

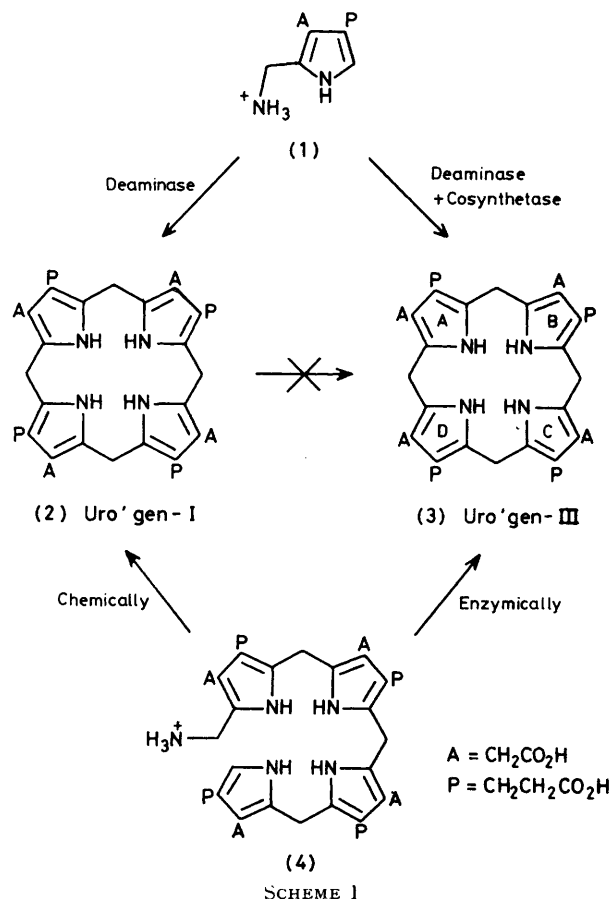
Biosynthesis of Porphyrins and Related Macrocycles. Part 18.^{1,2} Proof by Spectroscopy and Synthesis that Unrearranged Hydroxymethylbilane is the Product from Deaminase and the Substrate for Cosynthetase in the Biosynthesis of Uroporphyrinogen-III

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When the enzyme deaminase acts alone on porphobilinogen, it releases a transient intermediate into the medium which is unaffected by further treatment with a large excess of deaminase. The intermediate undergoes rapid ring-closure chemically ($t_{\frac{1}{2}}$ ca. 4 min) to form uroporphyrinogen-I. ¹³C Spectroscopic studies on the intermediate generated from ¹³C labelled porphobilinogen combined with synthesis of labelled standards for determination of chemical shifts establish its structure to be a linear tetrapyrrole, the unrearranged hydroxymethylbilane. Other workers deduced a different, cyclic structure (preuro'gen) which is shown here to be incorrect by chemical studies, ¹³C spectroscopy and ¹³C:¹⁵N double-labelling experiments. That the intermediate is the unrearranged hydroxymethylbilane is confirmed by its unambiguous synthesis. The natural and synthetic samples of this bilane are shown to be excellent and identical substrates for cosynthetase (free from deaminase) with production of uroporphyrinogen-III. Thus, deaminase is the enzyme for assembly of four porphobilinogen units to the linear tetrapyrrole stage and cosynthetase is the ring-closing and rearranging enzyme. Two proposals are discussed for the mechanism of inversion of the terminal ring-D of the hydroxymethylbilane in the formation of uroporphyrinogen-III.

THE formation of uroporphyrinogen-III (uro'gen-III) (3) and ammonia from four molecules of porphobilinogen (PBG) (1) is catalysed in living systems by two proteins, deaminase and cosynthetase. Cosynthetase is easily destroyed by heat-treatment and deaminase alone acts on PBG (1) to produce uroporphyrinogen-I (uro'gen-I) (2), an unnatural isomer. Uro'gen-I (2) is *not* enzymically isomerised to uro'gen-III (3) (Scheme 1). It was clear that cosynthetase must act on an intermediate produced by deaminase, or it must interact with deaminase at some stage to modify the way in which the PBG units are linked together. Parts 6,³ 16,⁴ and 17¹ of this Series covered the mass of evidence proving that the biosynthesis of uro'gen-III (3) involves a single intramolecular rearrangement which occurs *after* the construction of an unrearranged linear tetrapyrrole.

In support of this conclusion, it was shown⁴ that the synthetic aminomethylbilane (4), corresponding to head-to-tail assembly of PBG units (1), was a substrate for deaminase-cosynthetase and was converted by a single intramolecular rearrangement into uro'gen-III (3), essentially free of isomers (Scheme 1). This ring-closure of the aminomethylbilane (4) to uro'gen-III (3) was strongly accelerated by the enzyme system relative to the non-enzymic rate of ring-closure, which produced uro'gen-I (2). However, the preceding paper¹ reported that this enzymic rate was slower by a factor of ca. 15 than the rate at which uro'gen-III (3) was produced enzymically from the natural substrate, PBG (1). Thus, the aminomethylbilane (4) cannot be, in that exact form, a true intermediate on the biosynthetic pathway from PBG (1) to uro'gen-III (3). This kinetic discrepancy led to rate studies on the action of deaminase alone (no cosynthetase) on the aminomethylbilane (4). The important observation was made¹ that enzymic acceleration of ring-closure occurred but there was a clear lag



over the first few minutes of the run before formation of uro'gen-I (2) reached the maximum rate. This observation was in marked contrast to the results which had been obtained when deaminase and cosynthetase were

used together; then uro'gen-III (3) was reproducibly formed without any detectable initial lag.¹

The present paper describes research on the chemistry, structure and synthesis of the intermediate accumulated during this lag; the results from enzymic experiments can then be combined with the solid structural knowledge to clarify the roles of deaminase and cosynthetase in the biosynthesis of uro'gen-III (3).

Kinetic Studies.—The kinetic experiments outlined above were extended by examining the action of deaminase alone on PBG (1); an amount of enzyme was used (3 000 units) * which would consume all the substrate within a few minutes. Both the consumption of PBG

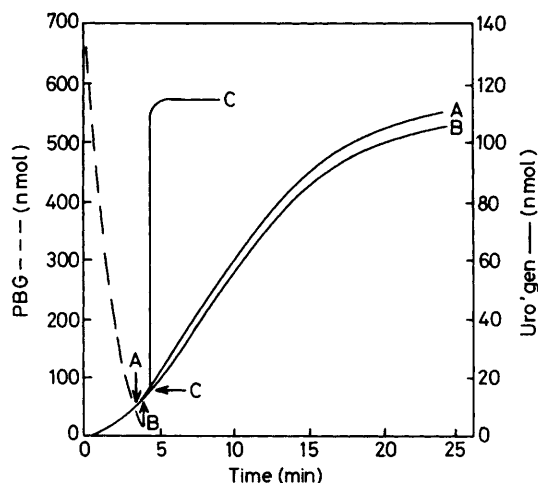


FIGURE 1 Conversion of PBG by deaminase into the hydroxymethylbilane (5) at 37 °C and pH 8.25, followed by ring-closure: (A) without additional deaminase; (B) in the presence of additional deaminase; (C) with added deaminase-cosynthetase

(measured spectroscopically by the Ehrlich reaction) and uro'gen-I production were monitored over the early stages of the run as done for the aminomethylbilane (4) above. The results (Figure 1) were striking. When virtually all of the PBG had been used (at arrow A, Figure 1), uro'gen formation (curve A) had reached only *ca.* 10% of its eventual maximum. Clearly an intermediate, with a half-life of *ca.* 5 min, had been formed which, after 25 min without consumption of additional PBG, had changed into uro'gen. As expected, isomer analysis on this final product by the improved h.p.l.c. method¹ showed it to be >99% uro'gen-I (2). The intermediate must be free rather than enzyme bound since far too little enzyme had been used to react stoichiometrically with the consumed PBG.

The foregoing experiment was then repeated and at arrow B (Figure 1), additional deaminase (9 000 units) was added; the rate of uro'gen-I formation was virtually unchanged (curve B, Figure 1). It was evident that uro'gen-I (2) had been formed *chemically* from the intermediate and that having built this product, deaminase had completed its task. The chemical formation of

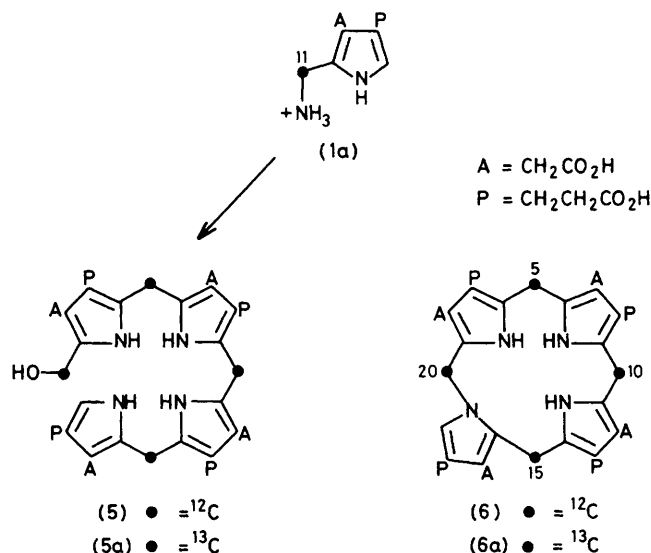
* 1 Unit is the amount of enzyme required to consume 4 nmol of PBG per hour.

uro'gen-I (2) from the intermediate strongly suggested a linear tetrapyrrole structure formed by head-to-tail assembly of four PBG units (1).

For the final kinetic experiments, the intermediate was again generated and at arrow C, Figure 1, deaminase-cosynthetase (12 000 units) was added; virtually instantaneous production of uro'gen occurred (curve C, Figure 1). In this case, isomer analysis¹ showed that all the uro'gen generated after addition of deaminase-cosynthetase was the type-III isomer (3). The great rapidity with which the intermediate was converted into uro'gen-III in this experiment explains why no lag had been observed when PBG (1) was treated with deaminase and cosynthetase together.

The foregoing results, in combination, showed that (a) deaminase converts PBG (1) into an unstable intermediate, (b) this intermediate transforms spontaneously at pH 8.25 to form uro'gen-I (2), and (c) the intermediate is converted into uro'gen-III (3) on treatment with deaminase-cosynthetase. Since deaminase has no apparent effect on the intermediate, it was likely that uro'gen-III (3) is formed from it by cosynthetase alone.

The following section covers our proof that the intermediate is the unrearranged hydroxymethylbilane (5). Shortly before this proof, we received preprints^{5a} from Professor A. I. Scott also reporting the detection (by n.m.r.) of an intermediate from deaminase said to have the cyclic structure (6) shown below to be incorrect. Since it was later claimed^{5b} that this structure had been proved and the claim has never been withdrawn,^{5c} it will be helpful to cover in this paper the evidence which firmly establishes the hydroxymethylbilane structure (5) and, in addition, that which unambiguously eliminates the cyclic structure (6).



Proof that the Intermediate is the Unrearranged Hydroxymethylbilane (5).—To gain information about the structure of the intermediate, it was generated as above but now from [11-¹³C]PBG⁶ (1a). When most of the sub-

strate had been consumed, the pH of the reaction mixture was adjusted to >12 with sodium hydroxide to quench the enzymic reaction and stabilise the product. The ^{13}C n.m.r. spectrum of the mixture was then determined using ^1H noise decoupling; the result is shown in Figure 2. With off-resonance decoupling, all four signals

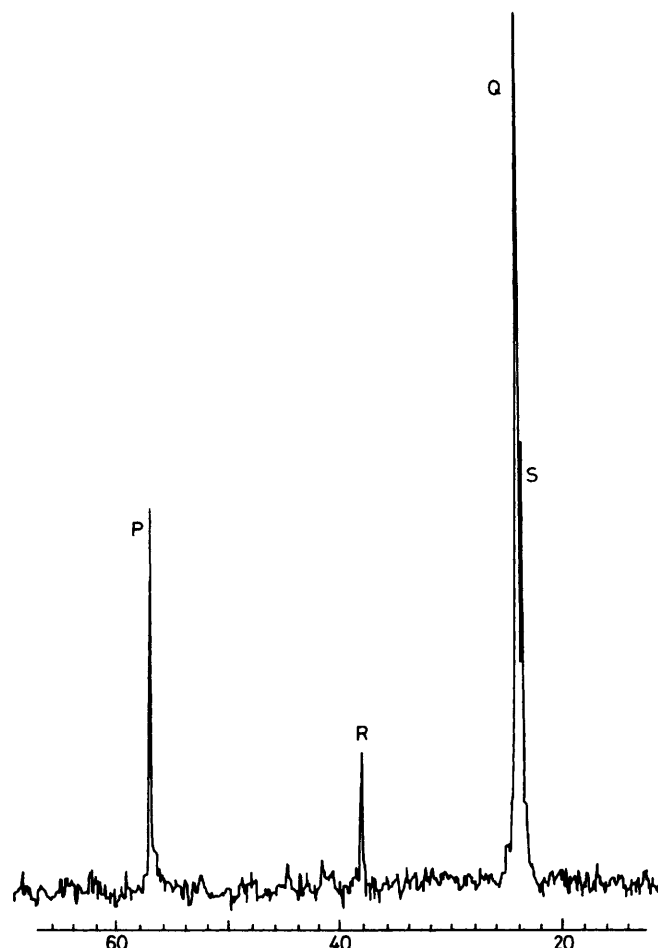


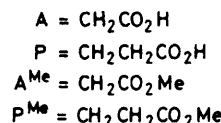
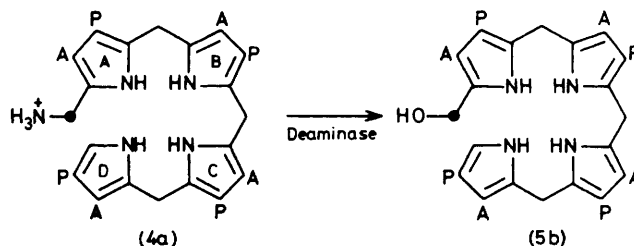
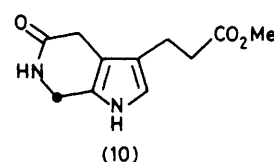
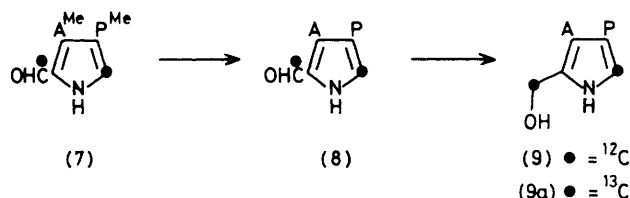
FIGURE 2 25.2 MHz ^{13}C n.m.r. spectrum of product from action of deaminase on $[11-^{13}\text{C}]$ PBG (1a) recorded with ^1H noise decoupling at 37 °C, pH >12. Signal P, HOCH_2 -bilane; Q, pyrrole- CH_2 -pyrrole of HOCH_2 -bilane; R, H_2NCH_2 of residual PBG; S, pyrrole- CH_2 -pyrrole of uro'gen-I. SW 5 000 Hz, 90° pulse, AT 0.8 s, NT 22 800; δ referred to $\text{Me}_3\text{SiCD}_2\text{-CD}_2\text{CO}_2\text{Na}$

appeared as triplets, so all arise from $^{13}\text{CH}_2$ groups. Signal S, δ 24.0 could be assigned from the chemical shift as arising from the four bridge methylene groups of uro'gen-I (2); this assignment is confirmed below. Signal R at δ 38.4 was familiar in being due to $^{13}\text{CH}_2\text{NH}_2$ of the residual $[11-^{13}\text{C}]$ PBG (1a). Thus the remaining two signals P and Q were the ones arising from the intermediate. The integrals P : Q were in the ratio 1 : 3 and since signal Q, δ 24.5 was known from experience with synthetic bilanes⁴ to correspond to the three bridge methylenes of a bilane, it was highly probable that signal P, δ 57.2 arises from the terminal pyrrole- $^{13}\text{CH}_2\text{Y}$ of the same bilane. Signal P is 18.9 p.p.m. downfield from

the signal corresponding to pyrrole- $^{13}\text{CH}_2\text{NH}_2$, δ 38.3, in the ^{13}C spectrum of the aminomethylbilane⁴ (4a). This difference is very close to that observed⁷ when the chemical shifts of a primary alcohol $\text{R-}^{13}\text{CH}_2\text{OH}$ and its amine counterpart $\text{R-}^{13}\text{CH}_2\text{NH}_2$ are compared. So these results clearly indicated that the intermediate released into the medium when deaminase acts on PBG (1) is the hydroxymethylbilane (5a). The unrearranged structure for this bilane follows from the knowledge that it ring-closed chemically to form uro'gen-I [as (2)].

