CONCERNING THE BIOSYNTHESIS OF VITAMIN B_{12}*

A. Ian Scott

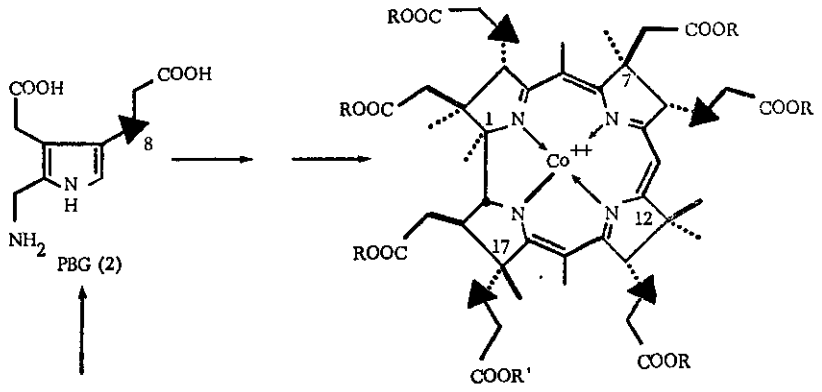
Sterling Chemistry Lab, Yale Univ., New Haven, Conn. 06520

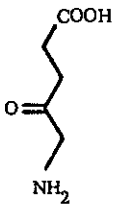
The use of ^{13}C FT-NMR has led to the observation that while 8 molecules of [2-^{13}C]-ALA are incorporated into vitamin B_{12} in P. shermanii, [5-^{13}C]-ALA labels only seven of the carbon atoms of cyanocobalamin i.e. one of the amino methyl groups of ALA is "lost" in the process. It has also been confirmed that seven of the methyl groups of B_{12} are derived from $^{13}CH_3$ enriched methionine and further that the chirality of the gem-dimethyl grouping at C_{12} labeled with [$^{13}CH_3$]-methionine is R. A soluble enzyme mixture from the 37,000 g supernatant of disrupted cells of P. shermanii converts both ^{14}C-labeled ALA and ^{14}C-uro'gen III to cobyrinic acid, the simplest corrinoid material on the pathway to vitamin B_{12} and the coenzyme in presence of NADPH, Co^{++}, Mg^{++}, S-adenosylmethionine and glutathione. A detailed hypothesis of the mechanism of corrin synthesis from uro'gen III has been developed.

A great deal of the ground work of corrin biosynthesis has been laid by Shemin[1] and Bogorad[2] for it is now certain that the "cobalt" pathway follows the great "iron"

* Preliminary communications on this topic have been published as follows: J. Amer. Chem. Soc., 1972, 94, 8267, 8269; 1973, 95, 5759, 5761.

and "magnesium" routes at least as far as porphobilinogen (PBG), the important building block for all of these systems. Furthermore the careful research of Bernhauer[3] has revealed the sequence of B_{12} biosynthesis from cobyrinic acid (3a) to the vitamin (3c) and the coenzyme.





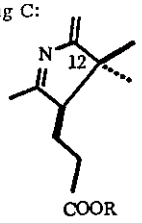
ALA (1)

(3a) R=R'=H

(3b) OR=NH_2 ; OR'=$NHCH_2CH(CH_3)OH$

(3c) OR=NH_2 ; OR'=aminoisopropanol-phosphate-ribose-5,6-dimethylbenzimidazole$\longrightarrow Co^{+}$—CN

(3d) as in 3b except ring C:



(3e) R=R'=CH_3

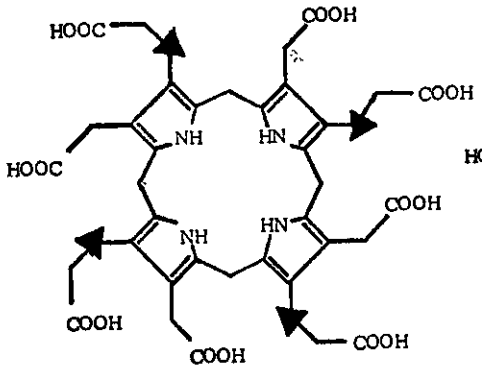
We shall discuss in this paper experimental and theoretical aspects bridging the gap between PBG and cobyrinic acid (3a). Using [8-^{14}C] - PBG (2), we first tested the premise that this pyrrole serves as a specific precursor of Vitamin B_{12} (Experiment 1). The probability had been assumed on the basis of the successful incorporation of δ-aminolevulinic acid (ALA, 1) into the vitamin; but apart from a preliminary report by Schwartz[4], no evidence regarding the specific nature of PBG

incorporation was available. Using resting young cells of Propionibacterium shermanii, incorporations of between 5 and 6% of PBG were obtained. Preliminary degradations of the recrystallized B_{12} (hydrolysis, Kuhn-Roth estimation) show that none of the ^{14}C label from a specimen of PBG labelled at C-8 (2) is found in the aminopropanol, ribose, or dimethylbenzimidazole segments or in those carbons (CH_3-C) which afford acetic acid on oxidation. Thus although the label is still only presumed to reside at the positions shown in (3c), the evidence is clearly in favor of specific and intact incorporations of PBG.

From Uro'gen III to B_{12}

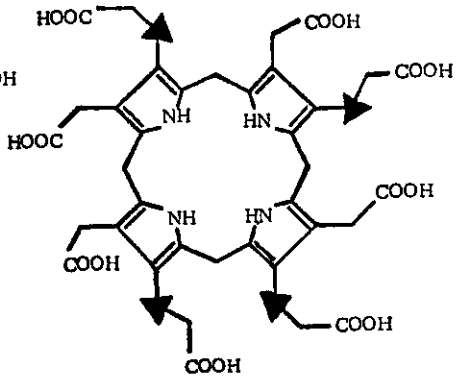
In considering the plausible mechanisms connecting PBG with corrinoids we have been attracted by the ideas expressed by Burnham[5,6] that the cobalt and iron pathways diverge after the formation of uro'gen III. The idea that the linear tetrapyrrole from head-to-tail condensation of four PBG units is transformed by uro'gen I synthetase in the presence of uro'gen III cosynthetase to give uro'gen III[7] is no longer tenable for the "switch" probably takes place at the earliest encounter of two PBG molecules in accord with the experiments of Frydman[8] et al. Regardless of the details of how uro'gen III is formed, an experimental distinction can be made between uro'gen intermediate and the corrin synthetase mechanism of Corwin which by-passes the uro'gens and forms the corrin link directly. Again, many of the numerous ideas concerning B_{12} biosynthesis which involve formation of the vital ring A → D linkage at an earlier assembly stage can be discarded if proof for the intervention of uro'gen III is forthcoming. Recent feeding experiments with whole cells of Propionibacterium shermanii have indicated that virtually no specific incorporation of enzymically or chemically synthesized ^{14}C-uro'gen could be observed.[9,10] We believe that these negative results may be attributed to the conditions of the feeding experiment and although valid for the concentrations and/or pH, aeration, heat treatment and cellular ages specified, may be contrasted

with the successful incorporations described below.



(5)

Uro'gen II: AP-PA-AP-PA
Uro'gen IV: AP-AP-PA-PA



(4)

A= $-CH_2COOH$
P= $-CH_2CH_2COOH$

Using freshly harvested cells of Propionibacterium shermanii (ATCC 9614) (25-30 g) suspended in degassed phosphate buffer (100 ml) containing $CoCl_2$ (1 mg) methionine (20 mg) 5,6-dimethylbenzimidazole (20 mg) and glucose (1.6 g), specific incorporations of chemically (Experiment 2) and enzymically (Experiments 5 and 6) synthesized ^{14}C-uro'gen III (4) were obtained as recorded in Table 1. Experiment 5 was run with 100 γ of uro'gen III/I mixture which approximates to the previously reported conditions in which very low (but not zero) incorporation occurs. The resultant cyanocobalamin (3c) after extensive purifications and recrystallization to constant radioactivity, was degraded by hydrolysis and Kuhn-Roth oxidation and showed that none of the original ^{14}C-label (from [8-^{14}C]-PBG) had been randomized into the nucleotide segment, or into those carbons (CH_3-C-) which afford acetic acid on oxidation. Although the ^{14}C-label was not directly located by this experiment it could be inferred that the positions shown in (3) are labelled and the

evidence is clearly in favor of specific and intact incorporation of uro'gen III.

