Biosynthesis of Porphyrins and Related Macrocycles. Part 21.[1] The Interaction of Deaminase and its Product (Hydroxymethylbilane) and the Relationship between Deaminase and Cosynthetase

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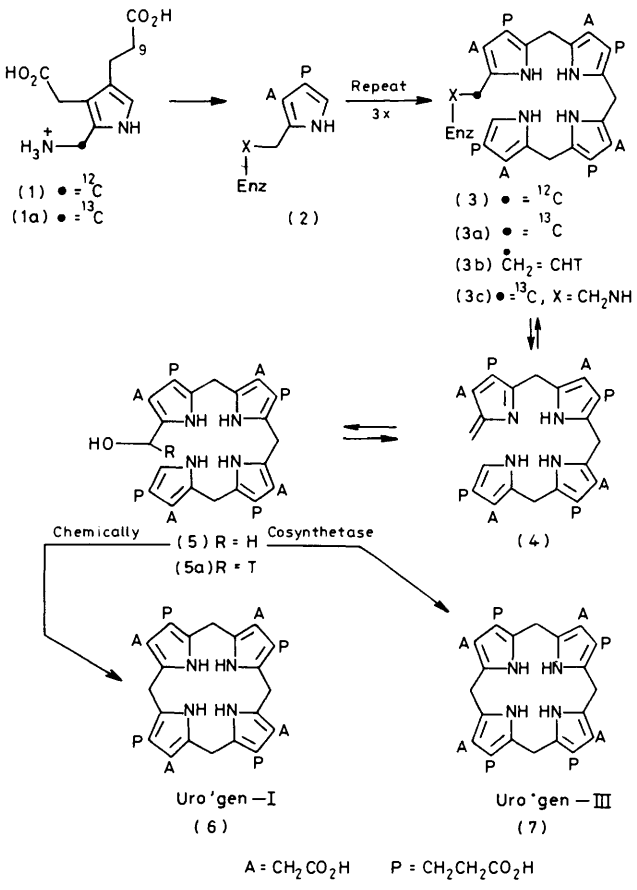
Experiments are described which show that (*a*) deaminase is inhibited by its own product, the linear hydroxymethylbilane; (*b*) this inhibition of uptake of porphobilinogen (PBG) is competitive, affecting K_M but not $V_{max.}$; (*c*) addition of synthetic hydroxymethylbilane to deaminase sets up the covalently bound linear tetrapyrrolic system on the enzyme; (*d*) it is the binding of the next PBG unit after construction of the linear tetrapyrrole which causes release of hydroxymethylbilane from deaminase; (*e*) the linear tetrapyrrole bound to deaminase is only slowly removed in the presence of a large excess of cosynthetase; (*f*) when the first PBG unit is taken up by deaminase it is covalently bound to the enzyme with release of one mole of ammonia; (*g*) the tentative conclusion is reached that this covalent binding occurs *via* the ε-amino group of lysine; (*h*) deaminase has no effect on the rate of formation of uro'gen-III from hydroxymethylbilane by cosynthetase.

Earlier parts of this series have described experiments which demonstrated unambiguously that the role of deaminase acting on porphobilinogen (1), PBG, is as an assembly enzyme to produce the linear hydroxymethylbilane (5).[2,3] This product ring-closes chemically to form uro'gen-I (6) but more importantly is the substrate for the second enzyme, cosynthetase, whose function is to ring-close the linear bilane [3] (5) with intramolecular rearrangement of ring-D [4] to form uro'gen-III (7).[5] The foregoing studies were based on the preparation of three materials *viz.* synthetic hydroxymethylbilane (5),[3] pure deaminase,[1] and cosynthetase which was free of deaminase.[6] This trio was also essential for the work described in the present paper which focusses on their relationships.

A puzzling and unexplained observation concerned the determination of the Michaelis constant K_M for PBG (1) with deaminase. When this enzyme was free from cosynthetase, a K_M value of 195 μM was found [7] but deaminase together with an excess of cosynthetase gave a K_M of 104 μM.[7] Values for K_M intermediate between these figures resulted when samples of partly purified deaminase, containing small amounts of cosynthetase were used. These experiments highlighted the problem but were not ideal because the true amount of deaminase was unknown for the runs with mixtures of deaminase and cosynthetase.

Accordingly, the standard procedure for determination of Michaelis constants was repeated using a known amount of pure deaminase to which cosynthetase was added. Figure 1 shows the linear double reciprocal plots of $1/V$ *vs* $1/S$; clearly, added cosynthetase had a marked effect on K_M (see intercepts on *x*-axis). However, it was evident that cosynthetase also had a considerable effect on the apparent $V_{max.}$ (a 65% increase for Expts *b* and *c* relative to *a*; see intercepts on *y*-axis). Deaminase activity was assayed for these experiments by measuring the rate of production of uro'gen. It should be emphasised, however, that the observations in Figure 1 are not complicated by the lag in uro'gen production by deaminase (which led to the initial detection of the hydroxymethylbilane) [2,3] because the experimental method was designed to overcome the lag. Moreover, when deaminase activity was measured by conversion of PBG (1) into product, there was again a striking rate enhancement by addition of cosynthetase (see Figure 2). The results in Figure 2 are related to Levin's observation [8] that cosynthetase stimulates deaminase activity under alkaline conditions (pH *ca.* 9) but not at lower pH values.

At this point several apparently disparate pieces of information or speculation fuse together. The first is the possibility raised by Levin [8] in 1971 that cosynthetase may be removing some deaminase-generated intermediate which inhibits deaminase activity. The second is the present knowledge that the product from deaminase is the hydroxymethylbilane (5).[3] Then the third is the fact that Levin found stimulation of deaminase activity by cosynthetase at alkaline pH's, where the hydroxymethylbilane (5) is now known to have enhanced



Scheme 1.

