Biosynthesis of Natural Porphyrins: Further Experiments with Hydroxymethylbilane

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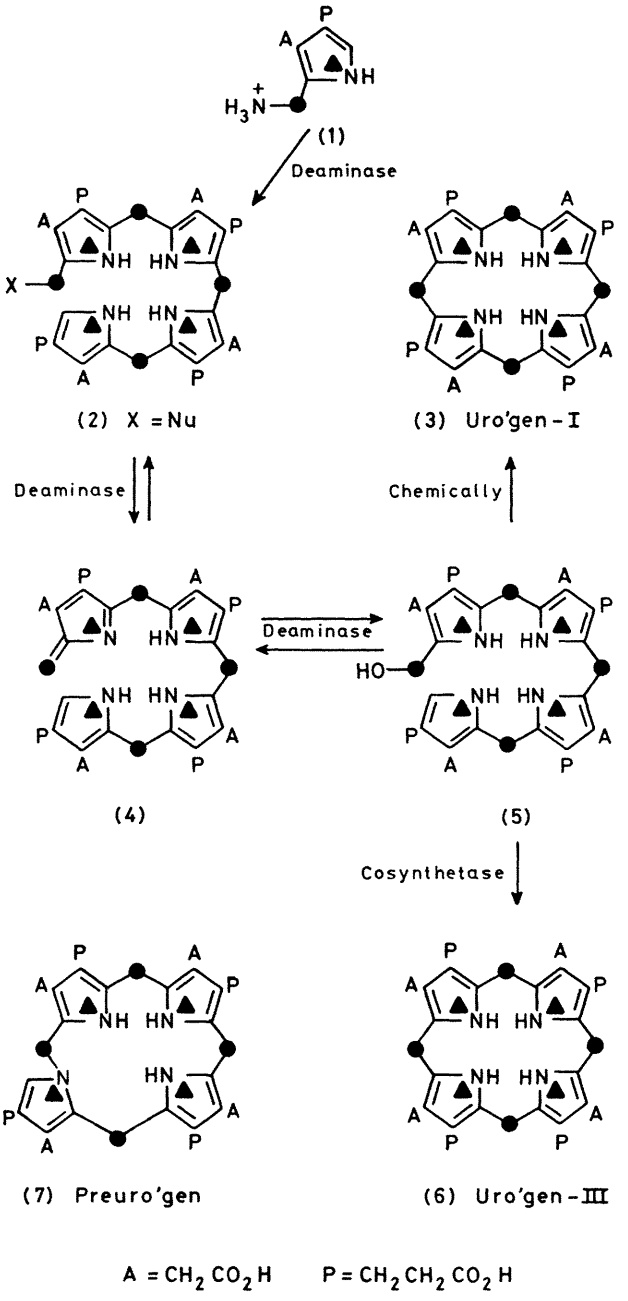
Summary It is shown that at low temperatures (as at higher ones) the enzyme deaminase converts porphobilinogen into the unrearranged hydroxymethylbilane (**5**), there is no evidence for formation of preuro'gen (**7**) nor for its involvement in porphyrin biosynthesis

IT had been firmly established[1] that uro'gen-III (**6**) is biosynthesised from porphobilinogen, PBG, (**1**) *via* processes mediated by the two enzymes deaminase and cosynthetase and, by 1977, work in this laboratory had proved[2,3a] that a single intramolecular rearrangement is involved at the unrearranged tetrapyrrole level, *i e* on bilane (**2**) These conclusions were drawn from rigorous $^{13}C_2$-labelling experiments on the one hand with PBG[2] (**1**) and on the other with the aminomethylbilane[3] (**2**, X = $^+NH_3$), both studies using deaminase with cosynthetase It was recognised[3b,4] that replacement of the amino function of (**2**, X = $^+NH_3$) and (**1**) by another nucleophile [shown as X in (**2**)] could occur enzymically before final ring-closure with rearrangement The nature of the actual replacing group X was found as a result of observing[5] that treatment of the aminomethylbilane (**2**, X = $^+NH_3$), or more strikingly, PBG (**1**), with deaminase alone (no cosynthetase) produces uro'gen-I (**3**) *only after a lag*, the structure of the intermediate accumulated during the lag [and which ring-closes chemically to uro'gen-I (**3**)] was proved by spectroscopic and synthetic studies[5,6] to be the unrearranged hydroxymethylbilane (**5**), thus the replacing nucleophile is simply hydroxide Both natural and synthetic samples of the hydroxymethylbilane (**5**) were shown[6] to be excellent and identical substrates for cosynthetase alone with production of uro'gen-III (**6**)

The intermediate was observed independently by Scott's group[7] but it was concluded[8] that its structure is the macrocycle preuro'gen (**7**) and further[8] that the foregoing hydroxymethylbilane (**5**) (i) was an artefact produced by ring-opening of preuro'gen (**7**) and (ii) was not a substrate for cosynthetase These three conclusions were shown to be incorrect[6] and it was subsequently accepted[9] that at 37 °C, the product from deaminase is indeed the hydroxymethylbilane (**5**) However, formation of preuro'gen (**7**) from PBG (**1**) by deaminase was again said to occur[9] and be observable if, *after generating the intermediate at* 37 °C, the n m r spectra were run at 0 °C The following experiments and arguments show that preuro'gen (**7**) is not formed in any significant quantity and that under low temperature conditions the product is hydroxymethylbilane (**5**) exactly as it is at 37 °C

