Biosynthesis of the Natural Porphyrins: Experiments on the Ring-closure Steps and with the Hydroxy-analogue of Porphobilinogen

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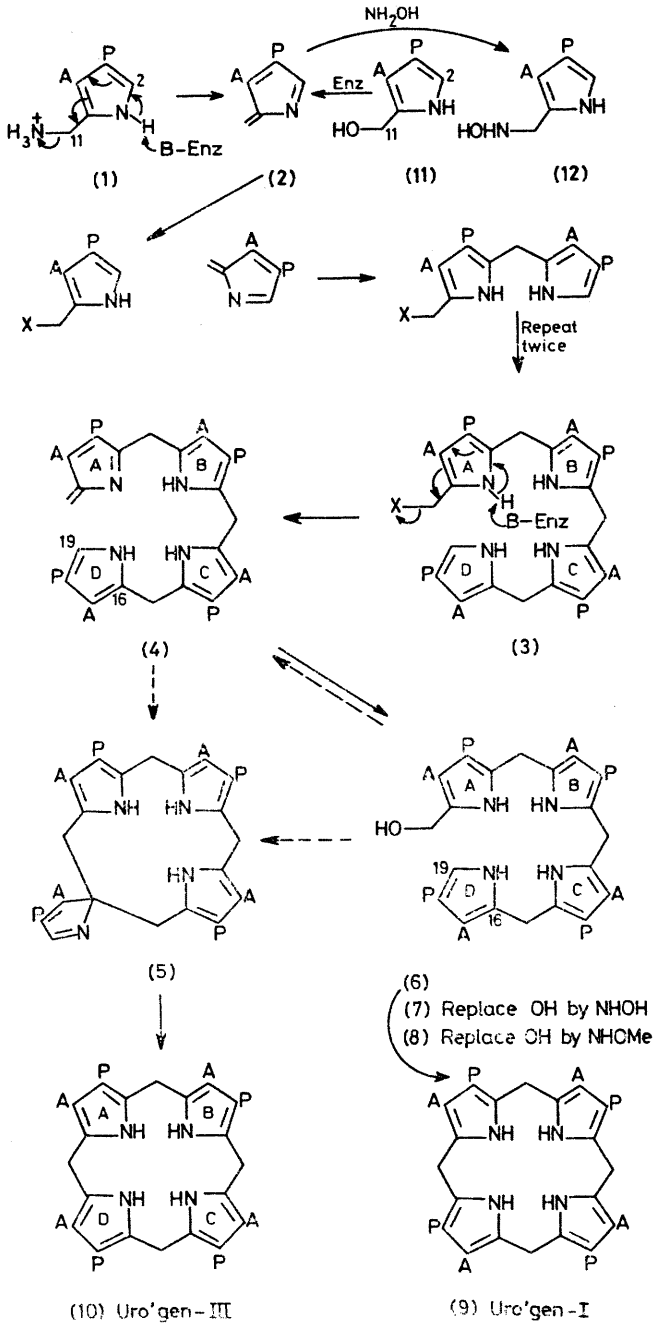
Summary Experiments on the conversion of porphobilinogen PBG (**1**) into uro'gens support formation of the pyrrolenine (**4**) which, at least for production of uro'gen-I (**9**), is converted next into the hydroxymethylbilane (**6**); the status of the latter for uro'gen-III (**10**) formation and the nature of the interaction between deaminase and cosynthetase are also studied.

IT is firmly established[1,2] that biosynthesis of uro'gen-III (**10**) involves head-to-tail assembly, by the enzyme system deaminase–cosynthetase, of 4 porphobilinogen units (**1**), PBG, *followed by* intramolecular rearrangement of the regular bilane (**3**) to reverse ring-D. We now outline experiments on the stages beyond (**3**) using enzymes isolated from *Euglena gracilis*.



SCHEME. X = H_3N^+ or Enz–Nu, A = CH_2CO_2H, P = $CH_2CH_2CO_2H$.

