Tetrahedron. Vol. 31, pp 2639 to 2653. Pergamon Press 1975. Printed in Great Britain

TETRAHEDRON REPORT

CONCERNING THE BIOSYNTHESIS OF VITAMIN B_{12}

A. IAN SCOTT
Sterling Chemistry Laboratory, Yale University, New Haven, CT 06520, U.S.A.

(*Received for publication* 22 *August* 1975)

Abstract—The use of ^{13}C-Fourier transform nuclear magnetic resonance (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 vitamin 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 or 100,000 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^{2+}, Mg^{2+}, *S*-adenosylmethionine and glutathione. Multiply-labeled uro'gens (^{13}C, ^{14}C and ^{3}H) have been used to show that incorporation takes place without randomization. A sequence for corrin synthesis from uro'gen III, involving as the first step decarboxylation of the ring-C acetic acid side chain, is proposed.

INTRODUCTION

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

In this discussion we deal with 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 embraced from the outset the idea expressed by Burnham[5,6] that the cobalt and iron pathways diverge *after* the formation of uro'gen III. The notion 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 an earlier encounter of two PBG molecules in accord with the experiments of Frydman *et al.*[8] Regardless of the details of how uro'gen III is formed, an experimental distinction can be made between uro'gen intermediacy and the corrin synthetase mechanism of Corwin which by-passes the uro'gens and to form 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.

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

†These conditions are critical! See e.g. Refs. 9, 10 and A. I. Scott, C. A. Townsend, K. Okada and M. Kajiwara, *J. Am. Chem. Soc.* **96**, 8054 (1974).

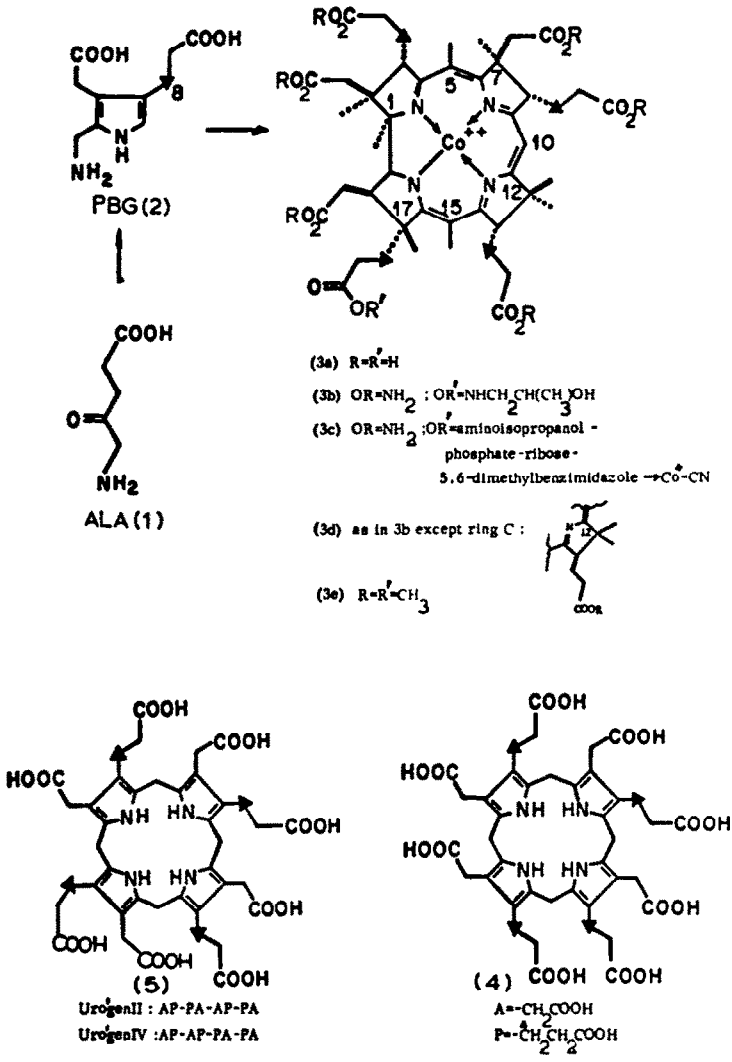


Fig. 1.

Table 1. ^{14}C-Feeding experiments using suspended cells of *P. shermanii* ATCC No. 9614 (30° g/100 ml phosphate buffer)

Experiment	Substrate Fed	mg Fed	Hr	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.

†In later experiments using about one half the cell weight specific incorporations of 2–4% were recorded.

synthetase procedure) gave either zero or very low incorporation when administered in carefully monitored parallel feeding experiments (Table 1; Experiments 3 and 4).

With proof for the inertness of uro'gen I in hand, the use of uro'gen III/I mixture (70/30–50) obtained from the wheat germ preparation or 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) can be justified. In conformity with heme and chlorophyll biosynthesis, 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 rigorously 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) according to the pattern shown above. The ^{13}C FT-NMR spectrum of [8-^{13}C]-PBG enriched vitamin B_{12} is reproduced in Fig. 2a. The spectrum contains three resonances at 37·8, 33·4 and 31·6 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 31·6 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* (~12 g cells/100 ml medium per flask) and the resultant vitamin B_{12} subjected to similar ^{13}C-NMR

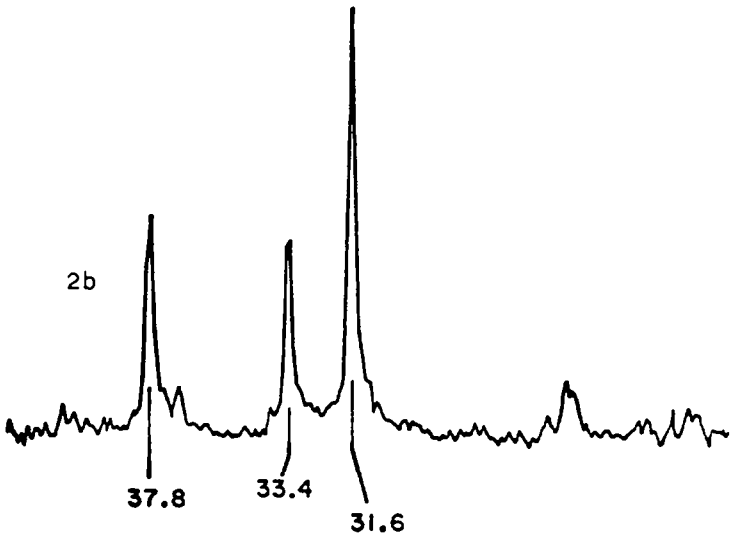


Fig. 2b(top). Portion, 44·7–17·2 ppm downfield from external HMDS, of the proton noise-decoupled ^{13}C FT spectrum of 40 mg [^{13}C]-uro'gen enriched cyanocobalamin in H_2O; same conditions as for spectrum 2a.

