The Bakerian Lecture, 1984

Biosynthesis of the pigments of life

BY A. R. BATTERSBY, F.R.S.

University Chemical Laboratory, Lensfield Road,
Cambridge CB2 1EW, U.K.

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Many vitally important functions in living systems are carried out by metal ions held as complexes within organic ligands, the organic part of the molecule being a tetrapyrrolic macrocycle. Chlorophyll, haemoglobin, the cytochromes and vitamin B\textsubscript{12} all fall into this family of ‘pigments of life’, a list that emphasizes their central importance in living systems.

Research on the biosynthesis of these pigments has involved the synergistic combination of synthesis, structure determination, carbon nuclear magnetic resonance and isotopic labelling with radioactive and stable isotopes in conjunction with enzymology and kinetics.

The lecture describes the logical series of experiments based on these approaches which have led to a step-by-step knowledge of the biosynthesis of the parent macrocycle (uroporphyrinogen-III) from which the other pigments are derived.

One main pathway from the parent macrocycle involves oxidative transformations and leads eventually to protohaem required \textit{inter alia} for haemoglobin and myoglobin. The second important pathway makes use of C-methylation to convert the parent macrocycle through many stages finally into vitamin B\textsubscript{12}.

The biosynthetic studies on vitamin B\textsubscript{12} are outlined with particular emphasis on the use of isotopic labelling with both radioactive and stable isotopes of carbon and hydrogen. Roughly two-thirds of the entire biosynthetic pathway to vitamin B\textsubscript{12} has now been elucidated. The scarcity of several of the known intermediates on the pathway severely hampers future researches and progress towards the total synthesis of these key materials is reviewed. Finally, the lecture brings out the evolutionary interest of what has been discovered about the biosynthesis of the pigments of life.

The aim of this Bakerian Lecture is to allow any scientist to know what are the pigments of life, to appreciate why they are so important and to be able to enjoy the amazing chemistry by which living systems biosynthesize these key substances. Since the lecture is not directed solely to experts in this field, the necessary background information will be given throughout and this means that most of the experimental detail must be stripped away. Only a small fraction of our total effort can be described; we will not be able to look together even at the tip of the iceberg, only at the top of the tip. But it is important to realize that all the detailed back-up
studies, including syntheses, spectroscopy, control experiments and the like are always there, unmentioned but providing nevertheless the main structure of the iceberg on which the tip stands. Our research on the biosynthesis of the pigments of life started in 1968 and this lecture will survey what has been achieved in Cambridge during those 16 years; recent reviews give full accounts including work from other laboratories (Battersby & McDonald 1975, 1979, 1982; Battersby et al. 1980).

Many vitally important functions in living systems are carried out by metal ions held as complexes within tetrapyrrolic macrocycles and these complexes are brightly coloured. A familiar example is the red pigment of blood which is the iron complex ($\text{Fe}^{2+}$) of the macrocycle protoporphyrin-IX $(1)$. This complex, called protohaem $(2)$, is responsible for the oxygen-carrying properties of haemoglobin and myoglobin and it is bright red in its oxygenated form. Protohaem $(2)$ has $\text{Fe}^{2+}$ held at the heart of an organic macrocycle composed of four five-membered nitrogenous rings joined by single carbon bridges, the so-called meso-bridges. The double bonds of the macrocycle are fully conjugated, a feature responsible for its aromatic character and its colour. Structure $(2)$ has a variety of groups around the periphery but particular notice should be taken of the two propionate groups on rings C and D which occupy adjacent positions on the periphery (see scheme 1).

![Scheme 1](http://rspb.royalsocietypublishing.org/)

The second example, chlorophyll $a$ $(3)$, is the fundamental pigment, for without photosynthesis, life on this planet as we know it would cease. The structure of chlorophyll $a$ $(3)$ is related to that of protohaem $(2)$. Again we see an organic
The macrocycle built from four five-membered rings with *meso*-bridges which sequesters a metal ion, now Mg\(^{2+}\). In this case, the propionate residue on ring C has been disguised by being cyclized to form a five-membered ring but this does not prevent one recognising that, as for protohaem (2), the two propionate groups on rings C and D are adjacent. The oxidation level of chlorophyll *a* (3) differs from that of protohaem (2) (10 double bonds for the former, 11 for the latter) and this changes its colour to the beautiful green of higher plants. The world would have a very different appearance if that extra double bond were present!

Vitamin B\(_{12}\) (4), the anti-pernicious anaemia factor, is the last example and is by far the most complex structure. The macrocycle, which here holds a Co\(^{3+}\) ion, shows the familiar features of the earlier systems including the adjacent propionate groups on rings C and D. However, the macrocycle of vitamin B\(_{12}\) differs by having a direct link between rings A and D and by carrying many methyl groups around its periphery (see scheme 2).

