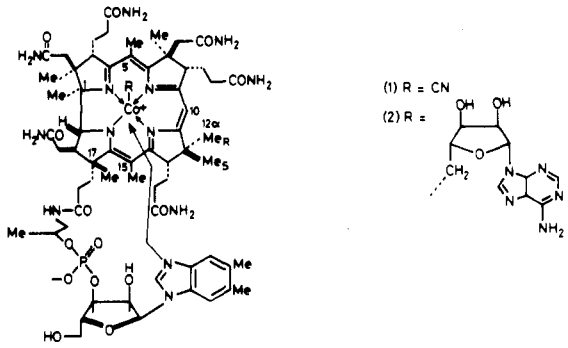
# Biosynthesis of Vitamin $B_{12}$[†]

ALAN R. BATTERSBY

*University Chemical Laboratory, Cambridge CB2 1EW, United Kingdom*

*Received October 30, 1985*

All chemists, especially those with interests in the organic field, are fascinated by the amazing assembly of structures produced by living systems. How are those molecules synthesized by the plant or the microorganism? Obviously building structures involves the making of bonds from carbon to another carbon or to oxygen or nitrogen, etc. and also involves the breaking of such bonds. These processes are the life blood of organic chemistry. One aim of this Account is to illustrate how the strengths of an organic chemist can be used to solve a complex biosynthetic problem. The one to be enjoyed here could hardly be more complex; we wish to discover the complete biosynthetic pathway to vitamin $B_{12}$ (1). The coenzyme form of this anti-pernicious-anemia vitamin has structure (2); the latter is responsible for the remarkable rearrangement reactions catalyzed by several enzymes which use (2) as cofactor.[1]




Study of the biosynthesis of a molecule such as vitamin $B_{12}$ (1) requires extensive use of labeling based on radioactive or stable isotopes, and many examples of these approaches will be given here. Steady progress in understanding biosynthesis started to be made in the 1950's when simple labeled organic compounds began to be available. But organic chemists were contributing ideas about biosynthesis even before that by suggesting possible biosynthetic routes to natural products.[2] There has been a transformation since then almost explosive in character.

Though isotopes are crucially important for biosynthetic research, equally important is the investigator's synthetic ability. The sequel will show how progess on the biosynthesis of vitamin $B_{12}$ often depended on synthesis. This has the enormous attraction that completion of a complex synthesis is not the end; synthetic success launches a new beginning, which is the use of the synthetic product to make discoveries in the living system.

Our researches in Cambridge on the biosynthesis of vitamin $B_{12}$ started in 1969, and a survey of the highlights of just that work will be given. Indeed, a comprehensive review of the field would use a complete issue of this journal. The important work of other investigators is covered elsewhere[1] and I refer particularly to Arigoni, Bernhauer, Bykhovsky, Müller, Scott, and Shemin; however, any of their results relevant to the main theme which are not so covered will be included here.

**Biosynthesis of Uroporphyrinogen-III (10).** It was known at the outset of our work on vitamin $B_{12}$ that two other pigments of life, protoheme (present in hemoglobin) and chlorophyll *a* are both derived in living systems from uroporphyrinogen-III (10), shortened to uro'gen-III. It will be seen later that this also holds true for vitamin $B_{12}$. So the foundation must be laid by focussing briefly on the biosynthesis of uro'gen-III (10). The pioneers (Bogorad, Granick, Neuberger, Rimington, and Shemin) had provided in the 1950's the knowledge[3] that uro'gen-III (10) is built from 4 molecules of porphobilinogen (3) by the cooperative action of two enzymes, usually called deaminase[4] and cosynthetase[4]; The techniques then available did not allow further progress. However, the advent of carbon-13 and of NMR spectrometers capable of detecting this isotope reopened the door and one could hardly imagine a more exciting problem in 1968 than to find out how uro'gen-III (10) is built.

The structure of uro'gen-III (10) is an unexpected one; straightforward head-to-tail assembly of 4 molecules of 3 would produce a macrocycle in which the side-chains appear in regular order around the periphery. But living systems do not build such a regular arrangement, and the two propionic acid residues which are adjacent on rings C and D of uro'gen-III (10) reveal that one or more rearrangement reactions must have occurred.

The pathway which was elucidated from porphobilinogen (3) to uro'gen-III (10) is shown in Scheme I. This scheme is the outcome of many experiments involving $^{14}C$ and $^{13}C$ labeling and enzyme isolation and

Alan Battersby is professor of organic chemistry at the University of Cambridge (since 1969) and fellow of St. Catharine's College, having previously held a chair in the Robert Robinson Laboratories of the University of Liverpool. He was educated at Leigh Grammar School and at the Universities of Manchester and St. Andrews, gaining his Ph.D. at St. Andrews in 1949. He is a Fellow of the Royal Society and holds the Davy and Royal Medals of that Society. The period 1950–1952 was spent in the United States at the Rockefeller Institute for Medical Research and the University of Illinois, and he has held many Visiting Professorships in the U.S. Professor Battersby's research interests are concerned with the chemistry of living systems, with biosynthetic research on the pigments of life, especially haem, the cytochromes, the chlorophylls, and vitamin $B_{12}$, and with studies of the individual enzymic reactions.

