during his sabbatical leave at Cornell University where this manuscript was written.

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Structure of Preuroporphyrinogen. Exploration of an Enzyme Mechanism by ^{13}C and ^{15}N NMR Spectroscopy

Sir:

We recently described[1,2] the discovery of preuroporphyrinogen (preuro'gen), a labile ($t_{1/2}^{37°C}$ = 4 min), tetrapyrrolic intermediate in the conversion of porphobilinogen (PBG, **1**) to uroporphyrinogen (uro'gen) I (**2**) catalyzed by the enzyme PBG deaminase. The importance of this substance, released from the enzyme and observed as a ^{13}C-enriched species at pH 8.5, resides in its proven role[2] as the first recognized substrate for the second enzyme of tetrapyrrole biosynthesis, uro'gen III cosynthetase, which has hitherto been considered as a synergistic companion for deaminase, since both enzymes[3] are required to convert PBG to uro'gen III (**3**), the precursor of heme,[4] sirohydrochlorin,[5] chlorophylls,[4] and vitamin B_{12}.[4-6]

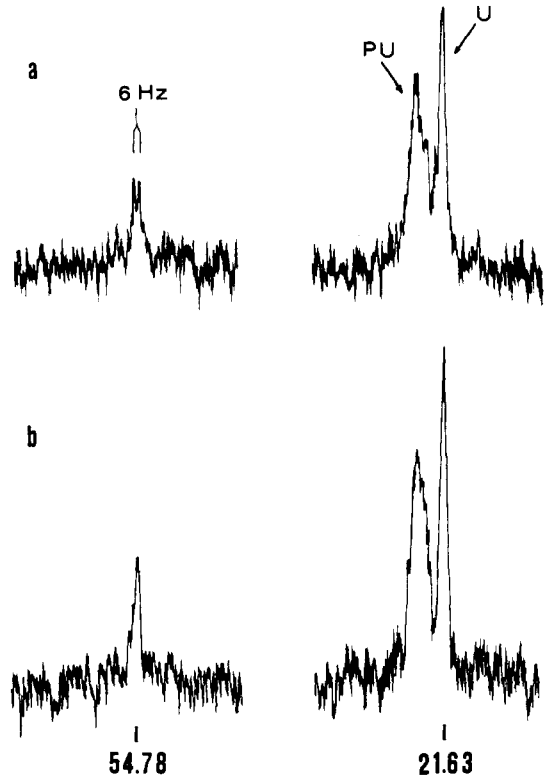

Figure 1. Proton decoupled 75.5-MHz ^{13}C spectra at 0 °C of 3-min incubations (37 °C, 85% conversion) of PBG deaminase with [11-^{13}C:1-^{15}N]-PBG (a) and [11-^{13}C]-PBG (b). Spectrum a is the result of 6000 and b of 2200 90° pulses accumulated over a spectral width of 7500 Hz while locked to internal D_2O (10%) with a repetition rate of 0.8 s. The lines were broadened 2 Hz by exponential multiplication of the FID.

By observing the ^{13}C NMR spectra of incubations of [11-^{13}C]- and of [2,11-$^{13}C_2$]-PBG with deaminase it was possible to detect, in addition to the signals assigned to uro'gen I (**2**), ^{13}C enrichments for four and eight carbons, respectively, of preuro'gen. Three structures, **4**, **5** (X = OH), and **6**, compatible with these chemical shifts and lack of ^{13}C–^{13}C coupling were proposed,[1] the N-alkylated macrocycle (**4**) being preferred for reasons stated elsewhere.[1,2] The structure **4** for preuro'gen has now been proved by using [11-^{13}C:1-^{15}N]-PBG as the substrate for deaminase and observing both ^{13}C and ^{15}N NMR spectra at the point of maximum preuro'gen formation.

The doubly enriched PBG was synthesized by modification of literature methods[7,8] and contained 90% ^{13}C at C-11 and 99% ^{15}N at N-1. Incubation[9] of 0.4 mg of this substrate with highly purified deaminase from *Rhodopseudomonas spheroides* (450 units/ml) for three minutes (37 °C; 85% conversion) gave the ^{13}C NMR spectrum shown in Figure 1a. In addition to the methylene signals (U) for uro'gen I at δ 21.63 ppm,[10] for C-11 of the remaining (15%) PBG at δ 34.95 ppm and for three carbons of preuro'gen (PU) at δ 22.00 ppm, the spectrum shows a doublet centered at δ 54.78 ppm (J = 6 Hz) which by comparison with the singlet observed for this resonance (see Figure 1b) in the non-^{15}N labeled experiment, must be ascribed to one bond ^{13}C–^{15}N coupling[11] with one of the enriched (^{15}N-1) pyrrolic nitrogens. Upon heating to 37 °C the latter signal disappears along with the three carbon methylene enrichment at δ 22.00 ppm, as preuro'gen is converted to uro'gen I. Confirmation of the above interpretation was secured by repeating the experiment whilst observing the ^{15}N NMR spectrum (at 8.1 MHz) which is shown in Figure 2. In addition to singlets for the four pyrrolic nitrogens of ur-