On the other hand uro'gen I (5) (prepared by the spinach synthetase procedure) gave either zero or very low incorporation when administered in carefully monitored parallel feeding experiments (Table I; Experiments 3 and 4).

With proof for the inertness of uro'gen I in hand, any concern over the use of uro'gen III/I mixture (70/30-50/50) obtained from the wheat germ preparation can be discounted as can the use (Experiment 2) of the chemically synthesized statistical mixture of the types I-IV isomers which contains 50% of the type III isomer together with 12.5% of uro'gen I, the remainder being the biologically inert types II and IV isomers. In conformity with heme and chlorophyll bio-

TABLE I ^{14}C-Feeding experiments using suspended cells of P. shermanii ATCC No. 9614.

Experiment	Substrate Fed	mg Fed	Hours	Spec. Incorp./C
1	PBG	21	70	5.1
2	Uro'gens I-IV	25	70	0.91
3	Uro'gen I	7	40	0.000
4	Uro'gen I*	25	70	0.017
5	Uro'gens III+I	0.1	70	0.0052
6	Uro'gens III+I	24	70	0.40
7	Uroporphyrins I-IV	34	60	0.000

* Paper chromatographic analysis of the isomer purity on the corresponding coproporphyrin indicated a small amount of the type III isomer arising from in vitro conversion of PBG to the mixture of uro'gens during the course of the enzyme incubation, thus accounting for the slight positive incorporation.

synthesis, no incorporation of the same statistical mixture of the ^{14}C-uroporphyrins I-IV (from which uro'gens I-IV were prepared) was observed (Experiment 7).

In order to confirm these results without recourse to the tedium of carbon-by-carbon degradation of the vitamin, the experiment was repeated using [8-^{13}C]-PBG and the uro'gen I-IV isomers labelled with ^{13}C (90% enrichment per carbon) as shown above. The ^{13}C FT-nmr spectrum of [8-^{13}C]-PBG enriched vitamin B_{12} is shown in Figure 1a. The spectrum contains three resonances at 37.75, 33.44 and 31.50 ppm arising from four enriched centers. That four enriched carbons were actually present was demonstrated by conversion of the vitamin to the dicyano form. The sharp signal at 31.50 ppm was cleanly resolved and the ^{13}C spectrum showed four enhanced peaks of equal intensity. When the [^{13}C]-uro'gen isomers were administered to P. shermanii (~ 12g cells/100 ml medium per flask) and the resultant vitamin B_{12} subjected to similar ^{13}C-nmr analysis, the enriched spectrum (Figure 1b) showed enhancement (~ 10-12%) of the same set of four methylene carbons as were labelled in the [^{13}C]-PBG experiment.

These results together with the enrichment data for [^{13}C]-ALA (see below) not only corroborate positive incorporation with carbon-14 but also provide unequivocal evidence for the location of the label, and strongly support the sequence PBG → uro'gen III → vitamin B_{12} in P. shermanii. Thus with the important proviso that sufficient substrate must be present to permeate the cell wall and to survive in vitro oxidative destruction it is our view that vitamin B_{12} is produced by a reductive contraction of uro'gen III.

Origin of the Methyl Groups in Vitamin B_{12}

Of the eight methyl groups attached to the periphery of (3) it was suggested[1] that those at C-1 and C-12 stem from C-5 and C-2 of ALA respectively, the latter by a well documented decarboxylation of acetate attached to the uro'gen system,

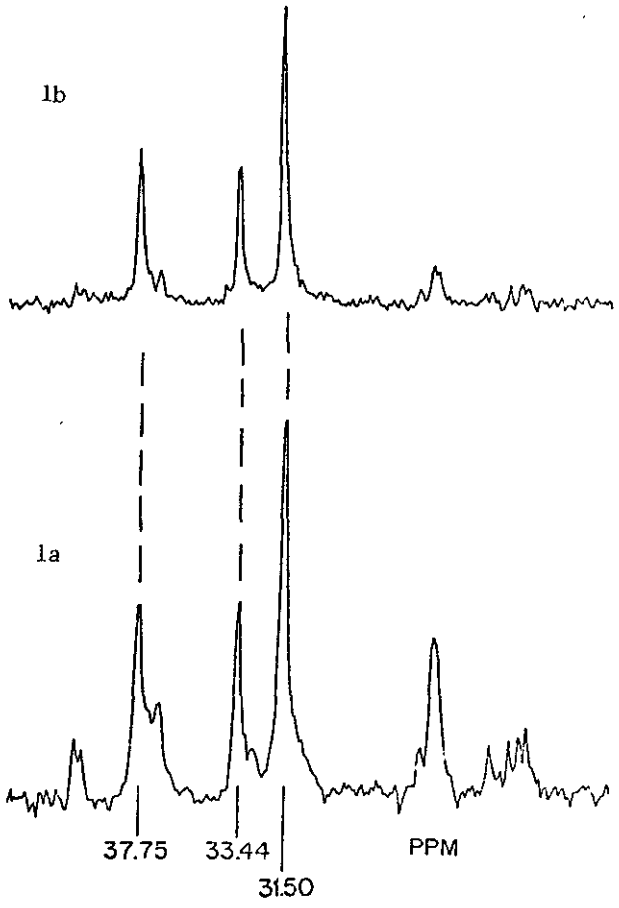


Figure 1a (bottom). Portion, 44.73 to 17.22 ppm downfield from external hexamethyldisilane (HMDS), of the proton noise-decoupled ^{13}C FT spectrum of 41 mg [8-^{13}C]-PBG enriched cyanocobalamin (vitamin B_{12}, 3c) in H_2O; data set = 4K points; digitizing rate = 10KHz; pulse width = 50 μsec; receiver skip = 100 μsec.[13]

Figure 1b (top). Portion, 44.73 to 17.22 ppm downfield from external HMDS, of the proton noise-decoupled ^{13}C FT spectrum of 40 mg [^{13}C]-uro'gen enriched cyanocobalamin in H_2O; same conditions as for spectrum 1a.

while the derivation of the former (C-1) methyl group could be envisioned either as a reduction of a $-CH_2-$ bridge of uro'gen III, or as a result of direct cyclization of a linear tetrapyrrole,[7] the six remaining methyl groups arising from methionine. Support for these ideas came from Kuhn-Roth oxidation of corrinoids labelled with [5-^{14}C]- and [2,3-^{14}C]-ALA and [$^{14}CH_3$]-methionine.[1]

We have now re-examined the problem using ^{13}C-Fourier transform nmr to determine the fate of [2-^{13}C]- and [5-^{13}C]-ALA in Propionibacterium shermanii. Administration of [2-^{13}C]-ALA to P. shermanii afforded a sample of vitamin B_{12} in which eight high-field signals in the $-CH_2-$ and $-CH_3$ region were enriched as shown in the proton noise-decoupled ^{13}C FT nmr spectrum (Figure 2a). Assignments of the eight ^{13}C resonances were made to the seven $-CH_2CONH_2$ methylenes and one of the gem-dimethyl groups of ring C in full accord with earlier ^{14}C studies. It is evident, however, that the methyl signal appears at lower field than the methyl region assigned by Doddrell and Allerhand.[11] A sample of B_{12} enriched by feeding [5-^{13}C]-ALA provided the surprising result that, of the eight anticipated enriched carbons, only seven signals appeared in the low field region associated with sp^2 (C=C and C=N) functions. The splitting pattern predicted for the distribution of label illustrated in(3c) was indeed obtained as shown in Figure 2b. Such an array is in harmony with current ideas on the mechanism of type III uro'gen formation. However, there was no ^{13}C enhanced signal above 95 ppm downfield from HMDS showing that no enrichment of the C-1 methyl had occurred. This indicates that one of the $-^{13}CH_2NH_2$ termini of ALA (and hence of PBG or uro'gen III) has been extruded in the formation of the vitamin. The origin of the "missing" C-1 methyl group has now been demonstrated to be methionine. Although the ^{13}C FT-spectrum of cyanocobalamin obtained by feeding [$^{13}CH_3$]-methionine (Figure 2c) revealed only 6 signals highly enriched above natural abundance, conversion of this sample to the dicyano form (Figure 2d) revealed seven well defined resonances. Hence the

