background and then covers very recent developments which have shown the importance of isobacteriochlorins in relation to the B_{12} biosynthetic pathway. These isobacteriochlorin systems are also key materials for sulphite and nitrite reductase enzymes, and thus they act as a bridge, presumably an evolutionary bridge, between these enzymes and vitamin B_{12}. Determination of the structures of the isobacteriochlorins opens up the central part of the biosynthetic pathway to the corrin macrocycle.

INTRODUCTION

The biosynthetic pathway (*1*) from 5-aminolevulinic acid, ALA (**1**),[2] to life's vital pigments of the heme and chlorophyll types is outlined in Scheme 1. This scheme also indicates that uroporphyrinogen-III, uro'gen-III (**3**), is a precursor of vitamin B_{12} (**5**), a fact which was rigorously established (*2, 3*) in 1975 when our earlier review (*1*) was at the proof stage. Since then, there has been considerable progress in defining part of the pathway from uro'gen-III (**3**) to cobyrinic acid (**7**), which is an established precursor (*4*) of vitamin B_{12}; this review is therefore a timely one.

INCORPORATION OF PRIMARY PRECURSORS

Pioneering experiments on the biosynthesis of vitamin B_{12} demonstrated that: (a) as for the porphyrins, the corrin nucleus is largely derived from ALA (**1**) (*5*); (b) at least six of the eight *C*-methyl groups of the vitamin (**5**) are provided by methionine (*5c, 6*); and (c) cobyrinic acid (**7**) is the parent corrin, which is successively amidated (*4*) and further modified to afford vitamin B_{12} (**5**) and coenzyme B_{12} (**6**).

The advent of ^{13}C-nmr allowed these observations to be considerably refined. Whereas the lack of suitable degradations had made it impossible to locate all the radioactivity in a multiple ^{14}C-labelled corrin, the labelling pattern of a ^{13}C-enriched corrin could be determined directly by nmr methods (*7*). This new approach confirmed that ALA (**1**) is incorporated into corrins (*8–11*) in essentially the manner found for the porphyrins, but it clearly showed that the methyl group at C-1 is derived *not* from ALA (*8–11*), via C-20 of a porphyrinogen, but from methionine (*9–12*). The methyl groups at

[1] Dedicated with warmest good wishes to Professor W. S. Johnson on the occasion of his 65th birthday.

[2] The boldfaced numbers in parentheses refer to the corresponding structures presented in this paper.

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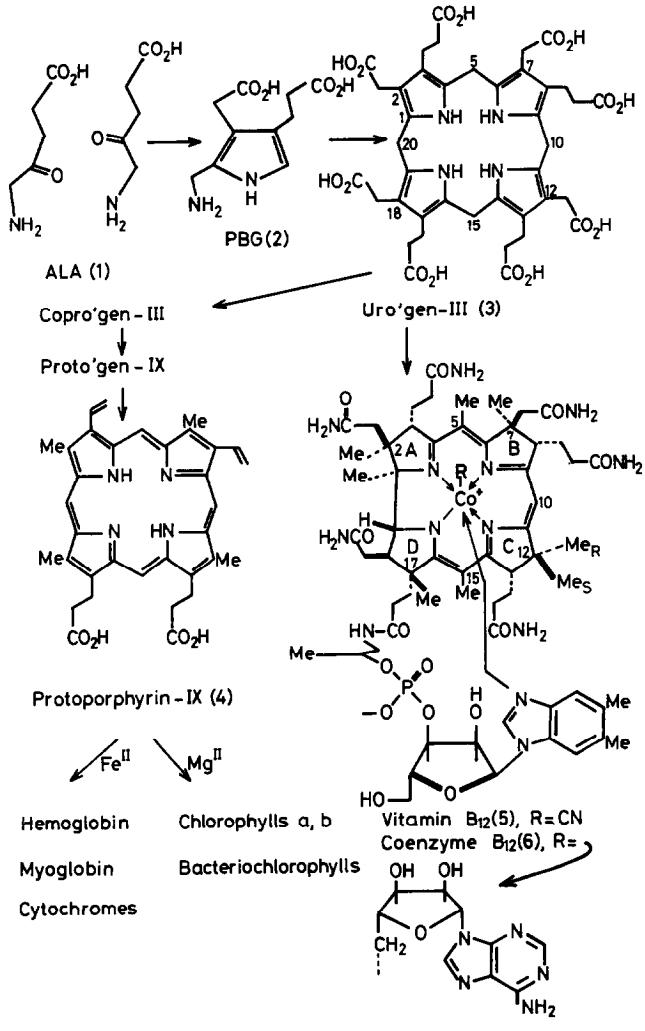


SCHEME 1

C-2, C-5, C-7, C-12 (R) (*10*, *11*, *13*), C-15, and C-17 [see (**5**)] are also methionine derived (*9–13*) and in each case the CH_3 group is transferred *intact* without proton exchange with the medium (*3b*, *14–17*).