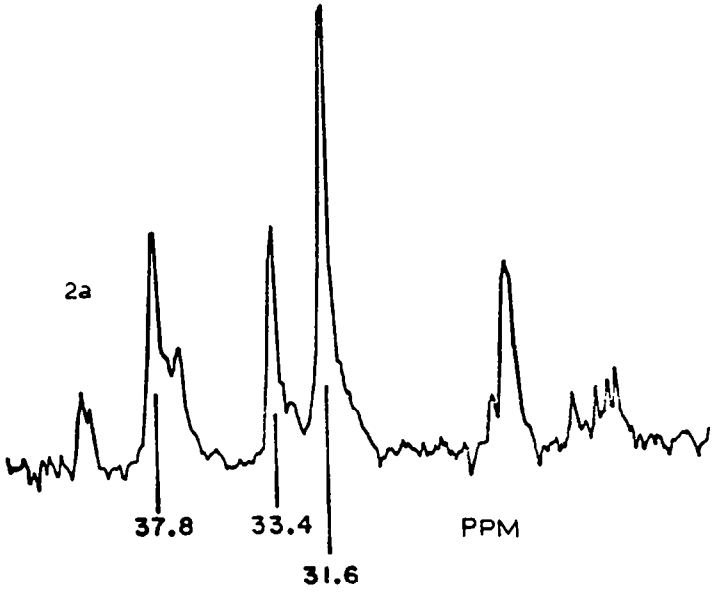


Fig. 2a(bottom). Portion, 44·7–17·2 ppm downfield from external hexamethyldisilane (HMDS), of the proton noise-decoupled ^{13}C FT spectrum of 41 mg [8-^{13}C]-PBG enriched cyanocobalamin (vitamin B_{12}, 3c) in H_2O; data set = 4K points; digitizing rate = 10 kHz; pulse width = 50 μsec.

analysis, the enriched spectrum (Fig. 2b) 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) confirm the positive incorporation using ^{14}C and also provide unequivocal evidence for the location of the label. However although the sequence PBG → uro'gen III → vitamin B_{12} now becomes even more attractive it must be recognised that the symmetrical nature of the labelling pattern of uro'gen III leaves open the possibility that a fragmentation recombination mechanism is operating. We shall return to this point in some detail after describing the development of both ^{13}C and cell free experiments.

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, 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 re-examined the problem using ^{13}C-Fourier

transform NMR to determine the fate of [2-^{13}C]- and [5-^{13}C]-ALA in *P. 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 (Fig. 3a). 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 Fig. 3b. Such an array is in harmony with current ideas on the mechanism of type III uro'gen formation and this result was simultaneously and independently confirmed in Shemin's laboratory.[12] However, there was no ^{13}C enhanced signal above 95 ppm downfield from HMDS showing that no enrichment of the C-1 methyl 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 (Fig. 3c) revealed only 6 signals highly enriched above natural abundance, conversion of this sample to the dicyano form (Fig. 3d) revealed seven well defined resonances. Hence the signal at 24·9 ppm (Fig. 3c) corresponds to two superimposed resonances. Inspection of the integrated spectrum (Fig. 3d) leaves no doubt that seven methionine methyl groups have been incorporated. Again this result, which is of considerable significance for the mechanism of corrin synthesis, received welcome confirmation from the work of Battersby and his group.[13]

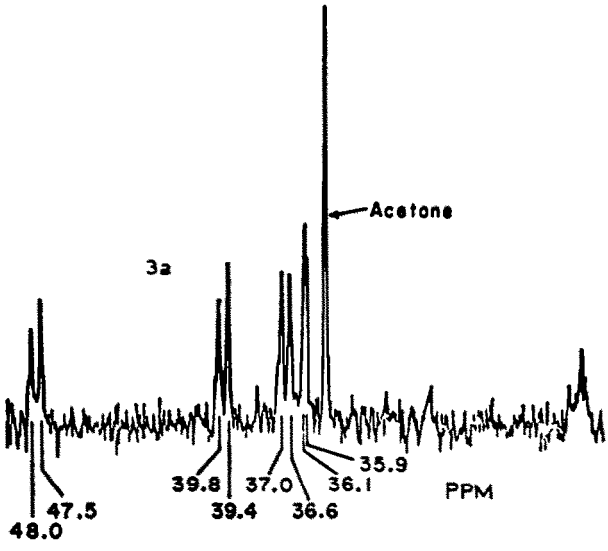


Fig. 3a. Proton noise-decoupled ^{13}C FT spectrum of [2-^{13}C]-ALA enriched cyanocobalamin (vitamin B_{12}, 26 mg) in H_2O. The methyl group of acetone, 35·1 ppm provides an internal reference. Only the range 50·5–19·6 ppm downfield of external HMDS is shown: Data set = 8K points; digitizing rate 10 KHz; pulse width = 50 μsec; receiver skip = 100 μsec.

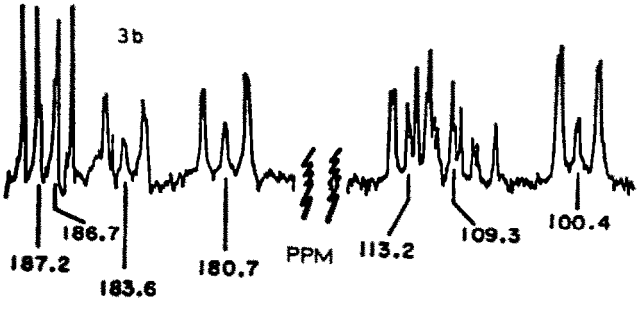


Fig. 3b. Proton noise-decoupled ^{13}C FT spectrum of [5-^{13}C]-ALA enriched cyanocobalamin (38 mg) in H_2O. Two portions of the spectrum, 188·5–117·5 ppm (left), and 117·8–95·7 ppm (right) downfield from external HMDS are shown: Data set = 4K; digitzing rate = 10 kHz; pulse width = 50 μsec; receiver skip = 100 μsec.