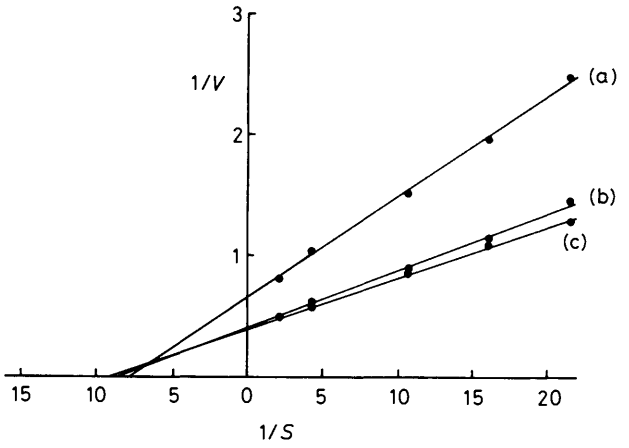


Figure 1. Double reciprocal plots showing effect of PBG concentration on the activity of deaminase in the absence and presence of cosynthetase. Scale for V, 1 = 10^4 units of deaminase activity per ml of stock solution; scale for S, 1 = 10^{-3} molar PBG. Units of cosynthetase added: (a) 0; (b) 365; (c) 3 650

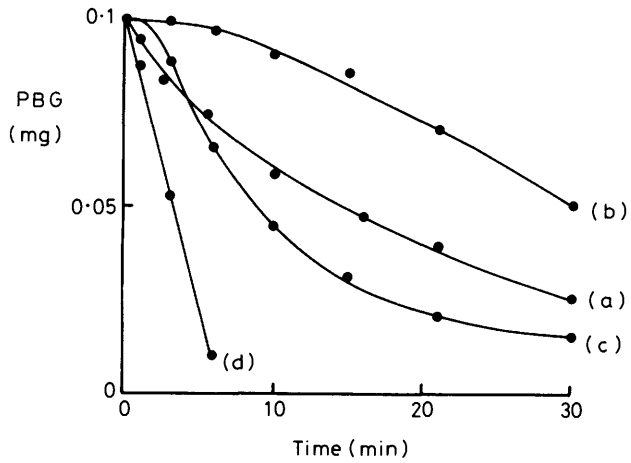


Figure 3. Effect of hydroxymethylbilane on the uptake of PBG by deaminase. PBG and deaminase were mixed with the following additions: (a) no addition; (b) hydroxymethylbilane; (c) hydroxymethylbilane and cosynthetase; (d) cosynthetase

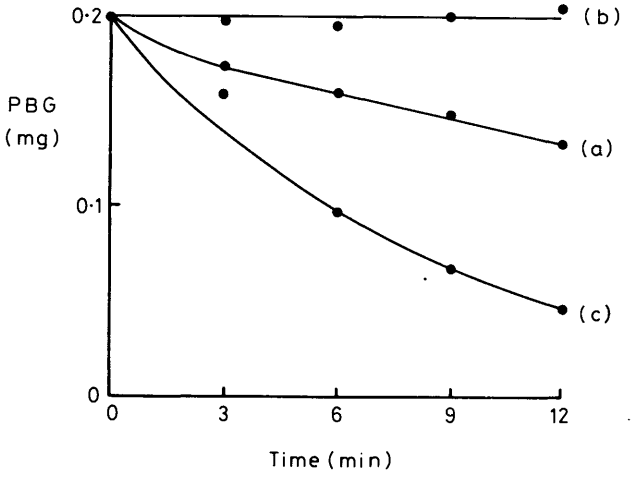


Figure 2. Effect of cosynthetase on the rate of uptake of PBG by deaminase. PBG was treated with (a) deaminase; (b) cosynthetase; and (c) deaminase with cosynthetase

stability [3] but not at low pH's where extremely rapid non-enzymic ring-closure of (5) occurs.[3] All three would fall into place if deaminase is inhibited by its product, the hydroxymethylbilane (5). The availability of synthetic (5) allowed appropriate experiments set out in Figure 3.

The standard curve (a) shows the rate of utilisation of PBG (1) by deaminase. Introduction of hydroxymethylbilane (5) caused severe restriction of uptake of PBG [curve (b)]; note that the rate of use of PBG gradually increases with time as the added inhibitory bilane (5) is removed by chemical ring-closure. The inhibition seen in curve (b) was overcome by adding cosynthetase with the bilane (5). After an initial brief lag, while the cosynthetase was converting the bilane (5) into uro'gen-III (7), the rate of PBG uptake [curve (c)] exceeded that of the PBG-deaminase control [curve (a)]. As in Figure 2, addition of cosynthetase alone at the outset to PBG-deaminase caused rapid uptake of the PBG [curve (d)].

It was clear from these results that the hydroxymethylbilane (5) inhibits deaminase. The nature of that inhibition was not clear nor was it obvious how it could be determined. The usual method of adding known quantities of inhibitor to standard mixtures of deaminase and PBG to attempt to obtain a range of double reciprocal plots is not possible in this case. The reason is that the highly reactive hydroxymethylbilane (5) is being lost by ring-closure to uro'gen-I (6) and deaminase itself is generating more inhibitor throughout the reaction period. So any straightforward attempts to achieve a constant rate will be frustrated. However, there *are* conditions under which the concentration of bilane (5) remains constant in incubations of PBG (1) with deaminase. These are when the rate of production of the bilane (5) from PBG equals the rate of its chemical conversion (non-enzymic) into uro'gen-I (6). This conversion is a unimolecular process and therefore, at a fixed pH, the rate of formation of uro'gen-I (6) is proportional to the concentration of hydroxymethylbilane (5). Thus, providing the assays are so arranged that an identical rate of uro'gen-I formation is measured over a range of PBG concentrations (by varying the amount of deaminase added), then a single double-reciprocal plot corresponding to a single (but unknown) concentration of hydroxymethylbilane can be obtained.

