Proof by Synthesis that Unrearranged Hydroxymethylbilane is the Product from Deaminase and the Substrate for Cosynthetase in the Biosynthesis of Uro'gen-III

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Summary The labile unrearranged hydroxymethylbilane (**5**) is synthesised unambiguously, is proved to be identical with the product from deaminase acting on porphobilinogen, and is shown to be the substrate for deaminase-free cosynthetase which quantitatively ring-closes (**5**) with rearrangement to uro'gen-III (**7**).

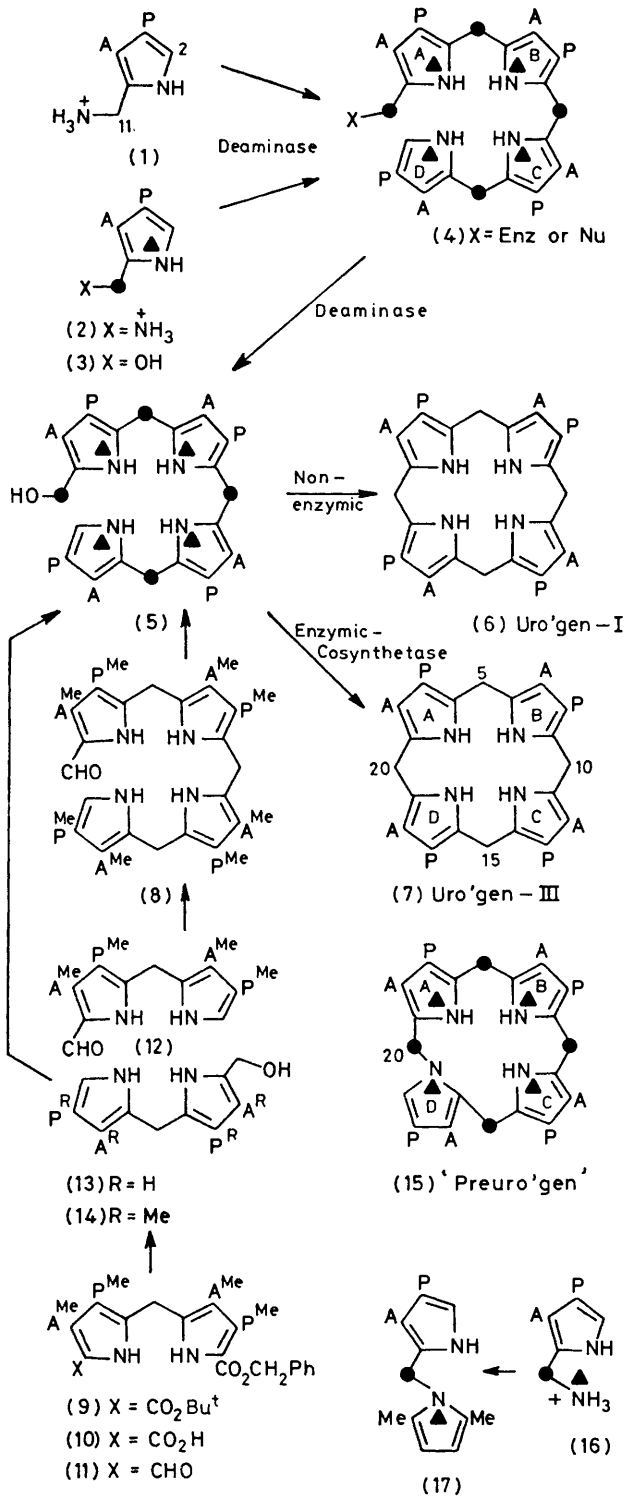
EARLIER work showed that uro'gen-III (**7**), the precursor of the natural porphyrins, chlorins, and vitamin B_{12},[1] is biosynthesised by head-to-tail assembly of 4 porphobilinogen units, PBG (**1**), starting at ring-A and building round to ring-D[2] to produce a bilane (**4**) followed by intramolecular rearrangement to reverse ring-D.[3] Two enzymes, deaminase and cosynthetase, work together to produce this result.[4] Recently it was found[5] that deaminase *is not* an enzyme for ring-closure and its function is to assemble a linear bilane (**4**) which in the absence of cosynthetase is released into the medium as hydroxymethylbilane (**5**). This cyclises *chemically* to uro'gen-I (**6**) (no rearrangement) but on addition of deaminase–cosynthetase the $HOCH_2$-bilane (**5**) is converted rapidly with rearrangement into uro'gen-III[5] (**7**).

