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Chemical and Enzymic Studies on Biosynthesis of the Natural Porphyrin Macrocycle: Formation and Role of Unrearranged Hydroxymethylbilane and Order of Assembly of the Pyrrole Rings

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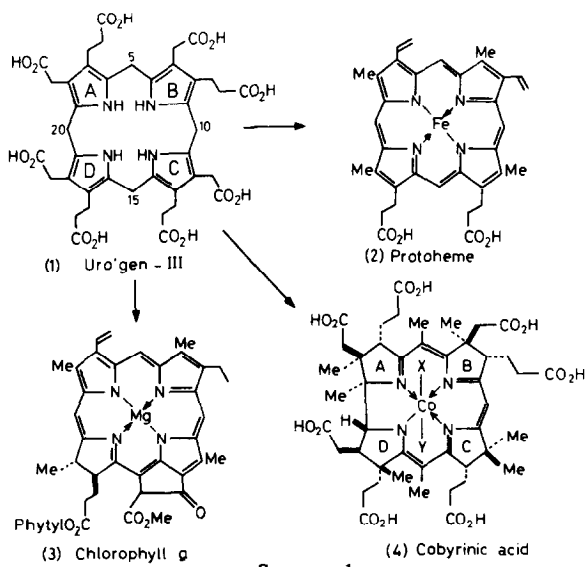
Following a review of the background to current research, it is shown that the action of deaminase alone on porphobilinogen leads to release into the medium of the unrearranged hydroxymethylbilane (**20**), which (*a*) ring closes chemically to uro'gen-I and (*b*) is very rapidly converted by deaminase–cosynthetase into uro'gen-III. The biosynthetic role of this $HOCH_2$-bilane is studied, and the results show that deaminase is a builder of the unrearranged bilane system but is *not* a cyclizing enzyme. The work also shows that deaminase and cosynthetase have independent active sites but that they may work in close physical association. Finally, it is established that the tetrapyrrole macrocycle is assembled starting at ring A and building around to ring D.

INTRODUCTION

The tetrapyrrole macrocycles of protoheme (**2**),[1] chlorophyll (**3**), the cytochromes and cobyrinic acid (**4**), the precursor of vitamin B_{12}, are all derived in living systems from uroporphyrinogen-III (**1**) (abbreviated to uro'gen-III throughout our paper).



SCHEME 1

[1] The boldface numbers in parentheses refer to the corresponding structures presented in this paper.

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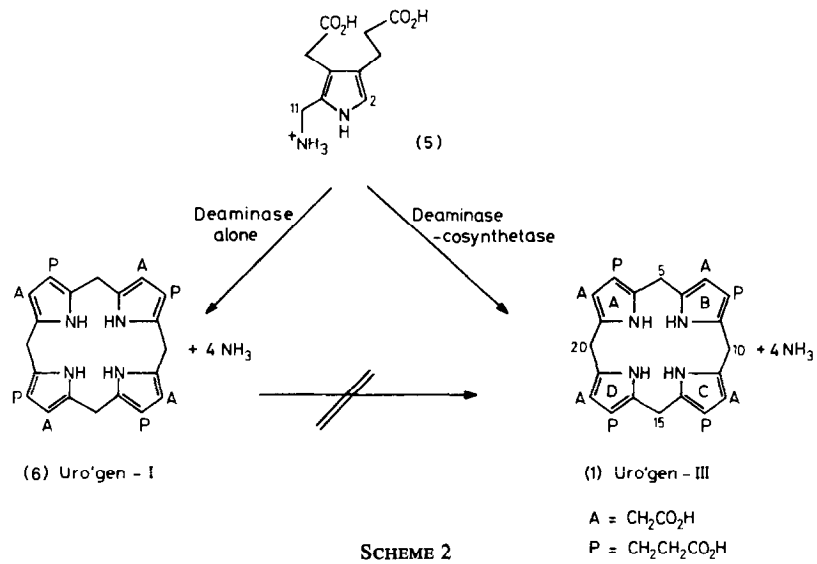
This central position for uro'gen-III in a family of immense biological importance has led to intense interest in its biosynthesis. The process has a special fascination for the organic chemist because of the remarkable rearrangement which must have occurred to produce the unexpected uro'gen-III structure (**1**). Knowledge to January 1978 has recently been reviewed (*1*) and the key discoveries are briefly as follows; these form the essential base for an understanding of the most recent work to be described here.

