conformation. Finally, converging lines of evidence indicate that the influence of absolute configuration on conformational dissymmetry requires that the effects of chirality and conformation be dealt with together in the analysis of the stereostructure–activity relationship of opioid ligands.

Biosynthesis of Vitamin B_{12}. In Search of the Porphyrin–Corrin Connection

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The history of corrin biosynthesis spans the relatively brief passage of 12 years, yet even in this period several distinct phases can be clearly recognized. By 1968, when our researches were inititated, the origin of the corrin nucleus had been defined, since the building blocks δ-aminolevulinic acid (ALA; **1**), porphobilinogen (**2**), and methionine were clearly involved[1,2] in the makeup of cobyrinic acid (**3a**), the "simplest" of the cobalt-containing corrins. To this key heptacarboxylic acid is added the nucleotide loop, whose components are derived from threonine, guanosine triphosphate, and the unusual base, dimethylbenzimidazole, and the primary amide functions.[3] The major technical problem in obtaining rigorous evidence of nonrandom incorporation of regiospecifically labeled carbon, however, was the lack of degradative chemistry of vitamin B_{12}, a void which had been created by solution of the structural problem by x-ray diffraction analysis.

In this Account we shall discuss both experimental and theoretical aspects developed recently in our laboratory and elsewhere which are attempting to solve several fascinating mechanistic problems in the unknown territory between the reduced type III porphyrin, uro'gen III (**4**), and the first fully corrinoid intermediate cobyrinic acid (**3a**). We begin our discussion with an account of the experiments designed to establish both the number and the mode of insertion of the methionine-derived methyl groups in the corrin nucleus as a background for the development of the mechanistic proposals for the uro'gen–corrin transformation.

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,[4] the six remaining methyl groups arising from methionine. Support for these ideas came from Kuhn–Roth oxidation of corrinoids labeled with [5-^{14}C]- and [2,3-^{14}C]ALA and [^{14}C-methyl]methionine.[1]

When the problem was reexamined using ^{13}C Fourier transform NMR, 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. 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.[5] 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** (Figure 2) was indeed obtained. Such an array is in harmony with current ideas on the mechanism of type III uro'gen formation, and this result was simultaneously discovered in Shemin's laboratory[6] in 1972. 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 was demonstrated to be methionine. Inspection of the integrated spectrum after feeding [^{13}C-methyl]methionine left no doubt that seven methionine methyl groups have been incorporated (see Figure 2). This result, which is of considerable significance for the mechanism of corrin synthesis, was to receive confirmation from the work of the Cambridge group,[7] and extension of these studies led to the ab-

A. Ian Scott was born in Scotland and received B.Sc., Ph.D., and D.Sc. degrees from Glasgow University, where he taught from 1957 to 1962, before holding professorships at the University of British Columbia, University of Sussex, and Yale University. In 1977, he was appointed a Distinguished Professor at Texas A&M University, where he continues his research on natural product biosynthesis in cell-free systems from fungi, bacteria, and plant tissue cultures. Professor Scott was recipient of the 1976 Ernest Guenther Award in the Chemistry of Essential Oils and Related Products sponsored by Fritzsche Dodge & Olcott Inc., and this Account is based on his award address. The author wishes to dedicate this work to the celebration of the birthday of another, but much younger, producer of the vitamin, R. B. Woodward.

(1) D. Shemin and R. C. Bray, *Ann. N.Y. Acad Sci.*, **112**, 615 (1964).
(2) L. Bogorad, *Ann. N.Y. Acad. Sci.*, **104**, 676 (1963).
(3) K. Bernhauer, F. Wagner, H. Michna, P. Rapp, and H. Vogelmann, *Hoppe-Seyler's Z. Physiol. Chem.*, **349**, 1297 (1968).
(4) J. H. Mathewson and A. H. Corwin, *J. Am. Chem. Soc.*, **83**, 135 (1961).
(5) D. Doddrell and A. Allerhand, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 1083 (1971).
(6) C. E. Brown, J. J. Katz, and D. Shemin, *Proc. Nat. Acad. Sci. U.S.A.*, **69**, 2585 (1972).
(7) A. R. Battersby, M. Ihara, E. McDonald, and J. R. Stephenson, *J. Chem. Soc., Chem. Commun.*, 404 (1973).

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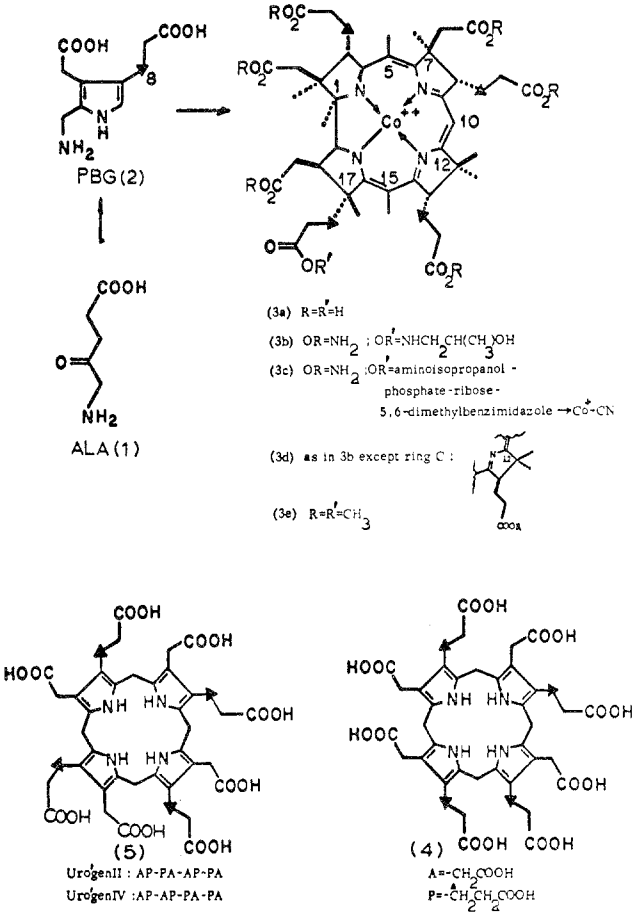

Figure 1.

