Biosynthesis of the Pigments of Life: Mechanistic Studies on the Conversion of Porphobilinogen to Uroporphyrinogen III

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Received March 5, 1990 (Revised Manuscript Received May 9, 1990)

Contents

I. Introduction

The tetrapyrrolic pigments of life include the hemes, chlorophylls, vitamin B_{12}, and the light-harvesting bilins of algae. They are all derived from the same tetrapyrrolic macrocycle, uroporphyrinogen III (3), which is generally abbreviated uro'gen III (Scheme I). In this review we are concerned with the mechanistic aspects of the biosynthesis of uro'gen III from its monopyrrolic precursor, porphobilinogen (2, known as PBG), catalyzed by the enzymes PBG deaminase and uro'gen III synthase (or cosynthetase). We will not mention, except in passing, the many studies on the isolation, properties, and genetics of these enzymes. These aspects and the remainder of the biosynthetic pathway to the pigments of life have been reviewed elsewhere.[1]

Historical Background. The first investigations into the biosynthesis of heme (7) were reported 1945 by Shemin, who, experimenting on himself, swallowed 66 g of [^{15}N]glycine over a period of 3 days.[2] Then, taking blood samples, he isolated the heme at various intervals and analyzed its ^{15}N content by mass spectrometry. He found that the label was rapidly incorporated and then the level of incorporation remained fairly constant until after about 120 days it started to decline (this being the life span of a red blood cell). Incorporation experiments with ^{14}C-labeled glycine and other precursors (using duck's blood) followed, and it was shown that heme is derived from glycine, with loss of C-1, and succinyl CoA. One likely product from these two precursors is 5-aminolaevulinic acid (1, known as ALA), and this was synthesized labeled with both ^{15}N and ^{14}C and shown to be a much better precursor of heme than glycine. The condensation reaction is catalyzed by the pyridoxal-dependent coenzyme ALA synthase. This pathway to ALA is known as the Shemin pathway, but it has been found that in plants, algae, and anaerobic bacteria another pathway is initially followed for the synthesis of chlorophylls and vitamin B_{12}. In these organisms ALA is formed not from glycine but from glutamate, with [1-^{14}C]glutamate labeling C-5 of ALA. All five carbons of glutamate are incorporated into ALA, and so this is termed the C_5 pathway.[3]

PBG (2) was first identified in the urine of patients suffering from a certain type of porphyria by Cookson and Rimington.[4] Labeled PBG was efficiently incorporated into heme in chicken blood. PBG is clearly formed by combination of two molecules of ALA as shown in Scheme I. This reaction, catalyzed by the enzyme ALA dehydratase (or PBG synthase) is very similar to a Knorr pyrrole synthesis, which is the main chemical reaction used for the synthesis of pyrroles similar to PBG.

In other types of porphyria, porphyrins such as uroporphyrin III (5) had been identified in the urine. It was natural to suppose that 5 was an intermediate between PBG and heme but, unexpectedly, labeled 5 could not be incorporated into heme. It was eventually realized that the true intermediate was the hexahydroporphyrin, uro'gen III (3), and the porphyrin had been formed by aerial oxidation of this porphyrinogen.

It is with this problem, then, that this review will deal: How is uro'gen III made from PBG and how is it that the acetate and propionate side chains on ring D are a different way round from the substituents on the other rings?

II. Relationship of the Two Enzymes

A. Nature of the Rearrangement

The foundation for the work carried out during the past 30 years was laid by an important series of investigations in the 1950s by the groups of Bogorad, Granick, Neuberger, Rimington, and Shemin.[2,5] This work showed that two enzymes are involved in the

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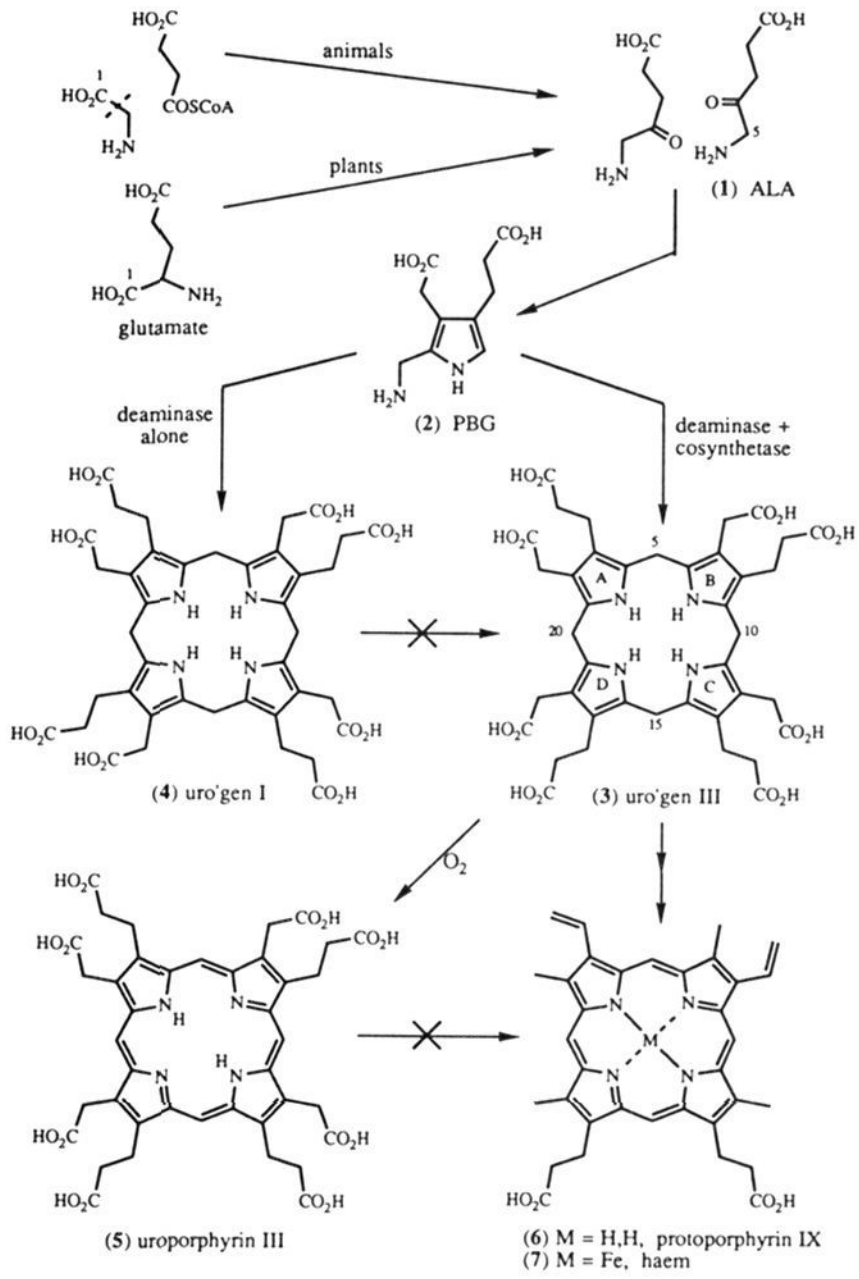
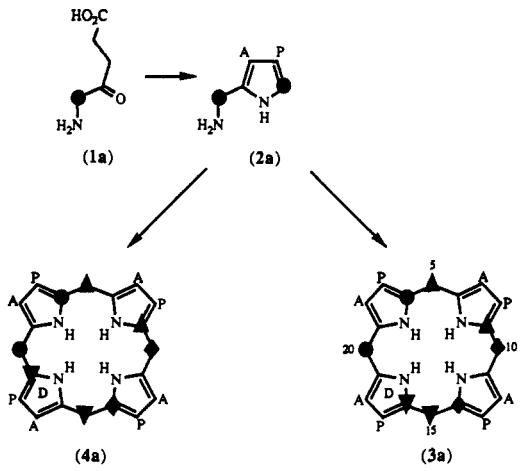
SCHEME I



conversion of PBG (**2**) into uro'gen III (**3**). These could not easily be separated, but one was much more heat-sensitive than the other. Whereas preparations containing both enzymes catalyze formation of uro'gen III from PBG, the final product detected at that time from the action of the heat-treated enzyme system on PBG was a different isomer, uro'gen I (**4**), in which no rearrangement of the substituents on ring D occurred. The two components were called PBG deaminase (for the heat-stable one) and uro'gen III cosynthetase (for the labile one). One possibility was that uro'gen I is the normal intermediate between PBG and uro'gen III, but it was soon shown that preparations containing active cosynthetase are unable to isomerize uro'gen I. Despite this, cosynthetase is sometimes referred to as uro'gen isomerase (the name used by Chemical Abstracts Service). It will be seen later that the name uro'gen I synthase, sometimes used for the other enzyme, is also incorrect.