To eliminate the possibility that the hydroxymethylbilane (5a) was an artefact produced from some true intermediate by reaction with the added hydroxide ions, $[11-^{13}\text{C}]$ PBG (1a) was converted by deaminase at pH 8.25 into the intermediate and a ^{13}C spectrum was run rapidly at low temperature without raising the pH. The same four signals of Figure 2 were present (and no others); the signal for pyrrole- $^{13}\text{CH}_2\text{OH}$ was at δ 57.1. The only change was that the signal from residual labelled PBG, which at pH 8.25 was now in the protonated form- $^{13}\text{CH}_2\text{NH}_3^+$, appeared at higher field (δ 37.2). During the accumulation of this spectrum, signal S (cf. Figure 1) from the interpyrrolic methylenes of uro'gen-I (2) increased at the expense of the two signals from the hydroxymethylbilane as expected.

Support for the hydroxymethylbilane structure (5) deduced for the intermediate came from synthesis of the hydroxymethylpyrrole (9), which is the hydroxy-analogue of PBG (1). Alkaline hydrolysis of the ^{13}C -



labelled formylpyrrole ⁴ (7) gave the corresponding diacid (8) which was reduced with sodium borohydride at room temperature. The ¹³C n.m.r. spectrum obtained from the resulting alkaline solution of the hydroxymethylpyrrole (9a) showed a strong signal at δ 57.3 for the ¹³CH₂OH group, in close agreement with the value found above for the natural intermediate. When the ¹³C spectrum of (9a) was determined at pH 7, the ¹³CH₂OH signal appeared at δ 57.2 but significant self-condensation of this labile substance occurred during the run. A spectrum taken after the solution had been kept overnight at room temperature showed no remaining signal from ¹³CH₂OH and only that corresponding to essentially complete formation of uro'gen. The chemistry of hydroxymethylpyrroles will be considered further below.

At this point, it was important to determine whether the aminomethylbilane (4) is converted by deaminase into the hydroxymethylbilane (5) for if that occurred, all the earlier data ^{3,4} from enzymic studies both on PBG (1) and on the various labelled forms of the synthetic aminomethylbilane (4) would fall into place. Accordingly, the [*aminomethyl*-¹³C]bilane (4a) was synthesised by the established route ⁴ from [11-¹³C]PBG lactam ester ⁶ (10) carrying 90 atom % ¹³C. This bilane (4a) was treated with a large amount of highly purified deaminase and the enzymic reaction was stopped before completion by adjustment of the mixture to pH 12. The ¹³C n.m.r. spectrum obtained from this solution (Figure 3) showed a considerable accumulation (15–20%) of [*hydroxymethyl*-¹³C]bilane (5b); the signal from the pyrrole-¹³CH₂OH group appeared at δ 57.2. The amount of hydroxymethylbilane accumulated in this case was, as predicted, much less than from PBG because the rate of its enzymic formation from the aminomethylbilane is much lower, whereas the rate of its chemical ring-closure to uro'gen-I (2) remains the same whether the hydroxymethylbilane is generated from PBG (1) or from the aminomethylbilane (4). At the end of the run which provided Figure 3, the n.m.r. sample was adjusted to pH 7 and after 10 min at 37 °C, the ¹³C n.m.r. spectrum was redetermined. As expected, the hydroxymethyl signal from bilane (5b) had disappeared and the signal from uro'gen-I (as 2) at δ 24.0 showed a corresponding increase in size.

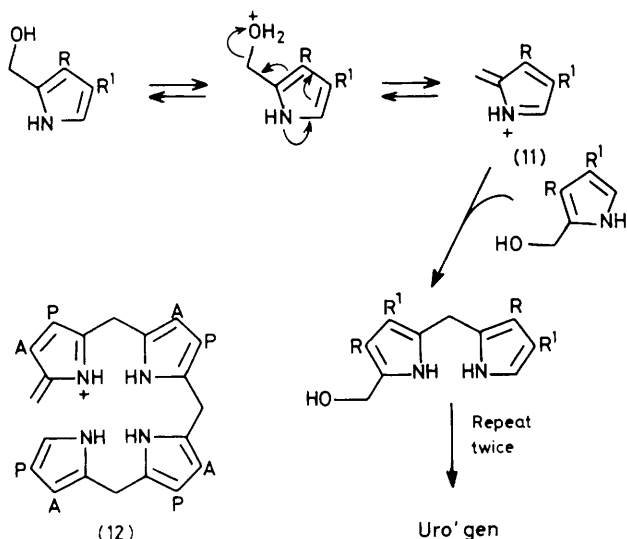
This formation of the hydroxymethylbilane (5b) from the synthetic aminomethylbilane (4a) is highly important. Firstly, it provides further support to the structural conclusions about the natural intermediate produced by deaminase which have been based so far on spectroscopic observations. Secondly, we now know that the ¹³C₁- and ¹³C₂-labelled samples of the aminomethylbilane [as (4)], extensively studied in Part 16,⁴ were enzymically converted first into the correspondingly labelled hydroxymethylbilane [as (5)]; all the conclusions drawn from these experiments thus gain further strength. Lastly, one can now understand the lag in formation of uro'gen-I (2) when the aminomethylbilane (4) is treated with deaminase. Clearly, the lag phase

represents the formation and build up of hydroxymethylbilane (5) until the pool size of this product is sufficient that its removal by unimolecular ring-closure to uro'gen-I (2) balances the rate of its enzymic formation from the aminomethylbilane (4). It has already been noted that the chemical ring-closure of the latter is a far slower process.



FIGURE 3 25.2 MHz ¹³C n.m.r. spectrum of product from action of deaminase on [*aminomethyl*-¹³C]bilane (4a) (1 mg) recorded with ¹H noise decoupling at 37 °C and pH 12. Signal P, HOCH₂-bilane; R, H₂NCH₂-bilane; S, pyrrole-CH₂-pyrrole of uro'gen-I. SW 5 000 Hz, 90° pulse, AT 0.8 s, NT 74 000

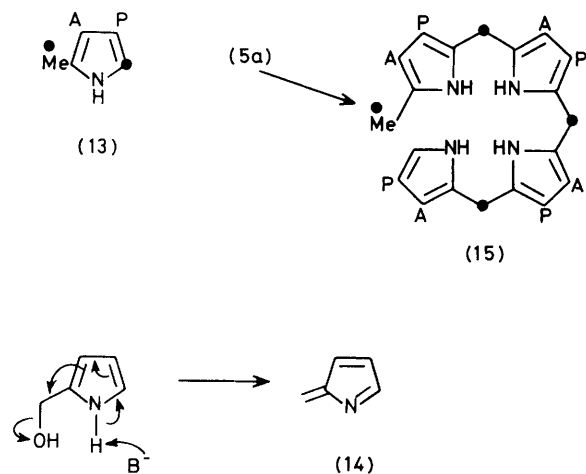
We can now comment on the chemistry of hydroxymethylpyrroles. The ease with which such molecules, which are unsubstituted at position-5, form porphyrinogens under mildly acidic conditions is well known.⁸ The reaction almost certainly involves protonation of the hydroxy-group followed by dehydration to give the electrophilic 1-azafulvenium ion (11) which can then react with another molecule of pyrrole (Scheme 2). Cyclisation of the hydroxymethylbilane (5) probably occurs by the same mechanism in which the rate-determining step is likely to be elimination of water to form the protonated azafulvene system (12). The rate of this step will be proportional to the concentration of the protonated hydroxymethyl species and so dependent on the pH of the medium. Our observation that the inter-



SCHEME 2

mediate produced by deaminase from PBG (1) forms uro'gen-I (2) rapidly at pH 8.25 but is relatively stable at pH 12 in keeping with this view and with the earlier structural conclusions.

When treatment of the alkaline solution of the $^{13}\text{C}_2$ -formylpyrrole (8) with borohydride was prolonged or run at higher temperatures, a small signal appeared at δ 12.9 in the ^{13}C -n.m.r. spectrum of the solution. If the reaction was run in 2M-potassium hydroxide for 30 min at



SCHEME 3

90 °C, this highfield resonance completely replaced that previously observed for the $^{13}\text{CH}_2\text{OH}$ group of the pyrrole (9a). With off-resonance decoupling, the signal at δ 12.9 appeared as a quartet, $J_{\text{red}} = 46.3$ Hz, and so must arise from the methylpyrrole (13). This reduction probably⁹ involves base-catalysed removal of the imino proton, elimination of hydroxide ion, and reduction of the azafulvene (14) (Scheme 3). The strongly alkaline conditions used here (2M-KOH) would favour formation of the methylpyrrole (13) since production of the azafulvene system (as 14) rather than its subsequent re-

duction is likely to be the difficult step. That this was so was demonstrated by carrying out the reduction in 0.2M-potassium hydroxide when only *ca.* 25% of the methylpyrrole (13) was formed, the other product being the hydroxymethylpyrrole (9a).

These results made it likely that the hydroxymethylbilane intermediate (5a) could be reduced by borohydride. Accordingly, $[11\text{-}^{13}\text{C}]\text{PBG}$ (1a) was briefly incubated with deaminase in the usual way and after the solution

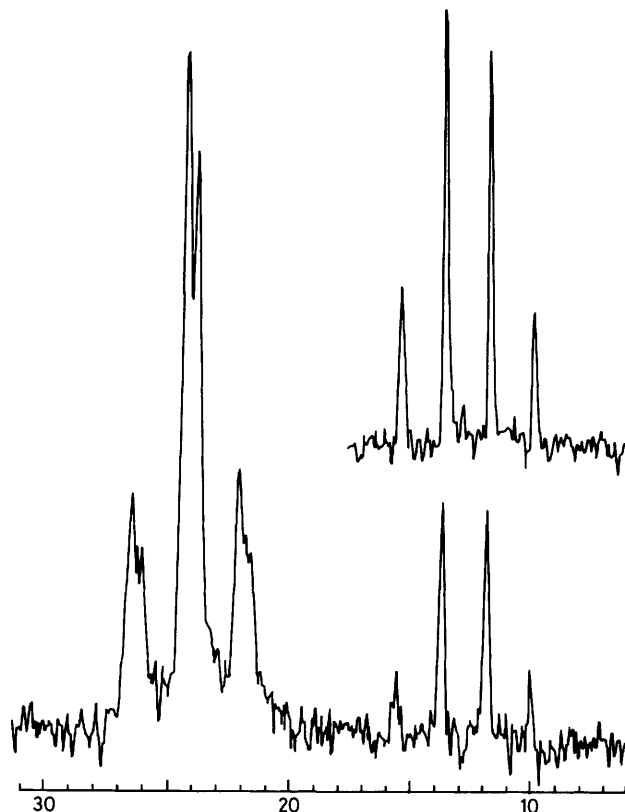


FIGURE 4 Off-resonance ^1H decoupled 25.2 MHz ^{13}C n.m.r. spectrum of methylbilane (15) from action of alkaline borohydride on the hydroxymethylbilane (5a) produced biosynthetically from $[11\text{-}^{13}\text{C}]\text{PBG}$ (1a). SW 5 000 Hz, 40° pulse, AT 0.8 s, NT 74 600. The two triplets *ca.* δ 24 are from the interpyrrolic methylene groups of uro'gen-I (2) and of the bilane (15). Inset: corresponding signal from methylpyrrole (13)

had been made 1.5 M with respect to sodium hydroxide, the labelled bilane (5a) was reduced at 90 °C with borohydride. The ^{13}C n.m.r. spectrum of the product (Figure 4) run with off-resonance decoupling showed a quartet at δ 12.9 with $J_{\text{red}} = 46.3$ Hz exactly as for the methylpyrrole (13). Clearly the intermediate had been converted into the methylbilane (15) thereby providing yet further support for the hydroxymethylbilane structure (5a). This reduction offers a way to isolate a derivative of the very labile intermediate (5a); methylbilanes similar to that of structure (15) have been synthesised¹⁰ and they are moderately stable.

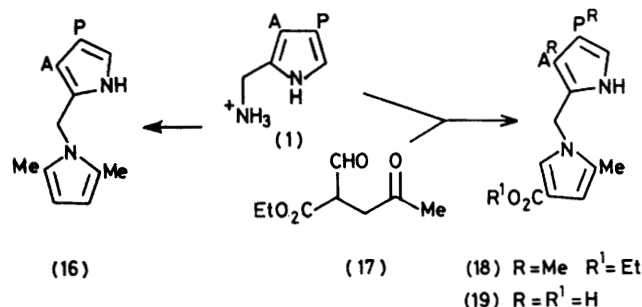
Elimination of the Cyclic Structure for the Intermediate.—The cyclic substance (6), so called preuro'gen, claimed

by Scott *et al*^{5b} to be the intermediate produced by deaminase can now be considered. *A priori*, structure (6) was highly unlikely since it failed to account for the most striking feature of the chemistry of the natural intermediate, *viz.* its rapid conversion ($t_{1/2}$ ca. 4 min at 37 °C, pH 8.25) *non-enzymically* into uro'gen-I (2). This change was said to occur^{5b} from the cyclic structure (6) by a sigmatropic rearrangement, N→C. However, it is evident that the orbitals involved in the postulated rearrangement are orthogonal so a very high activation energy would be expected. In agreement, no mild N→C rearrangements of *N*-alkylpyrroles are known and for those reported, temperatures in the range 500–600 °C are required.¹¹

Nevertheless, not only was the cyclic structure (6) claimed to be established^{5b} but the hydroxymethylbilane (5) was said to be a biologically valueless artefact (not a substrate for cosynthetase) derived by attack of hydroxide on the cyclic system^{5b} (6). The preceding sections have made it clear that our conclusions are quite different and therefore serious work was necessary to eliminate the cyclic preuro'gen structure (6).

There are two further inadequacies in structure (6). (a) This structure requires the ¹³C signal at δ 57.1* from the natural intermediate to be assigned^{5b} to the C-20 CH₂ group of (6). But simple shift calculations⁷ lead to the prediction that the signal from a CH₂ group in this environment should appear at least 12 p.p.m. to higher field at *ca.* δ 45 [calculated for the analogue of (16) lacking the methyl groups]. Indeed, the γ-effect⁷ from C-15 would probably cause the signal from C-20 in structure (6) to lie to even higher field than δ 45.

(b) The proposed formation of the hydroxymethylbilane (5) by base cleavage of preuro'gen^{5b} (6) requires this displacement to be extremely rapid since it must overwhelm the already rapid chemical conversion of the intermediate into uro'gen-I (2). *N*-Alkylpyrroles do not normally react in this way.



The foregoing inadequacies in structure (6) for the intermediate were studied by synthesising the model systems (16) and (19). The former was prepared by a Paal-Knorr condensation¹² between hexane-2,5-dione and PBG (1). The product was isolated as the dimethyl ester of pyrrole (16) and the diacid (16) was obtained by

* This value results from adjustment of the original figure^{5b} to match our scale (using the CH₂ signal from uro'gen-I as reference) to allow for different shift standards.

hydrolysis. It was stable over the pH range 2–12 and the ¹³C signal for the interpyrrolic CH₂ appeared at δ 42.0 as expected at somewhat higher field than predicted by calculation above, due to two γ-effects from the methyl groups.