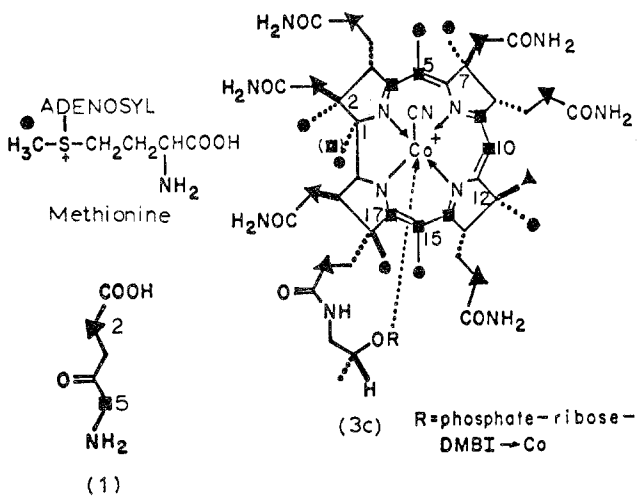

Figure 2.

solute configuration of the methylation process in ring C.[8-10] At this stage of the investigation (1972) the technique of cell-free corrin biosynthesis with *P. shermanii* was developed independently at Yale[11] and Cambridge.[12] The supernatant fraction (100000*g*) with

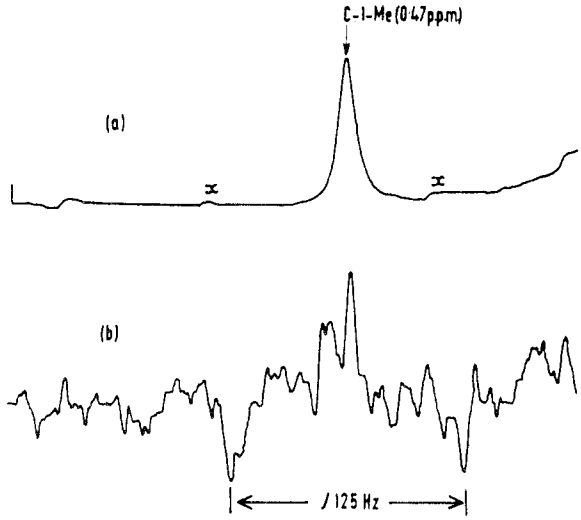

Figure 3.

appropriate additives[11,12] is capable of transforming ALA, methionine, PBG, and, as described below, uro'gen III to cobyrinic acid. A similar system from *Clostridium tetanomorphum*, which makes cobyrinic acid, but not heme, was developed simultaneously by Müller.[13]

Concerning the Fate of the Methyl Group Protons

Previous independent studies at Yale[14a,b] and at Cambridge[10] indicated that virtually intact methyl transfer from *S*-adenosylmethionine was taking place during corrin biosynthesis, and it could be shown[10,15] that this was indeed the case at C-7 (ring B) and C-12α (ring C). However, mass spectrometric[10,15] and $^{3}H/^{14}C$ results[14b] indicated that some minor exchange, possible at C-5 or C-15 or even at C_1, might be occurring. In order to achieve maximum sensitivity in a double labeled ($^{13}C^{2}H$) experiment, a sample (0.36 g) of [$^{2}H_3$,^{13}C-methyl]methionine (90% in ^{13}C; 98% in ^{2}H) was administered to resting whole cells of *P. shermanii* and the resultant purified cyanocobalamin (32 mg, **3c**) examined by the following technique.

First, ^{13}C and ^{2}H FT-NMR data indicated that (as shown in a simultaneous experiment with [^{14}C-methyl]methionine) good incorporation (20–25%) of the doubly labeled methionine had been achieved, with equal distribution of ^{13}C label to the seven "extra" methyl groups (at C-1, C-2, C-5, C-7, C-12α, C-15, and C-17) of **3**. As shown in Figure 3a the undecoupled ^{1}H FT spectrum of the enriched sample does not reveal any unusual ^{1}H–^{13}C coupling of the methyl signal centered at 0.47 ppm, which has been unambiguously assigned to C-1,[16,17] but only the spinning side bands (s,s in Figure 3a). However, quantitative confirmation that ^{1}H (^{13}C) coupling was present and *not* due to any ex-

(8) See A. I. Scott, C. A. Townsend, and R. J. Cushley, *J. Am. Chem. Soc.*, **95**, 5759 (1973), for a discussion of the use of *neo*-cobalamin for this assignment.
(9) H. Stoeckli-Evans, E. Edmond, and D. Crowfoot Hodgkin, *J. Chem. Soc. Perkin Trans. 2*, 605 (1972); R. Bonnett, J. M. Godfrey, V. B. Math, P. M. Scopes, and R. N. Thomas, *J. Chem. Soc., Perkin Trans. 1*, 252 (1973).
(10) A. R. Battersby, M. Ihara, E. McDonald, J. R. Stephenson, and B. T. Golding, *J. Chem. Soc., Chem. Commun.*, 458 (1974).
(11) A. I. Scott, B. Yagen, and E. Lee, *J. Am. Chem. Soc.*, **95**, 5761 (1973).
(12) A. R. Battersby, European Symposium in Bioorganic Chemistry, Gregynog, Wales, May 1973; see also ref 30.
(13) H. Dauner and G. Müller, *Hoppe-Seyler's Z. Physiol. Chem.*, **356**, 1353 (1975).
(14) (a) E. Lee, Ph.D. Thesis, Yale University, 1974; (b) B. Yagen, unpublished work, Yale University, 1973; (c) A. I. Scott, M. Kajiwara, T. Takahashi, I. M. Armitage, P. Demou, and D. Petrocine, *J. Chem. Soc., Chem. Commun.*, 544 (1976).
(15) (a) M. Imfeld, C. A. Townsend, and D. Arigoni, *J. Chem. Soc., Chem. Commun.*, 541 (1976); (b) A. Battersby, R. Hollenstein, E. McDonald, and D. C. Williams, *ibid.*, 543 (1976).
(16) C. E. Brown, D. Shemin, and J. J. Katz, *J. Biol. Chem.*, **248**, 8015 (1973).
(17) O. D. Hensens, H. A. O. Hill, J. Thornton, A. M. Turner, and R. J. P. Williams, *Philos. Trans. R. Soc. London, Ser. B*, **273** 353 (1976).

