Spectroscopic Evidence for a Porphobilinogen Deaminase–Tetrapyrrole Complex That Is an Intermediate in the Biosynthesis of Uroporphyrinogen III†

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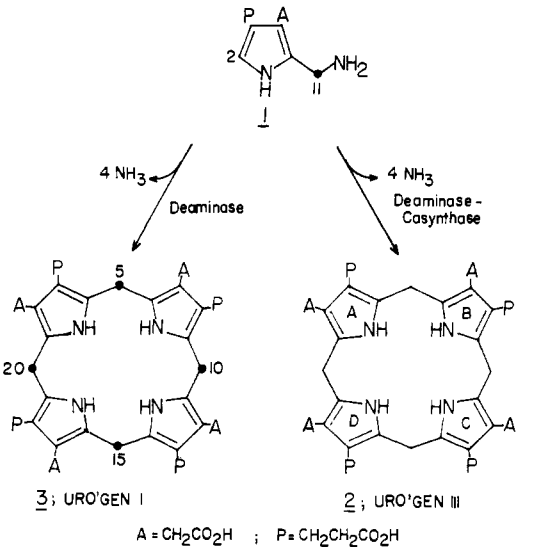
ABSTRACT: Incubation of porphobilinogen (PBG) with PBG deaminase from *Rhodopseudomonas sphaeroides* in carbonate buffer (pH 9.2) to total PBG consumption resulted in low yields of uroporphyrinogen I (uro'gen I). In the reaction mixture a pyrrylmethane accumulated, which at longer incubation periods was transformed into uro'gen I. The accumulated pyrrylmethane gave an Ehrlich reaction which was different from that of a 2-(aminomethyl)dipyrrylmethane or 2-(aminomethyl)tripyrrane. It resembled that of a bilane (tetrapyrrylmethane) but was different from that of a 2-(hydroxymethyl)bilane. The ^{13}C NMR spectra of incubations carried out with [11-^{13}C]PBG indicated that the pyrrylmethane was a tetrapyrrole with methylene resonances at 22.35–22.50 ppm. It was loosely bound to the deaminase, and when separated from the enzyme by gel filtration or gel electrophoresis, it immediately cyclized to uro'gen I. No enzyme-bound methylene could be detected by its chemical shift, suggesting that its line width must be very broad. When uro'gen III–cosynthase was added to the deaminase–tetrapyrrole complex, uro'gen III was formed at the expense of the latter in about 75% yield. The tetrapyrrole could only be partially displaced from the enzyme by ammonium ions, although a small amount of 2-(aminomethyl)bilane was always formed together with the tetrapyrrole intermediate. A protonated uro'gen I structure for this intermediate was ruled out by incubations using [2,11-^{13}C]PBG. Uro'gen III formation from 2-(hydroxymethyl)bilane (HMB) and from the deaminase–tetrapyrrole intermediate was compared by using deaminase–cosynthase and cosynthase from several sources. It was found that while the HMB inhibited uro'gen III formation at higher concentrations and longer incubation times, uro'gen III formation from the complex did not decrease with time.

Uroporphyrinogen III (**2**) (uro'gen III)[1] is the hexahydroporphyrin that is the natural precursor of heme, chlorophylls, and vitamin B_{12} (Frydman et al., 1979). It is formed in nature by the condensation of 4 mol of the pyrrole porphobilinogen (**1**)(PBG) in a reaction catalyzed by two enzymes, porphobilinogen deaminase[2] (EC 4.3.1.8) and uroporphyrinogen III cosynthase (EC 4.2.1.75). Under the action of deaminase four molecules of substrate (PBG) are condensed in a head-to-tail fashion (with liberation of 4 mol of ammonia) to give uro'gen I (**3**) (Scheme I). In the presence of cosynthase uro'gen III (**2**) is formed, although PBG is not a substrate of the cosynthase. The structural difference between uro'gen III (**2**) and uro'gen I (**3**) is the inversion in the order of the substituents in ring D found in uro'gen III (**2**), which is at variance with the alternate order of these substituents found in uro'gen I (**3**). This inversion has long been recognized as a mechanistic problem par excellence, and it has been the subject of long and intrincate studies (Frydman et al., 1979; Battersby, 1986; Scott, 1986). Two types of approaches have been used to examine the problem of how ring D inversion is produced when the enzymatic polymerization of PBG (**1**) is catalyzed by the action of deaminase in the presence of cosynthase. The older approach made use of synthetic 2-(aminomethyl)dipyrrylmethanes, 2-(aminomethyl)tripyrranes, and 2-(aminomethyl)bilanes, which were assayed as possible intermediates of the enzymatic reaction (Sburlati et al., 1983; Frydman et al., 1978a,b; Battersby et al., 1981; Scott et al., 1976; Franck et al., 1980). The results obtained in these studies were, however, inconclusive since they were bedeviled

Scheme I



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[1] Abbreviations: uro'gen, uroporphyrinogen; PBG, porphobilinogen; HMB, 2-(hydroxymethyl)bilane; PMSF, phenylmethanesulfonyl fluoride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; NMR, nuclear magnetic resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; λ_{max}, visible absorption maximum; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Trisacryl, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-2-propenamide.

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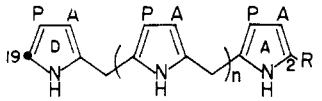
by very low incorporations of the synthetic (aminomethyl)-pyrrylmethanes into the uro'gens formed and by the fact that the chemical self-condensation of the former also led to the formation of uro'gens which had to be discriminated from the enzymatic reaction products. It was concluded, however, that the 2-(aminomethyl)bilane **4** formally derived from the



4; n = 2, R = CH_2NH_2
5; n = 2, R = CH_2OH
6; n = 2, R = =CH_2
7; n = 0, R = CH_2NH_2
8; n = 1, R = CH_2NH_2

A = CH_2CO_2H; P = $CH_2CH_2CO_2H$

head-to-tail condensation of PBG (**1**) was cyclized by the deaminase–cosynthase system in a reaction where an inversion of ring D took place and uro'gen III (**2**) was therefore formed (Battersby et al., 1981). The cyclization reaction of **4** with ring inversion to form uro'gen III (**2**) could not be detected at low enzyme concentrations (Díaz et al., 1979) but was confirmed when higher concentrations of deaminase–co-synthase were used (Sburlati et al., 1983). However, when large amounts of deaminase–cosynthase were used, 2-(aminomethyl)bilanes isomeric with **4** were also found to undergo a ring D inversion process during their cyclization to uro'gens, in several cases more efficiently than bilane **4** (Sburlati et al., 1983). The interaction of the 2-(aminomethyl)bilanes with the deaminase–cosynthase system was also strongly dependent of the enzyme's source.