For the second model (19), the known keto-aldehyde¹³ (17) was condensed with PBG (1) and the product was isolated as the ester (18) after treatment with diazomethane. The ester groups were then hydrolysed to allow the ¹³C n.m.r. spectrum of the product (19) to be determined under the conditions used for studying the natural intermediate. Again, this product was quite stable and the ¹³C signal for the interpyrrolic methylene group appeared in the expected position at δ 44.7; in this model, only one γ-effect is operative.

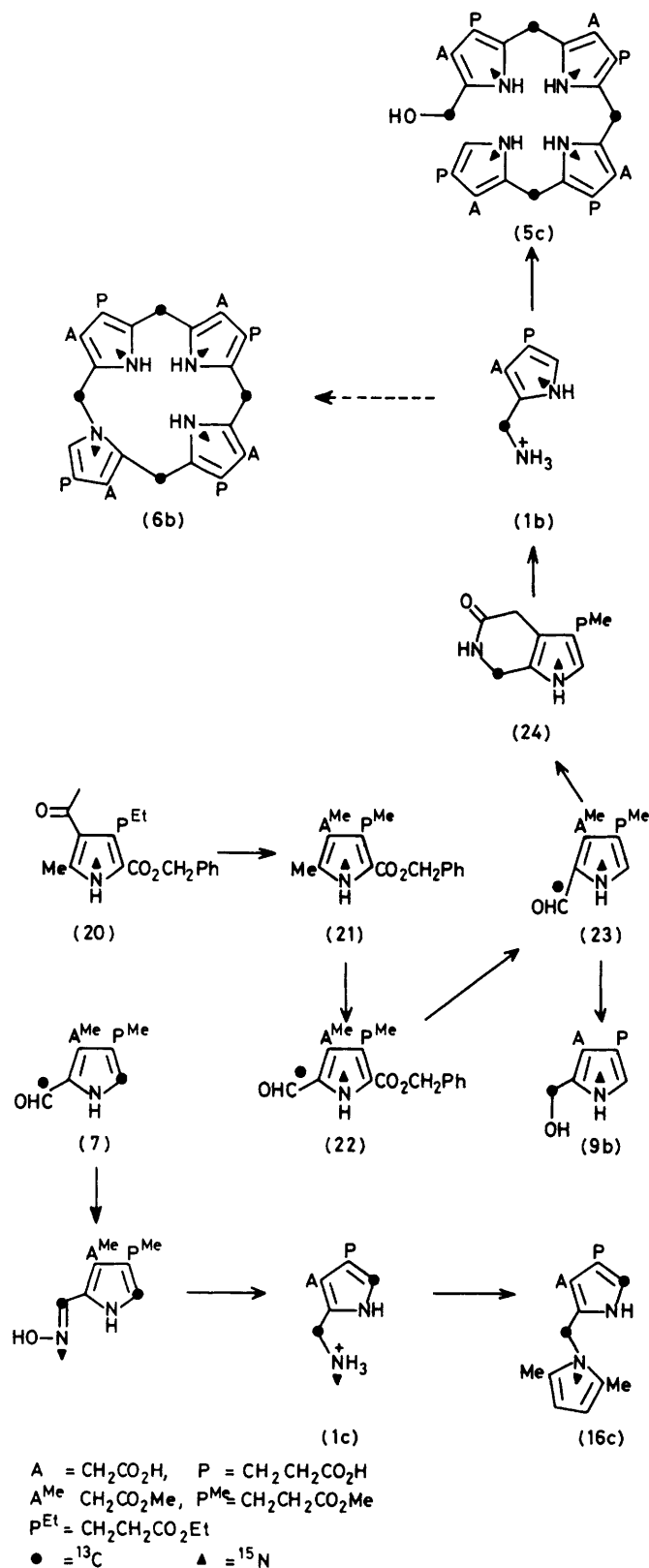
Thus the properties of the model *N*-alkylpyrrole systems (16) and (19) differ greatly from those of the intermediate produced by deaminase. These models (a) showed normal ¹³C n.m.r. signals, (b) did not undergo ready N→C rearrangement, and (c) were not cleaved by alkali. It was clear that structure (6) was incorrect.

In view of the overwhelming evidence against structure (6), it is important to examine that on which it was founded. This structure rested solely on the observation that when [1-¹⁵N,11-¹³C]PBG (1b) (Scheme 4) was converted by deaminase into the intermediate, the ¹H-decoupled ¹³C signal assigned to C-20 of structure (6b) appeared somewhat broadened and was claimed to be a 6 Hz doublet resulting from *one-bond* ¹³C–¹⁵N coupling.^{5b} The following synthetic and enzymic studies allowed a true understanding.

[*amino*-¹⁵N, 2,1-¹³C]PBG (1c) was prepared from the doubly-labelled aldehyde⁴ (7) *via* the oxime as in Scheme 4. This product was used in the Paal-Knorr reaction as above to afford the triply labelled system (16c) which showed a clear *one-bond* ¹³C–¹⁵N coupling of 10 Hz. The complementary experiment involved synthesis of the [1-¹⁵N,11-¹³C]PBG (1b) and of its hydroxy-analogue (9b) as in Scheme 4. The ¹⁵N was introduced from sodium nitrite (99 atom % ¹⁵N) at the stage of construction of the pyrrole (20). The ¹³C n.m.r. spectrum of the hydroxymethylpyrrole (9b) showed a *two-bond* coupling, ¹³C–¹⁵N, of 2.3 ± 0.2 Hz. This result made it essential to convert the doubly labelled PBG (1b) by deaminase into the intermediate to check the size of the coupling.

Four separate experiments with the doubly-labelled PBG (1b) were carried out at pH 8.25. In the first, incubation at 37 °C was followed by adjustment of the solution to pH > 12 before determination of the ¹³C n.m.r. spectrum of the product at 37 °C. The second and third experiments also involved incubations at 37 °C but the spectra were run at 20–25 °C and 4 °C, respectively, without pH adjustment. The final incubation was run at 13 °C [the lowest temperature at which the rate of formation of the intermediate compared favourably with the rate of its chemical conversion into uro'gen-I (2)] and the spectrum was recorded at 4 °C, without pH adjustment.

The four ¹³C spectra of the intermediate (5c) so ob-



SCHEME 4

tained were all identical, the key signal appeared at δ 57.1–57.2 as earlier and was a doublet for which the two-bond coupling ranged between 2.2 and 2.5 Hz (Figure 5*) exactly as had been found for the standard model compound (9b).

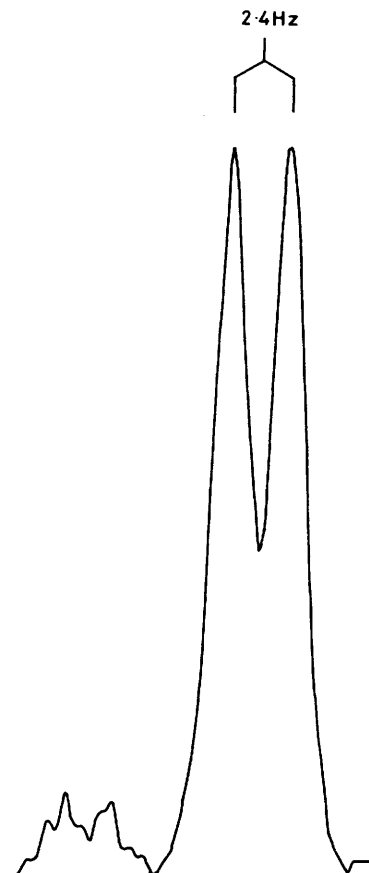


FIGURE 5 ^{13}C Signal from $HO^{13}CH_3$ -bilane (δ 57.1) in 1H noise decoupled ^{13}C n.m.r. spectrum, run at 4° , 25.2 MHz, of product (5c) from deaminase acting on $[1-^{15}N, 11-^{13}C]PBG$ (1b). See also footnote below

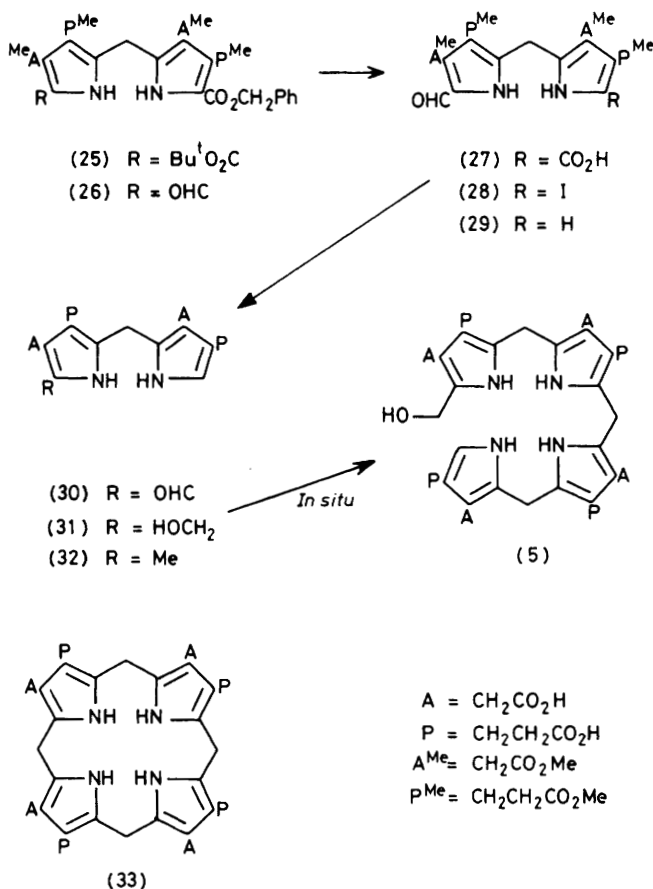
Thus again the structure of the intermediate released by deaminase is confirmed as the hydroxymethylbilane (5). The chemical, enzymic and spectroscopic properties of the intermediate determined in Cambridge are essentially identical with those reported by Scott *et al.*;⁵ it was clear to us that they were handling the same substance. Their so-called 6 Hz doublet on which structure (6) was based is spurious and can now be explained as a *ca.* 2.4 Hz two-bond ^{13}C – ^{15}N coupling which appeared in their work further broadened due to inadequate phasing in a spectrum of poor quality.

The foregoing results eliminate preuro'gen (6); it is not involved in porphyrin biosynthesis and with the

* Figure 5 shows the final result of using procedures, developed by Dr. R. G. Brereton, for computer handling of n.m.r. data to enhance resolution and signal-to-noise ratio (see Brereton *et al.*, *J. Magn. Reson.*, 1981, **43**, 224). The resulting spectra were significantly improved in quality but the *J* values remained essentially unaffected. The computer programs are available from Dr. Brereton, University Chemical Laboratory, Cambridge.

hydroxymethylbilane (5) being confirmed as the intermediate by unambiguous synthesis (next section) the cyclic structure ^{5b} (6) can be deleted from the literature. This is necessary because the false structure (6) leads to the conclusion that deaminase is a cyclising enzyme when clearly it is not.

Synthesis of the Hydroxymethylbilane (5).—Bearing in mind the great lability of natural hydroxymethylbilane (5), the plan for our first experiments was to synthesise the hydroxymethylpyrromethane (31) and to incubate this at pH 8 in the presence of cosynthetase. The ease, referred to earlier, with which a pyrrolic α -hydroxymethyl group undergoes nucleophilic attack by an α -free pyrrole residue promised that two molecules of the hydroxymethylpyrromethane (31) would combine to generate the hydroxymethylbilane (5) *in situ*; cf. the behaviour of the analogous aminomethylpyrromethane.¹⁴ This product was expected to be trapped by cosynthetase as it was formed to generate uro'gen-III (3). Such a scheme was not envisaged as a true synthesis but a successful outcome would encourage attack on the challenging problem of total synthesis and characterisation of the fugitive hydroxymethylbilane itself (5).



SCHEME 5

Scheme 5 shows the synthesis of the hydroxymethylpyrromethane (31). The route to the precursor (25) of starting material (26) was already established¹ and the

benzyloxycarbonyl group was removed by the standard sequence (27)→(28)→(29). Hydrolysis of the ester groups then gave the aldehyde (30) from which the required product (31) was obtained by borohydride reduction; it was characterised using ¹³C n.m.r. by comparison with standard synthetic pyrromethanes.¹⁴

For the first set of experiments, the alkaline solution of the hydroxymethylpyrromethane (31) was neutralised and aliquots were incubated (a) without enzymes (blank) (b) with deaminase alone, (c) with deaminase and cosynthetase, and (d) with cosynthetase alone. The isomer distribution in the uro'gens formed was determined as earlier.¹ The results (Table I) confirmed that deaminase

TABLE I

Uro'gen isomers formed from hydroxymethylpyrromethane (31) *via* bilane (5)

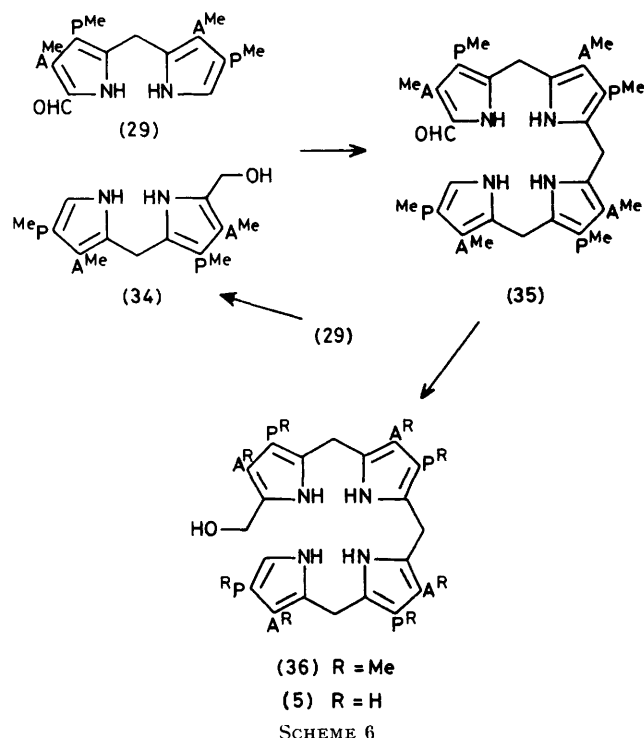
Expt. No.	Enzyme added	Uro'gens formed ^a (% of total)		
		Type-I	Type-III	Type-IV
1	None	83.5	5	11.5
2	Deaminase	82.5	4	13.5
3	Deaminase-cosynthetase	25	65	10
4	Cosynthetase	24	65.5	10.5
5	None	90.5	3	6.5
6	Cosynthetase (10 μ l) ^b	19	75.5	5.5
7	Cosynthetase (100 μ l) ^b	10.5	83.5	6.0
8	Cosynthetase (1 600 μ l) ^b	5	89.5	5.5

^a No Type-II could be detected. ^b Volume of standard preparation of cosynthetase.

does not apparently affect the hydroxymethylbilane (5) (experiments 1 and 2) but that deaminase-cosynthetase or cosynthetase alone (experiments 1, 3, and 4) greatly increase production of the rearranged type-III isomer (3) at the expense of the type-I system (2). Attention is also drawn to the finding that the proportion of uro'gen-IV (33), formed *chemically* by head-to-head combination of the hydroxymethylpyrromethane (31), remains roughly constant in all runs, as would be expected.

The rate-limiting step in the foregoing experiments could be either the chemical condensation step producing the bilane (5) or the enzymic conversion of this product into uro'gen-III (3). This aspect was studied by incubating a standard quantity of hydroxymethylpyrromethane (31) with increasing amounts of cosynthetase (10 to 1 600 μ l of enzyme solution). There was no appreciable difference in the rate of formation of uro'gen so the slow step is the chemical one. Improved methods were used in this second set of experiments (see Experimental section) which led to a very satisfying difference between the blank (experiment 5, Table I) and the one with a large amount of cosynthetase (experiment 8).

