Biosynthetic and synthetic studies on the pigments of life

Alan R. Battersby

University Chemical Laboratory, Lensfield Road,
Cambridge, CB2 1EW, UK.
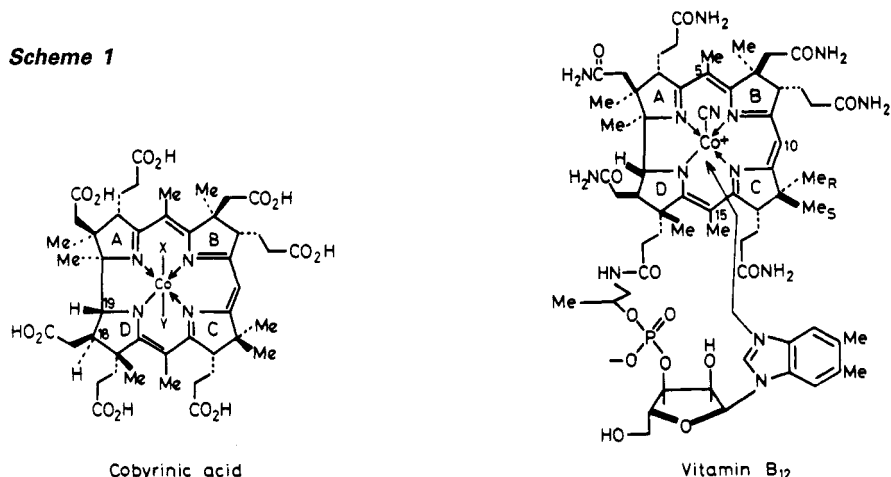
Abstract - Several examples will be described which illustrate
recent researches in Cambridge on the biosynthesis of vitamin B_{12}.
A brief historical survey to provide the background for the
latest work will be followed by the results of synthetic and
labelling experiments on the structure and oxidation level of
the first methylation product (precorrin-1) on the pathway. The
problem of when the 12-acetate residue undergoes decarboxylation
will be discussed together with experiments on pyrrocorphins,
the latter being putative intermediates on the biosynthetic
pathway. The final part of the lecture will focus on the chemistry
of hydroxymethylbilane synthase, the enzyme responsible for
assembly of four monopyrrolic building blocks into an open chain
system which is an early precursor of the macrocycle of vitamin B_{12}.
The mechanism of action of this enzyme has been proved to involve
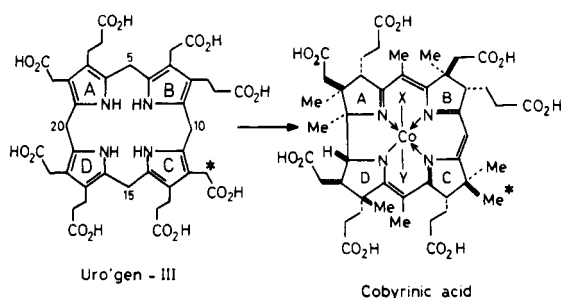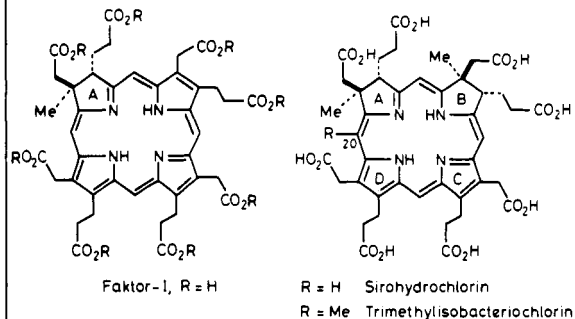a novel cofactor which has a pyrromethane structure.

INTRODUCTION

The full account of all the B_{12} biosynthetic work to be described would
require much more space than is available in this "long abstract". I
shall therefore use flow schemes of structures which are connected by
a highly condensed narrative. The first 10 years of work (before ca.
1978) by all groups in this area has to be cited via previous reviews
but full details of more recent studies can be found in the papers in
the reference list.