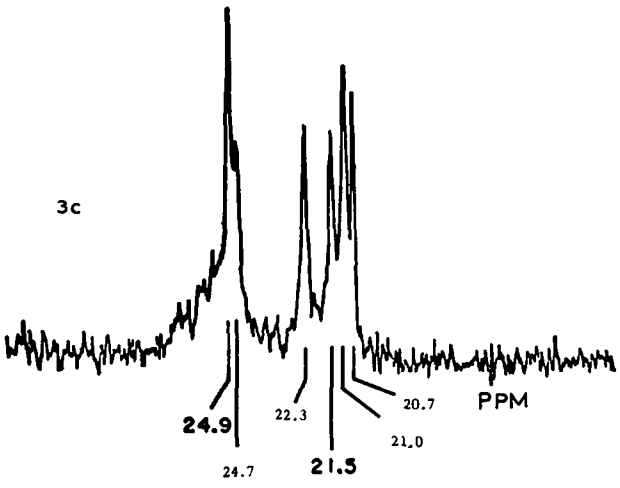


Fig. 3c. Portion, 35·1–8·6 ppm downfield of external HMDS, of the proton noise-decoupled ^{13}C FT spectrum of [$^{13}CH_3$]-methionine enriched cyanocobalamin (36 mg) in H_2O; conditions as in Fig. 2b.

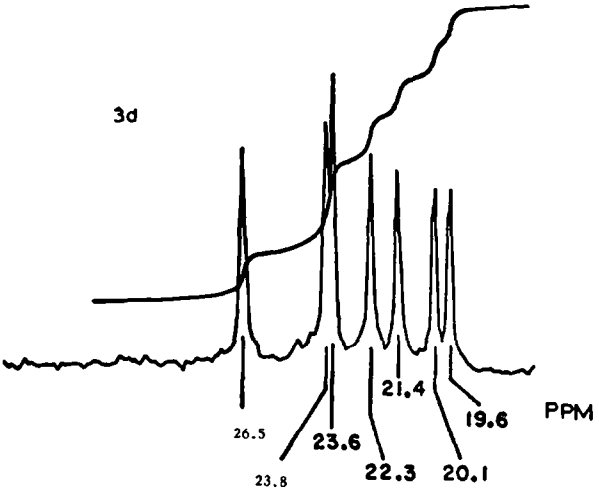


Fig. 3d. Portion, 29·9–16·6 ppm downfield from HMDS, of the proton noise-decoupled ^{13}C FT spectrum of dicyanocobalamin (36 mg) in 0·1 M KCN; conditions as in Fig. 2a.

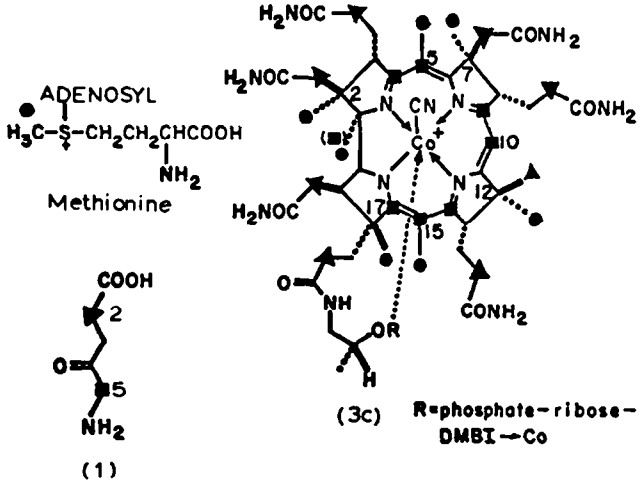


Fig. 4.

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 methl 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-enriched 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[14] of the C-ring of cobinamide (**3b**) places the α-methyl *syn-periplanar* to the adjacent axially oriented propionamide side chain at C-13 (Fig. 5a). 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,

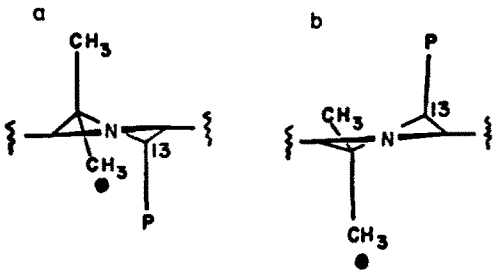


Fig. 5. Conformation of ring C in cobinamide (a) and neocobinamide (b) as viewed from cobalt on X-ray and CD/ORD data.

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 Fig. 6, (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.[15] Neocobinamide differs from cobinamide solely by virtue 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 (Fig. 5b). 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.[16] 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 (Fig. 6, 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

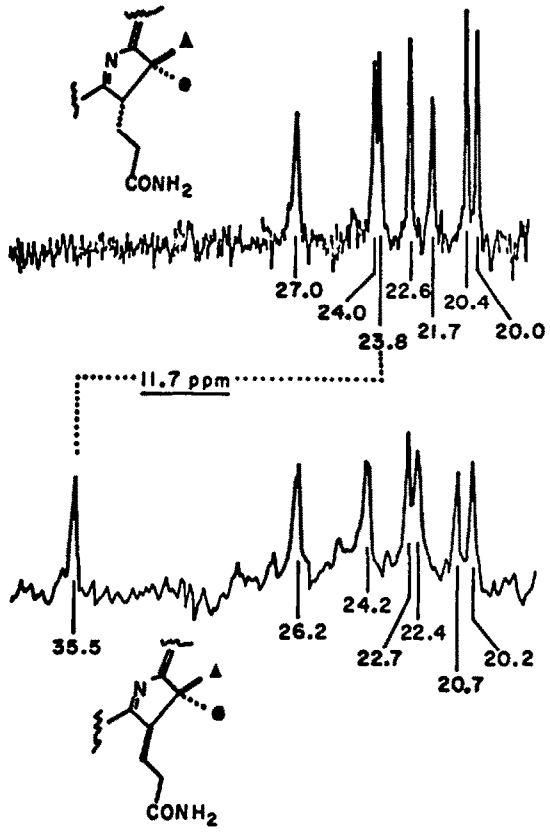


Fig. 6(top). Proton noise-decoupled ^{13}C FT spectrum of [$^{13}CH_3$]-methionine enriched dicyanocobinamide (9 mg) in 0·1 M KCN. Only the range 38·1–15·8 ppm downfield from HMDS is shown: data set = 8K points; digitizing rate = 10 kHz; pulse width 50 μsec; received skip = 100 μsec.

Fig. 6(bottom). Proton noise-decoupled ^{13}C FT spectrum of [$^{13}CH_3$]-methionine enriched dicyano*neo*cobinamide (2 mg) in 0·1 M KCN. Conditions and frequency range same as above except data set = 4K.

(*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. In this complex field of corrin biosynthesis it was again gratifying to find that an alternative proof for the absolute stereochemistry of the methylation at C_{12} was forthcoming from the work of the Cambridge group[16] who were also able to show by deuterium labelling that, as far as the methionine derived methyl group at C-12 (*R*) is concerned, there is no loss of hydrogen (^{2}H) during methyl transfer.