CONVERSION OF URO'GEN-III INTO COBYRINIC ACID

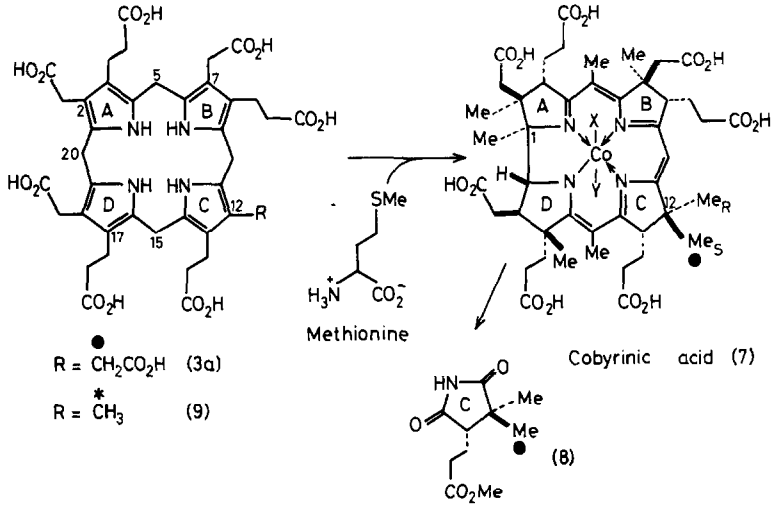
(*a*) *Specific Incorporation of Uro'gen-III* (**3**)

Clear proof that uro'gen-III (**3**) is a precursor of cobyrinic acid (**7**) came from experiments (*2*, *3*) in which nonsymmetrically labelled forms of (**3**) were incubated with enzyme preparations from *Propionibacterium shermanii*. It was found that [12-*methylene*-^{14}C]uro'gen-III (**3a**) (*3*) and [5, 15-$^{13}C_2$]uro'gen-III [as (**3a**)] (*2*) were incorporated well into cobyrinic acid (**7**). Degradation of the ester of cobyrinic acid

from the former experiment yielded the ring-C imide (**8**) and established specific labelling (*3*) as illustrated, while location of the ^{13}C-enriched sites was determined (*2*) by ^{13}C-nmr.

It is important to emphasize that the foregoing experiments used broken cells of *P. shermanii* or cell-free systems, and *specifically* labelled forms of uro'gen-III. Unambiguous labelling of the precursor and proof of labelling pattern in the product are always important in biosynthetic studies but especially so in the polypyrrole field, which is bedeviled by breakdown and chemical transformations. In addition, when whole cells of *P. shermanii* were used there were difficulties in achieving penetration of the labelled materials. Initially, no incorporation of uro'gen-III was observed into cobyrinic acid (*18*, *19*); but when symmetrically ^{14}C-labelled uro'gen-III [as (**3**)] was incubated in relatively large quantity with the cells, labelled cobyrinic acid (**7**) was produced (*20*).

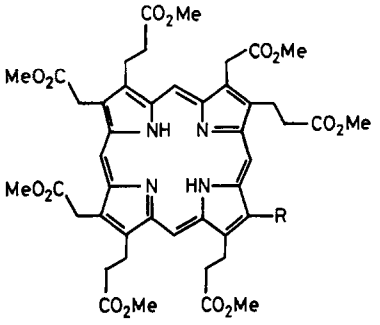
More recently (*21*), a cell-free enzyme system from *Clostridium tetanomorphum* has proved to be very effective in converting uro'gen-III (**3**) into the corrin nucleus (**7**).