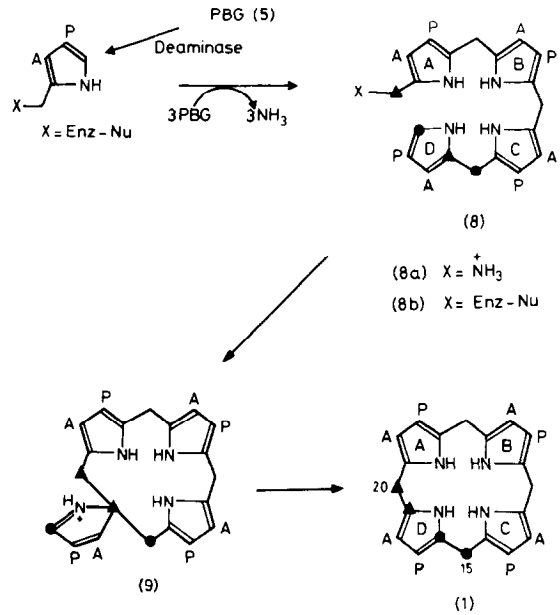
Shemin, Granick, Bogorad, Neuberger, and Rimington had shown in pioneering studies (*2*) that *two enzymes* called *deaminase* and *cosynthetase* are required to catalyse the conversion of 4 mol of the monopyrrole, porphobilinogen (PBG) (**5**) into uro'gen-III (**1**) and ammonia (Scheme 2).



SCHEME 2

In the absence of cosynthetase, deaminase catalyses the conversion of PBG into uro'gen-I (**6**), an unnatural isomer, though the one to be expected from simple head-to-tail combination of four PBG units. Importantly, uro'gen-I (**6**) is not transformed into uro'gen-III (**1**) by cosynthetase nor by deaminase–cosynthetase together. The crucial question thus was, "How does deaminase–cosynthetase build the unexpected type III isomer from PBG with rearrangement at some stage?"

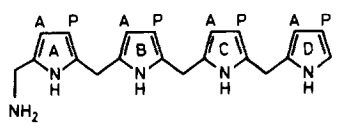
Extensive studies based heavily on ^{13}C-labelling in combination with nmr spectroscopy eventually established (*1*) that the biosynthesis of uro'gen-III, and so of the pigments of life listed at the outset, involves the following steps. Four PBG units (**5**) are enzymatically joined together head-to-tail to form the unrearranged bilane (**8**) where X could at this stage be either the original amino group (**8a**) or some nucleophile which has replaced the amino group; this nucleophile could be a group in the enzyme active site (**8b**). In the presence of cosynthetase, the bilane (**8**) is then rearranged as an intact molecule by an *intramolecular process* which directly affects only ring D and the two carbons which become C-15 and C-20 of uro'gen-III (**1**). Rings A, B, and C of uro'gen-III with their attached carbons C-20, C-5, and C-10, respectively, are provided from intact PBG units without rearrangement. During the rearrangement step which converts



the bilane (**8**) into uro'gen-III (**1**), it was proved that the atoms marked ● become bonded intramolecularly, as do the atoms ▲. The spiro system (**9**) was considered to be a likely intermediate for the rearrangement (*3*).

ENZYMIC STUDIES WITH ISOMERIC BILANES

The intact conversion of the unrearranged bilane (**8**) into uro'gen-III (**1**) by deaminase–cosynthetase was in sharp contrast to claims (*4*, *5*) that the rearrangement occurs at the dipyrrolic level. If these claims were well founded, then one of the *rearranged* bilanes (**10–14**) ought to be enzymically cyclized to uro'gen-III (**1**) far better than the unrearranged isomer (**8a**).



(8a)

P A A P A P A P (10)

A P P A A P A P (11)

A P A P P A A P (12)

A P A P A P P A (13)

P A P A P A A P (14)

TABLE 1

ACTION OF DEAMINASE–COSYNTHETASE FROM *Euglena gracilis* ON ISOMERIC BILANES

Substrate	V_{max}[a]	K_m (μM)	Uro'gen isomers produced (%)							
			Enzymic				Chemical			
			I	II	III	IV	I	II	III	IV
Unrearranged bilane (**8a**)	64 ± 1	5.1 ± 0.2	8	0	90	2	95	0	4	1
Reversed ring A (**10**)	—	—	3.5	0	91.5	5	3.5	0	94.5	2
Reversed ring B (**11**)	25 ± 1	72 ± 3	0	8.5	90	1.5	0	5.5	93	1.5
Reversed ring C (**12**)	—	—	0	2.5	90	7.5	0	2	94.5	3.5
Reversed ring D (**13**)	7.7 ± 0.2	11 ± 1	22	0	76	2	9.5	0	88	2.5
Reversed rings ABC (**14**)	—	—	2.5	0	94.5	3	2.5	0	95.5	2
PBG (**5**)	1000	104 ± 7								

[a] V_{max} is arbitrarily set at 1000 for PBG and the rest are referred to this; for further details see Ref. (*6*).

