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Mechanistic and evolutionary aspects of vitamin B_{12} biosynthesis

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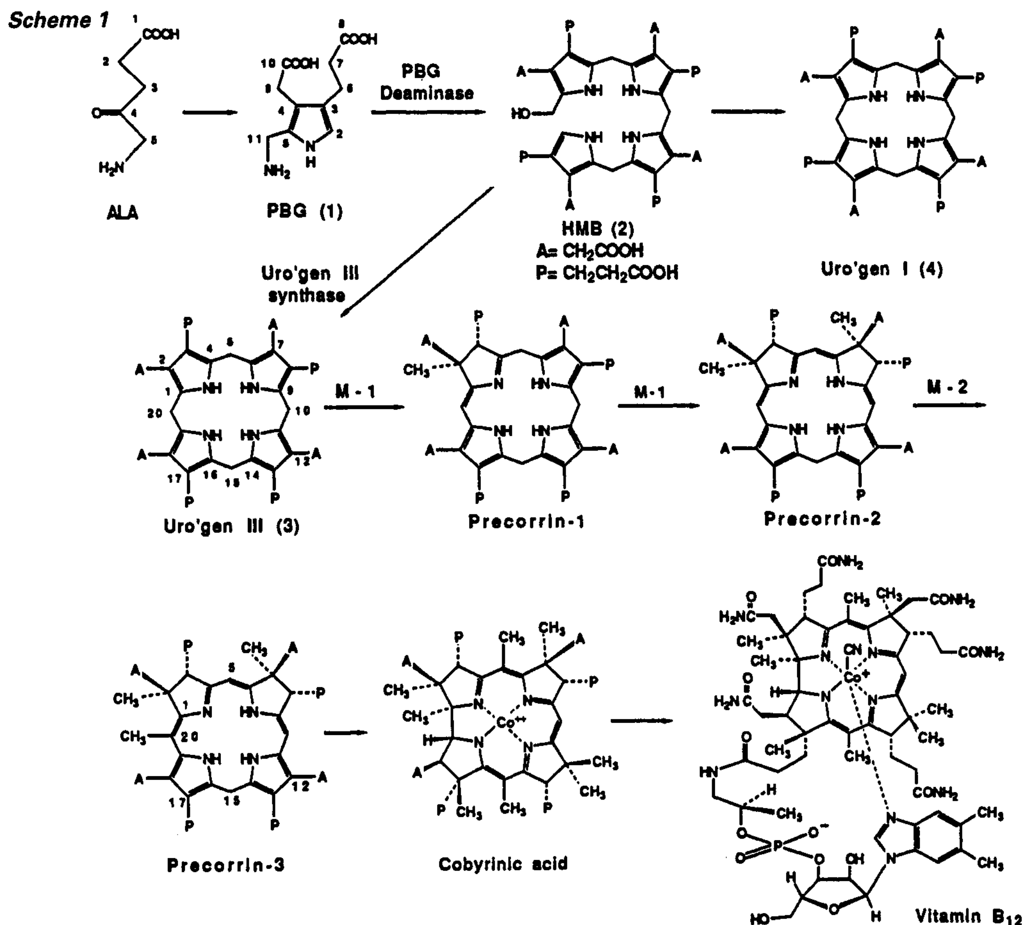
Abstract - The B_{12} biosynthetic pathway requires approximately 20 enzymes to convert aminolevulinic acid, methionine and cobalt to the corrinoids. Recently several of the genes encoding the enzymes of B_{12} biosynthesis have been expressed in *E. coli*. The availability of these biosynthetic catalysts in large amounts has revolutionized the study of the B_{12} and heme pathways. Recent progress on the elucidation of the mechanism of several of these enzymes will be described. The technique of choice for these studies is high resolution NMR spectroscopy which can be used to detect intermediates in the catalytic cycle and to follow the overall reaction kinetics. Genetic engineering of the cDNA encoding for the biosynthetic enzymes has been used to study the mechanism of catalysis and to produce enzymes with altered substrate specificity in order to elucidate the mechanistic details of processes such as porphyrin assembly and C-methylation on the path to B_{12}.

INTRODUCTION

For the past 20 years our laboratory has been engaged in the elucidation of the vitamin B_{12} biosynthetic pathway and an earlier review[1] described progress made in the first ten years of this endeavor. By that time (1977), it had been established that 5-aminolevulinic acid (ALA), porphobilinogen (PBG) uro'gen III and the three partially methylated isobacteriochlorins, Factors I-III were the sequentially formed intermediates on the way to the corrin nucleus exemplified by cobyrinic acid as summarized in Scheme 1. The ensuing decade has witnessed an exponential increase in the rate of the acquisition of pure enzymes using recombinant DNA techniques and these methods together with the exploration of new NMR pulse sequences have not only cast light on the mechanisms of