[†]This article provides an abbreviated and updated version of the Roger Adams Award Address given in Bozeman, MT, in 1983, by A. R. Battersby.

(1) For a full survey of all these aspects see: "$B_{12}$", Dolphin, D., Ed.; Wiley: New York 1982, Vol. 1 & 2 and specifically Vol. 1, p 107.

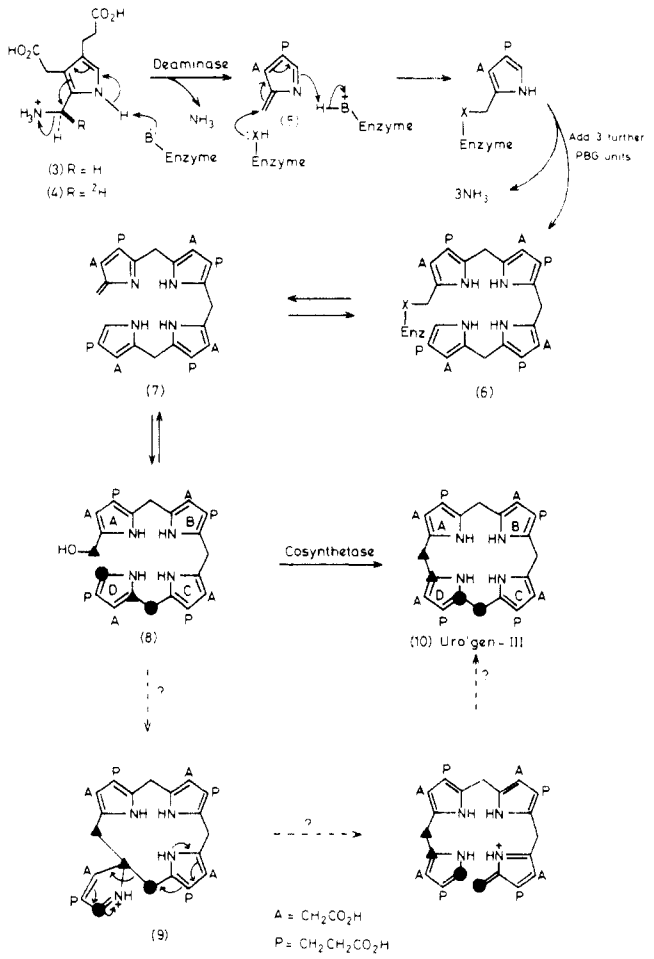
(2) Robinson, R. *The Structural Relations of Natural Products*; Clarendon: Oxford, 1955.

(3) Reviewed in the following: Battersby, A. R.; McDonald, E. In *Falk's Porphyrins and Metalloporphyrins*, 2nd ed.; Smith, K. M., Ed.; Elsevier: Amsterdam, 1975; p 61.

(4) The systematic name for deaminase (E.C.4.3.1.8) is hydroxymethylbilane synthase and for cosynthetase (E.C.4.2.1.75) is uroporphyrinogen-III synthase.

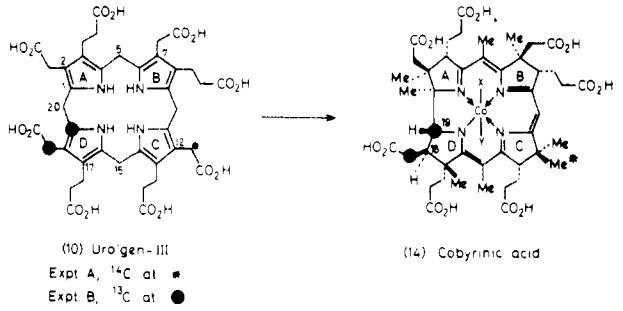
0001-4842/86/0119-0147$01.50/0 © 1986 American Chemical Society

**Scheme I**




kinetics, together with syntheses of the key intermediates.[5] Porphobilinogen (**3**) is first bound to deaminase through some nucleophilic group X on the enzyme,[6] with the release of 1 mol of $NH_3$; this binding probably occurs via the azafulvene (**5**). Clues as to the nature of X have been sought by $^{13}C$ NMR[6] and $^{3}H$ NMR[7] but in each case the signal quality was not good leaving the question still open. Following the attachment of the first pyrrole, three further pyrrole units are attached sequentially,[6] and 3 more mol of $NH_3$ are eliminated. This generates a covalently bound tetrapyrrole **6** called a bilane which is released from deaminase as the highly labile hydroxymethylbilane[8] (**8**) whose structure, based initially on the correct interpretation of $^{13}C$ NMR spectroscopy,[8] was rigorously established[8] by synthesizing **8**. That the azafulvene **7** is an intermediate for release of **8** from deaminase has strong experimental support.[6] Also recent stereochemical studies dependent on synthetic samples of (11*R*)-[11-$^{2}H_1$]porphobilinogen (**4**) and the (11*S*)-isomer have shown that the azafulvene **7** must be generated and undergo further reaction while still held in the active site of deaminase.[9] So deaminase catalyzes repetitive chemical processes which in essence are the same for the steps of binding, assembly of the bilane, and its release. *Deaminase is the assembly enzyme.*