[*pyrrole*-^{15}N, 11-^{13}C]PBG (**1**) was converted as earlier[5] during 7 min at 37 °C into the intermediate, the solution at pH 8 25 was immediately cooled to 0 °C and the proton-decoupled ^{13}C-n m r spectrum was run at 4 °C (measured in the tube with the decoupler on) It was known[6] that



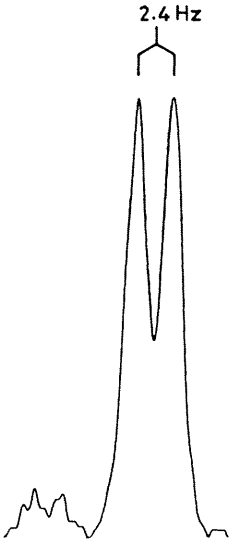


FIGURE. $HO^{13}CH_2$-Bilane signal (δ 57·1) in ^{1}H-noise decoupled ^{13}C-n.m.r. spectrum, run at 4 °C, of product from deaminase acting on [*pyrrole*-^{15}N,11-^{13}C]PBG (**1**). Computer aided methods were used to enhance signal/noise and resolution.

the $HO^{13}CH_2$-signal, δ 57·1, from the labelled bilane (**5**) appears as a 2·4 Hz doublet from two-bond ^{13}C–^{15}N coupling; the low temperature run gave a slightly broadened signal, δ 57·1, but again as a 2·4 Hz doublet (Figure). No signal having a larger J from one-bond ^{13}C–^{15}N coupling [as required by preuro'gen (**7**)] was present. This experiment (A) eliminates the possibility that preuro'gen (**7**) is formed at 37 °C but is only preserved by cooling to low temperature.

The ^{1}H-decoupled natural abundance ^{13}C-spectrum of synthetic hydroxymethylbilane (**5**) was then determined. It showed 26 separate signals of which 17 were distinguishable by chemical shift from the simpler natural abundance ^{13}C-spectrum of centrosymmetric uro'gen-I(**3**). A second sample of bilane (**5**) was mixed at 0 °C and pH 8·5 with deaminase (1·25 × 10^3 units per mg of bilane) and the natural abundance ^{13}C-spectrum of the solution was run at 4 °C. The resultant spectrum was simply the sum of the natural abundance spectra of uro'gen-I (**3**), formed chemically from the bilane (**5**) during the run, and that of hydroxymethylbilane (**5**) itself. This experiment (B) eliminates the claimed[9] but unlikely formation of preuro'gen (**7**) from hydroxymethylbilane (**5**) by cooling in the presence of deaminase.

Synthetic hydroxymethylbilane (**5**) was allowed to ring-close chemically in solution at pH 8·5 and 2 °C [the conditions used for experiment (B)]. A second equivalent solution was mixed at 2 °C with deaminase (1·25 × 10^3 units per mg of bilane). Uro'gen-I (**3**) was formed at the same rate in both solutions, as it had been earlier[5] at 37 °C. A third equivalent solution of bilane (**5**) at 2 °C was made 0·2M with respect to NH_2OH and then deaminase (quantity as above) was added. Formation of uro'gen-I (**3**) was inhibited (>90%) owing to trapping[6,10] of the hydroxymethylbilane (**5**) as (**2**; X = HONH), *via* enzymic formation of the putative methylenepyrrolenine[6] (**4**).* This experiment (C) demonstrates that sufficient deaminase was present in experiment (B) to activate the bilane (**5**) and also that the properties of (**5**) are unchanged by cooling to 2 °C apart from the expected rate differences.

The foregoing ^{13}C-spectra differ from those of the Texas group only by those above having greater signal-to-noise ratio and resolution; the chemical shifts are identical. It is now certain that both groups are handling the same intermediate proved in Cambridge[5,6] by spectroscopy and by its rational synthesis to be hydroxymethylbilane (**5**) *not* preuro'gen[8] (**7**). The latter structure was founded solely on one ambiguous spectroscopic observation.[8]

Our conclusions are therefore (i) deaminase acting alone assembles 4 PBG units head-to-tail and releases hydroxy-

methylbilane (**5**), over the temperature range studied, cooling does not affect the structure of this product in the presence or absence of deaminase (ii) There is no evidence at low or higher temperatures for the formation of preuro'gen (**7**) nor for its involvement in the biosynthesis of porphyrins The hydroxymethylbilane structure (**5**) is important because it allows further conclusions to be drawn concerning the *function* of the two enzymes These conclusions are (iii) since deaminase alone produces the hydroxymethylbilane (**5**) *it is not a ring-closing enzyme* and (iv) cosynthetase converts the open-chain hydroxymethylbilane (**5**) by a single intramolecular rearrangement into uro'gen-III (**6**), *cosynthetase is the ring-closing and rearranging enzyme* The preuro'gen structure (**7**) would wrongly lead to the opposite conclusions

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