Biosynthesis of Porphyrins and Related Macrocycles. Part 35.[1,2] Discovery of a Novel Dipyrrolic Cofactor Essential for the Catalytic Action of Hydroxymethylbilane Synthase (Porphobilinogen Deaminase)

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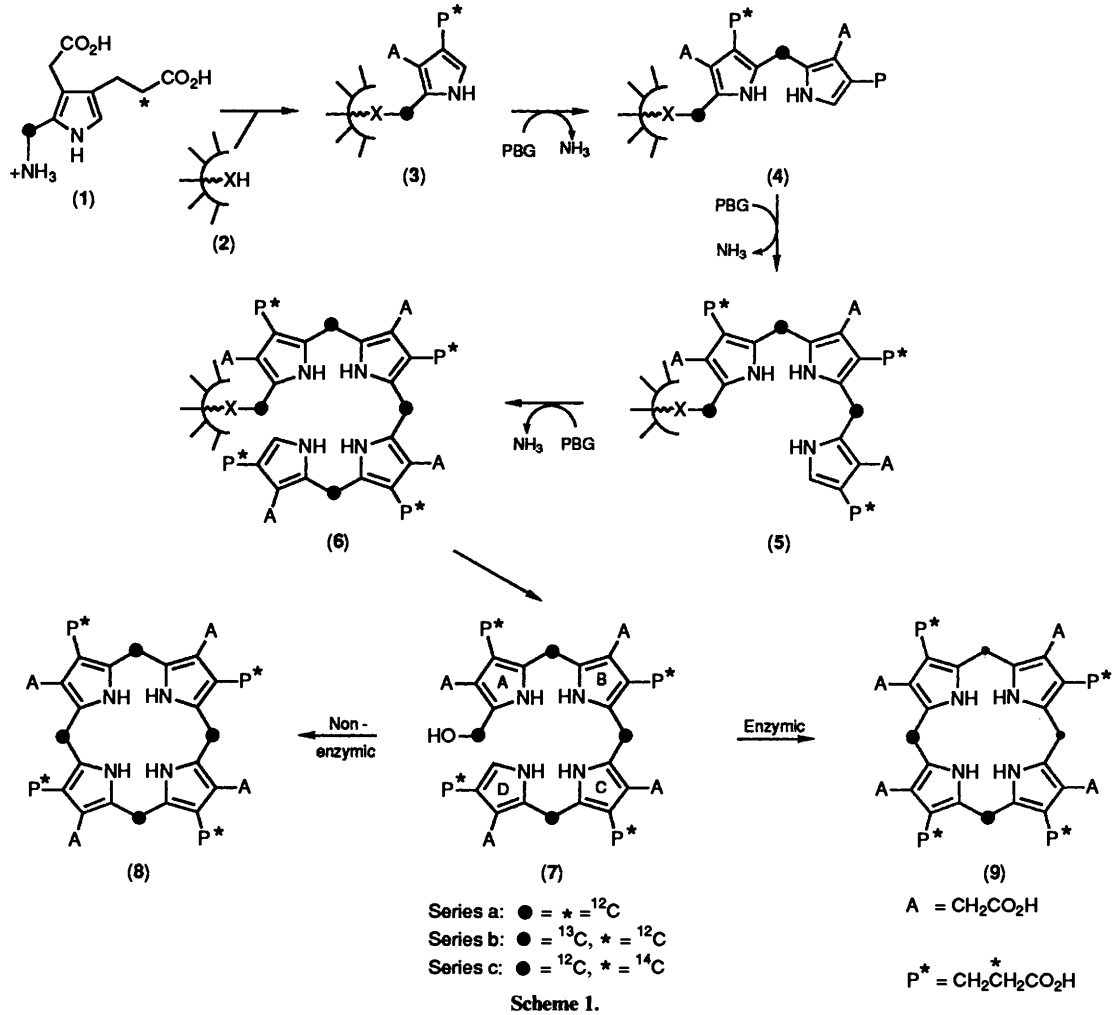
The enzyme hydroxymethylbilane synthase constructs the open-chain hydroxymethylbilane by assembly of four porphobilinogen units head-to-tail, the first of these being covalently bound to the enzyme through a group X. The surprising discovery is made that X is a novel dipyrromethane cofactor constructed from two porphobilinogen units and bound to the protein *via* the sulphur of cysteine. This cofactor does not turn over in the catalytic process but acts as an anchor for the assembly of a hexapyrrole from which the tetrapyrrolic hydroxymethylbilane is cleaved leaving the dipyrromethane cofactor in place for a further building cycle.

Hydroxymethylbilane synthase (HMBS), EC 4.3.1.8 (also known as porphobilinogen deaminase), acts in conjunction with uroporphyrinogen III synthase, EC 4.2.1.75, to convert porphobilinogen (PBG) (**1a**) into uroporphyrinogen III (uro'gen III) (**9a**), the precursor for haem, chlorophyll, and vitamin B_{12}.[3,4] HMBS assembles four PBG units, one after another, and it releases hydroxymethylbilane[5,6] (**7a**) into the medium; the growing oligopyrrolic chain is bound covalently[7,8] through some group X to HMBS during the building process (Scheme 1). Cosynthetase then converts the hydroxymethylbilane (**7a**) into uro'gen III (**9a**) by a process involving a single intramolecular rearrangement.[5,9]

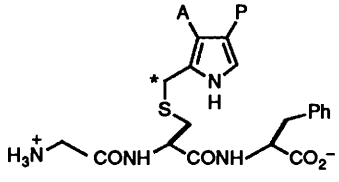
Our experimental approach for studying the nature of the X-group involved binding [11-^{13}C]PBG (**1b**) to HMBS [see



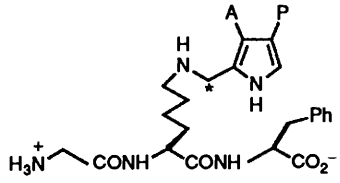
Scheme 1.

compounds (**3b**) and (**4b**)] when it should be possible, in principle, to prove what X is by observing the chemical shift of the ^{13}C signal from the carbon bound to X. All our early studies showed[8] that the approach, though simple in design, was extremely difficult in practice. The enzyme partially loaded with labelled PBG gave no recognisable ^{13}C NMR signals. Extensive enzymic degradation of this ^{13}C-loaded protein yielded pyrrole-carrying material of much lower molecular weight (*ca.* 3–4 000 Da) which still showed only very weak ^{13}C NMR signals, one family at δ 42–43 raising the possibility that the terminal nitrogen of lysine might be attached to the ^{13}C centre. However, no rigorous conclusions were possible nor claimed; these early studies have been reviewed.[10] Other workers, using tritium labelling, favoured the sulphydryl group of cysteine as the X-group.[11]

In all our foregoing experiments, the continual loss of pyrrolic material from the protein during handling and degradation emphasised the need for a study of the chemical properties of peptides with side-chains carrying -X-CH_2-pyrrole residues. We were familiar with the chemistry of related systems[5] having X = O and so the peptides (**10**; X = S) and (**11**; X = NH) were



(**10**)



(**11**)

synthesised.[1] These materials allowed the chemical shift of the ^{13}C signal from the starred centre in each peptide to be determined under the exact conditions to be used for the enzyme itself, but very importantly as it turned out, both peptides were found to be sufficiently stable if held at high pH, 12–14, to allow long NMR runs.[1]

A second decisive advance was the isolation of HMBS from *Escherichia coli*[12] followed by sequencing of the corresponding gene (*hem*C) for this enzyme[13] and over-expression of it in *E. coli*.[2a,14] The initial 200-fold over-expression[2a] has more recently been increased to 600–800 fold and a single protein is produced. Large amounts of HMBS thus became available for ^{13}C-labelling experiments. The sequences for the *hem*C genes from *E. coli*,[15] human,[16] rat,[17] and *Euglena gracilis*[18] have been published by other groups and the *E. coli* gene has also been over-expressed by one of these groups up to 100 times the wide-type level[19] to yield a mixture of five enzymically active proteins.

The foregoing synthetic studies and the success with over-production of HMBS opened the way to the following advances.

Discussion

Comparison of HMBS from Euglena gracilis *and* E. coli.—The proof[5] that HMBS is not a cyclising enzyme but an assembly enzyme which builds the open chain hydroxymethylbilane (**7a**) from four PBG molecules (**1a**) and all our other enzymic studies outlined above were carried out with enzyme isolated from *Euglena gracilis*. It was therefore important before using HMBS from *E. coli* for experiments on the nature of the X-group to demonstrate first that the enzyme from this source works in the same way.

[11-^{13}C]PBG (**1b**) was required for this study and the final stages of an earlier synthesis[8] were improved (see Experimental section). This product was incubated at pH 8 with sufficient *E. coli* HMBS to consume most of the substrate in 2 min. The pH of the mixture was then raised above 12 to quench the enzymic reaction and stabilize the product[5] for ^{13}C NMR analysis. The resultant ^1H-decoupled spectrum [Figure 1(*a*)] showed a singlet at δ 57.1 corresponding to HO-$^{13}CH_2$-pyrrole of the bilane (**7b**) and another at δ 24.5 arising from the three inter-pyrrolic methylene groups of this product (**7b**). The integral ratio of the former to the latter signal was 1:3 and the spectrum matched perfectly that observed when using the *E. gracilis* enzyme.[5] Uro'gen I (**8b**), produced by the known non-enzymic cyclisation of hydroxymethylbilane (**7b**) is responsible for the singlet at δ 24.0; this too was seen in the earlier study.[5] Final confirmation that the bilane (**7b**) had been formed was obtained by adjusting the pH of the solution used in Figure 1(*a*) to pH 3, which caused rapid cyclisation of hydroxymethylbilane (**7b**), followed by basification to pH > 12. The ^1H-decoupled ^{13}C spectrum from this solution in Figure 1(*b*) showed only the singlet at δ 24.0 from uro'gen I (**8b**). These results confirmed unambiguously that HMBS from *E. coli* produces the bilane (**7b**) by assembly of four PBG units (**1b**).

The earlier work on HMBS from *E. gracilis* had in addition used hydroxylamine in the incubation mixture to trap the assembled tetrapyrrole as hydroxyaminomethylbilane (**12**) (Scheme 2); such trapping had been done earlier with HMBS from spinach[20] and *Rhodopseudomonas sphaeroides*.[21] For the present studies, the experiment with the *Euglena* enzyme was repeated at pH 8.45 to provide a standard and the resultant spectrum [Figure 1(*c*)] matched the earlier one[8] in showing singlets at δ 51.2 from HONH-$^{13}CH_2$-pyrrole and δ 24.5 from the three interpyrrolic methylene groups. No signal appeared at δ 24.0 from uro'gen I (**8b**) showing the efficiency of trapping by hydroxylamine.

The equivalent experiment was then run at pH 8.5 with HMBS from *E. coli*. The expected signals at δ 51.2 and 24.5 appeared [Figure 1(*d*)] but the integral ratio was *ca.* 1:1 and now a small signal at δ 24.0 was observed proving that uro'gen I (**8b**) had been formed. The latter observation shows that trapping of the bilane is less efficient with the *E. coli* enzyme. Also, the foregoing 1:1 integral ratio suggested that hydroxylamine must be trapping materials on the enzyme which have not yet reached the tetrapyrrole level. Interestingly, when the hydroxylamine trapping experiment was repeated at pH 7, the ^{13}C spectrum in Figure 1(*e*) was obtained. The large signal at δ 51.2 corresponds to HONH-$^{13}CH_2$-pyrrole but there was only a very small signal at δ 24.5 from interpyrrolic methylenes of the bilane (**12**) or indeed of possible di-, or tri-pyrroles; the signal at δ 38.4 is due to unchanged [11-^{13}C]PBG (**1b**). So at pH 7, hydroxylamine is able to intervene almost quantitatively as the first PBG unit enters to bind to the X-group of HMBS (Scheme 2) generating the hydroxyamino analogue (**13**) of PBG (**1b**). The trapped product thought to be the monopyrrole (**13**) has been reported before[21] as has the detection (by radioactivity) of displacement by hydroxylamine of bound pyrroles from HMBS.[7]

A reasonable interpretation of our results is that at pH 7, the tetramerisation process leading to the bilane is slowed down allowing the monopyrrole to be trapped by path B as the hydroxyamine (**13**) before the normal attachment of a second PBG unit can occur (Scheme 2). At pH 8.5, however, a faster forward building rate leading to the tetrapyrrole, path a, means that a significant amount of trapped bilane (**12**) is formed.

The foregoing results made it important to study HMBS from *E. gracilis* in the same way. When this enzyme was incubated with [11-^{13}C]PBG (**1b**) and hydroxylamine at pH 7, the singlets at δ 51.2 and 24.5 had an intensity ratio of *ca.* 1:1.5 and a smaller signal appeared at δ 24.0 from uro'gen I (**8b**). So here again some trapping of early intermediates, probably as the monopyrrole (**13**), occurs at the lower pH but the effect is not so marked as with the enzyme from *E. coli*.