These results encouraged efforts on synthesis of the hydroxymethylbilane itself (5). Several approaches were studied; only the sole successful one is now described which again made use of the reactivity of hydroxymethylpyrroles (Scheme 6). The foregoing formylpyrromethane ester (29) was reduced with borohydride to give the hydroxymethyl analogue (34). This was then condensed with an excess of the same aldehyde (29) in methylene chloride-acetic acid to yield the formylbilane



octamethyl ester (35). It was the only material sparingly soluble in methanol and so was readily separated from the excess of aldehyde (29) and oligomeric by-products. The formylbilane (35) was reasonably stable and was fully characterised.

Alkaline hydrolysis of the bilane ester (35) followed by reduction with borohydride gave an alkaline solution of the hydroxymethylbilane (5). The ^1H -decoupled ^{13}C

n.m.r. spectrum of this material (Figure 6) confirmed its purity and, importantly, the signal from the CH_2OH group appeared at δ 57.1 (*cf.* δ 57.2 for the natural material).

For kinetic experiments with enzymes, it was found necessary to produce solutions of synthetic hydroxymethylbilane (5) free from boron-containing materials. Therefore the formylbilane ester (35) was reduced in chloroform-methanol containing triethylamine. After the unwanted materials had been washed out with water, the solution readily yielded pure hydroxymethylbilane ester (36) since this product, like the corresponding aldehyde (35), had low solubility in methanol. The esters were then saponified immediately to give an alkaline solution of synthetic hydroxymethylbilane (5).

It was now possible to make the crucial comparisons. The synthetic and natural samples of hydroxymethylbilane (5) were shown to be excellent and identical substrates for cosynthetase and the product in each case was essentially pure uro'gen-III (3) (Tables 2 and 3). The final column of Table 3 is particularly important in showing virtually identical V_{max} for cosynthetase alone acting on the synthetic and natural substrates.

Furthermore, the rate of chemical ring-closure to uro'gen-I (2) was the same for samples from both sources. Finally the opportunity was taken to demonstrate that the chemical and enzymic properties of the natural intermediate (5), generated from PBG (1) by deaminase at pH 8.25 were unchanged after incubation at pH > 12 (Table 3). Thus, the conclusion drawn from the earlier study based on ^{13}C n.m.r. that the natural intermediate is not affected by hydroxide ions is fully confirmed.

All these results rigorously establish that the intermediate produced from PBG by deaminase is the unarranged hydroxymethylbilane (5).

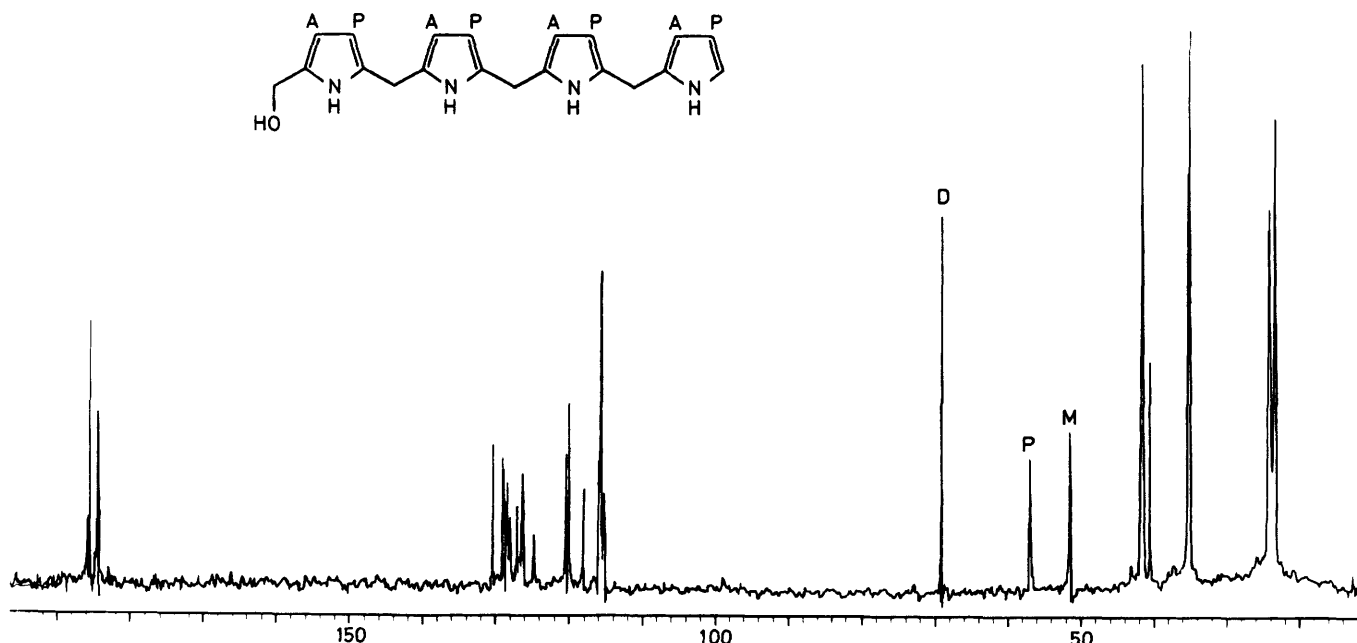


FIGURE 6 Natural abundance ^{13}C n.m.r. spectrum at 25 MHz of synthetic hydroxymethylbilane (37 mg) at pH > 12. Signal P, HOCH_2 -bilane; D, dioxan; M, methanol. SW 5 000 Hz, 40° pulse, AT 0.3 s, NT 164 500

TABLE 2

Uro'gen isomers formed from synthetic hydroxymethylbilane (5)

Expt. No.	Enzyme added ^a	Uro'gen formed (% of total)		
		Type-I	Type-III	Type-IV
1	None	100	0	0
2	Deaminase	100	0	0
3	Cosynthetase	7.5	92.5 ^b	0

^a Run at pH 8.25, 30 °C. ^b By allowing for the 6% uro'gen-I (2) shown to have been formed chemically before the cosynthetase was added, this corresponds to >98% conversion of hydroxymethylbilane (5) into uro'gen-III (3).

The Roles of Deaminase and Cosynthetase.—The foregoing experiments prove conclusively that deaminase joins four PBG units (1) head-to-tail and in the absence of cosynthetase releases the unrearranged hydroxymethylbilane (5) into solution; *deaminase is an assembling enzyme not a ring-closing enzyme.*

Cosynthetase then converts the hydroxymethylbilane (5) by an intramolecular rearrangement of ring-D ^{3,4} into uro'gen-III (3); *cosynthetase is the ring-closing and rearranging enzyme.*

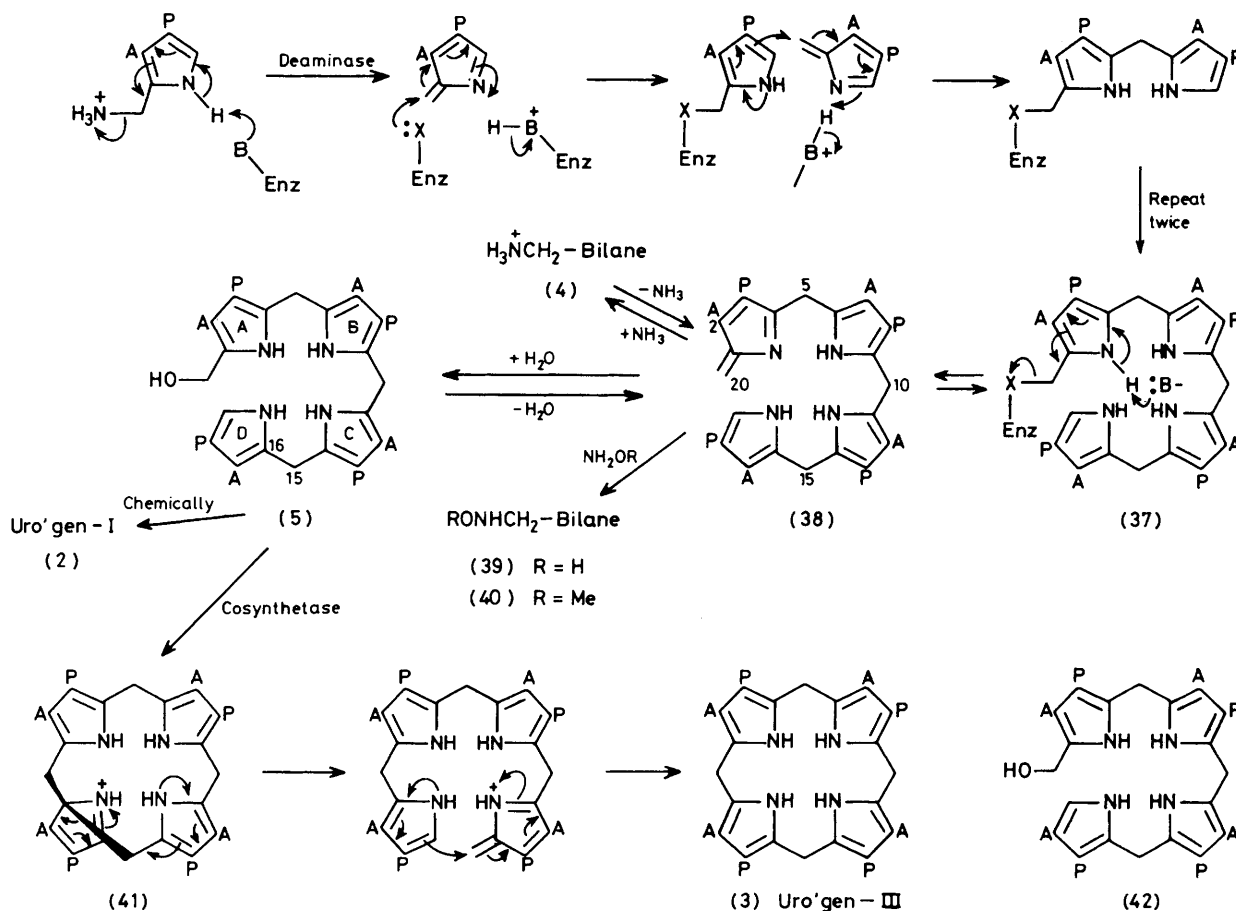
Present knowledge of how PBG (1) is transformed by

TABLE 3

Comparison of synthetic and natural HOCH₂-bilane (5)

	Main ¹³ C n.m.r. signals ^a (p.p.m.)	<i>t</i> ₁ (pH 8.25, 37 °C)/min	% Uro'gen-III formed by cosynthetase alone	<i>V</i> _{max} for cosynthetase ^c
Synthetic	δ 57.1 ^{b,c} , 24.4 ^d	4.0	>98	151
Natural (kept at pH 8.25)	δ 57.1, 24.5	4.2	94	148
Natural (after pH >12, 37 °C, 20 min)	δ 57.2 ^b , 24.5	4.1	98	148

^a All δ referred to internal Me₃SiCD₂CD₂CO₂Na. ^b Run at pH >12. ^c HO¹³CH₂-Pyrrole. ^d Pyrrole-¹³CH₂-pyrrole. ^e Uro'gen produced (μmol h⁻¹) at pH 8.25 and 25 °C, per ml of cosynthetase preparation; 1 μmol h⁻¹ equals 1 unit.



SCHEME 7

deaminase and cosynthetase into uro'gen-III (3) is in accord with Scheme 7. It is certain that the first PBG unit is covalently bonded to deaminase through a nucleophilic group X. Strong evidence for the existence of such covalently bound intermediates is provided by (a) the chromatographic separation of deaminase into different species carrying up to four units of bound PBG,¹⁵ (b) evidence for stable bound forms which are catalytically active from partial reaction of deaminase with unlabelled PBG and completion of the process using ¹³C-PBG,¹⁶ and (c) proof that ¹⁴C labelled PBG remains firmly bound even after deaminase has been denatured or degraded by proteolytic enzymes.¹⁷

With the linear tetrapyrrole (37) built (Scheme 7), elimination of X would form the azafulvene (38) which could react with water to give the hydroxymethylbilane (5). This addition of water must occur with (38) held on the active site of the enzyme since otherwise cyclisation, either to form a lactone with the acetate side-chain at position-2 or to give uro'gen-I (2) would be expected. Added small nucleophiles such as ammonia, hydroxylamine, and methoxyamine can compete with water in trapping the azafulvene (38) to yield the moderately stable bilanes (4), (39), and (40) detected in the important work of Bogorad¹⁸ and Neuberger.¹⁹ Similar trapping

and exchange experiments carried out in Cambridge will be described fully in Part 20.²⁰ The aminomethylbilane (4) is readily equilibrated with the azafulvene (38) on the surface of deaminase which allows the synthetic bilane (4) to act as a substrate for deaminase-cosynthetase and enter the normal biosynthetic pathway.

The hydroxymethylbilane (5) can cyclise chemically at considerable speed to give uro'gen-I (2) but its natural role is to act as the substrate for cosynthetase to form uro'gen-III (3). An attractive intermediate in this rearrangement is the spiro-system (41) similar to that first considered in 1961 by Mathewson and Corwin.²¹ The forward process to uro'gen-III (3) could then involve fission and recombination, as illustrated, but a series of [1,5]sigmatropic shifts is also possible around the periphery of ring-D.

A second possible mechanism involves fission of the C-15/C-16 bond of bilane (5) after protonation at C-16, while it is on the surface of cosynthetase, followed by inversion of ring-D involving strictly no exchange with the medium of the now separated pyrrole fragment. Recombination could then yield the ring-D reversed bilane (42) for straightforward cyclisation to uro'gen-III (3). A recent test of this idea using a synthetic sample of the ring-D reversed bilane (42) showed it to be a poor substrate for cosynthetase²² so eliminating this possibility.

There is, however, an attractive alternative to the spiro-mechanism which we refer to as the external methylene mechanism.* This is outlined in Scheme 8 where the external methylene group is illustrated as a quaternary imine. On this view, ring-D is held during the inversion sequence covalently attached to the external methylene group and finally the electrophilic methylene is regenerated when uro'gen-III (3) is formed. There are obvious minor variations of this basic principle.

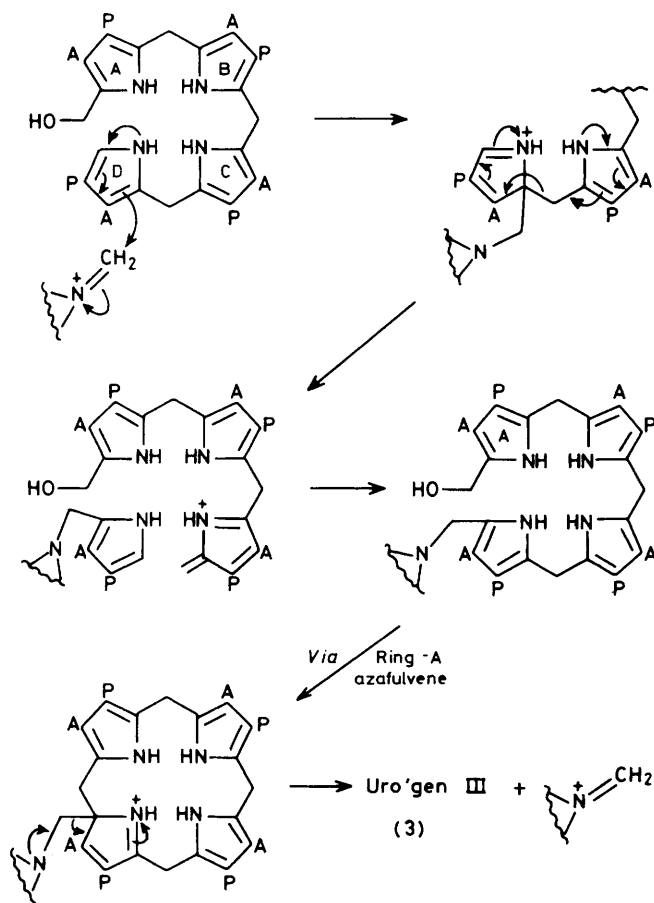
Knowledge of the structure and chemistry of the hydroxymethylbilane (5), its availability in specifically labelled forms by synthesis together with supplies of the two enzymes in separate preparations, allow further experiments. These are designed to decide between such alternatives as outlined above and so to fill in the last remaining gaps in understanding the biosynthetic pathway to uro'gen-III (3). This work is in progress.