The structure of vitamin B_{12} is shown in Scheme 1 and it was known (ref. 1)
before the outset of our work that the vitamin is formed from cobyrinic acid,
which acts as a late precursor, Scheme 1. So the problem of discovering
how the macrocycle of vitamin B_{12} is biosynthesised resolves itself into
that of how cobyrinic acid is constructed. Knowing (ref. 1) that cobyrinic

Scheme 1

Cobyrinic acid

Vitamin B_{12}

Scheme 2

Uro'gen - III

Cobyrinic acid

Scheme 3

Faktor-I, R = H

R = H   Sirohydrochlorin
R = Me   Trimethylisobacteriochlorin

acid, as for protohaem and chlorophyll, is biosynthesised from uroporphyrinogen III(uro'gen-III), Scheme 2, one can see the many steps (C-methylations, decarboxylation etc.) which are necessary to complete the transformation. It was established that the necessary decarboxylation of the acetate residue at C-12 of uro'gen-III is not the immediate next step on the pathway and this led on to experiments which proved that C-methylation is the next step (ref. 1). This conclusion depended on the isolation of three pigments from organisms which produce vitamin B$_{12}$; their structures are in Scheme 3. These materials are air-oxidised forms of the true biosynthetic intermediates and it was proved (ref. 2) that the true dimethylated intermediate is the dihydroisobacteriochlorin in Scheme 4 called precorrin-2 (ref. 3). It is probable that the illustrated trimethylated system, precorrin-3, is also produced and further transformed at this dihydro oxidation level, Scheme 4.

Scheme 4

R = H
R = Me
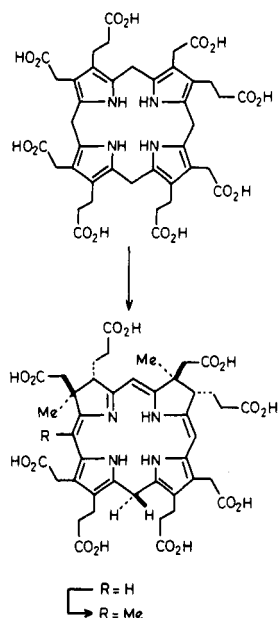
Scheme 5
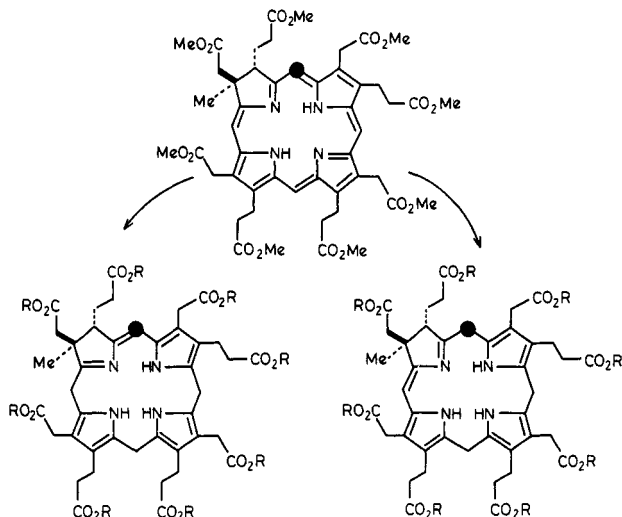
OXIDATION LEVEL OF THE MONOETHYLATED INTERMEDIATE

It was known (ref. 4) that some reduced form of the isolated mono-methylated system is involved in the biosynthesis but neither the oxidation level nor the structure was known. We had earlier developed a photochemical synthetic route to chlorins (ref. 5). Then 5-^{13}C-Faktor-I ester was synthesised (cf. ref. 6) shown as the top structure in Scheme 5. Reduction of this product produced two isomeric tetrahydrochlorins, Scheme 5, and n.m.r. showed that there had been a preference for the tautomer on the right hand side of Scheme 5. Moreover, incubation of the acids derived from these labelled tetrahydrochlorins in Scheme 5 with the cobyrinic

acid producing enzyme system gave cobyrinic acid ^{13}C-labelled specifically at C-5 (ref.7). Thus, precorrin-1 is a <u>tetrahydrochlorin</u> and the oxidation level of uro'gen-III is preserved as it is at the next stage of precorrin-2. Indeed there are steadily increasing indications that the entire biosynthesis of vitamin B_{12} is carried through without any involvement of external redox reagents.