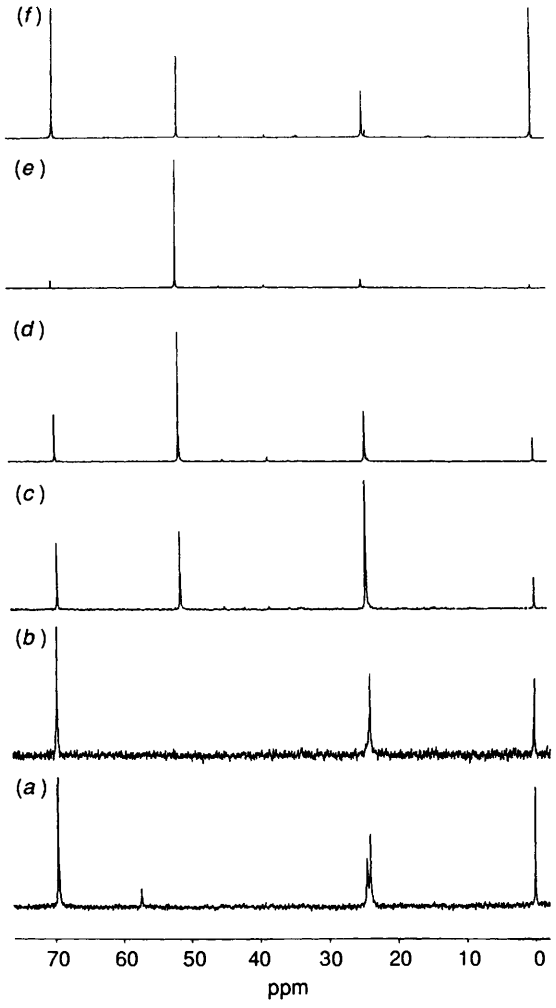


Figure 1. ^{13}C NMR spectra of (*a*) hydroxymethylbilane (**7b**) produced from [11-^{13}C]PBG by the action of HMBS from *E. coli*, in H_2O/D_2O at pH 12 (8 704 transients); (*b*) the solution as in (*a*) after acidification to pH 3 for 15 min and then rebasification to pH > 12 (7 712 transients); (*c*) the product from [11-^{13}C]PBG using HMBS from *Euglena gracilis* at pH 8.45 in the presence of NH_2OH (17 788 transients); (*d*) as for (*c*) but with HMBS from *E. coli* at pH 8.5 (59 923 transients); (*e*) as for (*d*) but at pH 7.0 (28 303 transients); (*f*) as for (*c*) but at pH 7.25 (12 459 transients). The signals at δ 69.3 (dioxane) and δ 0.00 (TSP) are the standards.

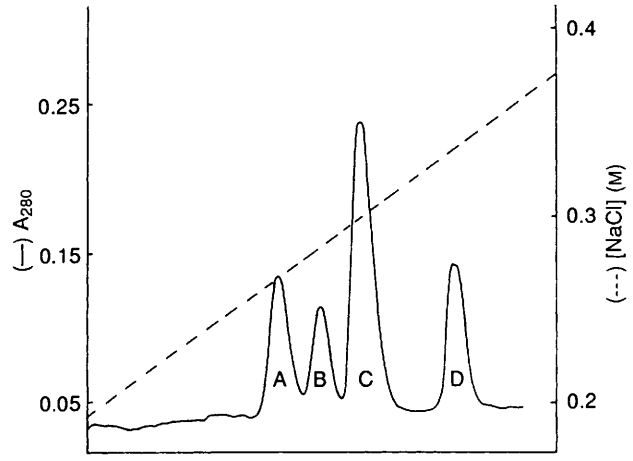


Figure 2. FPLC separation of HMBS from *E. coli* (10 mg) after mixing with [9-^{14}C]PBG; —— is the A_{280} value, – – – – is the concentration of NaCl in the eluant buffer.

In summary, these studies demonstrated that though HMBS enzymes isolated from *Euglena gracilis* and *E. coli* show some differences in kinetics, the major features of their mechanism of action are the same. Work on the X-group of the enzyme from *E. coli* could therefore be undertaken with confidence.

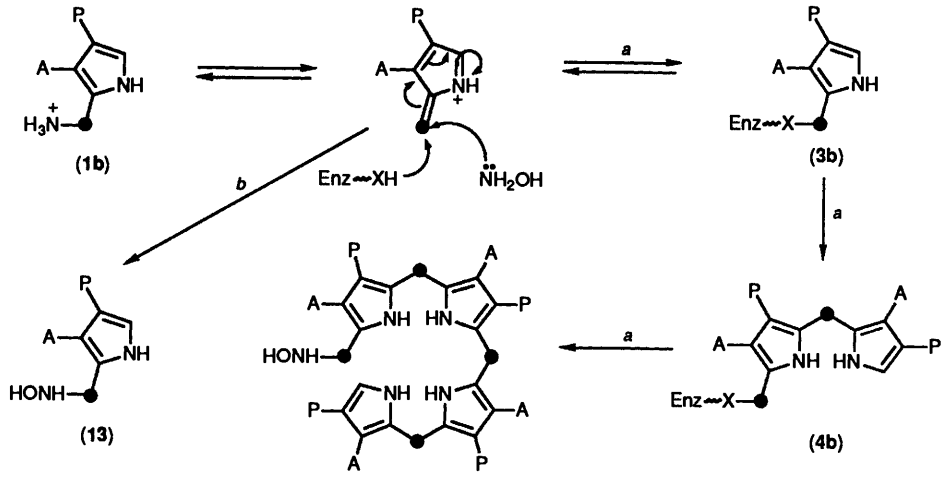
The Nature of the X-group of HMBS.—The final stages of purification of HMBS from wild-type *E. coli* used fast protein liquid chromatography[12] (FPLC). The same technique applied to HMBS from the overproducing strain[2a,14] of *E. coli* finally gave a single protein peak which precisely matched that from the wild-type source.[12] Enzyme of this quality was then partially loaded by rapid mixing with a solution of [9-^{14}C]PBG (**1c**) to generate the enzyme-PBG mono- (**3c**), di- (**4c**), and tri- (**5c**) complexes (peaks B, C, and D, respectively, Figure 2) which were separable by FPLC on a preparative scale;[22] the formation of these kinetically effective and separable complexes was known from earlier studies.[7,23–25] The ^{14}C-activities for the protein in peaks B and C (Figure 2) corresponded, within experimental error, to 1 and 2 mol of PBG per mole of enzyme respectively, so confirming that they represented the mono-, and di-complexes, whereas peak A carried no significant activity. Since the elution volume for peak A matched that for HMBS as isolated from *E. coli*, it followed that the enzyme as normally purified does not carry PBG substrate *i.e.* it is unloaded (**2**). This conclusion gains further support later. We shall refer to this unloaded enzyme (**2**) as holoenzyme.

Peak D in Figure 2, corresponding to the tri-complex (**5c**), gave a PBG/enzyme ratio consistently 16–17% lower than the expected value of 3. The most likely explanation is that a substantial conformational change occurs on binding the third PBG unit which alters the absorption coefficient of the protein to cause slight overestimation of the quantity of protein. Some support for this suggestion comes from the fact that whereas the spacings of peak A to B and peak B to C in Figure 2 are closely similar, there is a much larger separation of peaks C and D.

The next step was to study how the proportion of mono- (**3a**), di- (**4a**), and tri- (**5a**) complexes varied with the initial amount of PBG added at *ca.* 18 °C to the holoenzyme, HMBS. Analysis of the mixtures formed was by FPLC and Figure 3 shows the results. The maximum amounts of mono-, di-, and tri-complexes are produced with PBG:HMBS ratios of *ca.* 1; 2, and 3, respectively. More di-complex (**4a**) can be obtained than of mono- (**3a**) or tri- (**5a**) complexes and the di-complex is significantly more stable than the mono-complex.

We were now ready to investigate the nature of the X-group by ^{13}C NMR spectroscopy. Accordingly, 35 mg of holoenzyme was rapidly mixed with 1 mol equiv. of [11-^{13}C]PBG (**1b**) and after FPLC, *ca.* 10 mg each of the enzyme-^{13}C-PBG_1 mono-complex (**3b**) and enzyme-^{13}C-PBG_2 di-complex (**4b**) were isolated. Dialysis of these products was essential to remove Tris/HCl buffer and added protease inhibitors which otherwise interfered with the NMR spectroscopy; this dialysis was carried out against pH 12 sodium phosphate buffer so that the final concentrated solution of the enzyme-^{13}C-PBG mono- and di-complexes, (**3b**) and (**4b**), was stabilised[5] at pH *ca.* 12.

The technique used for the NMR studies deserves comment. Data were accumulated at 5–10 °C using a 45° pulse and an extremely short acquisition time of 0.086 s. Presumably a combination of the molecular size of the enzyme (34 245 Da[12]) and its at least partial denaturation[26] under the high pH conditions of measurement results in a correlation time (τ_c) which gives very short spin-lattice relaxation times (T_1) for the



Scheme 2. ● = ^{13}C.

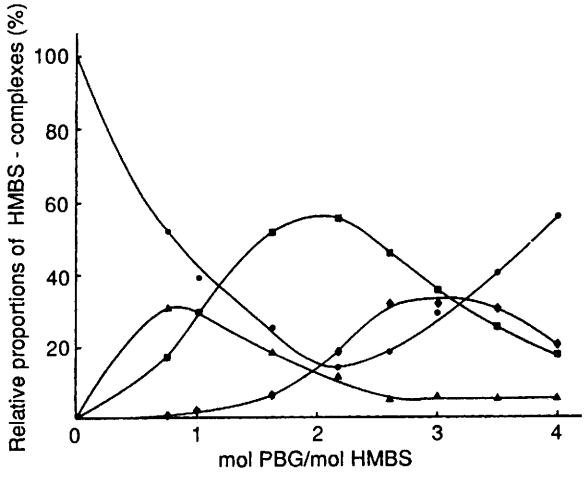


Figure 3. Graph of the relative amounts of holoenzyme (●) and mono- (▲), di- (■), and tri- (◆) complexes of HMBS from *E. coli* after mixing with various proportions of PBG. Proportions were determined from the areas of the peaks obtained from a separation by FPLC with detection at 280 nm.

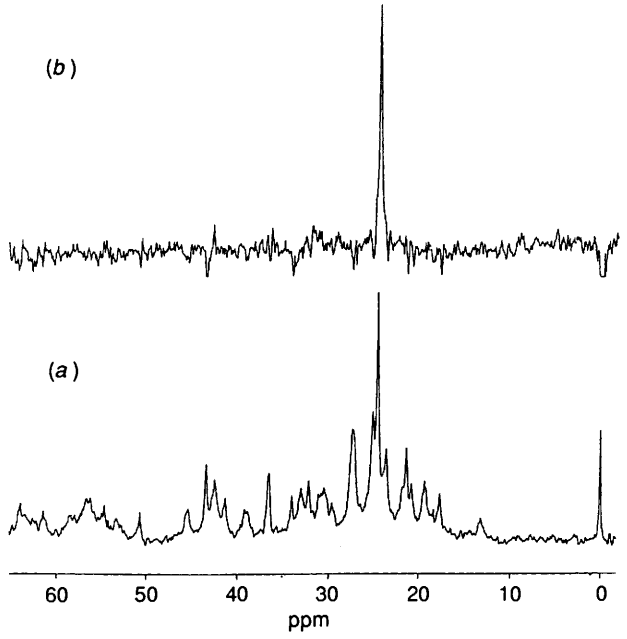


Figure 4. (*a*) ^{13}C NMR spectrum (0–65 ppm only) of the mono-complex (**3b**) of HMBS with [11-^{13}C]PBG at pH 12 and 5 °C (410 495 transients); (*b*) difference between the spectrum in (*a*) and a similar spectrum of unlabelled mono-complex (**3a**) (768 000 transients).

^{13}C nuclei. In this way, over 4 × 10^5 transients were collected in an overnight run and the resultant spectrum of the mono-complex (**3b**) [Figure 4(*a*)] showed high signal-to-noise ratio and well resolved signals. By repeating the above work but with unlabelled ^{12}C-PBG (**1a**), a natural abundance ^{13}C-spectrum was obtained from the enzyme-^{12}C-PBG_1 mono-complex (**3a**) that was almost identical to Figure 4(*a*) only lacking the strong signal at δ 24.6. The difference spectrum from the enzyme-^{13}C-PBG_1 minus enzyme-^{12}C-PBG_1 spectra showed a single strong signal at δ 24.6, Figure 4(*b*). The foregoing entire set of experiments was repeated twice more with fresh batches of HMBS from *E. coli* and the same strong signal was observed each time in the difference spectrum.