EXPERIMENTAL

General directions are given in ref. 1 where also are described the methods for assay of uroporphyrinogens and for separation by h.p.l.c. of isomeric coproporphyrin esters. Porphobilinogen was determined by the method of Mauzerall and Granick.²⁴ ¹³C N.m.r. spectra were run using a 12 mm tube usually fitted with a 0.9 ml micro-cell; the δ -values are referred to Me₃SiCD₂CD₂CO₂Na as standard.

Investigation of the Lag in Uro'gen Production when Deaminase Acts on Porphobilinogen.—PBG monohydrate (6.40 mg, 2.62 μ mol) was incubated at 37 °C with deaminase

* This proposal grew as a result of a valuable discussion with Dr. D. C. Williams (Trinity College, Dublin) who we warmly thank, on the possible involvement of methylene-tetrahydrofolate in porphyrin biosynthesis.



SCHEME 8

(11 250 units) in 0.5M-phosphate buffer, pH 8.25 (total volume, 1.50 ml). Consumption of PBG was followed using Ehrlich's reagent²⁴ (0.010 ml samples taken at 0.5 min intervals) and after 2.75 min, a sample (0.050 ml) was taken for uro'gen assay. The experiments were then continued as follows.

Experiment A. After 3.5 min, part (0.40 ml) of the incubate was mixed with water (0.60 ml) at 37 °C and the sample was held constant at this temperature; production of uro'gen was then monitored by taking samples (0.050 ml), initially at 3 min intervals, for the standard assay.

Experiment B. After 3.83 min, a second portion (0.40 ml) of the original solution was mixed with deaminase (9 000 units) in 0.025M-phosphate buffer (0.60 ml) at 37 °C and the experiment was continued as for Experiment A.

Experiment C. After 4.25 min, a third portion (0.40 ml) was mixed with deaminase-cosynthetase (12 000 units) in 0.025M-phosphate buffer (0.60 ml) at 37 °C. The experiment was then continued as for experiment A but in this case samples for uro'gen assay were taken every 0.25 min.

The results are plotted in Figure 1, in which the figures from the initial incubation have been adjusted to represent the amounts of PBG and uro'gen in each 0.40 ml aliquot used.

Isomer Analysis of Uro'gens Produced from PBG by Deaminase in the Lag Experiment.—Porphobilinogen monohydrate (0.42 mg, 1.72 µmol) was treated at 37 °C with deaminase (8 000 units) in 0.25 M-phosphate buffer, pH 8.25 (total volume, 1.0 ml) as above. After 6 min, when the solution contained 56.8 nmol of uro'gen per ml, portions (0.4 ml) were added to water (0.6 ml) and deaminase-cosynthetase (0.6 ml, 12 000 units) and the two mixtures were incubated at 37 °C.

After 3.25 min, the incubation mixture with deaminase-cosynthetase contained 115.2 nmol of uro'gen; it was treated with iodine and isomer analysis of the resulting mixture of porphyrins showed it to contain Type-III (78.6%) and Type-I (21.4%). Therefore, 98.3% of the uro'gen produced after addition of deaminase-cosynthetase was uro'gen-III (3).

After 33.5 min, the other incubation mixture (containing 95.9 nmol of uro'gen) was treated as above; >99% of the macrocyclic material present was uro'gen-I (2).

Incubation of [11-¹³C]Porphobilinogen with Deaminase: Identification of the Released Product as Hydroxymethylbilane (5a) by ¹³C N.m.r.—The [11-¹³C]porphobilinogen lactam methyl ester⁶ (10) (3.0 mg, 13.5 µmol) was hydrolysed in 2M-potassium hydroxide (50 µl) (65 °C, 5 min and 25 °C, 16 h) and the solution was adjusted to pH 8.25 with 1M-potassium dihydrogen phosphate. The resulting solution of [11-¹³C]PBG was incubated at 37 °C with deaminase (25 500 units) in 0.2M-phosphate buffer, pH 8.25 (total volume, 2.25 ml), and samples (5 µl) were withdrawn at 1-min intervals for standard PBG assay. After 7.5 min, when >90% of the PBG had been consumed, a determination of uro'gen showed that only 330 nmol (ca. 10% of the theoretical) had been formed. At this 7.5 min stage, part (0.8 ml) of the incubation mixture was withdrawn immediately and mixed with deuterium oxide (0.1 ml) for ¹³C n.m.r. analysis at 7 °C. The remainder (1.40 ml) was added to deuterium oxide (0.16 ml) containing sodium hydroxide (40 mg) for ¹³C spectroscopic study at 37 °C; the result from the latter run is shown in Figure 2.

The n.m.r. data at pH 8.25 were accumulated for 75 min

at 7 °C to give: δ_O 24.0 (pyrrole-CH₂-pyrrole of uro'gen), 24.5 (pyrrole-CH₂-pyrrole of bilane), 37.2 (CH₂NH₃⁺ of residual PBG), 57.1 (CH₂OH of bilane).

For the run at pH >12, data were accumulated for 5 h at 37 °C to give: δ_C 24.0 (pyrrole-CH₂-pyrrole of uro'gen), 24.5 (pyrrole-CH₂-pyrrole of bilane), 38.4 (CH₂NH₂ of residual PBG), 57.2 (CH₂OH of bilane). Ratio of peak integrals was 24 : 53 : 5 : 18. With off-resonance decoupling at δ_H - 5.0, all these signals were triplets, with *J*_{red} 36.6, 36.0, ca. 38 and 43.9 Hz respectively.

[aminomethyl-¹³C]Bilane (4a) and the Corresponding Lactam Heptamethyl Ester.—The starting material was the [aminomethyl-¹³C]tripyrrene, corresponding to rings A, B, and c of structure (4a); this had been previously synthesised.⁴ The labelled tripyrrene hydrobromide (35 mg) was condensed with the α-free formylpyrrole [as (7), unlabelled] (10.1 mg) in the usual way^{1,4} and the resulting biladiene dihydrobromide (42.8 mg, 98%) was reduced with sodium borohydride^{1,4} to give the lactam heptaester (90 atom % ¹³C) (25.1 mg, 68%) as a pale yellow powder, m.p. 224—228 °C (decomp). This was identical, apart from the ¹³C label, with the sample prepared earlier;⁴ δ (400 MHz) 2.33 (2 H, t, *J* 8 Hz), 2.47 (4 H, t, *J* 8 Hz), 2.51 (2 H, t, *J* 8 Hz), and 3.32 (8 H, m) (4 × CH₂CH₂CO₂), 3.35 (2 H, t, *J* 3.2 Hz, CH₂CONH), 3.32, 3.42, and 3.44 (each 2 H, s, 3 × CH₂CO₂), 3.56, 3.58, 3.60, 3.62, 3.64, 3.65, and 3.69 (each 3 H, s, 7 × OCH₃), 3.68, 3.71, and 3.73 (each 2 H, s, 3 × methane-CH₂), 4.38 (2 H, br d, *J* 141 Hz, ¹³CH₂NH), 5.92 (1 H, br, ¹³CH₂NH), 6.38 (1 H, d, *J* 2.4 Hz, pyrrole-H), and 8.65, 9.03, 9.05, and 9.19 (each 1 H, br, 4 × NH).

Material of 10 atom % ¹³C content at the aminomethyl carbon (243 mg, 65%) was synthesised from the same labelled tripyrrene hydrobromide above (35 mg) mixed with unlabelled tripyrrene hydrobromide (315 mg), the mixture being condensed with the α-free aldehyde [as (7)] (102 mg).

Formation of the [aminomethyl-¹³C]Bilane (4a) and its Study by ¹³C N.m.r. Spectroscopy.—The foregoing labelled bilane lactam ester (10 atom % ¹³C, 60 mg) was hydrolysed under nitrogen in aqueous 2M-potassium hydroxide (0.6 ml) for 3 h at 65 °C and 12 h at 25 °C. Deuterium oxide (0.1 ml) and water (to 0.9 ml) were added and the ¹³C n.m.r. spectrum of the resulting alkaline solution of the [aminomethyl-¹³C]bilane (4a) was determined at 37 °C: δ_O 23.8, 24.0, 24.5, and 24.7 (4 × CH₂CH₂CO₂ plus 3 × methane-CH₂), 35.4 (4 × CH₂CO), 38.4 (CH₂NH₂), 40.9 (1C) and 41.9 (3C) (4 × CH₂CH₂CO₂), 115.3, 115.8, 116.0, 119.7, 120.1, 120.5, 124.9, 126.4 (2C), 127.1, 127.7, 128.2, 128.5, 128.9, 130.5, and 131.2 (16 × pyrrole-C), and 184.2 and 185.1 p.p.m. (8 × CO₂).

When the ¹³C spectrum was redetermined, at pH 6, the chemical shifts of the highfield carbons were essentially unchanged, except for δ_O 37.4 p.p.m. (CH₂NH₃⁺). The pyrrole carbons now gave rise to signals at 115.3, 115.7, 116.1, 120.0, 120.3, 121.9, 124.8, 127.1, 127.7, 128.2, 128.9, 130.5, and 130.7 p.p.m.

After 4 days at 25 °C, pH 6, ca. 85% of the bilane had been converted into uro'gen-I: δ_O 24.0 (pyrrole-CH₂-pyrrole), 24.5 (4 × CH₂CH₂CO₂), 35.3 (4 × CH₂CO₂), 42.1 (4 × CH₂CH₂CO₂), 115.3, 120.4, 127.1, and 129.2 (each 4 C, 16 × pyrrole-C), and 184.4 and 185.3 (each 4 C, 8 × CO₂).

Conversion of [aminomethyl-¹³C]Bilane (4a) into [hydroxymethyl-¹³C]Bilane (5b) by Deaminase.—The foregoing [aminomethyl-¹³C]bilane lactam ester (90 atom %, 1.0 mg) was hydrolysed in 2M-potassium hydroxide (50 µl) (16 h at

25 °C) and the solution was adjusted to pH 8.25 with 1M-potassium dihydrogen phosphate. The resulting solution of [aminomethyl-¹³C]bilane (4a) was incubated at 37 °C for 15 min with highly purified deaminase (90 000 units, sp. act. > 20 000 units/mg) in 0.125M-phosphate buffer, pH 8.25 (total volume, 0.8 ml). Sodium hydroxide (20 mg) in deuterium oxide (0.1 ml) was then added and the mixture was examined by ¹³C n.m.r. spectroscopy (Figure 3): δ_C 24.0 (pyrrole-CH₂-pyrrole of uro'gen), 38.3 (CH₂NH₂ of aminomethylbilane), and 57.2 (CH₂OH of hydroxymethylbilane); ratio of peaks = 33 : 44 : 23.

After the n.m.r. measurements, the solution was adjusted to pH 7 with 2M-hydrochloric acid and kept at 37 °C for 10 min. The pH was then returned to >12 with sodium hydroxide (30 mg) and the ¹³C n.m.r. spectrum was re-determined. The hydroxymethyl signal (δ 57.2) had completely disappeared and the ratio of the signals from uro'gen relative to aminomethylbilane was now 57 : 43. Finally, the solution was adjusted to pH 8 and after the uro'gen had been oxidized with iodine, isomer analysis of the resulting porphyrin showed that it was >97% Type-I.

¹³C-Spectroscopic Studies on Hydroxymethylpyrroles: (a) the 2-[¹³C]Hydroxymethyl[5-¹³C]pyrrole (9a), (b) the 2-[¹³C]-Methyl[5-¹³C]pyrrole (13) and (c) the 2-[¹³C]Hydroxymethyl-[1-¹⁵N]pyrrole (9b).—(a) 2-[¹³C]Formyl-4-(2-methoxycarbonyl-ethyl)-3-methoxycarbonylmethyl[5-¹³C]pyrrole (7) (5 mg) was heated under nitrogen in aqueous 2M-potassium hydroxide (0.05 ml) for 15 min at 70 °C and the solution was then treated at 25 °C with sodium borohydride (3 mg) for 3 h. Deuterium oxide (0.05 ml), dioxan (0.02 ml), and water (to 0.9 ml) were added and the resulting solution of the pyrrole (9a) was examined by ¹³C n.m.r. spectroscopy [δ_C 57.3 (CH₂OH), 117.5 (C-5)].

(b) When the doubly labelled aldehyde (7) (2.25 mg) was hydrolysed in 2M-potassium hydroxide (0.50 ml) (90 °C, 10 min) and then treated with sodium borohydride (50 mg) at 90 °C for 0.5 h, there was further reduction. Examination of the solution by ¹³C n.m.r. spectroscopy after addition of deuterium oxide (0.05 ml), dioxan (0.01 ml), and water (to 0.9 ml), showed >90% formation of the methylpyrrole (13). With off-resonance ¹H decoupling (10 W) at δ_H - 5.0 p.p.m., the spectrum showed δ_C 12.9 (q, J_{red} , 46.3 Hz, pyrrole-CH₃), 115.1 (d, J_{red} , 97.6 Hz, 5-CH).

When the aldehyde (7) (2.25 mg) was hydrolysed in a smaller volume of alkali (0.05 ml) and the solution diluted with water (0.45 ml) before reduction, exactly as above, the ¹³C n.m.r. spectrum of the product showed that the methylpyrrole (13) and the hydroxymethyl analogue (9a) were present in a ratio of ca. 1 : 3.

(c) 2-[¹³C]Formyl-4-(2-methoxycarbonyl-ethyl)-3-methoxycarbonylmethyl[¹⁵N]pyrrole (23) prepared below (5 mg) was hydrolysed and reduced as in (a) above. Found for the alkaline solution, δ_C 57.3 (d, 2J 2.3 ± 0.2 Hz, ¹⁵NC¹³CH₂-OH). When the solution was adjusted to pH 8 with 1M-phosphoric acid, the chemical shift and coupling constant were essentially unchanged.

¹³C-Spectroscopic Studies on Borohydride Reduction of Hydroxymethylbilane (5a): Formation of Methylbilane (15).—[11-¹³C]PBG lactam methyl ester (10) (3.0 mg) was hydrolysed in the usual way (50 μ l of 2M-alkali) and the resulting solution of [11-¹³C]PBG, after adjustment to pH 8.25, was incubated at 37 °C with deaminase (50 400 units) in 0.2M-phosphate buffer, pH 8.25 (total volume 0.8 ml). After 8.25 min, when ca. 85% of the substrate had been consumed, sodium hydroxide (60 mg), sodium borohydride (100 mg) and deuter-

ium oxide (0.1 ml) were added and the mixture was kept at 90 °C for 1 h. The ¹³C n.m.r. spectrum (Figure 4), was then determined with off-resonance ¹H-decoupling (10 W) at δ_H - 5.0; δ_C 12.9 (q, J_{red} , 46.3 Hz, bilane-CH₃), 24.0 (t, J_{red} , ca. 55 Hz, pyrrole-CH₂-pyrrole of uro'gen), 24.4 (t, J_{red} , 55.5 Hz, pyrrole-CH₂-pyrrole of bilane), and 38.3 (t, J_{red} , 58.6 Hz, CH₂NH₂ of residual PBG).