There were a great number of early theories (more than 25) about the way the four molecules of PBG combine to give one molecule of uro'gen III. The number of rearrangement steps, their timing (e.g., at the mono-, di-, tri-, or tetrapyrrole stage), and their nature (e.g., turning of a whole pyrrole ring or interchange of its side chains or modification of the side chains) were all matters of speculation. Many of the theories differed in their predictions of which of the four bridging methylenes (C-5, -10, -15, and -20) of uro'gen III had been attached to each original pyrrole ring. This type of problem can be resolved by an appropriate

SCHEME II



double-labeling experiment. The one that was performed involved incorporation of [2,11-$^{13}C_2$]PBG (**2a**), synthesized from [5-^{13}C]ALA (**1a**) by the action of ALA dehydratase.[6] This was incubated first with deaminase alone to obtain the symmetrical uro'gen I, which was isolated as uroporphyrin I octamethyl ester. The labeling pattern of the uro'gen I (**4a**) that was deduced[6,7] from this is shown in Scheme II. All the meso carbons showed strong coupling (72 Hz) in the ^{13}C NMR spectrum due to an adjacent ^{13}C atom derived from a different molecule of PBG and a smaller long-range coupling (5.5 Hz) to the more distant ^{13}C atom belonging to the same PBG molecule. Clearly no rearrangement had occurred for the formation of uro'gen I, and all four PBG units had been incorporated intact.

To investigate the formation of uro'gen III, [2,11-$^{13}C_2$]PBG (**2a**) was diluted with four parts of unlabeled PBG and incubated with a cell-free system from the unicellular photosynthetic organism *Euglena gracilis*, which contained not only deaminase and cosynthetase but also the enzymes to convert uro'gen III further to protoporphyrin IX (**6**), isolated as its dimethyl ester. The labeling pattern of uro'gen III (**3a**) that could be deduced from the ^{13}C NMR spectrum of **6** obtained from **2a** is shown in Scheme II. Because of the dilution of the labeled PBG, most molecules of uro'gen III would only have had one pair of labeled atoms (●, ▲, ♦, ▼). Whereas the ^{13}C signals for three of the meso positions (C-20, -5, and -10) showed just the 5.5-Hz coupling, C-15 appeared as a doublet (72 Hz) due to the second ^{13}C atom from the same PBG molecule, which is now adjacent. Three important conclusions can be drawn from this result:

(i) Only one rearrangement occurs and that involves ring D; rings A–C arise from intact PBG units.

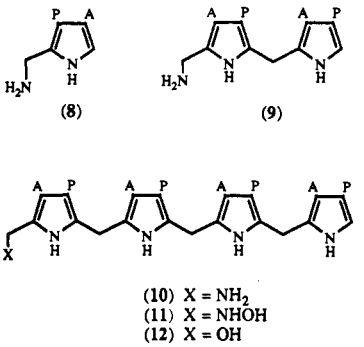
(ii) The rearrangement of ring D involves detachment of the pyrrole from the methylene that had been C-11 of PBG, turning round of the *whole* pyrrole ring, and reattachment to the methylene.

(iii) The rearrangement is intramolecular as the level of double-labeling in the ring D unit was no different from that of the PBG precursor; no exchange of part of the unit with unlabeled precursors has occurred.

The labeling patterns for uro'gens I and III derived from [5-^{13}C]ALA, shown in Scheme II, have been used for structure determinations (e.g., of precorrin-2,[8] a biosynthetic precursor of vitamin B_{12}) and more recently to determine from which of these isomers tetrapyrroles isolated from biological systems are derived.[9] If the isotopic label is not diluted, then all the marked atoms (●, ▲, ♦, ▼) are ^{13}C in the same molecule. Hence, for compounds derived from uro'gen I, all four meso carbons will appear as doublets in the proton-decoupled ^{13}C NMR spectrum, whereas for compounds derived from uro'gen III C-15 appears as a triplet and C-20 as a singlet. Some dilution of the label during biosynthesis leads to overlapping signals, e.g., a doublet superimposed on a singlet for C-5 and C-10. This method for assigning the signals was used in the proof that the third methyl group to be introduced in vitamin B_{12} biosynthesis is attached at C-20.[10]

B. Timing of the Rearrangement

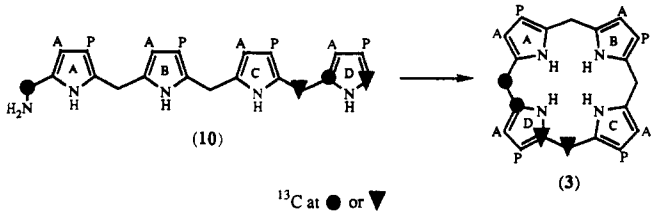
In principle, the rearrangement of ring D could occur at any stage in the building process from a monopyrrole to a tetrapyrrole. If it occurs at the monopyrrolic stage, it would be expected that PBG would be isomerized to isoPBG (**8**); however, no significant incorporation of isoPBG into protoporphyrin IX could be observed.[11]



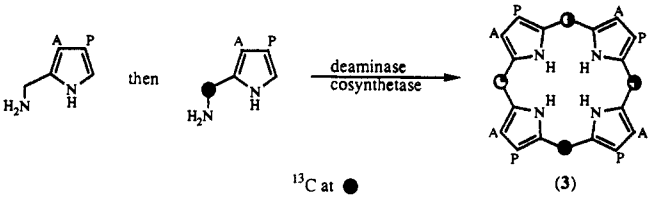
In order to test the possibility of rearrangement at the dipyrrolic stage, the unrearranged (aminomethyl)-dipyrromethane (**9**) and its three isomers in which one or both pyrrole rings are inverted were synthesized in ^{14}C-labeled form. Only the unrearranged dipyrrole (**9**) showed significant incorporation into protoporphyrin,[11] and degradation showed that the labeling was specific. This evidence would often be taken as sufficient to indicate that **9** is an intermediate in the biosynthetic pathway. However, it was later shown that **9** undergoes considerable nonenzymatic dimerization to give uro'gens, mainly of types I and IV.[12] In the presence of deaminase–cosynthetase, the major isomer becomes type III, produced at the expense of type I, while type IV is still present in the same proportions. The conclusion was that two molecules of the dipyrrole combine nonenzymically to form tetrapyrrolic bilanes (mostly of types I and IV), which in the absence of enzyme would cyclize to the corresponding uro'gen isomer. In the presence of deaminase–cosynthetase, however, the head-to-tail type I bilane is cyclized with rearrangement to uro'gen III while the type IV isomer remains unaffected. This conclusion was supported by the observation that high incorporations of **9** were only obtained with high concentrations and long incubations, conditions favoring the nonenzymic dimerization.[12,13]

The first evidence for the intermediacy of a bilane had come from studies of the reaction of PBG with

SCHEME III



SCHEME IV



deaminase in the presence of ammonia or hydroxylamine. This gave products identified as (aminomethyl)bilane (**10**) and its hydroxyamino analogue **11**.[14] (Aminomethyl)bilane (**10**) was an obvious candidate for the intermediate between PBG and uro'gen III, and it was synthesized by Müller's group and that at Cambridge, both of which showed[15] that deaminase–cosynthetase caused production of uro'gen III from this substrate, whereas nonenzymically only uro'gen I was formed. This (aminomethyl)bilane was then synthesized in two doubly ^{13}C-labeled forms, shown in Scheme III, which, after dilution with unlabeled material, were incubated with deaminase–cosynthetase.[16] The resultant labeling patterns of the uro'gen III demonstrate that (i) the rearrangement does occur at the tetrapyrrole stage, (ii) ring D of the bilane becomes ring D of uro'gen III, and (iii) the reaction is intramolecular, between the two ends of the same bilane molecule.

C. Order of Assembly of the Four Rings

The experiment just described indicated that deaminase–cosynthetase first produces an unrearranged bilane and then cyclizes it with rearrangement such that rings A–D of the bilane coincidentally become rings A–D of uro'gen III. The question then arises as to what order the four rings of the bilane were assembled. It could, for example, be that ring A was bound to the enzyme first and then ring B attached, and then C to these two, etc., or assembly could start at the other end with ring D, or two dipyrrolic units could be assembled and then joined together, or a random method of assembly could be conceived. In order to tackle this problem, a pulse-labeling experiment was planned.[17] This required a stoichiometric quantity of enzyme, and ca. 0.25 μmol of deaminase (with cosynthetase) was isolated from *E. gracilis*. This was incubated with a pulse of 0.50 μmol of unlabeled PBG, which is only half the amount required for one turnover of the enzyme. As a result, nearly all the enzyme molecules would have been loaded with at least one PBG and decreasing proportions would have had two, three, or four PBG molecules loaded. Addition of [11-^{13}C]PBG then caused the unlabeled PBG to be chased through to uro'gen III. In this way the ring in uro'gen III that is derived from the first ring to bind to deaminase would have the least ^{13}C label. Analysis of the labeling was achieved by oxidation of the uro'gen III to the corresponding uroporphyrin, decarboxylation of the acetate side chains by heating in hydrochloric acid, esterification of the resulting coproporphyrin III, and ^{1}H NMR spectroscopy in the presence of a shift reagent. This clearly showed that the least ^{13}C was at C-20 followed by C-5, C-10, and C-15, the latter being almost entirely ^{13}C-labeled (Scheme IV). Hence, the order of assembly of uro'gen III is ring A followed by B then C and finally D, and the order of assembly of the intermediate bilane is the same.