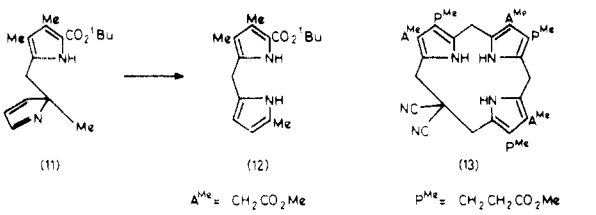
**Scheme II**




The pyrrole units of the hydroxymethylbilane **8** are assembled in a regular, unrearranged way and it is the second enzyme, cosynthetase, which carries out the surprising step of ring-closing **8** with rearrangement of ring-D[8] to generate uro'gen-III (**10**). The spots and triangles on **8** and **10** illustrate the $^{13}C$-double-labeling experiments which demonstated the intramolecular nature of the single rearrangement reaction.[10,11] Finally, other $^{13}C_2$-labeling studies proved that the rearrangement process only breaks the bond between ring-D and its attached $CH_2$ group in the bilane[5,12] **8**; the 3 pyrrolomethylene residues which make up the rest of the bilane **8** remain intact throughout.[12] *Cosynthetase is thus the ring-closing and rearranging enzyme.*

Two further fascinating results came from challenging cosynthetase with synthetic analogues of the natural system **8** in which the acidic side chains were positioned in a different order or were replaced by methyl or ethyl groups: (a) Cosynthetase was shown to be an enzyme which has evolved to turn around the terminal ring of an hydroxymethylbilane[13] and (b) the acetate group on ring-D of **8** plays an important role, as yet unknown, in the binding and/or rearrangement process.[14]

The $^{13}C_2$-labeling experiments (some summarized in Scheme I) put tight limits on possible mechanisms for the conversion of **8** with rearrangement into uro'gen-III **10**. Only two schemes fit the data. One, which is attractive to chemists by its neat simplicity, invokes the intermediacy of the spiro system[15] **9**. We have found (a) the synthetic system **11** undergoes rapid rearrangement under mild acidic catalysis to **12** showing



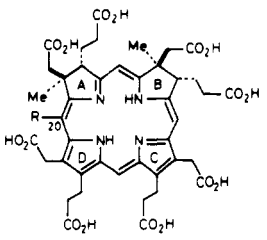

(5) Battersby, A. R.; Fookes, C. J. R.; Matcham, G. W. J.; McDonald, E. *Nature* **1980**, *285*, 17.
(6) Battersby, A. R.; Fookes, C. J. R.; Matcham, G. W. J.; McDonald, E.; Hollenstein, R. *J. Chem. Soc., Perkin Trans 1* **1983**, 3031. Battersby, A. R.; Fookes, C. J. R.; Hart, G.; Matcham, G. W. J.; Pandey, P. S. *J. Chem. Soc., Perkin Trans 1* **1983**, 3041.
(7) Evans, J. N. S.; Fagerness, P. E.; Mackenzie, N. E.; Scott, A. I. *J. Am. Chem. Soc.* **1984**, *106*, 5738.
(8) Battersby, A. R.; Fookes, C. J. R.; Gustafson-Potter, K. E.; McDonald, E.; Matcham, G. W. J. *J. Chem. Soc., Perkin Trans 1* **1982**, 2427.
(9) Neidhart, W.; Anderson, P. C.; Hart, G. J.; Battersby, A. R. *J. Chem. Soc., Chem. Commun.* **1985**, 924.
(10) Battersby, A. R.; Fookes, C. J. R.; Meegan, M. J.; McDonald, E.; Wurziger, H. K. W. *J. Chem. Soc., Perkin Trans. I* **1981**, 2786.
(11) Battersby, A. R.; Fookes, C. J. R.; Gustafson-Potter, K. E.; McDonald, E.; Matcham, G. W. *J. Chem. Soc., Perkin Trans 1* **1982**, 2413.
(12) Battersby, A. R.; Hodgson, G. L.; Hunt, E.; McDonald, E.; Saunders, J. *J. Chem. Soc., Perkin Trans. 1* **1976**, 273.
(13) Battersby, A. R.; Fookes, C. J. R.; Matcham, G. W. J.; Pandey, P. S. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 293.
(14) Battersby, A. R.; Fookes, C. J. R.; Pandey, P. S. *Tetrahedron* **1983**, *39*, 1919.
(15) Mathewson, J. H.; Corwin, A. H. *J. Am. Chem. Soc.* **1961**, *83*, 135.

that the postulated reactions 8 → 9 → 10 are feasible and facile;[16] (b) the tripyrrolic macrocycle present in 9 can be synthesized in the form 13 and its highly puckered conformation has been revealed by X-ray analysis.[17]

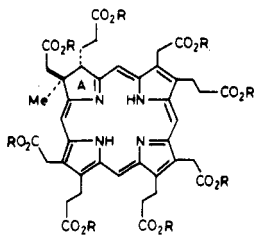
**Vitamin $B_{12}$: Pathway as far as the Trimethylated Intermediate.** The early work of Bernhauer's group[1] had led to the important finding that the vitamin 1 is biosynthesized from cobyrinic acid (14). Thus, the question posed at the outset now becomes, how is cobyrinic acid biosynthesized? This Account will start at the point where it had been rigorously established[18-20] that, as for the other pigments of life, cobyrinic acid (14) is built in living systems from uro'gen-III (10). Experiment A, Scheme II, shows one proof of this based on $^{14}C$ labeling.[19] This conversion, as for many others in the sequel, was carried out by using a cell-free enzyme preparation from the $B_{12}$ producer, *Propionibacterium shermanii*.