![Scheme 2](image)

The key roles played in living organisms by protohaem, the chlorophylls and vitamin B\(_{12}\) and by closely related systems such as the cytochromes have led to the title ‘pigments of life’. How are they constructed in Nature?

When our work started in this area, we were building on the admirable contributions of a group of pioneers, Bogorad, Granick, Neuberger, Rimington and Shemin working in Britain and the U.S.A. during the 1950s. They had found (Battersby & McDonald 1975) that protohaem (2) and chlorophyll *a* (3) are biosynthesized from the same parent uroporphyrinogen-III (6), usually shortened to uro’gen-III; we shall see later that this holds true also for vitamin B\(_{12}\). This central position for uro’gen-III (6) in a family of pigments of immense biological importance highlighted the primary need to understand its biosynthesis.

The foregoing workers had also found that uro’gen-III (6) is biosynthesized from four molecules of a monopyrrole called porphobilinogen, PBG (5) by the cooperative
action of two enzymes, deaminase and cosynthetase (scheme 3). The product from straightforward head-to-tail combination of four PBG units would be uro’gen-I (7) in which the acetic and propionic acid groups appear alternately in sequence around the macrocycle. Indeed, uro’gen-I is eventually (the significance of ‘eventually’ will become clear later) formed when PBG is treated with deaminase alone. However, in living systems, deaminase always works with cosynthetase and then the product is uro’gen-III (6). It is important to realize that uro’gen-I (7) is not rearranged to uro’gen-III (6) by cosynthetase or by deaminase with cosynthetase. So the rearrangement step or steps occur at some point or points along the right hand arrow in scheme 3. Living systems have thus developed over evolutionary time some highly specific way to generate enzymically the unexpected uro’gen isomer, namely, uro’gen-III (6) ready for modification into the pigments of life.

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Scheme 3. Enzymic formation of uro’gen-III (6) from porphobilinogen (5).

Close inspection of uro’gen-III (6) shows it to be a fascinating substance. Not only is its structure quite unexpected but uro’gen-III is not a pigment; it is a colourless reduced macrocycle. Moreover, it does not form complexes with the ions of divalent metals but yet it is the precursor of such complexes. Finally, we see that uro’gen-III (6) has the two propionic acid groups adjacent on rings C and D; this feature of the parent then appears in protohaem (2), chlorophyll a (3) and vitamin B$_{12}$ (4) which are the offspring.

It is clear that the unexpected structure for uro’gen-III (6) must arise by molecular rearrangement in which C-11 of a PBG unit (5, see scheme 3) separates from its original pyrrole ring and appears attached in some different way in the final uro’gen-III structure (6); one or more such rearrangements could be involved. Mathematically, there are 24 ways in which four PBG units can be broken up in this fashion and reassembled to form uro’gen-III (6). Since four identical initial building blocks are involved, a special approach was needed to discover what actually happens and a method was devised based on isotopic labelling with $^{13}$C with assay by $^{13}$C-nuclear magnetic resonance spectroscopy ($^{13}$C-n.m.r.).
The approach can be illustrated by considering the simple chain of carbon atoms shown top left in scheme 4; the circles represent carbon atoms which are not identical to the carbon atoms represented by squares. Imagine a reaction occurring in which the arrowed bond breaks and a new one forms between the two atoms at the ends of the chain to produce the top centre structure. If the two terminal carbons of the starting material are now replaced by $^{13}$C atoms (shown as full circle and square), then the proton-decoupled $^{13}$C-n.m.r. spectrum of this substance will show two strong singlets. When the reaction as above occurs, the two $^{13}$C-atoms become directly bonded with the result that each $^{13}$C-signal is split, so two doublets will be observed.

The final step in the planning was to consider the effect of mixing ca. one part of the $^{13}$C$_2$-starting material with ca. four parts of unlabelled starting material. If the illustrated reaction is intramolecular (occurring within one molecule) then the product will still be a mixture of unlabelled molecules and doubly labelled molecules with the $^{13}$C-atoms directly bonded. So the $^{13}$C-n.m.r. spectrum of the product will still show two doublets. In contrast, if the reaction is not intramolecular, then a ‘circles’ piece from a labelled molecule will usually become joined to a ‘squares’ piece from unlabelled starting material (because there is an excess of the latter). Hence the product will consist largely of the two species in the box, scheme 4. The $^{13}$C-atoms are no longer directly bonded and thus the $^{13}$C-n.m.r. spectrum will show two singlets. This was to be the approach in our studies of uro'gen-III biosynthesis and it worked superbly. The method allows the study of carbon–carbon bond breaking and bond formation and it rigorously distinguishes between inter- and intramolecular processes.

