^{13}C N.M.R. Evidence for a New Intermediate, Pre-uroporphyrinogen, in the Enzymic Transformation of Porphobilinogen into Uroporphyrinogens I and III

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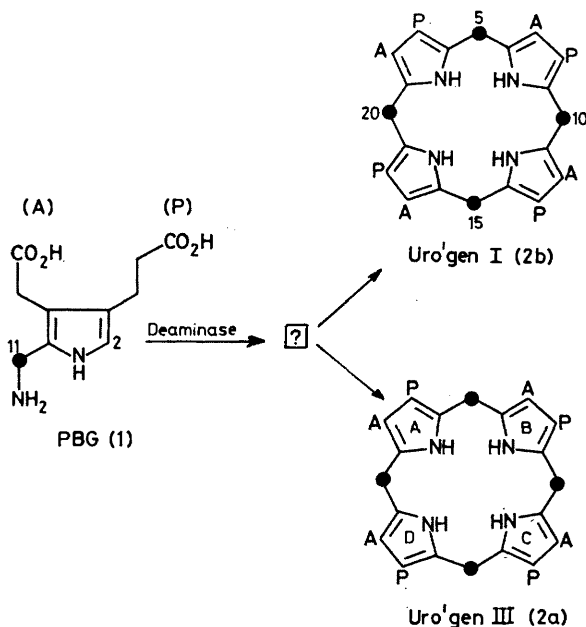
Summary Evidence is presented from ^{13}C n.m.r. spectroscopic studies which indicates that the enzymic transformation of porphobilinogen into uroporphyrinogens I and III occurs through a transient free intermediate, pre-uroporphyrinogen, produced by porphobilinogen deaminase (uroporphyrinogen I synthetase).

The enzyme-catalysed process by which porphobilinogen (PBG: **1**) is transformed into uroporphyrinogen (uro'gen) III (**2a**) has been extensively studied (for a review see ref. 1). The formation of uro'gen III, the biosynthetic precursor of haem, chlorophyll, and vitamin B_{12}, requires the participation of two enzymes, PBG deaminase (uro'gen I synthetase) and uro'gen III cosynthetase.[2] In the presence of deaminase alone, PBG is converted into uro'gen I (**2b**) which is not further involved in the pathway of tetrapyrrole biosynthesis.



Previous studies on the enzymic formation of uro'gens have revealed little evidence for the existence of free intermediates, although under conditions where the enzyme system has been inhibited by ammonia or hydroxylamine,[3] bilanes [as (**3a**)] are accumulated. The latter studies and incorporations with pyrromethanes and bilanes [as (**3a**)] suggest that any free intermediates between porphobilinogen and uroporphyrinogen would be short-lived and highly sensitive to conventional isolation procedures. We now describe the application of ^{13}C n.m.r. Fourier transform spectroscopy designed to trace the enzymic conversion of PBG (**1**), enriched with ^{13}C, into uro'gens in the n.m.r. tube, thus avoiding the isolation of potentially labile compounds.

Initial experiments focussed on the conversion of PBG into uro'gen I by highly purified PBG deaminase from *Rhodopseudomonas spheroides*.[4] In a typical experiment, the deaminase (80 units per mg) was incubated with 300 μg of 11-[^{13}C]-PBG[5] in tris buffer at 37 °C under anaerobic conditions. N.m.r. spectra were then recorded on a Varian SC-300 n.m.r. spectrometer using single frequency off resonance proton decoupling.

The spectrum of uro'gen I, obtained after the enzyme reaction had been allowed to proceed to completion, is shown in the Figure (a). The triplet centred at 21·63 p.p.m. [reduced $^1J(^{13}C-H)$ 110 Hz] results from the four equivalent *meso* carbon atoms [5, 10, 15, and 20 in structure (**2b**)]. When the enzyme reaction is allowed to proceed only until 47% of the PBG has been consumed (11 min), in addition to the remaining PBG signal [triplet centred at 34·95 p.p.m.; reduced $^1J(^{13}C-H)$ 125 Hz] and the uro'gen signal (21·63 p.p.m.), a complex signal is also present at 21·85—22·15 p.p.m. which integrated as 17% of the ^{13}C (Figure, b). In addition, a further signal (integral: 5% of ^{13}C) appears as a triplet at 54·78 p.p.m. [reduced $^1J(^{13}C-H)$ 130 Hz]. These latter signals disappear as the enzymic conversion proceeds to completion.† Treatment of the

† A detailed timecourse study substantiated these findings and revealed that the uroporphyrinogen signal at 21·63 p.p.m. was formed at the expense of the complex signal at 21·85—22·15 and the small signal at 54·78 p.p.m.