It is evident that the formation of 14 from 10 must be a multistep process with a dauntingly huge number of possible sequences. The investigator's best approach is to search for the *first* biosynthetic step beyond uro'gen-III (10). After eliminating some alternatives,[19,21] the breakthrough came[1] from the isolation of many new macrocyclic pigments from *P. shermanii* which had been grown strictly without cobalt, following Bykhovsky.[1]

Two pigments proved to be of decisive importance. The structures of their octamethyl esters were shown[22-24] to be 17 and 18 and were also worked out



(15) R = H Sirohydrochlorin
(16) R = Me Trimethylisobacteriochlorin
(17) Octamethyl ester of (15)
(18) Octamethyl ester of (16)

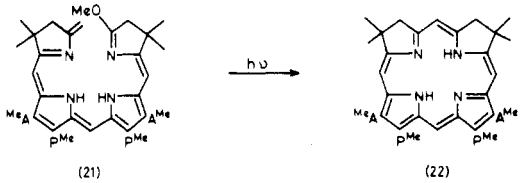


(19) Faktor-I, R = H
(20) R = Me

elsewhere.[1] Both pigments are based on the isobacteriochlorin (IBC) macrocycle and had a beautiful violet color. It was exciting to find that labeled forms of the octaacid (15) called sirohydrochlorin[25] and also of the trimethylated system[26,27] 16 were converted enzymically into cobyrinic acid[23] (14). These findings pinpointed the method Nature uses to switch the $B_{12}$ pathway away from that leading, for example, to protoheme; *the branching step involves C-methylation.* Finally, Arigoni and Müller[28] found that the first *C*-methyl group for vitamin $B_{12}$ is introduced into ring A of uro'gen-III (10), the monomethylated system being isolated in aromatized form as Faktor-I (19) (see also ref 29). Thus, the order of introduction of the first 3 *C*-methyl groups on the pathway from uro'gen-III (10) to cobyrinic acid (14) was clear, the first at C-2, the second at C-7, and the third, very surprisingly, at C-20.

The foregoing pigments, especially the trimethylated one 16, can be compared to the first flakes of gold found in panning; "if only we can find more, we will be rich". This is because 16 will be of immense value for future experiments and so stands out as a crucially important target for synthesis. Let us look now at that aspect.

**Synthesis of Isobacteriochlorins and Chlorins.** If synthesis is going to help the biosynthetic work, practical routes are needed capable of yielding workable quantities of products (ideally, hundreds of milligrams). Yet at the outset (1977) no rational route existed for building an isobacteriochlorin (IBC) even with simple substituents. Then methods were developed for synthesis of IBCs carrying alkyl side chains,[30,31] and the door was opened to the natural pigments 15 and 16 by a key observation. It was found[32] that the 18 $\pi$ seco system[33] 21 underwent smooth photochemical ring closure (antarafacial by the Woodward-Hoffmann rules) to afford 22. Importantly, this mild method was com-



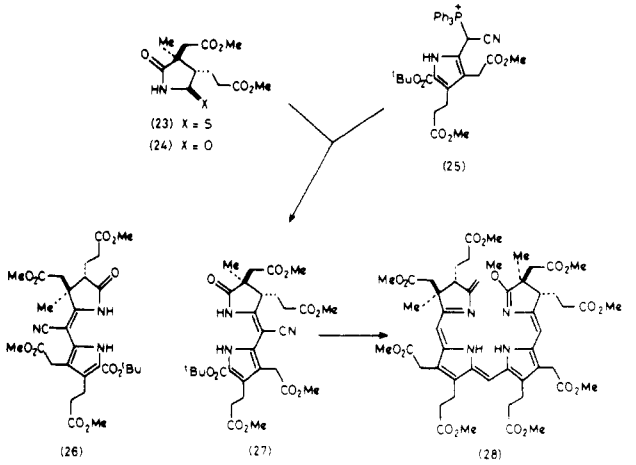
(21) (22)

patible with acetate and propionate residues (see 22) also present in the natural pigments 15 and 16.

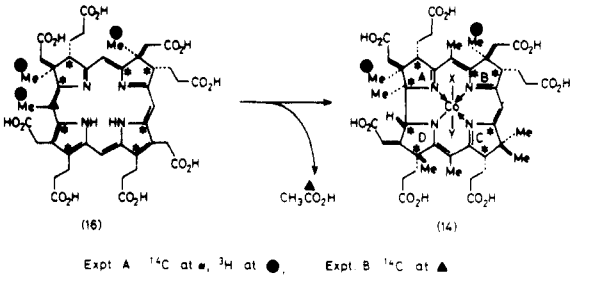
Exploitation of this photochemical method for synthesis of the octamethyl ester of sirohydrochlorin (17) involved treatment of the monothioimide 23 and phosphonium salt 25 with base to afford the ring-B/ring-C system 27. The ring-A/ring-D block 26 was built