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[17] 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* (ATCC 9614). Disruption by a French press in phosphate buffer [pH 7·6, 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 [^3H_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, nonradioactive sample.

The presence of ALA dehydratase, uro'gen I synthetase, uro'gen III cosynthetase, 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 [^3H_3C]-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 $^3H/^{14}C$ 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 ^3H from [^3H_3C]-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.

Further definition of the role of uro'gen III in corrin biosynthesis

All of the foregoing experiments employed *side chain* labelled samples of uro'gen III obtained both by chemical synthesis (in admixture with types I, II and IV uro'gens) and enzymatic preparation (together with uro'gen I). In experiments with ^{13}C-enriched substrates it was clearly shown that the label in the side chain propionate groups was carried to the corresponding positions in the appropriate corrin. However the intrinsic symmetry of these labeling patterns together with the problems of employing an isomer mixture left open the logical possibility that *in vitro* dissocation of the uro'gen into labeled fragments capable of assimilation by the enzyme system could give rise to the observed regiospecific enrichments *without mediation of the intact uro'gen III molecule.* In order to resolve this question of vital importance for the mechanism of corrin biosynthesis we have undertaken the regiospecific synthesis of a set of

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)		(<0·001)[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,k]
	[^3H_3C]-SAM[f]	1·1		0·26	(24)(^{3}H)
12	[$^{14}CH_3$]-SAM[g]	19	65	6·9	(36)[j]
13	[^3H_3C]-SAM[f]	1·1	65 (boiled enzyme)		(<0·001)[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, merceptoethanol 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.* (1951). *Specific activity*: [d] 26·2 mCi/mM; [e] 73·2 mCi/mM; [f] 4550 mCi/mM; [g] 52 mCi/mM; [h] The real yield should be based on uro'gen III; [i] $^3H/^{14}C$ ratio of the substrates 0·53, $^3H/^{14}C$ ratio of the product 5, 3; [j] Incubation conditions: (a) plus 1·25 mg ALA; [k] Incubation conditions: (a) plus 10 mg SAM.

uro'gens whose patterns of enrichment with both stable and radioisotopes are designed to provide unambiguous probes for intact bio-transformation and for the nature of the overall mechanism connecting the uro'gen and corrin structures.

The regiospecific syntheses of [α, γ-$^{14}C_2$]- and of ring B propionic acid [$^3H_2$]-uro'gen III were carried out by the procedures of MacDonald[18] and Franck[19] modified where appropriate for the introduction of radioisotope. Incubation of the doubly labelled uro'gen ($^3H/^{14}C$, 4·10; Fig. 7) in the cell-free system from *P. shermanii* gave after dilution with carrier, conversion to cobester, and crystallization to constant activity a sample of cobester with $^3H/^{14}C$, 4·05. Any randomization via fragmentation-recombination would have led, in the case of this unsymmetrically labelled substrate, to a profound change in the tritium–carbon ratio. To confirm this result and at the same time locate the site of label in the corrin, a specimen of α, γ-[$^{13}C_2$] uro'gen III was prepared via condensation of the dipyrromethane dialdehyde (6) and dipyrromethane (7), with introduction of ^{13}C from dimethyl formamide (90% ^{13}C), by a procedure established above for the synthesis of the ^{14}C-radiomer, to give finally a sample of the α, γ-^{13}C-enriched uro'gen (90% ^{13}C). Administration of 365 mg of this "north-south" labelled substrate to resting whole cells 340 g of *P. shermanii* gave (after the usual work-up) pure cyanocobalamin (11 mg) whose FT-CMR spectrum (Fig. 9a) on comparison with the natural abundance spectrum taken under identical conditions (Fig. 9b) revealed enhancement (4·5% specific incorporation) at only two resonances in the sp^2 region, viz. at 105·0 and 108·4 ppm downfield from TMS.† These signals had previously been assigned to C_{15} and C_5 respectively both by the correlations of Allerhand[11] and by biosynthetic labelling.[20,21] This experiment confirms the intact incorporation of doubly-labelled uro'gen III and unambiguously locates the labelled sites according to (Fig. 8) where ■C now denotes both ^{14}C and ^{13}C. Once again using a different ^{13}C-labelling pattern this result has recently been confirmed by Battersby.[22]

A complementary set of experiments was then conducted with a different multiply labelled version of uro'gen III obtained (in admixture with the types I, II, IV isomers) by *in vitro* condensation at pH 7·6 of [9-^3H]-porphobilinogen and [^{14}C]-formaldehyde followed by acidic equilibration[23] of the meso ^{14}C label (Fig. 10). Incubation of the substrate ($^3H/^{14}C$ 15·3 in recovered uroporphyrin) with the cell-free preparation, recovery and work up in the usual way gave crystalline cobester with $^3H/^{14}C = 17·8$ a value which is 10% below that

†Previous chemical shifts were cited downfield from HMDS, using a Bruker 90 instrument and the FT system described earlier. A complete set of corrected chemical shift values for the corrin series is now available (A. I. Scott and G. H. Temme III, *Tetrahedron Letters* (1975), in press.

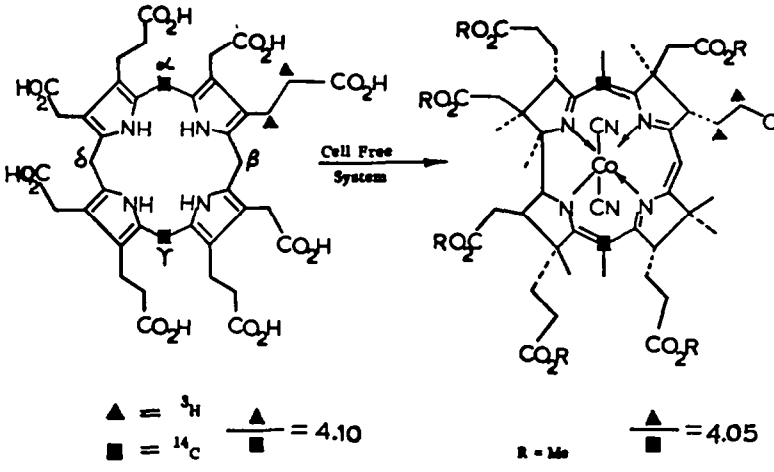


Fig. 7.

