Biosynthesis of Porphyrins and Related Macrocycles. Part 20.[1,2] Purification of Deaminase and Studies on its Mode of Action

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A procedure is described for isolation of essentially pure deaminase from *Euglena gracilis*. Experiments with this enzyme prove (*a*) that it assembles four *intact* units of porphobilinogen; (*b*) the intermediate mono-, di-, tri-, and tetra-pyrroles are covalently bound to deaminase before release as the hydroxymethylbilane; (*c*) this bilane is built starting with ring-A and ending with ring-D and each of the four units of porphobilinogen is built into the tetrapyrrole at the same rate; (*d*) a very reactive tetrapyrrolic intermediate is formed (even more reactive than the hydroxymethylbilane) and evidence is given from trapping experiments that this reactive species is the linear tetrapyrrolic azafulvene (6).

Porphobilinogen deaminase [E.C.4.3.1.8; systematic name: hydroxymethylbilane synthase] is usually referred to as *deaminase*. It functions in co-operation with a second enzyme, *cosynthetase* [E.C.4.2.1.75; uroporphyrinogen-III synthase] for the conversion of four molecules of porphobilinogen (1), PBG, into uroporphyrinogen-III [3,4] (9); normally the shortened name uro'gen-III is used. This macrocycle is the biochemical parent of all the pigments of life such as haem, chlorophyll, and vitamin B_{12}. When deaminase acts alone on PBG (1), an unnatural isomer, uroporphyrinogen-I (uro'gen-I) (8) is produced as the apparent product. However, this substance is formed chemically † from the true intermediate released by deaminase shown by spectroscopic [2a,5,7] and synthetic [6,7] studies in this Laboratory to be the hydroxymethylbilane (7) (Scheme 1). The present paper describes work aimed at discovering more about the way deaminase functions.

Most of the experiments in Cambridge since 1969 have made use of deaminase from *Euglena gracilis*. A procedure for partial purification of the enzyme from this source has been published [8] together with kinetic studies. The improved method which gave essentially homogeneous deaminase is outlined briefly below.

The crude soluble proteins from broken cells of *E. gracilis* were fractionated first by a combination of heat treatment (to inactivate cosynthetase) and ammonium sulphate precipitation. This was followed by chromatography of the active material by gel filtration and then by ion-exchange. The elution profiles for these two columns are shown as Figures 1 and 2, respectively. By careful selection of fractions from the ion-exchange column to exclude the peak of inactive protein which closely followed the deaminase, enzyme of activity 2.8×10^4 units/mg protein was obtained; that from the peak tube of the active fraction showed 3.1×10^4 units/mg protein. The Table shows the progress of purification for a run which combined all the best conditions. When the best quality

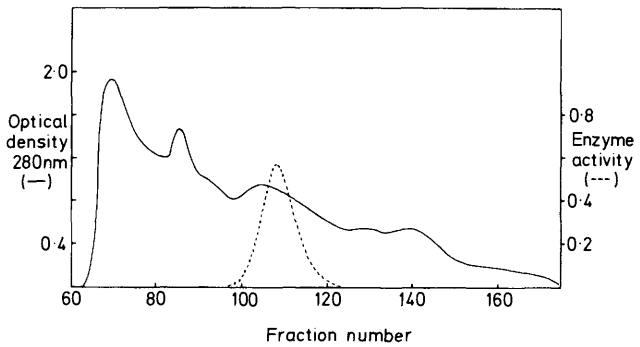


Figure 1. Elution profile (10 ml fractions) for fractionation of proteins from *Euglena gracilis* on Sephadex G-100; (——) optical density at 280 nm; (— — —) deaminase activity, arbitrary units

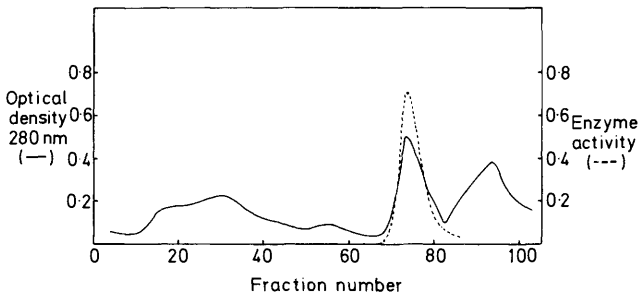


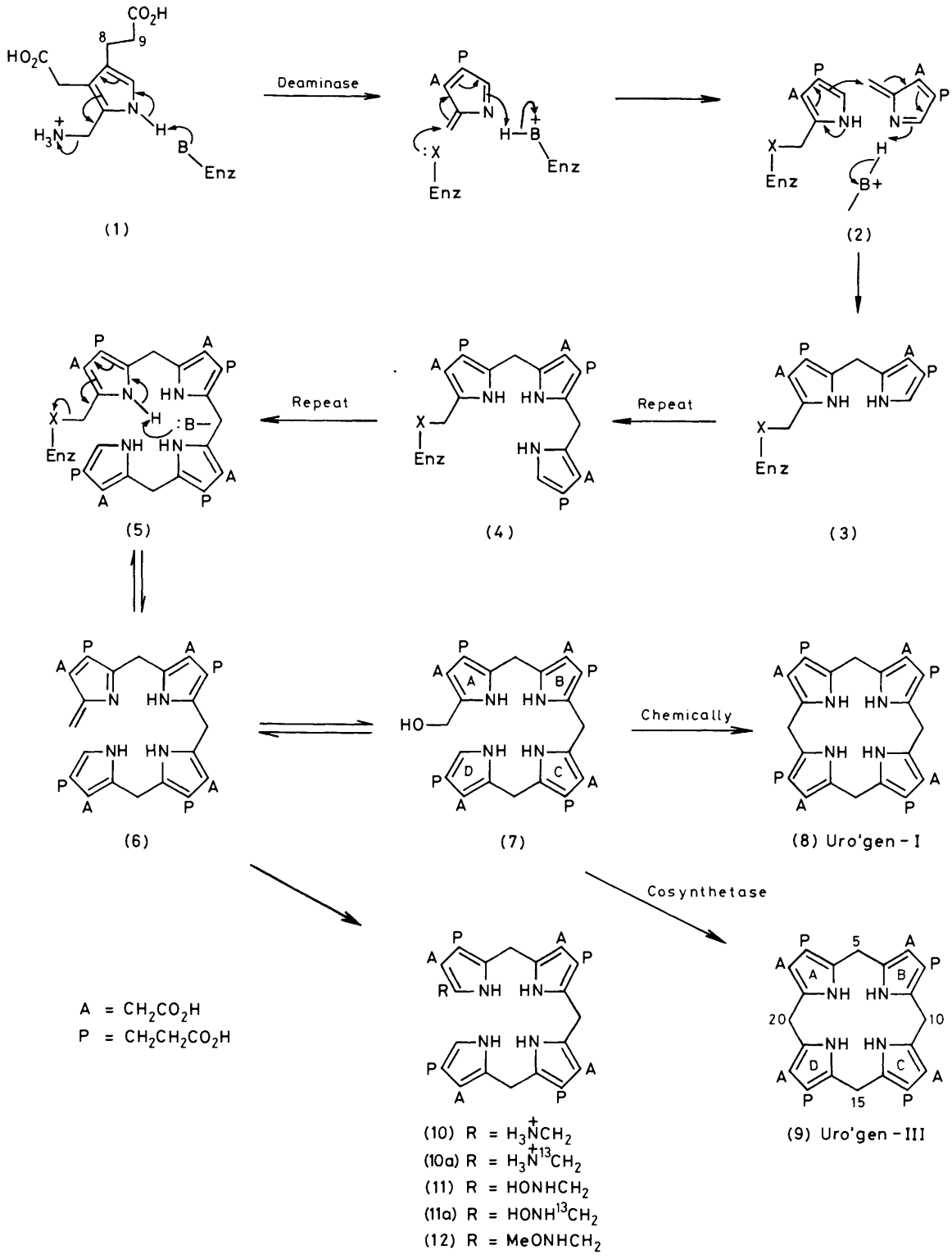
Figure 2. Elution profile (6 ml fractions) for final purification of deaminase on DEAE-cellulose; (——) optical density at 280 nm; (— — —) deaminase activity, arbitrary units

† The chemical formation of uro'gen-I from the intermediate was also shown independently (G. Burton, H. Nordlöv, S. Hosozawa, H. Matsumoto, P. M. Jordan, P. E. Fagerness, L. M. Pryde, and A. I. Scott, *J. Am. Chem. Soc.*, 1979, **101**, 3114) but the structure claimed for the intermediate was incorrect.