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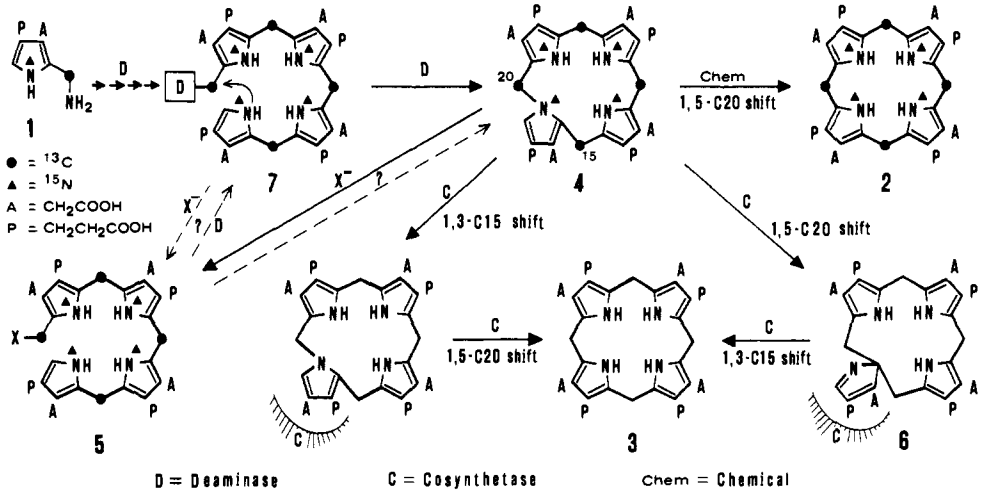
Scheme I



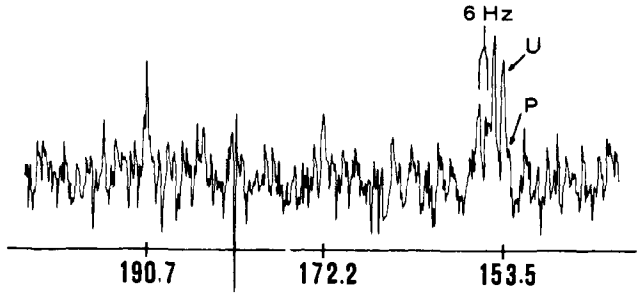


Figure 2. Proton decoupled 8.1-MHz ^{15}N spectrum at 0 °C of a 3-min incubation (37 °C, 85% conversion) of PBG deaminase with [11-^{13}C:1-^{15}N]-PBG; 21 200 90° pulses were accumulated over a spectral width of 4000 Hz while locked to internal D_2O (10%) with a repetition rate of 0.8 s. The lines were broadened 2 Hz by exponential multiplication of the FID and interpolated to 16K data points/kHz for better line-shape definition.[25]

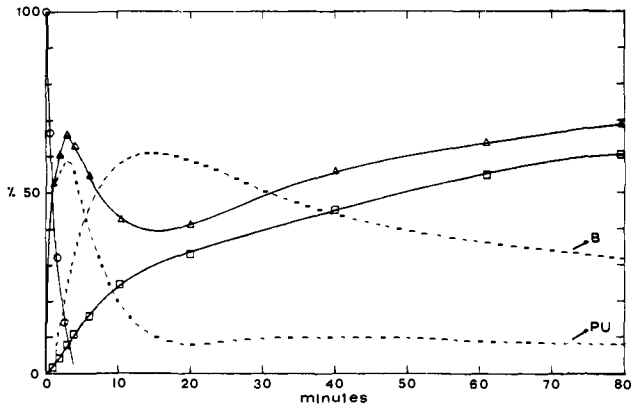


Figure 3. Kinetic profiles of uro'gen I formation from PBG by PBG deaminase. PBG (○) was determined by Ehrlich reaction, uro'gen I (□) spectroscopically by oxidation to uroporphyrin I with benzoquinone (λ_{max} 399 nm at pH 7.6), and uro'gen I plus preuro'gen (△) by conversion of preuro'gen to uro'gen III (20-s incubation with large excess of cosynthetase) and oxidation to uroporphyrins I plus III with benzoquinone. Broken lines show the calculated profiles for preuro'gen (PU) and bilane **5** (X = NH_2) plus its decomposition products (B). PU = (uro'gen I + preuro'gen) − uro'gen I; B = 100 − (PBG + uro'gen I + preuro'gen).