The experimental approach was as follows. A series of lines was determined which related the rate of uro'gen-I formation to the concentration of deaminase; one such line is shown as Figure 4. Each line was determined for a different concentration of PBG (1), the 5 chosen concentrations lying in the range 55—550 μM PBG. As was expected, increasing deaminase concentration did not lead to a linear increase in the rate of formation of uro'gen-I because this change also caused an increase in the concentration of the inhibitory hydroxymethylbilane (5).

Five convenient experimental rates of uro'gen-I formation were then selected and for each, the corresponding volume of deaminase stock solution was read off each line. This was done with the *same* five selected rates for all five lines, of which Figure 4 is one; it is emphasised that these five lines correspond to different concentrations of PBG. These data were then converted into a set of five double reciprocal plots, each plot being drawn on a separate graph to eliminate subjectivity. Finally the five double reciprocal plots, each corresponding to a different concentration of hydroxymethylbilane, were assembled to form a single presentation (Figure 5). The result clearly established that the inhibition of deaminase by hydroxymethylbilane (5) is competitive, affecting K_M but not $V_{max.}$

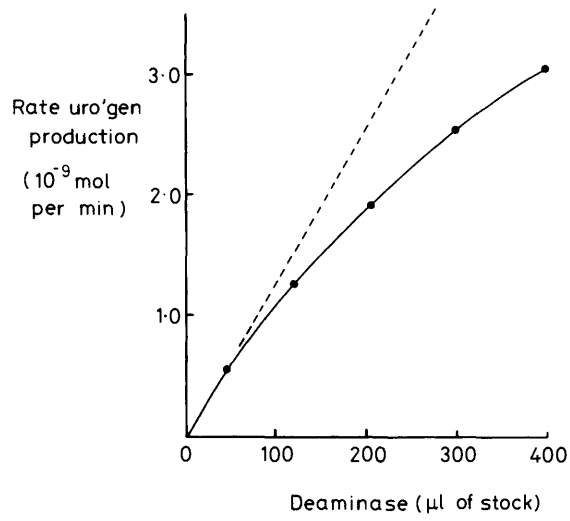


Figure 4. Effect of deaminase concentration on measured activity of enzyme

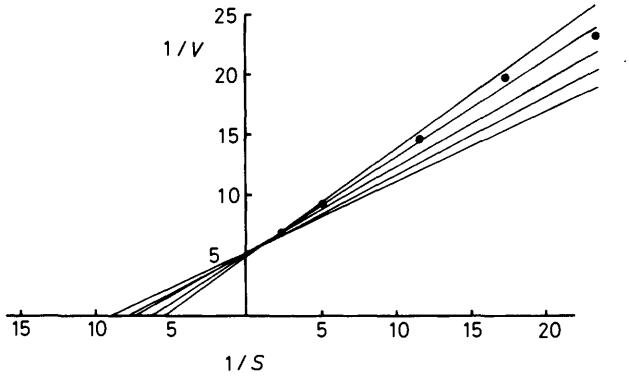


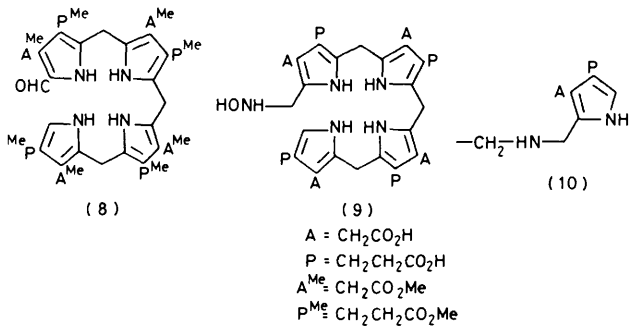
Figure 5. Double reciprocal plots showing the nature of inhibition by hydroxymethylbilane of deaminase activity on PBG. Scale for V, 1 = 10^4 units of deaminase activity per ml of stock solution; scale for S, 1 = 10^{-3} molar PBG. Each line corresponds to a different concentration of hydroxymethylbilane generated *in situ*. For clarity, the experimental points are illustrated only for the second line from the top; the data for the other lines gave as good or better fit

The foregoing work has several implications. (*a*) The true K_M for PBG can only be determined when the concentration of hydroxymethylbilane is essentially zero. This is best achieved by running the determination in the presence of an excess of cosynthetase. Thus, the K_M value from Figure 1 of 105 μM agrees with earlier determinations.[7]

(*b*) A double reciprocal plot of data from deaminase acting alone on PBG, where different rates of uro'gen-I formation are measured for a given quantity of deaminase, is only apparently linear because each data point [for example (a) of Figure 1] corresponds to a different concentration of inhibitor. Thus if the points for line (a) of Figure 1 could be extended, (a) would appear as a *curve* which bends to cross the y-axis at the same point as lines (b) and (c).

(*c*) Figure 4 suggests that assay of deaminase will give different results for different concentrations of enzyme. However, this exaggerated curve is only obtained at concentrations of PBG which are well below saturation; the standard assay for deaminase uses 500 μM PBG.

Our second main series of experiments probed the nature of



the binding of the linear tetrapyrrole (3) to deaminase before it is released as hydroxymethylbilane (5). Evidence was presented in Part 20 [1] that deaminase acts on PBG (1) to produce partly built oligopyrroles which are certainly covalently bound to the enzyme. There too it was shown that deaminase will equilibrate added hydroxymethylbilane with a highly reactive intermediate, probably the azafulvene (4). Hence, it seemed likely that by adding synthetic hydroxymethylbilane to deaminase, the covalently bound tetrapyrrolic intermediate (3) would be specifically set up. We felt that if this could be established, further important experiments were possible.

Accordingly, [1-$^3H_1$]hydroxymethylbilane (5a) was synthesised by reducing the aldehyde [3] (8) with sodium borotritiide followed by hydrolysis of the ester groups.[3] When this labelled bilane was treated with 1 molar equivalent of deaminase, it became covalently bound as shown by the following: (*a*) gel-filtration led to a protein fraction, separated from smaller molecular weight materials, which carried 72% of the original ^{3}H-activity; (*b*) ultrafiltration of the solution of the gel-filtered enzyme carrying the labelled tetrapyrrole caused no considerable loss of ^{3}H-activity as the volume was halved; (*c*) addition of sodium dodecyl sulphate (to 1% concentration) to denature the deaminase-tetrapyrrole system followed by gel-filtration gave most of the ^{3}H-activity still retained in the protein fraction. It was clear that covalent binding had been achieved.