The same order of assembly was also deduced later in the same year,[18] following essentially the same pulse-labeling approach except that ^{14}C-labeled PBG was used with an enzyme system from *Rhodopseudomonas sphaeroides* which converted it to protoporphyrin IX. The label was located by a degradation that only distinguished rings A + B from C + D.

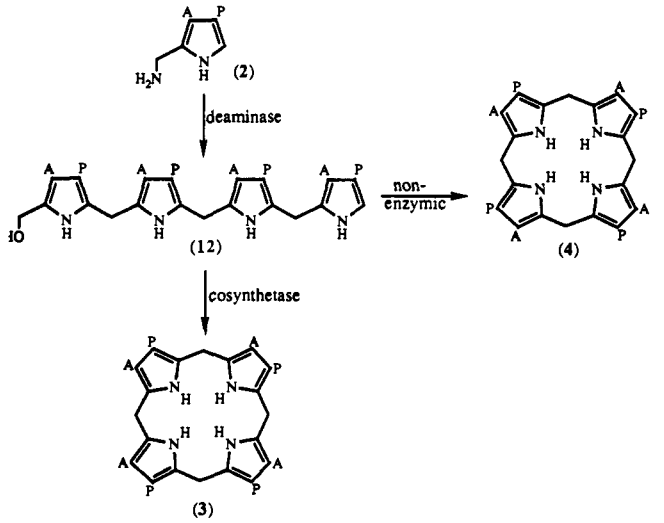
D. Direct Detection of the Product of Deaminase

Although the experiments described above with (aminomethyl)bilane (**10**) showed that it could be a substrate for deaminase–cosynthetase, it soon became apparent[19,20] that it could not be a normal intermediate in the process because (i) it is not detected as a normal product of deaminase except in the presence of a high concentration of ammonia, (ii) it is converted to uro'gens by deaminase (or deaminase–cosynthetase) 15 times slower than PBG, and (iii) it is not a substrate for cosynthetase but is converted by deaminase into some other compound that is a substrate for cosynthetase.

The time lag that was observed in the production of uro'gen I (**4**) from (aminomethyl)bilane (**10**) was also observed in Texas and Cambridge when PBG was incubated with a large quantity of deaminase.[19,20] Almost total consumption of PBG had occurred before 10% of the uro'gen I had been produced.[20] Clearly some intermediate accumulates and is then converted to uro'gen I. It was shown that this conversion is not accelerated by additional deaminase and is, therefore, a nonenzymic process whose half-life was measured as ca. 5 min at pH 8.25. Added cosynthetase, on the other hand, caused very rapid production of uro'gen III from this intermediate.

The nature of this intermediate was revealed by ^{13}C NMR spectroscopy following incubation of [11-^{13}C]PBG with deaminase. The spectra had to be recorded at 0 °C or after the addition of sodium hydroxide in order to stabilize the intermediate. This revealed methylene signals at δ 24.5 and 57.2 in a ratio of ca. 3:1, and there were differing views[19,20] on the structural interpretation of these signals. However, studies of a range of model compounds allowed the signals to be recognized[20,21] as belonging to three pyrrole–CH_2–pyrrole groups and one $HOCH_2$–pyrrole, respectively. It follows therefore that the intermediate must be (hydroxymethyl)bilane (**12**) (Scheme V). This structure was conclusively proved by an unambiguous synthesis of **12**.[21] The synthetic material had spectroscopic properties identical with those of the enzymic intermediate, cyclized nonenzymically in the same way to give uro'gen I, and was cyclized very rapidly by cosynthetase to give uro'gen III. It was

SCHEME V



later shown[22] that the accumulation and subsequent disappearance of 12 can be followed at pH 8 by ^1H NMR spectroscopy, the distinguishable peaks being at δ 6.4 (pyrrole α-H) and 4.4 (OCH_2-pyrrole).

It was soon shown that (hydroxymethyl)bilane (12) is converted into uro'gen III by cosynthetases from a wide range of sources including mammals, plants, yeast, and bacteria.[23]

These experiments clarified the relationship between deaminase and cosynthetase and indicated for the first time the true roles of two enzymes. Deaminase is the assembling enzyme but is not in any way responsible for ring closure, either to uro'gen I as had originally been assumed or to other macrocyclic structures as had been suggested. Cosynthetase is the ring-closing enzyme and is perfectly active in the absence of deaminase. Though it is possible that the two enzymes are associated in living systems, their mechanism of action does not *require* an interaction between them.

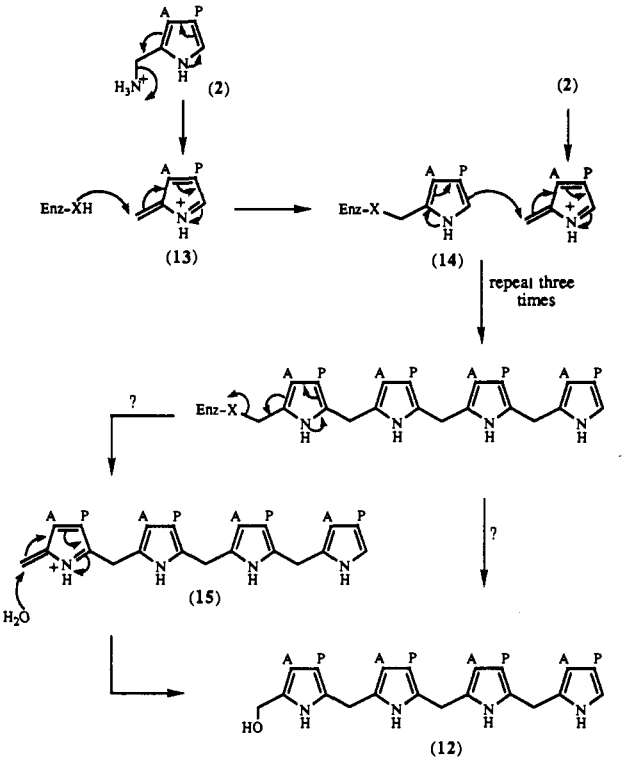
III. Porphobilinogen Deaminase

A. Attachment of PBG to Deaminase

The experiments described above mostly used either crude preparations or partially purified deaminase–cosynthetase. Although deaminase can be obtained free of cosynthetase activity by heat inactivation of the latter, further studies on deaminase or on cosynthetase alone required separation of the two enzymes. Deaminase (now alternatively known as (hydroxymethyl)bilane synthase, EC 4.3.1.8) has been purified from a number of different sources including *Rh. sphaeroides*,[24] spinach,[25] human red blood cells,[26,27] *Chlorella regularis*,[28] *E. gracilis*,[7,29] rat liver,[30] and *Escherichia coli*.[31,32]

It was noted, first by Anderson and Desnick for the human enzyme[27] and then for the one from *Rh. sphaeroides*,[33] that deaminase forms moderately stable complexes with PBG and the complexes in which one, two, three, and (for the human enzyme) four molecules of PBG are attached to one molecule of enzyme can be separated by electrophoresis. It was demonstrated that denaturation of these complexes did not cause release of the PBG, implying covalent attachment to the enzyme.[7,34] Also it was shown that binding of 1 mol of

SCHEME VI



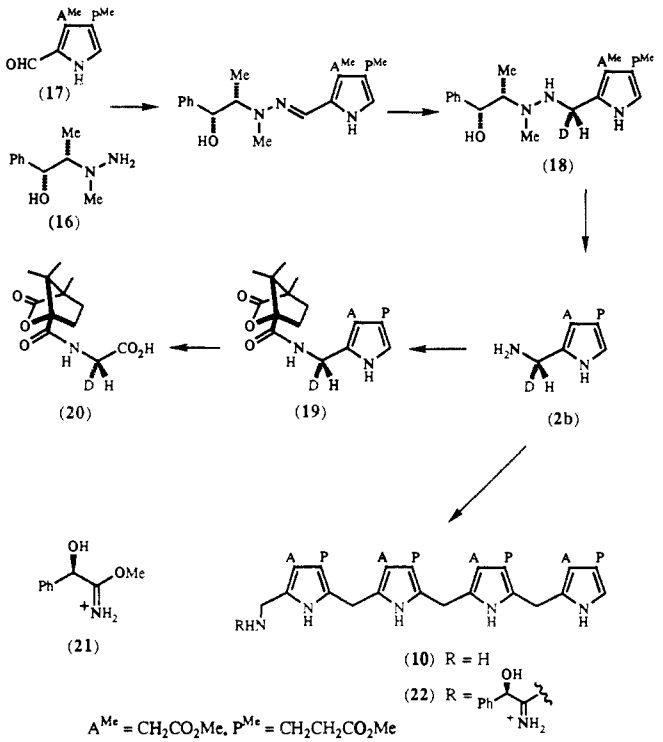
PBG/mol of enzyme results in release of 1 mol of ammonia.[35] Finally one can surmise that if binding of PBG to deaminase had been purely noncovalent and hence freely reversible, it is unlikely that the order of assembly experiments described above would have been successful.