Table. Purification of deaminase from *Euglena gracilis*

Fraction following	Volume (ml)	Protein (mg)	Activity [a] (10^3 units)	Specific activity (units/mg)	Purification	Recovery (%)
Initial centrifugation	647	4 270	276	64.6	1	100
Heat treatment	601	721	237	329	5	86
$(NH_4)_2SO_4$	13	295	230	780	12	83
Sephadex G100	148	45	186	4 133	64	67.5
DEAE-cellulose	65	4.35	122	28 060	434	44

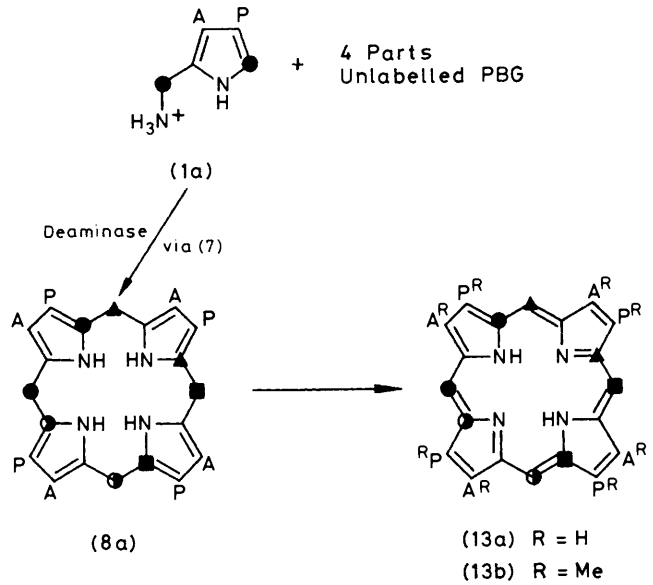
[a] One unit of enzyme is the amount required to produce 1 nmol of hydroxymethylbilane (7) per hour (assayed as uroporphyrin-I) at pH 8.0 and 37 °C



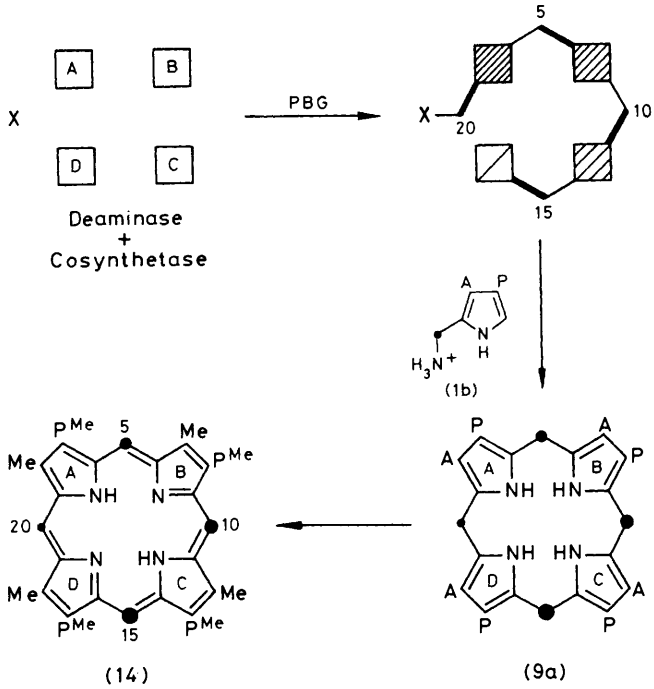
Scheme 1.

deaminase was checked by electrophoresis on polyacrylamide gel, a single band of protein was observed which exactly coincided with the band in a parallel gel demonstrated to have deaminase activity.[8] This enzyme preparation and the earlier one of partially purified deaminase-cosynthetase [9] provided the foundations for the following experiments.

Proof that Deaminase Joins Four Intact Units of Porphobilinogen.—Over the period 1973–75, it was rigorously proved [10] that for the biosynthesis of uro'gen-III (9) from PBG by deaminase-cosynthetase, ring-A and C-20, ring-B and C-5, and ring-C with C-10 are derived from *intact* PBG units (1) without rearrangement. The PBG unit which provides



Scheme 2.



Scheme 3.

ring-D is built in with rearrangement which is intramolecular with respect to that PBG unit. This was shown to hold true for enzymes isolated from an alga (*E. gracilis*) or from chicken's blood.[10] At that time (before knowledge of the hydroxymethylbilane), it was tacitly assumed that when uro'gen-I (8) was formed from PBG using deaminase alone, head-to-tail joining of four intact PBG units had occurred.

To test this, the earlier approach [10] was again used based on [2,11-$^{13}C_2$]PBG (1a) carrying 90 atom % ^{13}C at each enriched site. This was diluted with four parts of unlabelled PBG and then was converted into uro'gen-I (8a) by deaminase (Scheme 2). Aromatisation of this product gave labelled uroporphyrin-I (13a) which was purified as its ester (13b) and the derived acid (13a) was examined by ^{13}C n.m.r. The spectrum (Figure 3) displayed a strong 4.5 Hz doublet from the four bridge carbon atoms of the centrosymmetric structure (13). This shows that each bridge carbon atom experiences 3-bond coupling [10] to the atom given the same mark ‡ on structure (13) thus confirming that all four PBG units remain intact throughout. The doublet fine structure (4.5 Hz) of the small satellites proves that the large coupling (71 Hz) is not due to a rearranged PBG unit but that it appears because the probability is not negligible of two ^{13}C atoms having different marks on structure (13) becoming joined in the same molecule. The relative intensities of the large signal and the small satellites agree well with calculated values based on *ca.* 19% of doubly labelled PBG (1a) in the diluted sample used.

Order of Assembly of the Four Pyrrole Rings for Biosynthesis of the Hydroxymethylbilane (7).—Indications that the possibility existed to determine the order of assembly of the four pyrrole rings for the natural hydroxymethylbilane (7) came from studies described in Part 17.[9] A set of five isomeric aminomethylbilanes was synthesised, one was unrearranged (10) and the other four differed by having the acetic and propionic residues interchanged on one of the four pyrrole rings. These four modified bilanes were all bound less strongly and were transformed more slowly by deaminase-cosynthetase than was the unrearranged bilane (10). This suggested that there are four binding sites on deaminase which recognise the individual pyrrolic rings as the hydroxymethylbilane (7) is constructed from four PBG units (1). These putative empty sites are illustrated as A, B, C, and D in Scheme 3.