We can now analyse the chemical problems involved in transforming uro'gen-III (**3**) into cobyrinic acid (**7**). The following steps are required in a sequence yet to be determined: (i) decarboxylation of the acetic acid side chain at C-12; (ii) methylation at C-1, -2, -5, -7, -12 (R), -15, and -17; (iii) loss of C-20; (iv) bond formation between C-1 and C-19; (v) introduction of cobalt; (vi) redox adjustments. The number of possible sequences is immense, so there was a clear need to identify intermediates by isolation and, in parallel studies, by synthesis of hypothetical precursors for test in labelled form using the isolated enzyme system. Initially no clues were available from structurally related natural products, and we set ourselves the task of establishing what happens *first*. On mechanistic grounds, steps (iii, iv, v and vi) seemed unlikely to be first, and methylation at C-5 or C-15 was improbable because these carbon atoms are not appropriately activated in uro'gen-III (**3**). Last, introduction of the *pro*-R methyl group at C-12 was set aside, since this would appear to block the necessary decarboxylation step at this site. The remaining possibilities which thus seemed the most likely ones are now discussed in turn.

(b) Decarboxylation at C-12 as the First Step?

The possibility that decarboxylation of the acetic acid side chain at C-12 is the first step seemed attractive, since analogous decarboxylations certainly take place during the bioconversion of uro'gen-III (**3**) into copro'gen-III (*1*), as shown in Scheme 1. In the systems examined, the first side chain affected is that at C-18 (*22*), followed in sequence by those at C-2, C-7, and C-12 (*23*). For the studies on corrins, the heptacarboxylic porphyrin ester (**10**) was labelled with ^{14}C at the C-12 methyl group. It should be noted that this choice of labelling was made to avoid any risk of contamination by small amounts of the labelled ester of uroporphyrin-III (**11**) which might be formed by side-reactions in the synthesis; the presence of ^{14}C-labelled uroporphyrin-III (**11**) would have been disastrous. The corresponding heptacarboxylic porphyrinogen (**9**) was then incubated under a variety of conditions with broken cells of *P. shermanii*, but the incorporation of radioactivity into cobyrinic acid (**7**) was very small in every case; in parallel experiments, the incorporation of uro'gen-III (*3a*) into cobyrinic acid (**7**) was



R = *Me (10)

R = CH_2CO_2Me (11)

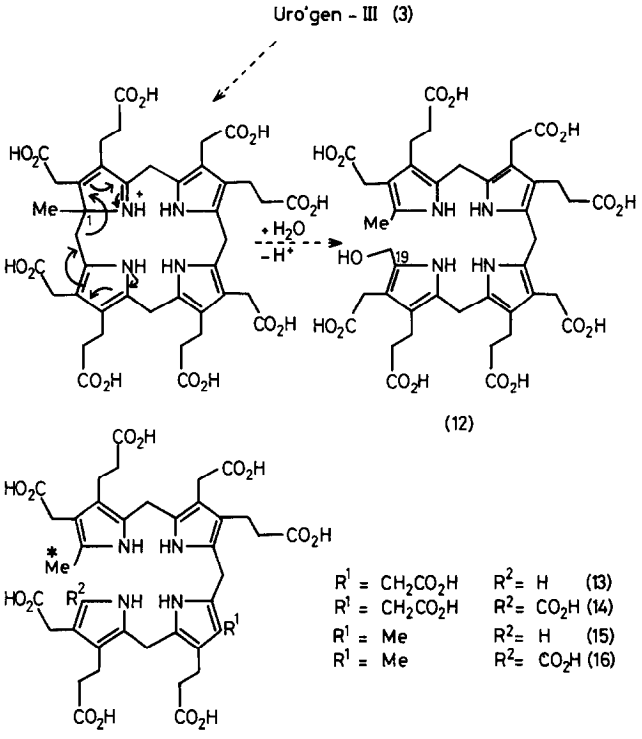
30–50 times greater (*3b*). The conclusion was that the heptacarboxylic porphyrinogen (**9**) is not a normal precursor of vitamin B_{12},[3] and this view (*3b*) has been confirmed by more recent research (see ahead).

(c) Methylation at C-1 as the First Step?

The loss of C-20 from uro'gen-III (**3**) during its conversion into cobyrinic acid (**7**) almost certainly involves cleavage of the macrocycle at some stage to form a *seco*-system. It was conceivable that this might occur at an early stage, triggered by C-methylation at C-1 of uro'gen-III (**3**), as in Scheme 2. Protonation of the tetrapyrrole system (**12**) at C-19 could then allow the carbon which was C-20 to be lost as formaldehyde, for which there is evidence (*24*), or as carbon dioxide after biochemical oxidation of the CH_2OH residue.