It is not possible in this lecture to cover details of the synthetic work and basic n.m.r. spectroscopy involved in applying this approach, nor can we deal with the isolation and purification of the necessary enzymes; we will leap forward to the result (Battersby et al. 1976). The outcome was that the three PBG molecules (5)
which provide ring A with C-20, ring B with C-5 and ring C with C-10 of uro'gen-III (6) are incorporated as intact units without rearrangement as indicated on the right hand side of scheme 5. In contrast, the arrowed PBG molecule which appears as ring D undergoes an intramolecular rearrangement at some stage in the assembly process such that the rearranged carbon atom forms the bridge at C-15 of uro'gen-III (see scheme 5).

Scheme 5. Mode of assembly of four molecules of porphobilinogen to form uro'gen-III.

These experiments established precisely what happens. However, they do not show when the single intramolecular rearrangement occurs and this we must now consider. I shall go directly to the decisive experiments. It is evident that the rearrangement could occur at the beginning of the assembly of the four PBG units or at the end or at any stage between these extremes. In brief, appropriate experiments eliminated the possibility of rearrangement at the outset (Battersby et al. 1981a) while many others (Battersby et al. 1981b) gradually strengthened the probability that the rearrangement must be occurring after assembly of a linear tetrapyrrole system. Such a system, called a bilane, was synthesized as indicated in scheme 6; it should be noted that this bilane (8) represents the unrearranged head-to-tail assembly of four PBG units (5). The bilane (8) underwent spontaneous non-enzymic ring-closure in solution to form uro'gen-I (7); so the non-enzymic reaction occurred without rearrangement. In contrast, treatment of the bilane (8) with the combination of deaminase and cosynthetase caused marked acceleration of the ring-closure which occurred with rearrangement to generate uro'gen-III (6) scheme 7. Naturally, the appropriate $^{13}$C-labelling experiments were carried out (illustrated in scheme 7) and as above these involved heavy dilution of the labelled sample with unlabelled bilane (8). The results proved (Battersby et al. 1981d) that during the ring-closure process, the terminal ring of the bilane system is enzymically rearranged by an intramolecular mechanism to form uro'gen-III (6). The circles and triangles in scheme 7 show exactly which atoms become bonded together in the rearranged product (6).

Five isomers of the unrearranged bilane (8) were also synthesized in which the order of the acetic and propionic acids on the pyrrole rings was changed relative to bilane (8). Only two of these analogues acted as substrates for deaminase-cosynthetase and they were enzymically transformed far less efficiently than was
In summary, the experiments so far had shown that the pigments of life are built by first assembling four PBG units (5) to generate a linear unrearranged tetrapyrrole; this subsequently undergoes a single enzyme-catalysed rearrangement (intramolecular) affecting ring D and the carbon atom which provides C-15 of uro'gen-III (6).

The next leap forward came as a result of studying the rates of several of the foregoing enzymic reactions. Though the rate of ring-closure of the aminomethylbilane (8) to uro'gen-III (6) was strongly accelerated by deaminase-cosynthetase, nevertheless this enzymic rate was slower by a factor of ca. 15 than the rate at
which uro’gen-III (6) was produced enzymically by PBG (5) under equivalent conditions (Battersby et al. 1982b). It follows that the aminomethylbilane (8) cannot be, in that exact form, a true intermediate on the biosynthetic pathway from PBG (5) to uro’gen-III (6). We were thus launched into a series of careful rate studies on the action of deaminase alone (no cosynthetase) on the bilane (8). The observation was made that enzymic acceleration of ring-closure occurred but there was a marked lag during the first few minutes of the run before formation of uro’gen-I (7) reached the maximum rate (figure 1); this observation proved to be highly important (Battersby et al. 1982b). It was in sharp contrast to the results we had obtained when deaminase and cosynthetase were used in combination; then uro’gen-III (6) was formed from the bilane (8) without any detectable initial lag.

The new intermediate being accumulated during this lag period was similarly formed during a very pronounced initial lag when PBG (5) was treated with deaminase alone (Battersby et al. 1982c). Figure 2 shows that by the time virtually all of the PBG had been consumed (at arrow A), uro’gen formation (curve A) had reached only ca. 10% of its eventual maximum. Clearly an enzyme-free intermediate with a half-life of ca. 5 min had been formed which after 25 min had changed into uro’gen without consumption of additional PBG; this uro’gen was proved to be
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**Figure 1.** Rate plots for incubation of the unrearranged aminomethylbilane (8) at pH 7.75 with increasing amounts of deaminase (free of cosynthetase); optical density at 406 nm is a measure of the uro'gen-I (7) formed.

**Figure 2.** Conversion of porphobilinogen by deaminase into the hydroxymethylbilane (9) at 37 °C and pH 8.25, followed by ring-closure: A, without additional deaminase; B, in the presence of additional deaminase; C, with added deaminase-cosynthetase.
uro'gen-I (7). Repetition of this experiment with addition of a second large quantity of deaminase at arrow B in figure 2 showed that the rate of uro'gen-I formation was virtually unchanged (curve B, figure 2). This showed that uro'gen-I (7) had been formed non-enzymically from the intermediate and that having built this intermediate, deaminase had completed its task. The non-enzymic formation of uro'gen-I (7) from the intermediate strongly indicated a linear tetrapyrrrole structure formed by head-to-tail assembly of four PBG units (5).