This signal at δ 24.6 was totally unexpected since the expected δ-values corresponding to the X-group being O, N, or S are [1,5] 57.1, 45.4, and 29.5, respectively; all three are therefore eliminated as candidates for the X-group. However, this δ 24.6 value corresponds precisely to an interpyrrolic methylene group,[5] *i.e.* a pyrrole-$^{13}CH_2$-pyrrole unit. This indicated that the X-group of HMBS possesses a terminal pyrrole residue to which the first substrate PBG molecule binds.

The enzyme-^{13}C-PBG_2 di-complex (**4b**) possesses its own interpyrrolic $^{13}CH_2$-group and thus provides an internal standard. In fact, the ^{13}C NMR difference spectrum, obtained using the same approach and conditions as for the above mono-complex, was essentially the same as Figure 4(*b*) with one sharp signal at δ 24.6 but of enhanced intensity. The integral ratio of this signal to that from the mono-complex in Figure 4(*b*) (measured in each case relative to the natural abundance signals of the protein) was *ca.* 1.7:1. This is satisfyingly close to the theoretical ratio of 2:1 bearing in mind the signal-to-noise ratios and the fact that the errors are inevitably greater for difference spectra.

That the ^{13}C signal at δ 24.6 from the enzyme-^{13}C-PBG_1 mono-complex (**3b**) was due to a methylene group was established by the ^{13}C NMR spin-echo spectrum[27] of this complex. This approach causes signals from the carbons of methyl and methine groups to appear as negative singlets and those from methylene groups and quaternary centres as positive singlets. A strong positive signal was again observed at δ 24.6 and the difference spectrum obtained by subtraction of the ^{13}C

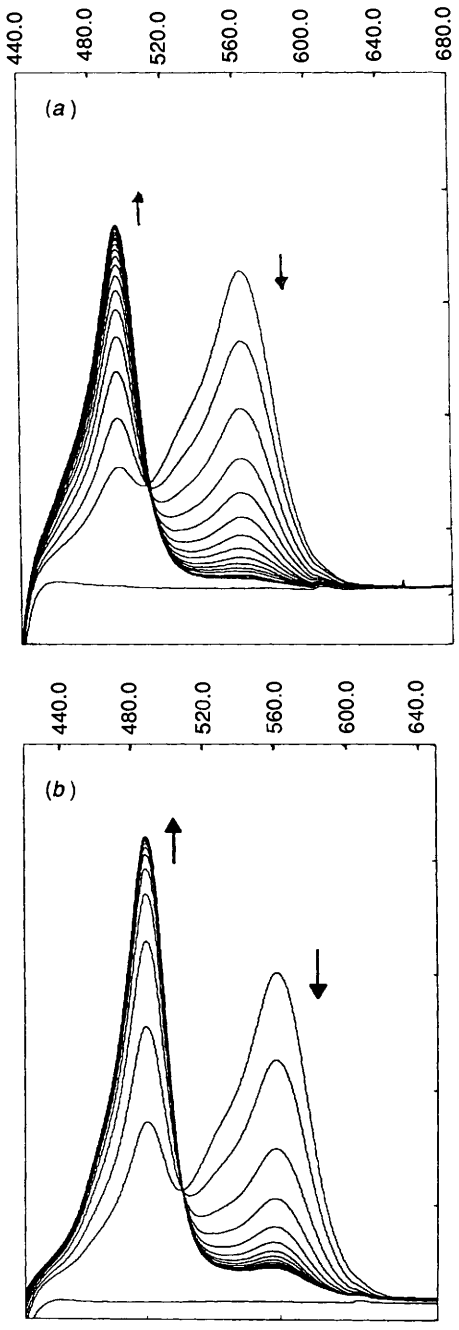


Figure 5. UV-VIS spectra of mixtures of modified Ehrlich's reagent with (*a*) HMBS and (*b*) dipyrromethane (**22**). The first spectrum was recorded after *ca.* 30 s after mixing and subsequent spectra at intervals of 2 min.

NMR spin-echo spectrum of the enzyme-^{12}C-PBG mono-complex (**3a**), acquired under identical conditions, showed the same signal as the only significant resonance. Both the sign and the chemical shift of the signal at δ 24.6 were in agreement with its origin from a CH_2 group between two pyrroles.

All these results demonstrated that the PBG molecule added first in the building process leading to the bilane (**7a**) binds to a tightly bound pyrrolic system already present in the holoenzyme.

Catalytic Competence of the Isolated Enzyme-^{13}C-PBG Complexes.—First, an attempt was made to obtain a ^{13}C spectrum from a sample of isolated enzyme-^{13}C-PBG_1 mono-complex (**3b**) at pH 8.5 and 5 °C (*cf.* pH > 12 used in the foregoing successful runs). No discrete ^{13}C signals were observed. Presumably this is because of the longer correlation times (τ_c) associated with molecular motions of the enzyme in its native conformation rather than partially denatured at pH > 12. The same sample was adjusted to pH 12 and even after only 2 h of acquisition at 5 °C, the key signal at δ 24.6 was clearly visible. Adjustment of this solution back to pH 8.5 was followed by incubation at 37 °C with unlabelled PBG and hydroxylamine. A catalytically competent enzyme-^{13}C-PBG_1 mono-complex (**3b**) should under these conditions continue the building process to the bilane stage which (see earlier) should be trapped as the hydroxyaminomethylbilane (**12a**), Scheme 3. The ^{13}C-spectrum from this experiment run at pH > 12 showed one major sharp singlet at δ 51.0, characteristic[25] of a HONH-$^{13}CH_2$-pyrrole. Finally, the solution was exhaustively dialysed at pH 12 to remove the bilane (**12a**) and the ^{13}C-spectrum of the enzyme was acquired as before. No signal at δ 24.6 was observed showing that the originally bound ^{13}C-labelled PBG unit had been quantitatively converted into bilane.

At least as striking as these experiments was the finding that when isolated enzyme-^{13}C-PBG_2 di-complex (**4b**) was carried through the turnover and trapping sequence, the ^{13}C spectrum of the final product showed two sharp signals at δ 51.0 and 24.4 with an integral ratio of 1.04:1.00. The results together demonstrate the formation of the doubly labelled bilane product (**12b**), Scheme 3.

These experiments establish that the first and second PBG units to bind to HMBS become ring A and ring B of the hydroxymethylbilane (**7a**) in full agreement with earlier experiments on the order of assembly of the tetrapyrrole system.[24,25,28] Further, the observation of the crucial signal at δ 24.6 from enzyme-^{13}C-PBG_1 mono-complex (**3a**) at pH 12 followed by successful turnover of the same preparation after adjustment to pH 8.5 to yield HONH-$^{13}CH_2$-bilane labelled at ring A (**12a**) gives powerful evidence that the observed ^{13}C signal arises from the product of binding the first PBG unit to the true X-group of HMBS. The conclusion from the previous section is thus greatly strengthened.

Finally, turnover of 11 mol of [9-^{14}C]PBG (**1c**) per mole of HMBS followed by fractionation of the enzymic mixture by FPLC as earlier gave the same pattern of four protein peaks as seen in Figure 2. However, since most of the ^{14}C-PBG had been converted into hydroxymethylbilane, the combined amount of mono-, di-, and tri-PBG-complexes were expected to be low; they represented only 22% of the amount of enzyme recovered. The holoenzyme was demonstrated still to be enzymically active and to carry the normal amount of pyrrolic material (by conversion to porphyrin as below) yet it was essentially radioinactive. Moreover a solution of holoenzyme under denaturing conditions was diluted, concentrated to low volume by ultrafiltration, diluted again and re-concentrated in the same way; this treatment would have removed any non-covalently bound small molecules. The protein was shown still to carry the pyrrolic material by Ehrlich's assay (see later) whereas the ultrafiltrate contained no detectable amount. Thus HMBS as normally isolated carries no PBG substrate but does possess a pyrrolic system which is covalently bound to the enzyme and is present in addition to the PBG units which are turned over during the assembly process.

The labelled hydroxymethylbilane (**7c**) produced in the foregoing turnover experiments cyclised non-enzymically to give uroporphyrinogen I (**8c**) which was oxidised by iodine and the resultant single porphyrin was identified as uroporphyrin I (**14c**); its specific molar radioactivity was measured at 4.4 times that of the [^{14}C]PBG initially used (within experimental error of the expected value, × 4).

Structure of the Pyrrolic Cofactor.—An earlier puzzling

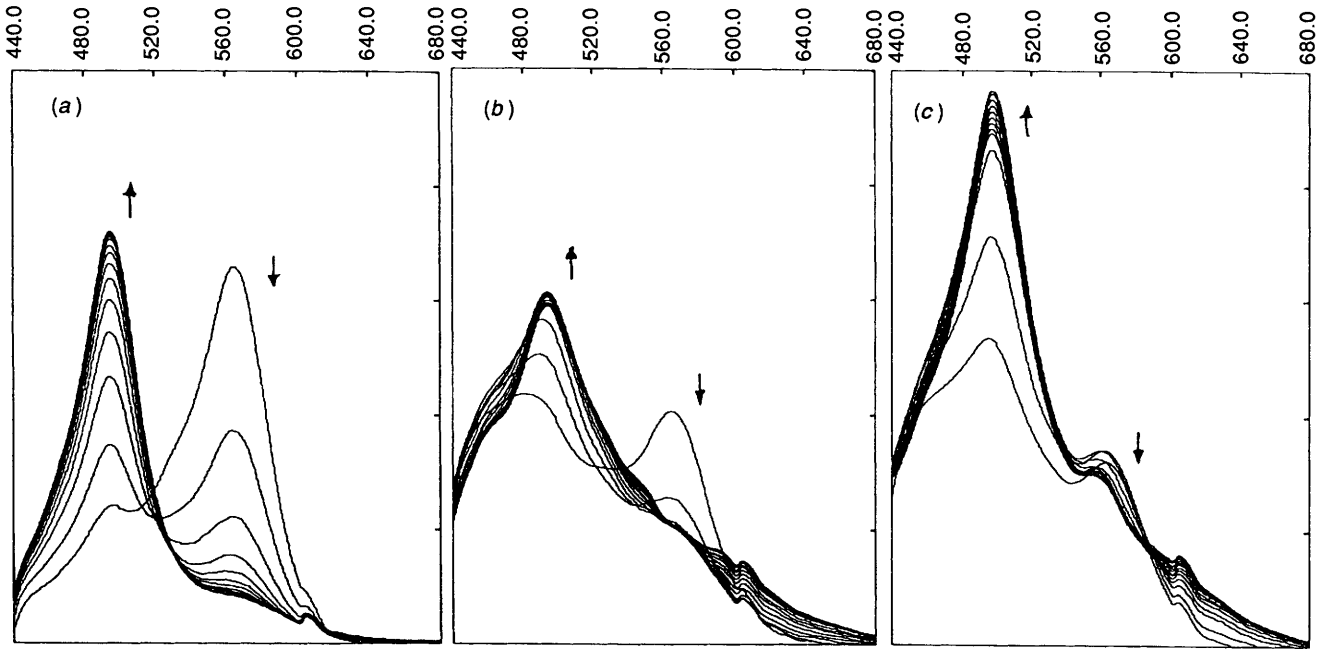
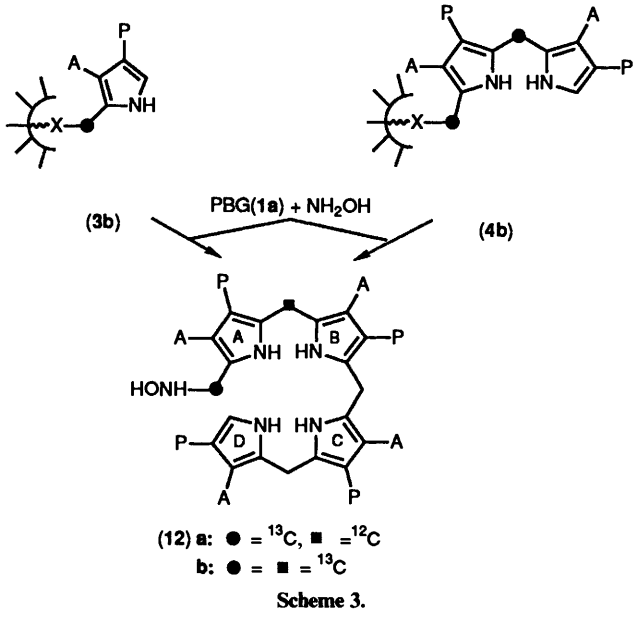


Figure 6. UV-VIS spectra of mixtures of modified Ehrlich's reagent with (*a*) HMBS-PBG mono-complex (final concentration 45 μg ml^{-1}); (*b*) di-complex (78 μg ml^{-1}), and (*c*) tri-complex (108 μg ml^{-1}), recorded as for Figure 5.