The second approach used to examine the problem of uro'gen III (**2**) formation was to study the enzymatic polymerization of [^{13}C]PBG by deaminase while the course of the reaction was monitored by ^{13}C NMR spectroscopy. This method allowed the identification of an intermediate released by the deaminase that was found to be a substrate of cosynthase (Burton et al., 1979; Jordan et al., 1979). The structure of this intermediate was shown to be 2-(hydroxymethyl)bilane **5** (HMB) (Battersby et al., 1979). The sequence of uro'gen III (**2**) formation from PBG (**1**) was therefore accepted to be a reaction catalyzed sequentially by the two enzymes; the deaminase that formed HMB **5** from PBG (**1**) (and was therefore called hydroxymethylbilane synthase), and the cosynthase that cyclized **5** with inversion of ring D to give uro'gen III (**2**). Formation of uro'gen I (**3**) was attributed to the fast ($t_{1/2}$ = 4 min at 37 °C) chemical cyclization of HMB **5** to give **3** in the absence of cosynthase. Recent work (Evans et al., 1986) where the polymerization of [^{3}H]PBG (**1**) by deaminase was monitored by using ^{3}H NMR spectroscopy showed, however, that uro'gen I (**3**) could be formed although HMB **5** was not detected. This was attributed to the high protein concentrations used in these experiments, which will stabilize an azafulvene species (**6**) released from the enzyme before **5** is formed.

It was known that under normal conditions no intermediates between PBG (**1**) and the tetrapyrrole were liberated into solution, the deaminase preferring to synthesize the latter from PBG by a mechanism involving only enzyme-bound intermediate species (Frydman et al., 1976). These enzyme-bound species were first identified by incubating deaminase from human erythrocytes with [^{3}H]PBG, which allowed detection by electrophoresis of the formation of charged isomers corresponding to pyrrylmethanes covalently bound to the enzyme (Anderson & Desnick, 1980). Four labeled protein bands were identified and attributed to mono-, di-, tri-, and tetrapyrrole intermediates. The existence of pyrrylmethane intermediates covalently bound to the deaminase was also detected by using enzyme from *Rhodopseudomonas sphaeroides*, although only the mono-, di-, and tripyrrylmethane enzyme-bound intermediates were detected (Jordan & Berry, 1981; Berry et al., 1981).

These enzyme–intermediate complexes where the pyrrylmethanes were covalently bound to the deaminase were found to be unusually stable and could be handled without liberation from the enzyme or further polymerization to give uro'gen I (**3**). However, in recent experiments carried out by using deaminase from the same source under conditions where mainly the monopyrrole complex was formed, the latter was found to be unstable and to rearrange to give uro'gen I (**3**) at room temperature (Evans et al., 1986).

In this paper we describe the detection by ^{13}C NMR spectroscopy of a loosely bound deaminase–tetrapyrrole complex formed by incubation of deaminase from *R. sphaeroides* with [^{13}C]PBG, which can serve as an efficient substrate of cosynthase to form uro'gen III (**2**). The formation of the latter from HMB **5** will also be discussed in connection with the mechanism of uro'gen III (**2**) formation.

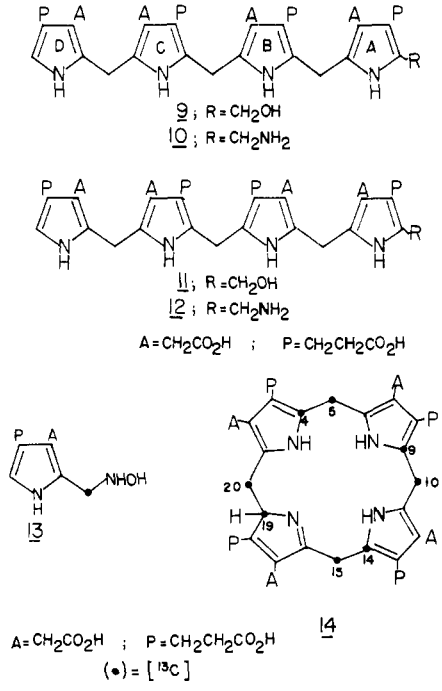
Experimental Procedures

Materials. All the reagents employed were of a high analytical grade. PBG (**1**) was prepared by synthesis (Frydman et al., 1969). [^{14}C]PBG and [^{13}C]PBG were prepared according to the methods described elsewhere (Valasinas & Díaz, 1978; Buldain & Valasinas, 1980). 5-Aminolevulinic acid (ALA) and PMSF were purchased from Sigma Chemical Co. [5-^{13}C]ALA (90% atom % ^{13}C) was prepared by synthesis (Tschudy & Collins, 1959). [2,11-^{13}C]PBG (80 atom % ^{13}C) was prepared by the action of partially purified ALA dehydratase from *R. sphaeroides* on [5-^{13}C]ALA. 2-(Aminomethyl)dipyrrylmethane (**7**), 2-(aminomethyl)tripyrrane (**8**), and 2-(aminomethyl)bilane **4** were obtained by synthesis as described elsewhere (Frydman et al., 1971; Valasinas et al., 1976; Díaz et al., 1979). HMB **5**, **9**, and **11** were obtained by synthesis following the described procedures (Battersby et al., 1982). 2-[(Hydroxylamino)methyl]porphobilinogen (**13**) was obtained by synthesis (Valasinas, unpublished). All the solvents used were of the highest analytical grade.

Analysis of Uro'gen Isomers. Uro'gen isomers were analyzed as their uroporphyrin derivatives. Uroporphyrin isomers I, II, and III were separated as the corresponding free-acid uroporphyrins following the HPLC procedure described by Wayne et al. (1979). Aliquots of the incubation mixtures to be analyzed for isomeric composition were oxidized with an aqueous 1% iodine solution to convert uro'gens to uroporphyrins, and the isomers were determined as described elsewhere (Sburlati et al., 1983).