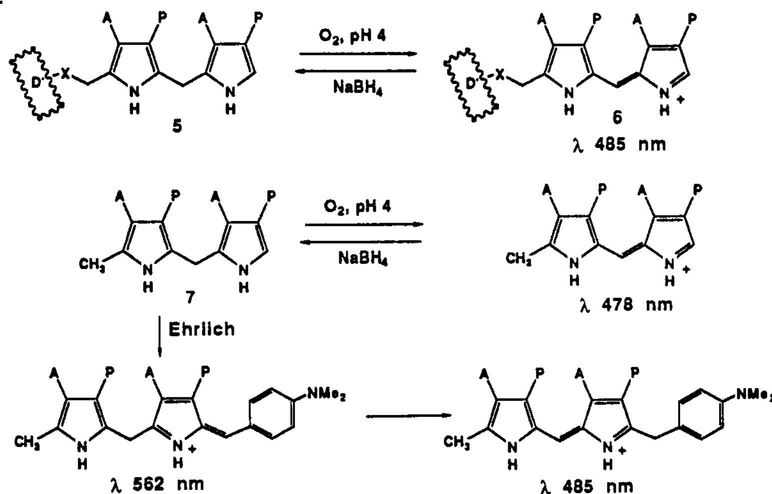


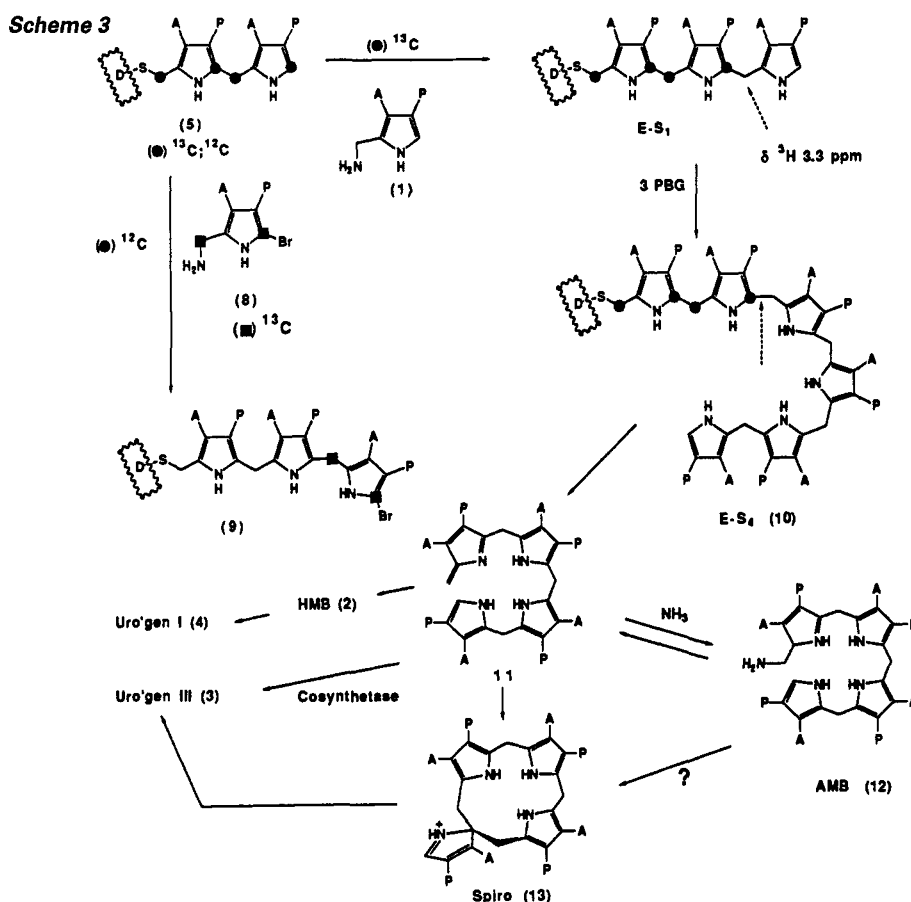
the B_{12} synthetic enzymes but have given us considerable optimism with regard to the discovery of the remaining intermediates and of the enzymes which produce them. This lecture will focus on three topics drawn from the "early" and "late" segments of the pathway. The first of these deals with mechanistic and structural proposals for PBG deaminase, the assembly enzyme for tetrapyrrole biosynthesis which, working together with uro'gen III synthase, is responsible for the synthesis of the unsymmetrical type-III macrocycle. The second topic encompasses the sequence of C-methylations connecting uro'gen III with the precorrins, leading finally to cobyrinic acid and the properties of the purified methyl transferase responsible for the first two methyl group insertions into uro'gen III. Thirdly, the discovery of a new class of corphinoids, which reflect a degree of non-specificity of the B_{12} synthesizing enzymes and the consequences of this finding for the biochemical evolution of the B_{12} structure will be discussed. The complex problems inherent in tetrapyrrole and corrin biosynthesis have occupied the attention of many laboratories over the last 20 years and although this article is concerned mainly with the author's contributions, key references to independent studies, notably from the laboratories of A. R. Battersby (Cambridge) G. Müller (Stuttgart) and P.M. Jordan (Southampton), which have provided valuable and stimulating results in the arena of heme and corrin biosynthesis, will be found in the text.