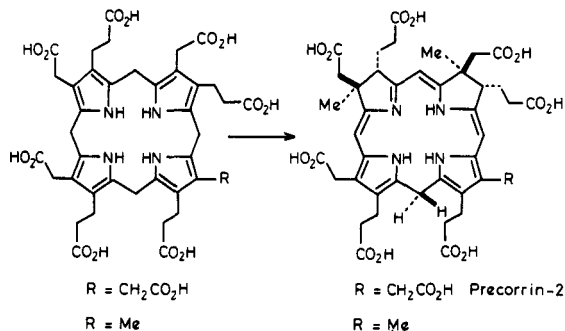**Scheme 6**

┌─ R = H
└→ R = Me

R = CH_2CO_2H or Me
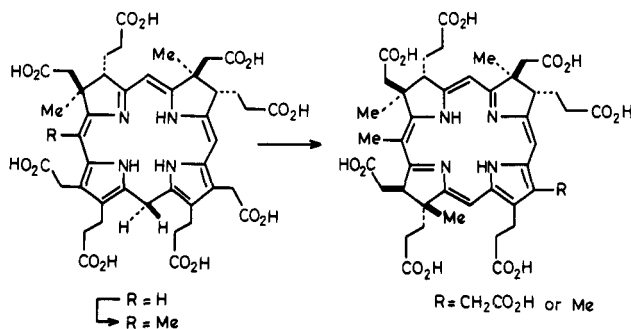
No intermediate resulting from the introduction of the fourth methyl group has so far been isolated. However, it was shown in 1982 by pulse-labelling (ref. 8) that this fourth methyl group is introduced at C-17 which should yield a pyrrocorphin, Scheme 6; for the chemistry of pyrrocorphins, see ref. 9. Scheme 6 illustrates that either the C-12 acetate residue is enzymically decarboxylated <u>before</u> the methylation step or <u>afterwards</u>. Depending on which sequence is used, there will either be one pyrrocorphin intermediate with R=Me or two, one having R= CH_2CO_2H and the other R=Me.

## TIMING OF DECARBOXYLATION OF THE 12-ACETATE GROUP

It was known (ref. 10) that the crude enzyme preparation from <u>Propionibacterium shermanii</u> would accept, rather than its normal substrate uro'gen-III with R=CH_2CO_2H, also the synthetic 12-methyl analogue with R=Me, Scheme 7. By using purified enzymes, it was possible also to carry out the next C-methylation at C-20 of the analogue with R=Me, Scheme 8. In this way, the analogue of precorrin-3 having R=Me, Scheme 8, was prepared in ^{14}C-labelled form and isolated as the aromatised isobacteriochlorin system (ref. 11).

Comparison was made of the incorporation of precorrin-3[†] (Scheme 8, R=CH_2CO_2H) relative to that of its 12-decarboxylated analogue (Scheme 8, R=Me) using the enzyme system which biosynthesises cobyrinic acid. Precorrin-3 was incorporated well into cobyrinic acid (up to 10%) whereas the 12-decarboxylated analogue did not act as a significant precursor (incorporation 0.025%), (ref. 11).
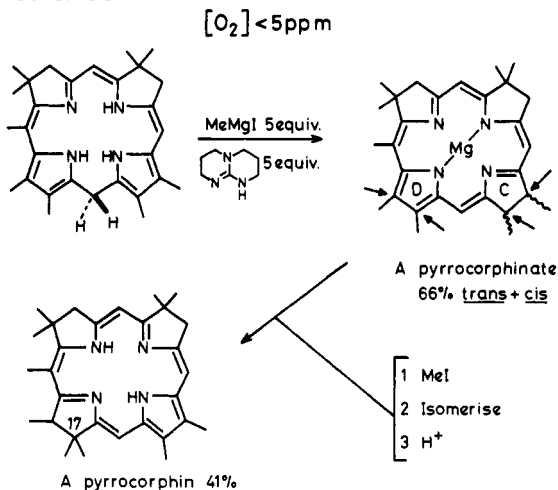
Scheme 7