Though knowledge of the biosynthesis of uro'gen-III (**7**) is now extensive,[6] the following key studies were still needed: (a) synthesis of the $HOCH_2$-bilane (**5**); and (b) study of $HOCH_2$-bilane (**5**) as substrate for cosynthetase alone. The intrinsic importance of (a) and (b) is obvious but was emphasised by publication[7] of a quite different view from that summarised above of the steps from the established[3] intermediate (**4**) to uro'gen-III (**7**) (see later).

TABLE 1. Uro'gen isomers formed from pyrromethane (**13**) or synthetic bilane (**5**)

Expt. no.	Substrate	Enzyme added	Uro'gens formed (% of total)			
			Type-I	Type-II	Type-III	Type-IV
1	(**13**)	None	83·5	0	5	11·5
2	(**13**)	Deaminase	82·5	0	4	13·5
3	(**13**)	Cosynthetase[a]	5	0	89·5	5·5
4	(**5**)	None[b]	100	0	0	0
5	(**5**)	Deaminase[b]	100	0	0	0
6	(**5**)	Cosynthetase[b]	7·5	0	92·5[c]	0

[a] At pH 8·25, 37 °C. [b] At pH 8·25, 30 °C. [c] If 6% uro'gen-I (**6**), shown to have been formed chemically before the addition of cosynthetase, is allowed for, this corresponds to >98% conversion of $HOCH_2$-bilane (**5**) into uro'gen-III (**7**).



SCHEME. A = CH_2CO_2H, P = $CH_2CH_2CO_2H$, A^{Me} = CH_2CO_2Me, P^{Me} = $CH_2CH_2CO_2Me$, A^R = CH_2CO_2R, P^R = $CH_2CH_2CO_2R$, ● = ^{13}C, ▲ = ^{15}N.

One pointer that $HOCH_2$-bilane (**5**) is a substrate for cosynthetase came from the synthesis of $HOCH_2$-pyrromethane (**13**) as follows. Treatment of pyrromethane (**9**) with $BF_3.Et_2O$–nitromethane following the method of R. J. Snow (Cambridge) gave the acid (**10**) which by Vilsmeier formylation yielded the aldehyde† (**11**). Standard hydrogenation, iodination, and hydrogenation gave the aldehyde† (**12**) which was hydrolysed and reduced with BH_4^- to the $HOCH_2$-pyrromethane (**13**). This self-condensed chemically at pH 9, 37 °C to form mainly uro'gen-I‡ (**6**) and some uro'gen-IV [(as **7**), reverse A,P on ring A] (Scheme) as expected[8] (expt. 1, Table 1). The former must arise *via* the unrearranged $HOCH_2$-bilane (**5**) and when the run was repeated in the presence of deaminase the isomeric composition of the product was unaffected (expt. 2, Table 1). In contrast, cosynthetase (no deaminase, see later) catalysed the formation of a large amount of uro'gen-III (**7**) (expt. 3, Table 1) presumably by intercepting the intermediate $HOCH_2$-bilane (**5**).

Synthesis of the $HOCH_2$-bilane (**5**) confirmed this interpretation and led to a full understanding. The half-life of natural $HOCH_2$-bilane (**5**) is 4 min at pH 8·25, 37 °C so the following mild approach was necessary. Condensation of the $HOCH_2$-pyrromethane (**14**), available from (**12**), with a 3 molar excess of the aldehyde (**12**) afforded the bilane† (**8**) (M^+ 964·3975, $C_{48}H_{60}N_4O_{17}$ M^+ 964·3953) which was readily separable from the excess of (**12**) and uro'gen esters formed as above from $HOCH_2$-pyrromethane (**14**). Borohydride reduction (buffered) of the aldehyde (**8**) gave the corresponding hydroxymethylbilane [octamethyl ester of (**5**)], from which (**5**) was obtained by alkaline hydrolysis (Scheme). The chemical, spectroscopic, and *quantitative* enzymic properties of this product were identical to those of the natural $HOCH_2$-bilane (**5**) produced by deaminase from PBG[5] (**1**), (see Tables 1 and 2).