The experimental tests of such possibilities were run in conjunction with the work described in the previous section. Accordingly, the materials corresponding to both

[3] Scott *et al.* reported (*2*) a 0.1% incorporation of the same porphyrinogen (**9**) [labelled with ^{14}C at C-5 and C-15] into cobyrinic acid (**7**) of unknown labelling pattern, and they drew the opposite conclusion; see also Ref. *24*.



SCHEME 2

structures (**3a**) and (**9**) were included in the plan. The four tetrapyrroles [(**13**)–(**16**)] were synthesized unambiguously (*25*) with a ^{14}C label at the terminal methyl group, and each one was tested in an enzyme system shown by control runs to be effective for converting uro'gen-III into cobyrinic acid. No incorporation into cobyrinic acid was observed (*25*) with any of the compounds [(**13**)–(**16**)]. These results pointed against methylation at C-1 of uro'gen-III (**3**) being the next step on the pathway, and rigorous proof came from studies to be described in the following sections.

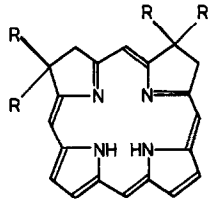
(d) Methylation at C-2 or C-7 or C-17 as the First Step?

An approach different from that used in the preceding section was necessary for this study because it would have been quite impractical to synthesize the many possible isomers of, say, one and two C-methylation steps on uro'gen-III (**3**). All the effort was therefore applied to a search for partially methylated materials from the B_{12}-producing organism. Two important clues were available in the literature to give invaluable help with this phase of the work.

One clue came from reports (*26*) of structural studies on the unusual heme prosthetic group of the sulphite reductase enzymes; this topic will be expanded later. The second clue was contained in reports from Moscow (*27*) of a mixture of new pigments from *P. shermanii* which were neither porphyrins nor corrins; a collaborative effort between Cambridge and Moscow was launched on the structure of these substances. This account of work on the new pigments will initially only be carried to the point where the

results fused with those obtained from research, run almost in parallel, based upon the sulphite reductase clue. Both lines of investigation will then be combined to drive forward to the final conclusion.

(i) *Isobacteriochlorins from P. shermanii.* The mixture of new pigments from *P. shermanii* had been obtained (*27*) by a growing regime which involved strict exclusion of cobalt and a starvation period. High-pressure liquid chromatography, h.p.l.c. (*28*), was highly effective for fractionation of the methyl esters of the pigments and the major components, corriphyrin-4 and corriphyrin-3, were isolated as their methyl esters in sufficient quantity for full spectroscopic study (*29*). Both showed the characteristic (*30*) isobacteriochlorin chromophore (**17**) and the assignment of this chromophore was confirmed by preparation of their Zn^{II} complexes for comparison with the Zn^{II} derivatives of a synthetic isobacteriochlorin. Field-desorption (FD) mass spectrometry (m.s.) established the molecular formulae of corriphyrin-4 and corriphyrin-3 methyl esters as $C_{48}H_{54}N_4O_{16}$ (*m/e* 942) and $C_{49}H_{56}N_4O_{16}$ (*m/e* 956), respectively, and both were hexacarboxylic esters. In addition, both esters showed strong absorption at 1775 cm^{-1} in the infrared, arising from γ-lactone residues in each structure. Finally, a small amount of a third isobacteriochlorin was isolated from the Moscow material and this proved (*29*) to be a mono-γ-lactone heptacarboxylic ester, $C_{49}H_{58}N_4O_{16}$ (*m/e* 958).



Isobacteriochlorin
(17)

The presence of lactone groups in these products strongly suggested that they were probably formed oxidatively during the isolation procedures; such oxidative ring closures are known in the corrin series (*31*). Accordingly, the handling and isolation methods were extensively modified and, under these conditions, the major product was an octacarboxylic isobacteriochlorin ($C_{50}H_{62}N_4O_{16}$, *m/e* 974, as octamethyl ester). It was accompanied by a smaller quantity of the same monolactone which had been the minor product in the material from Moscow. Reductive cleavage of the monolactone afforded, after esterification, the same octacarboxylic ester we had obtained above as the major product.