For the third experiment, the intermediate was again generated and at point C, figure 2, the deaminase–cosynthetase mixture was added; virtually instantaneous ring-closure was observed and the product was proved to be uro'gen-III (6), that is ring-closure and rearrangement had occurred. Since deaminase alone had no effect on the intermediate, it was highly likely that uro'gen-III (6) had been formed from it by cosynthetase.

![Scheme 8: Synthesis of hydroxymethylbilane (9)](http://rspb.royalsocietypublishing.org/)

\[ \text{Scheme 8. Synthesis of hydroxymethylbilane (9).} \]

The intermediate was proved to be the unrearranged hydroxymethylbilane (9) (scheme 8) by appropriate 13C-labelling experiments in combination with n.m.r. spectroscopy (Battersby et al. 1982c). Rigorous confirmation of this structure (9) came from the unambiguous synthesis of this highly labile substance as shown in scheme 9. The synthetic and natural samples of hydroxymethylbilane (9) were
found to be excellent and identical substrates for cosynthetase, the product in each case being uro’gen-III (6) (Battersby et al. 1982c).

At this stage, it was possible to define for the first time the roles of deaminase and cosynthetase in the biosynthesis of the pigments of life. Deaminase joins four PBG units (5) head-to-tail and in the absence of cosynthetase releases the unrearranged hydroxymethylbilane (9) into solution; deaminase is the enzyme for assembly and is not a ring-closing enzyme. Cosynthetase then converts the hydroxymethylbilane (9) by a single intramolecular rearrangement of ring D into uro’gen-III (6); thus cosynthetase is the ring-closing and rearranging enzyme.

The foregoing knowledge and synthetic methodology made it possible to probe the specificity and action of cosynthetase by synthesizing hydroxymethylbilanes which were isomeric with the natural substrate or slightly modified in some other way. Six bilanes were constructed including the reversed ring D bilane (10) (Battersby et al. 1981c) and the two bilanes (11) and (12) (scheme 10), each of the latter two lacking one of the carboxyl groups on the side chains of ring D (Battersby et al. 1983b). The action of cosynthetase on the reversed ring D bilane (10) gave the remarkable result that ca. 45% of the bilane which was ring-closed by the enzyme (allowance had to be made for the extent of non-enzymic ring-closure which occurs entirely without rearrangement) had undergone inversion of ring D to produce uro’gen-I (7). Incidentally, this result excludes the possibility that formation of uro’gen-III (6) from the regular hydroxymethylbilane (9) involves rearrangement of (9) into (10) as a first step. The bilane (12), lacking the carboxyl group of the propionic acid residue on ring D of the natural bilane (9), was a good substrate for cosynthetase but the efficiency of ring D inversion fell to ca. 65%; for the natural substrate (9), the inversion is quantitative. So though the propionic acid residue on ring D of the natural bilane (9) does not play an essential role in the interaction with the enzyme, it has, nevertheless, a contribution to make. In contrast, the bilane (11) lacking the carboxyl group of the acetic acid residue normally on ring D was an extremely poor substrate for cosynthetase thus emphasizing the importance of this acetic acid residue on ring D of the natural bilane (9).

† The systematic name for deaminase is hydroxymethylbilane synthase (EC 4.3.1.8) and for cosynthetase is uroporphyrinogen-III synthase (EC 4.2.1.75).
Recent studies have shown (scheme 11) that during the building of hydroxymethylbilane (9), the first PBG unit (corresponding to ring A of the bilane) becomes covalently bound to deaminase with the release of one mole of ammonia (Battersby et al. 1983g,f). The PBG units forming rings B, C and D of the bilane are then added sequentially in that order (Battersby et al. 1983g) and three more moles of ammonia are released. With the bound tetrapyrrole (13) now built, its release as hydroxymethylbilane (9) is triggered by the uptake of the next PBG unit required for construction of a new tetrapyrrole on the enzyme. At present, we do not know the nature of the group X on deaminase to which the growing oligopyrrole system is attached. There are pointers, no more than that (Battersby et al. 1983), indicating that X may be the terminal amino group of lysine but X may equally well be sulphur or oxygen. It is certain, however, that deaminase possesses an essential lysine residue which probably lies in or close to the active site (Hart et al. 1984).

The problem of how cosynthetase performs the conjuring trick of converting the hydroxymethylbilane (9) into uro'gen-III (6) is a fascinating one. The $^{13}$C-labelling experiments outlined earlier put strict limitations on possible mechanisms with the result that only two reasonable schemes fit the evidence (Battersby et al. 1982c). One of these hypotheses (scheme 12) invokes the spiro-system (14) as the key intermediate between the hydroxymethylbilane (9) and uro'gen-III (6). Though there is yet no direct evidence for the existence of the spiro-compound (14), we have found by synthesis of the model system (15) (scheme 13) that the chemistry that must be envisaged for involvement of the spiro-intermediate (14) is both feasible and facile (Battersby et al. 1983a).