Thus, it can be concluded that the first molecule of PBG binds to deaminase via some enzymic group X, with loss of the amino group to give the Enz–$(PBG)_1$ complex (14) (Scheme VI). The ease of displacement of the amino group in this system is to a large extent due to the electron-donating ability of the pyrrole ring. The same reaction in solution would certainly proceed by an S_N1-type mechanism via a protonated azafulvene (13), and accordingly this is shown as an intermediate in the enzymic reaction shown in Scheme VI. It should be recognized, however, that the distinction between S_N1 and S_N2 reactions becomes very blurred in the active sites of enzymes where the nucleophile is already present as the leaving group begins to leave. It is not possible to tell, therefore, whether 13 is a fully formed intermediate in the enzymic reaction or simply a structure that to some extent represents the transition state of a concerted substitution reaction.

Subsequent binding of three more molecules of PBG to each successive free pyrrolic α-position, via 13 or something close to it at each stage, leads to an Enz–$(PBG)_4$ complex. Finally, displacement of the X group by water, probably again via a protonated azafulvene (15) leads to the observed (hydroxymethyl)bilane (12). Alternatively, other nucleophiles such as ammonia or hydroxylamine, if present, can effect the displacement of the X group, leading to the (aminomethyl)- or [(hydroxyamino)methyl]bilanes, (10 or 11).

The stability of the Enz–$(PBG)_4$ complex clearly varies with the source of the enzyme because this complex was detectable after electrophoresis of the human enzyme but not with the enzyme from *Rh. sphaeroides*.

SCHEME VII



With deaminase from *Euglena* it was shown that the product **12** is a competitive inhibitor and binds covalently to give a reasonably stable complex.[35] The bound tetrapyrrole was only removed slowly by the addition of cosynthetase but was rapidly expelled from the enzyme by the addition of PBG.

In support of the mechanism shown in Scheme VI, the three complexes between ^{14}C-labeled PBG and deaminase from *Rh. sphaeroides* were separated by electrophoresis and each was converted to protoporphyrin IX with excess unlabeled PBG and the other necessary enzymes.[36] Degradation of the protoporphyrin showed that all the label from the Enz–$(PBG)_1$ and Enz–$(PBG)_2$ complexes was in rings A and B, whereas from the Enz–$(PBG)_3$ complex one-third of the label was found in ring C and/or D. This experiment confirmed both the nature of the complexes and the previously established order of assembly of the pyrrole rings.

B. Stereochemical Studies

The evidence in section II.D supports the view that (hydroxymethyl)bilane (**12**) is the true product from deaminase and also the normal substrate for cosynthetase. But the possibility has also been considered that the azafulvene (**15**) is the normal product from deaminase, which is then directly cyclized by cosynthetase to uro'gen III without the intervention of **12**. This possibility has been raised again recently.[37,38] If this is true, then the (hydroxymethyl)bilane is a side product formed by water trapping the released azafulvene (**15**). This would result in the loss of any stereochemistry that might have been present at the methylene attached to the X group due to isotopic labeling. It was therefore important to investigate whether any chirality was retained at the H_2NCH_2 or $HOCH_2$ groups of **10** or **12** derived from 11*R*- or 11*S*-deuteriated or -tritiated PBG.

In the first experiment, (11*R*)- and (11*S*)-[11-^2H]PBG were synthesized and incubated with deaminase in the presence of ammonia so as to generate the (aminomethyl)bilane (**10**).[39] This was because **10** does not cyclize to give uro'gen I nearly as fast as **12**, and it was therefore much easier to accumulate a sufficient quantity of product for the required NMR studies. The synthesis of the chirally deuteriated PBG is shown in Scheme VII. The key step that generates the required asymmetric center is the diastereoselective reduction (66% de) of the hydrazone formed between aminoephedrine (**16**) and pyrrolecarboxaldehyde (**17**). As both enantiomers of ephedrine are available, both enantiomers of the resulting hydrazine **18** could be made. Hydrogenolysis of the N–N bond and hydrolysis then gave the chirally deuterated samples of PBG (**2b**). The absolute configurations of these samples were determined by converting them into their camphanyl derivatives **19** and ozonolytic degradation to camphanyl glycine methyl ester (**20**) whose NMR spectrum had already been assigned.

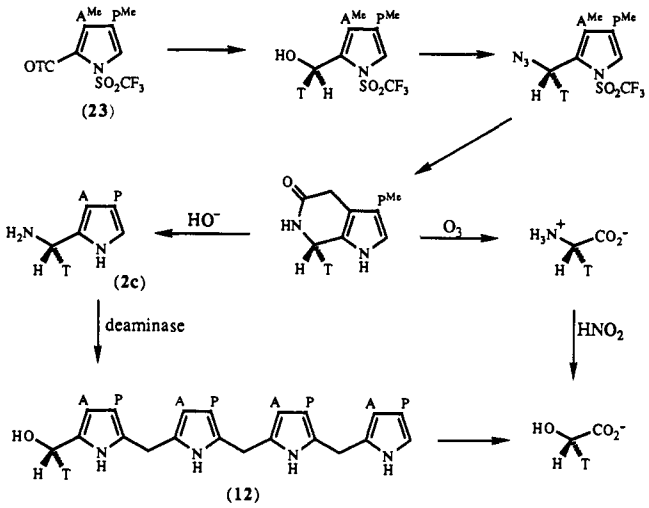
The samples of deuteriated PBG were converted into (aminomethyl)bilane (**10**) by both enzymatic and chemical procedures: The latter left the stereochemistry at the aminomethyl center unchanged. The stereochemistries of the enzymically and chemically synthesized samples were analyzed by reaction with chiral imidate **21** to give amidine **22** followed by ^1H NMR spectroscopy. Samples derived from the same enantiomer of deuteriated PBG by the two methods proved to have the same stereochemistry. Hence, the enzymic reaction proceeds with overall retention of configuration at this center. This implies that the two steps that affect this center (attachment of the first pyrrole to the X group and detachment of the bilane) proceed either both with retention or both with inversion.

It could be argued that formation of (aminomethyl)bilane (**10**) occurs by a mechanism different from that of formation of (hydroxymethyl)bilane (**12**) due to the increased nucleophilicity of ammonia compared to water. In order to study the stereochemistry of **12**, a different approach was required.[40] This was necessary because the lability of **12** means that it is more difficult to accumulate and handle reasonable quantities of this product and also the degradative reactions necessary for determination of the stereochemistry at the $HOCH_2$ site were expected to give low yields. Accordingly, it was necessary to employ the greater sensitivity of tritium rather than deuterium for the label.

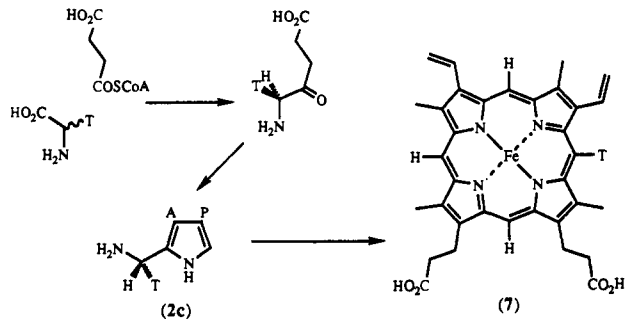
It was found that the previous synthesis of the deuteriated PBG could not be adapted for the synthesis of the tritiated compound without incurring an unacceptably low radiochemical yield. The alternative synthesis shown in Scheme VIII was therefore developed. In this, the chiral center is generated by reduction of pyrrole aldehyde **23** with pinylborane. The *N*-triflyl group is needed both to prevent the aldehyde being too much deactivated by the pyrrole and to stabilize the product.

The samples of chirally tritiated PBG (**2c**) from this synthesis were passed down a column containing deaminase immobilized on Sepharose, and the eluant was run into an alkaline solution in order to stabilize the **12** produced. In this way, considerably more **12** was

SCHEME VIII



SCHEME IX

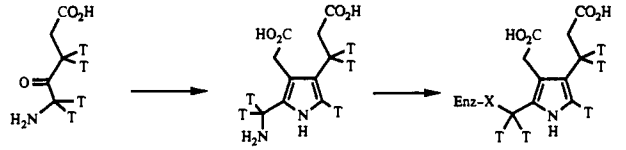


produced than could possibly have been obtained from the same amount of enzyme in solution. The (hydroxymethyl)bilane produced was derivatized and then degraded ozonolytically to give glycolic acid, and glycolic acid was also produced by direct degradation of the starting PBG to glycine followed by diazotization, which is known to proceed with retention of configuration. The configuration at the tritiated center in each sample of glycolic acid was analyzed by oxidation with glycolate oxidase, and again it was found that the enzymically and chemically produced samples from each enantiomer of [11-^{3}H]PBG had the same stereochemistry, indicating overall retention of configuration at this center during the reaction catalyzed by deaminase.[40]

The conclusion is clearly that the (hydroxymethyl)-bilane is not formed by trapping of some reactive species such as **15** free in solution but must be formed at the active site of the enzyme. The foregoing stereochemical studies do not, by themselves, rigorously confirm that (hydroxymethyl)bilane (**12**) is a true biosynthetic intermediate. But when they are combined with all other knowledge of deaminase and cosynthetase, the evidence supporting this view far outweighs that against it.