(ii) *Isobacteriochlorins from sulphite and nitrite reductases.* These enzymes catalyse the reductions shown in Scheme 3 and it was known that they contained an unusual heme of unknown nature as the prosthetic group. Important studies in 1973 showed (*26*) that the heme from *Escherichia coli* sulphite reductase gave, after reductive removal of iron, an octacarboxylic isobacteriochlorin which was named sirohydrochlorin. Such material was also observed by other workers (*32*). The composition and properties of this product ($C_{50}H_{62}N_4O_{16}$, *m/e* 974, for the octamethyl ester), agreed with its being derived from a uro'gen isomer by C-methylation on adjacent rings. No experimental evidence was available for deciding which isomer was involved, nor the

sites of methylation, and a structure tentatively proposed has more recently been ruled out. But the importance of this work should be made clear, and this research has revealed that isobacteriochlorins are fairly widely distributed in sulphite (*26*, *32*) and

$$SO_3^{2-} + 6H^+ + 6e \rightarrow S^{2-} + 3H_2O$$
$$NO_2^- + 6H^+ + 6e \rightarrow NH_4^+ + 2HO^-$$

SCHEME 3

nitrite (*33*) reductases. This discovery has linked up in a very satisfactory way with research on the biosynthesis of vitamin B_{12}.

(iii) *The link between the metabolites from P. shermanii and sirohydrochlorin.* The foregoing important lead was followed in Cambridge in parallel with the work on the pigments from *P. shermanii.* A good source of sirohydrochlorin was needed to provide sufficient pure material for ^{1}H-nmr studies. With pulsed F.T. equipment about 0.1 mg of sample would perhaps be sufficient, but even this amount was not easy to obtain initially. After screening a number of organisms, *Desulphovibrio gigas* was chosen as the best source, and its green sulphite reducing enzyme, desulphoviridin, was isolated [cf. Ref. (*26*)].

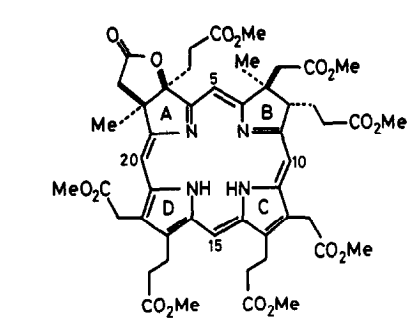
Release of the metal-free prosthetic group from this enzyme followed by esterification gave (*34*) a small quantity of sirohydrochlorin octamethyl ester, but the main product was a heptamethyl ester mono-γ-lactone, $C_{49}H_{58}N_4O_{16}$, *m/e* 958. Reductive cleavage of the lactone and esterification of the product gave sirohydrochlorin octamethyl ester.[4] It was very exciting to find that sirohydrochlorin octamethyl ester was identical with the octamethyl ester from the B_{12} producer, *P. shermanii* and the two samples of mono-γ-lactone from *P. shermanii* and *D. gigas* were also identical (*34*). These identities were established by ^{1}H-nmr, uv-visual, ir, FD–ms, h.p.l.c., and circular dichroism (CD); importantly, the identity of the CD curves for sirohydrochlorin ester from the two sources shows that they match in absolute configuration.

The link had thus been strongly forged (*29*, *34*) between the isobacteriochlorins of *P. shermanii* and those of the sulphite reductases. It seemed extremely likely that this link would have biosynthetic importance for vitamin B_{12}, and this turned out to be the case (see later). First, however, our full structural studies on all these isobacteriochlorins will be outlined.

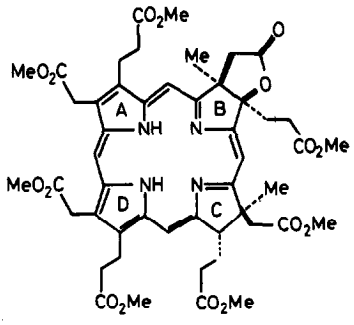
(*iv*) *The structures of sirohydrochlorin and its monolactone and of corriphyrin-4 and corriphyrin-3.* The reductive conversion of sirohydrochlorin monolactone into sirohydrochlorin was complemented by the observation that the former is produced from the latter during handling in air. Thus, structural information derived from studies on the lactone hold good for sirohydrochlorin itself. The key experiments (*29*, *34*) which led to structure (**18**) for the monolactone ester and to structure (**20**) for sirohydrochlorin ester are outlined below.