R = CH_2CO_2H

R = Me

R = CH_2CO_2H Precorrin-2

R = Me

Scheme 8

R = CH_2CO_2H
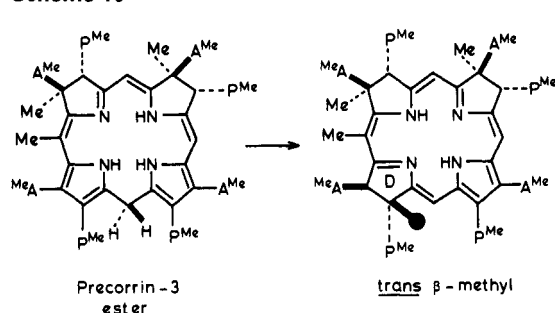
R = Me

R = CH_2CO_2H Precorrin -3

R = Me
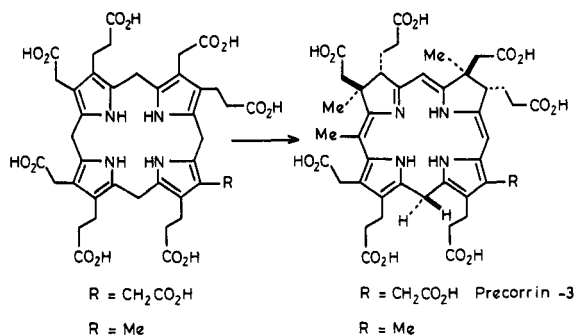
[†]The labelled materials are administered in the aromatised form (as isobacteriochlorins) to the crude enzyme system which is known (ref. 1) to be capable of reducing the aromatic forms back to the dihydro-forms.

For reasons to be discussed in the lecture, these results indicate that:
(a) precorrin-3 does not undergo decarboxylation of its 12-acetate group
(b) the 12-methyl analogue of precorrin-3 is not a biosynthetic intermediate
and (c) two pyrrocorphins are likely to exist with, respectively, R=CH$_2$CO$_2$H
and R=Me at the 12-position since for mechanistic reasons, the 12-acetate
residue is probably decarboxylated before the 12-ß-methyl group of cobyrinic
acid is introduced (see structure of cobyrinic acid, Scheme 1).

Scheme 9

[O$_2$]<5ppm

A pyrrocorphinate
66% trans + cis

[
1 MeI
2 Isomerise
3 H$^+$
]

A pyrrocorphin 41%
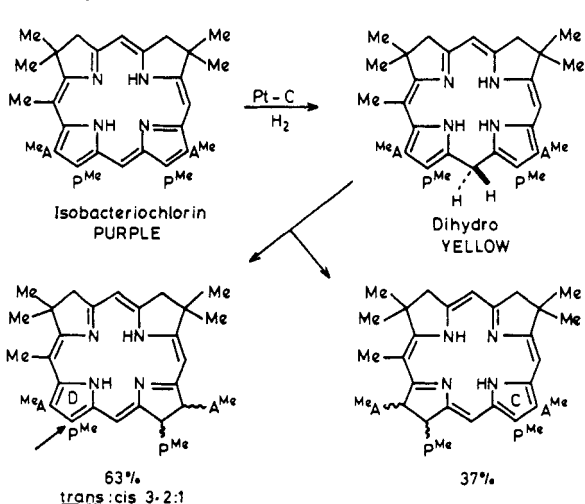
Scheme 10

Precorrin-3
ester

trans ß-methyl

SYNTHESIS OF PYRROCORPHINS WITH ESTER SIDE CHAINS

Scheme 9 shows one example of the outstanding work of Eschenmoser et
al (e.g. ref. 12) in which a dihydroisobacteriochlorin was isomerised
and C-methylated with a preference for C-17. It may thus be possible
to prepare the ester of the pyrrocorphin in Scheme 6 from precorrin-3
ester, see Scheme 10. There are two major problems (a) the conditions
using a Grignard reagent in Scheme 9 cannot be used in the presence of
esters and (b) precorrin-3 ester is chiral and so stereochemical problems
are superimposed on control of regio-chemistry; 16 isomers can arise.