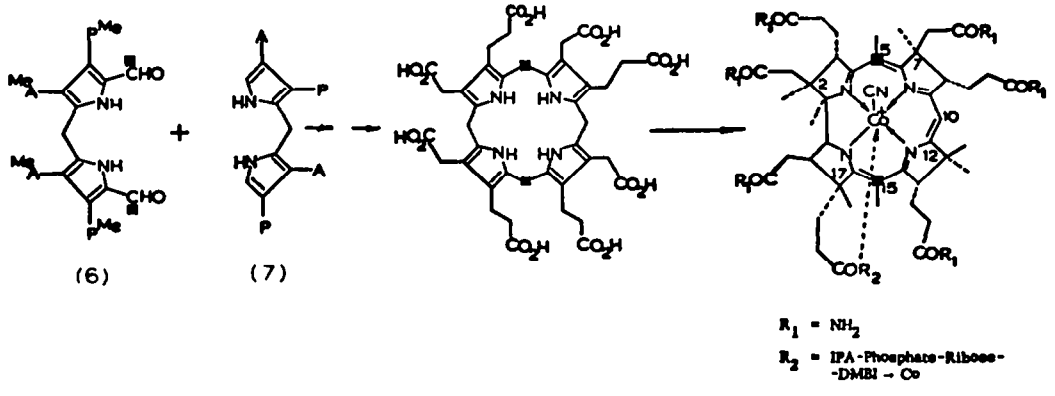


Fig. 8.

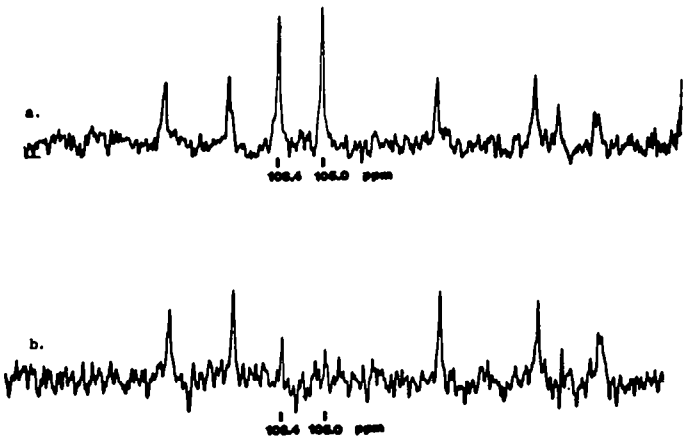


Fig. 9. (a) Proton noise decoupled ^{13}C-FT NMR spectrum of [^{13}C] uro'gen III enriched cyanocobalamin (D_2O; 4 K points) and assigned labelling patterns. (b) Proton noise decoupled ^{13}C-FT NMR spectrum of natural abundance cyanocobalamin (D_2O; 4 K points).

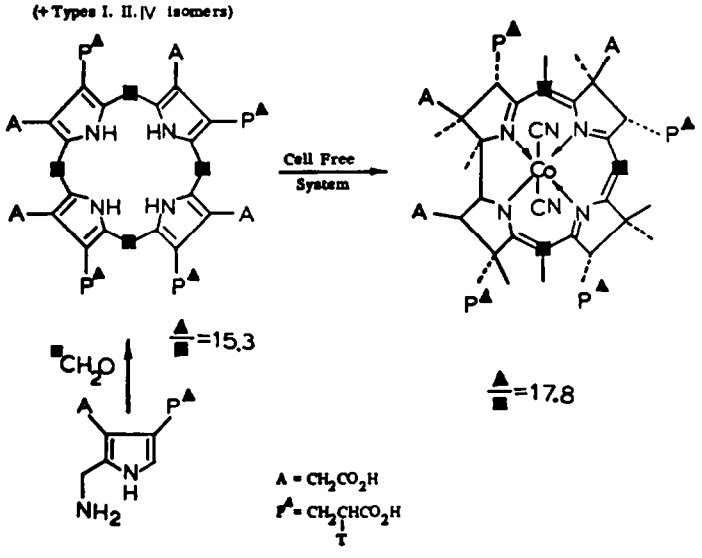


Fig. 10.

expected (20·4) on the basis of complete loss of the "western" (δ) meso-carbon from the system as formaldehyde, formate or possibly CO_2. Thus although strict stoichiometry was not realised in this experiment there is a clear trend towards a ratio representing the loss of the δ-meso carbon from uro'gen III.

Finally, in order to test the earlier hypothesis that decarboxylation of the acetic acid side chain in ring C takes place at the uro'gen level, a regiospecific total synthesis of the type III heptacarboxylic acid (**8**) was carried out as summarized in Fig. 11. The m.p. (238–240°) of the heptamethyl ester (**9**) was in excellent agreement with that reported by Battersby[24] *et al.* for this isomer (prepared by an analogous route) and spectroscopic and mixed m.p. comparison confirmed their identity. The synthesis was repeated (using ^{14}C-dimethyl formamide) and the resultant [α, γ-$^{14}C_2$]-hepta carboxylic uro'gen III incubated with the cell-free system to afford (after crystallization to constant activity) cobester (**3a**) (0·1% incorporation).† This experiment provides the first clear indication that the hepta-acid (**8**) is an intermediate in corrin biosynthesis (Fig. 11) and that uro'gen III suffers decarboxylation *prior to* the reductive methylation sequence necessary to generate the rearranged corrin structure. It is of considerable interest to note that since phyriaporphyrinogen III[24] (**10**) is considered to be the obligatory biosynthetic precursor for coprogen and heme, the new isomer (**8**) represents the branchpoint at which the heme and corrin pathways, having shared a common route from glycine and succinate to uro'gen III, diverge.

†Specific incorporations with the crude enzyme system cannot be calculated accurately. Using the whole cell technique the hepta acid (**8**) was converted to vitamin B_{12} (1) with a specific incorporation of 1%.