Most of the knowledge gained so far can now be summarized in scheme 14 with the reminder that evidence is still being sought for the nature of the group X on deaminase and for the existence of the hypothetical spiro-intermediate (14).

With uro'gen-III (6) having been biosynthesized in living systems as outlined above, enzymic modification of the structure then occurs. One pathway leads to protoporphyrin-IX (1), the precursor of protohaem (2) and chlorophylls a and b; the protohaem is required for the production inter alia of haemoglobin, myoglobin...
Scheme 12. Hypothesis which proposes the spiro-system (14) as an intermediate between the hydroxymethylbilane (9) and uro'gen-III (6).

Scheme 13.

Scheme 14. Biosynthesis of uro'gen-III (6) from porphobilinogen (5).
and cytochrome c. The sequence of reactions is illustrated in scheme 15 and briefly it involves first the decarboxylation of the four acetic acid residues to produce coproporphyrinogen-III (16) (Battersby & McDonald 1975). Oxidative modification of the propionate groups on rings A and B then takes place so generating two vinyl groups and the product, protoporphyrinogen-IX (17) is finally aromatized enzymically to give protoporphyrin-IX (1). Several of the steps in this sequence of reactions are oxidative and the enzymes involved generally require oxygen as the obligatory oxidizing agent; we will return to this topic later. It is important to notice that all the foregoing enzymic transformations take place on the reduced macrocycles (that is, on porphyrinogens); the macrocycle (1) at the correct oxidation level for metal ion complexation only appears at the end of the sequence.

With this picture in our minds of the biosynthesis of uro’gen-III (6) and of its transformation into those pigments of life based on protoporphyrin-IX (1), we can now consider a second major pathway that branches away from the one we have been considering so far. This second pathway leads to vitamin $B_{12}$ (4) and the aim of our work since 1969 has been to discover how Nature builds such a complex molecule; a recent comprehensive review of this field has appeared (Battersby & McDonald 1982).

We will deal very briefly with the early work that started with the demonstration by Bernhauer’s group that vitamin $B_{12}$ is biosynthesized from cobyrinic acid (18);
thus the various amide residues and the so-called nucleotide loop of the vitamin are added late in the sequence. This simplifies the biosynthetic problem somewhat to that of discovering how cobyrinic acid (18) is constructed. Evidence also steadily accumulated (Battersby & McDonald 1982) from the work of Shemin, Müller, Scott and our group in Cambridge supporting the view that the biosynthetic pathways to cobyrinic acid (18) and to protohaem (2) are identical as far as the formation of uro’gen-III (6). Once again uro’gen-III was found to be the parent macrocycle from which cobyrinic acid (18) is biosynthesized, a relationship proven by unambiguous labelling experiments (Battersby & McDonald 1982), see scheme 17 (Battersby et al. 1977b).

The conversion of uro’gen-III (6) into cobyrinic acid (18) obviously requires many steps summarized as follows: (i) introduction of seven methyl groups at carbons C-1, C-2, C-5, C-7, C-12, C-15 and C-17; (ii) decarboxylation of the acetic acid side chain at C-12; (iii) extrusion of C-20 from the skeleton of uro’gen-III; (iv) insertion of cobalt. We reasoned on chemical grounds that the most probable enzymic step to be carried out next on uro’gen-III (6) should be either C-methylation
or decarboxylation of the acetic acid residue at C-12. Appropriate tracer experiments (scheme 18) (Battersby et al. 1977b) firmly eliminated the possibility of decarboxylation as the next step, so our efforts were focused on seeking C-methylated intermediates. This involved searching through the pigments produced by the B$_{12}$-producer Propionibacterium shermanii when the organism was grown under the special cobalt-free conditions developed by Bykhovsky in Moscow (Bykhovsky et al. 1975, 1976; Bykhovsky & Zaitseva 1976). Many new substances were isolated but we will concentrate here on two beautiful pigments which proved to be of enormous importance (Battersby et al. 1977a, c, 1979b; Battersby & McDonald 1978); they were purified as their methyl esters and were both found to be based on the isobacteriochlorin macrocycle.

![Diagram](http://rspb.royalsocietypublishing.org/)

**Scheme 18.** Proof that 12-decarboxy-uro'gen-III does not follow uro'gen-III as the next intermediate on the biosynthetic pathway to vitamin B$_{12}$.

