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Biosynthesis of Uroporphyrinogens. Interaction among 2-Aminomethyltripyrranes and the Enzymatic System†

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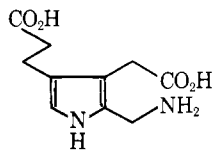
ABSTRACT: Many hypotheses on uroporphyrinogen biosynthesis advanced the possibility that 2-aminomethyltripyrranes formed by porphobilinogen deaminase are further substrates of uroporphyrinogen III co-synthase in the presence of porphobilinogen. These proposals were put to test by employing synthetic 2-aminomethyltripyrranes formally derived from porphobilinogen. None of them was found to be by itself a substrate of deaminase or of co-synthase in the presence of porphobilinogen. The tripyrranes chemically formed uroporphyrinogens by dimerization reactions, and the latter had to be deducted in control runs during the enzymatic studies. Two of the tripyrranes examined, the 2-aminomethyltripyrrane **7** and the 2-aminomethyltripyrrane **8,** were found to be incorporated into enzymatically formed uroporphyrinogen III in the presence of porphobilinogen and of the deaminase-co-synthase system. While the former gave only a slight incorporation, the latter was incorporated in about 16%. No incorporation of **8** into uroporphyrinogen I was detected. On the basis of these results, and of the previous results obtained with 2-aminomethyldipyrrylmethanes, an outline of the most likely pathway of uroporphyrinogen III biosynthesis from porphobilinogen is given.

Porphobilinogen (**1**) (PBG)[1] is transformed into uroporphyrinogen III (**2**) and into uroporphyrinogen I (**3**) in an enzymatic reaction which is catalyzed by two enzymes: porphobilinogen deaminase and uroporphyrinogen III co-synthase (Bogorad, 1958a,b).



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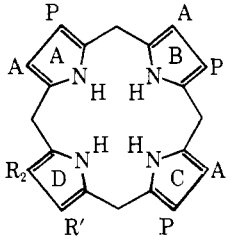

2, R′ = P; R_2 = A
3, R′ = A; R_2 = P

A = CH_2CO_2H; P = $CH_2CH_2CO_2H$

The deaminase is the substrate-consuming enzyme and it gives rise to uroporphyrinogen I (**3**). The enzymatic reaction proceeds with the loss of 4 mol of ammonia, and a stoichiometric relation of 4 mol of substrate consumed per mol of product formed is obtained. Hence, the reaction mechanism can be easily understood as a "head-to-tail" self-polymerization of the natural Mannich base. In the presence of the co-synthase, however, the reaction is driven toward the formation of uroporphyrinogen III (**2**). The mechanism of this reaction has been the subject of much speculation (see Frydman and Frydman (1975a) for a review of the problem). Bogorad (1958a,b) found that the co-synthase is not a substrate-consuming enzyme but takes part somehow in the process and drives it toward uroporphyrinogen III formation instead of uroporphyrinogen I formation. Since co-synthase affects the kinetic constants of the deaminase (Bogorad, 1958a,b; Frydman and Feinstein, 1974), many hypotheses on this reaction mechanism have advanced the idea that the deaminase forms a pyrrylmethane intermediate which serves as a further substrate of the co-synthase together with porphobilinogen. It is during this second enzymatic reaction that the rearrangement would take place leading to the formation of uroporphyrinogen III instead of uroporphyrinogen I. It has thus been proposed (Radmer and Bogorad, 1972; Dalton and Dougherty, 1969; Conford, 1964) that deaminase forms the tripyrrane **4** through a sequential "head-to-tail" polymerization. The tripyrrane **4** is then taken up by the co-synthase and condensed with the fourth molecule of porphobilinogen to give uroporphyrinogen III.

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[1] Abbreviations used are: PBG, porphobilinogen; TLC, thin-layer chromatography.