Enzyme Preparations and Purifications. PBG deaminase was prepared from *R. sphaeroides* (ATCC 17024) grown in medium S of Lascelles (1956) semianaerobically in light at 30 °C. The deaminase was purified as follows. *R. sphaeroides* cells (300-g wet weight) divided into six batches were suspended in 20 mM potassium phosphate buffer (pH 7.4) plus 10 mM 2-mercaptoethanol and sonicated in volumes of 100 mL for a total sonication time of 15 min in 30-s bursts with intervals of 1 min. The temperature of the cell suspension was

[2] The International Union of Biochemistry (IUB) has recently recommended the name hydroxymethylbilane synthase for this enzyme, but as is clear from the work discussed here, this name is not more informative of the enzyme's function than the old name porphobilinogen deaminase, which we think represents better the function of the enzyme and is therefore used in this paper.



maintained around 5 °C. The homogenate was centrifuged at 20000*g* for 30 min, and the supernatant was then heated at 65 °C for 7 min in 25-mL aliquots. The heated supernatant was cooled and centrifuged; the resulting supernatant was precipitated with ammonium sulfate up to 30% saturation and centrifuged, and the supernatant was brought to 60% saturation. The solution was centrifuged at 20000*g* for 20 min, and the precipitate was dissolved in ca. 60 mL of 20 mM potassium phosphate buffer (pH 7.4) and dialyzed against the same buffer plus 10 mM 2-mercaptoethanol. The dialyzed enzyme was ultracentrifuged at 150000*g* for 120 min, and the clear supernatant was applied to a DEAE-Trisacryl column (3.5 × 21 cm) equilibrated with 20 mM phosphate buffer–50 mM KCl plus 10 mM 2-mercaptoethanol. Elution was carried out with a linear salt gradient of 50 mM KCl [500 mL in 20 mM phosphate buffer (pH 7.4) plus 10 mM 2-mercaptoethanol] to 250 mM KCl (500 mL in the same buffer) at a velocity of 30 mL/h. The deaminase was eluted at around 100 mM KCl. The active fractions (180 units) were concentrated by ultrafiltration (Amicon YM-10 membranes) to a volume of 3 mL. The concentrate of two columns was applied on a Sephadex G-100 column (3.5 × 40 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.4) plus 10 mM 2-mercaptoethanol and was eluted with the same buffer at a flow rate of 20 mL/h. The active fractions were then pooled and concentrated (Amicon YM membranes). Another four batches were processed in the same manner. An aliquot (90 units) of the Sephadex G-100 concentrated eluates was applied to a DEAE-Trisacryl column (2 × 27 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 9.0) plus 10 mM 2-mercaptoethanol and eluted with a linear salt gradient of the above-mentioned buffer (250 mL) to 250 mM KCl in the same buffer (250 mL). The active fractions were concentrated by ultrafiltration and had a specific activity of 27 units/mg of protein. One unit is defined as the amount of enzyme required to consume 1 μmol of PBG/h in potassium phosphate buffer (pH 7.4).

R. sphaeroides cosynthase was prepared up to the ammonium sulfate precipitation step as described for the deaminase except that the heating step was omitted. The dialyzed 30–60% ammonium sulfate fraction was then applied to a hydroxylapatite column (3 × 13 cm) previously equilibrated with 10 mM potassium phosphate (pH 6.8) plus 2 mM 2-mercaptoethanol at a flow rate of 18 mL/h. The cosynthase was eluted with the same buffer while the deaminase was retained. The active cosynthase fractions were pooled, concentrated by ultrafiltration (Amicon YM-10 membranes), and filtered through a Sephadex G-100 (3.5 × 40 cm) freshly equilibrated with 50 mM potassium phosphate buffer (pH 7.4) plus 5 mM 2-mercaptoethanol. The active fractions (assayed by HPLC for uroporphyrin III formation) were pooled and concentrated to $^1/_{20}$ of the original volume. All columns were assayed for the deaminase and cosynthase activity. Spleen deaminase–cosynthase and cosynthase were prepared from spleens of phenylhydrazine-treated rats as described elsewhere (Sburlati et al., 1983). Spleens from 30 animals were homogenized in 20 mM potassium phosphate buffer (pH 7.4) plus 10 mM 2-mercaptoethanol, 1 mM EDTA, and 2 mM PMSF. The homogenate was centrifuged at 20000*g* for 30 min, and the supernatant was fractioned with ammonium sulfate. The fraction precipitating between 30 and 60% ammonium sulfate saturation contained the deaminase and the cosynthase, while the fraction precipitating between 60 and 80% saturation contained only the cosynthase. The precipitates were dissolved in a minimum volume of the same buffer. The ammonium sulfate fraction containing deaminase–cosynthase was applied to a DEAE-Trisacryl column (2 × 25 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 6.8), 10 mM 2-mercaptoethanol, 2 mM PMSF, and 1 mM EDTA. The column was washed with the same buffer to eliminate a great amount of hemoglobin and other proteins, and the enzymes were eluted with a linear gradient of KCl between 0 and 250 mM (300 mL each in the above-mentioned buffer but at pH 7.4). In this column deaminase was eluted at a 100 M KCl concentration while cosynthase was eluted at a 120 mM KCl concentration. The active fractions were pooled, concentrated by ultrafiltration, and either combined to have the deaminase–cosynthase complex or further purified on Sephadex G-100. In order to obtain pure cosynthase completely free of deaminase, the 60–90% ammonium sulfate saturation fraction was purified through a DEAE-Trisacryl column similar to that described above. The active fractions were concentrated and filtered through Sephadex G-100. The active fractions were pooled, concentrated by ultrafiltration, and used as cosynthase source.

Enzyme Assays. The deaminase was assayed routinely by estimating the consumption of PBG and the formation of uro'gen, which were measured as described elsewhere (Frydman et al., 1978a,b).

Incubations for ^{13}C NMR studies were typically prepared by dissolving [^{13}C]PBG (0.3 mg, 1.3 μmol) in buffer [0.25 mL of either sodium carbonate (pH 9.2) or potassium phosphate (pH 7.8) plus 50 μL of 90% D_2O]. The solution was divided into six aliquots, and each was incubated in a test tube with 120 μL of partially purified *R. sphaeroides* deaminase (6 units). Incubations were carried out at 37 °C for 5 min in the dark. The reaction was stopped by addition of 20 μL of a concentrated sodium hydroxide solution to each tube, and the incubated mixtures were pooled in a 10-mm NMR tube and examined under the conditions described below. PBG consumption and intermediate formation were monitored by taking aliquots (10 or 20 μL) from the incubation tubes at different times before the end of the incubation reaction. When the substrate activity of the intermediate was examined, this was done at the end of the incubation and before alkali addition. Two aliquots (20 μL each) were withdrawn and incubated in potassium phosphate buffer (pH 7.4) either alone

Table I: Effect of Buffer and pH on Uro'gen Yields in the Deaminase- and Deaminase–Cosynthase-Catalyzed PBG Polymerization

		enzymatic activity				
			uro'gen formed		isomers (%)	
enzymatic system[a]	buffer (pH)	PBG consumed (nmol)	nmol	%	I	III
deaminase	phosphate (7.4)	30.0 ± 1.0	5.4 ± 0.3	73	100	0
deaminase	phosphate (8.0)	29.5 ± 1.2	5.0 ± 0.2	67	100	0
deaminase–cosynthase	phosphate (7.4)	29.5 ± 1.0	5.6 ± 0.4	76	30	70
deaminase	Tris-HCl (8.2)	30.0 ± 0.5	5.9 ± 0.3	80	100	0
deaminase–cosynthase	Tris-HCl (8.2)	30.0 ± 1.0	5.9 ± 0.2	80	38	62
deaminase	Hepes (8.5)	30.0 ± 0.5	5.4 ± 0.3	73	100	0
deaminase	Hepes (9.1)	29.5 ± 1.0	2.3 ± 0.1	31	100	0
deaminase–cosynthase	Hepes (9.1)	29.5 ± 1.0	4.3 ± 0.3	58	18	82
deaminase	carbonate (9.2)	30.0 ± 0.5	1.8 ± 0.1	24	100	0
deaminase[b]	carbonate (9.2)	30.0 ± 0.7	2.7 ± 0.2	36	100	0
deaminase–cosynthase	carbonate (9.2)	29.5 ± 1.0	4.1 ± 0.2	56	20	80