In the experiments just described, the interpyrrolic methylenes of bilanes **10** and **12** would also presumably have been stereospecifically labeled but it is not yet possible to analyze this stereochemistry. However, the overall stereochemistry at C-11 of PBG during its incorporation into the four meso positions of protoporphyrin IX has been studied by two groups. In the first of these experiments by Akhtar's group[41] 11*S*-tritiated PBG was made enzymically from tritiated glycine and succinyl CoA. Degradation of the heme

SCHEME X



derived from this sample of PBG showed tritium only at C-10 (Scheme IX). In the alternative approach by Jackson et al.,[42] 11*R*-deuteriated PBG was synthesized by an approach very similar to the one above. Incorporation into protoporphyrin IX led to loss of the deuterium at C-10 and retention at the three other meso positions, as judged from the ^{1}H NMR spectrum. Thus, the two groups agree on the overall stereospecificity of the conversion. This is, however, a result of the stereospecificities of three separate enzymes, deaminase, cosynthetase, and protoporphyrinogen oxidase. The specificity of any one of these enzymes cannot be deduced from these results unless the specificities of the other two are known.

C. Nature of the X Group

There was a great deal of interest in establishing the identity of the enzymic group X, by which the first pyrrole ring becomes attached to deaminase. It was expected to be a nucleophilic atom (oxygen, nitrogen, sulfur) of one of the amino acid side chains. However, this proved more difficult to identify than expected. When a large quantity of deaminase (ca. 0.4 μmol) from *Euglena* was incubated with a slight excess of [11-^{13}C]PBG (0.52 μmol), no recognizable signal was observed in the ^{13}C NMR spectrum of the denatured enzyme.[32] This was assumed to be due to the large size of the enzyme, which would cause severe broadening of the peaks. After partial hydrolysis of the protein, either with alkali or a protease, a peak at δ 24.5, corresponding to pyrrole–CH_2–pyrrole, was observed along with some very small peaks at δ 42–43. It was tentatively suggested that the latter might be due to a pyrrole–CH_2 attached to the nitrogen atom of a lysine residue.

The presence of a lysine residue in or close to the active site was confirmed by inhibition studies with pyridoxal phosphate.[43] These showed that deaminase is competitively inhibited by pyridoxal phosphate due to imine formation with lysine residues and this inhibition is made irreversible by reduction of the imines with $NaBH_4$. Binding of the PBG protected the enzyme against inactivation and reduced the level of pyridoxal attachment by one molecule per molecule of enzyme. Hence, one molecule of pyridoxal can bind to a lysine in or near the active site. The apparent pK_a value of this active-site lysine was 6.7, which is abnormally low for the amino group of a lysine residue. It was suggested that this might be the X group.

With the failure of ^{13}C NMR spectroscopy to reveal any signals for labeled PBG attached to intact deaminase, another approach was tried by Scott's group using tritium.[44] PBG (**2d**), having four to five atoms of tritium per molecule, was made from [3,3,5,5-$^3H_4$]-ALA (Scheme X) and incubated with deaminase to give mainly the Enz–$(PBG)_1$ complex. The advantage of using tritium labeling was that there are no background signals from the enzyme in the ^{3}H NMR spectrum as the natural abundance is essentially zero. However, the

signals that were seen were very broad: a clear signal at δ 6.2 corresponded to the pyrrolic α-position and a hump between δ 1.5 and 4.2 appeared to consist of two broad peaks centered at δ 2.5 and 3.3. The former could be attributed to tritium on the propionate side chain, and it was suggested that the latter was due to the XCH_2–pyrrole position. The chemical shift of δ 3.3 was said to be consistent with linkage to a sulfur atom of a cysteine side chain, although an amino group (or indeed other possibilities) could not be excluded on account of the breadth of the signals.

D. Identification of a Bound Cofactor

Further advances in understanding the chemistry of deaminase did not come until after the introduction of two important new techniques.[45,46] First, the gene for deaminase in *E. coli* was cloned and overexpressed. Sequencing of the DNA by the groups of Jordan,[47] Sasarman,[48] and Abell[49] revealed the amino acid sequence consisting of 313 residues with a molecular weight of 33 857. Overexpression of the gene 200-fold was achieved, which allowed the production of much greater quantities of deaminase than had ever been available before. Second, fast protein liquid chromatography (FPLC) was introduced, which not only allowed more rapid purification of the enzyme but also enabled the separation of the various enzyme–PBG complexes on a preparative scale (up to 10 mg/run).

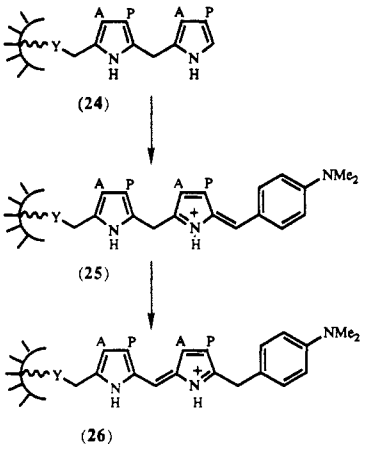
Because of these advances, it was now possible to incubate a large quantity of pure deaminase with 1 equiv of [11-^{13}C]PBG and purify the enzyme–$(PBG)_1$ complex.[45] The ^{13}C NMR spectrum of this complex at pH 8.5 showed only broad signals, which appeared identical with those of the native enzyme, but at pH 12 the signals were sharp, presumably due to denaturation of the protein. Studies on synthetic tripeptides having a PBG residue attached to either lysine or cysteine had shown that if such linkages were present at the active site, they would be sufficiently stable at pH 12 to allow acquisition of a ^{13}C NMR spectrum.[50]

When the ^{13}C NMR spectrum of the native enzyme was subtracted from that of the ^{13}C-labeled complex, the difference spectrum showed one single intense peak at δ 24.6. From synthetic work[50] it was known that this chemical shift did not correspond to attachment of C-11 of PBG to the sulfur atom of a cysteine residue (δ 29–30) nor to the ϵ-amino group of a lysine residue (ca. δ 45) and certainly not to an oxygen atom ($\delta \geq 57$). It did, however, correspond exactly to the chemical shift of a pyrrole–CH_2–pyrrole group.

This first successful observation of the ^{13}C NMR signal from [11-^{13}C]PBG bound to the enzyme established that the residue in deaminase involved in covalent binding of the substrate is a *pyrrole*. Previously, when this signal had been observed,[35] it had been thought to arise from the enzyme–$(PBG)_2$ complex (and later intermediates or products). In this latest work, however, it was certain, from the FPLC analysis, that the spectrum was of the pure enzyme–$(PBG)_1$ complex and was thus unambiguous.

The ^{13}C NMR spectrum of the purified enzyme–$([11\text{-}^{13}C]PBG)_2$ complex was also recorded, and as now expected it showed only the one peak at δ 24.6 in its difference spectrum but this was of nearly twice the size of the peak from the mono complex. Thus, both ^{13}C

SCHEME XI

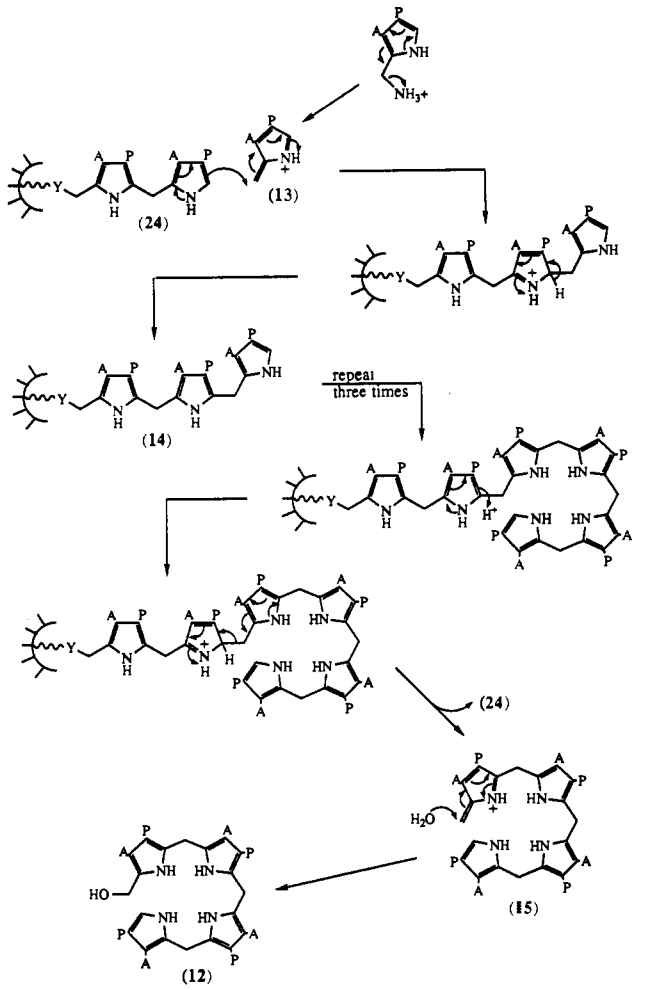


atoms are in the same type of pyrrole–CH_2–pyrrole group in this di complex. This confirmed the presence of a previously unsuspected and novel pyrrolic cofactor firmly attached to deaminase, to which the first PBG unit is bound.[45]