First, the ^{1}H-nmr spectrum of the lactone showed four well-spread signals (*30*) at low field corresponding to the four hydrogen atoms on the bridges, i.e., C-5, C-10, C-15, and C-20. Three of these hydrogens (between and adjacent to the reduced rings) underwent exchange with CF_3CO_2D, the signal at the highest field being most rapidly eliminated; this high-field signal arises from the C–H *between* the reduced rings (*30*).

[4] The remarkably specific isolation of the monolactone is most interesting and more work is needed before it will be fully understood.



(18)



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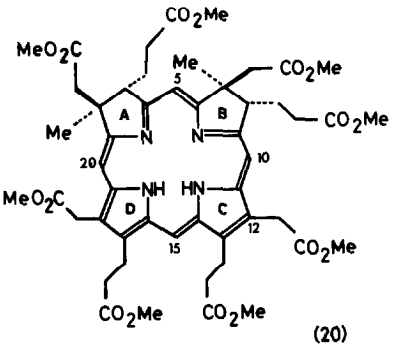
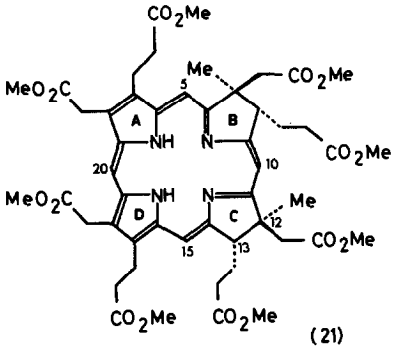


(20)

Sirohydrochlorin
octamethyl ester



(21)

Two 3H singlets at high field showed the presence of two *C*-methyl groups attached to quaternary carbon atoms.

An important feature of this ^{1}H-nmr spectrum was that one of the signals from a bridge C–H group *adjacent* to a reduced ring was split by long-range coupling to a single proton at δ4.3. Parallel synthetic work had shown that δ4.3 corresponds to a proton on the periphery of a reduced ring.

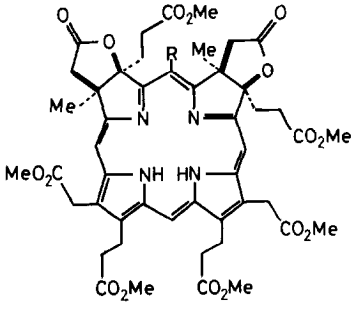
This information, coupled with the now very reasonable assumption that the monolactone and its relatives are trapped forms of intermediates on the pathway to cobyrinic acid (**7**), leads to structure (**18**) or (**19**) for the monolactone ester and to (**20**) or (**21**) for the ester of sirohydrochlorin itself. The first alternative in each case was preferred on the earlier argument that C-methylation at C-12 would block straightforward biochemical decarboxylation, which must occur subsequently at the attached acetic acid residue. Again unambiguous proof that the preferred structure (**20**) for sirohydrochlorin ester is indeed the correct one comes from later experiments; it will be accepted at this stage to simplify the presentation of the rest of the structural work.

Corriphyrin-4 hexamethyl ester (**22**) showed, in its ^{1}H-nmr spectrum, four low-field singlets from the four bridge C–H groups, similar to those described above; it also showed the same exchange behavior with CF_3CO_2D. Two 3H singlets were present at high field. Since corriphyrin-4 was found to be formed during handling of the monolactone (**18**), structure (**22**) follows from it, as accommodating all the data (*29*).

The ^{1}H-nmr spectrum of corriphyrin-3- hexamethyl ester (**23**) differed from that just described in two important respects: (a) Only three low-field signals appeared from

bridge C–H groups and none exchanged rapidly with CF_3CO_2D, thus, the position between the reduced ring is substituted; (*b*) an additional 3H singlet appeared at δ3.38. Bearing in mind that corriphyrin-3 ester contained one CH_2 more than corriphyrin-4 ester, then stucture (**23**) can be assigned (*29*) to corriphyrin-3 ester.

The structure of corriphyrin-3 ester (**23**) provides important information. Just as the acid corresponding to corriphyrin-4 ester (**22**) arises oxidatively from sirohydrochlorin [(**24**), Scheme 4], so the parent of corriphyrin-3 has structure [(**29**), Scheme 4]; the octamethyl ester of this material has recently been prepared from structure (**23**) by reductive cleavage of the lactone rings (*35*).