The plan was to load a large quantity of deaminase-cosynthetase [9] (*ca.* 0.3 μmol, see later) with a deficiency of unlabelled PBG (0.5 μmol). After a short time, the loading of the site onto which the first PBG unit binds should be highest, whilst the corresponding loadings for the other sites should decrease such that the last site to be filled carries least (see Scheme 3). The loading referred to includes all pyrrolic material on that site whether it be monopyrrole (2) or part of a di- (3), tri- (4), or tetra-pyrrolic species (5) (Scheme 1).

Five minutes after the first addition, the partially loaded enzyme was treated with an excess (3.5 μmol) of 90 atom % [11-^{13}C]PBG [11] (1b) to chase the bound pyrrolic species through to form uro'gen-III (9a) (Scheme 3). This was dehydrogenated with iodine to give uroporphyrin-III and after chemical decarboxylation and esterification, the resultant ^{13}C-enriched coproporphyrin-III tetramethyl ester (14) was studied by ^1H n.m.r. using a europium shift reagent. A proton attached to a bridge carbon in the ester (14) will give rise to a singlet in the ^1H n.m.r. spectrum if that carbon is ^{12}C but a widely spaced doublet will appear if it is a ^{13}C atom. Thus the relative sizes of the singlet and doublet for each bridge-proton signal will give an accurate measure of the $^{12}C/^{13}C$ ratio for that site. The signal assignments in Scheme 3 are rigorous by being based on samples of synthetic [12] [15-^{13}C]-, and [20-^{13}C]-coproporphyrin-III ester (15) and (16), respectively, and on [5-^{13}C]coproporphyrin-III ester (17) prepared by chemical decarboxylation of synthetic [5-^{13}C]uroporphyrin-III ester (18).[13]

The ^1H n.m.r. spectrum (Figure 4) from the biosynthetic

‡ Sites marked with the same sign carry ^{13}C-atoms within the *same* molecule; sites marked with different signs carry ^{13}C-atoms in *different* molecules (but see text for refinement of this broad statement).

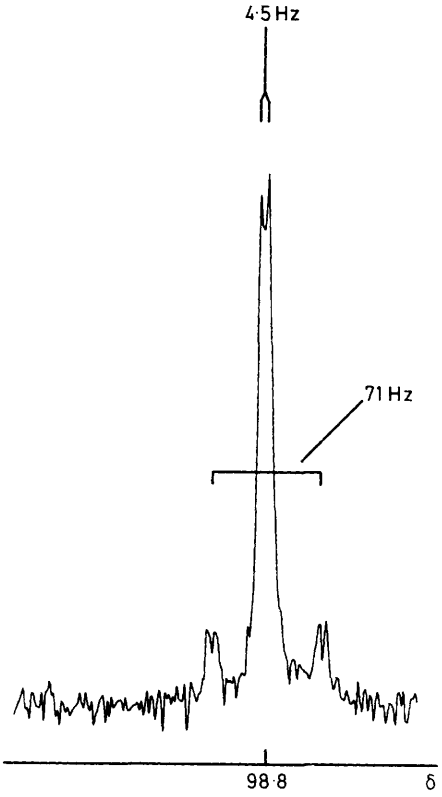


Figure 3. 25.2 MHz ^{13}C n.m.r. spectrum of uroporphyrin-I (13a) derived from [2,11-$^{13}C_2$]PBG (1a) recorded with ^1H-noise decoupling at 70 °C in D_2O–NaOH. SW 5 000 Hz, AT 0.8 s, NT 94 850

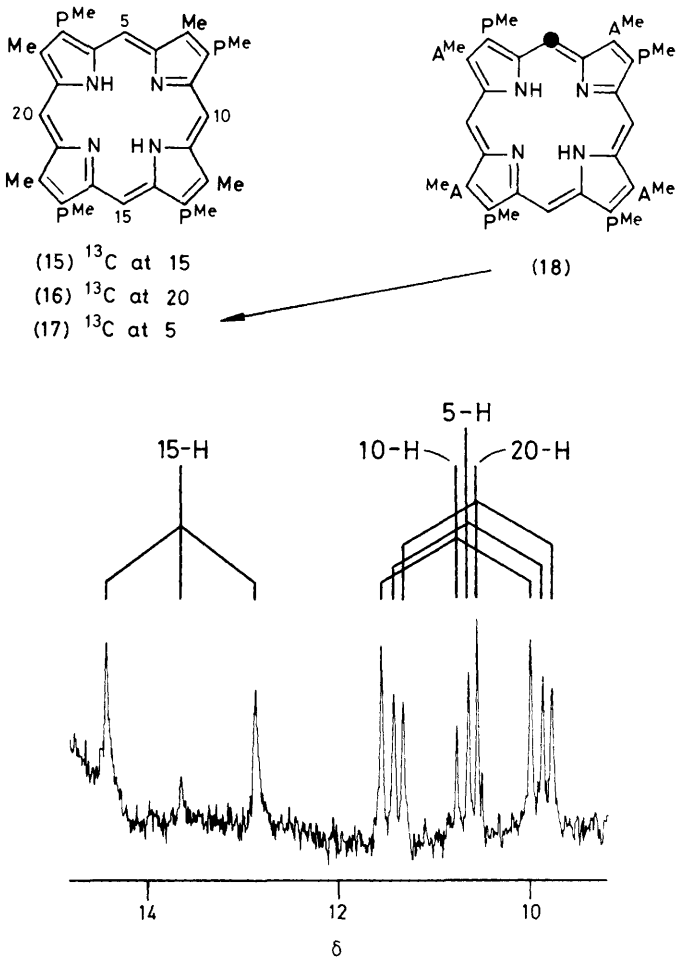


Figure 4. 100 MHz ^1H n.m.r. signals from protons at the bridges of coproporphyrin-III tetramethyl ester (14) derived from order experiment. Spectrum recorded in $CDCl_3$ with Eu([$^2H_9$]fod)$_3$ present; SW 2 000 Hz, 90° pulse, AT 2 s, NT 40 000

sample (14) clearly demonstrated that the ^{12}C content was greatest at C-20 and that the ^{12}C levels at the other bridges diminished in the order C-5 > C-10 > C-15. Integration of the signals showed that 30% of all the ring-A of the macrocycle (14), and therefore of the original uro'gen-III (9a) had been derived from the unlabelled PBG and similarly, 18%, 8% and 3 ± 1%, respectively, of rings B, C, and D. These values take into account that the enrichment of the [11-^{13}C]PBG was 90 atom %. It is thus established that the first PBG unit to bind becomes ring-A (with C-20) of uro'gen-III (9a), the second ring-B (with C-5), the third ring-C (and C-10), and the fourth ring-D (and C-15).[2b] Since the intermediate bilane system (7) was proved earlier [12] to register directly with the uro'gen-III macrocycle (9) as illustrated in Scheme 1, it follows that the hydroxymethylbilane (7) is built starting with ring-A and ending with ring-D. This order of building has been confirmed more recently by ^{14}C labelling.[14a]

Attention is drawn to this technique of ^{12}C labelling against a background of ^{13}C material because for this study it afforded a greater sensitivity of detection than the reverse approach. For example, the intensities of the three signals from the proton at C-10, which is labelled to the extent of 8% by the original [^{12}C]PBG, are 41.4, 17.2, and 41.4 units. If ^{13}C labelling had been used first followed by ^{12}C material, these signals would have been 3.6, 92.8, 3.6 units in size.