This remote possibility was tested by synthesizing (*6*) each of the bilanes (**10–14**) and comparing the rates of their conversion into uro'gens, both chemically and enzymically, by deaminase–cosynthetase. All these bilanes cyclized chemically to uro'gens of which 91 ± 3% in each case represented the structure expected from straightforward ring closure; the quantitative isomer analyses were carried out (*7*) by high-pressure liquid chromatography (hplc). The enzymic results (Table 1) showed that none of the rearranged bilanes matched the unrearranged system (**8a**) which had the highest V_{max} and the lowest K_m. At low substrate:enzyme ratio, bilane (**8a**) gave uro'gen-III (**1**) which was 98% pure. Also noteworthy is the observation that the bilane (**13**), with ring D already reversed, is enzymically transformed in part into uro'gen-I (**6**).

These findings confirm our earlier conclusion that rearrangement occurs on the unrearranged bilane (**8**), but the rate at which uro'gen-III (**1**) is produced from the aminomethylbilane (**8a**) is slower than the rate at which it is produced from PBG (**5**). Thus the $\overset{+}{N}H_3CH_2$-bilane cannot be a *free* intermediate; and the possibility that it could be enzymically modified to give the bound form (**8b**) had been stressed earlier (*1*, *2*, *6*), following the results of attempted incorporation of aminomethylpyrromethanes (*1*, *2*) by deaminase–cosynthetase into uro'gen-III (**1**). These results led us to propose a scheme (*1*, *2*) in which the first PBG binds to the enzyme with nucleophilic displacement of the amino group to give (**16**), which remains so bound until three further PBG units have been added to the first in a head-to-tail fashion. On this view, the tetrapyrrolic intermediate (**8b**) is generated more slowly from the $\overset{+}{N}H_3CH_2$-bilane (**8a**) than from four PBG molecules.

FORMATION OF UNREARRANGED HYDROXYMETHYLBILANE

Treatment of the $\overset{+}{N}H_3CH_2$-bilane (**8a**) with deaminase alone at pH 7.75 accelerated the ring closure to uro'gen-I (**6**) relative to the rate of chemical cyclization (*6*), but there

was a distinct lag in the formation of uro'gen-I (**6**). Accordingly, a similar study was made by treating PBG (**5**) with deaminase, wherein the lag was very pronounced (see Fig. 1); there was no lag in formation of uro'gen-III (**1**) from PBG or from the bilane (**8a**) when deaminase–cosynthetase was used. The lag with deaminase alone pointed to an intermediate substance being released into the medium, and its structure was determined (*8*) by the following experiments.

First, the substance was generated enzymically from PBG, and it was allowed to ring close chemically at pH 7.75. The product was >99% uro'gen-I (**6**); thus four pyrrole units have been joined head-to-tail. A second sample of the substance was similarly formed enzymically from [11-^{13}C] PBG (as **5**) by stopping the reaction after 7 min (adjustment to pH 12), when 82% of the original [^{13}C]PBG had been consumed. The

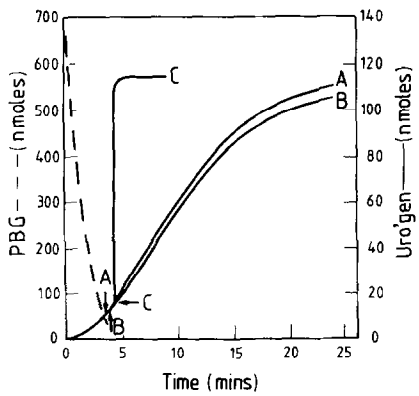


FIG. 1. Enzymic formation of hydroxymethylbilane (**20**) with subsequent ring closure: (A) without added enzyme; (B) with additional deaminase; (C) with added deaminase–cosynthetase.