THE ENZYMES OF TETRAPYRROLE SYNTHESIS: PBG DEAMINASE AND URO'GEN III SYNTHASE

PBG deaminase (EC4.3.1.8) catalyzes the tetramerization of PBG (**1**) to preuro'gen (hydroxymethylbilane, HMB; **2**)[2,3] which is cyclized with rearrangement to the unsymmetrical uro'gen III (**3**) by uro'gen III synthase[3,4,5] (EC4.2.1.75) (Scheme 1). In the absence of the latter enzyme, preuro'gen (**2**) cyclizes to uro'gen I (**4**), which, as discussed below, turns out to be a substrate for the methylases of the vitamin B_{12} pathway. We have used genetic engineering to construct a plasmid pBG 101 containing the *Escherichia coli hemC* gene[6,7] for deaminase. *E. coli* (TBI) transformed with this plasmid produces deaminase at levels greater than 200 times those of the wild strain[7] thereby allowing access to substantial quantities of enzyme for detailed study of the catalytic mechanism. Previous work with deaminase [8-11] had established that a covalent bond is formed between substrate and enzyme, thus allowing isolation of covalent complexes containing up to 3 PBG units (ES_1-ES_3). Application of ^3H-NMR spectroscopy to the mono PBG adduct (ES-1) revealed a rather broad ^3H chemical shift indicative of covalent bond formation with a cysteine thiol group at the active site.[12] However, with adequate supplies of pure enzyme available from the cloning of *hemC* we were able to show that a novel cofactor, derived from PBG during the biosynthesis of deaminase, is covalently attached to one of the four cysteine residues of the enzyme in the form of a dipyrromethane which, in turn, becomes the site of attachment of the succeeding four moles of substrate during the catalytic cycle. Thus, at pH < 4, deaminase (**5**) rapidly develops a chromophore (λmax 485 nm) diagnostic of a pyrromethene (**6**), whilst reaction with Ehrlich's reagent generates a chromophore typical of a dipyrromethane (λmax 560 nm) changing to 490 nm after 5-10 min. The latter chromophoric interchange was identical with that of the Ehrlich reaction of the synthetic model pyrromethane (**7**) and can be ascribed to the isomerization shown (Scheme 2) for the model system (**7**). Incubation of *E. coli* strain SASX41B (transformed with plasmid pBG 101: *hemA*⁻ requiring ALA for growth) with 5-^{13}C-ALA afforded highly enriched enzyme for NMR studies. At pH8, the enriched carbons of the dipyrromethane (py-CH_2-py) are clearly recognized at 24.0 ppm (py-CH_2py), 26.7 ppm (py-CH_2X), 118.3 ppm (α-free pyrrole) and 129.7 ppm (α-substituted pyrrole) (Scheme 3). Comparison with synthetic models reveals that a shift of 26.7 ppm is in the range expected for an α-thiomethyl pyrrole (py-CH_2 SR). Confirmation of the dipyrromethane (rather than oligo pyrromethane) came from the ^{13}C INADEQUATE spectrum taken at pH12 which reveals the expected coupling only between py-CH_2-py (δ 24.7) and the adjacent substituted pyrrole carbon (δ 128.5 ppm). When the enriched deaminase was studied by INVERSE INEPT spectroscopy, each of the 5 protons attached to ^{13}C-nuclei were observed. A specimen of deaminase was covalently inhibited with the suicide inhibitor [2,11-$^{13}C_2$]-2-bromo PBG (**8**, Scheme 3) to give a CMR spectrum (pH12) consistent only with structure **9**. The site of covalent attachment of substrate (and inhibitor) is therefore the free α-pyrrole carbon at the terminus of the dipyrromethane in the native enzyme, leading to the structural and mechanistic proposal for deaminase shown in Scheme 3. It was also possible to show that 2 moles of PBG are incorporated autocatalytically into the apoenzyme (obtained by cloning into an overexpression vector in *E. coli* which does not make PBG) before folding[13] and that the first (kinetic) encounter of PBG deaminase with substrate involves attachment of PBG (with

Scheme 2





loss of NH_3) to the α-free pyrrole position of the dipyrromethane to form the ES_1 complex[14,15] (Scheme 3). The process is repeated until the "tetra PBG" (ES_4) adduct (**10**) is formed. At this juncture site-specific cleavage of the hexapyrrole chain (at →) releases the azafulvene bilane (**11**) which either becomes the substrate of uro'gen III synthase, or in the absence of the latter enzyme, is stereospecifically hydrated[3] to HMB (**2**) at pH12, or is cyclized chemically to uro'gen I (**4**) at pH ≤ 8. Independent, complementary work from two other laboratories[16,17] has reached similar conclusions regarding the catalytic site. The ^{13}C-labeling defines the number of PBG units (two) attached in a head-to-tail motif to the native enzyme at pH8 and reveals the identity of the nucleophilic group (Cys-SH) which anchors the dipyrromethane (and hence the growing oligopyrrolic chain) to the enzyme. Site specific mutagenesis[7] and chemical cleavage[16,17,18] were employed to determine that Cys-242 is the point of attachment of the cofactor. Thus, replacement[7] of cysteine with serine at residues 99 and 242 (respectively) gave fully active and inactive specimens of the enzyme respectively. The use of the α-carbon of a dipyrromethane unit as the nucleophilic group responsible for oligomerization of 4 moles of PBG (with loss of NH_3 at each successive encounter with an α-free pyrrole) is not only remarkable for the exquisite specificity and control involved, but is, as far as we know, a process unique in the annals of enzymology in that a substrate is used not only once, but twice in the genesis of the active site cofactor!. Even more remarkable is the fact that the apoenzyme is automatically transformed to the active holoenzyme by addition of two substrate PBG units without the intervention of a second enzyme. Crystallization and X-ray diffraction studies of both the native enzyme and several of its genetically altered versions are now in progress.