(16) Battersby, A. R.; Broadbent, H. A.; Fookes, C. J. R. *J. Chem. Soc., Chem. Commun.* **1983**, 1240.
(17) Stark, W. M.; Baker, M. G.; Raithby, P. R.; Leeper, F. J.; Batterby, A. R. *J. Chem. Soc., Chem. Commun.* **1985**, 1294.
(18) Scott, A. I.; Georgopapadakou, N.; Ho, K. S.; Klioze, S.; Lee, E.; Lee, S. L.; Tomme, G. H., III; Townsend, C. A.; Armitage, I. A. *J. Am. Chem. Soc.* **1975**, *97*, 2548.
(19) Battersby, A. R.; Ihara, M.; McDonald, E.; Satoh, F.; Williams, D. C. *J. Chem. Soc., Chem. Commun.* **1975**, 436. Battersby, A. R.; McDonald, E.; Hollenstein, R.; Ihara, M.; Satoh, F.; Williams, D. C. *J. Chem. Soc., Perkin Trans. 1* **1977**, 166
(20) Dauner, H.-O.; Müller, G. *Z. Physiol. Chem.* **1975**, *356*, 1353.
(21) Battersby, A. R.; Kishimoto, S.; McDonald, E.; Satoh, F.; Wurziger, H. K. W. *J. Chem. Soc., Perkin Trans. 1*, **1979**, 1927.
(22) Battersby, A. R.; Jones, K.; McDonald, E.; Robinson, J. A.; Morris, H. R. *Tetrahedron Lett.* **1977**, 2213. Battersby, A. R.; McDonald, E.; Morris, H. R.; Thompson, M.; Williams, D. C.; Bykhovsky, V. Ya; Zaitseva, N.; Bukin, V. *Tetrahedron Lett.* **1977**, 2217.
(23) Battersby, A. R.; Thompson, M.; Bykhovsky, V. Ya *J. Chem. Soc., Chem. Commun.* **1978**, 150.
(24) Battersby, A. R.; Matcham, G. W. J.; McDonald, E.; Neier, R.; Thompson, M.; Woggon, W.-D.; Bykhovsky, V. Ya; Morris, H. R. *J. Chem. Soc., Chem. Commun.* **1979**, 185.
(25) A remarkable link was forged at this stage. The metal-free prosthetic group from the enzyme sulphite reductase had been isolated earlier by Kamin and Siegel[1] and called sirohydrochlorin but its complete structure was not known. The dimethylated pigment 15 from *P. shermanii*[22] proved to be identical with sirohydrochlorin and so elucidation of the structure of the *P. shermanii* pigment also settled that of the enzymic prosthetic group. See also ref 1.
(26) Battersby, A. R.; McDonald, E. *Bioorg. Chem.* **1978**, *7*, 161.
(27) Battersby, A. R.; Lewis, N. G.; Neier, R.; Matcham, G. W. J.; McDonald, E. *J. Chem. Soc., Chem. Commun.* **1979**, 541.
(28) Imfeld, M.; Arigoni, D.; Deeg, R.; Müller, G. In *Vitamin $B_{12}$*; Zagalak, B. J., Friedrich, W., Eds.; de Gruyter: Berlin, 1979; p 315.
(29) Battersby, A. R.; Seo, S. *J. Chem. Soc., Perkin Trans. 1*, **1983**, 3049.
(30) Montforts, F.-P.; Ofner, S.; Rasetti, V.; Eschenmoser, A.; Woggon, W.-D.; Jones, K.; Battersby, A. R. *Angew. Chem., Int. Ed. Engl.* **1979**, *18*, 675.
(31) Ofner, S.; Rasetti, V.; Zehnder, B.; Eschenmoser, A. *Helv. Chim. Acta* **1981**, *64*, 1431.
(32) Battersby, A. R.; Harrison, P. J.; Fookes, C. J. R. *J. Chem. Soc., Chem. Commun.* **1981**, 797.
(33) The critical steps for building such seco systems will be considered later when synthesis of the natural pigments is outlined.

**Scheme III**



**Scheme IV**



similarly (Scheme III). Several further steps were needed[34] to remove the nitrile function from **26** and **27** and so by further elaboration to generate the seco system **28** from those two key building blocks. These steps and the final photochemical cyclization proceeded smoothly to yield synthetic sirohydrochlorin ester **17**, identical with the natural pigment.[34]

This synthesis is well suited to its purpose because (a) there are only two starting materials **24** and **25** synthesizable in large quantity, (b) the route is convergent, (c) it can be modified to produce labeled forms of **15** or analogues of it. Thus the bottleneck in supplies of **15** has been cleared and with methodology already developed[35] for generating a C-methyl group at C-20 of an IBC, the same is expected soon to be true for the trimethylated system **16**.

A variation of this photochemical theme also allows the rational synthesis of chlorins[36] (chlorophyll is a chlorin) and has provided synthetic Faktor-I ester[37] **20** and synthetic bonellin,[38] a marine chlorin. To give the reader a feeling for the impact of these syntheses, more Faktor-I ester (**20**) was obtained from the first small synthetic run than the total we had isolated during 8 years from natural sources.

Returning now to direct biosynthetic experiments, we can survey what has been discovered about the later stages of $B_{12}$ biosynthesis.