total mixture was then examined by ^{13}C-nmr with ^{1}H-noise decoupling to give Fig. 2; when off-resonance decoupling was used, all four signals appeared as *triplets*.[2] Thus, all four ^{13}C-labelled carbons are methylenes,

$$^{13}CH_2\!<$$

The low-field signal P corresponded to HO^{13}CH$_2$-pyrrole and the signal Q to pyrrole-^{13}CH$_2$-pyrrole; the integral P:Q was 1:3. Signal R arises from unconsumed [11-^{13}C]-PBG (as **5**), and signal S was from the bridge methylenes of the symmetrical uro'gen-I (**6**). The foregoing signals were assigned unambiguously from the ^{13}C-spectra of synthetic bilanes (*9*, *10*), uro'gens, and hydroxymethylpyrroles (see later) which were specifically ^{13}C-labelled at known sites.

It follows from the chemistry and spectroscopy of the previous paragraph that the substance released into the medium during the lag in the formation of uro'gen-I (**6**) from

[2] Shortly before our proof of structure of the species accounting for the lag, Professor A. I. Scott *et al.* had studied by ^{13}C-nmr the action of deaminase on [2,11-^{13}C]PBG. The observed signals led to structures being proposed for the released substance which are quite different from that established here. Further, in their hands the released substance was converted into uro'gen-III by cosynthetase. We warmly thank Professor Scott for sending us preprints of his important work.

PBG (**5**) by deaminase alone is the *unrearranged hydroxymethylbilane* (**20**), Scheme 4 (*8*). Looking back to our suggestion (*1*, *2*, *6*) that the amino group of the ring-A PBG unit of bilane (**8b**) probably is replaced enzymically by a nucleophile during the building process, we now recognize clearly that the *final* nucleophile is water when deaminase acts alone.

It is important to realize that the $HOCH_2$-bilane (**20**) is not produced chemically from the amino analogue (**8a**) at the same pH when enzyme is omitted. However, the

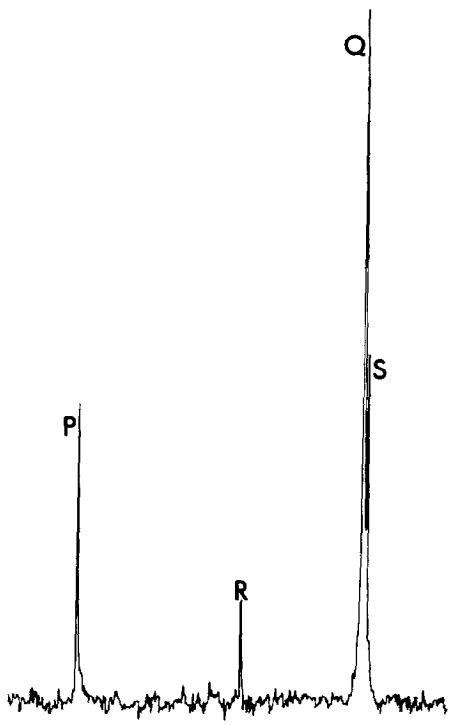


FIG. 2. ^{1}H-Noise decoupled ^{13}C-nmr spectrum of product from action of deaminase on [11-^{13}C]PBG. Signal P: $HOCH_2$·pyrrole, δ 57.21; signal Q: bilane CH_2 < $(pyrrole)_2$, δ 24.46; signal R: H_2NCH_2 of residual PBG, δ 38.35; signal S: uro'gen CH_2 < $(pyrrole)_2$, δ 23.97. All δ referred to $Me_3SiCD_2CD_2CO_2Na$.

$HOCH_2$-bilane (**20**) could conceivably have arisen from hydrolysis at high pH of an initially formed lactone (**23**). This was eliminated by showing that exactly the same four signals of Fig. 2 appeared when the ^{13}C-spectrum was run rapidly without raising the pH.

The $HOCH_2$-bilane (**20**) was shown to be the end product of the action of deaminase on PBG (**5**) by generating it as before and allowing it to ring close chemically from point *A* (Fig. 1). A second equivalent portion of (**20**) was treated at point *B* (Fig. 1) with additional deaminase. The two rates *A* and *B* of uro'gen-I formation were essentially the same. Clearly *deaminase alone is not an enzyme for ring closure* and the product of its reaction with PBG is the unrearranged hydroxymethylbilane (**20**).