Having the bilane-deaminase system (3b) thus available, it was possible to determine what causes the hydroxymethylbilane (5a) to be released from the enzyme. Four portions of the bilane-deaminase system (3b) were used for experiments at pH 8.0 and 37 °C. (*a*) One was incubated for 15 min; 84% of the ^{3}H-activity remained bound to the protein as shown by gel-filtration. Thus, no rapid release of hydroxymethylbilane (5a) occurs spontaneously though some of the bound tetrapyrrole is lost slowly from the enzyme to the medium. (*b*) A second portion was treated with an excess of PBG (1) and after 15 min, gel-filtration showed that >96% of the ^{3}H-activity had been released from the protein. (*c*) The third portion was mixed with the amount of cosynthetase needed to ring-close in <5 s the amount of bilane (5a) originally bound to the deaminase. After 15 min, 28% of the ^{3}H-activity was still bound to deaminase and the remainder emerged from the gel-filtration column appropriately for uro'gen. It is evident that this process involving cosynthetase is a relatively slow one. (*d*) Treatment of the last portion with hydroxylamine for 25 min released 50% of the ^{3}H-activity as material which eluted on gel-filtration as for the hydroxyaminomethylbilane (9).

For completeness, the uro'gens from experiments (*b*) and (*c*) were oxidised to the corresponding porphyrins which were analysed as usual [2] to confirm, as expected, that experiment (*b*) had yielded >90% uro'gen-I (6), with no detectable uro'gen-III (7) whereas experiment (*c*) had given 90% uro'gen-III (7) with 10% uro'gen-I. Most of the latter had arisen during

handling of the bilane-deaminase (3b) before the cosynthetase was added.

These results show that it is the binding of the next unit of PBG (1) to deaminase after construction of the linear tetrapyrrole (3) which leads to rapid release of hydroxymethylbilane (5). Without PBG, only a small amount of (4) and (5) are in equilibrium with the bound system (3) and the free bilane (5) slowly ring-closes to uro'gen-I (6). The effect of cosynthetase (experiment *c*) can be understood on this basis as being due to conversion of the small amount of free (5) into uro'gen-III; this removal of free bilane (5) pulls the equilibrium over to cause further loss of the bound bilane from deaminase.* The explanation of the result from experiment (*d*) is very similar save that the hydroxylamine presumably traps [1] and removes the azafulvene (4) which is also in equilibrium [1] with the low concentration of free hydroxymethylbilane (5). Finally, earlier kinetic studies [9] had indicated that deaminase works by a displacement-type mechanism, the overall reaction being dominated by a single rate-determining step; these previous findings are in keeping with the present results.

Two last pieces were then added to the mosaic picture of deaminase. For the first, we sought to demonstrate that when the first PBG unit (1) binds to the enzyme, 1 mol equiv. of ammonia is released. The experiments involved incubation of 0.9 equiv. of PBG with deaminase, the released ammonia being assayed using glutamate dehydrogenase [see equation (1), *cf.* ref. 9); this equilibrium lies almost completely to the righthand side. Triplicate runs with full controls showed that

$$HO_2CCH_2CH_2COCO_2^- + NH_4^+ + NADH + H^+ \rightleftharpoons HO_2CCH_2CH_2CH(NH_3^+)CO_2^- + NAD^+ + H_2O \quad (1)$$

1.2 ± 0.25 mol equiv. of ammonia had been released for each mol equiv. of bound PBG, again in agreement with covalent binding from the outset of the growing pyrrolic system [(2) ⟶ (3)] attached to deaminase.

The second mosaic piece involved binding [11-^{13}C]PBG (1a) to a large quantity of deaminase in the hope that the nature of the group X in Scheme 1 could be discovered using ^{13}C n.m.r. spectroscopy. This experiment, though simple in principle, has proved extremely difficult in practice; we report now our results so far which are the outcome of a major effort.

A large amount of deaminase was isolated (typically 5 × 10^5 units, *ca.* 0.4 μmol) which was incubated with [11-^{13}C]PBG (1a) (*ca.* 0.52 μmol) containing a little [9-^{14}C]PBG; then the protein was denatured with guanidine hydrochloride. The ^{13}C n.m.r. spectra of the material at this stage showed no recognisable signal corresponding to X-$^{13}CH_2$-pyrrole. Since the difficulty was judged to be the high molecular weight of the deaminase–oligopyrrole system, the product was degraded with pronase [1] and the peptides carrying the labelled pyrrolic material, isolated by gel-filtration (*M*, *ca.* 3 000—10 000) were again examined by ^{13}C n.m.r. spectroscopy. Still no clear ^{13}C signal from an enriched site could be observed. Further degradation was therefore carried out either by hydrolysis with 2M-potassium hydroxide or by thermolysin followed by subtilisin. Now the separated pyrrole-carrying peptides did show ^{13}C signals at δ 24.5 (corresponding to pyrrole-$^{13}CH_2$-pyrrole) and a set of closely spaced signals at δ 42—43. Though the latter signals were weak they appeared in both runs which had been taken through the above steps. The δ 42—43 chemical shift is in keeping with the partial structure (10). A set of close signals, rather than one, is not unexpected since a family of different peptides carrying the bound pyrrole residue(s) will almost certainly be present in the final hydrolysis product. Although at this stage rigorous conclusions cannot be drawn, the sum of evidence leads to the tentative proposal that X in Scheme 1 is the ε-amino group of lysine.