An explanation is needed of how the molar quantity of deaminase-cosynthetase used in the foregoing experiments was determined. It is known [15] that at normal pH values, cosynthetase does not affect the rate of deaminase-catalysed conversion of PBG into total uro'gens, providing PBG is at saturating concentration. So *n* units of deaminase-cosynthetase are equivalent to *n* units of deaminase. Knowing the specific activity of the purest deaminase from *E. gracilis* (31 000 units/mg protein), its molecular weight [8] (40 000), and the total number of units of deaminase-cosynthetase used, the quantity of enzyme can be calculated to be *ca.* 0.3 μmol of deaminase with sufficient cosynthetase to produce entirely uro'gen-III (9) from PBG (1).

The foregoing experiments not only establish the direction in which the hydroxymethylbilane (7) is built but they lead to three further conclusions. Firstly, earlier studies with the natural precursor, PBG, rigorously proved that uro'gen-III (9) is biosynthesised *via* a single intramolecular rearrangement of ring-D.[10] The present work, also based on PBG, has shown that ring-D is the last pyrrole to be built into the tetrapyrrole. It follows that the rearrangement *must* be at the tetrapyrrole level rather than earlier, which was precisely the conclusion shown from studies with synthetic aminomethylbilanes [12,16] [*e.g.* (10)].

Secondly, the success of the ' order experiment ' shows that catalytically active forms of deaminase can be set up carrying partly built pyrrolic chains shown as (2), (3), (4), and (5) in Scheme 1. This finding meshes with the chromatographic separation of such species first achieved by Anderson and Desnick.[17] The nature of the enzymic group X in Scheme 1 will be discussed in Part 21.[18]

Lastly, the way the deficiency of [^{12}C]PBG is distributed over the binding sites of deaminase in the order experiment depends on the relative rates of binding of the four PBG units. For example, if the first PBG unit had been incorporated

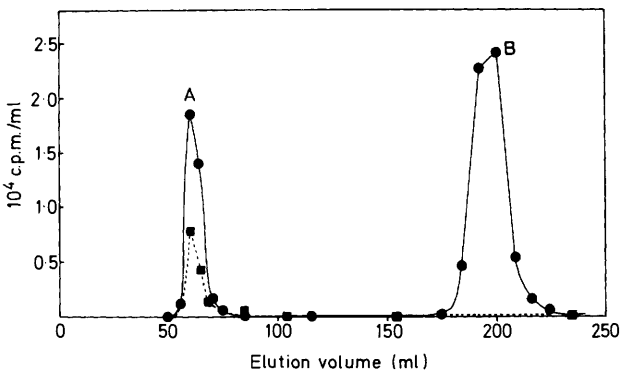
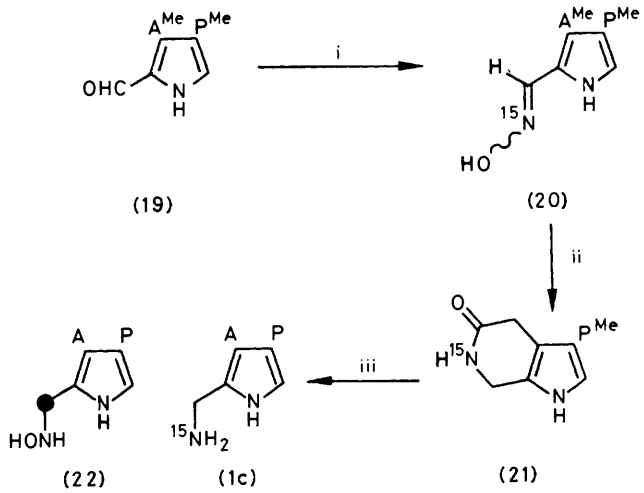


Figure 5. Chromatography of deaminase carrying bound pyrrole units derived from [^{14}C]PBG using Sephadex G50 (void volume: 60 ml; total column volume: 200 ml); additional details in Experimental section. (—●—●—●—) initial fractionation; (— — ■ — — ■) repeat chromatography of material in peak at void volume of initial run



Scheme 4. *Reagents:* i, $^{15}NH_2OH$; ii, H_2–Pt; iii, aqueous KOH

much more slowly than the remaining three, then the [^{12}C]-PBG would have been distributed approximately equally over the four rings of uro'gen-III (9a) (clearly *not* the result found). However, if the rates of incorporation of each of the four PBG units are taken to be equal then calculation shows that after 1.6*n* molecules of PBG have been accepted by *n* molecules of deaminase to occupy the sites in a strict A ⟶ D sequence, then the loading as defined earlier at A : B : C : D will be as 30 : 18 : 8 : 3. That these values correspond exactly to those observed above provides evidence that, starting with empty enzyme, each of the four PBG molecules is built into the tetrapyrrole (5) at the same rate.

Evidence for Covalent Binding of the Growing Pyrrolic Chain to Deaminase.—Scheme 1 shows the protonated amino group of the first PBG unit picked up by deaminase being replaced by a group X on the enzyme so that the extending pyrrolic chain is covalently bound. The results from the foregoing order experiments are clearly in keeping with such covalent attachment. To gain further support, deaminase (0.125 μmol) was incubated briefly with [9-^{14}C]PBG [19] as (1) (0.743μ mol) and after the solution had been made highly dissociating by addition of guanidine hydrochloride, the product was fractionated by gel filtration (Sephadex G50). Figure 5 shows the elution profile for radioactivity. The material in the void volume peak (A) [high molecular weight substances] carried the equivalent of 0.267 μmol of PBG whereas the uro'gen-I [as (8)] in the slow running peak (B) corresponded to 0.5 μmol of labelled PBG. Since 4 mol equivalents of PBG are required by deaminase to produce the hydroxymethylbilane [as (7)] which chemically yields uro'gen-I [as (8)], it was satisfying to find that the foregoing values correspond closely to the bound PBG representing the excess of PBG over 4 × mol equivalents of deaminase. On average, 2.14 molecules of PBG had been bound to each molecule of deaminase. This finding that the PBG in excess of 4 × mol equivalents of deaminase appears as the bound form requires the rate of binding of the first PBG unit to be not less than the rates of attachment of the later PBG units. So this result is in agreement with the earlier conclusions.

Ultrafiltration of this [^{14}C]PBG-deaminase followed by a repeat gel filtration left a considerable part of the labelled pyrrolic material still bound to the enzyme (see Figure 5). Finally, the loaded enzyme was subjected to proteolytic digestion (pronase) and gel filtration showed that the labelled pyrrolic material remained attached to peptides having a range of molecular weights but averaging *ca.* 3 500. These results demonstrate covalent attachment of the PBG residue(s) to deaminase and there is independent support based on denaturation.[14b]

Evidence for a Linear Tetrapyrrolic Azafulvene (6) *as a Highly Reactive Intermediate.*—The important studies of Bogorad [20] and Neuberger [21] showed that when PBG (1) was incubated with deaminase alone in the presence of ammonium ions or hydroxylamine or methoxyamine, the rate of PBG consumption was normal but formation of uro'gen-I (8) was strongly inhibited. Instead, the products were the aminomethylbilane (10) or the analogous hydroxyaminomethylbilane (11) or methoxyaminomethylbilane (12), respectively. These results must now be considered alongside the knowledge from Part 18 [7] and above that (*a*) the product released when deaminase acts on PBG (1) is the hydroxymethylbilane (7) and (*b*) the partly built intermediates are covalently bound to deaminase. All this information can only fit together if the amino group in the aminomethylbilane (10) isolated above is derived from the added ammonium ions rather than being the one originally attached to PBG.