4'-(2-Methoxycarbonyl-ethyl)-3'-methoxycarbonylmethyl-2,5-dimethyl-1,2'-dipyrrolylmethane, Dimethyl Ester of (16).—Porphobilinogen monohydrate (112 mg), hexane-2,5-dione (125 mg), methanol (3 ml), and aqueous 2M-potassium hydroxide (0.5 ml) were heated under reflux for 1.5 h and then cooled. After addition of methanol (2 ml) and pH adjustment to 2 with 3M-hydrochloric acid, excess of etheral diazomethane was added and, 5 min later, the solution was evaporated. Partition of the residue between water (25 ml) and dichloromethane (25 ml) gave the product in the organic layer which was chromatographed on silica (2.5 g) in dichloromethane; recrystallisation from ether-hexane gave the title compound as needles (105 mg, 65%), m.p. 76–77 °C (Found: C, 65.3; H, 7.4; N, 8.7. C₁₈H₂₄N₂O₄ requires C, 65.0; H, 7.3; N, 8.4%), δ_H 2.12 (6 H, 2 \times pyrrole-CH₃), 2.64 (4 H, m, CH₂CH₂CO₂), 3.43 (2 H, s, CH₂CO₂), 3.65 and 3.68 (each 3 H, s, 2 \times OCH₃), 4.94 (2 H, s, methane-CH₂), 5.81 (2 H, s, 2 \times pyrrole β -H), 6.35 (1 H, d, J 2.5 Hz, pyrrole α -H), and 7.19 (1 H, br, NH); δ_O 12.1 (2 \times pyrrole-CH₃), 20.4 (CH₂CH₂CO₂), 29.7 (CH₂CO₂), 34.6 (CH₂CH₂CO₂), 39.6 (methane-CH₂), 51.3 and 51.7 (2 \times OCH₃), 105.7 (C-3,-4), 113.8 (C-5'), 109.5, 121.9, 125.6 and 127.7 (C-2,-5,-2',-3',-4'), and 171.9 and 173.4 (2 \times CO₂); m/e 332 (44%, M^+) and 238 (100, M^+ - C₆H₈N).

4'-(2-Carboxyethyl)-3'-carboxymethyl-2,5-dimethyl-1,2'-dipyrrolylmethane (16) and [¹⁵N,¹³C₂] Labelled Material (16c).—The foregoing dimethyl ester (50 mg) was suspended in aqueous potassium hydroxide (0.5 ml) and argon was passed through for 1 min. The container was sealed and heated at 70 °C for 10 min after which the solution was evaporated under argon. The residue was dissolved in water (0.8 ml) plus deuterium oxide (0.1 ml) and the ¹³C n.m.r. spectrum run: δ_C 14.1 (2 \times pyrrole-CH₃), 24.3 (CH₂CH₂CO₂), 34.8 (CH₂CO₂), 40.6 (CH₂CH₂CO₂), 42.0 (methane-CH₂), 107.2 (C-3,-4), 116.4 (C-5'), 115.8, 125.3, 128.1 and 131.9 (C-2,-5,-2',-3',-4'), and 183.5 and 184.8 (2 \times CO₂).

[amino-¹⁵N,2,11-¹³C₂]PBG methyl ester, described later (97 atom % ¹⁵N, 10 atom % ¹³C, 10.0 mg), was hydrolysed in aqueous 2M-potassium hydroxide (0.10 ml) in the usual way and the solution was adjusted to pH 11 with 3M-hydrochloric acid. The residue from evaporation was heated for 2 h in methanol (0.30 ml) containing water (0.05 ml) and hexane-2,5-dione (7.6 mg, 1.5 equiv.). Evaporation of the solution then gave a residue of the [¹⁵N, ¹³C₂]labelled compound (16c) which was dissolved in deuterium oxide (0.10 ml) and 0.5M-potassium hydroxide (to 0.90 ml) for ¹³C n.m.r. as above. The bridge methylene, δ_C 42.0, appeared as a doublet with $^1J(^{13}C-^{15}N)$ 10 Hz.

4'-(2-Methoxycarbonyl-ethyl)-3'-methoxycarbonylmethyl-2-methyl-4-ethoxycarbonyl-1,2'-dipyrrolylmethane (18) and the Corresponding Tricarboxylic Acid (19) (with Dr. A. Pfenniger).—PBG lactam ester (111 mg, 0.5 mmol) was hydrolysed as above in aqueous 2N-potassium hydroxide (1 ml, degassed) under argon. The pH of the solution was adjusted to 9 with 2N-hydrochloric acid and the keto-aldehyde ¹³ (17) (110 mg, 0.64 mmol) in ethanol (1.5 ml) was added. After the mixture had been stirred at 20 °C for 1 h, the pH was adjusted to 2 and the collected precipitate was washed with

water. This product in acetone (5 ml) and methanol (5 ml) was treated with an excess of diazomethane to yield the *pyrrole ester* which was purified by chromatography on silica using ether and crystallisation at -20°C from ether-hexane, m.p. $77.5\text{--}78.5$ (84 mg, 43%) (Found: C, 61.4; H, 6.7; N, 7.2. $\text{C}_{20}\text{H}_{28}\text{N}_2\text{O}_6$ requires C, 61.5; H, 6.7; N, 7.2%). δ_{H} 1.25 (3 H, t, J 7 Hz, CH_2CH_3), 2.09 (3 H, s, CH_3), 2.33—2.93 (4 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.38 (2 H, s, CH_2CO), 3.62 (6 H, s, $2 \times \text{OCH}_3$), 4.18 (2 H, q, J 7 Hz, CH_2CH_3), 4.89 (2 H, s, N-CH_2), 6.25 (1 H, d, J 1.8 Hz, pyrrole-H), 6.44 (1 H, d, J 2.5 Hz, pyrrole-H), 7.06 (1 H, d, J 1.8 Hz, pyrrole-H), and 8.05 (1 H, br, NH); δ_{C} 173.6 (s), 172.0 (s), 164.9 (s), 129.7 (s), 125.5 (d), 123.5 (s), 121.9 (s), 115.4 (d), 114.6 (s), 112.4 (s), 108.4 (d), 59.5 (t), 51.9 (q), 51.5 (q), 42.8 (t), 34.8 (t), 29.9 (t), 20.6 (t), 14.5 (q), and 11.8 (q).

This ester (18) (80 mg, 0.21 mmol), suspended in degassed 2N-sodium hydroxide (0.6 ml), was warmed briefly until complete dissolution occurred; this solution was then kept at 20°C for 60 h under argon. The residue from freeze drying the solution was dissolved in D_2O for n.m.r. spectroscopic purposes (below) and was subsequently acidified to pH 4 to precipitate the crystalline triacid, m.p. $188\text{--}189^{\circ}\text{C}$ (decomp.), δ_{H} 2.17 (3 H, s, CH_3), 2.25—2.80 (4 H, m, $\text{CH}_2\text{CH}_2\text{CO}$), 3.35 (2 H, s, CH_2CO), 5.00 (2 H, s, N-CH_2), 6.21 (1 H, br, pyrrole-H), 6.63 (1 H, s, pyrrole-H), and 7.17 (1 H, d, J 2 Hz, pyrrole-H); δ_{C} 184.7 (s), 183.5 (s), 176.3 (s), 132.9 (s), 127.6 (d), 126.7 (s), 124.9 (s), 121.6 (s), 117.3 (s), 117.2 (d), 109.9 (d), 44.7 (t), 40.6 (t), 34.9 (t), 24.9 (t), and 13.6 (q). When the ^{13}C spectrum was determined with irradiation at δ_{H} 5.00, the signal at δ_{C} 44.7 became a singlet.

[Amino- ^{15}N , 2,11- $^{13}\text{C}_2$]Porphobilinogen Lactam Methyl Ester (3-Methoxycarbonyl-1,4,6,7-tetrahydropyrrolo[2,3-c]pyridin-5-one).—A solution of 2-[^{13}C]formyl-4-(2-methoxycarbonyl-3-methoxycarbonylmethyl-5- ^{13}C)pyrrole (7) (10.2 mg, 0.04 mmol) and the corresponding unlabelled material (91.1 mg, 0.36 mmol) in methanol (3 ml) containing [^{15}N]hydroxylamine hydrochloride (97.4 atom %, 35.5 mg, 0.50 mmol) and sodium acetate (41 mg) was heated under reflux for 0.75 h to yield the oxime. Adams' catalyst (90 mg) and additional methanol (7 ml) were then added to the mixture which was then stirred under hydrogen at 25°C for 24 h and then evaporated. A solution of the residue in methanol-chloroform (1:19) was filtered and chromatographed on silica (3 g), using the same solvent mixture as eluant, to give the *title lactam*, 97 atom % ^{15}N and 10 atom % ^{13}C (31.4 mg, 38%) as flakes, m.p. $247.5\text{--}249^{\circ}\text{C}$, from chloroform-hexane; it was identified by comparison with authentic unlabelled material.⁴

Benzyl 4-Acetyl-3-(2-ethoxycarbonyl-5-methyl- ^{15}N -pyrrole-2-carboxylate (20).—Sodium [^{15}N]nitrite (99 atom %, 350 mg) was added during 20 min to a stirred mixture of 1-benzyloxycarbonyl-4-ethoxycarbonylbutan-2-one (1.39 g), acetic acid (2 ml), and water (0.6 ml) at 0°C . After 20 h at room temperature, this solution, and a mixture of zinc dust (2 g) and ammonium acetate (2 g), were added simultaneously to a vigorously stirred solution of pentane-2,4-dione (0.75 g) in acetic acid (2 ml) at $90\text{--}100^{\circ}\text{C}$. After 1 h at this temperature, the mixture was poured into water (100 ml) and the product extracted into ether (100 ml and 2×50 ml). The ether extracts were washed (water, 100 ml; aqueous 0.5M-sodium hydroxide, 100 ml; water, 2×50 ml) and worked up to give a residue which was purified by chromatography on silica (15 g) with ether (0—30%) in dichloromethane-hexane (1:1); the major fraction afforded the *title pyrrole* (20) (1.24 g, 69%) as plates, m.p. $98\text{--}99^{\circ}\text{C}$ and

subsequently at $104.5\text{--}105^{\circ}\text{C}$ (from dichloromethane-ether-hexane). It was identical (apart from the isotope) with authentic unlabelled material; this also holds for the pyrroles (21), (22), (23), and (24) below (Found: M^+ , 358.1545. $\text{C}_{20}\text{H}_{23}^{15}\text{NO}_5$ requires M , 358.1546), δ 1.21 (3 H, t, J 7.1 Hz, OCH_2CH_3), 2.44 (3 H, s, COCH_3), 2.50 (3 H, d, 3J 3 Hz, $^{15}\text{NCCH}_3$), 2.51 (2 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.38 (2 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 4.08 (2 H, q, J 7.1 Hz, OCH_2CH_3), 5.31 (2 H, s, CH_2Ph), 7.36 (5 H, s, C_6H_5), and 9.05 (1 H, d, J 97.1 Hz, ^{15}NH); m/e 358 (100%, M^+), 313 (25, $M^+ - \text{C}_2\text{H}_5\text{O}$), 284 (24), 271 (34), 270 (20), 267 (40, $M^+ - \text{C}_7\text{H}_7$), 225 (36), 221 (49), and 207 (32).

Benzyl 3-(2-Methoxycarbonyl-4-methoxycarbonylmethyl-5-methyl- ^{15}N -pyrrole-2-carboxylate (21).—The foregoing labelled acetylpyrrole (1.23 g) in methanol (15 ml) was treated with a solution of thallium(III) nitrate trihydrate²⁵ (1.62 g) in methanol (5 ml) containing nitric acid (17M; 0.1 ml). After 48 h at room temperature, the precipitated thallium(I) nitrate was filtered off and washed with methanol, and the filtrate was diluted with water (100 ml). The product was extracted into dichloromethane (4×50 ml), washed with water (2×50 ml), recovered by evaporation, and chromatographed on silica (7 g) using ether (0—10%) in dichloromethane-hexane (1:1). It crystallised from ether-hexane to give the labelled pyrrole (21) (1.21 g, 94%) as needles, m.p. $78.5\text{--}80^{\circ}\text{C}$ (Found: M^+ , 374.1487. $\text{C}_{20}\text{H}_{23}^{15}\text{NO}_6$ requires M , 374.1495), δ_{H} 2.21 (3 H, d, 3J 2.6 Hz, $^{15}\text{NCCH}_3$), 2.51 (2 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.02 (2 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.42 (2 H, s, CH_2CO_2), 3.61 and 3.66 (each 3 H, s, $2 \times \text{OCH}_3$), 5.27 (2 H, s, CH_2Ph), 7.36 (5 H, s, C_6H_5), and 8.74 (1 H, d, J 97 Hz, ^{15}NH); δ_{C} 11.4 (d, 2J 1.8 Hz, 5- CH_3), 20.5 (d, 3J 0.9 Hz, $\text{CH}_2\text{CH}_2\text{CO}_2$), 29.5 (d, 3J 1.6 Hz, CH_2CO_2), 34.6 ($\text{CH}_2\text{CH}_2\text{CO}_2$), 51.2 and 51.7 ($2 \times \text{OCH}_3$), 65.6 (CH_2Ph), 114.0 (d, 2J 4.3 Hz) and 130.4 (d, 2J 4.5 Hz) (C-3,-4), 116.4 and 131.3 (each d, 1J 15.0 Hz, C-2,-5), 127.8 (2C), 127.9 (1C) and 128.2 (2C) (phenyl C-2,-3,-4,-5,-6), 135.8 (phenyl C-1), 160.4 (d, 2J 2.7 Hz, $^{15}\text{NCCO}_2$), 171.8 and 173.2 ($2 \times \text{CO}_2$); m/e 374 (57%, M^+), 342 (8), 316 (21), and 283 (100, $M^+ - \text{C}_7\text{H}_7$).

Benzyl 5-Formyl-3-(2-methoxycarbonyl-4-methoxycarbonylmethyl- ^{15}N -pyrrole-2-carboxylate [as (22), no ^{13}C].—The 5-methylpyrrole (21) (748 mg) was chlorinated with sulphuryl chloride (567 mg, 2.1 equiv.) in dichloromethane (14 ml) and then hydrolysed in the usual way.⁴ The product was chromatographed on silica (5 g) using ether (0—30%) in dichloromethane and recrystallised from ether-hexane to give the labelled pyrrolealdehyde (699 mg, 90%) as colourless prisms, m.p. $78\text{--}79^{\circ}\text{C}$ (Found: M^+ , 388.1284. $\text{C}_{20}\text{H}_{21}^{15}\text{NO}_7$ requires M , 388.1288); δ 2.55 (2 H, m, $\text{CH}_2\text{-CH}_2\text{CO}_2$), 3.04 (2 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.61 and 3.69 (each 3 H, s, $2 \times \text{OCH}_3$), 3.82 (2 H, s, CH_2CO_2), 5.33 (2 H, s, CH_2Ph), 7.38 (5 H, s, C_6H_5), 9.64 (1 H, d, J 99.7 Hz, ^{15}NH), and 9.76 (1 H, d, 3J 2.4 Hz, $^{15}\text{NCCHO}$); m/e 388 (24%, M^+), 357 (8), 356 (8), 329 (10), 328 (8), 297 (19, $M^+ - \text{C}_7\text{H}_7$), 269 (100, $M^+ - \text{C}_7\text{H}_7$, CO), and 265 (96, $M^+ - \text{C}_7\text{H}_7\text{CH}_2\text{O}$).