Scheme 3.

observation now fell perfectly into place. Treatment of HMBS with 70% aqueous formic acid led to the rapid formation of uroporphyrins, by air oxidation of the uroporphyrinogens initially generated. The same phenomenon was also observed when HMBS was dissolved in 0.1M hydrochloric acid but the process was considerably slower. The uroporphyrins from the formic acid treatment were shown as above to comprise type I:type III/IV:type II (see Scheme 4) in the ratio 57:40:3 and their total yield corresponded in different experiments to 0.8–1.2 mol of bound pyrrole per mole of enzyme. In contrast, treatment of PBG (**1a**) with formic acid under the same conditions did not lead to the formation of any significant amount of uroporphyrins whereas it was known that porphyrins can be formed from suitable dipyrromethanes (*e.g.* ref. 29). Since the non-enzymic conversion of dipyrromethanes[29] and bilanes[30] into uroporphyrins (*via* uroporphyrinogen) gave 40–50% yields, the amounts of porphyrins formed from HMBS were in keeping with there being a dipyrromethane bound to the protein. The illustrated structure (**18**) for this pyrrolic system tightly bound to a group Y on the protein, Scheme 5, is based on its conversion *via* uroporphyrinogens into uroporphyrins, mainly uroporphyrin I (**14a**); each pyrrole unit must carry one acetate and one propionate substituent in the illustrated order together with a one-carbon residue to form each methine bridge of the final porphyrin (**14a**).

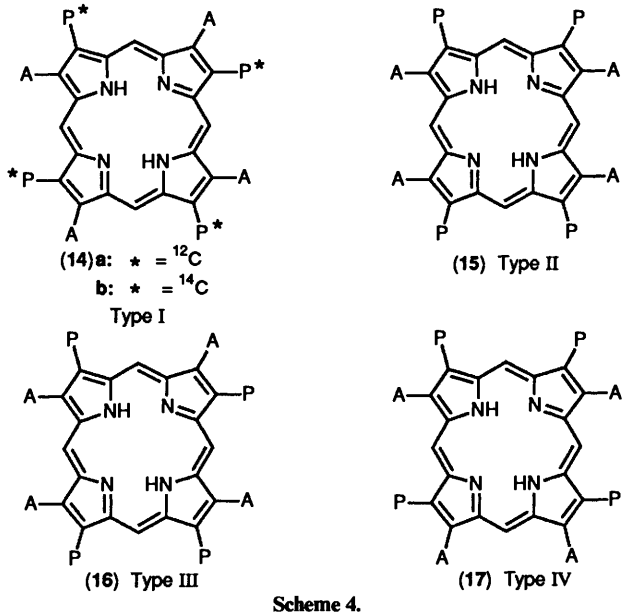
It is relevant that non-enzymic conversion of the aminomethyldipyrromethane, formed by hydrolysis of the lactam (**22**) into uroporphyrins afforded[29] a mixture of composition type I:type III/IV:type II in the ratio 68:31:1 (*cf.* the values above).

We wished to confirm that the covalently bound pyrrolic cofactor (**18**) possessed a free terminal α-positon and so HMBS was treated with acidic *p*-dimethylaminobenzaldehyde (Ehrlich's reagent). The purple pigment (λ_{max} 564 nm) characteristic of α-free pyrroles was generated rapidly but then decayed and was replaced by a second chromophore (λ_{max} 495 nm) which appeared orange; Figure 5(*a*) shows the clear isosbestic point from a series of sequential spectroscopic observations. This result was interpreted as being due to the initially formed Ehrlich pigment (**19**) undergoing tautomerisation to the more stable pyrromethene system (**20**), Scheme 5.

The foregoing interpretation was shown to be correct by synthesising the dipyrromethane (**21**) by modification of the earlier route,[31] followed by mild hydrolysis to yield the required standard substance (**22**), Scheme 5. When this was treated with Ehrlich's reagent as above, closely similar spectroscopic changes were observed [Figure 5(*b*)], the two maxima being 562 and 488 nm. It was clear that the enzyme HMBS possesses a covalently bound dipyrromethane cofactor (**18**).

Subsequently we found from the literature that similar spectroscopic changes on treatment of a synthetic dipyrromethane with Ehrlich's reagent had been observed 19 years ago[32] though the chemistry involved was not discussed at that time.

Our studies with the Ehrlich reaction were extended in the following ways. The HMBS used for most of the earlier work in



Scheme 4.

Cambridge [3] was from *Euglena gracilis* and this enzyme was also treated with Ehrlich's reagent. The striking spectroscopic changes shown in Figure 5(*a*) were again observed showing that *Euglena* HMBS also depends on the dipyrromethane cofactor.

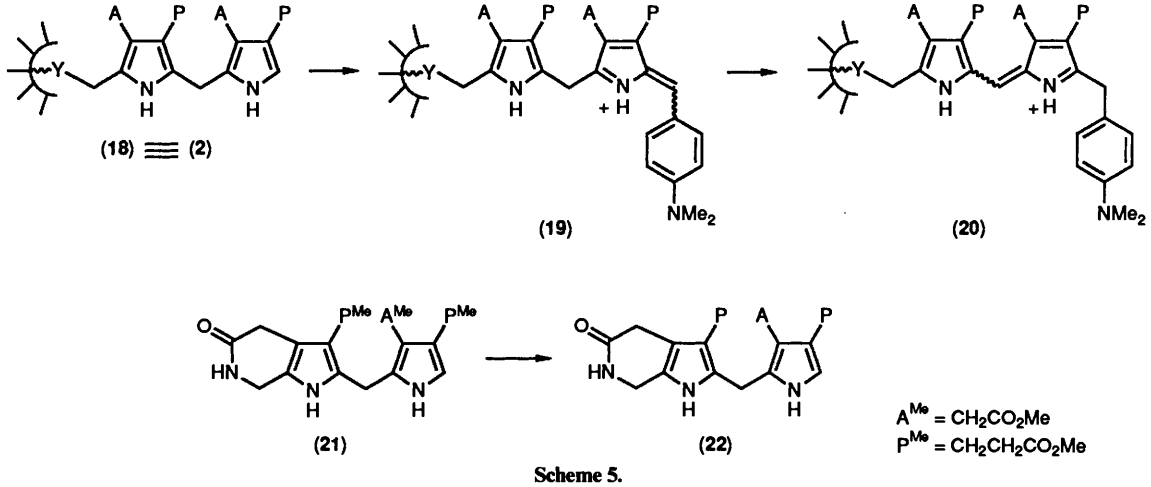
The pigment generated from *E. coli* HMBS by the tautomerisation above appeared orange but dialysis of the solution removed the excess of Ehrlich's reagent and the true colour of this second pigment was seen to be pink-to-red. Addition of Ehrlich's reagent to this solution re-established the orange colour; the 495–496 nm absorption was observed at the same intensity from both the pink-to-red and the orange solutions. So the true pink-to-red colour appears orange to the eye in the presence of the greenish yellow colour of Ehrlich's reagent.

Finally, the behaviour of the various enzyme-PBG complexes with Ehrlich's reagent was studied. Samples of the enzyme-PBG_1 mono-complex (**23a**), enzyme-PBG_2 di-complex (**24a**) and enzyme-PBG_3 tri-complex (**26a**), Scheme 6, were prepared and isolated as before by FPLC with further careful purification of the first two by FPLC just before use. Each was treated with Ehrlich's reagent as for the holoenzyme and the spectroscopic results are shown for the mono-, di-, and tri-complexes in Figure 6(*a–c*), respectively. The spectroscopic changes were more complex than were observed for the holoenzyme [Figure 5(*a*)] and a sharp isosbestic point was not seen for any of the three. However, some conclusions can be drawn. Tautomerisation of the initially formed Ehrlich products (**27**), (**29**), and (**30**), Scheme 6, again occurs leading for all the complexes to a pyrromethene chromophore, λ_{max} 495–497 nm. For the di-, and tri-complexes, less Ehrlich pigment (λ_{max} 564 nm) was detected in both cases and it disappeared quickly, so it seems that the tautomerisation is faster in these cases than for the holoenzyme or mono-complex.

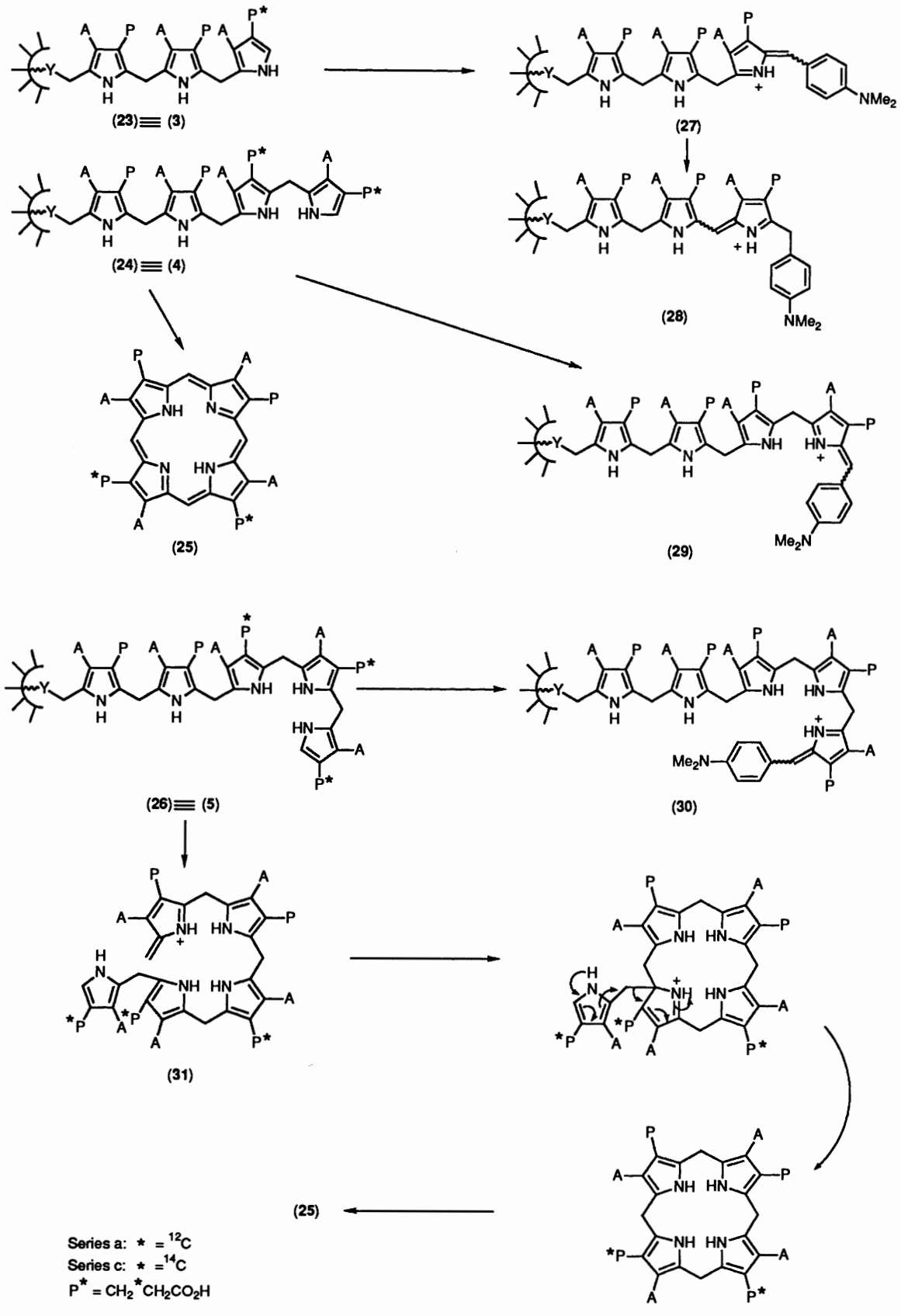
When the final steady absorbance values at *ca.* 495 nm had been reached in the three samples, each was dialysed for an extended period against pH 8 buffer containing 6M guanidine hydrochloride (denaturing conditions). In all three cases, the pigment passed into the dialysate and the retained protein showed almost no absorbance at *ca.* 495 nm. This is in sharp contrast to the properties of the final pyrromethene (**20**) formed from holoenzyme. This protein solution remained pink after the same dialysis step and retained 56% of the original 495 nm absorbance; for comparison, there is significant loss of absorbance from solutions of the pyrromethene (**20**) from holoenzyme simply on storage (*i.e.* without dialysis). Ultrafiltration of the dialysed pigmented protein solution similarly left the absorbing material attached to the enzyme. So the pyrromethene (**20**) from holoenzyme is reasonably stable to the acidic conditions of the Ehrlich reaction whereas the pyrromethenes formed from the mono-, di-, and tri-PBG complexes are cleaved from the protein. This indicates that the tautomerisation process for the Ehrlich products (**27**), (**29**), and (**30**) from the PBG complexes (**23a**), (**24a**), and (**26a**), respectively, does not in any of the three cases move the double bonds all the way until the pyrrole ring attached to the protein comes into conjugation, *i.e.* the final pyrromethene from the mono-complex has mainly structure (**28**), Scheme 6, and similarly for the di-, and tri-complexes.