To test this important point, [*amino*-^{15}N]PBG (1c) was synthesised by the novel route outlined in Scheme 4. The natural abundance ^{13}C n.m.r. spectrum of this product at pH >12 showed that the signal from the $^{15}NH_2{}^{13}$C-group appears as a doublet (*J* 3.6 Hz) at δ_C 38.4 relative to $Me_3SiCD_2CD_2CO_2Na$. Then [11-^{13}C]PBG [11] (1b) was incubated with deaminase at pH 8.25 in the presence of 0.2M $^{15}NH_4Cl$ the consumption of PBG being monitored with Ehrlich's reagent.[22] When PBG uptake was complete, the pH was adjusted to >12 to destroy the enzyme and to stabilise the resultant aminomethylbilane. The ^{13}C n.m.r. spectrum from this solution (Figure 6) showed essentially complete formation of $^{15}NH_2{}^{13}CH_2$-bilane (10b); the ^{13}C signal appeared as a doublet (*J* 3.7 Hz) at δ_C 38.3. Thus the amino group of the bilane (10) is indeed derived from the added ammonium ions.

The experiment was then repeated using 0.2M-hydroxylamine. Whereas in the run with ammonium ions, the aminomethylbilane (10b) had been accompanied by a considerable amount of uro'gen-I (signal at δ 24.0), no macrocyclic material was detected in the experiment with hydroxylamine. The n.m.r. signals (Figure 7) showed that the sole product was the hydroxyaminomethylbilane (11b). This difference is almost certainly a reflection of the differing basicities of the two nucleophiles. At pH 8.25, the concentration of $^{15}NH_3$ in

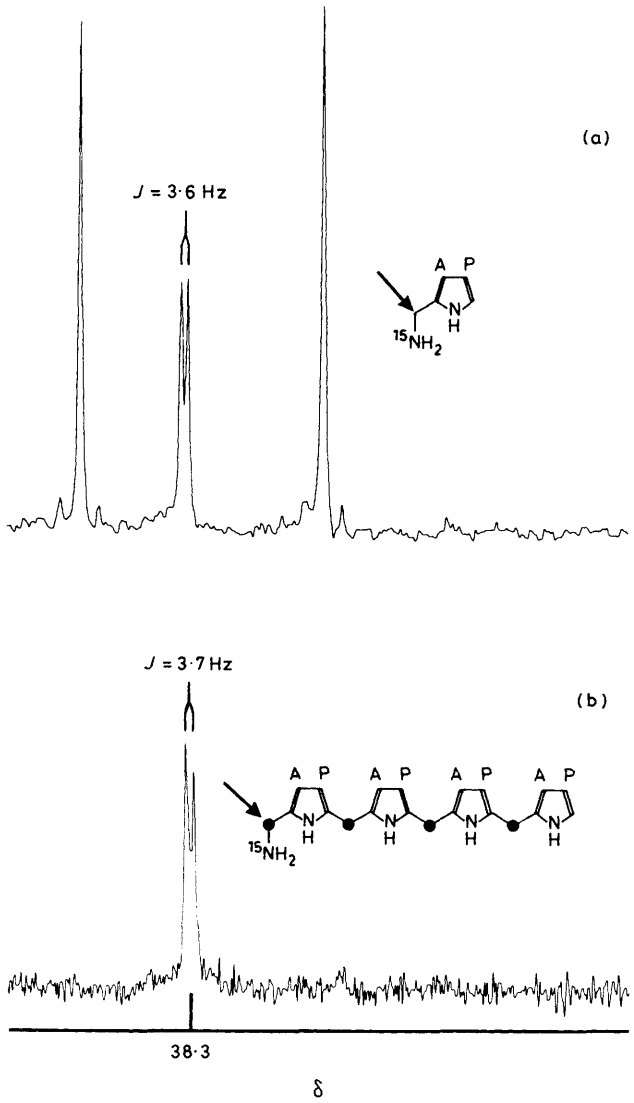


Figure 6. Doublet from arrowed carbon ($H_2{}^{15}N-{}^{13}CH_2$-pyrrole) in 25.2 MHz ^{1}H-noise decoupled ^{13}C n.m.r. spectrum of (*a*) [*amino*-^{15}N]PBG (1c) and (*b*) product from deaminase acting on [11-^{13}C]-PBG (1b) in presence of [^{15}N]ammonia. Both spectra run at pH > 12 in HOD–H_2O

Figure 7. ^{1}H Noise decoupled ^{13}C n.m.r. spectrum (25.2 MHz) of hydroxyaminomethylbilane (11b) from action of deaminase on [11-^{13}C]PBG in presence of hydroxylamine. Spectrum run at pH > 12 in HOD–H_2O. SW 5 000 Hz, 90° pulse, AT 0.8 s, NT 9 500. Signal A, HONHCH_2-pyrrole and B, pyrrole-CH_2-pyrrole

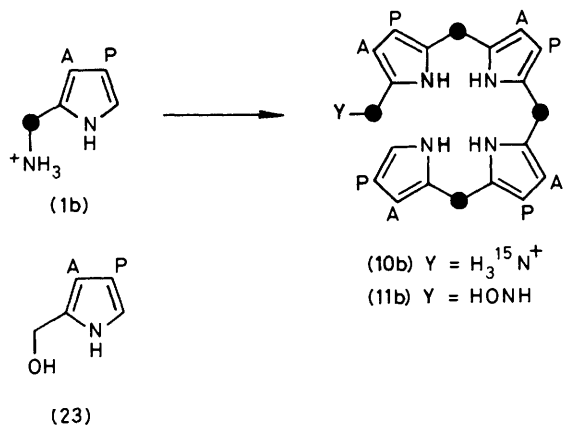
0.2M $^{15}NH_4Cl$ (pK_a *ca.* 9.2) is rather low whereas almost all the hydroxylamine (pK_a *ca.* 6.0) is in its free base form at this pH.

At this stage, it was necessary to demonstrate that no *chemical* (*i.e.* non-enzymic) displacement of the amino group from the aminomethyl residues of PBG (1) or the aminomethylbilane (10) could have occurred in the foregoing work. To this end, [*aminomethyl*-^{13}C]bilane [7] (10a) was treated with (*a*) 0.5M $^{15}NH_4Cl$ or (*b*) 0.33M hydroxylamine at pH 8.2—8.3 directly in the n.m.r. tube for 4 h at 37 °C; [11-^{13}C]PBG (1b) was subjected to the same conditions. The spectra accumulated over that period showed no splitting of the $H_3\overset{+}{N}$-^{13}C-signal in either case for the runs with $^{15}NH_4Cl$ and no significant formation of HONH$^{13}CH_2$-bilane (11a) nor of the hydroxyamino analogue of PBG (22). Neither did any chemical displacement by $^{15}NH_3$ or NH_2OH occur when representative examples from the foregoing experiments were repeated at pH > 12.

The evidence was completed by establishing that neither hydroxylamine nor ammonia cause chemical conversion of the hydroxymethylbilane (7) into the nitrogen analogues (11) and (10), respectively. This was studied initially by treating the hydroxymethylpyrrole (23) with 0.2M-hydroxylamine at pH 8.3; n.m.r. spectroscopy showed no significant chemical conversion (<3%) of the hydroxymethyl group to a hydroxyaminomethyl residue had occurred. When 0.5M-[^{15}N]ammonium ions were used in place of the hydroxylamine, less than 1% formation of [^{15}N]aminomethylpyrrole was detected. Next, the labelled hydroxymethylbilane [as (7)] was generated by brief treatment of [11-^{13}C]PBG (1b) with deaminase and the enzyme was demonstrated to be completely inactivated by raising the pH to >12 which also served to stabilise the bilane [7] [as (7)]. This deaminase-free solution was then adjusted to pH 8.25 and made 0.2M in hydroxylamine. After the solution had been kept for 20 min at 37 °C, ^{13}C n.m.r. spectroscopy showed that although extensive conversion of the bilane [as (7)] into uro'gen had occurred, there was no detectable formation of the hydroxyaminomethylbilane (11b).