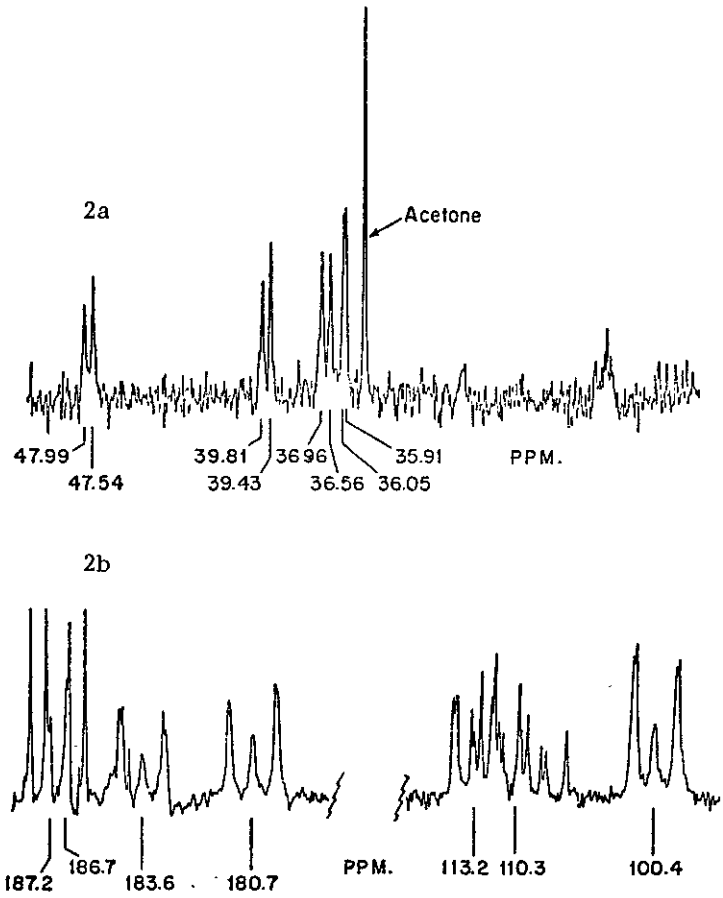


Figure 2a. Proton noise-decoupled ^{13}C FT spectrum of [2-^{13}C]-ALA enriched cyanocobalamin (vitamin B_{12}, 26 mg) in H_2O. The methyl group of acetone, 35.11 ppm provides an internal reference. Only the range 50.53 - 19.59 ppm downfield of external HMDS is shown: Data set = 8K points; digitizing rate 10 KHz; pulse width = 50 μsec; receiver skip = 100 μsec.

Figure 2b. Proton noise-decoupled ^{13}C FT spectrum of [5-^{13}C]-ALA enriched cyanocobalamin (38 mg) in H_2O. Two portions of the spectrum, 188.53 - 177.49 ppm (left), and 117.83 - 95.74 ppm (right) downfield from external HMDS are shown: Data set = 4K; digitizing rate = 10 KHz; pulse width = 50 μsec; receiver skip = 100 μsec.

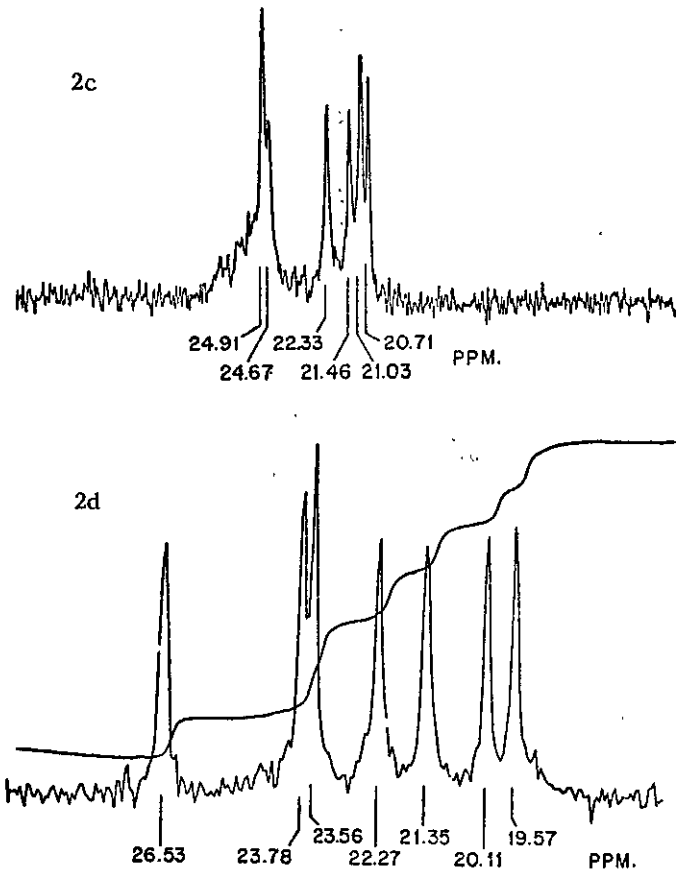
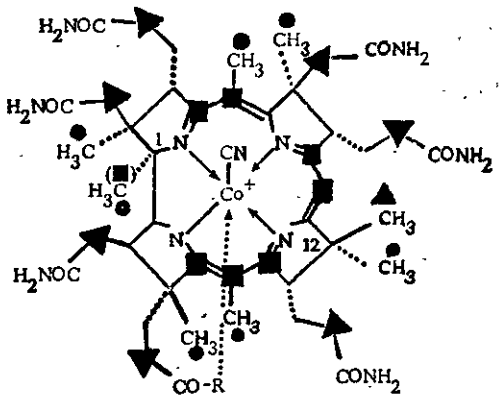


Figure 2c. Portion, 35.11 - 8.60 ppm downfield of external HMDS, of the proton noise-decoupled, ^{13}C FT spectrum of [$^{13}CH_3$] - methionine enriched cyanocobalamin (36 mg) in H_2O; conditions as in Figure 2b.

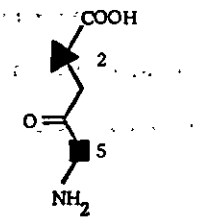
Figure 2d. Portion, 29.89 - 16.63 ppm downfield from HMDS, of the proton noise-decoupled ^{13}C FT spectrum of dicyanocobalamin (36 mg) in 0.1 M KCN; conditions as in Figure 2a.

signal at 24.91 ppm (Figure 2c) corresponds to two superimposed resonances. Inspection of the integrated spectrum (Figure 2d) leaves no doubt that seven methionine methyl groups have been incorporated.

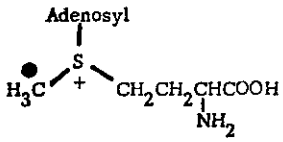


(3c)

R= aminoisopropanol-phosphate-ribose-DMBI → Co^{+}



(1)



S-Adenosyl methionine

Stereochemistry of Methyl Group Insertion in Corrinoid Biosynthesis

Before developing further mechanistic proposals for corrin biosynthesis, which appears to be controlled by both steric and electronic consequences of methyl group insertion via S-adenosyl methionine (SAM) (leading to α-orientation in rings A and B, β-in ring D), resolution of the problem of the stereochemistry of methylation at C-12 in ring C became necessary. Thus, although it has been rigorously demonstrated that one of the methyl groups at C-12 is derived from methionine and the other from C-2 of ALA (1), the stereospecificity of this process has not been established. The following experiments provide a ready solution to this problem with its attendant mechanistic implications, and also demonstrate the

particular usefulness of ^{13}C-chemical shifts for the determination of carbon isotope chirality.