It is beyond the scope of this lecture to deal in detail with our work on the structures and stereochemistry of these two new pigments. Only very small amounts of both were available so it was necessary to combine several different approaches (n.m.r., field desorption mass spectrometry, isotopic labelling, spectroscopy and degradative studies) to prize out the required information. As a result, one pigment called sirohydrochlorin† was proved to be the dimethylated isobacteriochlorin (19) (scheme 19) (Battersby et al. 1977a, c; Battersby & McDonald 1978) and the other turned out, very surprisingly (see later), to be the 20-methyl derivative (20) of the former pigment (Battersby et al. 1979b; Battersby & McDonald 1978); these structures were later derived independently by work from other laboratories (Battersby & McDonald 1982). When labelled forms of the octa-acids (19) and (20) were incubated with a cell-free enzyme system from P. shermanii, they were incorporated without scrambling of the labels into cobyrinic acid (18) (Battersby et al. 1978, 1979a). So these discoveries marked out the first part of the branching pathway leading to vitamin B$_{12}$ and they changed the whole course of research on this biosynthetic problem.

It is also known (Deeg et al. 1977; Imfeld et al. 1979) that the enzymic

† The metal-free prosthetic group from the enzyme sulphite reductase had been isolated (Murphy et al. 1973; Murphy & Siegel 1973; Siegel et al. 1973) and named sirohydrochlorin. Though the structure of sirohydrochlorin was not known at that time, it was shown to be an isobacteriochlorin; comparison of the dimethylated pigment (19) from the B$_{12}$-producing organism with sirohydrochlorin established the identity of the two substances, a structural link of considerable evolutionary interest (Battersby et al. 1977a, c; Battersby & McDonald 1978).
methylation of uro’gen-III (6) starts at ring A and the chlorin (21) was isolated which arises by aerial oxidation from the monomethylated intermediate (Battersby & McDonald 1982). The chlorin (21) was named Faktor-I (see scheme 20) and the chemistry and spectroscopic properties of this pigment have been explored (Imfeld et al. 1979; Battersby & Seo 1983). So the foregoing information shows that the first three C-methyl groups to be introduced enzymically into uro’gen-III (6) en route to cobyrinic acid (18) are added in the following order: first on ring A (at C-2), second on ring B (at C-7) and third at the meso-bridge between rings A and D (at C-20).

At this stage, we must look more closely at the enzymic C-methylation of uro’gen-III (6). This macrocycle contains eight double bonds and that oxidation level should not be changed by methylation on rings A and B. Yet sirohydrochlorin (19), the isolated form of the dimethylated system, possesses nine double bonds. It therefore seemed possible that the true biosynthetic intermediates on the pathway to vitamin B_{12} are dihydroisobacteriochlorins and that these are converted oxidatively, perhaps by air, into the isolated aromatic macrocycles (19) and (20). If so, a reducing system must be present in the cell-free enzyme extracts when sirohydrochlorin (19) is incorporated into cobyrinic acid (18) to regenerate the necessary dihydroisobacteriochlorin from the aromatized macrocycle (19).

Careful experiments under strictly anaerobic conditions in which uro’gen-III (6) was incubated with the P. shermanii enzyme preparation and S-adenosylmethionine (SAM) led to the isolation of a dimethylated dihydroisobacteriochlorin. This product was rapidly oxidized by air or iodine to yield sirohydrochlorin (19) and its structure was proved to be the 15,23-dihydro system (22) (Battersby et al. 1982d) (see scheme 21). This dihydroisobacteriochlorin (22) in labelled form was well incorporated into cobyrinic acid (18) and it proved to be a better precursor than was sirohydrochlorin itself (19). Another piece of the mosaic thus fell into place and it was clear that there is no change in oxidation level of the intermediates on the pathway from PBG (5), through uro’gen-III (6) and onto the dimethylated intermediate (22). It seems highly probable that the trimethylated intermediate is also generated and further transformed at the dihydro level (23) though at present there is no direct evidence for this.

I now want to focus attention on the unique feature of the structure of cobyrinic
acid (18) and vitamin B$_{12}$ (4), which is the direct link between rings A and D. In contrast, the dimethylated and trimethylated intermediates (22) and (23) still possess the meso-bridge between rings A and D and, very surprising at the time, the third C-methyl group of the trimethylated system was located on that meso-bridge. Whatever is Nature doing by methylating a carbon that has to be lost at some later stage on the pathway to cobyricinic acid (18)? One possibility was that the C-20 methyl group of intermediate (23) might undergo migration to C-1. This was tested by incorporating the illustrated multiply labelled form of the trimethyl system (20) into cobyricinic acid (18) in the normal way using the cell-free enzyme preparation, scheme 22. The results clearly demonstrated that the C-20 methyl group is lost when the trimethylated intermediate is converted into cobyricinic acid (18) (Battersby et al. 1979a; cf. Battersby & McDonald 1982).