Taken together, these results show that the action of deaminase on PBG (1) generates a highly reactive tetrapyrrolic intermediate which can be trapped by added small nucleophiles to generate unrearranged linear bilanes. The azafulvene structure (6) matches the properties of this intermediate which by reaction with water would generate hydroxymethylbilane (7), the normal product from deaminase.[7]

Finally, when the hydroxymethylbilane [as (7)], generated as above, was incubated with 0.2M-hydroxylamine in the presence of fresh deaminase, the ^{13}C n.m.r. spectrum now showed that all the hydroxymethylbilane [as (7)] had been



converted into the hydroxyamino analogue (11b). Thus the hydroxymethylbilane (7) in solution is in equilibrium with the reactive species (6) on deaminase.

Related experiments on this topic involved treating [11-^{13}C]PBG (1b) with enzyme in the presence of hydroxylamine but now using deaminase-cosynthetase rather than deaminase alone. Again, ^{13}C n.m.r. spectroscopy showed virtually complete formation of hydroxyaminomethylbilane with minimal production (<5%) of uro'gen. This bilane was cyclised [21] at pH *ca.* 8 and the isomeric composition of the resulting uro'gens (*ca.* 90% type-I), determined as earlier,[9] showed that here too the unrearranged bilane (11b) had been produced. Thus, the azafulvene (6) had been trapped very efficiently and this result demonstrates once again (*cf.* ref. 12) that the single intramolecular rearrangement effected by cosynthetase occurs *after* head-to-tail assembly of the unrearranged linear tetrapyrrole system.

In sum, the studies reported in this paper provide firm evidence for several important stages in Scheme 1 for which direct support was previously lacking.

Experimental

General directions are given in refs. 7 and 9.

Purification of Deaminase.—*Euglena gracilis* cells (475 g wet weight), frozen in buffer,[10] were thawed and centrifuged for 30 min at 23 000 *g* and 4 °C. The supernatant liquid (647 ml) was heat treated as follows. Portions (10 ml; pH 6.30) were placed in test tubes in a 60 °C water-bath for 12 min and then transferred to ice. The cooled contents were pooled and centrifuged for 10 min at 23 000 *g*. The clear supernatant liquid (601 ml) was stirred on ice, 2-mercaptoethanol added (1 ml/l) and the pH adjusted to 7.60 with ammonia solution. Solid $(NH_4)_2SO_4$ (enzyme grade) was added to 47% of saturation (27.1 g/100 ml) and stirring continued for 1 h. After centrifugation (10 min, 23 000 *g*, 4 °C), the supernatant liquid was again stirred on ice and the pH readjusted to 7.60. Solid $(NH_4)_2SO_4$ was added (10.6 g/100 ml of the 47% solution) to 65% of saturation. After the mixture had been stirred for a further 1 h, the pellets of precipitated protein were collected by centrifugation as before and could be stored frozen. All further handling was at 4 °C.

The protein pellets were resuspended in the minimum volume of 0.05M-phosphate buffer pH 7.0 and dialysed against the same buffer, containing 2-mercaptoethanol (1 ml/l) to give a clear solution (13 ml). This was applied to a column of Sephadex G100 (115 × 4.6 cm) and eluted with 0.015M-Tris/HCl buffer, pH 8.75, containing dithiothreitol (15 mg/l), 10 ml fractions being collected at 60 ml/h. The pooled fractions of deaminase activity (tubes 102—116, 148 ml) were immediately applied to a column of DEAE cellulose (DE52, Whatman, dimensions 10 × 1.2 cm) pre-equilibrated with the same buffer used on the previous column and washed on with more buffer (50 ml). Elution was then carried out with a linear gradient of 13.3 ⟶ 40 mM-NaCl in the same buffer (total vol. 600 ml). Fractions of 6 ml were collected at 36 ml/h and the pooled deaminase fractions were immediately concentrated by ultrafiltration to 3.3 ml; this solution was stored frozen.

Analysis of the purification procedure is shown in the Table. Deaminase activity was determined by an earlier method [8] modified to detect the linear rate of conversion of PBG into uro'gen-I following the initial lag.[17] 1 Unit is defined as the amount of enzyme required to produce 1 nmol of uro'gen per hour from PBG. Protein was determined by the method of Lowry [23] or Bradford [24] using bovine serum albumin as standard. The purity of the final preparation was confirmed by polyacrylamide gel electrophoresis under non-denaturing conditions, the single bands of protein and activity [8] being matched on corresponding gels.

Conversion of [2,11-$^{13}C_2$]*PBG* (1a) *into Uro'gen-I* (13a) *using Deaminase.*—[2,11-$^{13}C_2$]PBG [10] (90 atom % ^{13}C at each enriched site) diluted with four parts of unlabelled PBG (total 12.75 mg) was incubated with deaminase (14 800 units) for 4 h at 37 °C in pH 7.3 0.1M-phosphate buffer, containing 0.6 mM-EDTA (total 100 ml). The uro'gen-I was aromatised with iodine as usual [8] and the solution was shaken with ethyl acetate (150 ml) and glacial acetic acid (20 ml). The organic layer was equilibrated with saturated aqueous sodium acetate (50 ml) and the aqueous layer was adjusted to pH 3.2 to precipitate the uroporphyrin-I. This (6.5 mg) was collected by centrifugation, washed with water (4 × 20 ml) and acetone (2 × 20 ml), dried, and esterified in dry methanol (10 ml) containing 8% sulphuric acid at 20 °C for 18 h. The solution was poured into ammoniaical ice–water and the ester extracted into chloroform ready for recrystallisation from chloroform–methanol to give uroporphyrin-I octamethyl ester (4.7 mg). This was hydrolysed as usual [25] to the corresponding acid (13a) for study by n.m.r. spectroscopy (Figure 3).

Order of Assembly of the Four PBG Units.—Nitrogen was bubbled through a suspension of [11-^{13}C]PBG lactam methyl ester [11] (3.90 mg, 17.5 μmol) in 2M-aqueous potassium hydroxide (100 μl) for a few minutes and the sealed container was kept at 65 °C with swirling until the solid dissolved (5 min). After the solution had been kept at 20 °C for 16 h, the pH was adjusted to 7.5 with 1M-aqueous potassium dihydrogenphosphate and the mixture diluted with water (to 5.00 ml).

Unlabelled PBG monohydrate (122 μg, 0.5 μmol) in water (1 ml) was added over 20 s to deaminase-cosynthetase (396 000 units) in 10mM-phosphate buffer (pH 7.5, 6 ml), stirred at 4 °C. After 5 min, part of the [11-^{13}C]PBG solution prepared above (1.00 ml, 3.5 μmol) was added during 1.5 min and stirring was continued at 4 °C for 10 min and at 20 °C for 15 min. The solution was diluted with water (50 ml), porphyrinogens were oxidized by treatment with an excess of aqueous iodine [8] and, 5 min later, aqueous sodium hydrogensulphite was added to neutralise the remaining oxidant.[9] The precipitated protein was then removed by centrifugation; the pellet was resuspended in water (20 ml) and the mixture centrifuged again. The combined supernatant liquids were stirred for 5 min with Whatman DE 52 slurry (10 ml), after which the resin was collected, washed with water, and the uroporphyrin eluted with 3M-hydrochloric acid (20 ml). The

residue from evaporation of this solution was decarboxylated and esterified as usual [12] and chromatography of the product on silica (1 g), using methanol (0—1%) in dichloromethane, gave labelled coproporphyrin-III tetramethyl ester (14) (350 μg, 49% based on total PGB), >95% pure by h.p.l.c.;[9] δ (plus Eu([$^2H_9$]fod)$_3$) (partial spectrum) 10.52 (0.63H, d, *J* 155 Hz, 20-^{13}CH, plus 0.37H, s, 20-^{12}CH), 10.62 (0.74H, d, *J* 155 Hz, 5-^{13}CH, plus 0.26H, s, 5-^{12}CH), 10.74 (0.83H, d, *J* 154 Hz, 10-^{13}CH, plus 0.17H, s, 10-^{12}CH), and 13.61 (0.87H, d, *J* 155 Hz, 15-^{13}CH, plus 0.13H, s, 15-^{12}CH); see Figure 4.