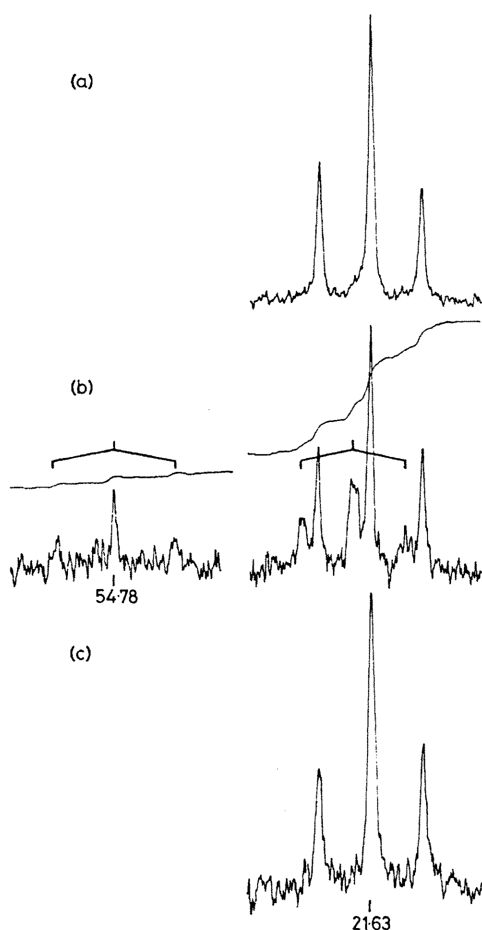
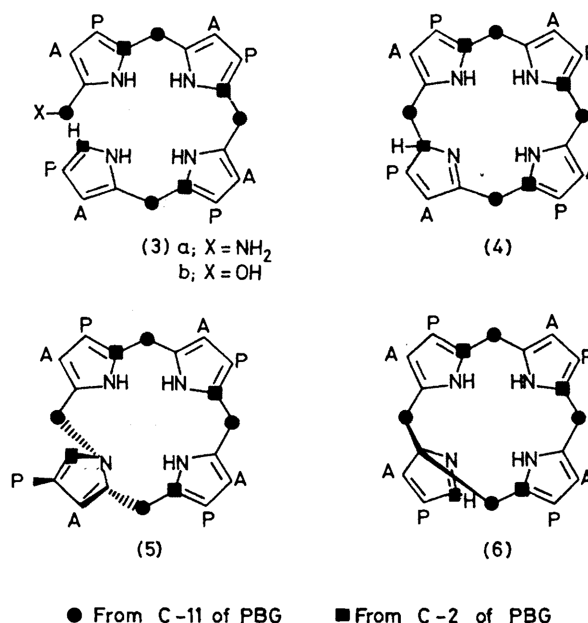


FIGURE. 75·5 MHz ^{13}C Spectra of enriched uro'gen I (a), uro'gen I, and pre-uro'gen (b), and uro'gen III (c) at 0 °C under single frequency off-resonance proton decoupling conditions. Typically, 35,000 90° pulses were accumulated over a spectral width of 9,000 Hz while locked to internal D_2O (10%) with a repetition rate of 0·6 s. The lines were broadened *ca.* 2 Hz by exponential multiplication of the F.I.D. (free induction decay).

incubation medium containing intermediate with either acid or base under conditions where the deaminase is inactive affords only uro'gen I. The final product of the reaction with deaminase in buffer is also exclusively uro'gen I. When the n.m.r. experiment is performed under identical conditions in the presence of sufficient cosynthetase[6] to produce 100% of uro'gen III,‡ after 47% of the PBG has been consumed (11 min), none of the complex signals at 21·85—22·15 p.p.m. or at 54·78 p.p.m. is observed, whilst the uro'gen III peak is proportionally increased§ (Figure, c). Under conditions where the concentration of the cosynthetase is reduced to produce decreasing ratios of uro'gen III/I, the signals at 21·85—22·15 and 54·78 p.p.m. increase to the same extent.