The sequence of corrin biosynthesis

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

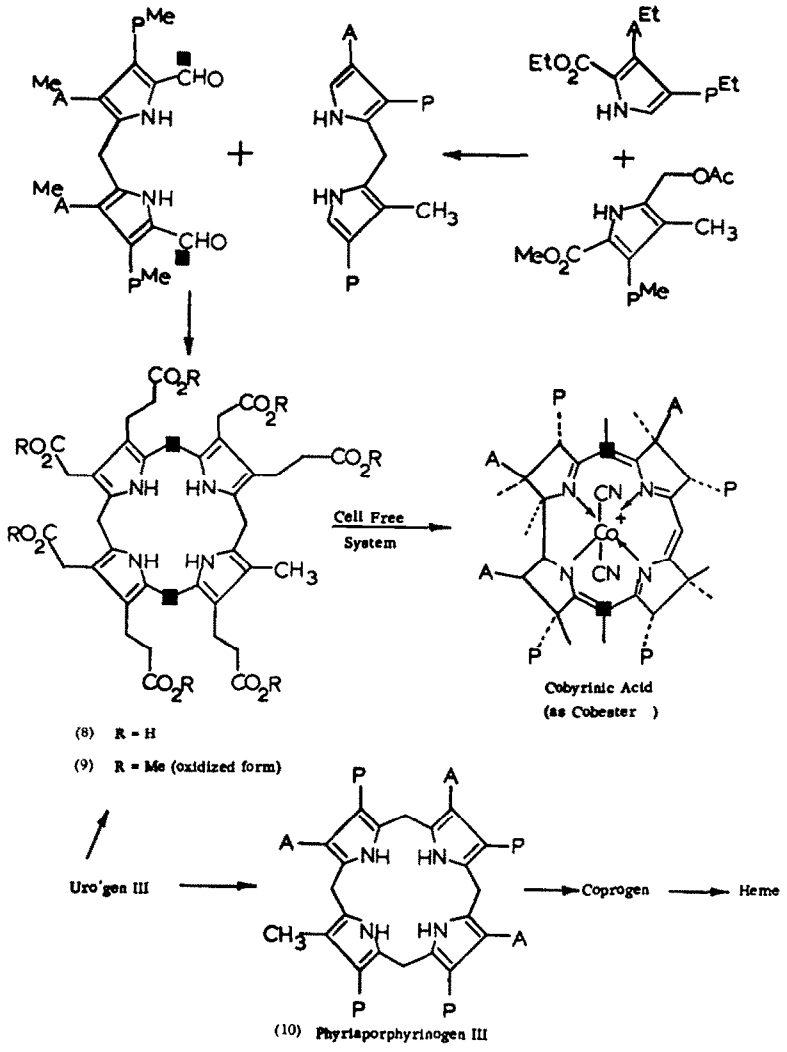


Fig. 11.

1. Decarboxylation of the acetic acid side chain at C-12.
2. Loss of the meso carbon at C-20 (as formaldehyde, formate or CO_2) 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.

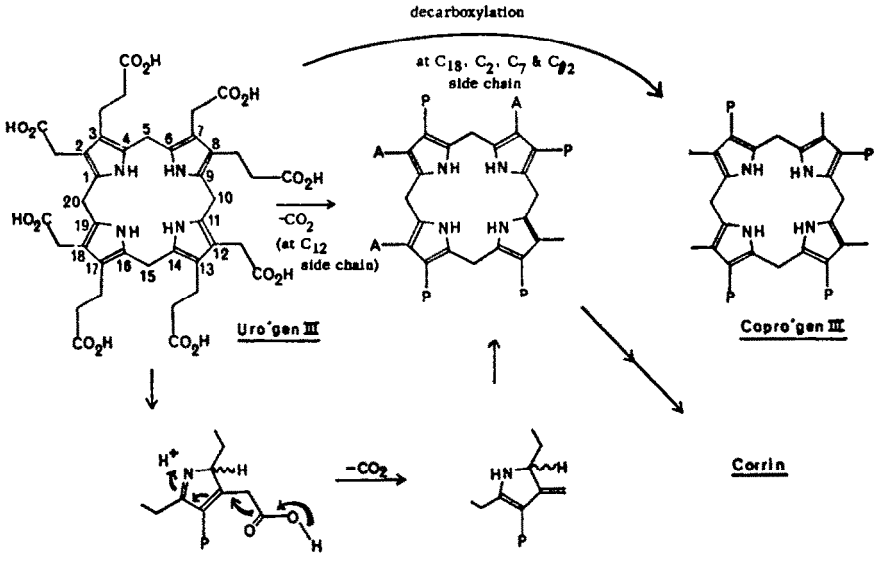
A set of mechanistic proposals for each of these events is outlined in the sequel.

1. *Decarboxylation.* With the demonstration of a cell-free system capable of transforming uro'gen III to cobyrinic acid we proposed in 1973 that the first event in this sequence be the decarboxylation of the acetic acid side chain at C_{12}. Since in all biological decarboxylations described hitherto the presence of an *electron sink* is obligatory, it was suggested that *decarboxyl ion 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, intermediates having been isolated in the partially decarboxylated form. With the recent discovery (*vide supra*) that the ring (C_{12}) decarboxylated uro'gen III serves as a good precursor for corrins this step now appears to be the control point for the branching of the corrin and heme pathways. Thus Jackson[25] has recently delineated the decarboxylation sequence (Rings D, A, B, C) from uro'gen to coprogen III (Scheme 1).

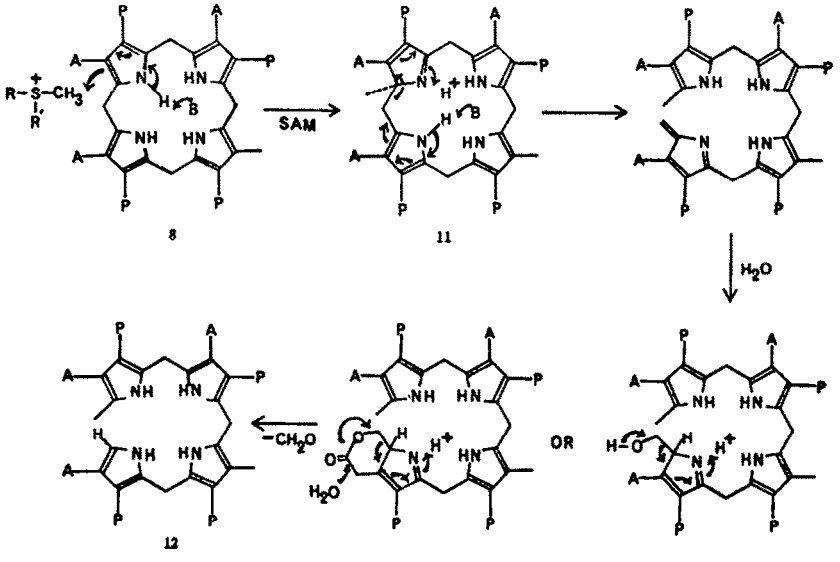
2. *Methylation and loss of* C_{20}. The heptacarboxylic porphyrinogen (**8**) can undergo the first methylation at C_1 by S-adenosylmethionine (SAM), presumably coupled with a base catalyzed hydrogen abstraction. The methylated species (**11**) is now poised for a rearrangement process in which formaldehyde is lost via hydration, generating the linear tetrapyrrole (**12**). We note that this step is equivalent to two electron reduction (Scheme 2) and also that loss of formaldehyde could be delayed for several steps or even take place before methylation at C_1.

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.