ACTION OF DEAMINASE–COSYNTHETASE ON HYDROXYMETHYLBILANE

The action of deaminase–cosynthetase on the hydroxymethylbilane (**20**) was in striking contrast to the above results with deaminase alone. Addition of deaminase–cosynthetase to the $HOCH_2$-bilane (**20**) at point *C* (Fig. 1) caused almost instantaneous *ring closure and rearrangement*, the product being uro'gen-III (**1**), shown by hplc to be 98% the type III isomer. It is because of this extremely rapid process that no lag is observed in the formation of uro'gen-III (**1**) when deaminase–cosynthetase acts on PBG (**5**) or on the aminomethylbilane (**8a**). Indeed, the hydroxymethylbilane (**20**) may be a true biosynthetic intermediate following Enz · Nu-bilane (**8b**) on the pathway to uro'gen-III (**1**); but one other possibility needs to be borne in mind, and this will be considered after exploring further the chemical and enzymic reactivity of the $HOCH_2$-bilane (**20**) in the next section.

REACTIVITY OF HYDROXYMETHYLBILANE (20)

The important studies of Radmer and Bogorad (*11*) and Davies and Neuberger (*12*) had shown that when PBG (**5**) was incubated with deaminase alone in the presence of NH_4^+, or NH_2OH or NH_2OMe, the formation of uro'gen-I (**6**) was inhibited and, respectively, the $\overset{+}{N}H_3CH_2$-bilane (**8a**) or the analogous $HONHCH_2$-bilane (**21**) or $MeONHCH_2$-bilane (**22**) was produced. In the light of our recent work outlined above these compounds could have arisen either from the $HOCH_2$-bilane (**20**) by chemical (nucleophilic) displacement or by trapping some more reactive enzyme-bound intermediate. The latter was shown to be correct by a series of double-labelling experiments.

First, [*amino*-^{15}N]PBG (as **5**) was synthesized from the corresponding ^{15}N-labelled oxime to show by natural abundance cmr that J for $^{13}C-^{15}N$ of PBG (as **5**) at pH > 12 was 3.64 Hz. Then incubation of [11-^{13}C]PBG, synthesized earlier (*10*), with deaminase and $^{15}NH_4^+$ under essentially Radmer and Bogorad's conditions (*11*) was followed by cmr of the resultant bilane at pH > 12 which showed essentially complete formation of $^{15}NH_2{}^{13}CH_2$-bilane (as **8a**) with $J = 3.68$ Hz. Thus at some stage in the experiment with deaminase and NH_4^+, the amino group of the ring-A PBG unit is replaced by $^{15}NH_3^+$, exactly as it is in the enzymic runs with NH_2OH and NH_2OMe outlined above.

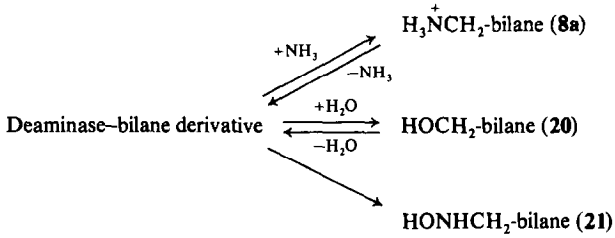
It was then necessary to determine whether chemical displacement of amino from the aminomethyl group could have occurred. Accordingly, [11-^{13}C]PBG (as **5**) and [*amino-methyl*-^{13}C]bilane (as **8a**) were treated separately with 0.5 *M* $^{15}NH_4^+$ or with 0.33 *M* $^{15}NH_2OH$ at pH 8.2–8.3 in the nmr tube for 4 h at 37°C. The spectra accumulated over that period showed no splitting of the $NH_3{}^+CH_2$-pyrrole signal in either case for the $^{15}NH_4^+$ runs and no significant formation of the HONH analogue of PBG (**18**) nor of the $HONHCH_2$-bilane (**21**).

Having thus eliminated the possibility of nonenzymic nucleophilic displacement causing $\overset{+}{N}H_3CH_2$-pyrrole → $HONHCH_2$-pyrrole, we turned our attention to the hydroxymethyl series. The [2,11-^{13}C]hydroxy analogue of PBG (as **17**) and the [^{13}C]-$HOCH_2$-bilane (**20**), generated from [11-^{13}C]PBG, were treated separately as above

with NH_2OH at pH 8.3 in the absence of deaminase. In neither case did any significant formation of $HONHCH_2$-pyrrole occur as shown by cmr.