Conditions were eventually found to allow pyrrocorphins with ester side
chains to be prepared, Scheme 11; the isomers were separated and their
structures determined (ref. 13). Excitingly, precorrin-3 has been converted
into a mixture of pyrrocorphinates, Scheme 12, ready for C-methylation
and subsequent incorporation experiments with labelled materials (ref.
13).

Scheme 11

Isobacteriochlorin
PURPLE

Pt-C / H$_2$

Dihydro
YELLOW

63%
trans:cis 3·2:1

37%

Scheme 12

Precorrin-3
ester
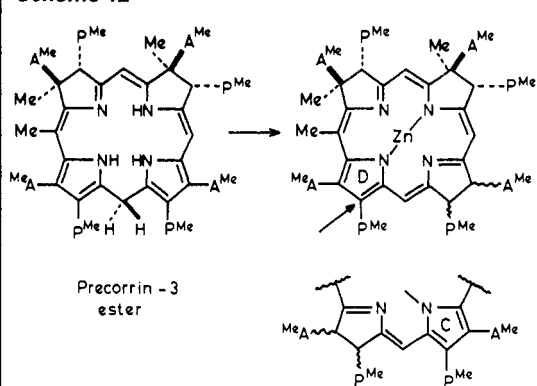
THE NOVEL COFACTOR USED BY HYDROXYMETHYLBILANE SYNTHASE

We have been concerned so far with that part of the biosynthetic pathway to vitamin B_{12} beyond uro'gen-III, Scheme 2. Now we turn to the enzyme hydroxymethylbilane synthase (HMBS), also called deaminase, which builds the hydroxymethylbilane (middle row, left) from four molecules of porphobilinogen, PBG (ref. 14), Scheme 13. A second enzyme, cosynthetase, carries out the remarkable conversion of hydroxymethylbilane into uro'gen-III, (ref. 14).

The first PBG unit to be bound to HMBS is connected covalently to the protein through some group X, Scheme 13; what is X? The gene for HMBS from <u>Escherichia coli</u> was cloned and overexpressed to allow production of more than 200 times the wild-type yield of enzyme (ref. 15). Now experiments were possible on the scale needed for ^{13}C-n.m.r. spectroscopy.