URO'GEN III SYNTHASE – THE RING D SWITCH

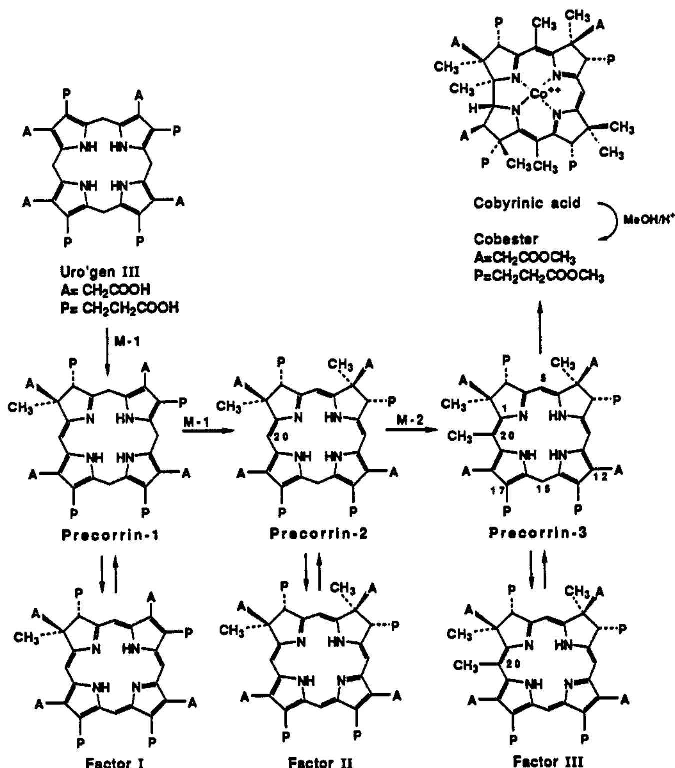
We now turn briefly to the rearranging enzyme Uro'gen III synthase. With the acquisition of substantial quantities of pure Uro'gen III synthase obtained by cloning the genes *hemC* and *D* together[19] and overexpression in *E. coli*, the substrate specificity of the synthase (often called cosynthetase) has been reinvestigated. Ever since its conception[20] by Mathewson and Corwin in 1961, the spiro compound (**13**) (Scheme 3) has been a favorite construct with organic chemists, since both its genesis through α-pyrrolic reactivity and its fragmentation - recombination rationalize the intramolecular formation of Uro'gen III from the linear bilane, preuro'gen (HMB;2). A careful search for the spiro-compound (**13**) was conducted at subzero temperatures in cryosolvent (-24° C; ethylene glycol/buffer) using various ^{13}C-isotopomers of HMB as substrate.[19] Although the synthase reaction could be slowed down to 20 hours (rather than 20 sec.) no signals corresponding to the quaternary carbon (δ ~80 ppm or to the α-pyrrolic methylene groups (▲; δ 35-40 ppm) could be observed. During these studies, however, it was found that AMB (**12**) served as a slow but productive substrate for Uro'gen III synthase at high concentrations (1 mmole in enzyme and substrate concentrations) and that if care is not taken to remove the ammonia liberated from PBG by the action of deaminase, not only is the enzymatic formation of AMB observed[21,22] but in presence of Uro'gen III synthase, the product is again Uro'gen III. This raises the interesting question of whether, under

certain conditions, the true substrate for Uro'gen III synthase is in fact AMB formed at the locus of deaminase by the ammonia released from the substrate. Present research is directed towards the solution of this problem by more sensitive, low temperature spectroscopy.

TEMPORAL RESOLUTION OF THE METHYLATION SEQUENCE

The bioconversion of Uro'gen III to cobyrinic acid is summarized in Scheme. 1 where it is postulated that the oxidation level of the various intermediates is maintained at the same level as that of Uro'gen III. The lability of the reduced isobacteriochlorins, the fact that they are normally isolated in the oxidized form, and the requirement for chemical reduction of Factor I (but not Factors II and III) before incorporation into corrin in cell free systems lent credence to this idea but rigorous proof for the *in vivo* oxidation state of Factors I and II has only recently been obtained as discussed below.[23,24] In order to distinguish between the oxidation levels of the isolated chlorins, isobacteriochlorins, corphins, etc.. and to avoid possible confusion in using the term "Factor" (which has other connotations in B_{12} biochemistry e.g. intrinsic Factor/Factor III) the term precorrin-**n** has been suggested[24] for the actual structures of the biosynthesized intermediates after Uro'gen III where **n** denotes the number of SAM derived methyl groups. Thus, although the names Factors I-III for the isolated species will probably survive(for historical reasons) all of the true intermediates probably have the same oxidation as Uro'gen III *viz.* tetrahydro Factor I (precorrin-1), dihydro Factor II (precorrin-2), and dihydro Factor III (precorrin-3) (Scheme 4). In spite of