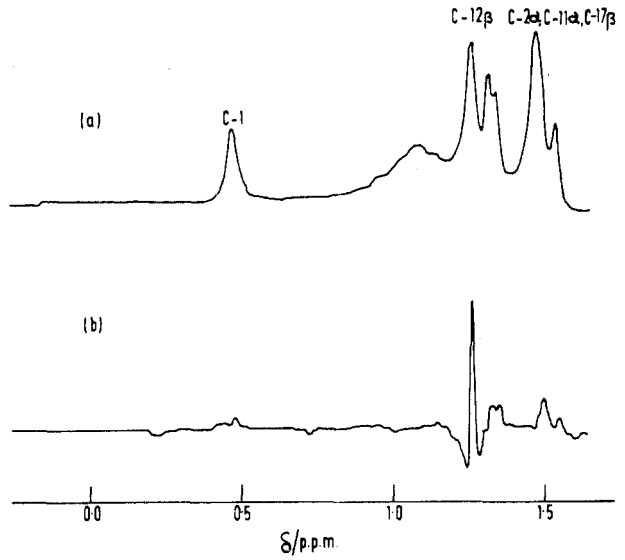

Figure 4.

change of the enriched C-1-methyl carbon ($\rightarrow {}^{13}CD_2H$, ${}^{13}CH_3$) was forthcoming by simultaneous subtraction of the *^{13}C-decoupled* spectrum to give the difference spectrum shown in Figure 3b. The latter technique reveals that: (a) all of the observable satellite peak intensity in Figure 3 is due to spinning side bands; (b) the ratio of ^{13}C satellite to ^{1}H resonance intensity at the C_1-methyl (1:200 for each satellite) (Figure 3b) is within experimental error (10%) identical with natural abundance (1.1%) ($J_{^1H-^{13}C} \approx 125$ Hz); (c) the analysis of the difference spectrum corresponding to the chemical shifts for the C-2α, C-12α, and C-17β methyls[17] (Figure 4b) shows no exchange of these $^{13}CD_3$ groups (natural abundance satellites only), thus confirming the earlier conclusion;[10] (d) a strong three-bond coupling (J = 4.0 Hz) centered at 1.26 ppm due to ^{13}C-(12α)–$^{1}H(12\beta CH_3)$ is clearly evident in Figure 4b, corresponding to a 20-fold enhancement of the ^{13}C-12α methyl signal which also is an internal standard. These results impose strict requirements on the mechanism of corrin synthesis, particularly with respect to *retention* of the C-1 methyl protons, a result achieved simultaneously by independent work at Zurich[15a] and Cambridge.[15b]

After considering plausible mechanisms for connecting PBG[18] with the corrin structure we decided to test the attractive idea particularly emphasized by Burnham[19,20] that the cobalt, magnesium, and iron pathways diverge *after* the formation of uro'gen III (**4**). The mechanism of the uro'gen III biosynthesis from PBG has been the subject of much debate, and most of the pertinent data regarding the switching of one of the PBG units (ring D) during the action of uro'gen I synthetase in the presence of uro'gen III cosynthetase has been summarized in two excellent reviews.[21,22a] Strong evidence implicating the head to head encounter

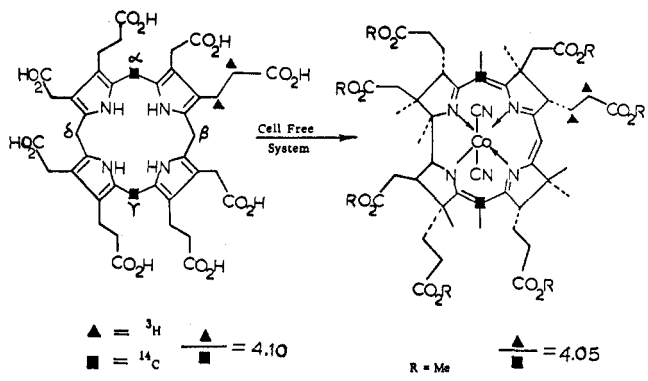

Figure 5.

of two PBG molecules has been presented,[21,23] but an alternative explanation involving the linear bilane derived from head to tail condensation of four PBG molecules is favored by the Cambridge group in a recent series of experiments.[22b] In this author's admittedly biased opinion, further experimental work is still needed by both the head–head and the head–tail schools before the question is finally resolved. 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 bypasses the uro'gens 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 could be discarded if proof for the intervention of uro'gen III could be obtained. The situation was complicated, however, by reports of work with *P. shermanii* cells which indicated that virtually no specific incorporation into vitamin B_{12} of enzymically or chemically synthesized [^{14}C]uro'gen could be observed.[24,25a] We now know 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 obtained in 1971 in our laboratory.

These early results, together with the enrichment data for [^{13}C]ALA (see below), confirmed positive incorporation using carbon-14 and also provided unequivocal evidence for the location of the label. However, although the sequence PBG → uro'gen III → vitamin B_{12} became, in mid-1972, even more attractive, it was recognized that the symmetrical nature of the labeling pattern of uro'gen III used in the first set of experiments left open the possibility of a fragmentation–recombination mechanism. In order to resolve this question of vital importance for the mechanism of corrin biosynthesis we next completed the regiospecific synthesis of a set of uro'gens whose patterns of enrichment with both samples and radioisotopes were designed to provide unambiguous probes for intact biotransformation and for the nature of the overall mechanism connecting the uro'gen and corrin structures.

(18) S. Schwartz, K. Ikeda, I. M. Miller, and C. J. Watson, *Science*, **129** 40 (1959).
(19) B. F. Burnham and R. A. Plane, *Biochem. J.*, **98**, 13c (1966).
(20) B. F. Burnham in "Metabolic Pathways", Vol. 3, 3rd ed, D. M. Greenburg, Ed., Academic Press, New York, N.Y., 1969, Chapter 18.
(21) B. Frydman and R. B. Frydman, *Acc. Chem. Res.*, **8**, 201 (1975).
(22) (a) A. R. Battersby and E. McDonald in "Falk's Porphyrins and Metalloporphyrins", 2nd ed, K. M. Smith, Ed., Elsevier, Amsterdam, 1975; (b) A. R. Battersby, E. McDonald, D. C. Williams, and H. K. W. Wurziger, *J. Chem. Soc., Chem. Commun.*, 113 (1977), and accompanying communications, 115, 117 (1977).
(23) A. I. Scott, K. S. Ho, M. Kajiwara, and T. Takahashi, *J. Am. Chem. Soc.*, **98**, 1589 (1976).
(24) G. Müller and W. Dieterle, *Hoppe-Seyler's Z. Physiol. Chem.*, **352** 143 (1971).
(25) (a) B. Franck, D. Gantz, and F. Hüper, *Angew. Chem., Int. Ed. Engl.*, **11**, 421 (1972); (b) B. Franck, D. Gantz, and F. Hüper, *ibid.*, **11**, 420 (1972).