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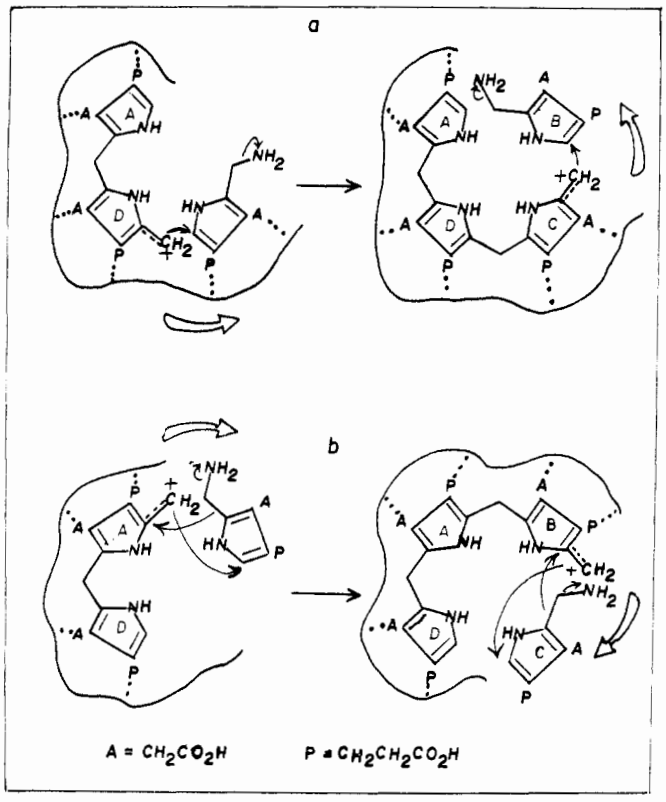
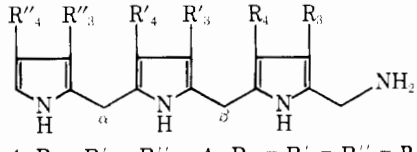


FIGURE 1.

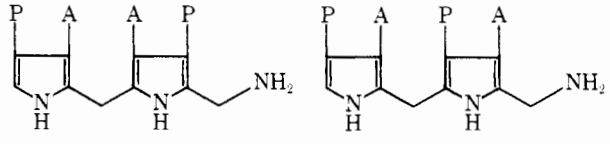
When we put this proposal to test by using synthetic tripyrrane **4** we found, however, that it was only incorporated into uroporphyrinogen I (**3**) (Frydman et al., 1974, 1975; Frydman and Frydman, 1975a). Tripyrrane **5** was suggested as an intermediate in the enzymatic formation of uroporphyringoen III (**2**) (Llambias and Battle, 1971). It has been recently prepared by a total synthesis (Valasinas et al., 1976), and its interaction with the deaminase–co-synthase system will be discussed below.



4, $R_3 = R'_3 = R''_3 = A$; $R_4 = R'_4 = R''_4 = P$
5, $R_3 = R'_4 = R''_4 = P$; $R_4 = R'_3 = R''_3 = A$
7, $R_3 = R'_4 = R''_3 = A$; $R_4 = R'_3 = R''_4 = P$
8, $R_3 = R'_3 = R''_4 = P$; $R_4 = R'_4 = R''_3 = A$

A = CH_2CO_2H; P = $CH_2CH_2CO_2H$

Since we found that dipyrrylmethane **6** was incorporated into **2** and not into **3** (Frydman et al., 1972, 1978), it follows that chain building to form uroporphyrinogen III (**2**) must start with dipyrrylmethane **6** (or its enzyme-bound equivalent), and grow through tripyrrane intermediates to form **2.**



6 **9**

A = CH_2CO_2H; P = $CH_2CH_2CO_2H$

Dipyrrylmethane **6** embodies the moiety of the uroporphyrinogen III molecule which carries rings D and A (Figure 1). Since it is the first intermediate of the process, the two further porphobilinogen units can became attached to it in only two possible ways. The first alternative is through an "anticlockwise" sequence, by which the 2-aminomethyl group (or its equivalent $-CH_2^+$ residue) of **6** is attached to the forthcoming ring D of the uroporphyrinogen molecule and condenses in a "head-to-tail" sequence with the remaining porphobilinogen units. The uroporphyrinogen III molecule would thus be constructed through an A–D → C → B ring sequence (Figure 1a). The second alternative is a "clockwise" type sequence of ring growth, where the 2-aminomethyl residue of **6** is attached to the forthcoming ring A of the uroporphyrinogen molecule, and is constantly displaced by the incoming porphobilinogen units to give first a D–A → B tripyrrane and finally the cyclic uroporphyrinogen III tetramer (Figure 1b).