Further Evidence for the Dipyrromethane Structure of the Cofactor.—The enzyme-[^{14}C]PBG_2 di-complex (**24c**) was prepared as usual from HMBS and [9-^{14}C]PBG (**1c**) and the entire tetrapyrrole was cleaved from the group Y using formic acid. The uroporphyrinogens first formed were oxidised with iodine to uroporphyrins which were separated to afford uroporphyrin I (**25c**), 75% of total porphyrin and uroporphyrin(s) III/IV, 25% of total. The illustrated labelling of uroporphyrin I (**25c**) shows that its specific molar activity should (since two unlabelled pyrroles have been cleaved from the enzyme) be only 50% that of uroporphyrin I (**14c**) produced by the normal action of HMBS on the same sample of [9-^{14}C]PBG (when all four pyrrole rings are derived from labelled substrate). The value found was 51%.

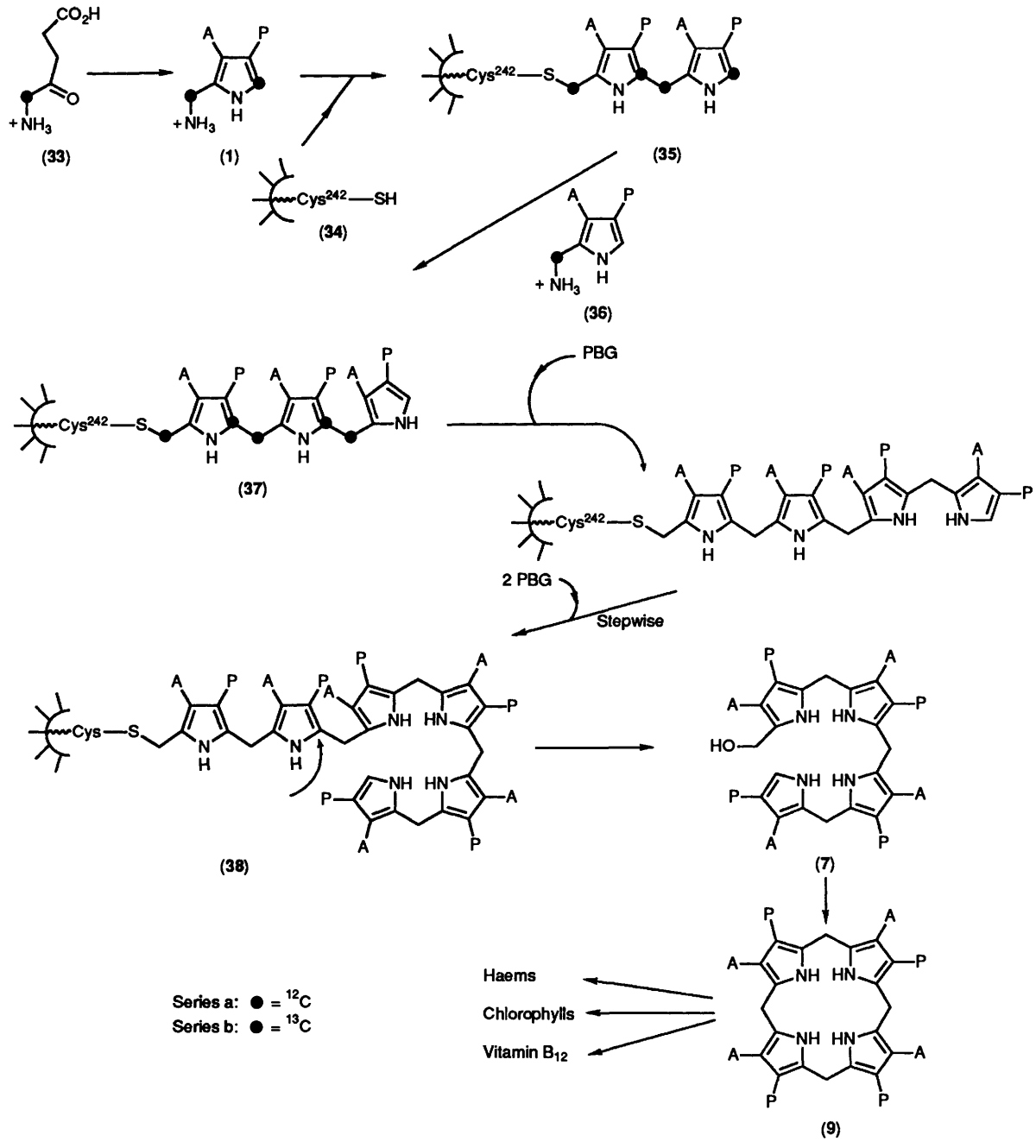
Similar acid-catalysed cleavages were carried out on



Scheme 5.


(23)≡ (3)
(27)
(24)≡ (4)
(28)
(25)
(29)
(26)≡ (5)
(30)
(31)
(25)
Series a: * = ^{12}C
Series c: * = ^{14}C
P* = CH_2 *CH_2CO_2H

Scheme 6.



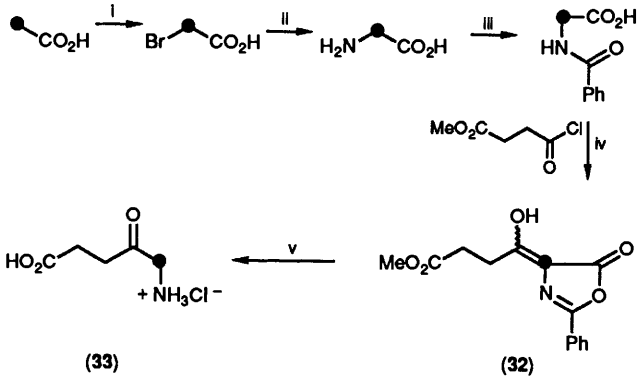
Scheme 7.

the enzyme-[^{14}C]PBG$_1$ mono-complex (**23c**) and enzyme-[^{14}C]PBG$_3$ tri-complex (**26c**). The former (**23c**) gave a ratio of uroporphyrin I:uroporphyrin III/IV of 3:2 and the specific activities were for type I, 36.5% and for type(s) III/IV, 34.8%, again relative to that of uroporphyrin I from normal turnover of the same [9-^{14}C]PBG. With one labelled PBG unit and two unlabelled units available [see compound (**23c**)], a statistical value of 33.3% could be expected.

The tri-complex (**26c**) gave a ratio of I:III/IV uroporphyrins of 69:31 with the relative specific activities (as above) for I being 50.4% and for III/IV being 52.5%. These activities are not significantly different from those found earlier for the di-complex (**24c**). For uroporphyrin I formation, we believe that the value of *ca.* 50% is due to acidic cleavage of the pentapyrrole (**26c**) from the protein to yield the azafulvene (**31c**) which specifically cyclises as illustrated in Scheme 6 with elimination finally of the terminal labelled pyrrole ring. The origin of the type III/IV product(s) will be studied.

The sum of all the foregoing evidence[2a] interlocked to establish for the first time* that (*a*) the enzyme HMBS makes use of a novel dipyrromethane cofactor (**18**) in its catalytic action; (*b*) the cofactor is covalently bound to the enzyme and its structure corresponds to the dipyrromethane formed by joining two units of PBG (**1a**) head-to-tail; and (*c*) the growing oligopyrrole system is covalently bonded to the cofactor. Thus

* This discovery was announced at the W. H. Perkin Jr. Centenary Symposium of the Royal Society of Chemistry, Heriot-Watt University, Edinburgh, 8 September, 1987.



Scheme 8. *Reagents:* i, P, Br_2; ii, NH_4OH; iii, PhCOCl; iv, RCOCL, 4-methylpyridine; v, 6M HCl, reflux.

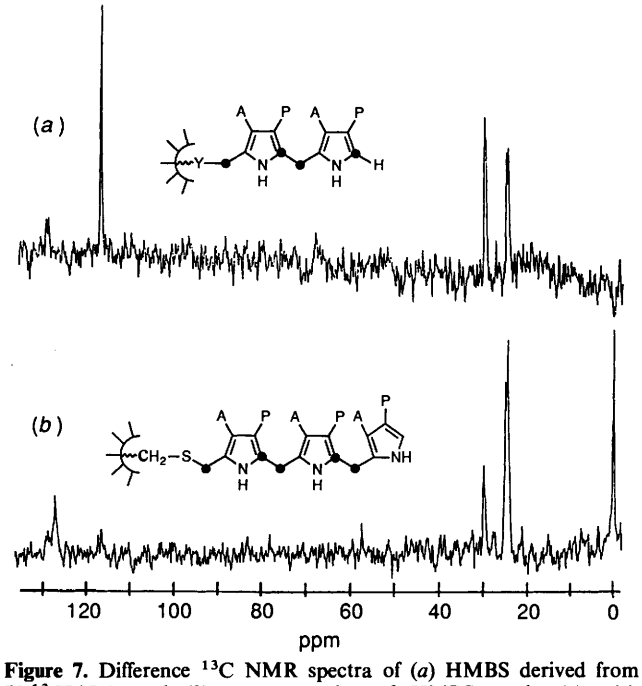


Figure 7. Difference ^{13}C NMR spectra of (*a*) HMBS derived from [5-^{13}C]ALA and (*b*) mono-complex of HMBS as in (*a*) with [11-^{13}C]PBG, in each case after subtraction of a spectrum of the corresponding unlabelled enzyme or complex.

the building of the tetrapyrrole system leading to release of hydroxymethylbilane (**7**) generates a hexapyrrole (**38**), see Scheme 7. Specific protonation of this system at the arrowed centre would lead to release of the assembled tetrapyrrole leaving the dipyrromethane cofactor ready for a new assembly cycle.

Following our initial publication,[2a] there were important developments from several groups and the main ones will be briefly summarised. The independent detection of the dipyrromethane cofactor in HMBS was reported[33] and the following year,[19] further evidence about its role appeared. In Cambridge, it was established that the Y-group to which the dipyrromethane is attached [see structure (**18**), Scheme 5] is the sulphur of cysteine[2b,22] (see also below) which was directly identified[34] as cysteine-242. In addition, the dipyrromethane cofactor was cleaved from HMBS without destroying the protein. This apoenzyme was shown to have the capability to rebuild its own cofactor from PBG and to attach it covalently so forming the holoenzyme.[22] Results in agreement with the findings on cysteine-242 were added by others[35] including valuable further studies by ^{13}C NMR spectroscopy and site-directed mutagenesis[36] which supported several of the structural features discussed above and below.

Our final experiments involved the incorporation of 5-amino-[5-^{13}C]laevulinic acid (**33**), ALA, into the dipyrromethane cofactor. First, suitable conditions were worked out using [4-^{14}C]ALA which was included at a concentration of 81 μM in the medium used for growing the overproducing strain of *E. coli*. The isolated HMBS was radioactive and its specific molar activity was 83% of the expected value calculated from the specific activity of the original ALA. Clearly there had been little dilution of the ALA (and any subsequent intermediates) by endogenous materials.

The labelled cofactor was cleaved with formic acid and isolated as previously in the form of uroporphyrins. Their specific activity was 93% of that expected from the initial specific activity of the labelled HMBS. It follows that essentially all the ^{14}C-activity of HMBS from [4-^{14}C]ALA was carried by the dipyrromethane cofactor and therefore that the cofactor is built from four ALA molecules.

To make the foregoing conclusions rigorous, [5-^{13}C]ALA (**33**) was synthesised from [2-^{13}C]acetic acid by a 5-step sequence, Scheme 8, in an overall yield of 42%; the key steps were based on the literature.[9b,37] Improvements in the preparation[37c] of the azlactone derivative (**32**) are described in the Experimental section. The intermediate [2-^{13}C]bromoacetic acid could also be conveniently prepared from sodium [2-^{13}C]acetate by Anker's method.[38]

The [5-^{13}C]ALA was then added at 93 μM to the growing medium for the overproducing *E. coli* strain and the HMBS was isolated and finally purified by FPLC. The ^{13}C NMR spectrum of this enzyme was run at pH 14 with the aim of increasing still further the mobility of the dipyrromethane residue.[34] As previously, the natural abundance ^{13}C spectrum of an equivalent sample of unlabelled HMBS, recorded under exactly the same conditions, was subtracted from the spectrum of the labelled sample leaving only the signals from the ^{13}C-labelled sites. Four remarkably sharp signals were obtained [Figure 7(*a*)], immediately confirming the incorporation of 4 ALA units into the dipyrromethane cofactor (**35b**), Scheme 7.