From these data we conclude that during the enzymic conversion of PBG into uro'gen a hitherto undetected intermediate is accumulated in solution which *ultimately* yields uro'gen I. The absence of ^{13}C signals at 21·85—22·15 and 54·78 p.p.m. under conditions where the enzymic system is forming only uro'gen III suggests that the intermediate, termed pre-uro'gen, responsible for these signals, may be involved in the normal pathway of uro'gen III biosynthesis, thus favouring the hypothesis of rearrangement at the tetrapyrrole level and the formation of pre-uro'gen from an enzyme-bound version of the bilane (**3a**).[7] Further biochemical data supporting this hypothesis are presented in the following communication.

The chemical shifts of pre-uro'gen are consistent with one of the intermediates on the energy surface represented by the uro'gen I tautomer (**4**), the *N*-alkylated macrocycle (**5**), and the spiro compound (**6**). The appearance of signals at 54·78 p.p.m. (integral 1Ċ) and at 21·85—22·15 (3Ċ) are also consistent with (**3b**) as the structure of pre-uro'gen, since acid or base treatment affords only uro'gen I, whereas the action of cosynthetase produces exclusively uro'gen III. The chemical shift data exclude the bilane (**3a**) (no $^{13}CH_2NH_2$ at 34·64 p.p.m.[8]) but still leave (**3b**), the spiro compound (**6**), and the uro'gen I tautomer (**4**) as formal possibilities. Structure (**3a**) is further excluded on the basis of the ratio of uro'gens reported for the non-enzymic cyclization of this species,[7] although type III uro'gen formation has been demonstrated during the combined action of deaminase and cosynthetase on (**3a**). These alternatives were further narrowed when 2,11-[$^{13}C_2$]-PBG served as substrate. In this experiment the resultant signal at 54·78 p.p.m. appeared as a broad triplet [reduced $^1J(^{13}C\text{–}H)$ 130 Hz], thus excluding the species (**4**) which would exhibit $^{13}\dot{C}\text{–}^{13}\dot{C}$ coupling for this carbon. The inherent instability of pre-uro'gen ($t_{1/2}$ 4 min at 37 °C) so far does not allow a final decision between (**3b**), (**5**), and (**6**). The latter



‡ Determined by h.p.l.c. analysis, as the uroporphyrin methyl esters (see following communication).

§ The four *meso* carbons of uro'gen III appear as a single peak owing to the similarity of the substituents in the pyrrole rings.

two species are formally connected by allowed [1,5]-sigmatropic shifts with the type I and type III uro'gens [*e.g.*, (**5**) → (**6**) → (**2a**) and (**6**) → (**5**) → (**2b**)]. The n.m.r. experiment may simply have revealed the most stable of the several possible or *actual* intermediates. There is, however, no doubt that the function of synthetase is much more complex than had previously been thought (see following communication) since it is capable of fashioning a highly reactive intermediate [*e.g.*, (**3b**), (**5**), or (**6**)] which is then either diverted chemically to uro'gen I or, in the presence of cosynthetase, is rearranged to uro'gen III. It now appears that the exact nature of the pathway from PBG to uro'gen III can be determined by further application of this methodology which has the advantage of using only the natural substrate, PBG. In our view, the earlier and sometimes conflicting results with pyrromethanes obtained in this laboratory and elsewhere (ref. 1) can be ascribed in part to chemical interaction of these species with pre-uro'gen and subsequent formation of uro'gen III. Studies are now in progress to elucidate the kinetics and equilibrium behaviour of pre-uro'gen in normal conditions and in the presence of 'non-physiological substrates' of the deaminase (pyrromethanes, bilane, *etc.*) as well as its biosynthetic implication in the enzyme system. Some of these experiments are described in the following communication.

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