Covalent Binding to Deaminase.—The hydrolysis of [9-^{14}C]PBG lactam methyl ester [19] (0.33 mg, 2.5×10^6 d.p.m./mg) was achieved by dissolving it in 2M-NaOH (0.1 ml) and leaving it at room temperature under nitrogen in the dark for 16 h. After addition of 1M-H_3PO_4 (0.1 ml), part of the neutralised hydrolysate (0.1 ml) was added to 0.025M-potassium phosphate buffer, pH 8.25 (1.25 ml) containing 1.5×10^5 units of deaminase, and the mixture was incubated at 37 °C for 10 min.

Guanidine hydrochloride solution (0.735 g/ml) containing dithiothreitol (0.46 mg/ml) in 0.2M-phosphate buffer (2 ml), pH 7.0, was then added and the incubation was continued for a further 30 min. The entire sample (3.35 ml in 4.6M-guanidine·HCl) was then applied to a column of Sephadex G50 (1.5 × 96 cm) which was eluted with 1M-guanidine·HCl in 0.1M-phosphate buffer pH 7.0, 4 ml fractions being collected. Samples (0.05 ml) from the various fractions were dispersed in aqueous scintillant and counted in a Packard liquid scintillation counter. The elution profile for radioactivity is shown in Figure 5. The void volume fractions (14—19) were pooled (21.5 ml); they contained 1.48×10^5 d.p.m., *i.e.* 2.67×10^{-7} mol of PBG. 1.5×10^5 Units of deaminase corresponds to 1.25×10^{-7} mol so an average of 2.14 molecules of PBG has been bound to each molecule of deaminase.

Rechromatography and Proteolytic Digestion of [^{14}C]*PBG-Deaminase*.—The [^{14}C]PBG-deaminase in the void volume fractions from a similar experiment (17 ml, 9.58×10^4 d.p.m.), was concentrated by ultrafiltration (substances >10 000 daltons retained) to 3 ml and rechromatographed as above on Sephadex G50 (Figure 5). The only peak of radioactivity detected was at the void volume, fractions 14—18, which were pooled (17 ml, 6.45×10^4 d.p.m.) and concentrated by ultrafiltration to 1.7 ml. Part (0.5 ml) was stored, frozen, for 5 months and then rechromatographed on Sephadex G50 to give the same peak of radioactivity at the void volume as the only radioactive material.

The remaining [^{14}C]PBG-deaminase solution (1.2 ml) was dialysed against 0.05M-potassium phosphate buffer, pH 7.0, (2 × 11 for 2 h at 4 °C) containing dithiothreitol (15 mg/l), to remove guanidine·HCl and then incubated with pronase (1 mg) for 24 h at 37 °C. The digest (2.8×10^4 d.p.m.) was applied to a column of Biogel P2 (1.1 × 92.5 cm) and eluted with 0.1M-potassium phosphate, pH 7.0, at 5 ml/h, 2 ml fractions being collected. The single radioactive peak, at the void volume (fractions 15—26, equivalent to molecular weight of $\geq 2 \times 10^3$), was pooled (28 ml, 2.4×10^4 d.p.m.), freeze-dried, and redissolved in 1M-guanidine·HCl in 0.1M-phosphate buffer (pH 7.0, 1.5 ml). Rechromatography on Sephadex G50 as above showed no radioactive peak at the void volume, but a broad peak of radioactivity centred on fraction 43 (172 ml) corresponding to molecular weight *ca.* 3.5×10^3.

Trapping Experiments

2-Formyl-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethylpyrrole [^{15}N]*Oxime* (20).—A solution of 2-formyl-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethylpyrrole (19) (101 mg) in methanol (2 ml) was heated under reflux while a solution of [^{15}N]hydroxylamine hydrochloride (97.4 atom %, 35.5 mg, 1.25 equiv.) and sodium acetate (41 mg) in water (0.25 ml) was added. After being heated for 0.75 h, the mixture was partitioned between dichloromethane and water. The organic layer was washed with water, dried, and evaporated and the residue was recrystallised from chloroform–hexane to give the [^{15}N]*oxime* (20) (104 mg, 97%) as needles, m.p. 127.5—128.5 °C. In spite of the sharp melting point, t.l.c. analysis (eluant, ether) clearly revealed that the compound was a mixture of *syn*- and *anti*-forms; *m/z* 269 (100%, M^+), 253 (13), 252 (36), 210 (55), 196 (39), 192 (31), and 178 (84). Unlabelled material, prepared as above, was fully characterised (Found: C, 53.9; H, 5.9; N, 10.7. $C_{12}H_{16}N_2O_5$ requires C, 53.7; H, 6.0; N, 10.4%; $\nu_{max.}$ 3 455, 3 350br, 1 728, and 1 712 cm^{-1}; $\lambda_{max.}$ 287 nm; δ 2.66 (4 H, m, $CH_2CH_2CO_2$), 3.48 (s, CH_2CO_2 of minor isomer), 3.51 (s, CH_2CO_2 of major isomer), 3.61 (6 H, s, 2 × OCH_3), 6.53 (br d, pyrrole-H of minor isomer), 6.66 (br d, pyrrole-H of major isomer), 7.24 (1 H, br, OH), 7.15 (s, C*H*NOH of minor isomer), 7.98 (s, C*H*NOH of major isomer), 9.04 (br, NH of minor isomer), 10.28 (br, NH of major isomer); *m/z* 268 (100%, M^+), 252 (12), 251 (73), 236 (25), 219 (38), 209 (66), 195 (38), 191 (43), and 177 (84).

[aminomethyl-^{15}N]*Porphobilinogen Lactam Methyl Ester* (21).—A solution of the foregoing oxime (20) (100 mg) in methanol (10 ml) containing Adams catalyst (50 mg) was stirred under hydrogen for 24 h and then evaporated to dryness; the residue was treated with methanol–chloroform (1 : 19) and the catalyst removed by filtration. The filtrate was chromatographed on silica (3 g), using the foregoing solvent mixture as eluant, to yield the *labelled lactam* (21) (37.9 mg, 45.7%) as flakes, m.p. 248—249.5 °C (from chloroform–hexane); δ (400 MHz) 2.54 (2 H, t, *J* 7.6 Hz, $CH_2CH_2CO_2$), 2.72 (2 H, t, *J* 7.6 Hz, $CH_2CH_2CO_2$), 3.41 (2 H, t, *J* 3.2 Hz, $CH_2CO^{15}NH$), 3.66 (3 H, s, OCH_3), 4.49 (2 H, m, $CH_2{}^{15}NH$), 5.83 (1 H, dt, ^1J 89 Hz, ^3J 2.1 Hz, $CH_2{}^{15}NH$), 6.55 (1 H, d, *J* 2.5 Hz, pyrrole-H), 7.69 (1 H, br, pyrrole-NH); *m/z* 223 (100%, M^+), 222 (75, $M^+ - H$), and 150 (85).