When the bilane (**3**) was treated with deaminase alone, the formation of uro'gen-I (**9**) was enzymically accelerated[1b] but there was a clear lag in formation of (**9**). This lag was even more evident when consumption of PBG (**1**) by deaminase and production of uro'gen-I (**9**) were determined

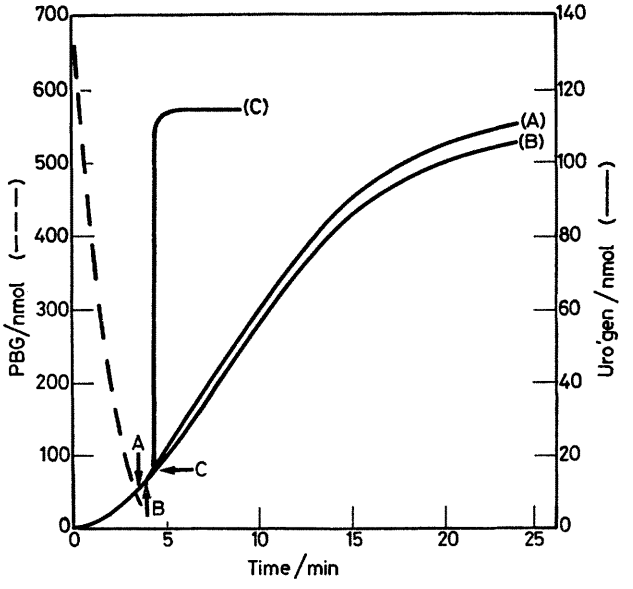


FIGURE 1. Enzymic formation of the hydroxymethylbilane (**6**) followed by ring-closure: (A) without added enzyme; (B) in the presence of additional deaminase; (C) with added deaminase–cosynthetase.

(see Figure 1); there was no lag when deaminase–cosynthetase was used. The lag in the former case indicates release into the medium of an intermediate substance.

The structure of the substance being produced by deaminase was established by: (a) showing that it ring-closed chemically to >98% uro'gen-I† (**9**); and (b) generation of the substance from [11-^{13}C] PBG[1] [as (**1**)] and stopping the enzymic reaction after 7 min by adjustment to pH >12 (>82% of original ^{13}C-PBG consumed) followed by ^{13}C n.m.r. spectroscopy with ^{1}H-noise decoupling to give Figure 2; with off-resonance decoupling, all four signals appeared as triplets (so all are from $^{13}CH_2<$).‡ Exactly the same signals appeared in a spectrum of lower quality run quickly without pH adjustment. The integral for Figure 2 was 1:3 for the $HOCH_2$.pyrrole signal: $CH_2<$(pyrrole)$_2$ bilane signal; the marked assignments are unambiguously based on ^{13}C n.m.r. analysis of synthetic bilanes[1] and hydroxymethylpyrroles (below). It follows that the product released into the medium during the lag is the unrearranged hydroxymethylbilane (**6**).

One further portion of the hydroxymethylbilane (**6**) generated as above was allowed, from point A, Figure 1 to ring-close chemically and a second equivalent portion was treated at point B, Figure 1 with additional deaminase; the two rates of uro'gen formation were essentially the same, Figure 1. However, addition of deaminase–cosynthetase to a third portion at point C, Figure 1 caused very rapid ring-closure to uro'gen-III (**10**), Figure 1. The latter statement is based on analysis, following iodine aromatisation of the uro'gens, before and after addition of deaminase–cosynthetase. This showed that 80 ± 2% of the final uro'gens had

† All isomer analyses were by h.p.l.c. as earlier.[1]

‡ Shortly before our proof of structure of the species accounting for the above lag, Professor A. I. Scott *et al.* had studied by ^{13}C n.m.r. spectroscopy the action of deaminase on [2,11-^{13}C] PBG but the observed signals were interpreted in a different way from ours. We warmly thank Professor Scott for sending us preprints covering his important work.