The signal at δ 29.5 stood precisely where the resonance appeared from the synthetic model peptide (**10**) carrying as a side-chain S-$^{13}CH_2$-pyrrole. It was thus certain[2b] (see also refs. above) that the dipyrromethane cofactor of HMBS produced normally from its natural precursor is bound to the sulphur of a cysteine residue. The doublet at δ 24.5 is the now familiar one from the interpyrrolic $^{13}CH_2$ group and the fact that it was a split signal proves that the interpyrrolic $^{13}CH_2$ is directly bonded to another ^{13}C atom. The splitting ($J = 45 \pm 5$ Hz) is consistent with an sp^3-sp^2 connection [see structure (**35b**)]. The signal from the sp^2 ^{13}C atom of this pair at δ 128.3 was weaker than the others as expected for a quaternary centre when rapid pulsing conditions are used; again, the splitting of this signal was quite clear ($J = 45 \pm 5$ Hz). Finally, the strong signal at δ 116.2 corresponded exactly to an unsubstituted α-carbon of a pyrrole [see structure (**35b**)]. These results confirmed all the main features of the dipyrromethane structure and its bonding to the protein.

The foregoing ^{13}C-labelled HMBS (**35b**) was then incubated with 1.06 mol equiv. of singly-labelled [11-^{13}C]PBG (**36b**) and the enzyme-[^{13}C]PBG_1 mono-complex (**37b**) was isolated. The ^{13}C NMR spectrum of this product was recorded at pH 14 and the by now standard difference spectrum was generated [Figure 7(*b*)]. If this spectrum is to confirm structure (**37b**) for the mono-complex, then it should show the following: (*a*) Most importantly (since the bonding site is thereby located), the strong singlet at δ 116.2 from the unsubstituted pyrrolic α-position of the HMBS cofactor [see Figure 7(*a*)] should not be present in Figure 7(*b*); (*b*) the signal at δ 24.5 from interpyrrolic $^{13}CH_2$ groups should have doubled in size; (*c*) the signal at δ 29.5 from the S-$^{13}CH_2$-pyrrole system should be un-

changed; and (*d*) the signal intensity at lowest field, *ca.* δ 126 should have doubled. Inspection of Figure 7(*b*) shows that all these requirements are met and thus structure (**37b**) for the mono-complex is rigorously confirmed.

All the foregoing studies show hydroxymethylbilane synthase, HMBS, to be a unique enzyme.[39] It is a protein that uses a novel dipyrromethane cofactor (**35a**) for assembly of a hexapyrrolic chain (**38**) from which the tetrapyrrolic hydroxymethylbilane (**7**) is cleaved as the product probably by protonation at the arrowed site. The apoenzyme (**34**) lacking its cofactor, possesses its own catalytic activity to construct and covalently bind the cofactor to itself through cysteine-242 using PBG (**1a**) as the building block;[22] PBG is also the normal substrate for the fully developed holoenzyme, The bound cofactor thereafter remains in place as an anchor for the assembly of four more PBG units and does not itself turn over. The full sequence is shown in Scheme 7. This remarkable enzyme deserves much further study.

Experimental

General.—General directions for the chemical syntheses are as given in ref. 1. PBG lactam methyl ester was hydrolysed in 2M aqueous KOH (0.1 ml mg^{-1}) for 16 h at 37 °C. Generally a larger amount of lactam than required was hydrolysed and a suitable aliquot of the hydrolysate was taken. General directions for the enzymic experiments are as given in ref. 22. One unit of enzyme produces 1 nmol of porphyrinogen per hour. All buffer solutions contained the added protease inhibitors and other additives described in ref. 12 unless otherwise stated. Concentration of enzymic solutions was performed by ultrafiltration using an Amicon ultrafiltration cell with a PM-10 membrane for larger volumes or an Amicon centricon 10 microconcentrator for smaller volumes. HMBS refers to hydroxymethylbilane synthase isolated from *E. coli* unless otherwise stated. The modified Ehrlich's reagent consisted of a solution of 4-dimethylaminobenzaldehyde (0.2 g) in formic acid (7 ml) and 2M hydrochloric acid (3 ml).

Preparative ion exchange FPLC was performed at ambient temperature on a Mono Q HR 10/10 column equilibriated with 15 mM Tris/HCl pH 8.3. The column was eluted at 3 ml min^{-1} with a linear gradient of 0–0.4M NaCl in the same buffer; the gradient volume was 110 ml and the eluant was continuously monitored at 280 nm. Analytical ion exchange FPLC was similar but used a Mono Q HR 5/5 column eluted at 1 ml min^{-1} and the gradient volume was 40 ml. Analytical separation of the isomers of uroporphyrin was achieved by reverse-phase FPLC on a PEP RPC HR 5/5 column, eluting with a gradient of 30 mM aqueous ammonium formate (containing 0.6 mM EDTA)–acetonitrile from 1:0–3:1 in 30 ml at 0.7 ml min^{-1} with continuous monitoring at 406 nm. Peaks were identified in each case by co-elution with standard samples of uroporphyrins I and III. Typical elution volumes were: I, 9.9 ml; III, 10.5 ml. By comparison with previous reports[40] it was assumed that the elution order of the other isomers would be IV with III, then II. Preparative separation of the uroporphyrins was performed in a similar manner using a PEP RPC HR 10/10 column, eluted with a gradient volume of 240 ml at 5.6 ml min^{-1}. Uroporphyrin concentrations were determined by the absorbance at 406 nm of solutions in 1M hydrochloric acid with ε_{max} 528 000.[41]

^{13}C NMR spectra were recorded on a Bruker AM400 spectrometer at 100 MHz in 5 mm tubes in water with added D_2O for the lock signal and sodium [$^2H_4$]-3-trimethylsilylpropionate (TSP) as a standard (0 ppm). For the bilanes, the spectra were acquired at ambient temperature over 16K data points with a spectral width of 207 or 292 ppm (acquisition time 0.39 or 0.28 s) using 90° pulses and Waltz proton decoupling; the FIDS were zero-filled to 32K and subjected to line-broadening of 2 Hz before Fourier transformation. The spectra of enzyme-bound intermediates were acquired at 5–10 °C over 4K data points with a spectral width of 237 ppm (acquisition time 0.086 s) using 45° pulses and Waltz proton decoupling; the FIDs were subjected to line-broadening of 10 Hz before Fourier transformation. Baseline correction was applied to the spectra where necessary. The spin-echo pulse sequence used a 90° pulse followed by a delay of 7.4 ms ($1/J_{C-H}$) with the decoupler off, then a 180° pulse and a chemical shift refocusing delay of 7.4 ms with full ^1H-decoupling before acquisition with full decoupling.

[11-^{13}C]*Porphobilinogen Lactam Methyl Ester.*—2-[^{13}C]-Formyl-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethylpyrrole[9b] was converted into its oxime as described in ref. 8(*a*). A solution of the oxime (297 mg, 1.10 mmol) in glacial acetic acid (3 ml) was diluted with water (3 ml) and then vigorously stirred at 0 °C for 1 h in the presence of pre-activated zinc dust[42] (234 mg, 3.58 mmol). The residual zinc was removed by filtration (Celite) and washed with acetic acid (7 ml) then water (7 ml). The combined filtrate and washings were carefully poured into a vigorously stirred two-phase system of saturated aqueous sodium carbonate (20 ml) and dichloromethane (40 ml) and the aqueous phase was then adjusted to > pH 9 with an excess of solid sodium carbonate. The organic phase was separated and the aqueous phase washed with methanol–dichloromethane (1:9; 5 × 20 ml). The combined organic extracts were dried (Na_2SO_4), filtered, and evaporated to give crude aminomethylpyrrole as a pale yellow solid. A solution of this aminomethylpyrrole and triethylamine (0.2 ml, 1.43 mmol) in warm methanol (30 ml) was heated under reflux for 2 h under an atmosphere of argon. The majority of the methanol was evaporated (on an oil bath) allowing the lactam to crystallise out of solution as plates. Further product was obtained by preparative TLC of the mother liquors (total 190 mg, 77%), m.p. 246–248.5 °C (lit.,[8a] 248–249.5 °C); δ_H(400 MHz; CD_3SOCD_3) 2.45–2.57 (4 H, m, $CH_2CH_2CO_2$), 3.14 (2 H, br s, CH_2CO_2), 3.57 (3 H, s, CO_2Me), 4.24 (2 H, dt, *J* 140, 2.6 Hz, $^{13}CH_2NH$), 6.46 (1 H, s, α-H), 7.71 (1 H, s, lactam-NH), and 10.31 (1 H, br s, pyrr-NH); *m/z* 223 (M^+, 100%) and 222 (92). The data showed 99 atom% ^{13}C.

[5-^{13}C]5-*Aminolaevulinic Acid Hydrochloride* (**33**).—[2-^{13}C]Bromoacetic acid[9b] was converted into [2-^{13}C]glycine by the method of Bloch[37a] and thence into [2-^{13}C]hippuric acid as in ref. 37(*b*). A stirred solution of [2-^{13}C]hippuric acid (692 mg, 3.84 mmol) in dry γ-picoline (4.35 ml) under argon was cooled to −10 °C and 3-methoxycarbonylpropionyl chloride (0.74 ml, 5.99 mmol) was added over 10 min so as to keep the temperature of the mixture < −5 °C. The mixture was stirred for 2.5 h at between −5 and 2 °C and then poured into a stirred mixture of ice (11 g) and concentrated hydrochloric acid (3.5 ml). The precipitate was collected and washed with water (22 ml) and ethyl acetate (22 ml) to give the crude azlactone (**32**) (620 mg, 58%).

The azlactone (620 mg, 2.24 mmol) was heated at reflux in 6M hydrochloric acid (16.6 ml) for 10 h. The solution was then cooled to 20 °C and filtered. The filtrate was boiled with activated charcoal (0.1 g) for 10 min, filtered through Celite while hot, and evaporated to dryness. The residue was dissolved in concentrated hydrochloric acid (0.2 ml) on a steam bath and acetone (2 ml) was added. After 12 h at 5 °C the crystals were collected, washed with cold acetone (3 × 1.5 ml), and dried *in vacuo* over potassium hydroxide to give [5-^{13}C]ALA hydrochloride (**33**) (320 mg, 49% for both steps); δ_H 4.1 (2 H, d, *J* 143 Hz, $^{13}CH_2$); $\delta_C(D_2O)$ 49.96. The data indicated 90 atom% ^{13}C.

Incubation of [11-^{13}C]PBG *with HMBS from* E. coli.—[11-

^{13}C]Porphobilinogen lactam methyl ester (1.0 mg, 4.5 μmol) was hydrolysed and the solution was added to 0.2M sodium phosphate buffer pH 8.0 (100 μl). The pH was adjusted to *ca.* 8 with 1M aqueous phosphoric acid (100 μl) and the solution was incubated at 37 °C with HMBS from *E. coli* (35 000 units) in 0.1M sodium phosphate buffer pH 8.0 (350 μl), for 2 min. A solution of sodium hydroxide (77 mg), with dioxane (10 μl) and [$^{2}H_4$]TSP (15 mg), in deuterium oxide (1.35 ml) was added to raise the pH above 12. The ^{13}C NMR spectrum [Figure 1(*a*)] showed δ_C 24.0 (pyrrole-$^{13}CH_2$-pyrrole of uro'gen), 24.5 (pyrrole-$^{13}CH_2$-pyrrole of bilane), and 57.1 (HO-$^{13}CH_2$- of bilane), ratio of peak integrals, *ca.* 35:30:9.

The above solution was acidified with 35% hydrochloric acid to pH 3, and after 15 min at room temperature was re-basified with sodium hydroxide (*ca.* 300 mg) in water (100 μl); δ_C 24.0 (pyrrole-$^{13}CH_2$-pyrrole of uro'gen I) [see Figure 1(*b*)].

Incubation of [11-^{13}C]PBG *with HMBS from* Euglena gracilis *and* E. coli *in the Presence of Hydroxylamine at* pH 8.5.—[11-^{13}C]Porphobilinogen lactam methyl ester (0.75 mg, 3.38 μmol) was hydrolysed and the solution was added to 1M aqueous potassium dihydrogen phosphate (150 μl), so adjusting the pH to *ca.* 8.

A solution of hydroxylamine hydrochloride (9.5 mg, 137 μmol) in 0.1M aqueous sodium pyrophosphate (275 μl) was carefully adjusted to pH 8.25 with NaOH and added to a solution of HMBS from *Euglena gracilis* (13 600 units) in 0.1M sodium pyrophosphate buffer pH 8.25 (200 μl) at 37 °C, followed by the solution of [11-^{13}C]PBG prepared above (total volume 700 μl; final hydroxylamine concentration *ca.* 0.2M; final pH 8.45). After 10 min at 37 °C, sodium hydroxide (40 mg) was added in deuterium oxide (100 μl), followed by dioxane (5 μl), [$^{2}H_4$]TSP (8 mg), and chromium(III) chloride hexahydrate (6 mg) as a paramagnetic relaxation agent to allow for accurate integration. The ^{13}C NMR spectrum [Figure 1(*c*)] showed δ_C 24.5 (pyrrole-$^{13}CH_2$-pyrrole of bilane) and 51.2 (HONH-$^{13}CH_2$-bilane), ratio of peak integrals, 2.3:1.