A labelled specimen of dicyanocobinamide (3b) was obtained, where one of the C-12 methyl groups was specifically enriched, by feeding [$^{13}CH_3$]-methionine to a vitamin B_{12} producing culture. The conformation[12] of the C-ring of cobinamide (3b) places the α-methyl syn-periplanar to the adjacent axially oriented propionamide side chain at C-13 (see Figure 3a). This juxtaposition would be predicted to produce a gamma effect on the ^{13}C-chemical shift of this methyl group (analogously this effect should be reflected in the chemical shifts of the methyls at C-1, C-2, C-7 and C-17). The ^{13}C-FT nmr spectrum of [$^{13}CH_3$]-methionine enriched (3b) derived from (3c) by treatment with CF_3COOH is shown in Figure 4 (top). The spectrum consists of seven methyl resonances 20-27 ppm downfield from HMDS. In addition to hydrolysis of the nucleotide, epimerization at C-13 also occurs in the same treatment of (3c) with CF_3COOH to yield neocobinamide (3d) which is easily separated from cobinamide (3b) above.[13] Neocobinamide differs from cobinamide

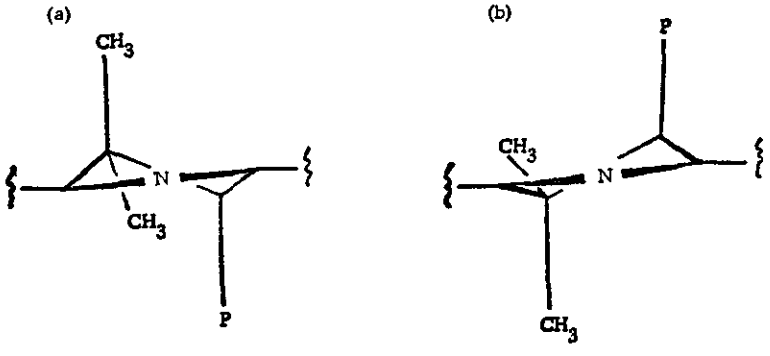


Figure 3. Conformation of ring C in cobinamide (a) and neocobinamide (b) as viewed from cobalt on X-ray[12] and CD/ORD[13] data.

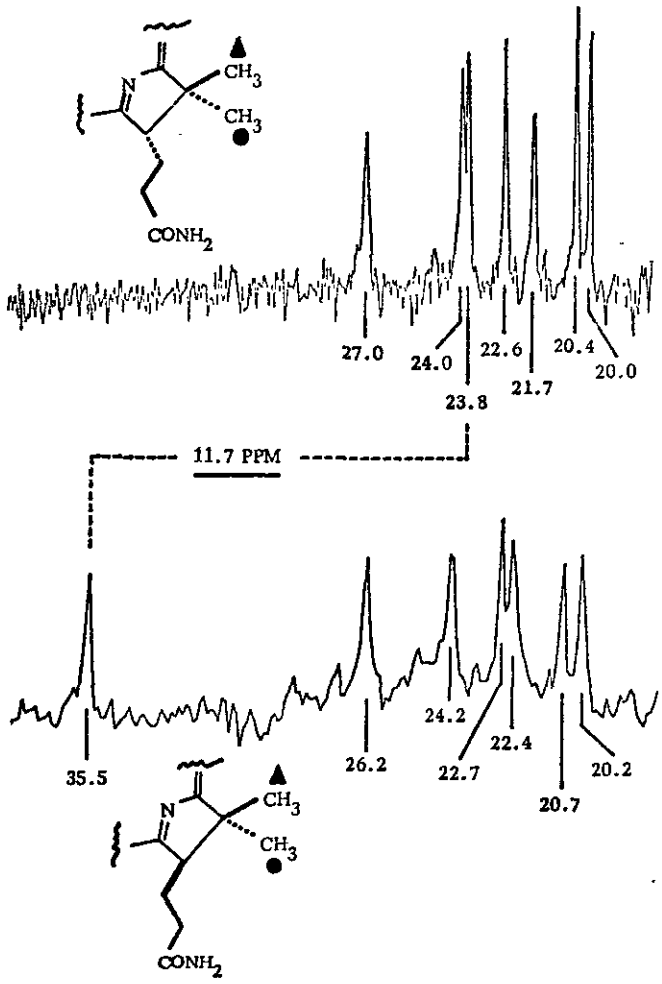


Figure 4 (top). Proton noise-decoupled ^{13}C FT spectrum of [$^{13}CH_3$]-methionine enriched dicyanocobinamide (9 mg) in 0.1 M KCN. Only the range 38.1 - 15.8 ppm downfield from HMDS is shown: data set = 8K points; digitizing rate = 10 KHz; pulse width 50 μsec; receiver skip = 100 μsec.

Figure 4 (bottom). Proton noise-decoupled ^{13}C FT spectrum of [$^{13}CH_3$]-methionine enriched dicyanoneocobinamide (2 mg) in 0.1 M KCN. Conditions and frequency range same as above except data set = 4K.

solely by virture of a configurational inversion of the propionamide group at C-13 accompanied by a conformational change in the skew of the C-12—C-13 bond (see Figure 3b). Thus, if the methionine derived methyl at C-12 is α-oriented in the neo series, it will bear an anti-periplanar relationship to the propionamide side chain and the concommitant removal of the gamma effect should result in a downfield shift of the methyl resonance signal.[14] That this is indeed the case is shown by the downfield shift of 11.7 ppm in the ^{13}C FT nmr spectrum for one of the methyl resonance lines in going from cobinamide to neocobinamide (Figure 4, bottom).

These results establish that the [$^{13}CH_3$]-methionine methyl (●) is inserted into the corrin nucleus at C-12 from the α-face and that the absolute configuration at C-12 is (R).. Furthermore, the ^{13}C results rationalize the apparent anomaly observed previously that the β-methyl group (▲) of the gem-dimethyl grouping at C-12, derived from C-2 of ALA (1), resonates at substantially lower field than the methyl region tentatively assigned by Doddrell and Allerhand.[11] It should be noted that all the remaining methyl groups at sp^3 carbons appear at higher field, i.e. within that region proposed by Doddrell and Allerhand, because of gamma interaction with adjacent syn groups.

A New Theory for Corrin Biosynthesis

In accord with all the published experimental evidence the following requirements must be met in the conversion of uro'gen III to cobyrinic acid:

1. Decarboxylation of the acetic acid side chain at C-12.
2. Loss of the meso carbon at C-20 and formation of a new bond between C-1 and C-19.
3. Introduction of the seven "extra" methyl groups from SAM.
4. Reduction (four electron equivalents).
5. Insertion of cobalt.

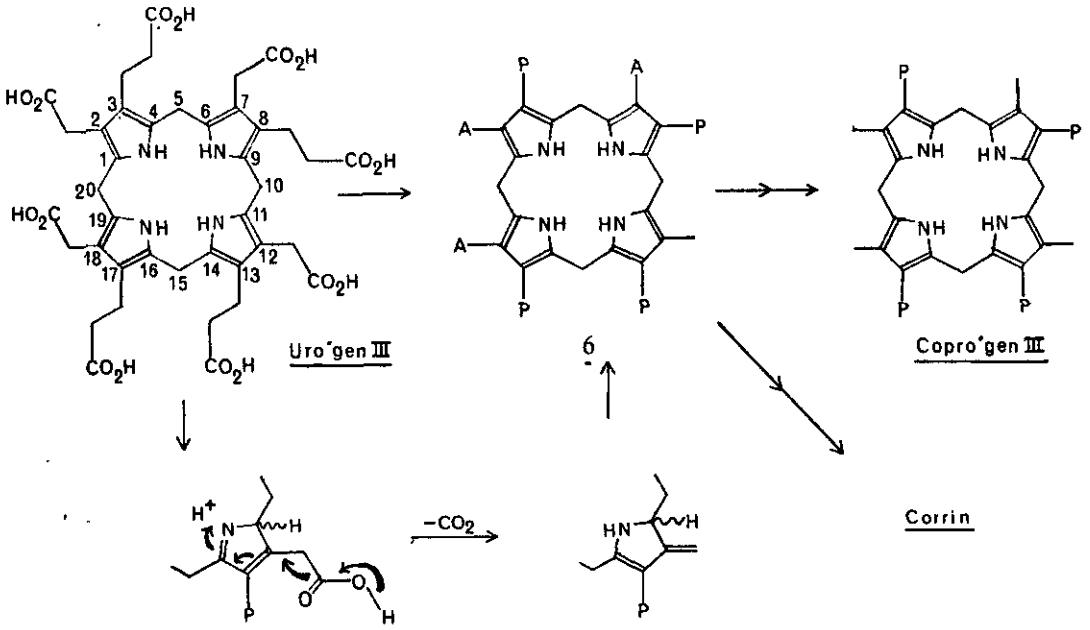
These requirements impose considerable limitations on any mechanistic proposals and at the same time render untenable many of the previous suggestions regarding *corrin biogenesis. In order to guide the design of future experiments in this area* and to fill the void left by the relinquishment of earlier ideas on corrin biogenesis, we have developed the following hypothetical sequence which may provide several interesting possibilities open to experimental test.