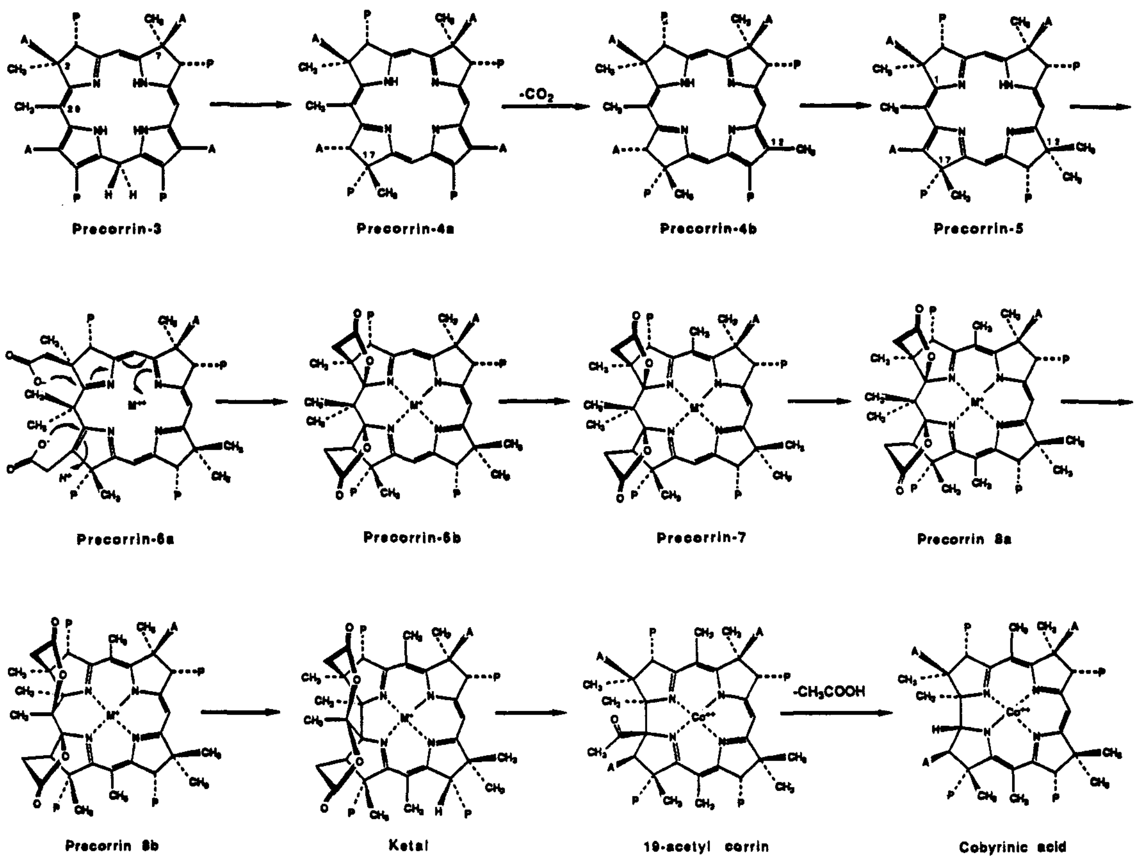
Scheme 4



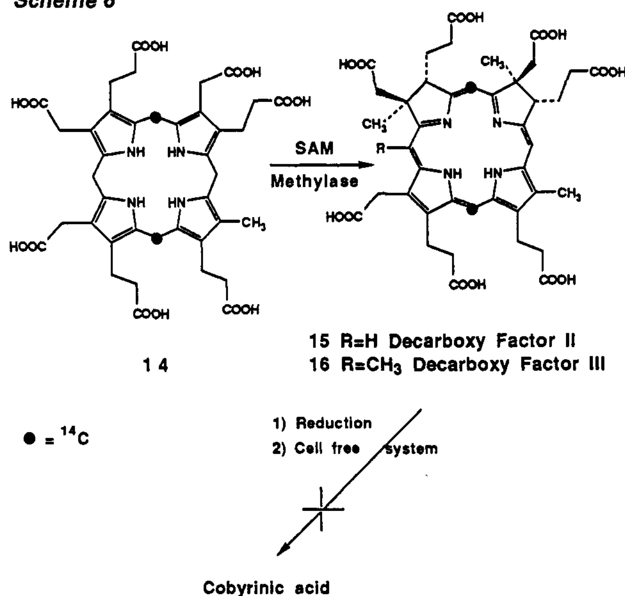
intensive search, no new intermediates containing four or more methyl groups (up to a possible total of eight) have been isolated, but the biochemical conversion of Factor III to cobyrinic acid must involve the following (see Scheme 4), not necessarily in the order indicated: (1) The successive addition of five methyls derived from S-adenosyl methionine (SAM) to *reduced* Factor III (precorrin-3). (2) The contraction of the permethylated macrocycle to corrin. (3) The extrusion of C-20 and its attached methyl group leading to the isolation of acetic acid.[25-28] (4) Decarboxylation of the acetic acid side chain in ring C (C-12). (5) Insertion of Co^{+++} after adjustment of oxidation level from Co^{++}. In order to justify the continuation of the search for such intermediates whose inherent lability to oxygen is predictable, ^{13}C pulse labeling methods were applied to the cell-free system which converts Uro'gen III (3) to precorrin-2 and thence to cobyrinic acid. By this approach it was possible to "read" the biochemical history of the methylation sequence as reflected in the dilution (or enhancement in the reverse experiment) of $^{13}CH_3$ label in the methionine-derived methyl groups of cobyrinic acid after conversion to cobester, whose ^{13}C-NMR spectra has been assigned.[29,30] The pulse experiments defined the complete methylation sequence, beginning from precorrin-2, as C-20 > C-17 > C-12α > C-1 > C-5 > C-15. Methylation at C-20 of precorrin-2 to give precorrin-3 is not recorded in the spectrum of cobester since C-20 is lost on the way to cobyrinic acid, together with the attached methyl group, in the form of acetic acid.[25-28] The sequence C-17 > C-1 > C-12α has also been found in *Clostridium tetanomorphum*[24] and further differentiation between C-5 and C-15 insertion suggests the order C-15 > C-5 in this organism for the last two methylations, i.e. opposite from the *P. shermanii* sequence. A rationale for these events is given in Scheme 5, which takes the following facts into account: (a) the methionine derived methyl group at C-20 of precorrin-3 does not migrate to C-1 and is expelled together with C-20 from a late intermediate (as yet unknown) in the form of acetic acid (b) neither 5,15-norcorrinoids[33] nor descobalto-cobyrinic acid[34] are biochemical precursors of cobyrinic acid (c) regiospecific loss of ^{18}O from [1-^{13}C, 1-$^{18}O_2$]-5-aminolevulinic acid-