o'gen I (U) (153.5 ppm)[10] and for N-1 of PBG (P) (152.8 ppm), a series of distinct pyrrole-nitrogen resonances is evident as three singlets—one at δ 154.5 ppm close to the observed resonance of uro'gen, another at δ 172.2 ppm and the third at δ 190.7 ppm. The latter two resonances are indicative of NH groups hydrogen-bonded[12] to a carboxylic acid side chain. The fourth ^{15}N resonance appears as a doublet ($^1J_{^{15}N\text{-}^{13}C}$ = 6 Hz) at δ 155.7 ppm which is assigned to the ring D pyrrole nitrogen covalently linked to the C-20 enriched methylene carbon ($^1J_{^{13}C\text{-}^{15}N}$ = 6 Hz). When the solution is warmed from 0 to 37 °C, the ^{15}N signals described above disappear in proportion to the increase in the ^{15}N uro'gen I singlet at δ 153.5 ppm.

These observations provide strong evidence for the unusual structure (**4**) for preuro'gen, whose genesis from the enzyme-bound bilane (**7**) is suggested as shown in Scheme I, which also portrays the further, spontaneous rearrangement of **4** to uro-'gen I (**2**)[1,2] and, with cosynthetase, to uro'gen III (**3**), events which may be mediated by allowed 1,3 and/or 1,5-sigmatropic shifts.[13] Thus, preuro'gen is the sole, enzyme-free intermediate between PBG and uro'gens observable under conditions which do not perturb the normal function of deaminase [at 0 °C ($t_{1/2}$ = 2 hr) or at 37 °C ($t_{1/2}$ = 4 min)], and serves as the substrate for cosynthetase.[2] In contrast, addition of nucleophilic reagents (ammonium ion,[14,15] hydroxylamines,[16] hydroxide ion[17]) to the incubation results in the accumulation of *bilanes*, e.g., **5** (X = NH_2; NHOR; OH), which are sufficiently stable to be observed in the NMR experiment at ambient temperature. Unlike preuro'gen, the bilane **5** (X = NH_2) which is available by total synthesis[18-20] or by trapping with ammonium ion[14,15] was not a substrate when incubated with highly purified cosynthetase, although converted by deaminase-cosynthetase to uro'gen III,[19] chemical equilibration with **7** presumably providing an entreé to the biosynthetic machinery. By analogy, the hydroxy bilane **5** (X = OH, available by trapping preuro-'gen with hydroxide) should behave in a similar way.[17]

The full kinetic profile of uro'gen synthesis can also be followed by chemical and NMR analyses of the incubation mixture at different times. Figure 3 shows the transient accumulation of preuro'gen (**4**) from PBG (**1**) on the way to ur-o'gen I (**2**). Bilane **5** (X = NH_2)[21] is formed by reaction of preuro'gen with the ammonia released during the enzymic polymerization of PBG. A change in the rate of formation of uro'gen I can be observed after 10 min as at this point the rate-limiting step is the slow deaminase-catalyzed conversion of bilane **5** (X = NH_2) into preuro'gen (**4**). This interpretation has been confirmed by carrying out the incubations in the presence of an "ammonia-consuming" enzyme system (glutamate dehydrogenase, α-ketoglutarate, NADH) in which case no bilane was formed, and by incubating deaminase with an authentic sample of bilane **5** (X = NH_2), which was transformed into preuro'gen (**4**) and finally into uro'gen I (**2**), 35 times more slowly than PBG.

Without detracting from the importance of many experiments involving the use of dipyrromethanes[3,4,22,23] and bilanes[3,19] which have provided excellent probes for the overall process via ^{13}C-labeling, it is now clear that the deaminase/cosynthetase enzymes can deal with both "normal" and rearranged species of bilane[3,19,24] and pyrromethane[4,22,23] which bear sufficient chemical reactivity to insinuate themselves into the biochemical machinery. We submit that the NMR method using the known, physiological substrate, PBG, provides an unequivocal, non-invasive view of the true enzyme process at work.