Scheme 13
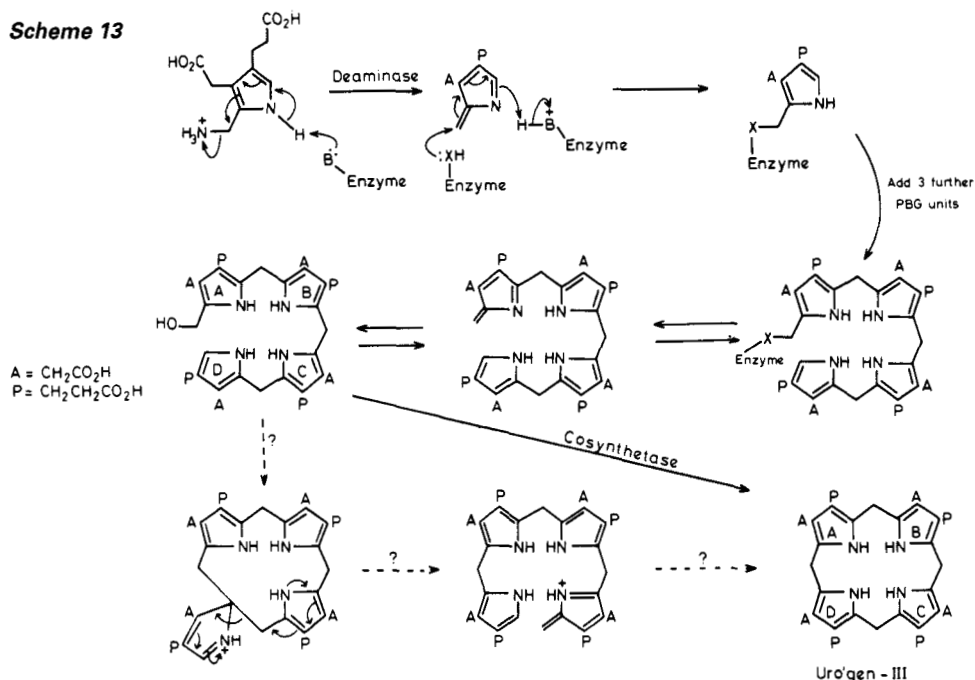
The isolated HMBS (called <u>holoenzyme</u>), shown to be unloaded, was loaded limited quantity of [11-^{13}C]PBG. Fast protein liquid chromatography yielded enzyme-^{13}C-PBG$_1$ mono-complex and enzyme-^{13}C-PBG$_2$ di-complex, Scheme 14. The ^{13}C-spectrum from the former complex showed sharp ^{13}C-signals, Figure 1, as did the natural abundance ^{13}C-spectrum of unlabelled enzyme-^{12}C-PBG$_1$ mono-complex. The difference between these two spectra, showed one strong signal at $\delta 24.6$ from the ^{13}C-enriched ^{13}CH$_2$ group attached to the X-group, Figure 2; the shift corresponded exactly to a pyrrole-^{13}CH$_2$-pyrrole system (ref. 16).

Scheme 14

Mono-complex

Di-complex
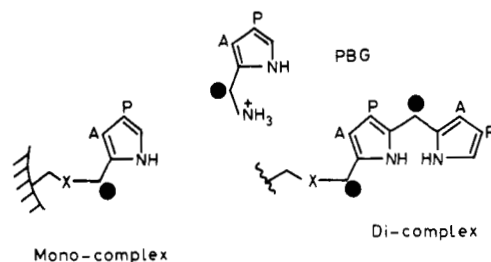
Thus, the PBG unit added first binds to a tightly bound pyrrole residue already present in the enzyme. Turnover of 12 mol ^{14}C-PBG per mol HMBS
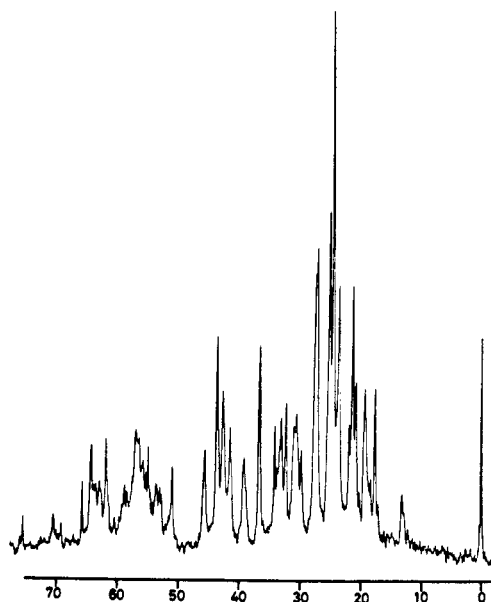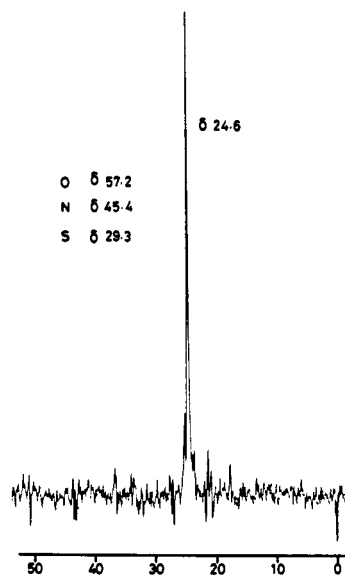
FIGURE 1

FIGURE 2

followed by reisolation of the holoenzyme gave <u>unlabelled enzyme</u> still
carrying the tightly bound pyrrolic system. Hence the latter is present
as a cofactor in the enzyme in addition to the PBG units which are turned
over (ref. 16).