**The Problem of Oxidation Level.** Comparison of the structures of uro'gen-III (**10**) and the di- and trimethylated pigments **15** and **16** shows that the latter two (with 9 double bonds) differ in oxidation level from the former (8 double bonds). Since C-methylation itself does not affect oxidation level, it seemed possible that the true intermediates on the biosynthetic pathway might be dihydro derivatives of **15** and **16**, these two being formed by oxidation during isolation. It the event, when the enzymic methylation of uro'gen-III (**10**) was run under strictly anaerobic conditions, a new yellow pigment was isolated[39] and shown to be **31**. Further, this dihydro-IBC **31** in labeled form was incorporated more efficiently[39] into cobyrinic acid (**14**) than was the aromatic relative[40] **15**. Thus it was established that there is no change of oxidation level on the $B_{12}$ pathway up to the dimethylated intermediate **31**, and it is highly probable that the trimethylated intermediate is produced and further transformed in the dihydro state **32**.

**Vitamin $B_{12}$: Steps Beyond the Trimethylated Intermediate. (a) The Direct Link between Rings A and D.** Rings A and D of vitamin $B_{12}$ (**1**) are directly bonded, whereas such pigments as protoheme, chlorophyll *a*, and the cytochromes all retain the single carbon between rings A and D present in the biosynthetic parent, uro'gen-III (**10**). This remarkable structural feature was explored by studying the incorporation of a multiply labeled sample of the trimethylated IBC **16** into cobyrinic acid (**14**), Experiment A, Scheme IV. The labeling of the latter product proved[41] that the methyl group at C-20 had been *lost* during the biosynthetic transformation. In what form? The answer was obtained by labeling **16** with $^{14}C$ at C-20, Expt. B, Scheme IV; the extruded fragment was found to be *acetic acid*.[42,43] This finding was of great value in giving clues about the likely mechanism of the ring-contraction process (see later) and also in yielding information about the question of oxidation level. When the oxidation level of cobyrinic acid (**14**) and acetic acid *taken together* is compared with that of the dihydrotrimethyl-IBC (**32**) from which they are formed, one finds that no external redox reagents are required for the conversion.

**(b) Decarboxylation of the Acetic Acid Residue at C-12.** Cobyrinic acid (**14**) carries two methyl groups at C-12. The *re*-methyl arises by C-methylation from *S*-adenosylmethionine (SAM) whereas it is decarboxylation of the 12-acetate group at some presently unknown point on the pathway between **10** and **14** which generates the 12-*si*-methyl group.[44] The stereochemistry of this decarboxylation was studied using δ-aminolevulinic acid (**29**) made chiral[45] at C-2 by isotopic substitution with $^2H$ and $^3H$. This substance is the

(34) Block, M. H.; Zimmerman, S. C.; Henderson, G. B.; Turner, S. P. D.; Westwood, S. W.; Leeper, F. J.; Battersby, A. R. *J. Chem. Soc., Chem. Commun.* **1985**, 1061.

(35) Arnott, D. M.; Battersby, A. R.; Harrison, P. J.; Henderson, G. B.; Sheng, Zhi-Chu. *J. Chem. Soc., Chem. Commun.*, **1984**, 525.

(36) Battersby, A. R.; Dutton, C. J.; Fookes, C. J. R.; Turner, S. P. D. *J. Chem. Soc., Chem. Commun.* **1983**, 1235.

(37) Turner, S. P. D.; Block, M. H.; Sheng, Zhi-Chu; Zimmerman, S. C.; Battersby, A. R. *J. Chem. Soc., Chem. Commun.* **1985**, 583.

(38) Dutton, C. J.; Fookes, C. J. R.; Battersby, A. R. *J. Chem. Soc., Chem. Commun.* **1983**, 1237.

(39) Battersby, A. R.; Frobel, K.; Hammerschmidt, F.; Jones, C. *J. Chem. Soc., Chem. Commun.* **1982**, 455.

(40) The complex mixture of enzymes in the cell-free enzyme preparation must be capable of reducing sirohydrochlorin (**15**) to the dihydro state **31** when the former pigment is used for incorporation experiments.

(41) Battersby, A. R.; Lewis, N. G.; Neier, R.; Matcham, G. W. J.; McDonald, E. *J. Chem. Soc., Chem. Commun.* **1979**, 541.