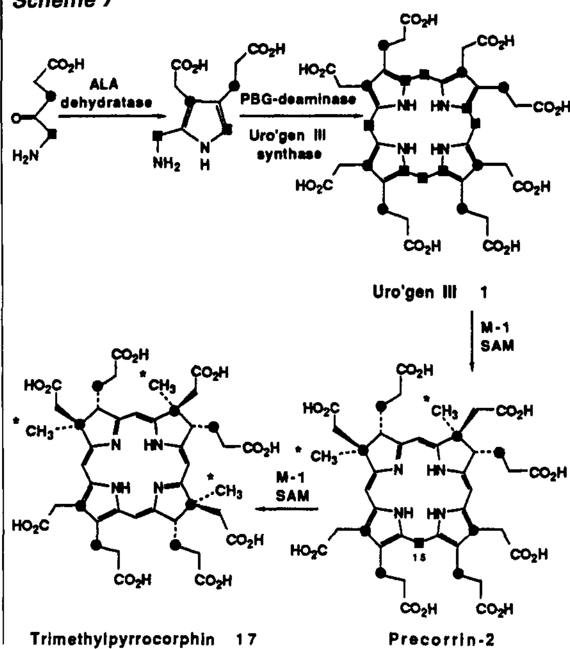
derived cyanocobalamin from the ring A acetate occurs,[35] in accord with the concept of lactone formation, as portrayed in Scheme 5, where precorrin-5 is methylated at C-20 followed by C-20 → C-1 migration and lactonization. If the latter mechanism is operative, the C-20 → C-1 migration must be stereospecific, since precorrin-3 labeled at C-20 with $^{13}CH_3$ is transformed to cobyrinic acid with complete loss of label, a less attractive

Scheme 5



alternative being direct methylation at C-1. It has also proved possible to define the point in the biosynthetic sequence where ring C-decarboxylation occurs, using synthetic [5,15-$^{14}C_2$]-12-decarboxylated uro'gen III (**14**) as a substrate for the non-specific methylases of *P. shermanii* to prepare the 12-methyl analogs (**15**) and (**16**) of factors II and III respectively (Scheme 6). Reduction and incubation of these possible intermediates with the corrin-synthesizing cell-free system in the presence of SAM afforded (after esterification) samples of cobester whose specific activities were compared in a control experiment with those of cobester derived from [^{14}C]-precorrin-**2** and from a mixture of [^{3}H]-precorrin-**3** and [^{14}C]-precorrin-**2**. It was found[32] that these ring C-decarboxylated analogs are not substrates for the enzymes of corrin biosynthesis (Scheme 6), leading to the conclusions that (a) in normal biosynthesis, precorrin-**3** is not the intermediate which is decarboxylated (b) decarboxylation occurs at some stage after the fourth methylation (at C-17) and by mechanistic analogy, before the fifth methylation at C-12. Hence, two pyrrocorphin intermediates *viz.* precorrins **4a**, **4b** should intervene between precorrins-**3** and -**5** i.e. precorrin-**3** is C-methylated at C-17 to give precorrin-**4a** followed by decarboxylation (→ **4b**) and subsequent C-methylations at C-12α, C-1, C-5, C-15, in that order as suggested in Scheme 5. Although the methylation sequence differs in the last two insertions from that reported for *C. tetanomorphum*,[24] consensus over the timing of decarboxylation in ring C has been reached since identical conclusions have been reported using *Pseudomonas denitrificans*.[36,37] We now return to the construction of a working hypothesis for corrin biosynthesis. The formulations precorrins **6b**, **7**, **8a**, **8b** take into account the idea of lactone formation using rings A and D acetate side chains. The migration C-20 → C-1 could be acid or metal ion catalyzed and the resultant C-20 carbonium ion quenched with external hydroxide or by the internal equivalent from the carboxylate anion of the C-2 acetate in ring A (precorrin **8a** → **8b**) as suggested by the results of labeling with ^{18}O discussed above.[35] In any event, the resultant dihydrocorphinol-bislactone precorrin-**8b**, is poised to undergo the biochemical counterpart of Eschenmoser ring contraction[38] to the 19-acetyl corrin. Before this happens we suggest that the final methyl groups are added at C-5 (precorrin-**7**), then C-15 (precorrin-**8a**) to take account of the non-incorporation of the 5, 15-norcorrinoids.[33] The resultant precorrin-**8b** (most probably with cobalt in place) then contracts to 19-acetyl corrin which, by loss of acetic acid as an irreversible step involving a deuterium isotope effect,[33,39-42] leads to cobyrinic acid. Cobalt insertion must precede methylation at C_5 and C_{15} since hydrogenocobyrinic acid does not insert cobalt enzymatically[34]. The valency change Co^{++} → Co^{+++} during or after cobalt insertion has so far received no explanation.