[a] The incubation mixture contained in a final volume of 100 μL 10 μmol of the indicated buffers, 30 nmol of PBG, partially purified *R. sphaeroides* deaminase (0.5 unit), and, where indicated, cosynthase (0.15 unit). Incubation time was 10 min. PBG, uro'gens, and isomer composition were measured as described under Experimental Procedures. The data represent the mean of four incubations. [b] Incubation time was 20 min.

or with partially purified (DEAE-Trisacryl step) spleen or *R. sphaeroides* cosynthase. Isomer analysis was performed as described above.

The incubations of the deaminase–cosynthase, deaminase, and cosynthase of different origins with the 2-(hydroxymethyl)bilanes or PBG were performed in a final volume of 650 μL. The incubation mixture contained 50 μmol of potassium phosphate buffer (pH 7.4), enzyme (16 units of deaminase or deaminase–cosynthase or 6 units of cosynthase), and the synthetic (hydroxymethyl)bilane (230 nmol) or PBG (400 nmol). Other substrate concentrations used are indicated in the text. Incubations were carried out at 37 °C. Each run included simultaneous incubations of the enzyme with PBG, of the enzyme with the synthetic substrates, and of the synthetic substrates in the absence of enzyme (chemical blank). Uro'gen formation and isomer analysis were carried out on aliquots (20 μL for the former and 50 μL for the latter) withdrawn at the times indicated in the text. One unit of cosynthase activity is defined as 1 μmol of uroporphyrin III.

NMR Spectroscopy. All the NMR spectra were performed on a Varian FT-80A spectrometer, and the ^{13}C NMR experiments were conducted at an observation frequence of 20 MHz in 10-mm tubes. Spectra were usually run at 28 °C. Spectra were collected every 0.189 s by using 8192 data points and 42° of flip angle (PW 90° = 27 μs). The resulting FID was weighted with a negative exponential function leading to a 2-Hz broadening of spectral lines. Proton decoupling was performed at 2 W. The spectra were referenced by using dioxane as an standard (67.4 ppm), and D_2O was used as internal lock.

Porphobilinogen and Pyrrylmethane Colorimetric Determinations. PBG and pyrrylmethanes were assayed colorimetrically with Ehrlich's modified reagent [2% *p*-(dimethylamino)benzaldehyde in glacial acetic acid/perchloric acid (84:16 v/v)]. The spectra were recorded by use of a double-beam Hitachi A 110 spectrometer.

Results

Effect of pH and Buffer on the Enzymatic Polymerization of PBG. When PBG was incubated in phosphate buffer at pH 7.4 or 8.0 with deaminase under conditions where total consumption of PBG is achieved in 5 min, uro'gen I (**3**) is formed in around 70% yield (Table I). When the same incubation was carried out in carbonate buffer at pH 9.2, the yield of uro'gen I (**3**) after total consumption of PBG was only 24%. At longer incubation periods the yield of uro'gen I (**3**) increased to 36%, although PBG was no longer available. The same incubation carried out in Tris-HCl buffer at pH 8.2 gave an 80% yield after total PBG consumption in 10 min. In Hepes buffer at pH 8.5, it gave around 70% yield of uro'gen, and in the same buffer at pH 9.1, a 30% yield was obtained (Table I). It is evident that at pH above 9.0 the enzymatic polymerization of PBG forms uro'gen in low yields, while the buffer composition affects to a lesser degree the enzymatic polymerization. When the same incubations were carried out from the start in the presence of cosynthase, substantially higher yields of uro'gen were obtained at the higher pH (Table I). It is obvious that at higher pH the presence of cosynthase accelerates uro'gen formation.

In the incubations where uro'gen yields were much lower than the expected stoichiometric values and especially in those carried out in carbonate buffer (pH 9.2), a pyrrylmethane intermediate accumulated that could be detected by using Ehrlich's reagent. The colored reactions indicated that the accumulated pyrrylmethane was different from the known ones (Figure 1). 2-(Aminomethyl)dipyrrylmethane (**7**) and 2-(aminomethyl)tripyrrane (**8**) gave Ehrlich derivatives with λ_{max} at 560 and 561 nm, respectively, which slowly changed to the pyrrylmethene derivatives with λ_{max} at 483.5 and 487 nm, respectively. The 2-(aminomethyl)bilane **4** and HMB **5** gave derivatives with λ_{max} at 562 nm that changed faster to give the pyrrylmethene derivatives with λ_{max} at 486 and 488 nm, respectively (Figure 1). The accumulated intermediate of the enzymatic reaction gave a derivative with a λ_{max} at 563 nm that changed very fast to the one with a λ_{max} at 494 nm. The rate of the methane–methene proton transfer (Frydman et al., 1976) indicated that the intermediate is a tetrapyrrole rather than a shorter chain polymer. Uro'gen also gave a slightly colored reaction with Ehrlich's reagent (Figure 1F) with a λ_{max} at 549 nm, very different from that of the open-chain pyrrylmethanes.

^{13}C NMR Studies. Incubation with [11-^{13}C]PBG (Scheme I) shed further light on the nature of the above-mentioned intermediate. In phosphate buffer (pH 7.4), the enzymatic reaction showed that sole formation of uro'gen I, which had a single resonance at 22.05 ppm corresponding to the meso positions 5, 10, 15, and 20 (Scheme I). The ^{13}C-enriched 2-aminomethyl signal of porphobilinogen (C-11) was at 36.30 ppm (Figure 2A). In carbonate buffer (pH 9.2) the spectra recorded immediately after total consumption of PBG (measured with Ehrlich's reagent) showed the presence of a small amount of free uro'gen I (**3**) (resonance at 22.05 ppm) and the presence of a larger amount of deaminase-bound uro'gen I at 21.97 ppm ($\nu_{1/2}$ = 3 Hz) (Figure 2B). Proof that the signal at 21.97 ppm corresponds to an enzyme-bound uro'gen I species was obtained by precipitation of the protein at acid