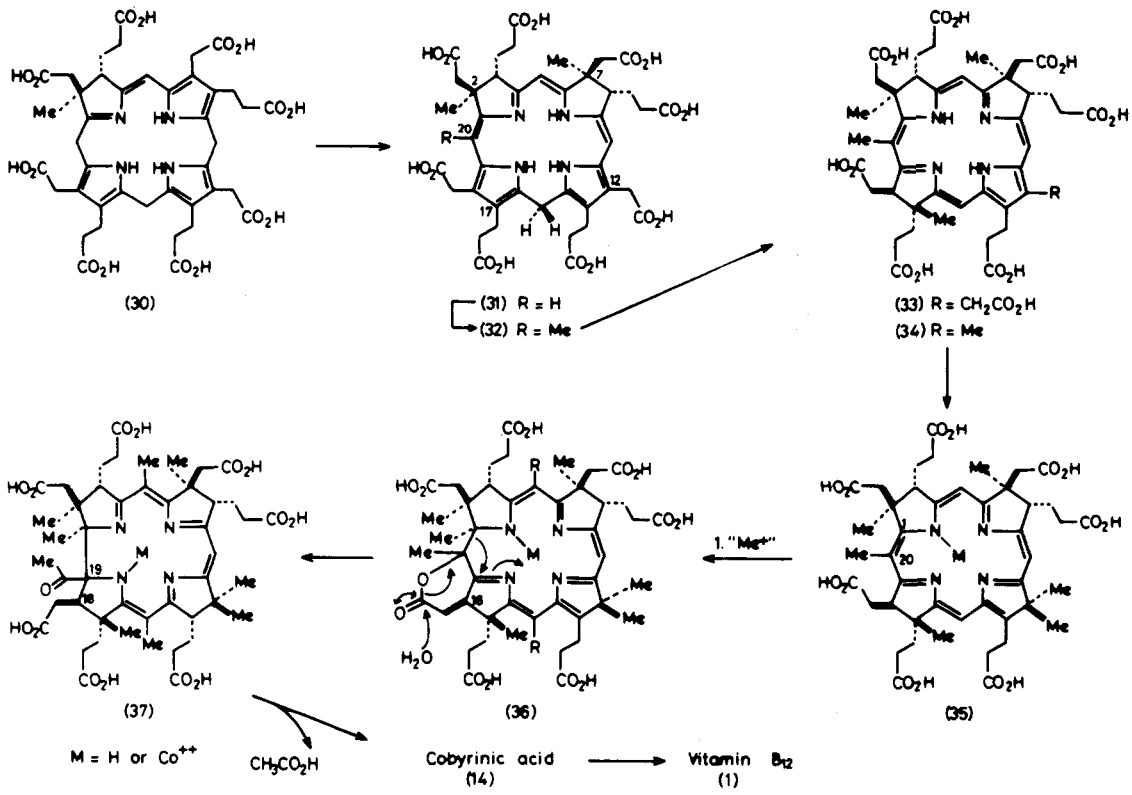
(42) Battersby, A. R.; Bushell, M. J.; Jones, C.; Lewis, N. G.; Pfenninger, A. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 13.

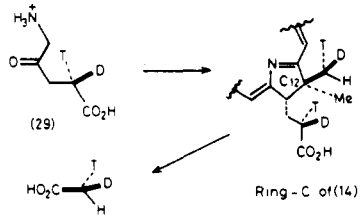
(43) Mombelli, L.; Nussbaumer, C.; Weber, H.; Müller, G.; Arigoni, D. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 9.

(44) Battersby, A. R.; Ihara, M.; McDonald, E.; Redfern, J. R.; Golding, B. T. *J. Chem. Soc., Perkin Trans. 1* **1977**, 158.

(45) Battersby, A. R.; Gutman, A. L.; Fookes, C. J. R. *J. Chem. Soc., Chem. Commun.* **1981**, 645.

**Scheme V**




immediate precursor of PBG (as **3**), and was incorporated into vitamin $B_{12}$ by growing *P. shermanii* cells. The C-12 methyl group, now carrying $^1H$, $^2H$, and $^3H$, was excised from **1** as acetic acid and the configuration of the chiral methyl group, determined by the Arigoni–Cornforth method, showed that the enzymic decarboxylation which generates the 12-*si*-methyl group occurs with overall *retention* of configuration.[46] This result matches what was found[46,47] for two similar decarboxylations of acetate residues so a uniformity seems to be emerging.

**(c) Origin of the Hydrogen Atoms at C-18 and C-19.** Though there is no change of oxidation level *overall* as the dihydro-IBC **32** is converted into cobyrinic acid (**14**), there is a possibility that the oxidation level might vary from one point to another in the multistage sequence. Indeed, there was a hint[48] that an intermediate with a C-18/C-19 double bond in **14**, or in the cobalt-free form, might be formed and subsequently reduced in vivo eventually to produce **14**. Therefore, the origin of the hydrogen atoms at C-18 and C-19 was studied in the following way.

When cobyrinic acid (**14**) was biosynthesized in $D_2O$ from the $^{13}C$-labeled[49] uro'gen-III (**10**), Experiment B, Scheme II, the $^{13}C$ NMR spectrum of the isolated ester of **14** showed (by $\beta$-shifts[50] of the $^{13}C$ signals) that the hydrogens at both C-18 and C-19 had been derived from the medium.[51] This result is fully discussed in ref 51 and the weight of evidence points strongly against the formation of a dehydrocobyrinic acid intermediate. Thus, it seems certain that for a large part of the pathway from PBG (**3**) to cobyrinic acid (**14**) external redox reagents are not required. Probably this holds true for the entire pathway.

**(d) Order of the Later C-Methylation Steps.** The biosynthesis of cobyrinic acid requires 8 *C*-methyl groups to be inserted (at C-1, 2, 5, 7, 12$\alpha$, 15, and 17 plus the one placed at C-20 and later lost as acetic acid). The sites for the first 3 methyls (at C-2, C-7, and C-20 in that order) were determined by isolation and structural work. This method could not be extended because, despite enormous efforts, no intermediates carrying 4, 5, or more methyl groups have been found. We therefore developed a different approach.