The nature of the tightly bound pyrrolic system was studied by treating
HMBS with acidic <u>p</u>-dimethylaminobenzaldehyde (Ehrlich's reagent). This
caused striking spectroscopic changes shown by studies with synthetic
model systems, to be characteristic of a <u>pyrromethane</u> group. The pyrromethane
cofactor is bound to the protein through a group Y, Scheme 15. Cleavage
of the pyrromethane from the Y-group by acid caused loss of enzymic activity
and the pyrromethane fragment reacted with another molecule of the same
fragment to generate mainly uroporphyrinogen-I, uro'gen-I, which is isomeric
with uro'gen-III (Scheme 13) but has the A & P groups running in sequence
around the periphery of the macrocycle. It is the formation of this
product which leads to knowledge of the acidic side-chains of the pyrromethane
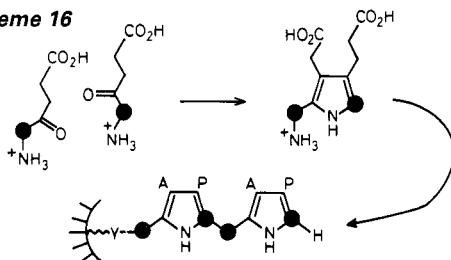cofactor.

The above enzymically inactive protein lacking the pyrromethane cofactor
(apoenzyme) can be restored to active enzyme by incubation with PBG
(ref. 17). By using 11-^{13}C-PBG (Scheme 15) in this incubation, HMBS
was regenerated with a ^{13}C-atom directly connected to the Y-group.
^{13}CN.m.r. difference spectroscopy, as for the X-group experiments, then
revealed that Y is <u>sulphur</u>. Thus, the pyrromethane cofactor is attached
to the protein <u>via</u> the sulphur atom of a cysteine residue (ref. 17).

It was also important to generate labelled HMBS in a natural way by growing
the <u>E. coli</u> which produces the enzyme in the presence of ^{13}C-labelled
aminolaevulinic acid, the precursor of PBG. The labelled precursor was
incorporated well into HMBS to generate the labelling pattern shown in
Scheme 16. ^{13}C-N.m.r. difference spectroscopy confirmed unambiguously

Scheme 15
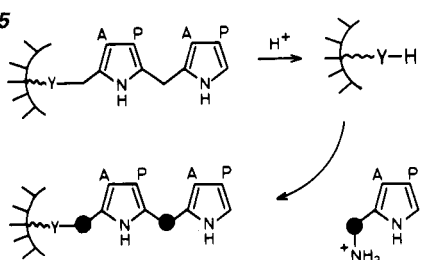
Scheme 16

that Y is sulphur (ref. 18) and showed signals of the correct chemical shift for the other three ^{13}C-labels in that cofactor, Scheme 16 (ref. 18).

All these results interlock to prove that the assembly process on HMBS involves construction of a hexapyrrole from which the hydroxymethylbilane is cleaved, Scheme 17. The bound pyrromethane of HMBS represents a unique enzymic cofactor which the apoenzyme can self-assemble from PBG onto a cysteine residue; this cofactor is essential for the catalytic process (but is not turned over) yet is built from the substrate for that enzyme. As shown in Scheme 17, the hydroxymethylbilane is ring-closed <u>with rearrangement</u> to form uro'gen-III. There is strong evidence from synthetic studies (ref. 19) that the dashed pathway in Scheme 13 <u>via</u> the spiro-system is used to achieve this formation of uro'gen-III.