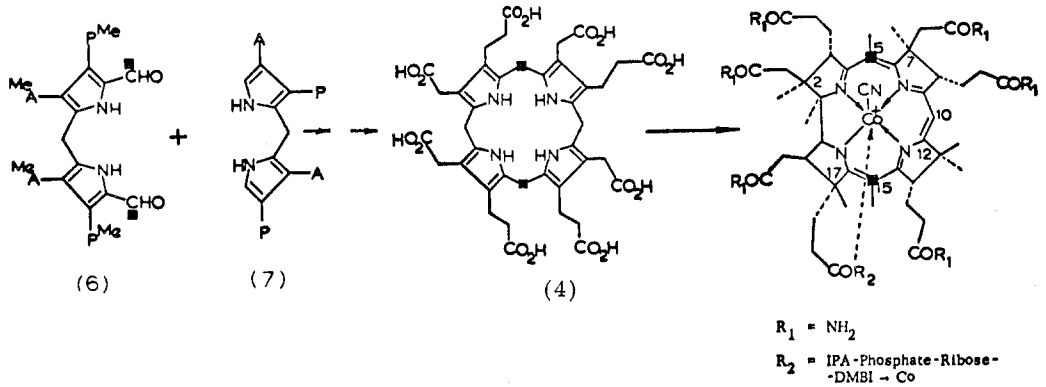

Figure 6.

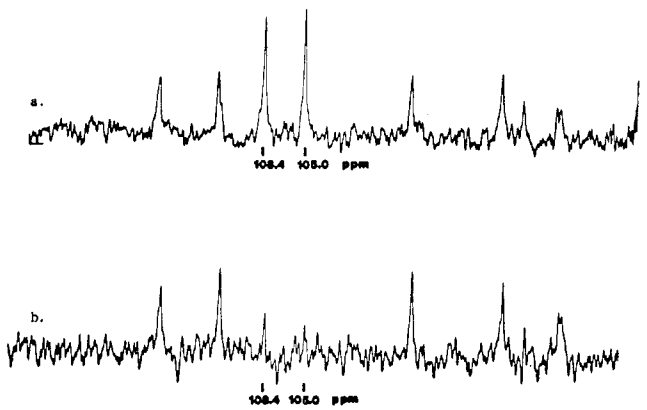

Figure 7.

The regiospecific synthesis of [5,15-$^{14}C_2$]uro'gen III and of ring B propionic acid [$^3H_2$]uro'gen III were carried out by the procedures of MacDonald[26] and Franck,[25b] modified where appropriate for the introduction of radioisotope. Incubation of the doubly labeled uro'gen ($^3H/^{14}C$, 4.10; Figure 5) 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 labeled substrate, to a profound change in the tritium–carbon ratio. To strengthen the evidence and at the same time locate the site of label in the corrin, a specimen of [5,15-$^{13}C_2$]uro'gen III was prepared via condensation of the dipyrromethanedialdehyde (**6**) and dipyrromethane (**7**), with introduction of ^{13}C from dimethylformamide (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 this "north–south" labeled substrate to resting whole cells of *P. shermanii* gave pure cyanocobalamin whose FT-^{13}C NMR spectrum on comparison with the natural abundance spectrum taken under identical conditions revealed (Figure 7) enhancement (4.5% specific incorporation) at only two resonances in the sp^2 region, viz., at 105.0 and 108.4 ppm downfield from Me_4Si.[27] These signals had previously been assigned to C-15 and C-5, respectively, both by the correlations of Allerhand[5] and

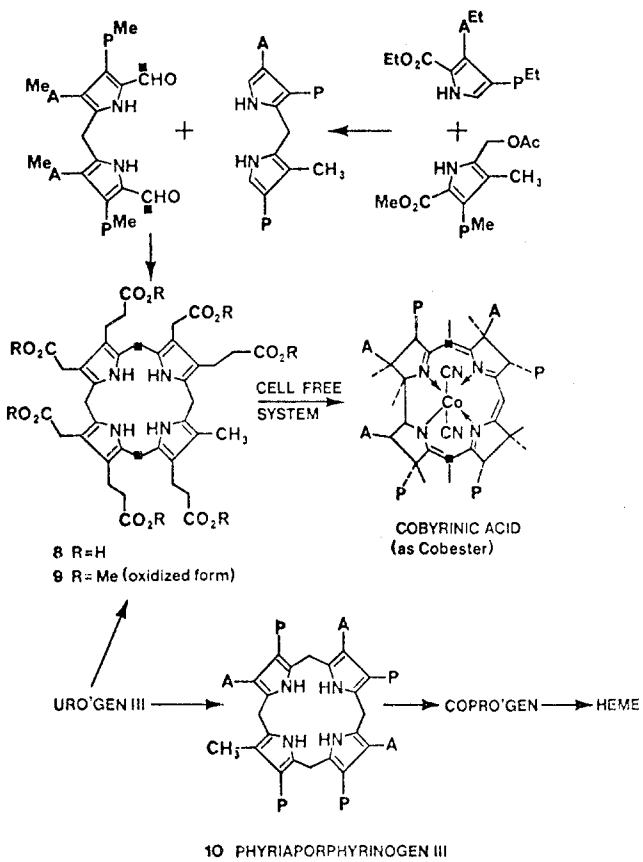

Figure 8.

by biosynthetic labeling.[28,29] This experiment revealed the intact incorporation of doubly labeled uro'gen III and unambiguously located the labeled sites according to Figure 5 where C■ now denotes both ^{14}C and ^{13}C.[29] Battersby,[30] using a different ^{13}C-labeling pattern ($^{13}CH_2CO_2H$ in ring C), reached the same conclusion. The methyl label used in the Cambridge experiment served to locate the C-12β methyl group in ring C.