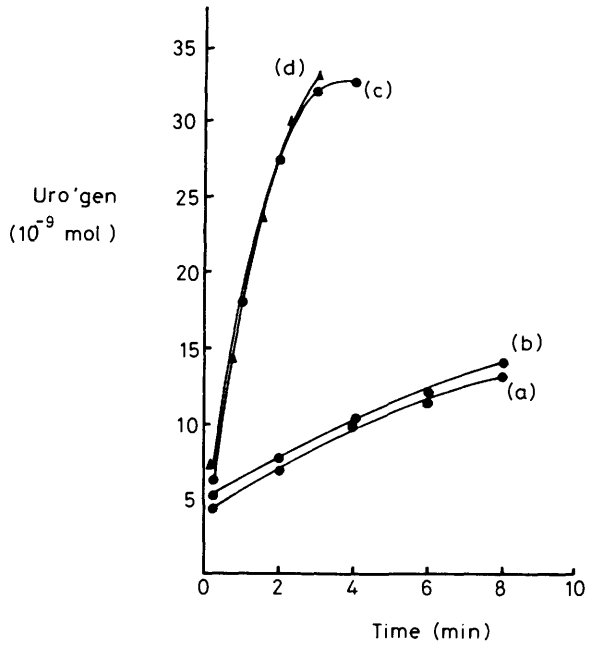


Figure 6. Effect of deaminase on rate of uro'gen formation from hydroxymethylbilane. The enzyme and substrate were mixed with the following additions (a) no addition; (b) deaminase; (c) cosynthetase; (d) deaminase and cosynthetase

Our experience of aminomethylbilanes [2,4] indicates that the stability and properties of the system (3c) [*cf.* (10)] would satisfactorily match those of the mono-, (2) to tetra-pyrrolic (3) species covalently bound to deaminase during the assembly of the hydroxymethylbilane (5). The outcome of further work to confirm or eliminate this proposal will be interesting.

This paper has been much concerned with the effect of cosynthetase on deaminase. The approach was then reversed to examine whether deaminase affects the activity of cosynthetase. Figure 6 is self-explanatory showing that deaminase has no effect on the rate of formation of uro'gen-III (7) from hydroxymethylbilane by cosynthetase.

The foregoing studies have considerably clarified the relationship of deaminase to its product, the hydroxymethylbilane (5) and also the relationship of both to cosynthetase. The advances are listed in the summary to this paper. In addition, the new knowledge gained shows that whilst deaminase and cosynthetase may be physically close together in their natural state, or even associated, such association is not an essential feature of their mode of action.

Experimental

For general directions, see ref 2 and 3.

Effect of Cosynthetase on K_M *and* $V_{max.}$ *Determinations for Deaminase Acting on PBG* (1).—Incubates at 37 °C contained PBG (0.204, 0.274, 0.410, 1.025, and 2.044 nmol), deaminase (112.5 units, or 75 units when cosynthetase was added) and

* Gel filtrations and ultrafiltrations were run throughout all these experiments at 4 °C to retard restoration of the equilibrium between bound bilane and free hydroxymethylbilane. Without this precaution, there could have been serious loss of bound bilane during the analyses.

cosynthetase (0, 365, or 3,650 units [2]) in 0.1M-potassium phosphate buffer (3.75 ml), at pH 8.0. Samples (0.75 ml) were withdrawn at intervals up to 15 min, and transferred directly into 7% trichloroacetic acid solution which ring-closed any remaining hydroxymethylbilane (5). Uro'gen was then measured as uroporphyrin at 406 nm after oxidation with iodine as usual. Enzyme activity was calculated from the linear rate of uro'gen production. The results are collected in Figure 1.

Effect of Cosynthetase on the Rate of Uptake of PBG by Deaminase.—PBG (0.2 mg) was incubated at 37 °C in 0.2M-Tris/HCl buffer at pH 8.90, (1.5 ml) with (*a*) deaminase (3×10^3 units),* (*b*) cosynthetase (3 650 units),* and (*c*) deaminase with cosynthetase. Samples (0.1 ml) were withdrawn at intervals up to 12 min and added immediately to Ehrlich's reagent [10] (0.6 ml) plus distilled water (0.5 ml). After 15 min, PBG was estimated by measuring the extinction at 554 nm. The time courses for disappearance of PBG in the three runs are shown in Figure 2.

Effect of Hydroxymethylbilane on PBG Uptake by Deaminase.—PBG (0.1 mg) was incubated with deaminase (1.5×10^3 units) in 0.1M-potassium phosphate buffer (0.75 ml), pH 8.0, at 25 °C with the following additions: (*a*) none; (*b*) hydroxymethylbilane (0.3 mg); (*c*) hydroxymethylbilane and cosynthetase; (*d*) cosynthetase (7 575 units). Samples (0.08 ml) were removed and the PBG was estimated as above. The time courses for disappearance of PBG are shown in Figure 3.

Effect of Deaminase Concentration on Measured Activity of the Enzyme.—PBG (0.067 mg) was incubated at 37 °C in 0.1M-potassium phosphate buffer (3.75 ml) at pH 8.0, containing deaminase (67.5, 180, 305, 450, and 600 units). Samples (0.75 ml) were withdrawn at 6, 9, 12, and 15 min and added immediately to 7% trichloroacetic acid solution. Uro'gen was determined as uroporphyrin at 406 nm after oxidation with iodine.[3] Linear rates are plotted against μl of stock deaminase added (Figure 4).