At this stage several different observations interlocked. It was observed[45] that when native deaminase was treated with aqueous formic acid, a pink coloration developed, which proved to be a mixture of uroporphyrin isomers, presumably formed by aerial oxidation of uro'gens. This could be expected of enzyme–PBG complexes, but it could be demonstrated by FPLC that the native enzyme was free of these complexes so the uroporphyrins must have arisen from the cofactor. Further information about the pyrrolic species bound to the enzyme was revealed by its reaction with Ehrlich's reagent (acidic *p*-(dimethylamino)benzaldehyde), which gives a pinkish purple color (λ_{max} 560–570 nm) with free α-pyrroles. Native deaminase gave this coloration (λ_{max} 564 nm) with Ehrlich's reagent initially, but over a few minutes it changed to an orange-red color (λ_{max} 495 nm), which is more typical of a conjugated dipyrromethene. This type of spectroscopic change had been noted earlier for free α-dipyrromethanes and bilanes.[51] It is thought[45] to be caused by tautomerization of the initial Ehrlich's product, such as **25**, to the pyrromethene (**26**), as shown in Scheme XI. These changes in the absorption spectrum for the Ehrlich reaction and also the formation of porphyrins from deaminase were also observed independently by Jordan and Warren[46] who interpreted them similarly. Finally, deaminase from a number of different sources behaves in the foregoing way with Ehrlich's reagent.[45,52]

The evidence above shows the presence on native deaminase of a dipyrromethane cofactor (**24**) made up of two PBG units. Further, the ^{13}C NMR results establish the binding of the growing oligopyrrole chain to the enzymic cofactor. The full mechanism of action of deaminase can now be drawn as shown in Scheme XII. It is apparent that the attachment of the first substrate PBG molecule occurs by a mechanism identical with that for the attachment of the second, third, and fourth molecules. It may be that the enzyme can use the same catalytic groups for all four steps. This possibility for catalytic efficiency may be the reason why use of this unique cofactor has evolved. It should be noted that the cofactor remains in place throughout the catalytic cycle and does not turn over.

SCHEME XII



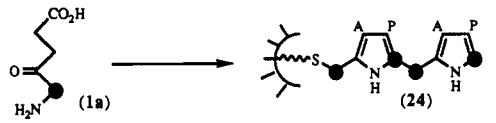
The foregoing developments led to a period of high activity in several laboratories, and the results will be outlined in the following section. However, the findings of this valuable subsequent work[53-56] were in full agreement with the conclusions set out above.

E. Site of Attachment of the Cofactor

Once it was discovered that the X group is dipyrromethane **24**, the question immediately arose as to what the group Y is to which the cofactor is attached. Clearly, ^{13}C NMR spectroscopy could give the answer to this question, just as it had for the X group, as long as a method could be found for labeling the cofactor at the appropriate carbon atom. Two approaches suggested themselves: either reconstitute the apoenzyme in vitro with labeled cofactor or arrange for the cofactor to be labeled in vivo by incorporation of a suitable precursor. In practice, both of these approaches were undertaken and both proved successful.

The first reported experiment used the in vitro reconstitution approach.[57] The cofactor was cleaved from the protein by treatment with hydrochloric acid. The precipitated protein was resolubilized in 6 M aqueous urea, and the urea was then slowly removed by dialysis. At this stage the apoenzyme was devoid of catalytic activity and showed no interaction with Ehrlich's reagent. Incubation with [11-^{13}C]PBG for 4 h at 5 °C then reconstituted the cofactor and restored 43% of the original activity. The reconstituted enzyme was then

SCHEME XIII



repurified by FPLC. It is very interesting that the apoprotein of deaminase is capable of assembling its own dipyrromethane cofactor and does not require any additional enzyme to perform the attachment, as is the case for the linking of heme to apocytochrome *c*, for example. This reconstitution has subsequently been confirmed.[53,54]

In order to obtain good ^{13}C NMR spectra of this labeled cofactor, the sample had to be made more alkaline than before (pH 14), presumably because the region of the protein around the cofactor is more resistant to denaturation. The difference spectrum between this labeled enzyme and unlabeled enzyme showed just two significant peaks: one was at δ 24.5, as expected for the interpyrrolic methylene, and the other appeared at δ 29.5. This latter chemical shift is exactly that found for PBG attached to a cysteine residue of a model tripeptide.[50] It was concluded, therefore, that the cofactor is attached to one of the cysteine residues of the enzyme. This conclusion was supported by the finding that only three cysteine residues could be modified in the denatured protein despite the fact that the DNA sequence shows that there are four cysteine residues in *E. coli* deaminase.[55,57]

The other approach to introducing ^{13}C labels into the cofactor was by biosynthetic incorporation. This approach was taken, more or less simultaneously by three different groups.[55,56,58] It was first established that deaminase derived from *E. coli* grown in the presence of ^{14}C-labeled ALA contained radioactivity that was released when the cofactor was cleaved from the protein using formic acid.[46] Incorporation of radioactive ALA or PBG into deaminase has also been observed in pea chloroplasts.[59] For the enzyme from *E. coli* this radioactivity is not released during normal turnover of the enzyme nor does the enzyme become radioactive when ^{14}C-labeled PBG is turned over by unlabeled enzyme.[45,52,53] There is no interchange, it appears, between the pyrrole units of the cofactor and those of the substrate.

The biosynthetic origin of the cofactor from ALA and PBG has also been shown by a different method. Mutants of *E. coli* that are deficient in ALA synthase (*hem*A) or ALA dehydratase (*hem*B) have very low deaminase activity, but this activity can be restored by the addition of ALA or PBG to the growth medium.[53,54,60,61]

The overproducing strain of *E. coli* was next grown on [5-^{13}C]ALA, and the ^{13}C NMR spectrum of the purified enzyme as observed in Cambridge,[58] Southampton,[55] and Texas[56] revealed four enriched signals as expected for the biosynthesis of a dipyrromethane from ALA (see Scheme XIII). In addition to the two previously mentioned peaks at δ 24.5 and 29.5, peaks were also observed at δ 116.2 and 128.3 for the two labeled pyrrolic carbons. The latter peak was smaller than the other three as expected for a quaternary carbon and was a clear doublet coupled to the carbon at δ 24.5 (J = 45 Hz).[58] These results both confirm the attachment of

the cofactor to a sulfur atom and prove its structure beyond any reasonable doubt.

As mentioned above, there are four cysteines in *E. coli* deaminase, but the site of attachment of the cofactor could be narrowed down because only two of these are conserved in the sequence of human deaminase, which otherwise shows considerable homology.[62] Treatment of deaminase from *E. coli* with 70% formic acid for 30 h caused specific cleavage of the peptide between Asp-103 and Pro-104. Surprisingly, after this strongly acidic treatment, a small amount of ^{14}C-labeled cofactor was still found attached to one of the two peptides.[55] The only conserved cysteine residue in this moiety is Cys-242, and hence it was suggested that this is the site of attachment of the cofactor.

A more rigorous proof that Cys-242 is the binding site came from degradation of the enzyme with a protease, endoproteinase Glu-C.[63] The cofactor-bearing peptide was purified by reversed-phase FPLC and sequenced. The sequence was identical with that deduced from the DNA sequence for amino acids 240–251, which contains only one cysteine residue, at position 242.

Site-directed mutagenesis of the two conserved cysteine residues in deaminase from *E. coli* by Scott's group has also confirmed the location of the cofactor attachment. Changing Cys-99 for Ser had no significant effect on the activity of the enzyme, whereas changing Cys-242 caused complete deactivation.[37]

F. Evidence about the Active Site of Deaminase

The identification of the dipyrromethane cofactor at the active site and its attachment to Cys-242 has been a great advance in understanding the mechanism of deaminase, but as yet little is known for sure about the other catalytic and binding groups present. Such information will be extremely valuable for deducing the fine details of the mechanism once further details of the structure are available. It has been reported that crystals have been obtained from a mixture of five forms of deaminase from an overproducing strain of *E. coli*.[32] Presumably the crystals represent one of these proteins, and so it can be hoped that the X-ray crystal structure of this material will soon be available.

It is sometimes possible to deduce catalytically important groups by comparing the amino acid sequences from a wide variety of different organisms. Currently the sequences of deaminase from *E. coli*,[49] humans,[62] rats,[64] and *E. gracilis*[65] are available. However, there are a large number of amino acids that are conserved over all four of these enzymes. Therefore, it is not yet possible to assign the residues involved in the active site. One possibly relevant observation is that there are 12 conserved arginine residues, a surprisingly large number. It is interesting to speculate that these arginines are used to bind the carboxyl residues of the cofactor and four substrate molecules, which also number 12 (perhaps just coincidentally).

Chemical modification studies mentioned earlier indicated the presence of a lysine residue close to or at the active site of deaminase from *E. gracilis* and *Rh. sphaeroides*,[43] and this has also proved to be the case for the wheat germ[66] and *E. coli* enzyme.[31] The location of this lysine in the *E. coli* enzyme has been investigated by inactivation with pyridoxal phosphate and sodium borotritiide, proteolytic digestion of the enzyme, and purification and sequencing of the resulting radiolabeled peptides.[67] It transpired that it was not just one lysine residue that was labeled but one of two lysines close togther in the sequence (Lys-55 and -59). Both are also conserved in all four known sequences and so probably have some important function. These two lysine residues have been changed for glutamine residues by the technique of site-directed mutagenesis.[68] While changing Lys-59 had a major effect on the enzymic activity, mainly by raising the K_M value, changing Lys-55 had much less overall effect. The fact that the effect of changing Lys-59 was mainly on K_M and not k_{cat} implies that this residue is more involved in substrate binding than in the catalytic mechanism.