The key experiment (no. 6) involved chromatographic isolation of cosynthetase, *free from deaminase*, from *Euglena gracilis*.[9] This cosynthetase catalysed rapid ring-closure of the natural[5] and the synthetic $HOCH_2$-bilane (**5**) to uro'gen-III (**7**). It is thus confirmed that deaminase is not a ring-closing enzyme; it produces the $HOCH_2$-bilane (**5**) from PBG (**1**). Further, it is established that cosynthetase carries out ring-closure of (**5**) with rearrangement to form uro'gen-III (**7**).

Valuable enzymic work in this area has been reported independently by Scott's group[7] but the chemistry and conclusions are completely different from ours. Their claims are: (i) deaminase *is* a cyclising enzyme which produces the *N*-alkyl macrocycle (**15**), named preuro'gen; (ii) this rearranges *chemically* to uro'gen-I (**6**); whereas (iii) it is rearranged to uro'gen-III (**7**) by cosynthetase; (iv) that our $HOCH_2$-bilane (**5**) is an artefact formed by displacement of the pyrrole residue from C-20 of (**15**) by hydroxide and hence it was predicted[7] that the $HOCH_2$-bilane (**5**) will not be a substrate for cosynthetase. If point (iv) is considered first, the latter part has been shown above not to be true. If the former part holds, the natural intermediate will be changed by hydroxide. This does not occur; the enzymic and spectroscopic properties

† Combustion analysis or accurate mass, with full spectroscopic data, is available for all new compounds.

‡ All isomer analyses were obtained by an improved h.p.l.c. method (*cf.* A. R. Battersby, D. G. Buckley, G. L. Hodgson, R. E. Markwell, and E. McDonald, in 'High Pressure Liquid Chromatography in Clinical Chemistry,' eds. P. F. Nixon, C. H. Gray, C. K. Lim, and M. S. Stoll, Academic Press, London, 1976, p. 63) which separates the esters of the four derived coproporphyrins in one run (A. R. Battersby, C. J. R. Fookes, and E. McDonald, in preparation).

of the intermediate as freshly generated by deaminase at pH 8·25 were unchanged by adjustment of the mixture first to pH >12 and back to pH 8·25 (Table 2). Evidence against points (i) → (iii) is outlined below.

bilane (**5**) apart from the reported 6 Hz 'doublet' mentioned above. It is highly probable that the Texas group is also handling the $HOCH_2$-bilane (**5**); all the data fit together if their 6 Hz 'doublet' is regarded as an artefact.

TABLE 2. Comparison of synthetic and natural $HOCH_2$-bilane (**5**).

	Main ^{13}C n.m.r. signals[a] /p.p.m.	$t_{\frac{1}{2}}$ (pH 8·25, 37 °C) /min	% Uro'gen-III formed by cosynthetase alone	V_{max} for cosynthetase[e]
Synthetic	δ 57·1[b,c] δ 24·4[d]	4·0	>98	151
Natural (kept at pH 8·25)	δ 57·0 δ 24·3	4·2	98	148
Natural (after pH >12, 37 °C, 20 min)	δ 57·1[b] δ 24·4	4·1	94	148

[a] All δ referred to internal $Me_3SiCD_2CD_2CO_2Na$. [b] Run at pH >12. [c] $HO^{13}CH_2$-Pyrrole. [d] Pyrrole-$^{13}CH_2$-pyrrole. [e] μmol uro'gen produced at pH 8·25, 25 °C per hour, per ml of cosynthetase preparation.

If preuro'gen (**15**) is the correct structure for the intermediate, it follows that: (a) N → C alkyl migration for (**15**) → (**6**) must occur *chemically* at high speed ($t_{\frac{1}{2}}$ 4 min at 37 °C); (b) chemical displacement of the ring-D pyrrole from C-20 of (**15**) by hydroxide under these mild conditions must be even faster; (c) δ 54·8[7]§ must be accepted as correct for C-20 in the unexceptional environment of (**15**) when analogy suggests it should be at least 12 p.p.m. to higher field; (d) the 6 Hz 'doublet' observed for C-20 of the intermediate[7] when [*pyrrole*-^{15}N, 11-^{13}C]PBG (**2**) is the substrate for deaminase must be accepted as being from *one bond* ^{15}N–^{13}C coupling rather than two bond ^{15}N–C–^{13}C coupling.