Nature of Inhibition of Deaminase Activity by Hydroxymethylbilane with PBG as Substrate.—Exactly the foregoing procedure was used to determine the effect of deaminase concentration on activity at a range of PBG concentrations. For 0.05 mg PBG in 3.75 ml as above, 90, 225, 405, 630, and 900 units of deaminase were used; at 0.067 mg PBG, 67.5, 180, 315, 450, and 600 units; at 0.1 mg PBG, 52.5, 112.5, 187.5, 277.5, and 390 units; at 0.25 mg PBG, 37.5, 90, 157.5, 240, and 337.5 units; at 0.5 mg PBG, 37.5, 82.5, 145, 195, and 262.5 units. Five figures analogous to Figure 4 were then drawn, plotting for each figure, activity (rate of increase in extinction at 406 nm) against volume of stock deaminase used for one of the five PBG concentrations used. From each of these figures, the volume required to give a rate of 0.02 (ΔE_{406}/min) was read off and from this the activity of the stock deaminase, at each PBG concentration, calculated. Thus, for a given measured rate of activity (corresponding to a constant concentration of hydroxymethylbilane) a double reciprocal plot was drawn. This was then repeated for rates of 0.04, 0.06, 0.08, and 0.10 (ΔE_{406}/min). The five double reciprocal plots were drawn separately and then combined to give Figure 5. The result is a series of double reciprocal plots at different hydroxymethylbilane concentrations.

* One unit of enzyme activity is that producing 1 nmol of uro'gen per hour at 37 °C from PBG (for deaminase) or from hydroxymethylbilane (for cosynthetase) at pH 8.0 and 8.25, respectively. This differs from the unit used for cosynthetase in Part 18,[3] the change being made to set both enzymes on the same basis.

Preparation of Bound Tetrapyrrole (3b) *and Proof of Covalent Binding.*—The aldehyde [3] (8) (10 mg) in dichloromethane (0.6 ml) and methanolic 0.01M-potassium carbonate (0.6 ml) was stirred under argon with sodium borotritiide (*ca.* 10 mCi, 25 μmol) for 8 h at 25 °C. Unlabelled sodium borohydride (10 mg) was then added to reduce the remaining aldehyde (8) and after 10 min, chloroform (2 ml) was added. The solution was washed with saturated brine (3 × 1 ml), and the residue from evaporation was crystallised several times from chloroform–methanol to yield the ^{3}H-labelled hydroxymethylbilane octamethyl ester (6 mg, specific activity 6.3×10^7 d.p.m. mg^{-1}).

This labelled hydroxymethylbilane ester [2 mg, octamethyl ester of (5a)] was hydrolysed at room temperature for 16 h under nitrogen in the dark by mechanically shaking it with aqueous 2M-potassium hydroxide (0.08 ml).

Deaminase was isolated from *Euglena gracilis* by the improved method [1] save that the heat treatment was omitted. It was dialysed against 50 mM-phosphate buffer, pH 8.0, containing 100 μM-dithiothreitol. This preparation was demonstrated by isomer analysis [2] of the product from its action on PBG to be free of cosynthetase.

The foregoing alkaline solution of the bilane (3b) was diluted with water (1 ml) and a portion of this was diluted further 1 : 10 with water. Part of this 10-fold diluted solution (146 μl) was added at 0 °C to a stirred solution of the above deaminase (31 900 units, 0.027 μmol) together with 0.015M-HCl (146 μl) to neutralise the excess of alkali used for hydrolysis. The ratio of enzyme : bilane was *ca.* 1 : 1. After 10 min, the solution was gel-filtered at 4 °C on a column (1.8 × 40 cm) of Sephadex G-50 which had been previously equilibrated with 50 mM-phosphate buffer, pH 8.0, containing 0.6 mM-EDTA and 100 μM-dithiothreitol. Fractions (5 ml) were collected at a flow rate of *ca.* 50 ml h^{-1}.

Those fractions which emerged from the gel column at the correct point for 'deaminase-protein' were combined; they carried 72% of the ^{3}H-activity added originally to the deaminase. This solution was concentrated to half-volume by ultrafiltration (Amicon UM-10 membrane) and 97% of the radioactivity present before filtration was retained in the concentrate.

A solution (4 ml) of the deaminase-bilane (3b) prepared and separated by gel-filtration as above was mixed with 5% aqueous sodium dodecyl sulphate (1 ml) and incubated at 37 °C for 15 min. The solution was then gel-filtered through a column of Sephadex G-50 (1.8 × 40 cm) which had been equilibrated with 50 mM-phosphate buffer, pH 8.0, containing 0.1% sodium dodecyl sulphate. The protein fraction still carried *ca.* 85% of the original ^{3}H-activity, demonstrating covalent binding.

(*a*) *Stability of bound tetrapyrrole* (3b). A sample of the deaminase-bilane (3b) prepared as above (from 3 900 units of deaminase) was incubated at 37 °C in a total volume of 0.4 ml for 15 min. An aliquot (0.1 ml) was then cooled to 0 °C and fractionated on Sephadex G-50 (1.2 × 8 cm) equilibrated as above. The peak corresponding to deaminase carried 84% of the ^{3}H-activity present at the start of the incubation. A second portion of deaminase-bilane was kept at 4 °C for 20 h and then gel-filtered again, *ca.* 33% of the labelled bilane was still covalently bound and the remainder had been released.

(*b*) *Displacement of hydroxymethylbilane* (5a) *by PBG* (1). Half of the concentrated solution above from ultrafiltration (3.5 ml) was mixed with PBG (3.1 mg) and incubated at 37 °C for 15 min. Gel filtration as before showed that the enzyme now retained 4% of the original ^{3}H-activity, with 96% emerging at the position expected for uro'gen; the latter material was examined further below.

(*c*) *Treatment of bound tetrapyrrole* (3b) *with cosynthetase.* To the remaining half of the above concentrated solution of

deaminase-bilane was added the amount of cosynthetase required to ring-close in <5 s all the hydroxymethylbilane originally added to deaminase. The solution was incubated for 15 min at 25 °C and then gel-filtered as for (*b*) above. The deaminase fraction still carried 28% of the ^{3}H-activity, the remainder being in the fraction which emerged appropriately for uro'gen; the latter fraction was examined further below.

The peak fraction from the deaminase emerging from this column (which also contains an excess of cosynthetase) was kept at 4 °C for 20 h and then gel-filtered again. Of the ^{3}H-labelled bilane covalently bound to the enzyme before the storage period, 53% had been released as uro'gen by the end of storage. Control experiments in which the amount of cosynthetase used above was mixed with labelled hydroxymethylbilane and later gel-filtered showed no significant radioactivity in the protein fraction. Further work will be needed to check whether this result (sufficient for the current work) means that cosynthetase does not carry bound bilane or that it does, but the radioactivity is too small to measure reliably because the molar quantity of cosynthetase used is so minute.