Inactivation of deaminase by modification of cysteine residues has been observed for enzyme from some sources[24,69] but not others.[66,70] Interestingly, the native enzyme from *E. coli* is relatively resistant to thiol-directed reagents, but the complexes with one, two, and three molecules of PBG are increasingly sensitive to these reagents.[53] Quantitative measurements indicate one thiol becomes exposed, presumably by some conformational change of the protein, and modification of this thiol completely deactivates the enzyme.

Other chemical modification experiments have indicated that both arginine and carboxylate-containing residues are important for the activity of deaminase.[66,70]

G. Remaining Questions on the Mechanism

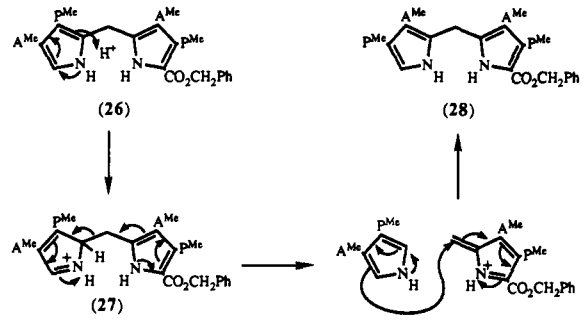
Two main questions about the mechanism of deaminase remain unanswered. First, what is the arrangement of the binding/catalytic sites? Are there six individual binding sites for the six pyrrole rings that are present at the end of the catalytic cycle or does the catalysis all occur at one position between a pair of binding sites and the oligopyrrole chain then moves along to accomodate the next incoming PBG molecule? Second, by what mechanism is the enzyme able to bind to a monopyrrole (i.e., PBG) or a tetrapyrrole (i.e., the hydroxymethylbilane) but apparently not any externally provided intermediate di- or tripyrroles? Similarly the enzyme does not normally release di- or tripyrroles, but there is evidence that enzyme–PBG complexes can slowly release their terminal pyrrole unit.[53] It seems as if the conformational change detected in the enzyme complexes may be important in answering the second question, but further evidence is required before we can give any conclusive answers to either question.

IV. Uroporphyrinogen III Cosynthetase

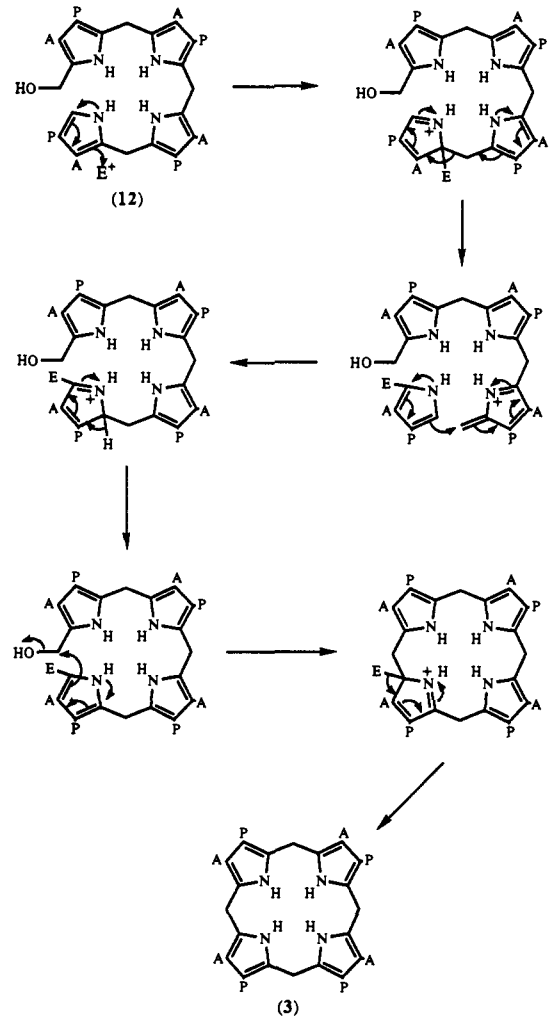
Studies on cosynthetase (uro'gen III synthase, EC 4.2.1.75) alone have lagged behind those on deaminase for a number of reasons: Its true substrate was not known until 1979 and is unstable and difficult to prepare in quantity either enzymically or chemically; the enzyme itself is also unstable and is deactivated by a brief heat treatment or by leaving at room temperature for a short period. Therefore, it is only recently that cosynthetase has been purified to a high degree, from rat liver,[71] *E. gracilis*,[72] and *E. coli*.[73]

The gene for cosynthetase (*hem*D) in *E. coli* was found immediately following that of deaminase (*hem*C); in fact, the first base pair of the former is the last one of the latter. Both this gene[49,74] and the corresponding

SCHEME XIV



SCHEME XV



human one[75] have recently been sequenced, and the *E. coli* enzyme has been overexpressed up to 1000-fold, which has allowed the isolation of milligram quantities for the first time.[76] Following the genes for deaminase and cosynthetase on the genome of *E. coli* are two further genes that appear to be part of the same operon (i.e., their transcription is controlled by the same promoter).[48,49] This would often mean that the corresponding enzymes are part of the same biosynthetic pathway, but no firm evidence has as yet been obtained for the function of these two genes. These recent advances in the molecular biology will no doubt facilitate future studies of cosynthetase, but our current knowledge of the mechanism of this enzyme comes entirely from work with cosynthetase isolated from normal wild-type strains.

The identification of (hydroxymethyl)bilane (**12**) as the substrate for cosynthetase and the elucidation by isotopic labeling of the nature of the rearrangement to give uro'gen III (**3**) have been described above. We will deal here with the possible mechanisms for this reaction.

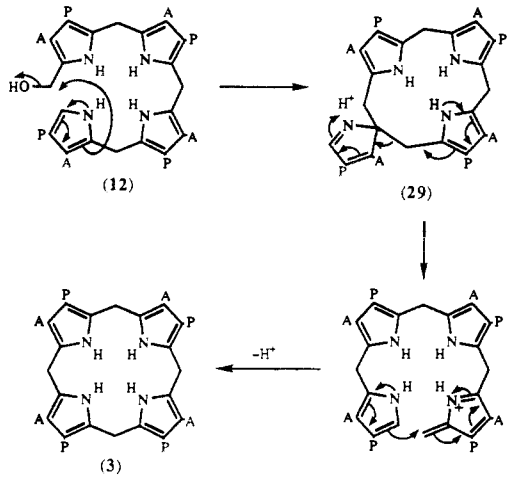
A. Possible Mechanisms

Rearrangement reactions of dipyrromethanes and porphyrinogens in acid have been recognized for a long time.[77] In fact, acid-catalyzed self-condensation of PBG (**2**) leads to uroporphyrinogens in which the orientation of the rings has been totally randomized. Interestingly, the statistical mixture that results contains mostly uro'gen III (50%) and smaller amounts of uro'gens IV (25%), II (12.5%), and I (12.5%). The mechanism assumed for the rearrangement of dipyrromethanes such as **26** ⇄ **28** is shown in Scheme XIV. It has recently been demonstrated by a crossover experiment that this type of rearrangement does indeed proceed by fragmentation followed by recombination as shown rather than a series of intramolecular [1,5]-sigmatropic rearrangements of the pyrrolenine (**27**) formed by the initial protonation.[78]

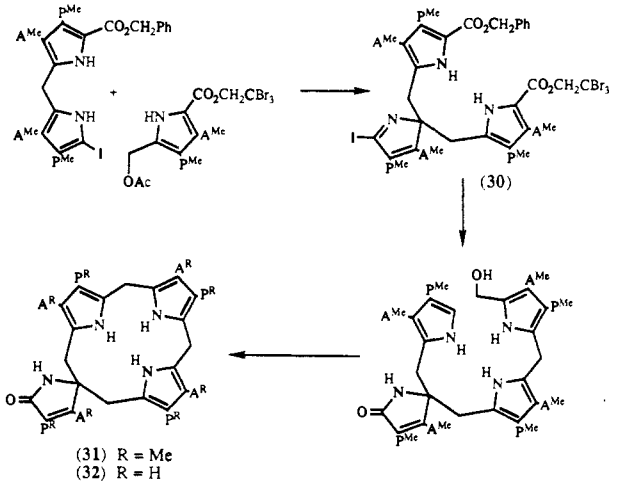
Although in these chemical models rearrangement is always initiated by protonation, in principle any electrophile could replace the proton. Therefore, a possible mechanism for the rearrangement catalyzed by cosynthetase is shown in Scheme XV. The nature of the electrophile has been the subject of considerable debate. It could be a proton, but if this were the case, it would be possible that the monopyrrole that gets detached would be able to leave the enzyme and be replaced by another identical molecule. No such crossover was observed in the double-^{13}C-labeling experiments described earlier. Furthermore, experiments with isomeric bilanes as substrates also make this possibility seem unlikely. We will describe these experiments briefly.