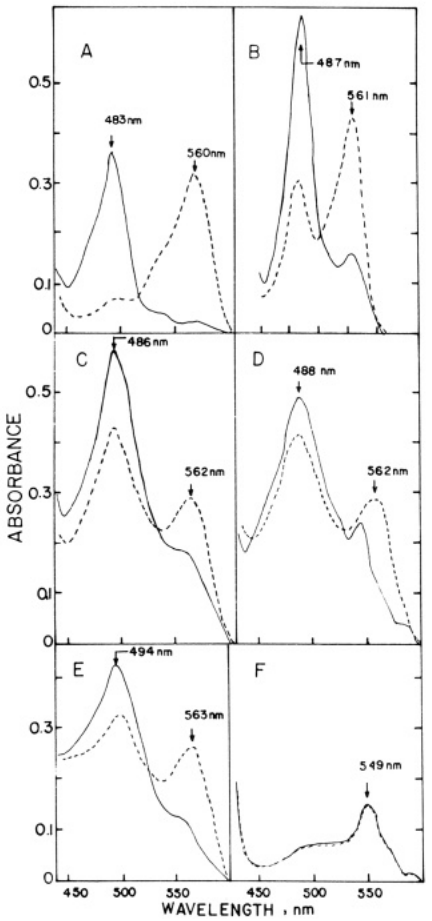


FIGURE 1: Absorption spectra of the Ehrlich derivatives of (A) dipyrrylmethane (**7**), (B) tripyrrane (**8**), (C) (aminomethyl)bilane (**4**), (D) (hydroxymethyl)bilane **5**, (E) pyrrylmethane intermediate accumulated by incubation of PBG with deaminase in carbonate buffer (pH 9.2), and (F) uro'gen I. Spectra were recorded immediately after addition of Ehrlich's reagent (—) and after (---) (A) 40 min, (B) 30 min, (C and D) 10 min, (E) 5 min, and (F) 5–60 min. These spectra did not change further after these time intervals.

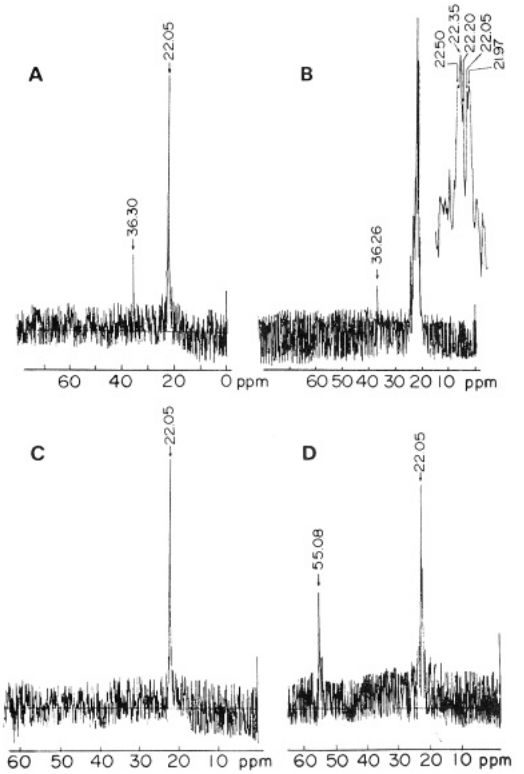


FIGURE 2: ^{1}H-decoupled (2 W continuous) 20-MHz ^{13}C NMR spectra: (A) Uro'gen I and [11-^{13}C]PBG derived from incubation of 90% [11-^{13}C]PBG (0.3 mg) in potassium phosphate buffer (pH 7.8) with deaminase (see Experimental Procedures) before the total consumption of PBG (3 min); (B) same incubation as in (A) but carried out in carbonate buffer (pH 9.2) to total PBG consumption as measured with Ehrlich's reagent (7 min); (C) same incubation as in (B) but after addition of trypsin (1 mg) and further incubation for 3 min; (D) incubation of HMB **5** (R = $^{13}CH_2OH$, 2 mg) with deaminase (18 units) in carbonate buffer (pH 9.2) and in the presence of 0.3 mg of [^{12}C]PBG. All spectra were recorded with an acquisition time of 0.819 s and NS = 25 000.

pH, which led to the transformation of this resonance into a mixture of a uro'gen resonance at 22.05 ppm and a uroporphyrin resonance at 99.8 ppm. A signal with $\nu_{1/2}$ = 4 Hz at 22.35–22.50 ppm corresponded to the methane bridges of a pyrrylmethane. A small amount of 2-(aminomethyl)bilane **4** was also detected by its 2-aminomethyl resonance at 36.26 ppm (C-2) and at 22.20 ppm (methane bridges). The latter was hidden behind the broader signal of the aforementioned pyrrylmethane (Figure 2B). These compounds were stable at alkaline pH (pH 12) and could be recorded over a period of 6 h. If the incubation mixture was not adjusted to pH 12, the reaction proceeded at the probe temperature (28 °C) and finally reached a stage where only free and deaminase-bound uro'gen I was detected. The methylene signals at 22.35–22.50 ppm correspond to a pyrrylmethane structure since they were downfield from the uro'gen meso methylenes and in the region where the methylene bridges of the former could be expected.

When trypsin was added to the incubation mixture containing the pyrrylmethane intermediate (Figure 2B) and the mixture was incubated for 3 min, the ^{13}C NMR spectra showed that the intermediate had disappeared and only free uro'gen I could be detected (Figure 2C).

The above-mentioned spectrum of the intermediate at 22.35–22.50 ppm (Figure 2B) corresponds very likely to an enzyme-bound bilane. Its bilane (tetrapyrrylmethane) structure is evident from the fact that it was transformed into uro'gen I by the deaminase in the absence of any PBG present. Its enzyme-bound structure is supported by the fact that no signal which could be attributed to a C-2 methylene residue was detected in the spectra. It should therefore be assumed that it is bound to a high molecular weight structure of slow tumbling which prevents the detection of the 2-methylene resonance at 20 MHz, while the pyrrylmethylene resonances of greater mobility were detected as a 4-Hz broad signal (see Discussion). Even at higher fields ^{13}C NMR spectroscopy did not detect the C-2 methylene resonance in enzyme-bound PBG (Evans et al., 1986). The binding of the intermediate to the enzyme was crucial for its stability since it was rapidly converted into the cyclic uro'gen I when the enzyme was hydrolyzed with trypsin. It was also impossible to isolate the intermediate by gel filtration or by gel electrophoresis, since during the workup it was rapidly transformed into uro'gen even at low temperatures (4 °C). The bilane is therefore loosely bound to the deaminase, at variance with the stable shorter chain intermediates detected by other methods (see introduction).