(*d*) *Treatment of bound tetrapyrrole* (3b) *with hydroxylamine.* The deaminase-bilane prepared as earlier (0.2 ml of solution) was mixed with 0.14M-hydroxylamine hydrochloride in 0.2M-phosphate buffer pH 8 (50 μl) and incubated at 37 °C for 25 min. Part (125 μl) was then gel-filtered on Sephadex G-50 (1.2 × 8 cm) equilibrated as under (*a*). The protein fraction still carried *ca.* 50% of the original ^{3}H-labelled material and the released ^{3}H-activity appeared from the column at the point expected for the hydroxyaminomethylbilane (9).

Nature of the Uro'gens Produced in Foregoing Experiments (b) *and* (c).—The uro'gen fraction from experiment (*c*) above was mixed with uro'gen-I (2 mg) (freshly prepared from PBG) and with uroporphyrin-III octamethyl ester (3 mg) and then oxidised with iodine as usual [2] before being freeze-dried. The residue was stirred at 25 °C with methanol (2 ml), aqueous 1M-sodium hydrogen carbonate (2.5 ml), Hünig's base (0.5 ml), and trimethyloxonium tetrafluoroborate (0.4 g). After 10 min, more 1M-sodium hydrogen carbonate (1 ml) and the oxonium salt (0.2 g) were added and 10 min later the products were extracted into dichloromethane (3 × 5 ml). The residue from evaporation of the solvent was treated at 25 °C in dichloromethane (1 ml) and methanol (2 ml) with Hünig's base (0.25 ml), solid sodium hydrogen carbonate (100 mg), and trimethyloxonium tetrafluoroborate (200 mg). After 30 min, the organic solution was washed with water, dried, and evaporated to give the uroporphyrin octamethyl esters (3 mg). These were dissolved in dichloromethane (10 ml) and part (1.45 ml) was separated by multiple injections (50 μl each) on a Kontron semi-preparative silica column (500 mm × 12 mm, 5 μ Spherisorb) using hexane–ethyl acetate (1 : 1) [11] at a flow rate of 1.3 ml/min. The separated bands of uroporphyrin-III ester and uroporphyrin-I ester carried, respectively, 90% and 10% of the total ^{3}H-activity.

The product from one run of experiment (*b*) was oxidised with iodine and esterified as above without added carrier; h.p.l.c. showed this product to be essentially pure uroporphyrin-I octamethyl ester. This was confirmed in a second run where unlabelled uro'gen-I (2.5 mg) was added at the end of the ^{3}H-experiment to act as carrier (total activity at this stage 2.84 × 10^5 d.p.m.). Oxidation, esterification, and purification by semi-preparative h.p.l.c. as above showed that only one band appeared containing uroporphyrin-I ester which carried all the ^{3}H-activity (1.3 mg; 1.3 × 10^5 d.p.m. total activity).

Determination of Ammonia Released when PBG Binds to Deaminase.—The materials used were as follows: α-ketoglutarate (Sigma); glutamate dehydrogenase (solution in 1 : 1 water-glycerol, Boehringer) which was dialysed before use against 0.1M-phosphate buffer, pH 7.0; AnalaR ammonium sulphate; deaminase, purified by best procedure [1] and dialysed against 0.1M-sodium phosphate buffer, pH 7.4, containing 0.6 mM-EDTA and 100 μM-dithiothreitol.

The system was calibrated using a standard solution of ammonia, an aliquot of which (50 μl) was added to the assay mixture containing α-ketoglutarate (14.2 μmol), and NADH (0.107 μmol) to which was added glutamate dehydrogenase (0.5 mg) in 0.18M sodium phosphate buffer, pH 7.4 (1 ml) in a 1 cm path-length semi-micro cuvette. The concentration of ammonia in the mixture at the onset of the run was 25 μM; the decrease in absorbance at 340 nm was followed.[9]

Deaminase (0.025 μmol) solution above was mixed with PBG (0.0225 μmol, 0.9 equiv.) at 0 °C and after 30 min at 20—22 °C, the ammonia released was determined using the method and quantities of materials described for the calibration experiments. The reaction was initiated by addition of the glutamate dehydrogenase.

Control experiments were carried out in a strictly parallel way with omission of PBG. All runs were done in triplicate using 3 different preparations of deaminase and all measurements within a given run were at least in duplicate. The runs gave the ammonia released on binding PBG as 1.40; 0.94; 1.37 mol NH_3 per mol of PBG, *i.e.* 1.2 ± 0.25 mol per mol.

N.m.r. Experiments on the Covalent Binding Group of Deaminase.—(*a*) *Enzymic followed by alkaline hydrolysis.* Deaminase [1] (5×10^5 units) in 0.1M-potassium phosphate buffer (1 ml) at pH 8.0 was treated with [11-^{13}C, 9-^{14}C]PBG (0.577 mg in 32.5 μl of solution). This PBG was prepared by mixing [9-^{14}C]PBG lactam ester (0.42 mg, 2.5 × 10^6 d.p.m./mg) with [11-^{13}C]PBG lactam ester (3.15 mg, 90 atom %) followed by hydrolysis as usual in aqueous 2M-potassium hydroxide (0.1 ml); the solution was then adjusted to pH 6.5 by addition of 1M-phosphoric acid (0.1 ml). After the deaminase and PBG had been incubated at 37 °C for 10 min, part (2 ml) of a solution of guanidine hydrochloride (9.55 g) and dithiothreitol (6 mg) in 0.2M-phosphate buffer (12 ml) at pH 7.0 was added. The guanidine concentration in the mixture was 5.5M. The mixture was kept at 37 °C for 30 min after which the major portion (3.012 ml) was applied to a column of Sephadex G-50 (200 ml, 1.5 × 96 cm) and eluted with 0.1M-potassium phosphate buffer, pH 7.35, containing 1M-guanidine hydrochloride and dithiothreitol (15 mg/l); fractions of 4 ml were collected.