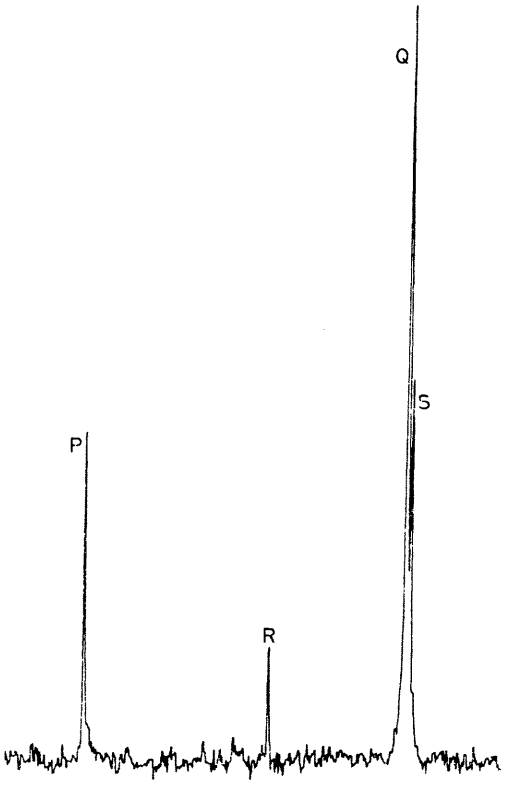


FIGURE 2. ^{1}H-Noise decoupled ^{13}C n.m.r. spectrum of product from action of deaminase on [11-^{13}C] PBG. Signal P, $HOCH_2$.-pyrrole, δ 57·21; Q, bilane $CH_2<(pyrrole)_2$, δ 24·46; R, H_2NCH_2 of residual PBG, δ 38·35; and S, uro'gen $CH_2<(pyrrole)_2$, δ 23·97 p.p.m. All δ in this paper referred to $Me_3SiCD_2CD_2CO_2Na$.

been formed by enzymic ring-closure of the hydroxymethylbilane (**6**) and that the product so formed was uro'gen-III (**10**).

The biosynthetic status of the hydroxymethylbilane (**6**) was studied by enzymic and control experiments with added nucleophiles. Radmer and Bogorad[3] and Davies and Neuberger[4] had found that when NH_4^+, $HONH_2$, or $MeONH_2$ were added to deaminase as it acts on PBG (**1**), the products were aminomethylbilane (**3**), or the HONH-, or MeONH-analogues, (**7**) and (**8**) respectively. These important observations were extended here by synthesis first of [*amino*-^{15}N] PBG [as (**1**)] *via* the corresponding ^{15}N-oxime to show by natural abundance ^{13}C n.m.r. spectroscopy that J for ^{13}C–^{15}N of (**1**) at pH $>$ 12 was 3·64 Hz.

Incubation of [11-^{13}C] PBG[1] [as (**1**)] with deaminase and $^{15}NH_4^+$ (conditions as ref. 3) followed by ^{13}C n.m.r. spectroscopy of the resultant bilane [as (**3**)] at pH $>$ 12, showed essentially complete formation of $^{15}NH_2{}^{13}CH_2$-bilane (δ 38·31 p.p.m., J 3·68 Hz). Thus at some stage in the deaminase experiment with $^{15}NH_4^+$, the amino group of PBG is replaced by $^{15}NH_3^+$, exactly as it is for $HONH_2$ and $MeONH_2$ outlined above.

To determine whether chemical displacement could occur at any stage, the following studies were made. Treatment of [11-^{13}C] PBG [as (**1**)] and separately, [*aminomethyl*-^{13}C] bilane[1] [as (**3**)] with 0·5 M $^{15}NH_4^+$ or 0·33 M $HO^{15}NH_2$ at pH 8·2—8·3 in the n.m.r. tube for 4 h at 37 °C showed, in the spectra accumulated over that period, that no splitting of the $^+NH_3CH_2$ pyrrole signal occurred in either case for the $^{15}NH_4^+$ runs and no significant (<1%) formation of HO-^{15}NH-pyrrole in the hydroxylamine runs. Exactly comparable experiments with the [2,11-$^{13}C_2$] hydroxy analogue [as (**11**)] of PBG showed no appreciable (<3%) chemical conversion of the hydroxymethyl group into aminomethyl or hydroxyaminomethyl residues (though considerable chemical formation of uro'gens had occurred). Finally, the hydroxymethylbilane (**6**) was generated enzymically as above, the protein was removed by ultrafiltration at pH 11, and the recovered bilane (**6**) was treated with 0·2 M $HO^{15}NH_2$ at pH 8·3 during accumulation of the ^{13}C n.m.r. spectrum over 16 h. Again, there was no production (<5%) of $HO^{15}NH$-CH_2.pyrrole.