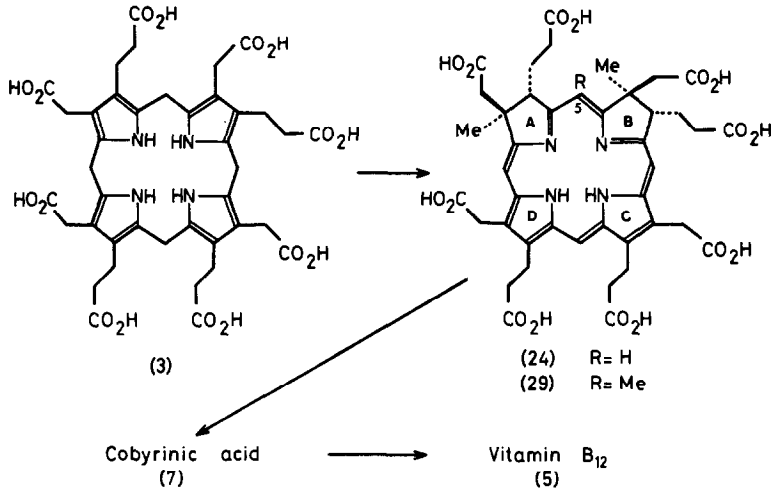
(**22**) R=H

(**23**) R=Me

A further thread came into the story during the later stages of the foregoing work. The pigments called Faktors I, II, and III were isolated in Stuttgart (*36*) from *P. shermanii* and labelled forms of Faktors II and III gave radioactive cobyrinic acid (**7**) when they were incubated with an enzyme preparation from *Clostridium tetanomorphum*. It was evident to us from the clearly reported properties of Faktor II that it must be sirohydrochlorin; and this was confirmed by comparing a sample of its ester, kindly provided by Professor Müller, with our material. The more recent Faktor III (*36b*) will almost certainly be identical with the above reduction product from corriphyrin-3.

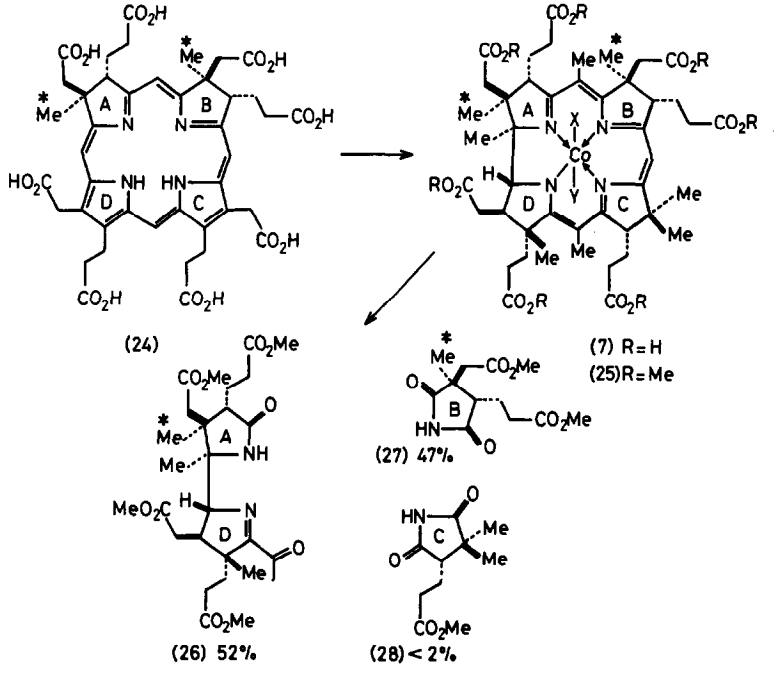
(*v*) *Specific incorporation of labelled sirohydrochlorin into cobyrinic acid; proof of A–B structure.* We can now cover the rigorous proof of structure (**24**) for sirohydrochlorin. The origin of its two *C*-methyl groups from *S*-adenosylmethionine had been indicated by experiments with ^{14}C-labelled methionine (*34*, *36*); this was confirmed by incorporation of [*methyl*-^{13}C]methionine using *D. gigas* and examination of the product by ^{13}C-nmr (*37*). It was then possible to produce highly radioactive sirohydrochlorin (**24**), labelled solely at the two *C*-methyl groups, by incubating *P. shermanii* with [*methyl*-^{14}C] methionine under appropriate conditions. The radiochemically pure ester [as (**20**)] was then hydrolysed, and the labelled sirohydrochlorin (**24**) was incorporated into cobyrinic acid (**7**) using the broken-cell system (*3*) from *P. shermanii*. Degradation of the labelled cobester (**25**) by ozonolysis[5] gave the fragments (**26**), (**27**), and (**28**), and the percentages of the original radioactivity carried by each

[5] The ozonolysis of cobester and isolation of ring-B and ring-C imides was carried out according to the method of T. L. Bogard and A. Eschenmoser (unpublished work); the A–D fragment was first identified by D. Arigoni and co-workers, Zurich. We thank Professor Arigoni and Professor Eschenmoser for kindly informing us of their work.