Next, in order to test a hypothesis[31] that decarboxylation of the acetic acid side chain in ring C takes place at the uro'gen level, a regiospecific total synthesis of the

(26) E. J. Tarlton, S. F. MacDonald, and E. Baltazzi, *J. Am. Chem. Soc.*, **82**, 4389 (1960).

(27) Previous chemical shifts were cited downfield from HMDS, using a Bruker 90 instrument and the FT system described earlier. A complete set of correlated chemical shift values for the corrin series is now available (G. H. Temme III, D. Petrocine, and A. I. Scott, manuscript in preparation).

(28) A. I. Scott, C. A. Townsend, K. Okada, M. Kajiwara, P. J. Whitman, and R. J. Cushley, *J. Am. Chem. Soc.*, **94**, 8267, 8269 (1972); A. I. Scott, C. A. Townsend, K. Okada, M. Kajiwara, R. J. Cushley, and P. J. Whitman, *ibid.*, **96**, 8069 (1974).

(29) A. I. Scott, B. Yagen, N. Georgopapadakou, K. S. Ho, S. Klioze, E. Lee, S. L. Lee, G. H. Temme III, C. A. Townsend, and I. M. Armitage, *J. Am. Chem. Soc.*, **97**, 2548 (1975); see **98**, 2371 (1976), for a correction.

(30) A. R. Battersby, M. Ihara, E. McDonald, F. Satoh, and D. C. Williams, *J. Chem. Soc., Chem. Commun.*, 436 (1975).

(31) A. I. Scott, E. Lee, and C. A. Townsend, *Bioorg. Chem.*, **3**, 229 (1974); A. I. Scott, *Tetrahedron*, **31**, 2639 (1975).

Table I
Conversion of Uro'gen Substrates to Corrin, Formaldehyde, and CO_2[a]

Expt	Substrate	CO_2-hyamine Disintegrations/min	Disintegrations/min	CH_2O-dimedone adduct % incorporation	Disintegrations/min	Cobester % incorporation
1	[5,14,20-$^{14}C_3$]Uro'gen III	470	2.29×10^5	3.81	2.67×10^5	3.98
2[b]	[5,15,20-$^{14}C_3$]Uro'gen III	667	732	0.012	246	0.0037
3	[5,15,20-$^{14}C_3$]Hepta-carboxylic uro'gen	485	3476	0.17	19250	0.21
4[b]	[5,15,20-$^{14}C_3$]Hepta-carboxylic uro'gen	700	135	0.006	574	0.0062

[a] M. Kajiwara, K. S. Ho, H. Klein, A. I. Scott, A. Gossauer, J. Engel, E. Neumann, and H. Ziech, *Bioorg. Chem.* 6, in press. [b] Boiled enzyme.

type III heptacarboxylic acid (8) was carried out as summarized in Figure 8. The melting point (238–240 °C) of the heptamethyl ester **9** was in excellent agreement with that reported by Battersby et al.[32] for this isomer (prepared by an analogous route), and spectroscopic and mixed melting point comparison confirmed their identity. The synthesis was repeated using [^{14}C]dimethylformamide, and the resultant [5,15-$^{14}C_2$]heptacarboxylic uro'gen III incubated with the cell-free system to afford (after crystallization to constant activity) cobester (**3e**) (0.52% incorporation). This experiment provided an indication that the heptaacid 8 is an intermediate in corrin biosynthesis (Figure 8) 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[33] (**10**) is considered to be the obligatory biosynthetic precursor for coprogen and heme, the new isomer (8) could represent the branchpoint at which the heme and corrin pathways, having shared a common route from glycine and succinate to uro'gen III, diverge. Bearing in mind that a rather nonspecific metabolic grid operates between cobyrinic acid (**3a**) and vitamin B_{12} (**3c**) it was necessary to obtain proof that the incorporation of the heptaacid 8 was a specific biotransformation. Before carrying out the requisite synthesis of a multiply labeled version of 8, we next addressed ourselves to the problem of the "disappearing" C-20 or *δ-meso*-methylene group. This information becomes vital not only for the mechanistic argument but also as a test of the role of the heptaacid 8[29] and an understanding of the nature of the putative intermediates, the secocorrins.

On the Identity of the "Disappearing C_1 Unit"

In principle, the "C_1 unit" derived from the western (C-20) position could depart from the system at any oxidation level between methanol and carbon dioxide. However, many attempts in this laboratory[34] to obtain a stoichiometric relationship between capture of such a C_1 unit and cobyrinic acid synthesis in a cell-free system were frustrated by the well-known in vitro release and recapture of formaldehyde during the equilibration of the types I–IV isomers of the reduced (uro'gen) series at pH 4. Thus the demonstration that "formaldehyde" or its biochemical equivalent is formed enzymically becomes dependent on the development of

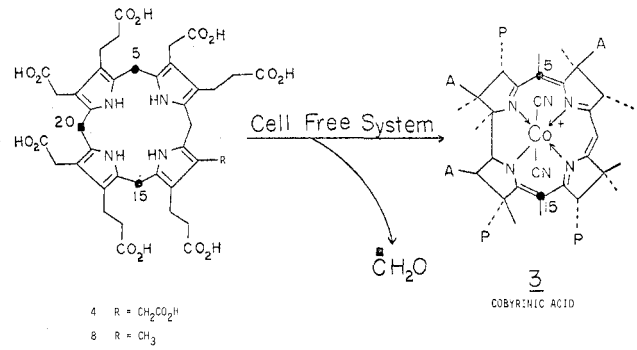

Figure 9. (a) Proton noise decoupled ^{13}C FT-NMR spectrum of [^{13}C]uro'gen III enriched cyanocobalamin (D_2O; 4K points) and assigned labeling patterns. (b) Proton noise decoupled ^{13}C FT-NMR spectrum of natural-abundance cyanocobalamin (D_2O; 4 K points).