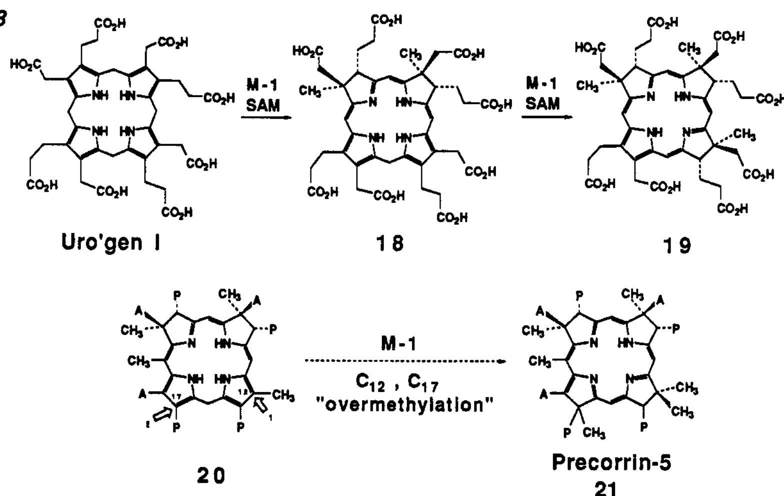




THE METHYL TRANSFEREASES

The first of the methylase enzymes catalyzes the sequential formation of Factors I and II and has been named S-adenosyl methionine Uro'gen III methyl transferase (SUMT). SUMT was first partially purified from *P. shermanii* by G. Müller[43] and recently has been overexpressed in *Pseudomonas denitrificans*.[44] In *E. coli* it was found that the *CysG* gene encodes Uro'gen III methylase (M-1) as part of the synthetic pathway to siroheme, the cofactor for sulfite reductase, and overproduction was achieved by the appropriate genetic engineering.[45] Although, SUMT and M-1 appear to perform the same task, it has beeen found that their substrate specificities differ. Thus, it has been possible to study in detail the reaction catalyzed by M-1 directly using NMR spectroscopy and to provide rigorous proof that the structure of precorrin-2 is that of the dipyrrocorphin tautomer of dihydro-Factor II (dihydrosirohydrochlorin). Uro'gen III (enriched from [^{13}C-5-ALA] at the positions shown in Scheme 7) was incubated with M-1 and [$^{13}CH_3$]-SAM. The resultant spectrum of the precorrin-2 revealed an sp^3 enriched carbon at C-15, thereby locating the reduced center. By using a different set of ^{13}C-labels (• from ^{13}C-3 ALA) and [$^{13}CH_3$]-SAM) the sp^2 carbons at C_{12} and C_{18} were located as well as the sp^3 centers coupled to the pendant ^{13}C-methyl groups at C_2 and C_7. This result confirms an earlier NMR analysis[23] of precorrin-2 isolated by anaerobic purification of the methyl ester, and shows that no further tautomerism takes place during the latter procedure. The two sets of experiments mutually reinforce the postulate that precorrins-**1**, -**2**, and -**3** all exist as hexahydroporphinoids and recent labeling experiments[46] have provided good evidence that precorrin-**1** is discharged from the methylating enzyme (SUMT) as the species with the structure shown (or an isomer).

However, prolonged incubation (2 hr.) of Uro'gen III with M-1 provided a surprising result for the UV and NMR changed dramatically from that of precorrin-2 (a dipyrrocorphin) to the chromophore of a pyrrocorphin, hitherto only known only as a synthetic tautomer of hexahydroporphyrin. At first sight, this event seemed to signal a further tautomerism of a dipyrrocorphin to a pyrrocorphin catalyzed by the enzyme but when $^{13}CH_3$-SAM was added to the incubation, it was found that a <u>third</u> methyl group signal appeared in the 19-21 ppm region of the NMR spectrum. When Uro'gen III was provided with the ^{13}C labels (•) (as shown in Scheme 7) 3 pairs of doublets appeared in the sp^3 region (δ 50-55 ppm) of the pyrrocorphin product. The necessary pulse labeling experiments together with appropriate FAB-MS data finally led to the structural proposal (**17**)[47,48] for the novel trimethyl pyrrocorphin produced by "overmethylation" of the normal substrate, uro'gen III, in presence of high concentration of enzyme. Thus M-1 has been recruited to insert a ring C methyl and synthesizes the long sought "natural" chromophore corresponding to that of the postulated precorrin-4 although in this case the regiospecificity is altered from ring D to ring C. This lack of specificity on the part of M-1 was further exploited to synthesize a range of "unnatural" isobacteriochlorins and pyrrocorphins based on isomers of Uro'gen III. Thus, Uro'gen I produces 3 methylated products corresponding to precorrin I, precorrin-**2** (**18**), and the type-I pyrrocorphin (**19**) (Scheme 8). Uro'gens II and IV can also serve as substrates for M-1 but not for SUMT! These compounds are reminiscent of a series of tetramethyl type I corphinoids, Factors S_1-S_4 isolated from *P. shermanii*[49,50] which occur as their zinc complexes. When uro'gen I was incubated with SUMT,[51] isolation of Factor II of the type I (Sirohydrochlorin I) family revealed a lack of specificity for this methyltransferase also, although in the latter studies no pyrrocorphins were observed. This may reflect a control mechanism in the *P. denitrificans* enzyme (SUMT) which does not "overmethylate" precorrin-2 as is found for the *E. coli* M-1 whose physiological function is to manufacture sirohydrochlorin. The fact that *E. coli* does not synthesize B_{12} could reflect an evolutionary process in which the C-methylation machinery has been retained, but is only required to insert the C-2 and C-7 methyl groups. The sites of C-methylation in both the type I and III series are also reminiscent of the biomimetic C-methylation of the