1. Decarboxylation

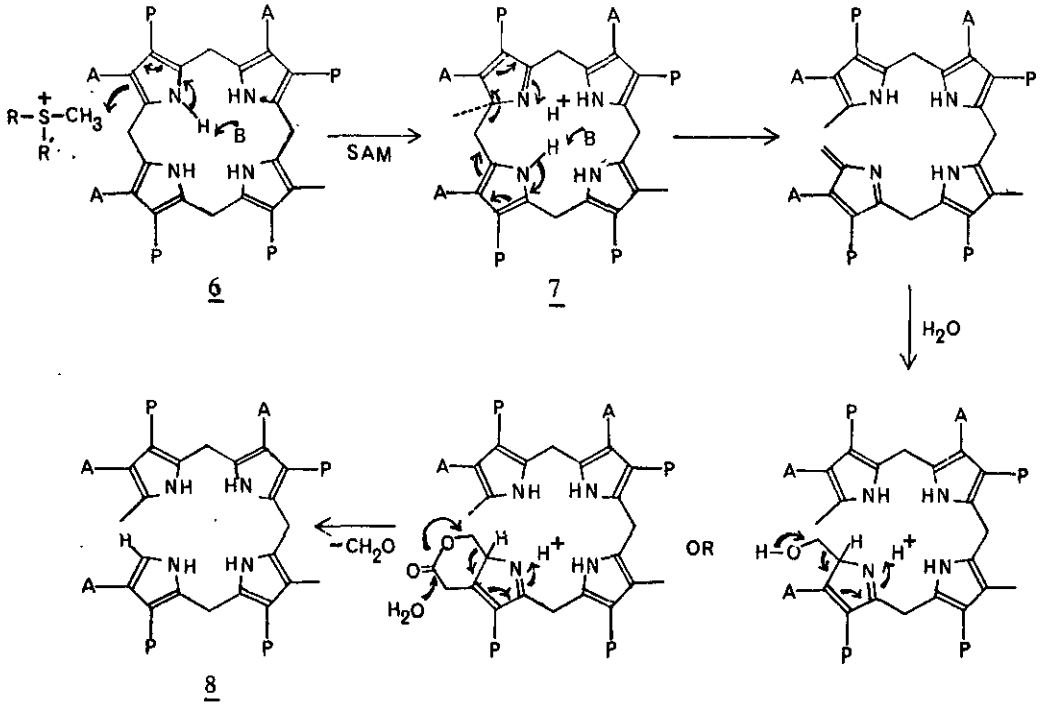
We propose that the first event in this sequence be the decarboxylation of the acetic acid chain at C-12. Since in all biological decarboxylations described hitherto to the presence of an electron sink is obligatory, it is suggested that decarboxylation should occur prior to methylation at C-12. The enzymatic decarboxylation of uro'gens to coproporphyrinogens (copro'gens) by the ubiquitous uro'gen decarboxylase is a well known process,[15, 16] intermediates having been isolated in the partially decarboxylated form. Although the exact structures of the heptacarboxylic porphyrins have not been defined, the low substrate specificity of these decarboxylases implies that there is probably formed an equal amount of each possible geometric isomer, one of which will be implicated in the biosynthesis of vitamin B_{12} (Scheme 1).

2. Methylation and Loss of C-20

The heptacarboxylic porphyrinogen 6 can undergo the first methylation at C-1 by SAM, presumably coupled with a base catalyzed hydrogen abstraction. The methylated species 7 is now poised for a rearrangement process in which formaldehyde is lost via hydration, generating the linear tetrapyrrole 8. We note that this step is equivalent to two electron reduction (Scheme 2).

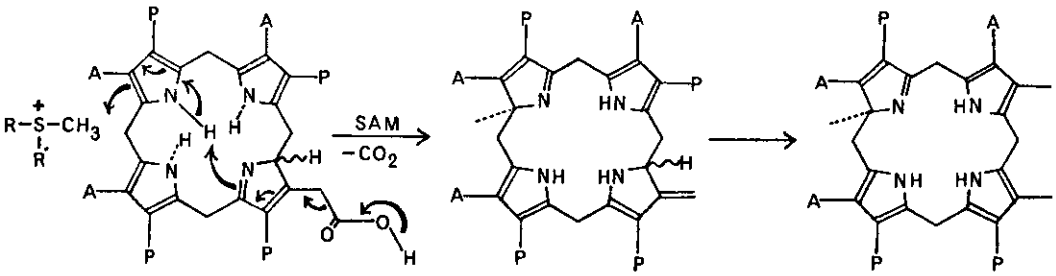


(SCHEME 1)



(SCHEME 2)

It is also instructive to consider that these first steps, viz decarboxylation and methylation, could occur simultaneously in favorable conditions (Scheme 3). By operation of such a push-pull mechanism, it is possible to reduce further the arbitrary nature of the scheme, which would then require a specific decarboxylase/ methyl transferase.

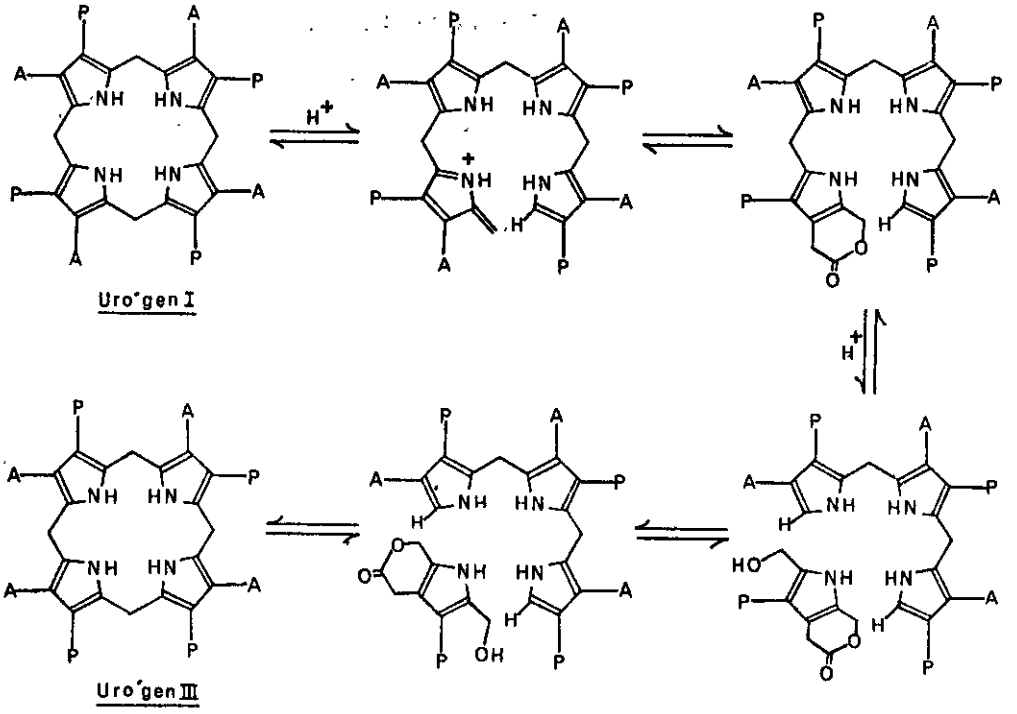


(SCHEME 3)