Scheme 22. Proof that the C-20 methyl group is lost when the trimethylated precursor is converted into cobyricinic acid (18).
Here was the opportunity to gain clues about the mechanism of the ring-contraction process that generates the smaller corrin macrocycle of cobyrinic acid (18) from a ring which is larger by one carbon atom. We must find out in what form the C-20 meso-bridge and its attached methyl group are extruded as the corrin ring is formed. By labelling the trimethylated system at C-20, or alternatively at the C-20 methyl group, it was clearly shown that the extruded fragment is acetic acid, scheme 23 (Mombelli et al. 1981; Battersby et al. 1981f). The C-20 methyl group of the macrocycle (23) provides the methyl group of the acetic acid and the C-20 meso-bridge appears as the acetate carboxyl group. When the oxidation level of cobyrinic acid (18) and acetic acid taken together is compared with that of the dihydrotrimethylisobacteriochlorin (23) from which they are formed, one finds that no external redox reagents are required for the conversion. Bearing in mind the earlier finding that there is no change of oxidation level over that part of the pathway that sees PBG (5) eventually converted into the dihydrotrimethyl system (23), it now seems probable that the entire biosynthetic sequence through to cobyrinic acid (18) is not dependent on external redox reagents; we shall return to this aspect at the end of the lecture.

Our earlier analysis of the steps required for the conversion of uro'gen-III (6) into cobyrinic acid (18) pinpointed seven sites for C-methylation. However, that was before the discovery of the trimethylated intermediate that carries a methyl group at C-20 shown above to be subsequently lost. Therefore, the order of insertion of eight methyl groups needs to be elucidated (together with other knowledge) before the biosynthetic problem is solved. So far, the order for the first three methyl groups is known. Naturally, we have invested a huge effort trying to detect intermediates carrying four, five or more methyl groups. None have been found. Accordingly, we changed tack and aimed to gain information about the order of methylation by pulse labelling.

The plan was to incubate unlabelled the dimethylated sirohydrochlorin (19) with a cell-free enzyme system (this time from Clostridium tetanomorphum) capable of biosynthesizing cobyrinic acid (18) but to add initially only a small amount of unlabelled S-adenosylmethionine (SAM) as methylating agent. After a period of

![Scheme 23. Proof that acetic acid is extruded during the ring contraction process which generates cobyrinic acid (18).](image-url)
incubation to allow methylated intermediates to be produced from the dimethylated precursor, a large excess of (methyl-\textsuperscript{13}C)SAM was to be added and the incubation continued to produce cobyrinic acid (18). This product was expected to have a lower \textsuperscript{13}C-content in the methyl groups introduced early in the sequence of methylations than in those added later; labelling levels were to be determined by \textsuperscript{13}C-n.m.r. spectroscopy on the heptamethyl ester of cobyrinic acid (18), the so-called cobester. This approach requires assignment of the signals in the \textsuperscript{13}C-spectrum of cobester and this was done (Ernst 1981; Battersby \textit{et al.} 1982\textit{a}).

The \textsuperscript{13}C-spectrum of the product from the pulse labelling experiment gave the striking result shown in figure 3. The signal from the methyl group at C-17 was of considerably lower intensity than the rest (ca. 70\% of standard) (Battersby & Uzar 1982; Battersby \textit{et al.} 1983\textit{c}). It is obvious that this pulse labelling experiment can be run in the reverse sense, that is, with the (methyl-\textsuperscript{13}C)SAM added first followed by a large excess of unlabelled SAM to complete the biosynthesis. This inverse experiment gave the highly satisfying result (H. C. Uzar and A. R. Battersby, unpublished) that the signal from the C-17 methyl group was now the largest of the set of methyl signals. So the evidence is extremely strong that the fourth methyl group for the biosynthesis of cobyrinic acid (18) is introduced at C-17.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.pdf}
\caption{Proton-noise decoupled \textsuperscript{13}C-spectra showing enhanced signals from (lower spectrum) the heptamethyl ester of cobyrinic acid uniformly \textsuperscript{13}C-labelled at the methyl groups derived from \textit{S}-adenosylmethionine (SAM) and (upper spectrum) the heptamethyl ester of cobyrinic acid from the pulse labelling experiment with \textsuperscript{13}C[SAM].}
\end{figure}
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The effect of methylating the dihydroisobacteriochlorin (23) at C-17 should now be considered. As shown in scheme 24, this methylation generates (Battersby & Uzar 1982) a pigment (24) of a type not previously observed on the pathway to vitamin B\textsubscript{12}; this new pigment is based on the parent macrocycle, called pyrrocorphin. Various alkyl substituted pyrrocorphins have been synthesized to allow their chemistry and spectroscopic properties to be explored (Johansen et al. 1981; Schwesinger et al. 1982; C. J. R. Fookes & A. R. Battersby, unpublished). It is possible that the substance (24) is the next intermediate beyond the trimethylated stage on the pathway to vitamin B\textsubscript{12}. But there is a second possibility. If decarboxylation of the acetic acid residue at C-12 of (23) precedes C-methylation at C-17, then the dihydroisobacteriochlorin (25) and the pyrrocorphin with a methyl group at C-12 (26) will be the next two intermediates following the initially formed trimethyl system (23).