hexahydroporphyrins discovered by Eschenmoser[52] and the regiospecificity is in accord with the principles adumbrated[53] for the stabilizing effect of a vinylogous ketimine system. In principle the methylases of the B_{12} pathway, which synthesize both natural and unnatural pyrrocorphins and corphins can be harnessed to prepare several of the missing intermediates of the biosynthetic pathway, e.g. (**20**) → (**21**) (Scheme 8). It is of note that the instability towards oxygen rationalizes our earlier inability to isolate any new intermediates under aerobic conditions (> 5 ppm O_2).

EVOLUTIONARY ASPECTS AND FURTHER OUTLOOK

Genetic mapping of the loci of the B_{12}-synthesizing enzymes has been reported for *Pseudomonas denitrificans*.[44] This complements a most interesting study on the genetics of *Salmonella typhimurium* which cannot make B_{12} when grown aerobically.[54] A mutant requiring methionine, cobinamide or cyanocobalamin when grown anaerobically produces B_{12} *de novo* thus leading to the isolation of other mutants blocked in B_{12} synthesis including one which cannot make Factor II required for siroheme production. All of the cobalamin mutations lie close together on the chromosome and a cluster of several methyl transferases maps at 42 min. Thus rapid progress can be expected in the isolation of the remaining biosynthetic enzymes from *Salmonella*. Until quite recently it had been assumed that the Shemin pathway (glycine-succinate) to ALA was ubiquitous in bacterial production of porphyrins and corrins. However it is now clear that in many archaebacteria (e.g. *Methanobacterium thermoautotrophicum*,[55] *Clostridium thermoaceticum*[56-57]) the C_5 pathway from glutamate is followed. Phylogenetically the C_5 route is conserved in higher plants, and it appears from recent work[58] that *hemA* of *E. coli* (and perhaps of *S. typhimurium*) encodes the enzyme for the glutamate → ALA conversion, i.e. the C_5 pathway is much more common than had been realized. In *C. thermoaceticum* we have shown[57] that the B_{12} produced by this thermophilic archaebacterium is synthesized from ALA produced from glutamate. Although *E. coli* does not seem to be able to synthesize B_{12}, the enzyme M-1, a close relative of SUMT, is expressed as part of the genetic machinery (*CysG*) for making siroheme, as discussed above. Eschenmoser has speculated that corrinoids resembling B_{12} could have arisen by prebiotic polymerization of hydrogen cyanide and has developed an impressive array of chemical models[53] to support this hypothesis, including ring contraction of porphyrinoids to acetyl corrins, deacetylation and the C-methylation chemistry discussed earlier, which provide working hypotheses for the corresponding biochemical sequences. A primitive form of corrin stabilized by hydrogen,[53] rather than by methyl substitution may indeed have existed > 4 x 10^9 years ago, before the origin of life[59] or the genetic code[60] and could have formed the original "imprint" necessary for the evolution of enzymes which later mediated the insertion of methyl groups to provide a more robust form of B_{12}. Since B_{12} is found in primitive anaerobes and requires no oxidative process in its biogenesis (unlike the routes to heme and chlorophyll which are oxidative) an approximate dating of B_{12} synthesis would be 2.7 - 3.5 x 10^9 years, i.e. after DNA but before oxygen-requiring metabolism.[61] With the genetic mapping of B_{12} biosynthesis now under way, it should at last be possible to discover the remaining intermediates beyond-precorrin-3, together with the enzymes which mediate the methyl transfers, decarboxylation, ring contraction, deacetylation, and cobalt insertion. It is anticipated that the powerful combination of molecular biology and NMR spectroscopy which has been essential in solving the problems in B_{12} synthesis posed by the assembly and intermediacy of Uro'gen III and the subsequent C-methylations leading to precorrin-3, will again be vital to the solution of those enigmas still to be unraveled in the fascinating saga of B_{12} biosynthesis.

Acknowledgements: The work described in this lecture has been carried out by an enthusiastic group of young colleagues whose names are mentioned in the references. Financial support over the last 20 years has been generously provided by the National Institutes of Health, National Science Foundation, and the Robert A Welch Foundation. It is a special pleasure to thank Professors Gerhard Müller (Stuttgart) and Peter Jordan (London) for their continued stimulating collaboration.

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