Scheme 17
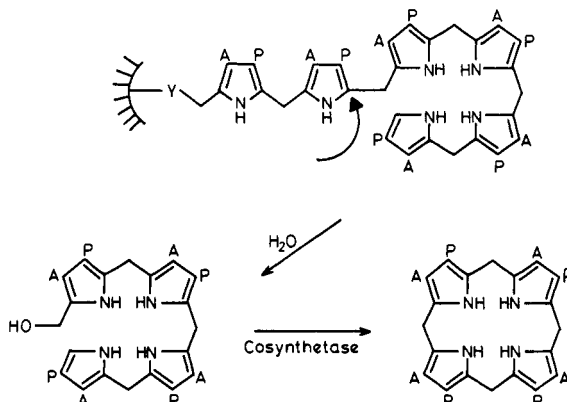
I have shown in this lecture how research on the biosynthesis of vitamin B_{12} requires a broad-ranging approach involving demanding total synthesis, sophisticated spectroscopy, structural studies, enzymology and molecular biology. The pathway has been elucidated rather clearly as far as the third or fourth C-methylation stage and there is considerable information about the later ring-contraction processes. Advances have been made by many research groups and this lecture has covered the latest advances from Cambridge. The reader interested in full coverage should consult ref. 1, the book cited in ref. 4 and ref. 20 together with references given in these review series.

Though work on the unknown part of the B_{12} biosynthetic pathway is now experimentally very demanding, future studies will surely lead to a full understanding of how this marvellous molecule is built in Nature.

REFERENCES

1. Reviewed by A. R. Battersby & E. McDonald, in "B_{12}", ed. D. Dolphin, p. 107, Wiley, New York, (1982).

2. A. R. Battersby, K. Frobel, F. Hammerschmidt & C. Jones, <u>J. Chem. Soc., Chem. Commun.</u>, 455, (1982).

3. H. C. Uzar, T. A. Carpenter, F. J. Leeper & A. R. Battersby, <u>J. Chem. Soc., Perkin Trans. 1</u>, 1689, (1987).

4.   M. Imfeld, D. Arigoni, R. Deeg and G. Müller, in "Vitamin B_{12}",
      eds. B. J. Zagalak & W. Friedrich, p. 315, de Gruyter, Berlin,
      (1979).

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

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

7.   R. D. Brunt, F. J. Leeper & A. R. Battersby, unpublished work.

8.   H. C. Uzar & A. R. Battersby, J. Chem. Soc., Chem. Commun., 1204
      (1982).

9.   e.g. J. E. Johnson, V. Piermattie, Ch. Angst, E. Diener, Ch. Kratky
      and A. Eschenmoser, Angew. Chem. Int. Ed. Engl., 20, 261, (1981).

10.   A. R. Battersby, C. Bladon, J.-J. Huang & S. Seo, J. Chem. Soc.,
      Chem. Commun., 1492, (1986).

11.   F. Blanche, S. Handa, D. Thibaut, C. L. Gibson, F. J. Leeper &
      A. R. Battersby, J. Chem. Soc., Chem. Commun., in press.

12.   C. Leumann, K. Hilpert, J. Schreiber & A. Eschenmoser, J. Chem.
      Soc., Chem. Commun., 1404, (1983).

13.   C. L. Gibson & A. R. Battersby, unpublished work.

14.   A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham & E. McDonald,
      Nature, 285, 17, (1980); A. R. Battersby, C. J. R. Fookes, K. E.
      Gustafson-Potter, E. McDonald & G. W. J. Matcham, J. Chem. Soc.,
      Perkin Trans. 1, 2427, (1982).

15.   C. Abell, P. R. Alefounder, A. R. Battersby and N. Crockett, in
      press.

16.   G. J. Hart, A. D. Miller, F. J. Leeper & A. R. Battersby, J. Chem.
      Soc., Chem. Commun., 1762, (1987).

17.   G. J. Hart, A. D. Miller & A. R. Battersby, Biochem. J., in press.

18.   U. Beifuss, G. J. Hart, A. D. Miller & A. R. Battersby, Tetrahedron
      Lett., in press.

19.   W. M. Stark, G. J. Hart & A. R. Battersby, J. Chem. Soc., Chem.
      Commun., 465, (1986).

20.   A. R. Battersby, Acc. Chem. Res., 19, 147, (1986).