Though the timing of the decarboxylation step at C-12 is not yet known, it has been shown by recent experiments with precursors stereospecifically labelled with both \textsuperscript{2}H and \textsuperscript{3}H, that the decarboxylation occurs with retention of configuration, (Battersby et al. 1983b), that is, the incoming hydrogen occupies the same space as was previously filled by the carboxyl group.

The available hard-won knowledge of the biosynthesis of cobyrinic acid (18), the precursor of vitamin B\textsubscript{12}, can now be collected in scheme 25 which starts with uro'gen-III (6). The full biosynthetic pathway leading to uro'gen-III was shown earlier in scheme 14. C-Methylation on ring A (at C-2) of this parent macrocycle generates (27), or a tautomer, and serves to channel material along the B\textsubscript{12} pathway. The second and third methyl groups are then inserted, respectively, into ring B (at C-7) and at the C-20 meso-carbon. Then two possibilities are still open. One involves decarboxylation at the C-12 acetate residue of (23) as the next step to generate (25) which by methylation at C-17 is converted into the heptacarboxylic pyrrocorphin (26). The alternative is that decarboxylation is a later step in which case the tetramethylated intermediate is the octacarboxylic pyrrocorphin (24). Also included in scheme 25 is the knowledge that when ring-contraction to the

Scheme 24. Formation of the pyrrocorphin macrocycle (24) or (26) by C-17 methylation of the dihydroisobacteriochlorin system.
Scheme 25. Summary of what has been discovered about the biosynthetic pathway from uro'gen-III (6) to vitamin B$_{12}$ (4).

corrin nucleus of cobyriciic acid (18) occurs, the meso-bridge at C-20 and its attached methyl group are extruded together as acetic acid. At the end of the pathway, the side-chain amide groups are added and the nucleotide loop is attached to the C-17 propionate chain to produce vitamin B$_{12}$ (4).

Some feeling for the current state of research can be gained by mentally combining schemes 14 and 25. Roughly two thirds of the entire pathway to vitamin B$_{12}$ is now known; much remains to be discovered. The next few years will surely see the discovery of novel macrocycles as intermediates between the pyrrocorphin stage (24 or 26) and cobyriciic acid (18). Certainly these future years will not be dull!

Looking ahead from our present vantage point, one thing is clear; future work would be greatly helped by having available greater quantities of the very scarce dimethylated and trimethylated macrocycles (19) and (20), especially the latter. One way to clear this bottle neck would be to develop an effective total synthesis of the pigment (20) which would yield tens or hundreds of milligrams rather than
The hundreds of micrograms which are available from natural sources. Accordingly, we are investing a major effort in Cambridge aimed at the synthesis of the trimethylisobacteriochlorin (20). This has already led to the discovery of a mild photochemical method for construction of the isobacteriochlorin macrocycle (Battersby et al. 1981c). A similar photochemical route is also effective for the synthesis of chlorins (Battersby et al. 1983e), including the synthesis of the marine natural product bonellin (Battersby et al. 1983d). More recently, methods have been developed (Battersby et al. 1984) which are well suited to the problems one faces in applying the photochemical approach to the natural pigment (20). So there is real hope that the bottle neck due to shortage of material may be cleared during the coming year or so.

This lecture has concentrated largely on the chemistry and biosynthesis of uro’gen-III, protohaem and vitamin B12. But the advances made in our understanding of the biosynthesis are also fascinating in relation to evolution. It was stressed earlier that the parent macrocycle, uro’gen-III (6), does not form complexes with metal ions. It is now clear from what has been described in my lecture that living systems have developed two ways for transforming uro’gen-III (6) into metal complexing macrocycles. One is oxidative and leads to the chlorophylls and to protohaem (for haemoglobin and the cytochromes) and the obligatory acceptor for some of the enzymic oxidations is oxygen. The second method is non-oxidative and makes use of methylation; this pathway is found in anaerobic organisms and in the case of vitamin B12 affords a macrocycle that is perfectly fitted to hold the cobalt ion. It seems likely that as the complete pathway to vitamin B12 is uncovered, there will be more evolutionary clues and connections. These will add a bonus to our main reward which will be the complete understanding of the biosynthesis of the pigments of life.

I am glad to have the opportunity to thank all my young colleagues, now many in number, who have worked with me on these demanding problems. Today’s lecture could not have been given without their skilled and enthusiastic efforts; their names are given on the papers collected in the references. I also wish to record my debt to my senior colleagues who over these past 16 years have been Dr E. McDonald, Dr C. J. R. Fookes and Dr G. W. J. Matcham and now Dr F. J. Leeper; they have all made invaluable contributions to the work. Finally, the financial support of the Nuffield Foundation, S.E.R.C. and Roche Products Ltd is gratefully acknowledged.

References


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