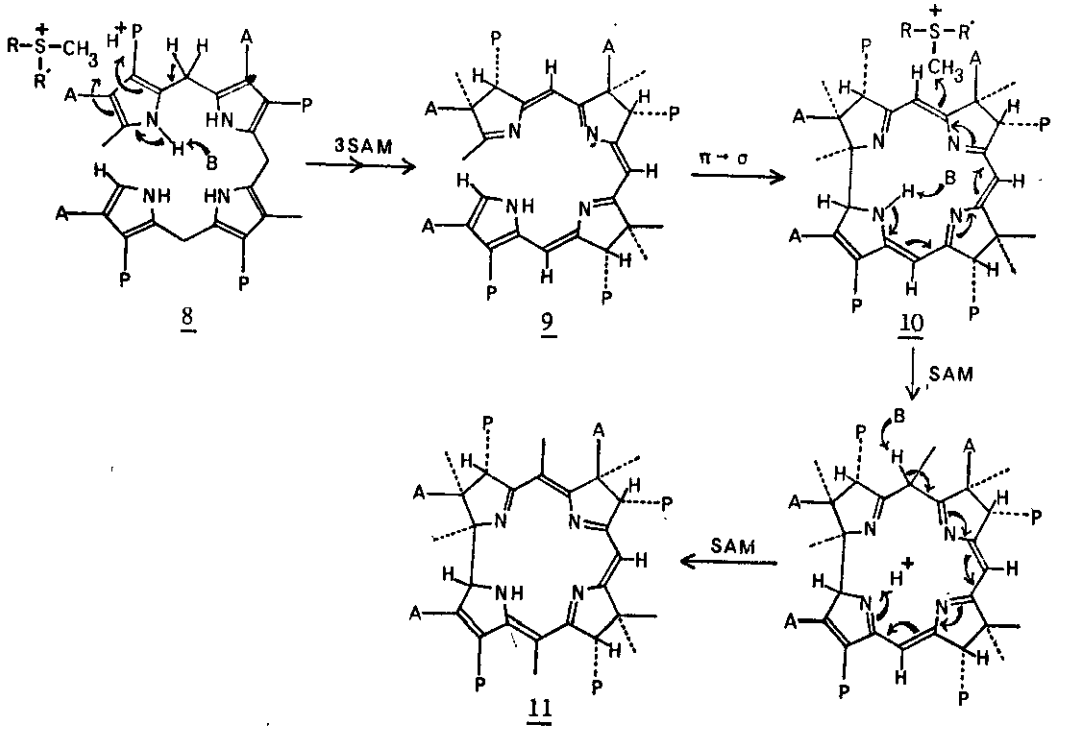
To explain the acidic in vitro isomerization of uro'gens, Mauzerall has proposed[17] ring cleavage coupled with protonation as a working hypothesis as illustrated in Scheme 4. At the same time, the latter author established that the yield of uro'gen dropped markedly when the reaction was performed in the presence of dimedone, an efficient formaldehyde trapping agent, whereas exogenous formaldehyde equilibrated freely with the meso carbons. Here we find a close analogy to Scheme 2, viz methylation, ring cleavage and loss of formaldehyde.

3. Further Methylations and Cyclization

Subsequent consecutive methylations at C-2, C-7, and C-12, in which the conformation of the substrate and the direction of approach of the incoming methyl groups are apparently controlled by the methylating enzyme, provide a conjugated 16π-electron array 9, which may now undergo an orbital-symmetry-allowed π-σ rearrangement in a concerted manner to produce a dehydrocorrin chromophore 10 upon which C-5 and C-15 methylations may operate (Scheme 5).

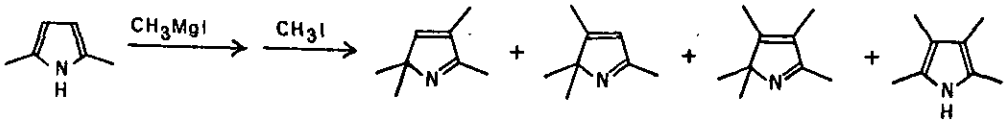


(SCHEME 4)



(SCHEME 5)

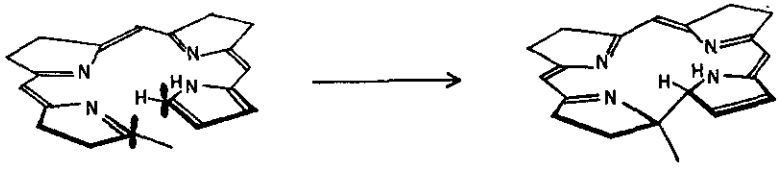
Electrophilic substitution usually favors the α-position rather than the β-position of pyrroles[18, 19] but the preference is not overwhelming and certainly much less in pyrroles than in furans and thiophenes. For instance, low, but comparable yields of both α- and β-methylated products were obtained from 2,5-dimethylpyrrolylmagnesium iodide with methyl iodide[20] (Scheme 6).



(SCHEME 6)

In the case of substrate 8, the energy stabilization resulting from an extensive delocalization of π-electrons should facilitate β-methylations on pyrrole units. An interesting outcome of the above scheme is the possibility of C-12 methylation from he α-face in unison with C-2 and C-7 methylations, in accord with the observed chirality of the methylation process.

Close examination of the model structures reveals that the electrocyclic reaction calls for a minimum alteration in atomic arrangements, i.e., the delocalized π-orbital has an ideal Möbius overlap for a conrotatory ring closure to achieve the observed trans stereochemistry of the C-1 methyl and C-19 hydrogen (Scheme 7).

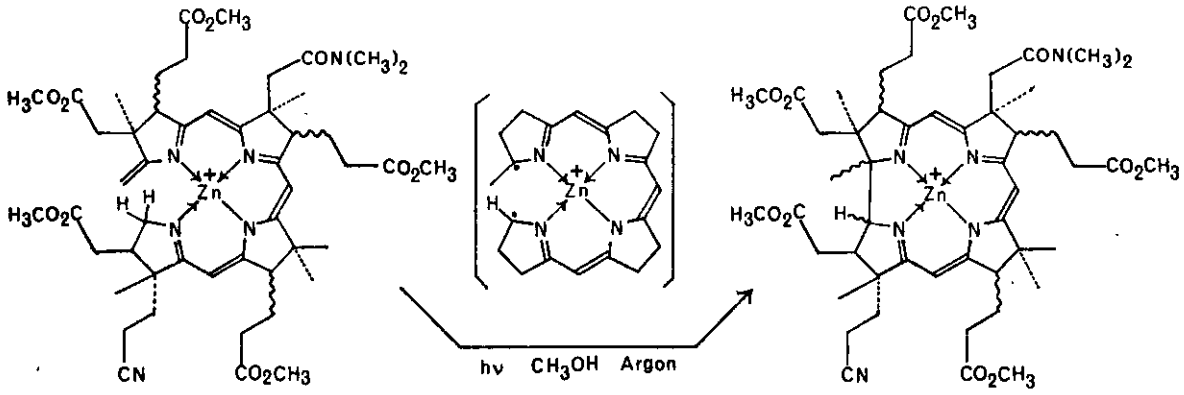


9 10

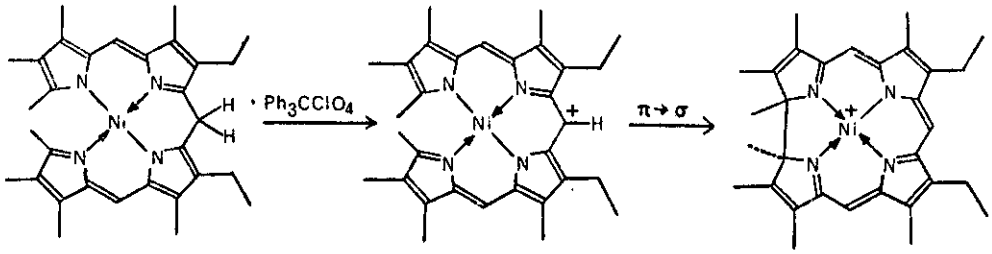
(SCHEME 7)

Such a process is reminiscent and isoelectronic with the second (thermally allowed) part of Eschenmoser's remarkable photochemical synthesis of corrins[21] (Scheme 8) and also finds excellent analogy in the elegant cyclization studies of tetradehydrocorrins by Johnson[22] (Scheme 9).

Both authors cite the template effect of the central metal atoms in their substrates which ensure close proximity of the interacting centers and prevent severe distortions of the π-electron system. Although the possible involvement of cobalt or any other metal ions in this process cannot be ruled out it seems reasonable to assume that the conformation of an intermediate may depend totally on enzyme specificity without the aid of a template metal atom.