Finally, the ^{13}C-labelled $HOCH_2$-bilane (**20**) was treated with deaminase and NH_2OH and, strikingly, the cmr spectrum showed essentially complete formation of $HONHCH_2$-bilane (**21**).

The foregoing results show that for deaminase alone, the nucleophiles NH_3, NH_2OH, and NH_2OMe are trapping an intermediate, probably enzyme bound, which is more reactive than the $HOCH_2$-bilane (**20**). The relationships of the various species are shown in Scheme 3. We propose that the reactive bilane derivative is the enzyme-stabilized methylenepyrrolenine (**19**).



SCHEME 3

The extremely rapid conversion of $HOCH_2$-bilane (**20**) into uro'gen-III (**1**) by deaminase–cosynthetase was described above. It is so rapid that when the $HOCH_2$-bilane (**20**) is mixed with 0.2 *M* NH_2OH and then treated with deaminase–cosynthetase, ca. 60% of it is converted into uro'gen-III (**1**) [>98% type III] and ca. 40% appears finally as trapped $HONHCH_2$-bilane[3] (**21**), which was proved to be the *unrearranged* bilane. Yet when PBG (**5**) was treated under the same conditions with NH_2OH and deaminase–cosynthetase, only the trapped unrearranged $HONHCH_2$-bilane (**21**) was formed in agreement with Davies and Neuberger (*12*). These results, like those above, are important for the later discussion of the mode of action of deaminase–cosynthetase. First, however, there is some additional information from studies with the hydroxy analogue of PBG (**17**).