The weak signal at 36.26 ppm (Figure 2B) can be attributed to the presence of a small amount of 2-(aminomethyl)bilane **4** formed by a transamination reaction that takes place between the ammonia released in the enzymatic reaction with the enzyme-bound bilane or with a reactive species such as the azafulvene **6**. The possibility that **4** was formed by a transamination reaction which took place between a possible HMB **5** formed in the enzymatic reaction and the ammonia released during the same reaction was excluded by incubating [^{13}C]-

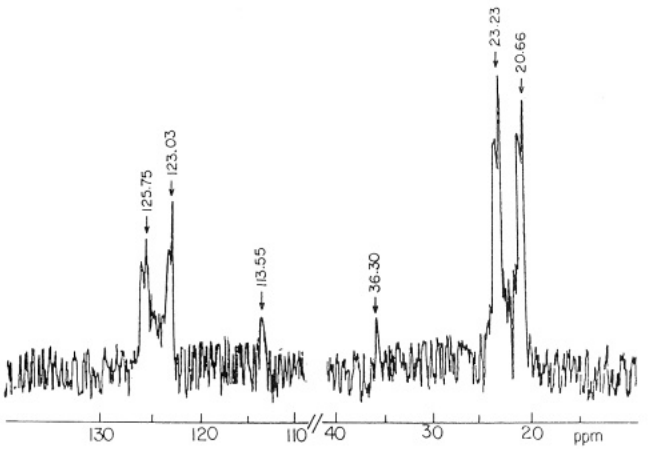


FIGURE 3: ^{1}H-decoupled (2 W continuous) 20-MHz ^{13}C NMR spectrum of [2,11-$^{13}C_2$]PBG (0.3 mg) incubated with deaminase in carbonate buffer (pH 9.2) following the procedure described under Experimental Procedures NMR conditions are the same as in Figure 2.

HMB **5** (R = $^{13}CH_2OH$) with deaminase in the presence of [^{12}C]PBG. The spectrum showed only the formation of [^{13}C]uro'gen I (Figure 2D). Ehrlich's reaction, however, showed the formation of the aforementioned pyrrylmethane intermediate. There was always the possibility that the signal at 36.26 ppm was due to the formation of the protonated uro'gen macrocycle **14**, since the C-20 methylene signal bound to the sp^3 carbon could be expected around 36.00 ppm (Johansen et al., 1980). To eliminate this possibility, [2,11-^{13}C]PBG (see Scheme I) was incubated with deaminase in carbonate buffer. The obtained spectrum (Figure 3) showed the expected *meso*-methylene doublets arising from the direct coupling ($J_{^{13}C^{13}C}$ = 51.4 Hz) in (4,5,9,10,14,15,19,20-$^{13}C_8$) deaminase-bound uro'gen I centered at 21.95 ppm and a broader doublet centered at 22.40 ppm corresponding to the pyrrylmethane intermediate and also to a small amount of **4**. The aromatic region corresponding to the α-pyrrole carbons of uro'gen I (**3**) gave doublets centered at 124.30 ppm, and the doublets corresponding to the pyrrole carbons of the intermediate (and of **4**) centered at 124.8 ppm could also be seen. A broad signal at 113.55 ppm corresponding to the C-19 carbon of the intermediate and of the 2-(aminomethyl)bilane **4** was also detected, while the signal at 36.26 ppm was a singlet. If this latter resonance would correspond to the C-20 methylene of the protonated uro'gen structure **14**, a doublet should be expected. Since PBG had been entirely consumed (measured with Ehrlich's reagent), the weak singlet at 36.26 ppm must be attributed to the noncoupled 2-aminomethyl residue of the small amount of 2-(aminomethyl)bilane **4** formed.

The amount of 2-(aminomethyl)bilane **4** formed during these incubations could be reduced to a minimum and even completely avoided if the incubations were carried out under a constant stream of nitrogen. When the enzymatic reaction was run in the presence of a high concentration of ammonium ions (0.2 M final concentration), the formation of 2-(aminomethyl)bilane **4** was increased and could be easily detected in the spectrum at 36.26 and 22.20 ppm (Figure 4). However, a large amount of the enzyme-bound intermediate was still present in the incubation mixture (22.50 ppm), indicating that in carbonate buffer the former was not easily displaced from the enzyme by the ammonium ions. The relative small effect of the exogenously added ammonium ions suggests that the 2-(aminomethyl)bilane **4** formed in the incubation of PBG with deaminase in carbonate buffer is due to the action of an endogenous ammonium source, presumably protein-trapped ammonium.

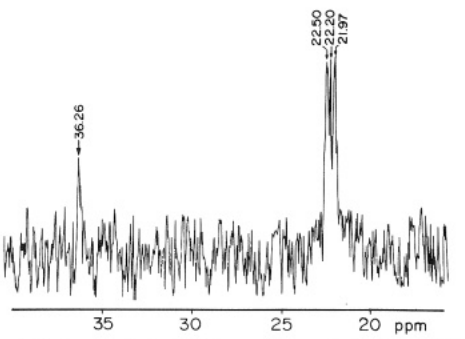


FIGURE 4: ^{1}H-decoupled (2 W continuous) 20-MHz ^{13}C NMR spectrum of the incubation mixture used in Figure 2B but carried out in the presence of 0.2 M ammonium chloride. All NMR conditions are as in Figure 2.

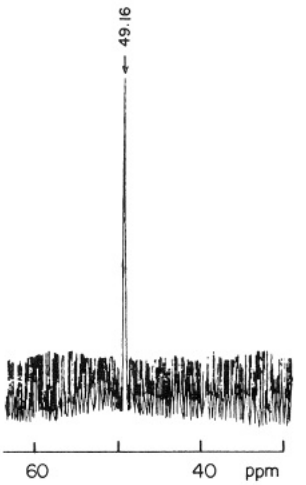


FIGURE 5: ^{1}H-decoupled (2 W continuous) 20-MHz ^{13}C NMR spectrum of the incubation mixture of Figure 2B but carried out in the presence of hydroxylamine (0.2 M) added at the start of the incubation. All NMR conditions are the same as in Figure 2B, but the NS = 13 000.

When hydroxylamine was added to the incubation mixture at the start of the enzymatic reaction, the only resonance detected was that of 2-[(hydroxylamino)methyl]porphobilinogen (**13**) at 49.14 ppm (Figure 5). This indicates that deaminase catalyzed the transhydroxylamination of PBG at a higher rate than that of its polymerization. When hydroxylamine was added after PBG consumption, a spectrum similar to that of Figure 2B was obtained, indicating that hydroxylamine did not displace (in carbonate buffer, pH 9.2) the enzyme-bound bilane to give a 2-[(hydroxylamino)methyl]bilane. Ehrlich's reaction carried out on 2-[(hydroxylamino)methyl]porphobilinogen (**13**) gave λ_{max} at 556 nm, which changed to a stable color with λ_{max} at 486 nm, similar to those obtained with pyrrylmethanes.