Acknowledgments. We thank the National Institutes of Health (Grant AM 20528) for support of this work, the Consejo Nacional de Investigaciones Cientificas y Tecnicas de la Republica Argentina (G.B.) for a fellowship, and Professor B. Frydman for a reference sample of bilane (**5**, X = NH_2). The 75-MHz ^{13}C NMR spectra were obtained at the National Institutes of Health Division of Biotechnology Resources at the University of Utah, supported by Grant RR-00574-07.

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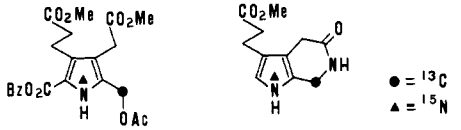

[^{13}C]-DMF, had the following: ^1H NMR δ 5.01 (dd, $-CH_2OAc$, $^1J_{H-^{13}C}$ = 149.2, $^2J_{H-^{15}N}$ = 2.8 Hz), 9.22 (d, NH, $^1J_{H-^{15}N}$ = 98.0 Hz); ^{13}C NMR δ 56.90 (d, $-^{13}CH_2OAc$, $^2J_{^{13}C-^{15}N}$ = 1.9 Hz). i was converted to PBG lactam methyl ester (ii), *m/e* 224 (97%, M^+), 223 (100%, $M^+ - 1$), to give a pure sample of PBG 99% in ^{15}N and 90% in ^{13}C.
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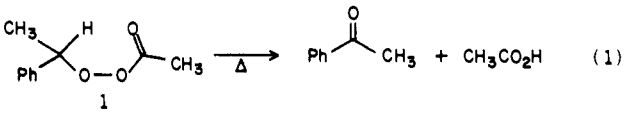
Investigation of the Mechanism of the Unimolecular and the Electron-Donor-Catalyzed Thermal Fragmentation of Secondary Peroxy Esters. Chemiluminescence of 1-Phenylethyl Peroxyacetate by the Chemically Initiated Electron-Exchange Luminescence Mechanism

Sir.

Our interest in highly exergonic thermal reactions of organic peroxides led us to the investigation of 1-phenylethyl peroxyacetate (**1**). Thermolysis of **1** in benzene solution gives a quantitative yield of acetic acid and acetophenone,[1] a small fraction of which is electronically excited. The reaction of **1** is catalyzed by a wide range of easily oxidized substances. In



(1)

this case, the electronically excited state of the catalyst (activator) is formed apparently by the recently described chemically initiated electron-exchange (CIEEL) mechanism.[2] We report herein our examination of the mechanism of both the unimolecular and catalyzed reaction of **1**.

Perester **1** was prepared by the acid-catalyzed reaction of ketene with 1-phenylethyl hydroperoxide in CH_2Cl_2 and purified by distillation.[3] The thermolysis of **1** in argon purged benzene can be followed conveniently by the indirect or activated[4] chemiluminescence that results upon addition of biacetyl or any one of several easily oxidized fluorophores (see below), respectively. The rate at which the perester reacted showed apparent first-order kinetic behavior. However, the observed rate constants and derived activation parameters for solutions 1×10^{-2} M and above are dependent upon the initial perester concentration, indicating the likely involvement of a radical induced homolysis path.[5] At low initial perester concentration (1×10^{-5} to 1×10^{-3} M) the rate of reaction is independent of concentration. Moreover, the activation parameters for the reaction, $\Delta H^{\ddagger} = 33.2 \pm 0.7$ kcal/mol, $\Delta S^{\ddagger} = 11.0 \pm 1.9$ eu (see Figure 1), under these conditions indicate a unimolecular process.[6]

In contrast to the modified Russell mechanism[7] suggested by Hiatt and co-workers[8] for the thermolysis of secondary peresters, our findings are more consistent with a stepwise process in which oxygen–oxygen bond homolysis is followed by rapid in-cage hydrogen atom abstraction. In particular, the activation enthalpy indicates a transition state in which bond cleavage is uncompensated by bond formation,[6] and the quantitative yield of acetic acid rules out escape from the solvent cage of a significant amount of the so formed acetyloxy radical.[9] The calculated heat of reaction for the process shown in eq 1 is −58 kcal/mol.[10] Thus, the transition state for this reaction lies some 94 kcal/mol above ground-state products. Sufficient energy is released therefore to populate electronically excited states of acetophenone.[11] Indeed, we detect a low

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