(SCHEME 8)



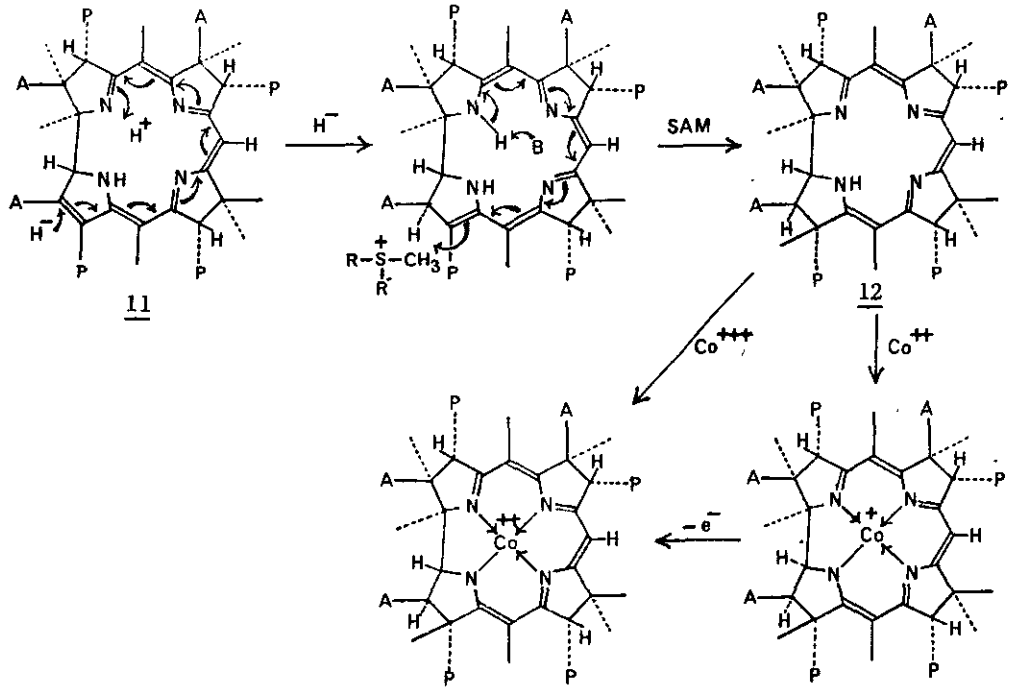
(SCHEME 9)

4. Reduction and Methylation

The resultant 18-dehydro-17-desmethyl cobalt-free cobyrinic acid 11 is now aligned for a final reductive methylation sequence. Following the conjugative attack of hydride (possibly from NADPH) at C-18 a study of models suggests that α-side of the nascent corrin is more sterically encumbered than the β-face and the attack of SAM should occur from the less hindered β-face at C-17 which is now activated towards electrophilic substitution (Scheme 10). The product, cobalt-free cobyrinic acid 12 may well be a precursor of the family of naturally occurring cobalt-free corrins.[23, 24]

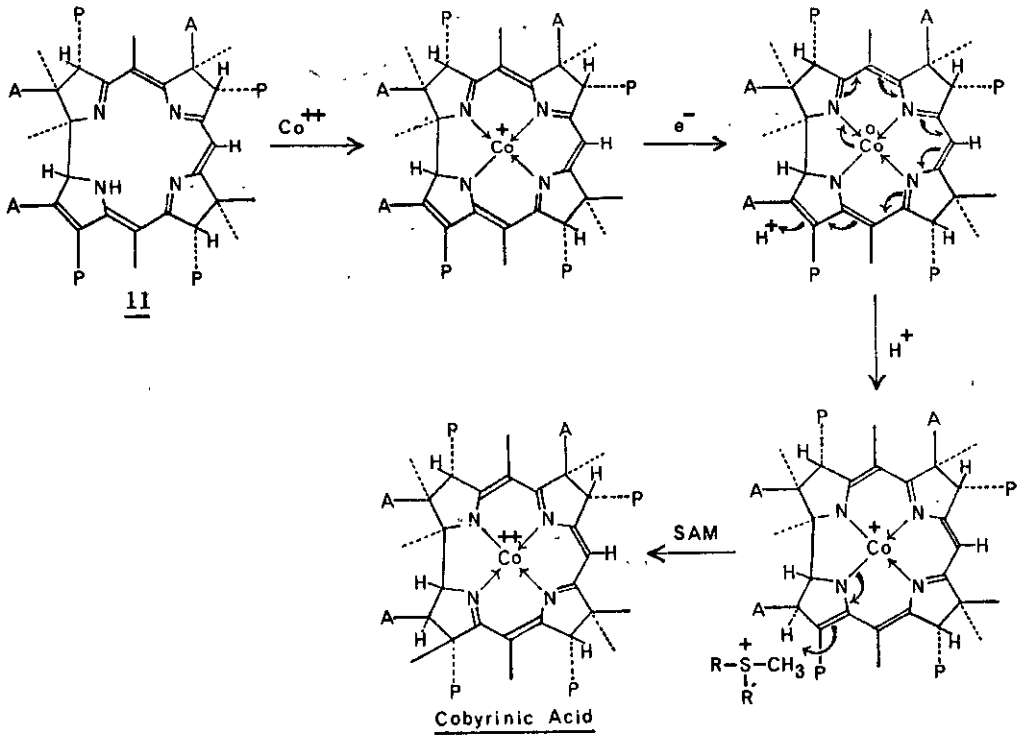
5. Insertion of Cobalt

The incorporation of cobalt atom poses the last problem in the sequence. The cobaltous ion (Co^{++}) may be oxidized before or after incorporation into the ring (Scheme 10).



(SCHEME 10)

On the other hand, the possible utilization of the extra electron in Co^{++} for C-17 methylation cannot be discounted. For instance, the addition of Co^{++}, a methyl group and a proton with supply of one electron to the precorrin system 11 will produce cobyrinic acid (Scheme 11). One electron reductions are well known in vitamin B_{12} enzymology in conjunction with the biological formation of co-enzyme B_{12}.[25]



(SCHEME 11)

A Cell-free System from Propionibacterium shermanii

Recognizing the difficulties experienced by ourselves and other workers in achieving uniformly substantial, reproducible incorporations of an advanced intermediate such as uro'gen III, especially where microgram quantities of this

sensitive substrate are incubated in whole cell suspensions for long periods, we have recently developed a stable, cell-free preparation from P. shermanii which carries out reproducible biosynthesis of corrins from appropriate precursors at the sub-micromolar level.

The crude mixture of "corrin synthetases" was prepared from freshly harvested wet cells of P. shermanii (ATCC9614). Disruption by a French press in phosphate buffer [pH7.6, 0.01 M] and centrifugation at 37,000 g afforded an active supernatant fraction which could be stored at -30° without measurable loss of activity for up to 4 months. Incubations of [5-^{14}C]-ALA, [$^{14}CH_3$]- and [$^{3}H_3C$]-SAM and [α, β, γ, δ-meso-^{14}C]-uro'gen I-IV mixture were carried out with the cofactors and additives as shown in Table 2. Post-incubation mixtures were freeze-dried and subjected to exhaustive methanolysis after dilution with various combinations of corrins. Cobyrinic acid heptamethyl ester (cobester, 3e) was the sole corrinoid product of methanolysis, and was purified by three different TLC separations, coupled with autoradiography, and finally recrystallized to constant activity after dilution with authentic, non-radioactive sample.

The presence of ALA dehydratase, uro'gen I synthetase, uro'gen III co-synthetase, as well as the complete methylative, reductive and cobalt inserting enzymes in the crude enzyme mixture can be inferred from the remarkably efficient conversion (ca 23%) of [5-^{14}C]-ALA to corrins isolated as cobester (Experiment 8).

Confirmation of the role of uro'gen III is evident from Experiments 10 where the observed radiochemical yield corresponds to at least 6-7% incorporation of uro'gen III, since the synthetic mixture contains 50% of the type III isomer. Isolation and identification of the methyl esters of penta-, hexa-, and hepta-carboxylporphyrins as well as the copro- and uro-porphyrins were carried

out by established procedures and these were found to be radioactive, indicating that the decarboxylative enzymes of uro'gen metabolism are also present in the cell-free system, in common with similar preparations from bacteria, avian red cells, mammalian reticulocytes, and mouse spleen.