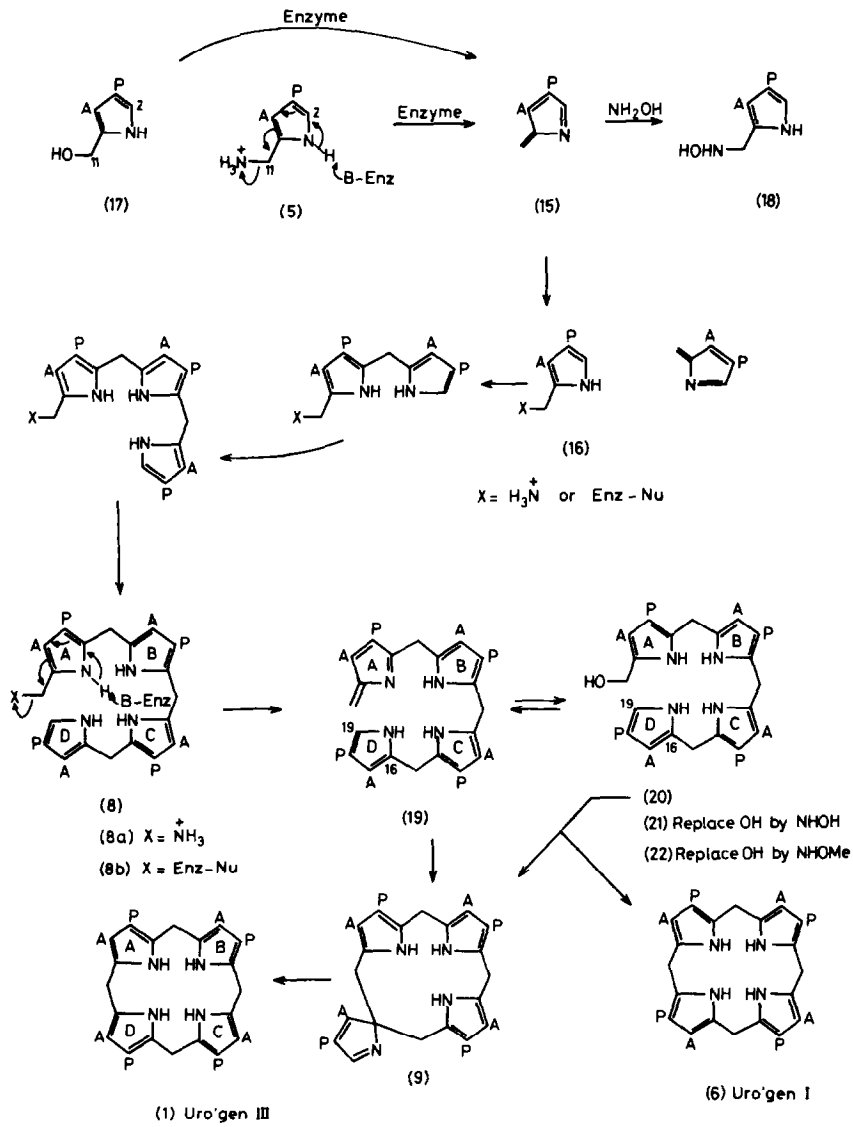
THE HYDROXY ANALOGUE OF PBG

This was synthesized in 2,11-^{13}C-labelled form (as **17**) from the corresponding aldehyde to show that the ^{13}C-signal for $HOCH_2$-pyrrole appears at pH >12 at δ 57.27. The hydroxy analogue acts as a good substrate for deaminase–cosynthetase, the rate of ring closure being ca. one-third that of PBG itself; the product was uro'gen-III (**1**), >98% type III. The hydroxy analogue was also converted into uro'gens by deaminase, with a clear lag in keeping with formation once again of the $HOCH_2$-bilane (**20**). When the hydroxy analogue of PBG was allowed to cyclize chemically at pH 7.5, it produced uro'gens at ca. 4% of the rate catalysed by deaminase–cosynthetase and the isomer composition was type I (65%), type II (0%), type III (24%), and type IV (11%).

[3] Some of this trapping could have arisen because of lack of certainty that an excess of cosynthetase was present; work continues.

NATURE OF CATALYSIS BY DEAMINASE AND COSYNTHETASE

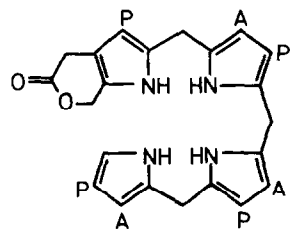
All the results described here and earlier (*1, 2, 11, 12*) are in accord with Scheme 4. Deaminase is not a ring-closing enzyme, and its role is to assemble four PBG units in a head-to-tail manner. It is probable[4] that the first PBG unit is covalently bonded to the enzyme through a nucleophilic group X; and isolation (*12*) of the PBG analogue (**18**) from the action of deaminase on PBG (**5**) in the presence of NH_2OH, but not in its absence, supports the enzymic formation of pyrrolenine (**15**) ready for such a binding



SCHEME 4

[4] Covalent bonding to an enzymic X group does not yet have *overwhelming* support but it seems likely.

to the enzyme. A second, third, and fourth unit of (**15**), generated as before from PBG, can then be added sequentially to give the bound bilane (**8b**), which by a fourth repetition of the same elimination could form pyrrolenine (**19**). In the absence of cosynthetase, (**19**) reacts with water, the water probably being held in the active site (since otherwise lactone formation to (**23**) would be expected to predominate) to form the $HOCH_2$-bilane (**20**), which cyclizes chemically to uro'gen-I (**6**).



(23)

Added small nucleophiles such as NH_3, NH_2OH, or NH_2OMe probably enter this region of the enzyme to trap the reactive tetrapyrrole with release of the various unrearranged bilanes already described (**8a**, **21**, **22**).

With the linear tetrapyrrole system having been built by deaminase, the stage is set for cosynthetase to play its part. There are three possible ways in which deaminase and cosynthetase could function:

(*a*) Deaminase could be the only true enzyme, and its active site builds the regular bilane. Cosynthetase could be a "modifier" protein which does not act on a substrate but rather binds to deaminase to change its conformation, so allowing uro'gen-III (**1**) to be formed rather than uro'gen-I (**6**).

(*b*) Deaminase and cosynthetase could be independent enzymes such that *in their natural state* the product from deaminase is released into the medium to be picked up by cosynthetase for ring closure with rearrangement.

(*c*) Deaminase and cosynthetase have independent active sites but the two enzymes are closely associated by physical binding, the formation of uro'gen-III taking place within the complex.