an incubation/assay system which is virtually devoid of a chemical blank. Using totally synthetic [5,15,20-$^{14}C_3$]uro'gen III (Figure 9) we have devised a reproducible protocol in which the unconsumed uro'gen III in an aliquot of the postincubation mixture is oxidized to uroporphyrin III and the formaldehyde–dimedone adduct isolated subsequently from this preparation at pH 4. A second aliquot is analyzed in the usual way for cobyrinic acid synthesis (conversion to cobester 3, crystallization to constant activity). The results of these experiments are shown in Table I where it can be seen (experiment 1) that not only do the formaldehyde–dimedone and cobester conversions correlate well but, most importantly, the removal of enzyme (experiment 2) generates a negligible amount of "C_1 unit". These results are to be contrasted with typical runs where dimedone is added to the incubation mixture and the pH adjusted to 4 without prior oxidation. In these experiments no correlation of the formaldehyde number with cobester yield was observed. It is now safe to conclude that the C-20 carbon of uro'gen III is trapped at the oxidation level of formaldehyde. Since the evolution of free formaldehyde is a rare biochemical event, the intervention of a tetrahydrofolate-mediated reaction[35] becomes an attractive possibility which can now be tested, albeit by indirect biochemical methodology.[36]

With regard to the timing of the loss of the second carbon from uro'gen III, as CO_2 from the ring C acetate side chain, the studies cited above[29] had shown (without location of the final ^{14}C label) that the heptacarboxylic acid 8 labeled at C-5 and C-15 was consistently, but

(32) A. R. Battersby, E. Hunt, M. Ihara, E. McDonald, J. B. Paine III, F. Satoh, and J. Saunders, *J. Chem. Soc., Chem. Commun.*, 994 (1974).
(33) A. H. Jackson, H. A. Sancovich, A. M. Ferramola, N. Evans, D. E. Games, S. A. Matlin, G. H. Elder, and S. G. Smith, *Philos. Trans. R. Soc. London, Ser. B*, **273**, 191 (1976).
(34) N. Georgopapadakou, Ph.D. Thesis, Yale University, 1975.
(35) M. Friedkin, *Annu. Rev. Biochem.*, **32**, 185 (1963).
(36) P. R. Farina, L. J. Farma, and S. J. Benkovic, *J. Am. Chem. Soc.*, **95**, 5409 (1973), and references cited therein.

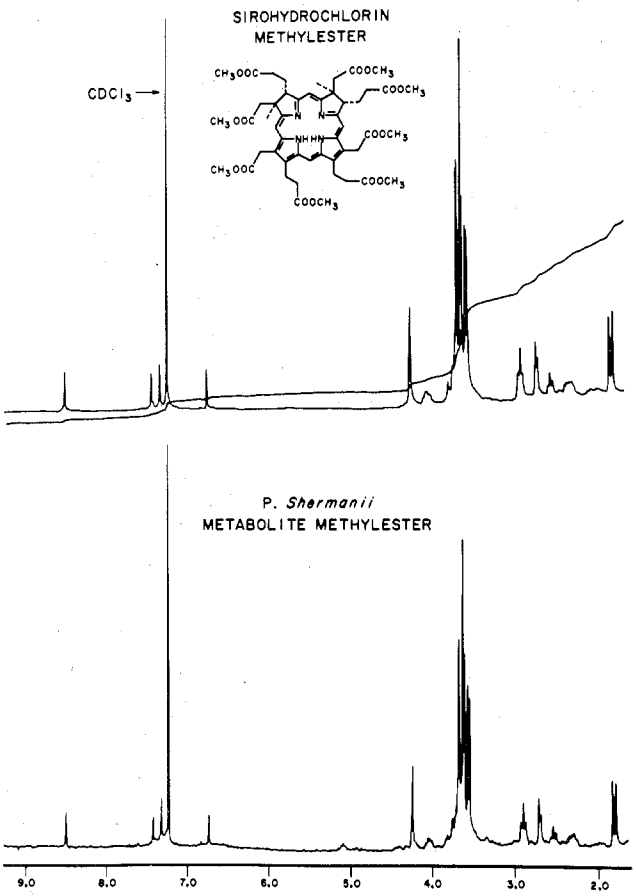

Figure 10.

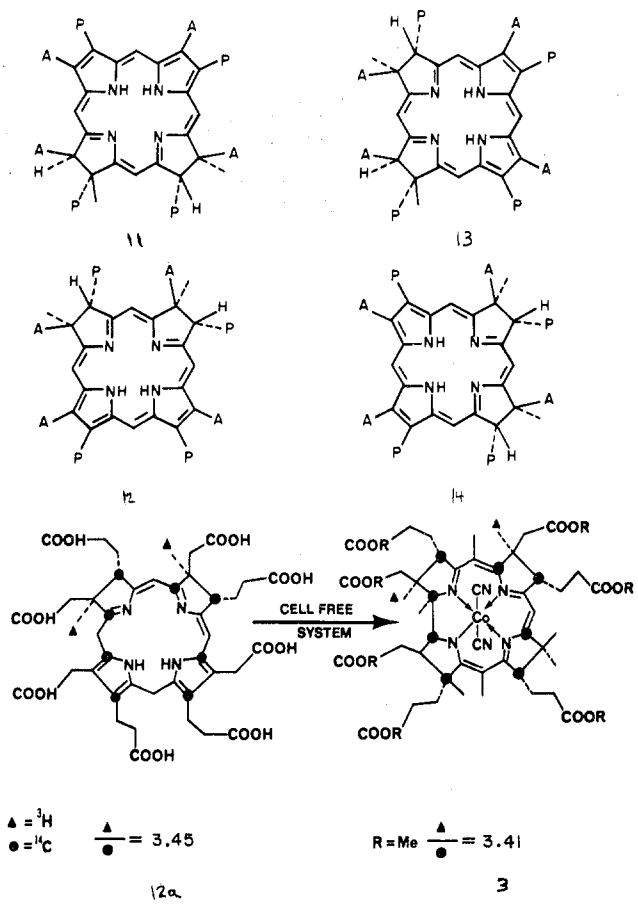

Figure 11.

rather inefficiently, incorporated into cobyrinic acid. We therefore carried out the total synthesis of triply labeled 8 (^{14}C at C-5, C-15, and C-20) and incubated this substrate with the cell-free system (Figure 9). If incorporation of 8 is controlled by the same enzymic mechanism utilized by 4 then the radiochemical yield of methylene–dimedone adduct and cobester should match, regardless of the efficiency of the process. Reference to Table I indicates that, although the bioconversion of uro'gen heptaacid 8 to cobyrinic acid (3) is an order of magnitude less than that of uro'gen III (0.2%), the enzymic release of formaldehyde (0.17%) from this substrate ($^{14}C_{20}$) and its otherwise intact conversion (^{14}C-5 and ^{14}C-15) are no longer in doubt. However, the discovery of sirohydrochlorin has added a new dimension to the problem.