SCHEME 4

fragment are indicated. These results prove (a) specific incorporation of sirohydrochlorin[6] (**24**) into cobyrinic acid (**7**) and (b) that sirohydrochlorin has the A–B methylation pattern (**24**), i.e., the earlier preferred structure is correct.



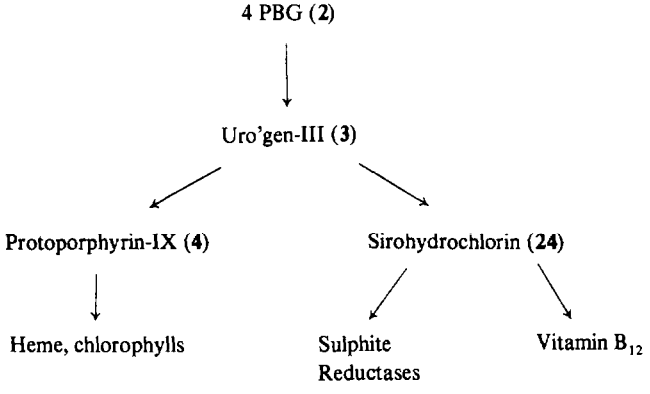
[6] It should be mentioned that two C-methylations of uro'gen-III (**3**) would produce a dihydroisobacteriochlorin. So, either (a) the dihydro system is dehydrogenated *in vivo* to the isobacteriochlorin which lies on the pathway and adjustment of the oxidation level occurs at a later stage or (b) the dihydro system is the true intermediate but the oxidized form is isolated; if this is true, the oxidized form must be reduced *in vivo* to the dihydro state to account for its specific incorporation into cobyrinic acid. These aspects require further study.

After completion of the foregoing studies, we learned from Professor A. I. Scott that structure (**20**) for sirohydrochlorin ester has been independently established by an entirely different (nmr) method (*38*) and that the corresponding acid was incorporated into cobyrinic acid.

The studies outlined above on the set of isobacteriochlorins, including sirohydrochlorin (**24**) have opened up the central part of the biosynthetic pathway to vitamin B_{12} in a most satisfactory way. With the proviso given in Footnote 6, the sequence from uro'gen-III can now be seen as (**3**) → (**24**) → (**29**) → (**7**) → (**5**), as illustrated in Scheme 4.[6]

PROSPECT AND EVOLUTIONARY INTEREST

The challenge now is to uncover, step by step, the remainder of the pathway. This will undoubtedly involve new and exciting chemistry, and it will be fascinating to learn the route nature uses from the many ways by which corrins are now known to be formed *in vitro* (*39*).



SCHEME 5

In addition, it is interesting to compare from the evolutionary viewpoint, the findings so far for heme and chlorophyll, on the one hand, and isobacteriochlorins and corrins, on the other (see Scheme 5). For both sets of pigments, uro'gen-III (**3**), a colourless compound incapable of chelating metal ions effectively, is transformed into a conjugated macrocycle which can form stable complexes with M^{II} and M^{III} ions. The pathway to heme and chlorophyll involves several *oxidative* steps (e.g., removal of 6H to aromatize proto'gen-IX to protoporphyrin-IX, Scheme 1) to produce the chelating macrocycle. For the other pathway, reviewed here, most of the necessary chemistry to generate the organic ligand is achieved by C-methylation. Sulphite and nitrite reducing organisms are ancient on the evolutionary time scale, and it is probable that the methylation pathway evolved when the earth's atmosphere lacked oxygen. It is fascinating to find that the type-III isomer, uro'gen-III (**3**), had already been selected for the biosynthesis of life's pigments at that stage of evolution [for review of the type-III problem see Ref. (*40*)]. Many questions, such as, "what triggers the ring contraction to form the corrin nucleus," remain to stimulate exciting future work in this area.

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