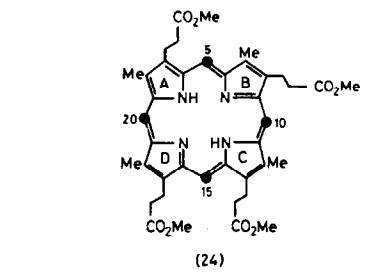
The experiments with deaminase alone and deaminase–cosynthetase on PBG plus NH_2OH as compared with those in parallel studies with the $HOCH_2$-bilane (**20**) and NH_2OH point against possibility (*a*), and this has been confirmed by experiments in progress (*13*) with deaminase-free cosynthetase which establish that deaminase and cosynthetase can function independently *in the test tube*. The distinction between the closely related possibilities (*b*) and (*c*), though real, is subtle. Under normal conditions *in vivo*, the two proteins may be associated, since there is physical evidence (*14*, *15*) that they do mutually bind. Also, we find K_m for deaminase with PBG is $195 \pm 10\,\mu M$, whereas for deaminase–cosynthetase the K_m is $104 \pm 7\,\mu M$, and that K_m with deaminase for H_2NCH_2-bilane (**8a**) is ca. twice that for the combined enzymes. But further work is in hand to check whether these K_m determinations are being affected by competitive inhibition by $HOCH_2$-bilane. So a clear decision between the close possibilities (*b*) and (*c*) *in vivo* cannot yet be made. If it turns out that possibility (*c*)

holds, the tetrapyrrole transferred from deaminase to cosynthetase could be either the pyrrolenine (**19**) or the unrearranged $HOCH_2$-bilane (**20**) ready for cosynthetase to complete the ring closure with rearrangement. The most attractive intermediate for this final stage is the spiro system (**9**).

ORDER OF ASSEMBLY OF THE FOUR PBG UNITS

The problem is to discover whether the first PBG unit bound to deaminase–cosynthetase eventually becomes ring A of uro'gen-III (**1**) followed by addition sequentially of ring B, ring C, and finally ring D or vice versa.

The solution depended on developing ways to obtain purified, concentrated preparations of deaminase–cosynthetase in large amounts so that we could add significantly less than one equivalent of unlabelled PBG. The aim was to ensure that the major species present was the putative enzyme-bound pyrrole (**16**) (Scheme 4), and that a smaller quantity of the dipyrromethane species (Scheme 4) would be present. Still less would be expected of the tripyrrole system, and least material of all would be in the form of the bilane (**8b**). An excess of 90 atom% [11–^{13}C]PBG (as **5**) was then added to chase through the bound PBG to form uro'gen–III (**1**). This was oxidized to the corresponding porphyrin which was decarboxylated chemically to coproporphyrin-III, and the corresponding tetramethyl ester (**24**) was examined by ^{1}H-nmr in the presence of Eu(fod)$_3$.



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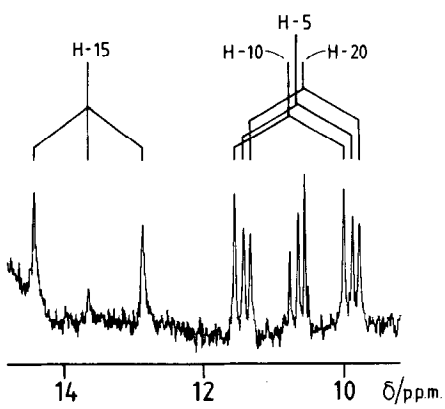

FIG. 3. ^{1}H-Signals from *meso* bridges of coproporphyrin-III tetramethyl ester (**24**) in presence of Eu(fod)$_3$ from experiment to determine order of assembly of rings.

The signal assignments in Fig. 3 are unambiguously based on synthetic, specifically ^{13}C-labelled samples of (**24**). The spectrum shows clearly that ^{13}C enrichment is greatest at C-15, and the ^{13}C level at the other bridge carbons falls in the order C-10 > C-5 > C-20. Thus the first PBG to be bound becomes ring A and C-20, the second ring B and C-5, the third ring C and C-10, with the last, largely derived from the added [11-^{13}C] PBG becoming ring D and C-15.

Not only does this order experiment have its intrinsic interest, but it also showed that the bound bilane, built naturally from PBG, is registered as in (**8b**) relative to the final uro'gen-III (**1**). This is exactly the way in which the $\overset{+}{N}H_3CH_2$-bilane (**8a**) is bound and registered; so all the double-labelling data from that source gained additional strength from this complementarity.

CONCLUSIONS AND PROSPECT

This survey has added the latest findings on the way living systems synthesize the uro'gen-III macrocycle (**1**), and an almost complete picture is now available. Scheme 4 briefly presents the pathway but the reader will have appreciated that much detailed knowledge from precise double-labelling experiments is also available (see (*1*)).

There are, however, a few outstanding points. For example, direct information is needed about the nature of the intermediate in the rearrangement step [spiro system (**9**)?] and also the nature of the X group in Scheme 4.

There will be considerable interest in the outcome of current research on these aspects, and the answers will fill in the last few strokes on what is by now an essentially complete canvas.

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