Benzyl 3-(2-Methoxycarbonyl-4-methoxycarbonylmethyl- ^{15}N -pyrrole-2-carboxylate.—The foregoing [^{15}N]-labelled aldehyde (694 mg) was decarbonylated as earlier⁴ with tris(triphenylphosphine)rhodium (I) chloride (1.737 g, 1.05 equiv.) in toluene (75 ml). Chromatography of the product on silica (15 g), using dichloromethane-petroleum (1:1) containing ether (0—10%) as eluant, followed by recrystallisation from ether-hexane afforded the α -free pyrrole (566 mg, 88%) as needles, m.p. $55\text{--}57^{\circ}\text{C}$ (Found: M^+ , 360.1331. $\text{C}_{19}\text{H}_{21}^{15}\text{NO}_6$ requires M , 360.1339); δ 2.52

(2 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.01 (2 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.49 (2 H, s, CH_2CO_2), 3.61 and 3.67 (each 3 H, s, $2 \times \text{OCH}_3$), 5.29 (2 H, s, CH_2Ph), 6.83 (1 H, dd, 2J 4.1 Hz, 3J 3.1 Hz, $\text{HC}^{15}\text{N}-\text{H}$), 7.36 (5 H, s, C_6H_5), 8.92 (1 H, dd, 1J 98.3 Hz, 3J 3.1 Hz, HC^{15}NH); m/e 360 (27%, M^+), 329 (14), 328 (30), 301 (24), 269 (100, $M^+ - \text{C}_7\text{H}_7$), 237 (97, $M^+ - \text{C}_7\text{H}_7, \text{CH}_4\text{O}$), and 209 (62).

Benzyl 5-[^{13}C]Formyl-3-(2-methoxycarbonylethyl)-4-methoxycarbonylmethyl[^{15}N]pyrrole-2-carboxylate (22).—The labelled α -free pyrrole (560 mg) was formylated, as earlier,⁴ with dimethyl[^{13}C]formamide (90 atom %, 173 mg, 1.50 equiv.) and phosphoryl chloride (358 mg) in acetonitrile (2.5 ml). After 3.5 days at room temperature, work-up as before⁴ afforded a product which was purified by chromatography on silica (5 g), using dichloromethane-petroleum (1:1) containing ether (0–20%) and by recrystallisation from ether-hexane to give the doubly labelled aldehyde (22) (540 mg) as prisms, m.p. 79–80 °C. P.l.c. of mother liquors and mixed fractions raised the yield to 576 mg (95%) (Found: M^+ , 389.1315. $^{13}\text{C}_{12}\text{C}_{10}\text{H}_{21}^{15}\text{NO}_7$ requires M , 389.1322); δ_{H} 2.54 (2 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.03 (2 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.61 and 3.69 (each 3 H, s, $2 \times \text{OCH}_3$), 3.82 (2 H, s, CH_2CO_2), 5.32 (2 H, s, CH_2Ph), 7.38 (5 H, s, C_6H_5), 9.64 (1 H, dd, 1J 99.6 Hz, 3J 0.9 Hz, $\text{H}^{15}\text{NC}^{13}\text{CHO}$), 9.76 (1 H, dd, 1J 179 Hz, 3J 2.2 Hz, $\text{H}^{15}\text{NC}^{13}\text{CHO}$); δ_{C} 179.1 (d, 2J 1.9 Hz, $^{15}\text{NC}^{13}\text{CHO}$); m/e 389 (16%, M^+), 358 (6), 357 (10), 330 (10), 329 (5), 298 (15), 270 (100, $M^+ - \text{C}_7\text{H}_7, \text{CO}$), and 266 (88, $M^+ - \text{C}_7\text{H}_7, \text{CH}_4\text{O}$).

2-[^{13}C]Formyl-5-iodo-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethyl[^{15}N]pyrrole.—The foregoing doubly labelled pyrrole (22) (570 mg) was debenzylated as earlier⁴ to give the corresponding acid (389 mg, 88%) as colourless flakes, m.p. 134–136 °C (Found: M^+ , 299.0826. $^{13}\text{C}_{12}\text{C}_{12}\text{H}_{15}^{15}\text{NO}_7$ requires M , 299.0852); δ (CD_3OD), 2.54 (2 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.01 (2 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.61 and 3.67 (each 3 H, s, $2 \times \text{OCH}_3$), 3.88 (2 H, s, CH_2CO_2), and 9.72 (1 H, br dd, 1J 177 Hz, 3J 1.0 Hz, $^{15}\text{NC}^{13}\text{CHO}$).

A solution of iodine (377 mg) and potassium iodide (456 mg) in water (8 ml) was added during 1 min to a refluxing, vigorously stirred mixture of the foregoing carboxylic acid (383 mg), sodium hydrogen carbonate (310 mg), ethanol-free chloroform (9 ml), and water (5 ml). Five minutes later, the mixture was worked-up in the usual way⁴ and the product chromatographed on silica (5 g) using ether (0–20%) in dichloromethane-petroleum (1:1). Appropriate fractions were recrystallised from ether-hexane to give the iodopyrrole (383 mg, 78%) as needles, m.p. 103–104.5 °C (Found: M^+ , 380.9909. $^{13}\text{C}_{12}\text{C}_{11}\text{H}_{14}\text{I}^{15}\text{NO}_5$ requires M , 380.9921); δ 2.63 (4 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.67 and 3.70 (each 3 H, s, $2 \times \text{OCH}_3$), 3.80 (2 H, s, CH_2CO_2), 9.43 (1 H, dd, 1J 177 Hz, 3J 2.6 Hz, $^{15}\text{NC}^{13}\text{CHO}$), 9.50 (1 H, d, J 100.2 Hz, ^{15}NH); m/e 381 (27%, M^+), 350 (10), 349 (11), 262 (18), and 254 (100, $M^+ - \text{I}$).

2-[^{13}C]Formyl-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethyl[^{15}N]pyrrole (23). A mixture of the foregoing iodopyrrole (381 mg), sodium acetate (400 mg), Adams' catalyst (50 mg), and methanol (25 ml) was stirred under hydrogen for 40 min. The product was isolated as earlier⁴ and purified by chromatography on silica (5 g), using ether (0–40%) in dichloromethane-hexane (1:1), and by recrystallisation from ether-hexane to give the α -free doubly labelled aldehyde (23) (241 mg, 94%) as needles, m.p. 97.5–98.5 °C (Found: M^+ , 255.0949. $^{13}\text{C}_{12}\text{C}_{11}\text{H}_{15}^{15}\text{NO}_5$ requires M , 255.0954); δ_{H} 2.67 (4 H, m, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.66 and 3.69 (each 3 H, s, $2 \times \text{OCH}_3$), 3.75 (2 H, s, CH_2CO_2), 6.90 (1 H, br

dd, 2J 3.7 Hz, 3J 3.1 Hz, H^{15}NCH), 9.45 (1 H, ddd, 1J 98.5 Hz, $^3J_{\text{H}^{15}\text{NCH}}$ 3.1 Hz, $^3J_{\text{H}^{15}\text{NCO}}$ 0.7 Hz, ^{15}NH), 9.62 (1 H, ddd, 1J 174.9 Hz, 2J 2.5 Hz, 5J 0.9 Hz, $\text{HC}^{15}\text{NC}^{13}\text{CHO}$); δ_{C} 177.6 p.p.m. (d, 2J 2.3 Hz, $^{15}\text{NC}^{13}\text{CHO}$); m/e 255 (26%, M^+), 223 (19), 196 (32), 195 (31), 173 (26), 172 (20), 136 (48), 124 (60), 107 (100), 106 (17), and 105 (33).

[1- ^{15}N , 11- ^{13}C]Porphobilinogen Lactam Methyl Ester (24).—A mixture of the foregoing aldehyde (23) (102 mg), hydroxylamine hydrochloride (42 mg, 1.5 equiv.), sodium acetate (50 mg), and methanol (2 ml) was stirred at room temperature for 2 h. The product was extracted with methylene chloride and recrystallised from chloroform-hexane to give the oxime derivative (107 mg, 99%) as needles, m.p. 127.5–128.5 °C (Found: M^+ , 270.1046. $^{13}\text{C}_{12}\text{C}_{11}\text{H}_{16}^{15}\text{N}^{14}\text{NO}_5$ requires M , 270.1063); m/e 270 (100%, M^+), 254 (30), 253 (48), 252 (14), 238 (23), 211 (40), 201 (15), 197 (36), 195 (15), 193 (43), 189 (11), 179 (74), 178 (14), and 173 (45).

The oxime (105 mg) in methanol (25 ml) was hydrogenated over Adams' catalyst (50 mg) for 20 h. Chloroform (50 ml) was added to dissolve the precipitated lactam, the filtered solution was evaporated, and the residue chromatographed on silica (5 g), using methanol (5%) in chloroform as eluant. Recrystallisation of appropriate fractions from chloroform-hexane gave the doubly labelled lactam (24) (40.2 mg, 46.2%) as flakes, m.p. 248–249 °C (Found: M^+ , 224.1002. $^{13}\text{C}_{12}\text{C}_{10}\text{H}_{14}^{15}\text{N}^{14}\text{NO}_3$ requires M , 224.1008); δ (400 MHz) 2.55 (2 H, t, J 7.6 Hz, $\text{CH}_2\text{CH}_2\text{CO}_2$), 2.72 (2 H, t, J 7.6 Hz, $\text{CH}_2\text{CH}_2\text{CO}_2$), 3.41 (2 H, t, J 3.4 Hz, CH_2CONH), 3.66 (3 H, s, OCH_3), 4.48 (2 H, br dd, 1J 140 Hz, 3J 2 Hz, $^{15}\text{NC}^{13}\text{CH}_2$), 5.93 (1 H, br, $^{13}\text{CH}_2\text{NH}$), 6.55 (1 H, m, pyrrole-H), and 7.71 (1 H, dd, 1J 96 Hz, 3J 3 Hz, H^{15}NCH); m/e 224 (100%, M^+), 223 (81), 151 (76), 122 (72), and 121 (54).

[1- ^{15}N , 11- ^{13}C]Porphobilinogen (1b).—The lactam methyl ester (24) (2.0 mg) was hydrolysed in aqueous 2M-potassium hydroxide (0.10 ml) in the usual way (5 min at 70 °C, 16 h at 25 °C) and the resulting solution of the labelled PBG was diluted with deuterium oxide (0.05 ml), dioxan (0.01 ml), and water (to 0.9 ml) for examination by ^{13}C n.m.r. spectroscopy; δ_{C} 38.4 (d, 2J 2.2 \pm 0.2 Hz, $^{15}\text{NC}^{13}\text{CH}_2\text{NH}_2$).

The solution was adjusted to pH 8.3 with 1M-phosphoric acid to give δ_{C} 37.4 (d, 2J 2.4 Hz, $^{15}\text{NC}^{13}\text{CH}_2\text{NH}_3^+$).

Formation of Hydroxymethylbilane (5c) from [1- ^{15}N , 11- ^{13}C]Porphobilinogen (1b).—The deaminase used in the following experiments had been purified²⁰ to specific activity above 20 000 units/mg; four runs were carried out.

(a) [1- ^{15}N , 11- ^{13}C]Porphobilinogen lactam methyl ester (24) (1.5 mg) was hydrolysed in the usual way (50 μl of 2M-alkali) and the resulting solution of [1- ^{15}N , 11- ^{13}C]PBG (1b) after adjustment to pH 8.25, was incubated at 37 °C with deaminase (25 000 units) in 0.2M-phosphate buffer, pH 8.25 (total volume, 0.8 ml). After 7 min, when PBG consumption exceeded 90%, sodium hydroxide (20 mg), deuterium oxide (0.1 ml) and dioxan (5 μl) were added and the ^{13}C n.m.r. spectrum was recorded over 5 h at 37 °C: δ_{C} (partial spectrum) 57.2 [d, 2J 2.3 \pm 0.2 Hz, $^{15}\text{NC}^{13}\text{CH}_2\text{OH}$ of hydroxymethylbilane, (5c)].

(b) The previous experiment was repeated but no sodium hydroxide was added at the end of the incubation. The ^{13}C -n.m.r. spectrum was recorded over 1.75 h at 20–25 °C: δ_{C} (partial spectrum) 57.1 [d, 2J 2.3 Hz, $^{15}\text{NC}^{13}\text{CH}_2\text{OH}$ of hydroxymethylbilane (5c)].

(c) Experiment (b) was repeated (29 000 units of deaminase) and the ^{13}C n.m.r. spectrum was recorded at 3–4 °C (measured in the tube with the decoupler on). The doublet

from the hydroxymethyl group was broader than in the higher temperature runs and hence the signal-to-noise ratio was poor; the coupling constant was estimated to be 2 Hz (minimum) to 2.5 Hz (maximum).

(d) [^{15}N , ^{13}C]PBG (from 4.5 mg of lactam methyl ester) was mixed at 0 °C with deuterium oxide (50 μl) and deaminase (90 000 units) in 0.04 M-pyrophosphate buffer, pH 8.25 (total volume 0.95 ml). After 2 h at this temperature, the solution was transferred to the n.m.r. spectrometer at 3–4 °C but a spectrum recorded between 3.75 and 4.25 h after mixing indicated that only 25% of the PBG had been consumed. This figure increased to 35% in the next 2.25 h, but the uro'gen signal was now larger than that from the bridge methylenes of the bilane (5c). The probe temperature was therefore increased to 13 °C for 80 min and then returned to 3–4 °C. A spectrum accumulated over 2 h showed the signal from the hydroxymethyl group at δ 57.2, as a doublet, $^2J = 2.4 \pm 0.2$ Hz.

Computer Assisted Manipulation of the N.m.r. Data Derived from the [^{15}N , ^{13}C]Labelled Hydroxymethylbilane (5c) (with Dr. R. G. Brevelton).—The Free Induction Decays (FIDs) from experiments (a) (b) and (d) above, were transferred via punched paper-tape from the Varian 620-L mini-computer to the Cambridge IBM 370/165 mainframe machine. The procedure used in each case is described for the spectrum from experiment (d).

The 8 K FID, which was recorded over a spectral width of 2 500 Hz (1.6 s acquisition time) was first trapezoidally apodized from 1.4 to 1.6 s. After exponential multiplication, with a time constant of -1 s, the FID was zero filled to 16 K data points (3.2 s) and then Fourier transformed. A region of the resulting frequency domain spectrum 78 Hz wide which included the doublet from the hydroxymethyl group was inverse transformed and after the pseudo FID had been trapezoidally apodized from 1.4 to 1.6 s, it was multiplied by a sine bell function of the form $\sin(360 Ft + \phi)$.²⁶ In this case, F (frequency) was 0.7 Hz and ϕ , the phase shift, was set at 10°; the FID was then zero filled to 13 s (0.08 Hz/data point) and Fourier transformed. After phasing, a 25 point Golay smoothing function, of order 3, was applied and to improve presentation, the spectrum was squared. The resulting doublet (Figure 5) had 2J 2.44 ± 0.08 Hz. This coupling ranged between 2.35 and 2.48 Hz when the sine bell frequency and the number of points in the Golay smoothing operation were varied.

*Benzyl 5'-Formyl-4,3'-di(2-methoxycarbonyl)ethyl-3,4'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole-5-carboxylate*²⁷ (26).—A solution of the pyrromethane **1** (25) (7 g) in dry nitromethane (20 ml) was treated at -15 °C with boron trifluoride–diethyl ether²⁸ (1 ml) under nitrogen. After the mixture had been stirred at -15 °C for 1 h, it was diluted with acidified water (20 ml) and the product was extracted into dichloromethane (3×10 ml) and washed with water. This acid crystallised from dichloromethane–diethyl ether–hexane to give *benzyl 5'-formyl-4,3'-di(2-methoxycarbonyl)ethyl-3,4'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole-5-carboxylic acid* as needles (4.03 g, 63%), m.p. 160–161.5 °C (Found: C, 59.9; H, 5.8; N, 4.2. $\text{C}_{32}\text{H}_{36}\text{N}_2\text{O}_{12}$ requires C, 60.0; H, 5.7; N, 4.4%), m/e 596.2385, $M^+ - \text{CO}_2$ requires 596.2400, δ 10.73 (1 H, s, NH), 10.49 (1 H, s, NH), 7.37 (5 H, Ph), 5.31 (2 H, s, CH_2Ph), 3.99 (2 H, s, pyrrole- CH_2 -pyrrole), 3.85 (2 H, s, CH_2CO), 3.75, 3.71 (each 3 H, s, OCH_3), 3.62 (8 H, 2OCH_3 , CH_2CO), and 2.40–3.01 (8 H, m, CH_2 - CH_2).