Experiment 11 was carried out with [$^{3}H_{3}C$]-SAM using [^{14}C]-uro'gen as internal standard. The by-products of the incubation, viz uro-, copro- and the partially decarboxylated-porphyrins (as their methyl esters) contained ^{14}C but no tritium isotope. On the other hand, repeated crystallization of cobester from Experiment 11 gave a constant $^{3}H/^{14}C$ ratio (in agreement with the separate feeding Experiments 10 and 12) providing an internal check that both decarboxylating and methylating systems were operative. Since all of the cell-free assays depend on the purification of cobyrinic acid as the crystalline heptamethyl ester, this removes any ambiguity of in vitro methyl transfer in the esterification process, and also shows that no secondary incorporation of ^{3}H from [$^{3}H_{3}C$]-SAM occurs during the incubation.

Experiment 12 demonstrates that, in presence of added ALA, the incorporation of [$^{14}CH_{3}$]-SAM reaches 36% while the methylating enzyme system is inactivated by boiling (Experiment 13).

Using an entirely different assay procedure, the actual biosynthesized corrin (Experiment 10) was found to be cobyrinic acid (3a). The post-incubation mixture was subjected to phenol extraction after treatment with corrin mixture (as carrier) and the purified solution was separated by electrophoresis (Whatman 3MM and ET 81) and ion exchange paper chromatography (Whatman ET 81). Autoradiographs showed cobyrinic acid to be the only detectable radioactive corrin in all of these separations. With the establishment of the comparatively rapid assay technique described herein, separation of the component synthetase(s) responsible for the intriguing steps between uro'gen III and cobyrinic acid is now in progress.

TABLE 2. Incorporation of ALA, Uro'gen and SAM into Cobyrinic Acid by a Cell-free System

Expt.	Substrate	Weight per incubation (nmole)	Incubation conditions[a] (mg protein[c])	Radiochemical yield[b] nmole	(%)
8	[5-^{14}C]-ALA[d]	390	400	89	(23)[k]
9	[5-^{14}C]-ALA[d]	110	65 (boiled enzyme)		(<.001)[k]
10	[^{14}C]-uro'gen[e]	490	65	16	(3.4)[h,k]
11	[^{14}C]-uro'gen[e] +	130	65	3.9	(3.1)(^{14}C)[h,i]
	[$^{3}H_3$C]-SAM[f]	1.1		0.26	(24) (^{3}H)
12	[$^{14}CH_3$]-SAM[g]	19	65	6.9	(36)[j]
13	[$^{3}H_3$C]-SAM[f]	1.1	65 (boiled enzyme)		(< .001)[j]

(a) Incubation mixture contains amount of protein indicated and the following components in final volume of 10 ml of phosphate buffer pH 7.6, 0.02 M: GSH 4 mg, ATP 3 mg, $NADP^+$ 3 mg, NADH 2 mg, NADPH 6 mg, $CoCl_2$ 1 mg, 5,6-dimethylbenzimidazole 1 mg, cystein 1 mg, mercaptoethanol 5 μL, 16 hr 37° C.

(b) Total radioactivity in isolated cobester divided by total radioactivity in the substrate multiplied by 100.

(c) The protein concentration of crude enzyme solution was estimated by the procedure of Lowry et al.[26]

Specific activity:

(d) 26.2 mCi/mM

(e) 73. 2mCi/mM

(f) 4550 mCi/mM

(g) 52 mCi/mM

(h) The real yield should be based on uro'gen III.

(i) $^{3}H/^{14}C$ ratio of the substrates 0.53, $^{3}H/^{14}C$ ratio of the product 5,3

(j) Incubation conditions: (a) plus 1.25 mg ALA

(k) Incubation conditions: (a) plus 10 mg SAM.

ACKNOWLEDGMENT The development of the experimental technique necessary for the solution of the problems of corrin biosynthesis was only made possible by the prodigious ef. +s of my colleagues Drs. K. Okada, M. Kajiwara, B. Yagen, P. J. Whitman, S. Klioze, K. Obata, R. J. Cushley and Messrs C. A. Townsend and Eun Lee. I should like to express a special debt of gratitude to the latter two graduate students who not only shared much of the experimental burden but also contributed to the theoretical aspects of the problem.

REFERENCES

1 D. Shemin and R. C. Bray, Ann. N.Y. Acad. Sci., 1964, 112, 615.

2 L. Bogorad, Ann. N.Y. Acad. Sci., 1963, 104, 676.

3 K. Bernhauer, F. Wagner, H. Michna, P. Rapp and H. Vogelmann, Hoppe Seyler's Z. Physiol. Chem., 1968, 349, 1297.

4 S. Schwartz, K. Ikeda, I. M. Miller and C. J. Watson, Science, 1959, 129, 40.

5 B. F. Burnham and R. A. Plane, Biochem. J., 1966, 98, 13c.

6 B. F. Burnham, In Metabolic Pathways, D. M. Greenburg, Ed. 3rd ed., 3, Ch. 18, Academic Press, New York, N.Y. and London, England, 1969.

7 J. H. Mathewson and A. H. Corwin, J. Amer. Chem. Soc., 1961, 83, 135.

8 (a) The exact nature of the linear polypyrroles of porphyrin and corrinoid intermediary metabolism cannot yet be defined.[8b, c, d, e]

(b) L. Bogorad, In The Chlorophylls, 1966; L. P. Vernon and G. R. Seeley, Ed., Academic Press, New York, N. Y.

(c) L. Bogorad and J. Pluscec, Biochemistry, 1970, 9, 4736.

R. Radmer and L. Bogorad, Biochemistry, 1972, 11, 904.

(d) B. Frydman, S. Reil, A. Valasinas, R. Frydman and H. Rapoport, J. Amer. Chem. Soc., 1971, 93, 2738.

R. Frydman, A. Valasinas, H. Rapoport and B. Frydman, FEBS Letters, 1972, 25, 309.

(e) A. R. Battersby, In "The 23rd International Congress of Pure and Applied Chemistry, Special Lectures", 1971, Vol. 5, 1.

9 G. Müller and W. Dieterle, Hoppe-Seyler's Z. Physiol. Chem., 1971, 352, 143.

10 B. Franck, D. Gantz and F. Hüper, Angew. Chem., (Int'l ed.) 1972, 11, 421.

11 D. Doddrell and A. Allerhand, Proc. Nat. Acad. Sci. (U.S.), 1971, 68, 1083.

12 H. Stoeckli-Evans, E. Edmond and D. Crowfoot Hodgkin, J. Chem. Soc. Perkin Trans., 1972, II, 605.

13 R. Bonnett, J. M. Godfrey, V. B. Math, P. M. Scopes and R. N. Thomas, J.Chem.Soc., Perkin Trans., 1973, I, 252.

R. Bonnett, J. M. Godfrey and V. B. Math, J.Chem.Soc., 1971, (C), 3736.

14 D. K. Dalling and D. M. Grant, J.Amer.Chem.Soc., 1972, 94, 5318.

D. M. Grant and B. V. Cheney, J.Amer.Chem.Soc., 1967, 89, 5315.

15 L. Bogorad in "Methods in Enzymology", 1962, Vol. V, S. P. Colowick and N. O. Kaplan eds., 893.

16 G. Romeo and E. Y. Levin, Biochim.Biophys. Acta, 1971, 230, 330.

17 D. Mauzerall, J.Amer.Chem.Soc., 1960, 82, 2601.

18 G. Marino, In "Advances in Heterocyclic Chemistry", 1971, Vol. 13, 235.

19 R. A. Jones, In "Advances in Heterocyclic Chemistry", 1970, Vol. 11, 383.

20 H. Booth, A. W. Johnson, E. Markham and R. Price, J.Chem.Soc., 1959, 1587.

21 A. Eschenmoser, In "The 23rd International Congress of Pure and Applied Chemistry, Special Lectures", 1971, Vol. 2, 69.

22 R. Grigg, A. P. Johnson, A. W. Johnson and M. J. Smith, J.Chem.Soc. (c), 1971, 2457.

23 J. I. Toohey, Fed. Proc., 1966, 25, 1628.

24 K. Sato, S. Shimizu and S. Fukui, Biochem.Biophys.Res.Commun., 1970, 39, 170.

25 G. A. Walker, S. Murphy and F. M. Huennekens, Arch.Biochem.Biophys., 1969, 134, 95.

26 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J.Biol.Chem., 1951, 193, 265.

Received, 14th November, 1973