This involved biosynthesising cobyrinic acid (**14**) from the dimethylated system **15** using a cell-free enzyme preparation with the addition of only a limited amount of unlabelled SAM. After incubation to allow methylated intermediates to accumulate, an excess of [*methyl*-$^{13}C$] SAM was added to complete the formation of cobyrinic acid (**14**). Those methyl groups of **14** introduced early in the sequence of methylations should have a lower $^{13}C$ content than those inserted later and

(46) Battersby, A. R.; Deutscher, K. R.; Martinoni, B. *J. Chem. Soc., Chem. Commun.* **1983**, 698.
(47) Barnard, G. F.; Akhtar, M. *J. Chem. Soc., Perkin Trans. 1* **1979**, 2354.
(48) Dresow, B.; Ernst, L.; Grotjahn, L.; Koppenhagen, V. B. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 1048.
(49) The sample consisted of a 1:1 mixture of the *singly* $^{13}C$-*labeled molecules* produced synthetically. This was because the experiment depended on shifted $^{13}C$ NMR signals and we wanted to have no doubts about the origin of the observed shifts.
(50) Abell, C.; Staunton, J. *J. Chem. Soc., Chem. Commun.* **1981**, 856.
(51) Battersby, A. R.; Edington, C.; Fookes, C. J. R. *J. Chem. Soc., Chem. Commun.* **1984**, 527.

this can be analyzed by NMR.[52]

In the event, the signal from the C-17 group was the small one, a result which showed[53] that the fourth methyl group for the biosynthesis of **14** is added at C-17. If this occurs before **32** is decarboxylated at C-12, then the product is the system **33** based on the pyrrocorphin macrocycle.[54] But if decarboxylation precedes C-methylation, then the tetramethylated intermediate will be **34**.

With the above pulse-labeling approach shown to be successful, the experiment was run in reverse with the [*methyl*-$^{13}$C]SAM added first followed by an excess of unlabelled SAM. Now the NMR spectrum of the isolated cobyrinic acid (**14**) (as its ester) showed the C-17 methyl signal to be the largest one confirming the result above. In addition, careful integration of all the signals and standardization showed[55] that, following C-17, the order of methylation is 12$\alpha$, 1, 15, 5.[56]

**Present State and Prospect.** The biosynthesis of uro'gen-III (**10**) was illustrated in Scheme I and the post uro'gen-III stages to cobyrinic acid (**14**) are summarized in Scheme V. The first methylation on ring-A of **10** produces **30** or a tautomer and so channels material along the $B_{12}$ pathway. Further methyl groups are placed sequentially at C-7 and C-20 to yield **31** and **32**, respectively. Then, depending on the timing of decarboxylation of the C-12 acetate group, the tetramethylated macrocycle is either **33** or **34**.

Finally, knowledge of the order of the later C-methylations allows some likely intermediates beyond the tetramethylated one to be pinpointed as targets for future research. Thus, the fifth methylation at C-12 yields the corphin[57] **35** and subsequent methylation at C-1 generates **36** ready for ring-contraction. The illustrated mechanism is based on Eschenmoser's model studies[58] together with the knowledge that acetic acid is liberated (from **37**) as a result of ring-contraction. The stage on the pathway where cobalt is inserted is as yet unknown so this has been left as an open question in Scheme V. Our future aim is to answer not only this question but also to discover and establish the structures of the last remaining unknown intermediates on the long and beautiful pathway to vitamin $B_{12}$ (**1**).

*The progress outlined in this Account would not have been made without the efforts of many skillful and courageous young colleagues who have tackled the $B_{12}$ problem with me. All their names are given in the literature references and my debt to them is huge. I am also grateful to the Science and Engineering Research Council for financial support.*

(52) For the $^{13}$C-methyl signal assignments see: Battersby, A. R.; Edington, C.; Fookes, C. J. R.; Hook, J. M. *J. Chem. Soc. Perkin Trans. 1* **1982**, 2265. Ernst, L. *Liebigs Ann. Chem.* **1981**, 376.

(53) Uzar, H. C.; Battersby, A. R. *J. Chem. Soc., Chem. Commun.* **1982**, 1204. Doddrell, D. M.; Uzar, H. C.; Battersby, A. R. *J. Org. Chem.* **1983**, *48*, 3615.

(54) Johnson, J. E.; Piermattie, V.; Angst, Ch.; Diener, E.; Kratky, Ch.; Eschenmoser, A. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 261.

(55) Uzar, H. C.; Battersby, A. R. *J. Chem. Soc., Chem. Commun.* **1985**, 585.

(56) The reverse experiment was also run by others with essentially the same result: Scott, A. I.; Mackenzie, N. E.; Santander, P. J.; Fagerness, P. E.; Müller, G.; Schneider, E.; Sedlmeier, R.; Worner, G. *Bioorg. Chem.* **1984**, *12*, 356.

(57) For corphin chemistry see: Johnson, A. P.; Wehrli, P.; Fletcher, R.; Eschenmoser, A. *Angew. Chem., Int. Ed. Engl.* **1968**, *7*, 623.

(58) Rasetti, V.; Pfaltz, A.; Kratky, C.; Eschenmoser, A. *Proc. Nat. Acad. Sci. U.S.A.* **1981**, *78*, 13.