A solution of the foregoing acid (4.8 g) in dichloromethane

(100 ml) was mixed with a solution of toluene-*p*-sulphonic acid (2.7 g) in methanol (15 ml) and stirred under nitrogen for 20 h at 20 °C in the dark. The resultant red solution was treated with sodium borohydride (0.6 g) in ethanol (3 ml) and kept until it was pale yellow. Water (100 ml) was then added followed by ammonia solution (d 0.88) until the solution was alkaline after which the product was extracted into dichloromethane and recovered as a gum by evaporation.

This was dissolved directly in dry *N,N*-dimethylformamide (30 ml), benzoyl chloride (3 ml) was added, and the solution was stirred under nitrogen at 20 °C for 2 h. The mixture was then partitioned between 10% aqueous sodium carbonate and dichloromethane and the product from the combined, washed organic layers was crystallised from dichloromethane–diethyl ether–hexane to yield the *formylpyrromethane* (26) (3.4 g, 72%), m.p. 119–120.5 °C (lit.,²⁷ 120–121 °C), m/e 624.2276, $\text{C}_{32}\text{H}_{36}\text{N}_2\text{O}_{11}$ requires 624.2319, δ 9.92 (1 H, s, NH), 9.60 (1 H, s, CHO), 7.36 (5 H, Ph), 5.28 (2 H, CH_2Ph), 4.01 (2 H, s, pyrrole- CH_2 -pyrrole), 3.84, 3.71, 3.65, and 3.56 (each 3 H, s, OCH_3), 3.76 and 3.61 (each 2 H, s, CH_2CO), and 2.44–3.13 (8 H, m, CH_2CH_2).

5'-Formyl-4,3'-di-(2-methoxycarbonyl)ethyl-3,4'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole (29).—A solution of the foregoing formylpyrromethane (2.23 g) in tetrahydrofuran (20 ml) and triethylamine (1 ml) was shaken with 10% palladium on charcoal (40 mg) and hydrogen under ambient conditions until uptake ceased. The filtered solution was evaporated and the residue was recrystallised from aqueous methanol to yield the acid (27) as prisms (1.49 g, 75%), m.p. 196–196.5 °C, m/e 505 ($M^+ - \text{CO}_2$).

A solution of this acid (0.1 g) in a mixture of dichloromethane (1.5 ml) and water (1 ml) containing sodium hydrogen carbonate (0.1 g) was vigorously stirred while a solution of iodine (55 mg) and potassium iodide (56 mg) in water (0.5 ml) was added dropwise during 2 min. The excess of iodine was then removed by addition of sodium metabisulphite and the product was extracted with dichloromethane. It was chromatographed on silica first in dichloromethane followed by dichloromethane–diethyl ether (1 : 19) and the pure iodopyrromethane (28) was crystallised from diethyl ether–hexane (79 mg, 69%), m.p. 132.5–134 °C (Found: M^+ , m/e 616.0913, $\text{C}_{24}\text{H}_{29}\text{IN}_2\text{O}_9$ requires M^+ , 616.0918), δ 10.4 (1 H, s, NH), 9.61 (1 H, s, CHO), 9.32 (1 H, s, NH), 3.93 (2 H, s, pyrrole- CH_2 -pyrrole), 3.85, 3.73, 3.72, and 3.71 (each 3 H, s, OCH_3), 3.78 and 3.60 (each 2 H, s, CH_2CO), and 2.53–2.86 (8 H, m, CH_2CH_2).

A solution of the iodopyrromethane (0.2 g) in methanol (10 ml) and triethylamine (0.5 ml) was shaken with Adams' platinum oxide (20 mg) and hydrogen at ca. 760 mm/20 °C until uptake ceased (45 min). The solution was filtered, 10% aqueous sodium carbonate (10 ml), and sodium chloride (0.3 g) were added and the product was extracted into dichloromethane. The recovered *pyrromethane* (29) crystallised from dichloromethane–diethyl ether–hexane as prisms (0.11 g, 72%), m.p. 101–102 °C (Found: C, 58.5; H, 6.3; N, 5.5%; M^+ , m/e 490.1940. $\text{C}_{24}\text{H}_{30}\text{N}_2\text{O}_9$ requires C, 58.7; H, 6.2; N, 5.7%; M^+ , 490.1951), ν_{max} 314 nm; δ 9.59 (1 H, s, CHO), 8.77 (1 H, s, NH), 6.48 (1 H, d, pyrrole-H), 3.93 (2 H, s, pyrrole- CH_2 -pyrrole), 3.83, 3.72, 3.70, and 3.67 (each 3 H, s, OCH_3), 3.78 and 3.55 (each 2 H, s, CH_2CO), and 2.50–2.88 (8 H, m, CH_2CH_2).

Enzymic Studies with the Hydroxymethylpyrromethane (31).—(a) *Studies on type-isomers formed.* A suspension of the foregoing pyrromethane ester (29) (50 mg) was stirred at

20 °C under nitrogen for 6.5 h with 2*N*-potassium hydroxide (0.5 ml). Sodium borohydride (40 mg) was added to the clear solution which was then stirred for 1 h after which there was no significant absorbance at 314 nm. The mixture was then adjusted to pH 8 with 1*M*-potassium dihydrogen phosphate (9.5 ml) and four aliquots [0.5 ml, containing 2.2 mg of hydroxymethylpyrromethane (31)] were each incubated at 37 °C in 0.2 *M*-phosphate buffer (0.95 ml), pH 8 which was 6 mM with respect to EDTA, with the following additions: (i) with 50 mM-phosphate buffer pH 8.25 (0.45 ml) and 15 mM-Tris buffer, pH 8.9 (1 ml); (ii) with deaminase (16 300 units in 0.45 ml) and 15 mM-Tris buffer (1 ml), pH 8.9; (iii) with deaminase (16 300 units in 0.45 ml) and a solution of an excess of cosynthetase (1 ml); (iv) with cosynthetase solution (1 ml) and 50 mM-phosphate buffer pH 8.25 (0.45 ml).

The four incubations were run for 1.75 h and then worked up and analysed for content of isomeric porphyrins in the way already described. The results are collected in Table 1, experiments 1–4.

(b) *Kinetic studies.* Four further experiments were carried out with a fresh preparation of hydroxymethylpyrromethane (31). Four aliquots of the solution of this material in 2*N*-potassium hydroxide [0.1 ml containing 0.68 mg of (31)] were each mixed with 2*M*-phosphate buffer, pH 8.25 (1.4 ml) and bovine serum albumin (10 mg) in the same buffer (0.5 ml) containing 3*N*-hydrochloric acid (9.4 μ l). These were incubated at 37 °C with the following additions: (i) water (0.1 ml); (ii) water (0.09 ml) and cosynthetase solution (10 μ l); (iii) cosynthetase solution (0.1 ml); and (iv) cosynthetase solution (1.6 ml). Samples (50 μ l) were taken after 30 s and 3, 5, 10, 15, 22, 30, 40, 55, and 76 min and these were analysed as usual for uro'gen content and for the type-isomers produced. Runs (i)–(iii) were the comparable ones for kinetic analysis. The results on isomer analysis are collected in Table 2, experiments 5–8.

Synthesis of Hydroxymethylbilane

1-Formyl-3,8,13,18-tetra-(2-methoxycarbonylethyl)-2,7,12,17-tetrakis(methoxycarbonylmethyl)bilane (35).—Sodium borohydride (20 mg) was added during 2 min to a stirred solution of the above 5-formyl-3,4'-di(2-methoxycarbonylethyl)-4,3'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole (29) (20 mg) in methanol (0.5 ml) containing triethylamine (3 drops). Five minutes later, dichloromethane (5 ml) was added and the solution was washed with dilute brine (3 \times 2 ml). Evaporation of the dried dichloromethane solution gave a residue of hydroxymethylpyrromethane (34) (one spot on t.l.c.) which was redissolved in dichloromethane (2 ml) containing triethylamine (2 drops) and used immediately in the next step.

A solution of the same aldehyde (29) (60 mg) in dichloromethane (5 ml) containing acetic acid (1 ml) was stirred at 20 °C under argon and all the foregoing hydroxymethylpyrromethane solution was added dropwise during 5 min. Ten minutes later, the solution was washed with dilute brine (20 ml) and carefully neutralised with solid sodium hydrogen carbonate; the organic phase was washed with water (2 \times 10 ml), dried, and evaporated with a stream of argon in a Craig tube. Methanol (2 ml) was added to the residue which was triturated and evaporated with argon. The residue was resuspended in methanol (2 ml) and the solid collected on a small plug of cotton wool in a Pasteur pipette. After washing with methanol (3 \times 1 ml) (the filtrate contained all the excess of formylpyrromethane), the solid was

washed through into a Craig tube with methanol–chloroform (1 : 9). The residue from evaporation was triturated with methanol (2 ml), centrifuged, and dried *in vacuo* to give the *formylbilane* (35) (22 mg, 56%) as a glass which was virtually pure by t.l.c. (4% MeOH in CHCl₃, green-brown colour with bromine vapour). It was further purified by p.l.c. on silica, eluant methanol (5%) and triethylamine (0.1%) in chloroform; the plate was run in the dark under an atmosphere of argon. The product was recovered with minimum exposure to air and further purified as above using methanol to give 17.3 mg (44%) of pale yellow solid, m.p. 157–160 °C (decomp.) (Found: M^+ , 964.3975. $C_{48}H_{60}N_4O_{17}$ requires M , 964.3953), ν_{\max} 3 330br, 1 730, and 1 643 cm^{-1} ; λ_{\max} 312.5 nm; δ 2.26–2.94 (16 H, m, 4 \times CH₂CH₂CO₂), 3.38 (4 H, 3.44 (2 H) (each s, 3 \times CH₂CO₂), 3.56, 3.61, 3.64, 3.66, 3.68, 3.70, 3.72, 3.75, and 3.80 (32 H, 8 \times OCH₃, 3 \times methane-CH₂ and CH₂CO₂), 6.38 (1 H, d, J 2.1 Hz, pyrrole-H), 8.58 (1 H, br, D-ring NH), 9.04 and 9.22 (each 1 H, br, B,C-ring NH), 9.51 (1 H, s, CHO), and 9.80 (1 H, br, A-ring NH); m/e 964 (100%, M^+), 948 (16), 936 (15), 933 (11), 905 (14), 891 (15), 877 (10), 875 (9), 739 (20), 727 (58), 726 (38), 699 (45), and 698 (25).

3,8,13,18-Tetra-(2-carboxyethyl)-2,7,12,17-tetrakis(carboxymethyl)-1-hydroxymethylbilane (5) (*The Hydroxymethylbilane*).—(a) *For spectroscopic study.* The foregoing formylbilane octamethyl ester (35) (37 mg) was suspended in nitrogen-saturated aqueous 2*M*-potassium hydroxide (0.40 ml) and the mixture was kept in the dark at 70 °C for 15 min. The cooled solution was treated with sodium borohydride (20 mg) at 25 °C for 3 h, after which dioxan (0.002 ml), deuterium oxide (0.050 ml), and water (to 0.90 ml) were added. The resulting alkaline solution of the *hydroxymethylbilane* (5) was examined by ¹H decoupled ¹³C n.m.r. spectroscopy (Figure 6): δ_c 23.7 (4 \times CH₂CH₂CO₂), 24.4 and 24.6 (3 \times pyrrole-CH₂-pyrrole), 35.4 (4 \times CH₂CO₂), 40.8 (1C) and 41.8 (3C) (4 \times CH₂CH₂CO₂), 57.1 (CH₂OH), 115.6 (C-19), 115.2, 115.9, 118.0, 119.9, 120.4, 124.7, 126.4, 127.0, 128.0, 128.4, 128.7, 128.9, 129.0, and 130.3 (15 \times ring-C), 184.0 (1C), 184.4 (3C), 185.4 (3C), and 185.7 (1C) (8 \times CO₂).

(b) *For enzymic work.* Sodium borohydride (20 mg) was added during 2 min to a stirred solution of the formylbilane octamethyl ester (35) (5.0 mg) in chloroform (0.5 ml) containing methanol (0.2 ml) and triethylamine (3 drops). Five minutes later, chloroform (2 ml) was added and the solution was washed with dilute brine (3 \times 1 ml). The dried organic solution was then concentrated with argon (to ca. 0.2 ml), diluted with methanol (2 ml), and concentrated as before. The residue was re-suspended in methanol (0.5 ml) containing triethylamine (2 drops); the solid was then collected by centrifugation and dried *in vacuo*. As soon as the resulting hydroxymethylbilane octamethyl ester (36) had reached constant weight (3.5–4.0 mg, 70–80%), it was hydrolysed under argon in 2*M*-potassium hydroxide (0.20 ml) for 16 h at 25 °C. The alkaline solution of the hydroxymethylbilane (5) was then adjusted in pH as required for the enzymic experiments.

Enzymic Studies with the Hydroxymethylbilane (5).—(a) *Studies on type-isomers formed.* Hydroxymethylbilane (5) (0.6 mg) prepared as above, was (i) incubated at 30 °C and pH 8.25 in phosphate buffer (0.14*M*). Otherwise identical incubates included (ii) deaminase (430 units) or (iii) cosynthetase (90 units, as Table 3). Samples (50 μ l) were taken to determine the uro'gen present, by the standard method, at intervals up to 30 min [(i) and (ii)] and 2 min (iii). At

these times, the residual solutions were worked up and analysed for content of isomeric porphyrins in the way already described. The results are in Table 2.

(b) *Comparison of synthetic and natural hydroxymethylbilane (5).* Natural hydroxymethylbilane (5) was generated as described above. Typically, PBG (0.2 mg) was treated with deaminase (14 000 units) in phosphate buffer (0.35 ml; 0.02 M) for 2 min at pH 8.25 and 37 °C. This solution was either used immediately or the pH was adjusted to >12 by the addition of 5M-NaOH (25 µl). In the latter case, the pH was adjusted back to 8.25 after 20 min at 37 °C by addition of 1M-HCl (0.125 ml) and the buffer for the subsequent incubation.

To determine $t_{\frac{1}{2}}$ (chemical ring closure) hydroxymethylbilane (5) (1 mg synthetic in 5 ml incubate), or natural bilane (from 0.2 mg PBG in 1 ml incubate) was incubated at 37 °C and pH 8.25 in Tris-HCl buffer (0.25 M) and 50 µl samples were removed to determine the rate of uro'gen formation by the standard method.

To examine the effect of cosynthetase on the uro'gen isomers produced, incubations of synthetic (0.6 mg) or natural (from 0.2 mg PBG, with and without alkali treatment) hydroxymethylbilane (5) with cosynthetase (150 units/mg bilane equivalent) were as described for (iii) in the previous section. Uro'gen production was monitored and that present before the addition of cosynthetase (type-I) was deducted before calculation of the percentage of type-III produced by cosynthetase.

To determine V_{\max} for cosynthetase, five portions of hydroxymethylbilane (5) (0.1 mg) were incubated at 25 °C with cosynthetase (5 µl of enzyme preparation) in 0.5, 1.0, 1.5, 2.0, and 2.5 ml of Tris-HCl buffer (0.16M; pH 8.25), thus producing a range of concentration of the bilane. Samples corresponding to one tenth of each of these solutions (50, 100, 150, 200, and 250 µl, made up to 0.75 ml) were taken for uro'gen determination at intervals up to 4 min. From each initial experimental rate was subtracted the rate of chemical formation of uro'gen from hydroxymethylbilane (5) (0.1 mg in 0.5 ml same buffer). A similar experimental approach was used for examining the effect of varying the concentration of naturally produced hydroxymethylbilane (5) on cosynthetase activity. There was no variation in the initial enzymic rates so obtained, over the range of bilane concentrations studied and thus the enzyme was taken to be at V_{\max} , in all cases. The results of these comparisons are shown in Table 3.

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