A number of isomeric (aminomethyl)bilanes were synthesized and tested as substrates for deaminase and cosynthetase together,[20,79] but these experiments are difficult to interpret because they depend on the substrate specificity of both enzymes. More direct studies on the substrate specificity of cosynthetase have used (hydroxymethyl)bilanes.[80,81] In testing such compounds it is not always easy to distinguish the enzymic cyclization from the nonenzymic one, which is rapid at physiological pH. Nevertheless, it was shown that (hydroxymethyl)bilanes isomeric to **12** having rings C or D reversed can act as substrates for cosynthetase but ones having ring B reversed cannot.[80] The enzymic reaction on the ring C reversed bilane proceeded with almost complete inversion of ring D, and on the ring D reversed bilane almost 50% inversion was observed, giving uro'gen I. This last result is the most significant one because it shows that while inversion of ring D on the enzyme is not an obligatory part of the mechanism (or not the only mechanism that can occur), it does seem to be quite a strong tendency. If, for example, the mechanism were as shown in Scheme XV with H^+ as the electrophile, then the detached monopyrrole would be the same from the ring D reversed bilane as from **12**. Accordingly, the same (almost exclusively type III) product would be obtained from both, which is not what is observed.

SCHEME XVI



SCHEME XVII



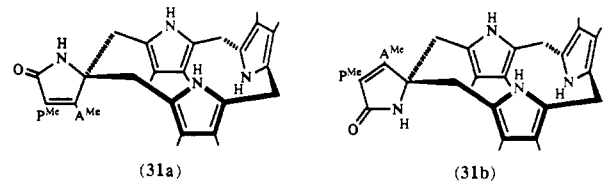
A few other bilanes have also been incubated with cosynthetase. Analogues of 12 in which the acetate and propionate side chains on ring D were separately replaced by methyl and ethyl groups, respectively, have been synthesized.[81] The former was a very poor substrate, but the latter was much better, reacting at 25% of the rate for the normal substrate (12) with 65% inversion of ring D. Analogues of 12 in which the α-position of ring D is substituted with methyl or cyano groups proved to be powerful inhibitors of cosynthetase with K_i values (10 μM) approximately equal to the K_M value for 12.

Returning to the mechanism shown in Scheme XV, another electrophile that has been suggested is a methyleneiminium ion ($R_2^+N{=}CH_2$). The most likely source of this would be methylenetetrahydrofolate, and several reports have indicated that tetrahydrofolates can increase the reactivity of cosynthetase.[71,82,83] However, purified cosynthetase from *E. gracilis*[72] and rat liver[84] does not contain any detectable folate but is still active. It is just possible that folates act allosterically to activate cosynthetase in some organisms, but is seems certain that they are not directly involved in the mechanism.

B. The Spiro Mechanism

An alternative to using an external electrophile is that the electrophile is the hydroxymethyl group in ring A of the bilane. This would result in the formation of a spiropyrrolenine (29) as shown in Scheme XVI. This mechanism also involves fewer steps than one using an external electrophile as the bond to the hydroxymethyl carbon had to be made anyway. When this mechanism was first proposed in 1961, it was suggested that formation of the macrocycle of this spiro intermediate (29) would not be possible because of ring strain.[85] Instead, it was suggested that protonation on the α-positions of the pyrrole rings would have to occur to give more flexibility. This has now been proved to be unnecessary through synthesis of several compounds having the same macrocyclic ring.[86,87] The closest of these compounds to the proposed intermediate 29 is a spiro lactam 32, which only differs in the replacement of the imine by an amide in the five-membered spiro ring.

The synthesis of spiro lactam 32 was based on a novel reaction of an iodopyrrole with an (acetoxymethyl)-pyrrole to give iodopyrrolenine 30 as shown in Scheme XVII.[87] The iodopyrrolenine was hydrolyzed to the lactam, and this was then elaborated to add the fourth ring and cyclized to form the macrocycle. The final cyclization provided not one but two compounds of structure 31. These are thought to differ in the orientation of puckering of the three pyrrole rings with respect to the orientation of the lactam ring. These two atropisomers are illustrated in structures 31a and 31b.



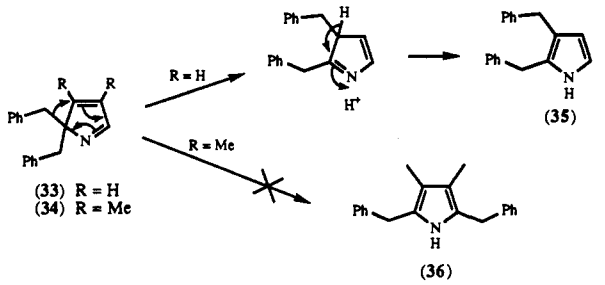
They are separable by chromatography and do not interconvert. It is thought that the space in the center of the macrocycle is so constricted that it is not possible for the NH groups of the pyrrole rings to pass from above the plane to below or vice versa. This interpretation is supported by an X-ray crystal structure of a related macrocycle, which has two cyano groups in the place of the lactam ring.[86]

Both isomers of 31 were separately hydrolyzed to free acids 32. In one case, the product had no effect on cosynthetase but the other isomer proved to be a very strong inhibitor of cosynthetase with a K_i value (ca. 1 μM), which is 1 order of magnitude lower than the K_M of the substrate.[87] This strength of binding strongly suggests that 32 resembles a high-energy intermediate stabilized on the enzyme and indicates that the spiro mechanism is almost certainly correct.

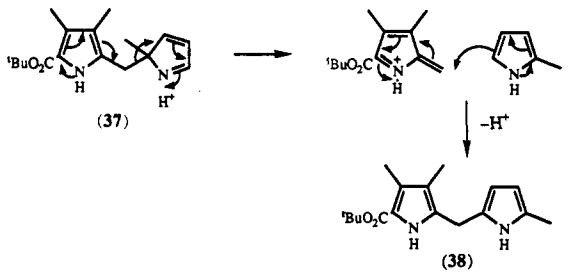
C. Model Studies

As mentioned before for the rearrangement of dipyrromethanes, there are two mechanisms by which an intermediate pyrrolenine such as 29 can rearrange, either by fragmentation and then recombination (as shown in Scheme XVI) or by a series of [1,5]-sigmatropic rearrangements. The possibility of the latter was demonstrated by the synthesis of a simple dibenzylpyrrolenine (33).[88] This rearranged with heating or mild acid treatment to 2,3-dibenzylpyrrole (35), presumably by the [1,5]-sigmatropic mechanism shown in Scheme

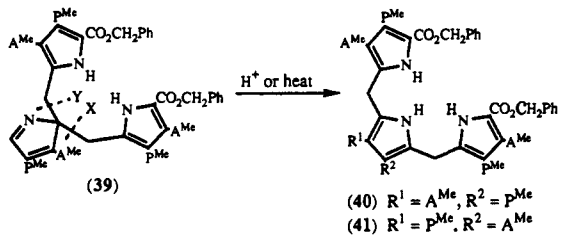
SCHEME XVIII



SCHEME XIX



SCHEME XX



XVIII (fragmentation is not likely because the phenyl ring is not nearly as electron donating as a pyrrole). However, introduction of substituents onto the pyrrolenine as in **34** made the compound much more stable, and there was no sign of the 2,5-dibenzylpyrrole (**36**) which would be produced by a series of three [1,5]-sigmatropic rearrangements.

In contrast to the behavior of benzylpyrrolenine **33**, (pyrrolylmethyl)pyrrolenine (**37**) was very labile and rearranged with the slightest trace of acid to give the 2,5-disubstituted pyrrole **38** as the major product.[88] The substitution pattern of this pyrrole, as well as the structure of some of the minor products of this reaction, indicates that the reaction proceeds by the fragmentation–recombination mechanism shown in Scheme XIX. The rearrangement of bis(pyrrolylmethyl)pyrrolenine (**39**), which is much closer to spiropyrrolenine (**29**), has also been investigated.[89] In this case, the two major products were tripyrroles **40** and **41** (Scheme XX) but significant amounts of crossover products were also formed, thus confirming the expected fragmentation–recombination mechanism. Most interesting was the observation that between 3 and 5 times as much of **40** was formed as of **41**, indicating that cleavage of the bond marked X occurred more readily than that of the bond marked Y. If applied to spiropyrrolenine (**29**), the cleavage at X would lead to uro'gen III whereas that marked Y would lead to uro'gen I.

In summary, these model studies indicate that [1,5]-sigmatropic rearrangements of a compound such as **29** would be most unlikely whereas fragmentation–recombination should be facile. Therefore, it is virtually certain that the mechanism of action of cosynthetase is essentially as shown in Scheme XVI. This might finally be proved by the synthesis of the proposed spiro intermediate **29**, a task which is in hand in the Cambridge laboratories. But its difficulty should not be underestimated, and it is possible that **29** is so labile that it rearranges before it can be brought into contact with the enzyme.

This synthesis and the answers to the questions raised in this review depend on future research.

Registry No. PBG deaminase, 9074-91-3; uro'gen III synthase, 37340-55-9.

V. References

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