Similarly, a solution of hydroxylamine hydrochloride (8.7 mg, 125 μmol) in 0.1M aqueous sodium pyrophosphate (250 μl) was carefully adjusted to pH 8.25 with NaOH and was added to a solution of HMBS from *E. coli* (13 600 units) in 0.1M sodium pyrophosphate buffer pH 8.25 (125 μl) at 37 °C, followed by a solution of [11-^{13}C]PBG prepared as above (total volume 600 μl; final hydroxylamine concentration *ca.* 0.2M; final pH 8.50). After 10 min at 37 °C, the solution was treated as above. The ^{13}C NMR spectrum [Figure 1(*d*)] showed δ_C 24.5 (pyrrole-$^{13}CH_2$-pyrrole of bilane) and 51.2 (HONH-$^{13}CH_2$-bilane/pyrrole), ratio of peak integrals *ca.* 1:1.3.

Incubation of [11-^{13}C]PBG *with HMBS from* Euglena gracilis *and* E. coli *in the Presence of Hydroxylamine at pH* 7.0–7.25.—[11-^{13}C]Porphobilinogen lactam methyl ester (**1b**) (0.75 mg, 3.38 μmol) was hydrolysed and the solution was added to 0.2M sodium phosphate buffer pH 8.0 (100 μl) and adjusted to a pH of *ca.* 7 with 1M phosphoric acid (100 μl).

A solution of hydroxylamine hydrochloride (11.3 mg, 163 μmol) in 0.1M aqueous sodium phosphate (325 μl) was carefully adjusted to pH 7.5 with NaOH and added to a solution of HMBS from *Euglena gracilis* (13 500 units) in 0.1M sodium phosphate buffer pH 8.0 (300 μl) at 37 °C, followed by the solution of [11-^{13}C]PBG prepared above (total volume 900 μl; final hydroxylamine hydrochloride concentration *ca.* 0.2M; final pH 7.25). After 10 min at 37 °C, sodium hydroxide (40 mg) was added in deuterium oxide (100 μl), followed by dioxane (10 μl) and [$^{2}H_4$]TSP (15 mg). The ^{13}C NMR spectrum, acquired using a relaxation delay of 4.7 s [see Figure 1(*f*)], showed δ_C 24.5 (pyrrole-$^{13}CH_2$-pyrrole of bilane) and 51.1 (HONH-$^{13}CH_2$-bilane) (ratio of peak integrals, 1.38:1), and a small additional peak at 24.0 ppm (pyrrole-$^{13}CH_2$-pyrrole of uro'gen I). The spectrum was recorded again (with no relaxation delay) after the addition of chromium(III) chloride hexahydrate (6 mg) and the same peaks were observed in a ratio of 1.67:1.

Similarly, a solution of hydroxylamine hydrochloride (8.7 mg, 125 μmol) in 0.1M aqueous sodium phosphate (250 μl) was carefully adjusted to pH 7.5 with NaOH and added to a solution of HMBS from *E. coli* (12 500 units) in 0.1M sodium phosphate buffer pH 8.0 (125 μl) at 37 °C, followed by a solution of [11-^{13}C]PBG prepared as above (total volume 650 μl; final hydroxylamine hydrochloride concentration *ca.* 0.2M; final pH 7.0). After 10 min at 37 °C, sodium hydroxide (40 mg) was added in deuterium oxide (350 μl), followed by dioxane (5 μl) and [$^{2}H_4$]TSP (8 mg). The ^{13}C NMR spectrum, acquired with the relaxation delay as before [see Figure 1(*e*)], showed δ_C 24.5 (pyrrole-$^{13}CH_2$-pyrrole of bilane) and 51.2 (HONH-$^{13}CH_2$-pyrrole) (ratio of peak integrals *ca.* 1:6.5), and very small additional peaks at 24.0 ppm (pyrrole-$^{13}CH_2$-pyrrole of uro'gen I) and 38.4 (H_2NCH_2-pyrrole of PBG).

This experiment was repeated and gave the same peaks at δ_C 24.5, 38.3, and 51.2 but in a ratio of *ca.* 1:3:7.

Preparation of the Complexes of HMBS with [11-^{14}C]PBG.—A solution of HMBS (10 mg, 0.29 μmol) in 15 mM Tris/HCl buffer pH 8.3 (5 ml) was rapidly mixed at 18 °C with a solution of [9-^{14}C]PBG (*ca.* 0.58 μmol) of known specific activity and then fractionated by FPLC.[22] Fractions corresponding to peaks A–D (see Figure 2) were collected. The protein concentrations of the fractions were determined by their absorbance at 280 nm[22] and their PBG content was determined from their radioactivity measured by liquid scintillation counting. The results for mol PBG/mol enzyme averaged over several runs were: peak A (two runs), 0.02 ± 0.005; peak B (four runs), 0.94 ± 0.04; peak C (four runs), 1.98 ± 0.10; peak D (two runs), 2.51 ± 0.01.

Proportion of the Complexes of HMBS Produced with Various Amounts of PBG.—Solutions of HMBS (1 mg, 29 nmol) in 15 mM Tris/HCl buffer pH 8.3 (1 ml) were rapidly mixed at 18 °C with solutions of PBG containing up to 4 mol PBG/mol enzyme. After 20 min the complexes were separated by analytical FPLC. The peaks obtained were twice traced, cut out, and weighed to determine their relative areas, which are plotted in Figure 3.

Preparation and ^{13}C *NMR Spectroscopy of the Complexes of HMBS with* [11-^{13}C]PBG.—[11-^{13}C]PBG lactam methyl ester (1.9 mg, 8.56 μmol) and [9-^{14}C]PBG lactam methyl ester (0.30 mg, 1.35 μmol) were both hydrolysed and added to 0.2M aqueous sodium phosphate pH 8.0 (1 ml). The pH was adjusted to *ca.* 8 with 1M phosphoric acid (220 μl). This stock solution of ^{13}C/^{14}C-PBG (85 atom% ^{13}C; 6.15 mM by quantitative colorimetric Ehrlich assay[43]) contained 89.7 dpm nmol^{-1}.

HMBS (35.8 mg, 1 045 nmol) in 15 mM-Tris/HCl pH 8.5 (19 ml) was rapidly mixed at 0 °C with an aliquot of this ^{13}C/^{14}C-PBG stock solution (160 μl, 984 mmol) and then allowed to warm to room temperature. The mixture was fractionated in two batches by preparative ion-exchange FPLC. Each main peak was collected on ice using manual peak cutting. Enzyme having no bound PBG (9.0 mg, 263 nmol) was re-treated with ^{13}C/^{14}C-PBG stock solution (40 μl, 246 nmol) and fractionated in the same way. The fractions containing the mono-complex (**3b**) (10.2 mg, 296 nmol) were found, by radioactive counting, to contain 0.94 mol PBG/mol enzyme. The di-complex (**4b**) (14.5 mg, 418 nmol) was found to contain 1.88 mol PBG/mol enzyme. Both solutions were concentrated to *ca.* 3 ml and dialysed overnight against two changes of nitrogen-saturated and additive-free 15 mM aqueous sodium phosphate, pH 12, to

remove the Tris/HCl and additives. Both solutions were further concentrated to *ca.* 0.3 ml by ultrafiltration and then prepared for ^{13}C NMR spectroscopy by the addition of deuterium oxide (100 μl) and [$^{2}H_4$]TSP (*ca.* 1 mg) [see Figure 4(*a*)].

The ^{13}C labelled mono-complex (**3b**) for the spin-echo experiment (8.9 mg, 258 nmol, 0.89 mol PBG/mol enzyme) was prepared from HMBS (34.1 mg, 996 nmol) in the usual way using the $^{13}C/^{14}C$-PBG stock solution. The solution of the ^{13}C mono-complex was concentrated in the usual way and dialysed overnight against two changes of nitrogen-saturated and additive-free 15 mM sodium pyrophosphate buffer pH 8.5 at 0 °C, further concentrated to *ca.* 280 μl by ultrafiltration, adjusted to pH 12 by the addition of 0.2M aqueous sodium dihydrogen phosphate (50 μl) and 2M aqueous sodium hydroxide (7 μl), and prepared for ^{13}C NMR spectroscopy in the usual manner.

Unlabelled mono-complex (**3a**) (6.7 mg, 194 nmol) was prepared in the same way from HMBS (31.3 mg, 914 nmol) and unlabelled PBG monohydrate (198 μg, 811 nmol) in 0.2M sodium phosphate buffer pH 8.0 (37 μl). The solution was concentrated to *ca.* 3 ml, dialysed overnight against two changes of nitrogen-saturated and additive-free 15 mM aqueous sodium pyrophosphate pH 8.5 at 0 °C, further concentrated by ultrafiltration to *ca.* 380 μl, and prepared for ^{13}C NMR spectroscopy in the usual manner. After acquiring a spectrum at pH 8.5 (*ca.* 2 h), the pH was adjusted to 12 by the addition of 0.2M aqueous sodium dihydrogen phosphate (30 μl) and 2M aqueous sodium hydroxide (6 μl). First a standard spectrum (768 000 transients, 18.4 h) and then a spin-echo spectrum (634 535 transients, 17.6 h) was acquired.

Demonstration of the Catalytic Competence of the Complexes of HMBS with [11-^{13}C]PBG.—^{13}C Mono-complex (**3b**) (10.6 mg, 307 nmol, 1.02 mol PBG/mol enzyme) was prepared from HMBS (12 100 units mg^{-1}; 30.5 mg, 890 nmol) in the usual way using the $^{13}C/^{14}C$-PBG stock solution. The solution was concentrated to *ca.* 3 ml by ultrafiltration and dialysed overnight at 0 °C against two changes of nitrogen-saturated and additive-free 15 mM aqueous sodium pyrophosphate pH 8.5. An aliquot (80 μl) of the solution of the ^{13}C mono-complex was diluted into 15 mM Tris/HCl buffer pH 8.5 (600 μl) for analysis by analytical ion-exchange FPLC. The remaining solution of the ^{13}C mono-complex was further concentrated to *ca.* 250 μl by ultrafiltration, and then prepared for ^{13}C NMR spectroscopy by the addition of deuterium oxide (100 μl) and [$^{2}H_4$]TSP (*ca.* 1 mg). After acquisition of the spectrum (72 514 transients, 1.75 h), the solution was adjusted to pH 12 by the addition of 0.2M aqueous sodium dihydrogen phosphate (30 μl) followed by 2M aqueous sodium hydroxide (6 μl) and a further spectrum was acquired (48 000 transients, 1.15 h).

The solution was then re-adjusted from pH 12 to 8.45 by the addition of 0.23M aqueous disodium dihydrogen pyrophosphate (38 μl) (total volume 486 μl) and treated with a solution of hydroxylamine hydrochloride (10.7 mg, 154 μmol) in 0.2M aqueous sodium phosphate (185 μl) which had been adjusted to pH 8.0 with NaOH, followed by a solution of unlabelled PBG (**1a**) monohydrate (663 μg, 3.00 μmol) in 0.2M aqueous sodium phosphate buffer pH 8.0 (31 μl). After incubation at 37 °C for 8 min, the solution was cooled on ice and 2M aqueous potassium hydroxide (40 μl) was added. The ^{13}C NMR spectrum, recorded at 10 °C, showed δ_C 51.0 (HONH-$^{13}CH_2$-bilane) and small additional peaks at 24.5 (pyrrole-$^{13}CH_2$-pyrrole of bilane) and 50.8 ppm (HONH-$^{13}CH_2$-pyrrole).

Finally the solution was dialysed overnight against 15 mM aqueous sodium phosphate pH 12 and then repeatedly concentrated by ultrafiltration and diluted to *ca.* 2 ml until the yellow-coloured material (from bilane) had been removed. After final concentration to 500 μl, the solution was prepared for ^{13}C NMR spectroscopy by the addition of deuterium oxide (100 μl) and [$^{2}H_4$]TSP (*ca.* 1 mg). No signal at δ 24.6 was observed.

^{13}C Di-complex (**4b**) (10.9 mg, 314 nmol; 2.04 mol PBG/mol enzyme) was prepared from HMBS (12 100 units mg^{-1}; 30.5 mg, 890 nmol) in the usual way using the $^{13}C/^{14}C$-PBG stock solution. The solution was concentrated to *ca.* 3 ml by ultrafiltration and dialysed overnight against two changes of nitrogen-saturated and additive-free 15 mM aqueous sodium pyrophosphate buffer pH 8.5 at 0 °C. An aliquot (70 μl) of the solution was analysed by analytical ion-exchange FPLC to show that the complex had withstood dialysis and concentration. The remaining solution was concentrated further to *ca.* 250 μl by ultrafiltration and then prepared for ^{13}C NMR spectroscopy in the usual way. After acquisition of the spectrum, the solution was treated with solutions of hydroxylamine and unlabelled PBG monohydrate, incubated at 37 °C for 8 min, and quenched with 2M aqueous potassium hydroxide as in the previous experiment. The ^{13}C NMR spectrum showed: δ_C 24.4 (pyrrole-$^{13}CH_2$-pyrrole of bilane) and 51.0 (HONH-$^{13}CH_2$-bilane), ratio of peak integrals 1:1.04.