Uro'gen III (**2**) *Formation from the Intermediate Formed at pH 9.2.* When PBG was incubated with the deaminase–cosynthase system, no intermediate was detected either with Ehrlich's reagent or by the ^{13}C NMR spectra, even in carbonate buffer. It was therefore conceivable that the intermediate accumulated in carbonate buffer could serve as a substrate for the cosynthase. When cosynthase was added to an incubation mixture where PBG had been exhausted and that gave a ^{13}C NMR spectrum such as that depicted in Figure 2B (Table II, 7 min) and the mixture was further incubated for 10 min, the isomer composition of the uro'gen mixture showed about a 50% content of isomer III. The substrate of

Table II: Formation of Uro'gen III from the Deaminase-Bound Intermediate as a Function of Time

incubation time with deaminase[a] (min)	incubation time with cosynthase[b] (min)	uro'gen III formed[c] (%)	conversion of intermediate into uro'gen III[d] (%)
	10	49	75
7	20	50	75
	30	50	75
	10	35	73
15	20	35	73
	30	32	73
	10	22	73
30	20	20	71
	30	23	71

[a] Samples of the PBG incubation were withdrawn at the indicated times and cosynthase was added. [b] Incubation times of the deaminase–intermediate complex with cosynthase after total consumption of PBG. [c] Determined by HPLC (see Experimental Procedures); the difference was uro'gen I. When buffer was added instead of cosynthase, 100% of uro'gen I was formed. [d] Uro'gen III formation from the intermediate as calculated after subtraction of the amount of uro'gen I already present in the incubation mixture at the time of cosynthase addition.

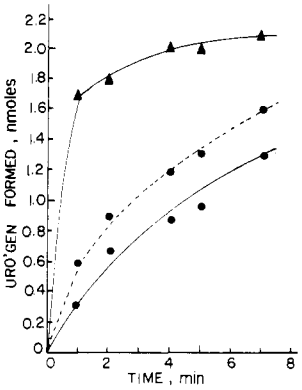


FIGURE 6: Rates of chemical and ezymatic polymerization of HMB **5**. The incubation mixtures were as described under Experimental Procedures. Aliquots (20 μL) of the incubation mixtures were withdrawn at the indicated times, and uro'gen formed was estimated after oxidation with iodine as described under Experimental Procedures. (●–●) Chemical polymerization; (●---●) incubated with *R. sphaeroides* deaminase; (▲) incubated with *R. sphaeroides* cosynthase.

the cosynthase was therefore the deaminase-bound intermediate, since apart from the latter, only uro'gen I and a small amount of 2-(aminomethyl)bilane **4** were present in the incubation mixture (see Figure 2B). Longer incubation periods with cosynthase did not significantly alter the isomer composition. If the original proportion of uro'gen I (**3**) in the mixture before the addition of cosynthase is considered (about 35%), then uro'gen III (**2**) formation at the expense of the intermediate could be estimated to be about 75%. If the cosynthase was added 8 min after total PBG consumption, the proportion of isomer III in the final product decreased to 35% (Table II, 15 min). However, if the correction is made for the amount of preformed uro'gen I (**3**), then 73% of the intermediate was transformed into uro'gen III (**2**). When the cosynthase was added about 20 min after PBG exhaustion, the proportion or uro'gen III (**2**) in the mixture fell to about 20% (Table II). These data indicate that the intermediate described above behaved as a substrate of the cosynthase to give uro'gen III (**2**). As the intermediate disappeared at longer incubation times (and was transformed into uro'gen I), the addition of cosynthase formed less uro'gen III.

Interaction of 2-(Hydroxymethyl)bilanes **5**, **9**, *and* **11** *with Deaminase and Cosynthase.* 2-(Hydroxymethyl)bilanes **5**, **9**, and **11** readily cyclized (at pH 7.6–8.2) to form the corresponding uro'gen isomers. Addition of deaminase to HMB **5** accelerated the rate of ring closure to uro'gen (Figure 6). It is therefore conceivable that **5** and deaminase interact to form a more reactive species than **5** itself. Such an interaction was already demonstrated by using deaminase from *Euglena gracilis* (Battersby et al., 1983). Addition of cosynthase also accelerated uro'gen formation, especially at short incubation times (Figure 6). The interaction of **5** and cosynthase is well-known and leads to the formation of uro'gen III (**2**) (Battersby et al., 1983).

Table III: Interaction of HMB **5** with Deaminase, Deaminase–Cosynthase, and Cosynthase

enzymatic system[a]	substrate	uro'gen III formed[b] (%) as a function of time: 2 min	5 min	7 min	15 min
R. sphaeroides					
deaminase	HMB	0	0	0	0
deaminase–cosynthase	HMB	46	42	28	20
cosynthase	HMB	43	40	30	20
deaminase–cosynthase	PBG	80	83	77	83
spleen					
deaminase	HMB	0	0	0	0
deaminase–cosynthase	HMB	65	52	45	35
cosynthase	HMB	65	50	45	35
deaminase–cosynthase	PBG	100	100	100	98

[a] The incubation mixtures and conditions are as described under Experimental Procedures. [b] Determined by HPLC; the difference was uro'gen I.

Table IV: Effect of Increasing HMB **5** Concentration on the Amount of Uro'gen III Formed

system[a]	HMB **5** (nmol)	uro'gen III (%) formed as a function of time: 2 min	15 min
deaminase–cosynthase	35	65	34
	70	47	28
	120	27	24
deaminase–cosynthase + PBG		100	100

[a] The incubation mixture contained in a final volume of 150 μL 10 μmol of potassium phosphate buffer (pH 7.8), partially purified (DEAE-Sephacryl step) spleen deaminase (1 unit) plus spleen cosynthase (0.5 unit), and either HMB **5** or PBG (60 nmol). Incubations were carried out at 37 °C during the indicated times. Isomer analysis was performed by HPLC (see Experimental Procedures).

The formation of uro'gen III (**2**) from **5** using enzyme preparations from *R. sphaeroides* and spleen was examined (Table III). The amount of uro'gen III (**2**) formed from **5** varied with the origin of the cosynthase used and decreased at longer incubation times. In our hands the best yields of uro'gen III (**2**) from **5** were obtained with spleen cosynthase at short incubation times. The decrease in uro'gen III (**2**) formation from **5** at longer incubation times could be attributed to the inactivation of cosynthase with time, but this could be discarded since it was not observed when PBG was used as a substrate (Table III). A possible inhibitory effect of **5** on the cosynthase was examined by increasing the concentration of HMB **5**. The results indicated that at increasing concentrations of **5** the amounts of uro'gen III (**2**) formed by the cosynthase decreased (Table IV). There are two explanations for these results. One of them is that cosynthase was inhibited by increasing concentrations of **5**. The other is that unless a very large excess of cosynthase was used, the chemical cyclization of **5** to give uro'gen I (**3**) competed with the enzymatic action of the cosynthase to give uro'gen III (**2**). The work that reports more than 90% formation of uro'gen III (**2**) from **5**

made use of a very large excess of cosynthase (Battersby et al., 1982).