To examine both sequences, and if possible to distinguish between them, a study was carried out of the interaction of the enzymatic system with the tripyrrane **7** (the possible intermediate in the "anticlockwise" sequence), and with the tripyrrane **8** (the possible intermediate in the "clockwise" sequence). These studies were also expected to afford valuable information about the interaction of the 2-aminomethyltripyrranes with the enzymatic system.

Materials and Methods

Porphobilinogen (**1**) and porphobilinogen-^{14}C were prepared by synthesis (Frydman et al., 1969). The label was at the C-3 of the propionic acid side chain. 2-Aminomethyltripyrranes were prepared by synthesis (Valasinas et al., 1976). When labeled, the ^{14}C was at the (CH_2) α bridge (see **4–8**). All the chemicals used were reagent grade. Porphobilinogen deaminase and uroporphyrinogen III co-synthase were isolated and purified from either wheat germ or human erythrocytes (Frydman and Frydman, 1970; Frydman and Feinstein, 1974), and were recombined to afford the desired isomer composition in the reaction products. The chemical polymerizations of the 2-aminomethyltripyrranes were carried out by heating during 60 min at 37 °C a tripyrrane solution in 0.1 M phosphate buffer (pH 7.4) in a final volume of 100 μL. The standard incubation mixtures contained (in a final volume of 100 μL), 10 μmol of phosphate buffer (pH 7.4), 9 nmol of porphobilinogen-^{12}C or 8 nmol of porphobilinogen-^{14}C (sp act. 7500 cpm/nmol), 25 μL of porphobilinogen deaminase, 50 μL of uroporphyrinogen III co-synthase (when needed to form isomer III), and the indicated amount of tripyrrane used in each case. The incubations were carried out at 37 °C during 60 min; the reaction was stopped by addition of a 1% aqueous iodine solution and the solution was evaporated to dryness in vacuo at 20 °C. When the total amount of uroporphyrinogen formed in the reaction was estimated, the evaporation to dryness was omitted; the excess of iodine was destroyed by adding a 2% sodium thiosulfate solution, and uroporphyrins were estimated spectrophotometrically as described elsewhere (Frydman and Frydman, 1970). When isomers were estimated, the residue obtained in the evaporation described above was esterified with 5% sulfuric acid in methanol, and the octamethyl esters were extracted and decarboxylated to the corresponding coproporphyrins (Edmondson and Schwartz, 1953). The coproporphyrin isomers of types I, II, and III/IV were separated by TLC on cellulose-coated plates (E. Merck, DC-Fertigplatten, Schichtdicke 0.1 mm) using 2,6-lutidine–11 N ammonia (40:28, v/v). Coproporphyrins III and IV were distinguished using TLC on cellulose as described elsewhere (Frydman and Frydman, 1975b). The coproporphyrin isomers were located by fluorescence and eluted with a 0.7 M ammonia solution. The porphyrin concentration in the eluates was determined by a

spectrophotometric method; the eluates were then plated on planchets, and counted in a gas-flow counter. The control runs for the incorporation experiments described in Tables III and V were done by preparing two separate incubation mixtures (for a discussion of this method see Frydman et al., 1978). One contained (in a final volume of 100 μL), 10 μmol of phosphate buffer (pH 7.4), 8 nmol of porphobilinogen-^{14}C, 25 μL of porphobilinogen deaminase, and 50 μL of uroporphyrinogen III co-synthase. The second contained the indicated amount of tripyrrane, porphobilinogen-^{14}C (8 nmol), and 10 μmol of phosphate buffer (pH 7.4) in a final volume of 100 μL. Both mixtures were incubated separately at 37 °C during 60 min and stopped by addition of a 1% aqueous iodine solution, and the resulting uroporphyrin solution was worked up as described above. The obtained octamethyl esters were mixed, and the mixture was decarboxylated as described. The controls prepared in this way accounted for the uroporphyrinogens formed enzymically from porphobilinogen-^{14}C and the uroporphyrinogens formed by the chemical polymerization of the tripyrranes. When the incorporation of tripyrrane-^{14}C into both uroporphyrinogen isomers was measured (Table IV) the incubation mixtures and the control runs were prepared as follows. The incubated system (see Table IV) was obtained by mixing two separate incubation mixtures. One contained deaminase (25 μL), co-synthase (50 μL), porphobilinogen (9 nmol), phosphate buffer (10 μmol), and the indicated amount of tripyrrane-^{14}C (1300 cpm/nmol). The second contained porphobilinogen (9 nmol), the indicated amount of tripyrrane-^{12}C, and phosphate buffer (10 μmol). Both were incubated separately as usual, and the obtained octamethyl esters were mixed and decarboxylated to coproporphyrins. The control system (see Table IV) was also obtained by mixing two separate incubation mixtures. One contained deaminase (25 μL), co-synthase (50 μL), porphobilinogen (9 nmol), and the indicated amount of tripyrrane-^{12}C. The second contained tripyrrane-^{14}C (1300 cpm/nmol) and porphobilinogen (9 nmol). Both mixtures were incubated separately as usual; the obtained octamethyl esters were mixed and then decarboxylated to coproporphyrins. By doing so the inhibitory effect of tripyrrane **7** on the enzymatic formation of uroporphyrinogen III (Frydman and Frydman, 1975a) was accounted for, and the amount of uroporphyrinogens-^{14}C formed in the chemical polymerization of **7** could be subtracted from the enzymatic reaction by including it in control runs (for a discussion of this method see Frydman et al., 1973).