Sirohydrochlorin: Prosthetic Group of Sulfite and Nitrite Reductase Enzymes and Its Relation to an Intermediate of B_{12} Biosynthesis

A novel hemelike prosthetic group in a rather widespread class of enzymes which catalyze the six-electron reduction of sulfite to sulfide was first characterized in 1973[37] and named siroheme. Removal of the iron from this species afforded an orange fluorescent compound, sirohydrochlorin, which formed octacarboxylic methyl and ethyl esters and displayed a UV spectrum typical of a reduced porphyrin of the isobacteriochlorin class. High-resolution mass spectrometry permitted assignment of the molecular formula, and a tentative structure was proposed.[38] It did not escape the notice of Siegel et al. that such a structure was notably reminiscent of the oxidized version of a doubly β-methylated uro'gen III but further that "(sirohydrochlorin) could represent an early intermediate in the biosynthesis (of B_{12})".[38] From another quarter[39] came brief reports of a modified (mono?) methylated-tetrapyrrole-containing cobalt obtained from whole cells of *P. shermanii* and which appeared to have a similar structure, incorporated radioactive methionine into its methyl groups, and furthermore increased B_{12} production in the organism.

By modifying our cell-free technique it has been possible to isolate almost 1 mg of an orange fluorescent substance close in R_f on the usual TLC systems to uroporphyrin III. Comparison of UV, mass, CD, and ^1H NMR spectra (Figure 10) and TLC R_f values of methyl ester of the new isolate and of sirohydrochlorin octamethyl ester established complete identity. Furthermore, administration of [4-^{14}C]ALA and of [$^3H_3$-methyl]methionine to the 37 000*g* supernatant synthetase mixture, isolation, and purification yielded radiochemically pure, doubly labeled material ($^3H/^{14}C$ = 3.45). This experiment confirmed the derivation of sirohydrochlorin from ALA and methionine with sufficient incorporation to allow refeeding of a *reduced* version (**12a**) to the corrin synthetase mixture. The resultant cobester was formed in 3% radiochemical yield and moreover *retained* the $^3H/^{14}C$ ratio (3.41), thus indicating intact incorporation of reduced sirohydrochlorin. The structural proposals (**11**–**13**) for

(37) L. M. Siegel, M. J. Murphy, and H. Kamin, *J. Biol. Chem.*, **248**, 251 (1973).

(38) M. J. Murphy, L. M. Siegel, H. Kamin, and D. Rosenthal, *J. Biol. Chem.*, **248**, 2801 (1973).

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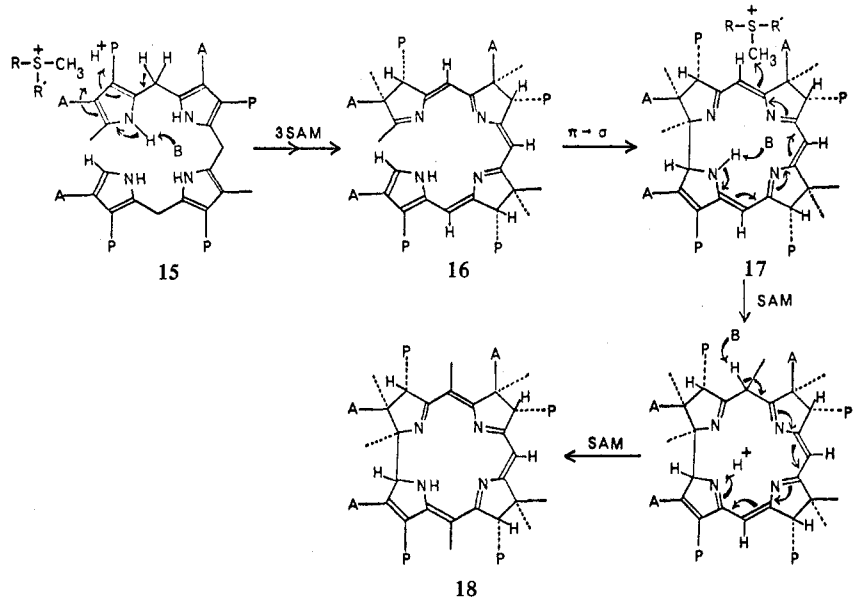

Figure 12.

sirohydrochlorin must now be confined to the *stereo* structures **12** and **13** not only bearing evidence of methylation on two contiguous rings of uro'gen III but also endowed with the same absolute stereochemistry as that of cobyrinic acid (Figure 11), since ring C cannot be methylated *before* decarboxylation. Further structural analysis by proton-decoupling experiments at 270 MHz allowed a decision in favor of **12** (lack of coupling between $-CH_2CO_2H$ and methine proton as required by **12**). Unexpectedly incubation of unreduced **12** also gave cobyrinic acid.[40] The discovery of sirohydrochlorin and its role in corrin biosynthesis sheds new light on the intermediary steps and requires reassessment of the part played by the heptaacid **8**. The utilization of a substrate such as **8** within a metabolic grid is not without precedent (cf. the latter stages of B_{12} biosynthesis), but the final definition of true intermediacy still awaits separation of the enzymes (synthetases) involved.

The Sequence of Corrin Biosynthesis

In accord with all of the above evidence, the following requirements must be met in the conversion of uro'gen III to cobyrinic acid (not necessarily in the order shown): (a) decarboxylation of the acetic acid side chain at C-12 *prior* to methylation at C_{12}; (b) loss of the meso carbon at C-20 (as formaldehyde, or its equivalent) and formation of a new bond between C-1 and C-19, a process which does not exchange the protons of the C_1-methyl group; (c) introduction of the seven "extra" methyl groups from SAM; (d) reduction (four electron equivalents); (e) insertion of cobalt.

Within the scope of this account it is neither possible nor appropriate to develop in depth a full set[31] of mechanistic proposals for the most likely sequence of the uro'gen–corrin pathway. However, the following salient features and problems associated with the above requirements serve both as signposts for future work and as reminders that many mysteries remain to be solved.