Demonstration that HMBS Does Not Remain Labelled After Turnover of [19-^{14}C]PBG.—A solution of HMBS (2.5 mg, 73 nmol) in 15 mM Tris/HCl buffer pH 8.3 (2.5 ml) was incubated at 18 °C with [9-^{14}C]PBG (680 dpm nmol^{-1}; 800 nmol) for 50 min and then fractionated by analytical FPLC. Holoenzyme (78% of the mixture) was separated from the mono-, di-, and tri-complexes and shown to be still active by enzymic assay. Samples (0.24 mg by A_{280}) showed *ca.* 162 dpm, which corresponds to incorporation of only 0.034 mol PBG/mol enzyme and is probably due to slight contamination by mono-complex (**3c**). The uroporphyrinogen produced in the incubation (partially oxidized to uroporphyrin) was eluted from the FPLC column with 1M NaCl in the eluant buffer and oxidized to the porphyrin with I_2 and KI. Excess I_2 was removed with aqueous sodium metabisulphite. The porphyrin was purified by reverse-phase FPLC and eluted as a single peak corresponding to uroporphyrin I. The quantity of porphyrin was calculated from the A_{406} measurement[41] and its radioactivity was *ca* 3 000 dpm nmol^{-1} (4.4 times the activity of the PBG).

Confirmation that HMBS Contains Covalently Bound Pyrrolic Material.—A solution of HMBS (0.8 mg) in 15 mM Tris/HCl buffer pH 8 (0.2 ml) was mixed with 6M guanidine hydrochloride in the same buffer (0.8 ml). After 30 min at 37 °C the solution was concentrated to 0.1 ml by ultrafiltration, diluted with the same guanidine hydrochloride solution (0.9 ml), and again concentrated to 0.1 ml. Tris/HCl buffer pH 8.5 (15 mM; 1.9 ml) was added and the solution was dialysed for 16 h at 18 °C against the same buffer containing 0.1% SDS. An aliquot (0.6 ml) of the resulting solution was mixed with modified Ehrlich's reagent (0.6 ml), giving an immediate intense purple colour and a precipitate. Membrane filtration showed the colour was only associated with the precipitate. The filtrates from the earlier ultrafiltration steps gave no colour when mixed with the modified Ehrlich's reagent but a strong purple colour was observed when HMBS (0.16 mg) was added to the mixture.

Treatment of HMBS and its Complexes with Acid.—HMBS (0.5 mg) was dissolved in 0.1M hydrochloric acid. After 16 h the solution was pink (a similar intensity was obtained in 1–2 h in 70% formic acid). The total quantity of uroporphyrins produced was measured by the A_{406} value after dilution of aliquots with 1M hydrochloric acid.[41] The relative amounts of the uroporphyrin isomers were measured by reverse-phase FPLC (type I, 57%; types III and IV, 40%; type II, 3%). It was found that more prolonged acid treatment did not increase the quantity of porphyrins produced.

A solution of HMBS-PBG_2 complex (**4c**) (12.5 mg; 21 ml) was prepared in the usual way using [9-^{14}C]PBG, concentrated to 4 ml by ultrafiltration, dialysed for 16 h against nitrogen-saturated 15 mM sodium pyrophosphate buffer pH 8.5, and then further concentrated to 0.55 ml. Analytical FPLC showed that the di-complex was still intact. Formic acid (0.55 ml) was added, followed, after 20 min at 4 °C, by a solution of I_2 (0.5%) and KI (1%) in water (60 μl). After 2 min at room temperature, excess I_2 was reduced by the addition of 1% aqueous sodium metabisulphite (25 μl). The solution was evaporated under reduced pressure and resuspended in 30 mM aqueous ammonium formate (3 ml). The pH was adjusted to *ca.* 6.5 by the addition of 2M aqueous potassium hydroxide (20 μl) and insoluble material was removed by centrifugation. The relative proportions of the uroporphyrin isomers were determined by analytical reverse-phase FPLC (type I, 75%; types III/IV, 25%). The isomers were separated and their specific radioactivities, determined as before, were compared with the radioactivity of a sample of uroporphyrin I (**8c**) prepared by normal turnover of the same [9-^{14}C]PBG (0.18 mg) using HMBS (2.5 mg) in 15 mM Tris/HCl buffer pH 8.3 (2.5 ml) at 18 °C for 50 min. The uroporphyrin I from the di-complex (**4c**) contained 51% of the radioactivity of the standard (**8c**) and the uroporphyrin III contained 48% (average of two runs in each case, maximum variation ±3%).

Similar experiments were carried out for the mono- (**3c**) and tri-complexes (**5c**) with the following results. Monocomplex (**3c**) gave a uroporphyrin I to III/IV ratio of 60:40, with specific radioactivities relative to the standard being: I, 36.5%; III/IV, 34.8%. Tri-complex (**5c**) gave a uroporphyrin I to III/IV ratio of 69:31, with specific radioactivities relative to the standard being: I, 50.4%, III/IV, 52.5%.

Treatment of HMBS and its Complexes with Ehrlich's Reagent.—Solutions of HMBS and its mono-, di-, and tri-complexes (*ca.* 0.1–0.2 mg ml^{-1}) were prepared as before except that the mono- and di-complexes were each repurified by a second FPLC run. The solutions were mixed with equal volumes of the modified Ehrlich's reagent and the UV spectra were recorded as soon as possible after mixing and every 2 min thereafter [see Figures 5(*a*) and 6]. Once the final steady absorbance values had been reached, the samples were dialysed against several changes of 15 mM Tris/HCl buffer pH 8.0 containing 6M guanidine hydrochloride. After dialysis the solution derived from the holoenzyme was still pink and the absorbance at 495 nm was 56% of the value before dialysis. This solution was concentrated (× 10) by ultrafiltration, which gave a colourless filtrate with negligible A_{495} value while the retentate was still pink. The solutions from the complexes, on the other hand, were colourless after dialysis with negligible A_{495} values. A solution derived from the tri-complex (**5a**) which was not dialysed, still had a significant A_{495} value after 4 days.

Hydrolysis of Dipyrromethane Lactam Trimethyl Ester (**21**) *and Reaction with Ehrlich's Reagent.*—The α-free pyrromethane (**21**) (13 mg, 31 μmol) was vigorously stirred in methanol (500 μl) and 1M aqueous potassium hydroxide (500 μl) at room temperature, in the dark, under an atmosphere of argon for 1 h. The mixture was then neutralised by careful addition of concentrated hydrochloric acid followed by 0.5M hydrochloric acid and freeze-dried. The residue was purified on a reverse-phase HPLC column, eluted with 50 mM aqueous ammonium hydrogen carbonate (50 ml) at 5.6 ml min^{-1}. Appropriate fractions were collected on ice and freeze-dried twice to give the pyrromethane tri-acid (**22**) as a solid (10 mg, 77%), which was stored in a deep freeze, in the dark, and under argon to limit decomposition of the sensitive product; δ_H(400 MHz; D_2O at pD 4) 2.36 (2 H, t, *J* 7.7 Hz, $CH_2CH_2CO_2$), 2.48 (2 H, t, *J* 8.0 Hz, $CH_2CH_2CO_2$), 2.63–2.71 (4 H, m, 2 × $CH_2CH_2CO_2$), 3.37 (2 H, s, CH_2CO_2), 3.42 (2 H, t, *J* 3.8 Hz, CH_2CO lactam), 3.84 (2 H, s, pyrr-CH_2-pyrr), and 4.37 (2 H, t, *J* 3.8 Hz, CH_2NH lactam); *m/z* (FD) 418 (MH^+, 100%).

A solution containing the pyrromethane tri-acid (**22**) (1.3 μg) in 50 mM sodium phosphate buffer pH 8.0 (60 μl) was added to a 3 ml cuvette containing 50 mM sodium phosphate buffer pH 8.0 (1.5 ml) and modified Ehrlich's reagent (1.5 ml). After thorough mixing (30 s), the absorbance spectrum was recorded over the range 650–400 nm against a 3 ml reference cuvette containing the same solutions as above. The recording was repeated at 2 min intervals thereafter for 30 min [see Figure 5(*b*)].

Production of HMBS from [4-^{14}C]ALA *and Cleavage of the Cofactor.*—The overproducing strain of *E. coli* was grown as previously described [22] in an 11 l fermenter with the addition of [4-^{14}C]5-aminolaevulinic acid hydrochloride (122.5 mg, 0.89 mmol; 11.6 μCi). After 17 h the cells were harvested (24 g wet weight) and the HMBS (26.3 mg) was purified as usual. The total radioactivity in the HMBS was 73 000 dpm (0.29% total incorporation) which corresponds to 43.2 μCi $mmol^{-1}$—a specific incorporation of 332% or 83% of that required for the incorporation of 4 mol ALA/mol enzyme.

A solution of this HMBS (1.65 mg, 48 nmol) in 15 mM Tris/HCl buffer pH 8.3 (0.5 ml) was mixed with formic acid (0.5 ml). After 21 h in the dark at 18 °C, the solution was evaporated to dryness under reduced pressure. The residue was purified by reverse-phase FPLC on an analytical column as described in the general directions but with a gradient volume of 60 ml. The specific activity of the uroporphyrin mixture, determined from the A_{406} value and radioactive counting, was calculated to be 80.16 μCi $mmol^{-1}$, which accounts for 93% of the radioactivity from the HMBS on the basis of two molecules of the cofactor generating one of porphyrin.

Production of HMBS from [5-^{13}C]ALA *and* ^{13}C *NMR Spectroscopy.*—The overproducing strain of *E. coli* was grown as previously described [22] in a 20 l fermenter with the addition of [5-^{13}C]5-aminolaevulinic acid hydrochloride (315 mg, 1.87 mmol), yielding 55 g wet weight of cells. HMBS (20.6 mg) was purified as before and the solution obtained from FPLC was dialysed against two changes of nitrogen-saturated, additive-free 15 mM sodium phosphate buffer pH 12 at 0 °C for 15 h. The solution was then concentrated to 0.5 ml by ultrafiltration, and treated with D_2O (0.1 ml), [$^2H_4$]TSP (1.7 mg), and, immediately before the acquisition of the ^{1}H NMR spectrum, 12M aqueous potassium hydroxide (50 μl) at 0 °C under argon to adjust the pH value to 14. The difference ^{13}C NMR spectrum between this sample (**35b**) and unlabelled HMBS (**2a**) under the same conditions is given in Figure 7(*a*).

Reaction of HMBS Derived from [5-^{13}C]ALA *with* [11-^{13}C]PBG.—The overproducing strain of *E. coli* was grown as previously described [22] in a 20 l fermenter with the addition of [5-^{13}C]5-aminolaevulinic acid hydrochloride (287 mg, 1.7 mmol; 99.4 atom%), yielding 62 g wet weight of cells. HMBS (**35b**) (59 mg) was purified as before. A portion of this HMBS (34.25 mg, 1.00 μmol) dissolved in 15 mM Bistris/HCl buffer pH 6.0 (7 ml) was mixed with 15 mM Tris/HCl buffer pH 8.5 (28 ml) and then rapidly mixed with a solution of [11-^{13}C]PBG (1.06 μmol) in 0.2M sodium phosphate buffer pH 8.0 (164 μl) at 0 °C. After 20 min the mixture was fractionated in three batches by preparative ion-exchange FPLC and the fractions corresponding to the mono-complex (**37**) were collected. More material was obtained by treatment of a further portion of HMBS (24.1 mg, 0.70 μmol) with [11-^{13}C]PBG (0.746 μmol) and then by treatment of the holoenzyme (12.3 mg, 0.36 μmol) obtained from the fractionation of this run with [11-^{13}C]PBG (0.38

μmol). The combined mono-complex fractions were repurified by FPLC to yield a solution containing pure mono-complex (**37**) (6.0 mg, 0.17 μmol). This solution was concentrated to 3 ml by ultrafiltration, dialysed against two changes of nitrogen-saturated additive-free 15 mM sodium phosphate pH 12 at 0 °C, further concentrated to 440 μl, and treated with D_2O (0.1 ml), [$^2H_4$]TSP (1.7 mg), and, immediately before the acquisition of the ^{13}C NMR spectrum, 12M aqueous potassium hydroxide (50 μl) at 0 °C under argon to adjust the pH value to 14. The difference ^{13}C NMR spectrum between this sample (**37**) and unlabelled mono-complex (**3a**) under the same conditions is given in Figure 7(*b*).

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