[aminomethyl-^{15}N]*Porphobilinogen* (1c).—The foregoing lactam ester (36 mg) was hydrolysed under nitrogen in aqueous 2M-potassium hydroxide (0.35 ml) as for the ^{13}C-labelled sample above. The pH was adjusted to *ca.* 12 with 1M-aqueous potassium dihydrogenphosphate, and the solution diluted (to 0.9 ml) with deuterium oxide (0.1 ml) and water for examination by ^{13}C n.m.r. spectroscopy; δ_C 24.5 ($CH_2CH_2CO_2$), 35.2 (CH_2CO_2), 38.4 (d, *J* 3.6 Hz, $CH_2{}^{15}NH_2$), 40.8 ($CH_2CH_2CO_2$), 116.5 (C-2), 116.0, 124.9, and 132.7 (3 × pyrrole-C), and 184.1 (2 × CO_2).

Incubation of [11-^{13}C]*Porphobilinogen with Deaminase in the Presence of* [^{15}N]*Ammonium Ions*.—[11-^{13}C]*Porphobilinogen* lactam methyl ester [11] (90 atom %, 3.0 mg) was hydrolysed as earlier. After the pH had been adjusted to 8.5 with aqueous 1M-potassium dihydrogenphosphate (*ca.* 0.10 ml), the solution was added to deaminase (12 000 units) in 0.3M-phosphate buffer, pH 8—8.5 (1.65 ml) containing [^{15}N]-ammonium chloride (99.7 atom %, 21 mg, *i.e.* 0.2M), and the mixture was incubated at 37 °C. Consumption of PBG was monitored by treating aliquots (5 μl) of the incubate with modified Ehrlich reagent [22] and when <8% of the substrate remained (25 min from the start of incubation), sodium hydroxide (40 mg) and deuterium oxide (0.2 ml) were added. The ^{13}C n.m.r. spectrum (Figure 6) was then determined: δ_C 24.0 (pyrrole-CH_2-pyrrole of uro'gen-I), 24.3 (1C) and

24.5 (2C) (3 × pyrrole-CH_2-pyrrole of bilane), and 38.3 (d, *J* 3.7 Hz, bilane-$CH_2{}^{15}NH_2$).

Incubation of [11-^{13}C]*Porphobilinogen with Deaminase in the Presence of Hydroxylamine.*—[11-^{13}C]PBG obtained as above from the lactam ester (1.5 mg) was incubated at 37 °C with deaminase (20 000 units) in 0.2M-phosphate buffer, pH 8.25 (total volume, 0.9 ml) containing hydroxylamine hydrochloride (12.5 mg, to 0.2M). Consumption of substrate was monitored as in the previous experiment and after 10 min, when less than 10% of the PBG remained, deuterium oxide (0.1 ml) and sodium hydroxide (20 mg) were added to the incubate. The ^{13}C n.m.r. spectrum of the solution (Figure 7) showed that the product was the hydroxyaminomethylbilane (11b): δ_C 24.4 (3C, pyrrole-CH_2-pyrrole of bilane), 50.9 (1C, bilane-CH_2NHOH). The presence of more than very small amounts of the hydroxyamino analogue of PBG (22) was excluded by the integral ratio.

Incubation of Hydroxymethylbilane (7) *with Deaminase in the Presence of Hydroxylamine.*—[11-^{13}C]PBG (from 6 mg of lactam ester) was incubated at 37 °C in 0.2M-phosphate buffer, pH 8.25 with 60 000 units of deaminase (total vol. 1 ml). After 15 min, 5M-NaOH (50 µl) was added to raise the pH to *ca.* 13 and the sample was kept at 37 °C for 20 min. It was cooled to 0 °C, adjusted to pH 8.25 with 1M-KH_2PO_4 (total vol. now 1.3 ml), and one sample (40 µl) was used to show (using PBG) that no deaminase activity remained. A second sample (400 µl) was treated at 37 °C for 25 min with deaminase (20 200 units) in a solution (total vol. 0.85 ml) made 0.2M in NH_2OH. ^{13}C N.m.r. spectroscopy as above showed that the product was largely the hydroxyaminomethylbilane (11b).

Treatment of [aminomethyl-^{13}C]*Bilane* (10a) *with* [^{15}N]-*Ammonia and with Hydroxylamine.*—(*a*) [*aminomethyl*-^{13}C]-Bilane lactam ester [7] (10 atom %, 50 mg) was hydrolysed under nitrogen in aqueous 2M-potassium hydroxide (0.5 ml) for 3 h at 65 °C and 12 h at 25 °C after which the solution was adjusted to *ca.* pH 10 with 1M-potassium dihydrogenphosphate. It was then concentrated under nitrogen (to *ca.* 0.7 ml), and after [^{15}N]ammonium chloride (99.7 atom %, 25 mg) and D_2O (0.1 ml) had been added, the pH was adjusted to 8.3. The resulting solution of [*aminomethyl*-^{13}C]bilane (10a) which was 0.5M in $^{15}NH_4{}^+$, was examined by ^{13}C n.m.r. spectroscopy at 37 °C. The spectrum, accumulated over 4 h, showed no splitting of the signal from the aminomethyl group, δ_C 37.4.

(*b*) The experiment above was repeated, but with 0.3M-hydroxylamine instead of [^{15}N]ammonia. Before the spectroscopic study, the solution was incubated at 37 °C for 1.5 h; the pH was then adjusted to >12 by the addition of sodium hydroxide (20 mg). A ^{13}C n.m.r. spectrum of the product, accumulated over 1 h, showed no signal attributable to hydroxyaminomethylbilane (11a).

Treatment of [11-^{13}C]*Porphobilinogen with* [^{15}N]*Ammonia and with Hydroxylamine.*—(*a*) [11-^{13}C]Porphobilinogen lactam methyl ester [11] (90 atom %; 5 mg) was hydrolysed as usual (0.1 ml of 2M-alkali) and the solution was then made 0.5M in [^{15}N]ammonium chloride, at pH 8.25, as above. A ^{13}C n.m.r. spectrum of this solution, recorded over 4 h at 37 °C, showed no splitting of the signal from the aminomethyl group.

The same result was observed when a similar solution, at pH 6, was left at 25 °C for 4 days before its ^{13}C n.m.r. spectrum was determined and the same again when this solution was adjusted to pH > 12, and another spectrum accumulated over 20 h at 37 °C.

(*b*) A solution (0.9 ml) of [11-^{13}C]PBG (from 2.5 mg of lactam methyl ester) in phosphate buffer, pH 8.2, containing hydroxylamine hydrochloride (21 mg, to 0.3M) and deuterium oxide (0.1 ml) was placed in the n.m.r. spectrometer at 37 °C. A ^{13}C spectrum accumulated over 4 h showed no detectable formation of the hydroxyaminomethylpyrrole (22).

Incubation of [11-^{13}C]*Porphobilinogen with Deaminase-Cosynthetase in the Presence of Hydroxylamine.*—The earlier experiment was repeated on the same scale but with deaminase-cosynthetase (12 700 units) instead of deaminase alone. This time a small amount of uro'gen was detected: 40, 67, and 74 nmol after incubation for 4, 8, and 12 min, respectively; the theoretical maximum is 1.68 µmol. After 15 min, D_2O and sodium hydroxide were added and the solution was examined by ^{13}C n.m.r. spectroscopy; only hydroxyaminomethylbilane (11b) (δ_C 24.4 and 50.9) was detected. The solution was then adjusted to pH 8 and then kept at 37 °C for 18 h to cyclize the bilane. Isomer analysis [9] of the resulting uro'gens showed 89% of Type-I and 11% Type-III were present.

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