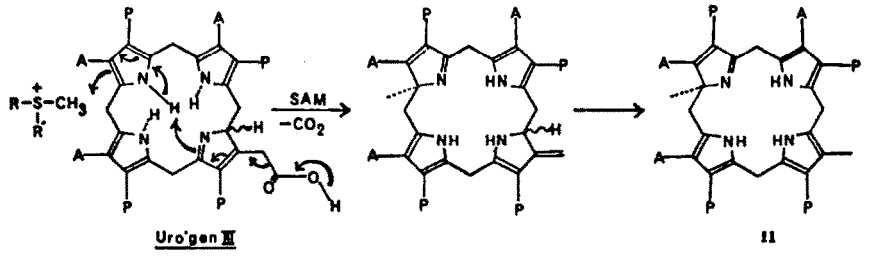
To explain the acidic *in vitro* isomerization of uro'gens. Mauzerall has proposed ring cleavage coupled with



Scheme 1.



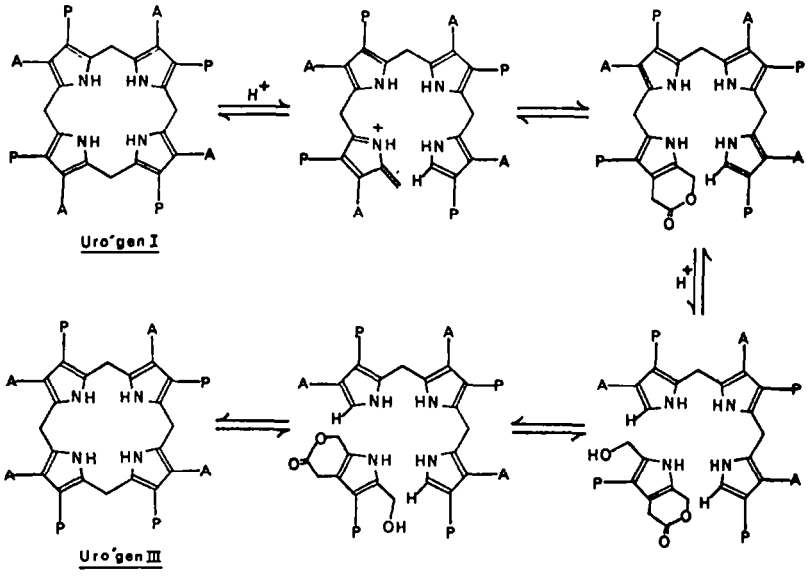
Scheme 2.



Scheme 3.

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

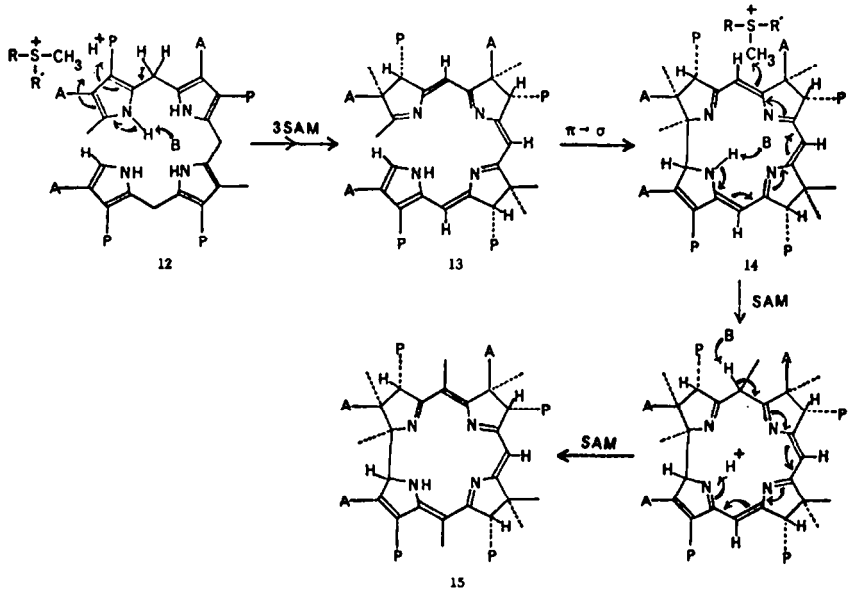


Scheme 4.

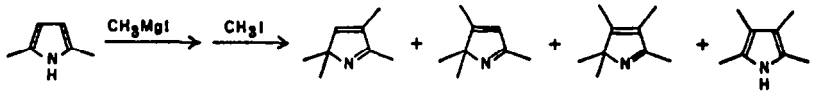
controlled by the methylating enzyme, provide a conjugated 16π-electron array (**13**), which may now undergo an orbital-symmetry-allowed $\pi - \sigma$ rearrangement in a concerted manner to produce a dehydrocorrin chromophore (**14**) upon which C_5 and C_{15} methylations may operate (Scheme 5).

Electrophilic substitution usually favors the α-position rather than the β-position of pyrroles 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 (Scheme 6). The recently disclosed structure of sirohydrochlorin is also instructive in this regard.[26]

In the case of substrate **12**, the energy stabilization resulting from an extensive delocalization of π-electrons should facilitate β-methylations on pyrrole units. An



Scheme 5.



Scheme 6.

interesting outcome of the above scheme is the possibility of C_{12} methylation from the 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 Möbious overlap for a conrotatory ring closure to achieve the observed *trans* stereochemistry of the C_1 methyl and C_{19} hydrogen (Scheme 7).

Such a process is reminiscent and isoelectronic with the second (thermally allowed) part of Eschenmoser's remarkable photochemical synthesis of corrins (Scheme 8) and also finds excellent analogy in the elegant cyclization studies of tetradehydrocorrins by Johnson (Scheme 9). Very recently Eschenmoser[27] has provided a striking *in vitro* example of the thermal cyclization of the 16π system of **13** to a dehydrocorrin of type **14**.

The above 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 (cf. the cyclization of the linear tetrapyrrole system to uro'gen).

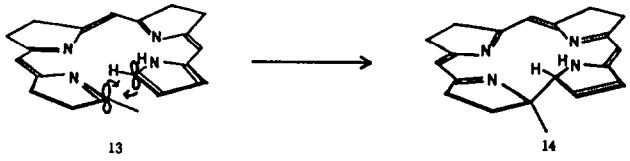
4. *Reduction and methylation.* The resultant 18 - dehydro - 17 - desmethyl cobalt-free cobyrinic acid (**15**) is now aligned for a final reductive methylation sequence. Following the conjugative attack of hydride (possibly from NADPH) at C_{18} a study of models suggests that α-side of the nascent 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 (**16**) may well be a precursor of the family of naturally occurring cobalt-free corrins.

5. *Insertion of cobalt.* The incorporation of cobalt atom poses the last problem in the sequence. The cobaltous ion (Co^{2+}) may be oxidized before or after incorporation into the ring (Scheme 10).