These requirements were studied as follows. [*Amino*-^{15}N,11-^{13}C]PBG[5] (**16**) with hexane-2,5-dione gave the labelled *N*-alkylpyrrole (**17**); this was stable and no pyrrole displacement occurred over the pH range 8—>12. Under our standard conditions, the signal from the enriched ^{13}C-site of (**17**) was at δ 42·0, *i.e.* as expected, to far higher field than reported[7] for preuro'gen (**15**); the one bond $^1J(^{13}C–^{15}N)$ was 10 Hz.

The complementary experiment was to synthesise [*pyrrole*-^{15}N, 11-^{13}C]hydroxy-PBG (**3**) (*cf.* ref. 5) which showed a *two bond* $^2J(^{13}C–^{15}N)$ of 2·3 ± 0·2 Hz. Finally, [*pyrrole*-^{15}N, 11-^{13}C]PBG (**2**) was synthesised and was converted by deaminase at pH 8·25 into $HOCH_2$-bilane (**5**) which gave the $HO^{13}CH_2$-pyrrole signal shown in the Figure. This corresponds to that reported earlier[5] save that *two-bond* coupling now produces a doublet, $^2J(^{13}C–^{15}N)$ of 2·4 ± 0·1 Hz. The same coupling was observed at both pH 8·25 and >12.

Thus, the chemical and spectroscopic properties of a synthetic *N*-substituted pyrrole differ considerably from those described by the Texas group[7] for the intermediate produced by deaminase (from *R. spheroides*). Further, the chemical, spectroscopic, and enzymic properties of the *R. spheroides* intermediate are identical to those of the $HOCH_2$-

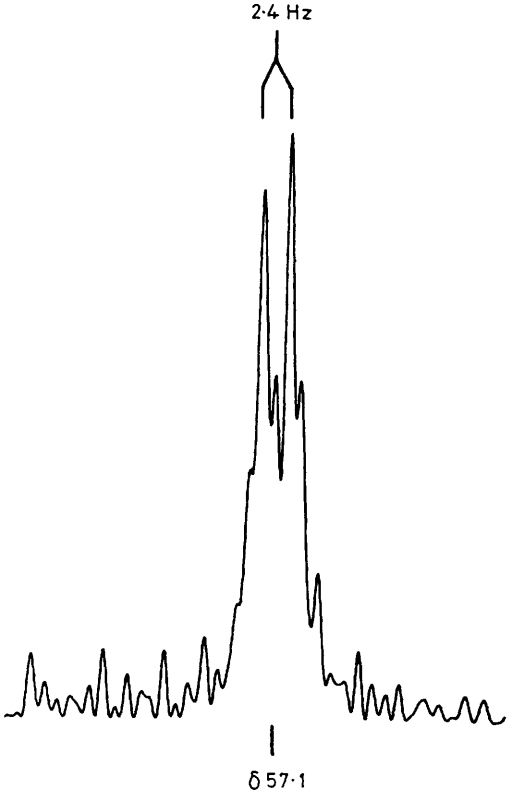


FIGURE. $HO^{13}CH_2$-Bilane signal in ^1H-noise decoupled ^{13}C n.m.r. spectrum of product from deaminase acting on [*pyrrole*-^{15}N,11-^{13}C]PBG. Computer aided techniques were used to enhance resolution (R. G. Brereton and J. K. M. Sanders, unpublished work).

§ This value needs adjustment to match our scale (using the CH_2 signal of uro'gen as reference) to allow for different shift standards; it then becomes δ 57·1; *cf.* Table 2 for $HOCH_2$-bilane (**5**).

In summary, the experiments reported here and earlier[5] prove that deaminase from *E. gracilis* converts PBG (**1**) not into preuro'gen (**15**) but into the previously identified[5] unrearranged hydroxymethylbilane (**5**), shown to be an excellent substrate for deaminase-free cosynthetase which quantitatively converts it into uro'gen-III (**7**).

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