Decarboxylation, C_{20} Loss, Methylation, and Reduction. Although the heptacarboxylic acid **8** is specifically incorporated into cobyrinic acid (**3a**), the radiochemical conversion is an order of magnitude less than that of both uro'gen III (**4**) and reduced sirohydrochlorin (**13**). Perhaps there is some lack of enzyme specificity at this stage and also in the reduction of sirohydrochlorin (**12**) to **13**. Secondly, if the reduced sirohydrochlorin is an obligatory intermediate, does it lose formaldehyde at once or undergo further methylation/decarboxylation? In either case a modified bilane or secocorrin structure must surely be implicated. A suggestion for the secocorrin → corrin recyclization utilizing the 16π system of **15** → **18** (Figure 12) was made earlier[31] and has recently enjoyed excellent in vitro analogy in Eschenmoser's laboratory.[41] We note that the biochemical counterpart of this process does not require exchange of protons at the vital C_1–CH_3 group.

The *disadvantage* of the electrocyclic closure **16** → **17** as a model is the production of a *dehydrocorrin* (**18**) which now must be reduced and methylated in ring D. A proposal was made some time ago involving the Co^{2+} → Co^{3+} change as an alternative to NADPH-mediated reduction. Recent experiments with NAD^3H[40] indicate negligible incorporation of tritium during cell-free corrin synthesis. At this juncture many possibilities remain for this redox change, the Co^{2+} → Co^{3+} oxidation [followed by addition of a proton in ring D (**18** → **21** → **3**)] being only one of these (Figure 13).

Insertion of Cobalt. The isolation of cobalt-free corrins from *Chromatium*[42] and *Streptomyces*[43] spp. indicates that a descobalto corrin (as **18**) may be the penultimate intermediate. Clearly, it will be necessary to prove this at the enzyme level since cobalt insertion can occur nonenzymically. However, no known method

(40) A. I. Scott, A. J. Irwin, and L. M. Siegel, submitted for publication. The incorporation of labeled "Faktor II" from *Clostridium tetanomorphum* and *P. shermanii* into cobyrinic acid has recently been described in an independent study at Stuttgart [R. Deeg, H-P. Knemler, K-H. Bergmann, and G. Müller, *Z. Physiol. Chem.*, **358**, 339 (1977)]. "Factor II" appears to be identical with sirohydrochlorin (**12**). We thank Dr. Müller for informing us of his results. Structure **12** has now been confirmed by ^{13}C NMR spectroscopy.

(41) v. B. Kräutler, A. Pfaltz, R. Nordmann, K. O. Hodgson, J. D. Dunitz, and A. Eschenmoser, *Helv. Chim. Acta*, **59**, 924 (1976).

(42) J. I. Toohey, *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **25**, 1628 (1966).

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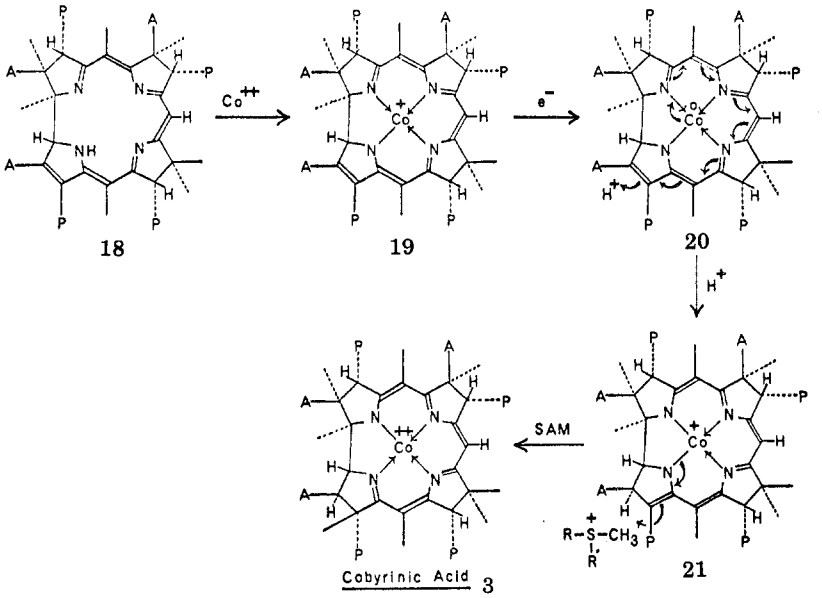

Figure 13.

exists for *removing* the cobalt ion (at any valency level) from corrins. It would appear, therefore, that Nature can fashion the cobalamin structure without the use of cobalt as a template. This does not exclude other species (e.g., Mg, Zn) from consideration but rather indicates a further set of necessary experiments.

Epilogue

The story of corrin biosynthesis so far has yielded many surprises. One cannot cease to marvel at Nature's method of synthesizing this complex macrocyclic ligand by first forming uro'gen III, the precursor of heme and chlorophyll, and then undoing the ring system by ejecting a C_1 unit under the "pressure" of β-methylation, and finally recyclizing the secocorrin to reveal the cobalt-free ligand with its full panoply of stereochemistry, the overall process being that of *reductive* methylation. Indeed, on the evolutionary scale B_{12} may be a much more venerable compound than heme, for a study of the most ancient microorganisms reveals[34,44] that several archaic anaerobes produced B_{12} but no heme, whereas the more developed aerobic bacteria oft-times synthesize heme but not vitamin B_{12}. It is also clear that much remains to be clarified before the mechanistic details of the uro'gen–corrin connection are finally settled.

The work described in this Account was only made possible by the skilled efforts of the interdisciplinary group of colleagues mentioned in the references and by generous support of the National Science Foundation (Grant MPS72-04601) and the National Institutes of Health (Grant AM17014) in the period 1968-1977.[45]

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Radiation-Induced Organic Hydrogen Isotope Exchange Reactions in Aqueous Solution

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A complete understanding of the chemical reactions which take place in a given system should incorporate a consideration of possible exchange reactions in which there is no net chemical change. Such identity reactions are usually not directly detectable unless a suitable isotope label is present. Kinetically, these reactions are almost invariably of first order with respect to the concentration of the isotope label.[1] Low concentrations of tritium can easily be estimated by liquid scintillation counting, and this technique permits even exceedingly

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