It follows that in the above *enzymic* experiments involving nucleophiles and in the earlier ones[3,4] the isolated aminomethylbilane (**3**), and its hydroxyamino and methoxyamino analogues, are not formed by chemical displacement on the aminomethylbilane (**3**) or the hydroxymethylbilane (**6**) but are formed by trapping a more reactive intermediate, shown in the Scheme as the methylenepyrrolenine (**4**).

This Scheme rationalises all the foregoing and earlier findings. It involves enzymic conversion of PBG (**1**) into the methylenepyrrolenine§ (**2**) ready for bonding to a nucleophile X on the enzyme. A second, third, and fourth unit of (**2**), generated as before from PBG can then be added sequentially to give the extensively studied bilane[1,2] (**3**)¶ which by a fourth repetition of the same elimination could form the pyrrolenine (**4**). In the absence of cosynthetase, (**4**) is converted into the hydroxymethylbilane (**6**) which cyclises chemically to uro'gen-I (**9**). Deaminase alone is not an enzyme for ring-closure.

With deaminase–cosynthetase, either the pyrrolenine (**4**) is ring-closed directly or the hydroxymethylbilane (**6**) is a subsequent intermediate, the cyclisation being directed to C-16 to form the natural uro'gen-III (**10**) *via* the spiro intermediate (**5**). If the pyrrolenine (**4**) is ring-closed directly by deaminase–cosynthetase, then this enzyme system can convert (**6**) back into the pyrrolenine (**4**) very efficiently.

Experiments related to the last point involved synthesis of the [2,11-^{13}C] hydroxy analogue (**11**) of PBG from the corresponding aldehyde ester[1] to show that the ^{13}C-signal for $HOCH_2$.pyrrole appears (pH $>$ 12) at δ 57.27 p.p.m. This analogue (**11**) acts as a good substrate for deaminase–cosynthetase, the rate being *ca.* one third that of PBG itself (**1**); (**11**) is also converted into uro'gens by deaminase (again with a clear lag). The proportions of the four isomeric uro'gens produced at pH 7·5 from the hydroxy analogue (**11**) were: chemically (at *ca.* 4% of rate with deaminase–cosynthetase) type-I (65), II (0), III (24), and IV (11%); with deaminase–cosynthetase, type-I (1·5),

§ The isolation[4] of the PBG analogue (**12**) from the action of deaminase on PBG (**1**) in the presence of $HONH_2$ (but not when enzyme is absent) supports the formation of the pyrrolenine (**2**).

¶ The direction of building could be ring-D → ring-A rather than the illustrated A→D sequence, without affecting the main features of the chemistry. Also an enzymic X group is not obligatory for building A → D and this is indicated in the Scheme.

II (0), III (98·5), and IV (0%). Presumably the hydroxymethylbilane (**6**) is again assembled from (**11**) for rapid enzymic ring-closure as above.

Finally, the following facts cast some light on whether or not, in the normal joint operation of deaminase–cosynthetase, deaminase produces and releases into the medium the substrate for cosynthetase. (a) Earlier physical experiments[5,6] support association of deaminase with cosynthetase. (b) We find K_M for deaminase with PBG is 195 $\pm$ 10 μM whereas for deaminase–cosynthetase[1b] K_M is 104 $\pm$ 7 μM. Further, K_M for the regular aminomethylbilane (**3**, X=H_3N^+) with deaminase is also *ca.* twice that for deaminase–cosynthetase.[1b] (c) The rate of ring-closure of the ring-D reversed bilane[1b] [as (**3**) with A and P interchanged on ring-D] is not increased by deaminase alone but is significantly accelerated with deaminase–cosynthetase. All these data indicate that deaminase and cosynthetase normally work in close association rather than as independent enzymes.

Our conclusion is that the sequence (**1**) → (**2**) → stepwise → (**3**) → (**4**) → (**5**) → uro'gen-III (**10**) [with the hydroxymethylbilane (**6**) either lying between (**4**) and (**5**) or being a trapped form of (**4**)] is in accordance with the results outlined here and with all that is known[1–4] about the ring-closure process which produces the natural porphyrins.

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