Fractions 13—17 contained the deaminase carrying bound pyrroles (total activity 6.55 × 10^4 d.p.m.) and the pooled fractions were concentrated to 1 ml by overnight vacuum dialysis. ^{13}C N.m.r. spectroscopy at this stage showed no signals from enriched ^{13}C-sites. The whole solution was therefore dialysed at 4 °C for 2 × 2 h periods against 0.05M-potassium phosphate buffer (1 l each period), pH 7.0, containing dithiothreitol (15 mg/l). Protein precipitated during the dialysis. This suspension was incubated for 11 h at 37 °C under nitrogen with pronase (2 mg, Boehringer) and dithiothreitol (1 mg). An almost clear solution resulted which was unchanged by further incubation for 3 h with additional pronase (1 mg). The clear supernatant liquid (total 3 ml), after centrifugation of the final product and washing the small pellet (centrifuge again), was fractionated as earlier [1] on a column of Biogel P2 at 4 ml/h, collecting 2 ml fractions. The central part (fractions 16—23) of the band carrying ^{14}C-activity (total 3.15 × 10^4 d.p.m.) was combined and freeze-dried. Examination of the residue by ^{13}C n.m.r. spectroscopy still revealed no clear signals from enriched sites.

The recovered peptide mixture in water (5 ml) was treated

with aqueous 5.6M-potassium hydroxide which rendered the solution 2M in potassium hydroxide (some base used by phosphate). Dithiothreitol (1 mg) was added and the solution was kept at 30 °C under nitrogen for 13.5 h; it was then adjusted to pH *ca.* 6.5 with phosphoric acid. The residue from freeze drying the solution was mixed with water (3 ml), filtered, and then gel-filtered on Sephadex G-25, with 10 mM-phosphate buffer at pH 7 as eluant. The radioactive fractions were combined and freeze dried; the residue contained 2.4×10^4 d.p.m. total activity. It was dissolved in the absolute minimum of water and diluted with methanol, and after removal of the precipitated salts by filtration, the clear solution was evaporated to dryness. ^{13}C N.m.r. spectroscopy (see refs. 1—3 for conditions) now showed signals from enriched sites at δ_C 24.5 (pyrrole-$^{13}CH_2$-pyrrole) and a set of closely spaced signals at δ_C 42—43 (assigned to $-CH_2-NH-^{13}CH_2$-pyrrole).

(*b*) *Sequential enzymic hydrolysis.* Deaminase (3.6×10^5 units) prepared as for the experiments to generate (3b) described above, was treated in solution (26 ml) with [11-^{13}C 9-^{14}C]PBG (471 μl of 834 μM soln.) at 0 °C. The molar ratio of PBG : deaminase was 1.3 : 1. After 35 min, dithiothreitol (15 mg) was added and the temperature was raised to 37 °C. Guanidine hydrochloride was then slowly added to a final concentration of 5.1M and the pH was maintained 7.0—7.5 by dropwise addition of 1M-sodium hydroxide. After the mixture had been incubated at 37 °C for 1 h to denature the enzyme, it was dialysed for 5 h against pH 7.0 50 mM-phosphate buffer (4×1 litre changes) containing dithiothreitol (100 μM). The contents of the dialysis bag, including the precipitate, were incubated with dithiothreitol (5 mg) and pronase (5 mg) for 15 h at 37 °C under nitrogen. Then thermolysin (2 mg, Sigma) and dithiothreitol (2 mg) were added and incubation was continued for 4 h. The clear supernatant liquid from mild centrifugation was checked for radioactivity (94% of the original ^{14}C still present) and freeze dried. A solution of the residue in the minimum of water was centrifuged to remove a little insoluble matter and then gel-filtered on Sephadex G-50 equilibrated with 5 mM-phosphate buffer pH 7.5 containing 50 mM-sodium chloride and 100 μM dethiothreitol. Fractions (6 ml) were collected and the radioactive band contained 75% of the original ^{14}C-activity. The solution of these peptides with attached pyrroles was freeze dried, and the residue dissolved in water (4 ml), and mixed with dithiothreitol (4 mg) and sodium dodecyl sulphate (4 mg in 0.2 ml water). Subtilisin Carlsberg (3 mg, Sigma) was added and the solution was incubated at 37 °C under nitrogen for 17 h. This solution, after centrifugation, was gel-filtered on Sephadex G-50 as above and the radioactive fractions were combined and freeze dried; they contained 62% of the original ^{14}C-activity.

The freeze-dried residue in water (2.5 ml) was fractionated on Sephadex G-25 (1.2×40 cm) when two radioactive bands were observed. One emerged immediately following the void volume and the other at a position shown by control experiments to correspond to a molecular weight of 800—1 000 daltons. This second peak again showed the peak-splitting phenomenon when re-run; this is almost certainly due to salt effects.[12] Suitable re-runs and combination of bands finally allowed all the fractions containing pyrrole carrying peptides of *M* 800—1 000 to be collected together and freeze dried. These carried 24% of the original radioactivity and were examined by ^{13}C n.m.r. spectroscopy. The same set of signals was observed in the δ_C 42—43 region as had appeared in (*a*) above.

Effect of Deaminase on Cosynthetase Activity.—Hydroxymethylbilane (0.1 mg) was incubated at 25 °C in 0.175M-Tris/HCl buffer pH 8.25 (0.5 ml) to which was added (*a*) nothing, (*b*) deaminase (675 units), (*c*) cosynthetase (5 000 units), or (*d*) deaminase and cosynthetase. Samples (0.05 ml) were withdrawn and placed immediately into 7% trichloroacetic acid containing iodine. The uro'gen produced was thus estimated as uroporphyrin at 406 nm. The results are shown in Figure 6.

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Received 1*st June* 1983; *Paper* 3/877