On the other hand, the possible utilization of the extra electron in Co^{2+} for C_{17} methylation cannot be discounted. For instance, the addition of Co^{2+}, a methyl group and a proton with supply of one electron to the precorrin system **15** will produce cobyrinic acid (Scheme 11). One electron reductions are well known in vitamin B_{12} enzymology in conjunction with the biological formation of coenzyme B_{12}.

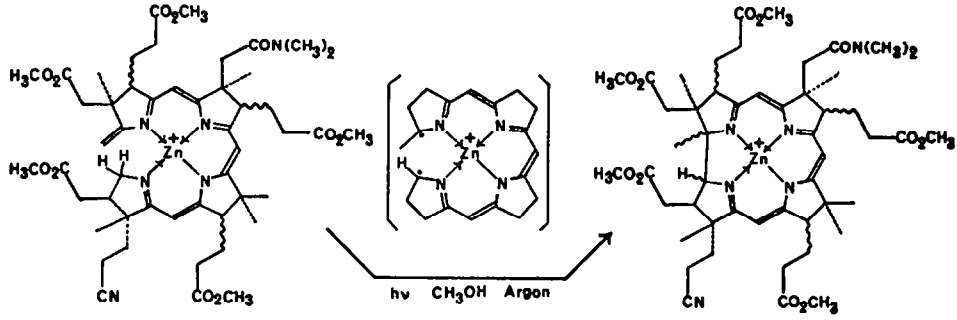
The further metabolism of cobyrinic acid to the cobalamins and coenzyme B_{12} has been exhaustively studied by Bernhauer in *P. shermanii*[3] and the full sequence of successive amidation and addition of the aminoisopropanol and nucleotide segments have been determined.

In conclusion, we feel that the above hypothesis offers a rationale for the uro'gen → corrin transformation which

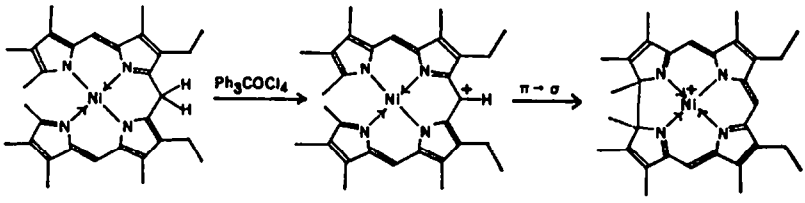


13 14

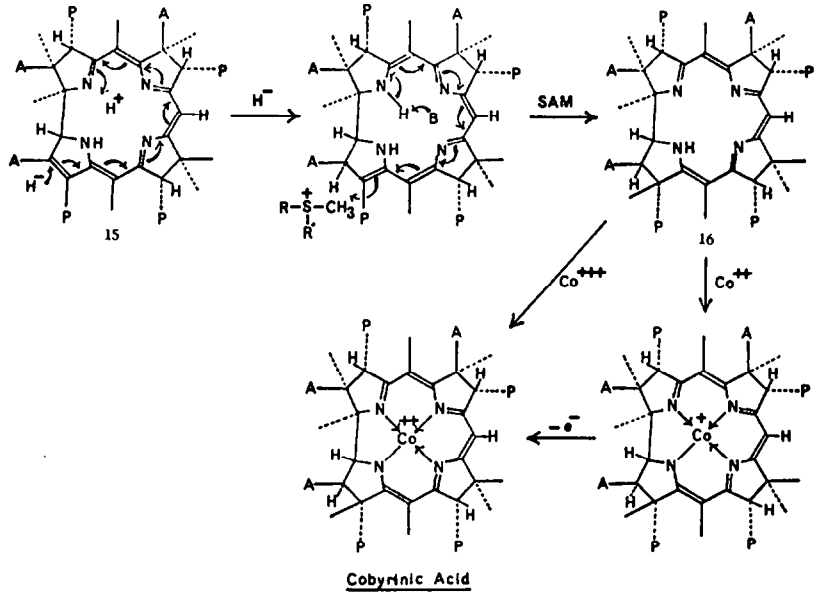
Scheme 7.



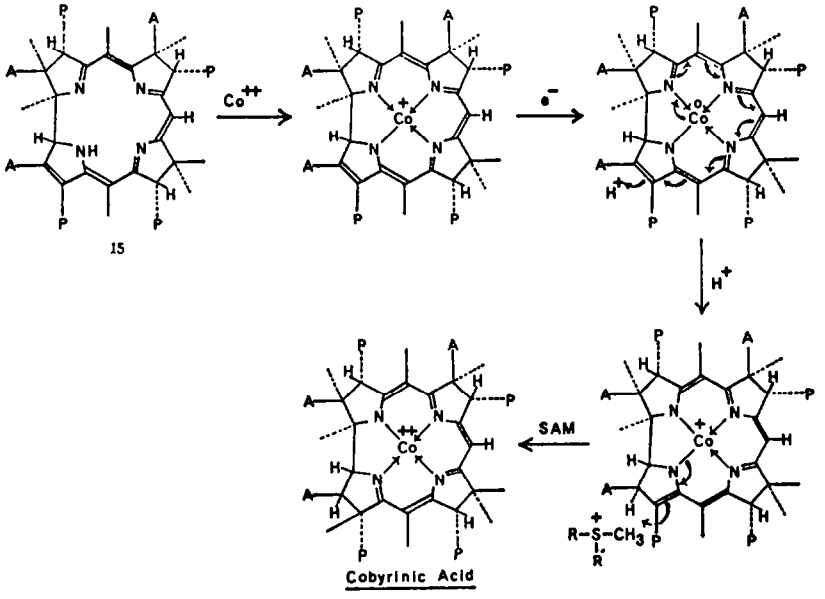
Scheme 8.



Scheme 9.



Scheme 10.



Scheme 11.

can be tested at those points where the stability of certain of the putative intermediates would allow inhibition and/or feeding studies to be conducted, a case in point being the recent demonstration that the hepta-acid (**8**) represents the first intermediate after uro'gen III.

Acknowledgements—The work carried out at Yale is the result of a splendid team effort (1968–75) to which the following colleagues devoted much energy, skill and thought: C. A. Townsend, E. Lee, K. Okada, B. Yagen, K. S. Ho, G. H. Temme, N. Georgopapadakou, S. Klioze, P. A. Bays, P. J. Whitman, R. J. Cushley, N. Obata, W. K. Chan, W. Fitch, P. Sampathkumar, I. M. Armitage, D. Brownstein, A. Brown, J. Petrillo and M. Kajiwara. We thank the National Institutes of Health and the National Science Foundation for generous financial support; Profs. H. Rapoport, A. R. Battersby and Drs. S. F. MacDonald and A. Brossi for gifts of samples.

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