By incubation of [^{14}C]PBG together with **5** using a deaminase–cosynthase system from *R. sphaeroides* (data not shown), it was found that **5** inhibited the enzymatic polymerization of PBG as well as uro'gen III (**2**) formation from the latter. The inhibitory effect of **5** on deaminase was also reported by other authors (Battersby et al., 1983).

The 2-(hydroxymethyl)bilanes **9** and **11** did not react with cosynthase when assayed under the same conditions as **5**. (Hydroxymethyl)bilane **9** gave uro'gen III (**2**) by chemical cyclization as well as in the presence of cosynthase (or deaminase–cosynthase), while cyclization with ring D inversion should have resulted in the formation of uro'gen I (**3**). (Hydroxymethyl)bilane **11** gave uro'gen II by chemical cyclization in the presence of cosynthase or deaminase–cosynthase, while ring D inversion would have led to uro'gen III (**2**) formation.

Discussion

Recent work showed that deaminase polymerizes four units of PBG to produce HMB **5**, which serves as a substrate of cosynthase to give uro'gen III (see introduction). Although this finding was an important breakthrough to understand the mechanism of uro'gen formation, many important questions remain unanswered. To cyclize **5** to uro'gen III (**2**) by cosynthase, a large excess of the enzyme and short incubation times are needed in order to obtain good yields of uro'gen III (**2**) (Tables III and IV). At longer incubation times or at higher HMB concentrations, the chemical cyclization of **5** formed substantial amounts of uro'gen I (**3**). This was not the case when PBG was used as a substrate of deaminase–cosynthase (Table III), since in this case a slight excess of cosynthase guaranteed the sole formation of uro'gen III (**2**). Although in normal biological systems there is an excess of cosynthase over deaminase (Stevens et al., 1968), this fact alone cannot be the only reason that under normal conditions only uro'gen III (**2**) is formed. Liberation of HMB **5** into the medium would always lead to formation of uro'gen I (**3**), while the latter is only found under pathological conditions.

In this paper we describe the detection of an intermediate formed by the deaminase from PBG (Figure 1E and Figure 2B) that was converted by the cosynthase into uro'gen III (**2**). This intermediate is a tetrapyrrole loosely bound to the deaminase. It is readily converted into uro'gen I (**3**) when separated from the enzyme by gel filtration or gel electrophoresis and is therefore a tetrapyrrole of "type I". Its existence lends support to the proposal that the inversion of ring D to form uro'gen III (**2**) takes place after a tetrapyrrole is built by the deaminase (Battersby, 1986).

In order to gain information about the possible nature of the detected intermediate, some considerations should be taken into account the ^{13}C NMR experiment. Ehrlich's reaction as well as its behavior indicated that the intermediate is very likely a tetrapyrrole, but it only gives rise to one signal (22.35–22.50 ppm) in the NMR spectrum. Two different possibilities may be proposed to explain this fact. One is that the four methylenes of the tetrapyrrole system that are detected are almost equivalent. It is therefore conceivable that the signal at 22.35–22.50 ppm arises from a cyclic tetrapyrrole system in which all the meso carbons should be equivalent due to symmetry considerations. The difference in chemical shifts between uro'gen I (**3**) and the intermediate may be explained by assuming that they are two conformers of the same structure. Conformers of porphyrinogens have been characterized (von Maltzan, 1982). A main drawback in this model is that since the signals of uro'gen I and of the intermediate are only 7 Hz appart, the simultaneous observation of the two signals requires that the rate of interconversion should be slower than 7 Hz. Otherwise, a coalescence process would be observed with a single resonance somewhere between that of the uro'gen I (**3**) and the intermediate peaks. Such a slow rate of interconversion is not expected in the NMR time scale used. According to a second model, the tetrapyrrole intermediate is a bilane bound to the enzyme, and therefore the four bridge methylene carbons are nonequivalent. The first methylene (the one directly bound to the enzyme) is expected to be shifted from the other three but was not observed. This could be due to its different relaxation behavior as compared to the other three methylenes. It has been shown that the line width that can be expected in the ^{13}C NMR spectrum of a deaminase-bound PBG under extreme narrowing conditions is ca. 300 Hz and is therefore beyond the sensitivity of the experiment (Evans et al., 1986). It is, however, reasonable to assume that similar considerations cannot be applied to the rest of the chain pyrroles. Although in the pyrrole bound to the enzyme the movements are very restricted by the latter, pyrrole rings removed from the site of binding will possess movements characterized by shorter correlation times. Similar cases have been reported in the literature where the rigid-body model was found to be too crude to explain the relaxation behavior of macromolecules (Ribeiro et al., 1980; Otvos & Armitage, 1980; Birdsall et al., 1982; Jaffe & Markham, 1987). If this is the case, broadening due to relaxation effects will not affect too strongly the line width of the carbons not directly bound to the enzyme, whose signals will be observable. Small inequivalences in the chemical shifts of these carbons would probably be "masked" by the line width of these resonances (4–5 Hz).

Under the experimental condition used in this paper a deaminase-bound intermediate was mainly detected. Any unbound intermediate was present in its cyclic form as uro'gen I (**3**), and a small amount of the intermediate escaped from the enzyme as the 2-(aminomethyl)bilane **4** (Figure 2B). What makes this deaminase-bound intermediate particularly attractive is that its transformation into uro'gen III (**2**) by cosynthase was found to be independent of incubation times (Table II). Since the loose association between the intermediate and the deaminase avoids its fast transformation into uro'gen I (**3**), it facilitates its interaction with the cosynthase.

In previous work (Sburlati et al., 1983) we showed that not only 2-(aminomethyl)bilane **4** interacted with deaminase–cosynthase to cyclize to uro'gen III (**2**) with inversion of ring D but that the isomeric 2-(aminomethyl)bilanes **10** and **12** also cyclized with ring D inversion. The reported cyclization of **4** to give uro'gen III (**2**) in the presence of deaminase–cosynthase was shown to be due to a transamination reaction catalyzed by the deaminase that transformed **4** into **5** and by the known fact that **5** is a substrate of cosynthase (Battersby, 1986). However, when the (hydroxymethyl)bilanes **9** and **11** were assayed as substrates of deaminase–cosynthase (see above), it was found that they did not cyclize with ring D inversion. It is therefore conceivable that for a 2-(aminomethyl)bilane or a 2-(hydroxymethyl)bilane to undergo ring closure with ring D rearrangement a prior formation of a bilane–deaminase or bilane–cosynthase complex is necessary. It was recently found that HMB **5** and deaminase can form a stable complex which can serve as a substrate for cosynthase (Battersby et al., 1983).

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