Results

Interaction of 2-Aminomethyltripyrrane **5** *and the Enzymatic System.* The proposal that tripyrrane **5** is an intermediate in the biosynthesis of uroporphyrinogen III was put to test by using synthetic **5**. Under the usual incubation conditions the tripyrrane formed uroporphyrinogens by its chemical self-polymerization (Table I). If it were enzymatically incorporated into uroporphyrinogens by a deaminase-co-synthase system, or by deaminase in the presence of porphobilinogen, then this incorporation should be reflected in higher uroporphyrinogen values then the combined values obtained from the chemical reaction and the enzymatic reaction at the expense of porphobilinogen. When the tripyrrane **5** was incubated with a deaminase-co-synthase system which was forming uroporphyrinogens from porphobilinogen, the amount of uroporphyrinogen formed was less than the combined amounts formed by incubating the tripyrrane with co-synthase (chemical reaction), and the amount of uroporphyrinogen formed by the enzymatic reaction in the absence of **5** (Table I). Similar results were obtained when the tripyrrane was incubated with deaminase in the presence of porphobilinogen, and the amount of total product formed was compared with the values of the chemical reaction and the enzymatic reaction in the absence of **5** (Table I). The tripyrrane **5** was thus not behaving as an intermediate in the biosynthesis of either uroporphyrinogen III or uroporphyrinogen I. Further evidence in this sense was obtained when the effect of **5** on the enzymatic formation of each isomer was examined (Figure 2). The most striking effect was the strong inhibitory effect of **5** on the enzymatic formation of uroporphyrinogen III, which led to its total inhibition in the presence of larger amounts of **5** (Figure 2b). The same inhibitory effect was also exerted on the formation of uroporphyrinogen I by the deaminase, although it was not so pronounced (Figure 2a).

A more elaborate analysis of the interaction of **5** with the enzymatic system leading to uroporphyrinogens III and I can be seen in Table II. The chemical self-condensation of tripyrrane **5** alone or in the presence of porphobilinogen led to the formation of uroporphyrinogen III (or IV) as the main product, together with a smaller amount of uroporphyrinogen I and a minor amount of uroporphyrinogen II. The amount of isomer III in the reaction product was not affected when the incubation was carried out in the presence of deaminase, deaminase

TABLE I: Chemical Polymerization of Tripyrrane **5** in the Presence of the Enzymatic System.[a]

Incubation mixture	Uroporphyrins formed (nmol)	
Tripyrrane	3.57	
Tripyrrane + porphobilinogen	4.06	
Tripyrrane + co-synthase	2.94	
Tripyrrane + co-synthase + porphobilinogen	3.54	→4.57
Deaminase + co-synthase + porphobilinogen	1.63	
Deaminase + co-synthase + porphobilinogen + tripyrrane	3.21	
Deaminase + porphobilinogen	1.79	→4.73
Deaminase + porphobilinogen + tripyrrane	4.29	

[a] The incubation mixtures and conditions were as described under Materials and Methods. Tripyrrane **5** (100 nmol) and porphobilinogen (8 nmol) were used. Deaminase and co-synthase from wheat germ were used.

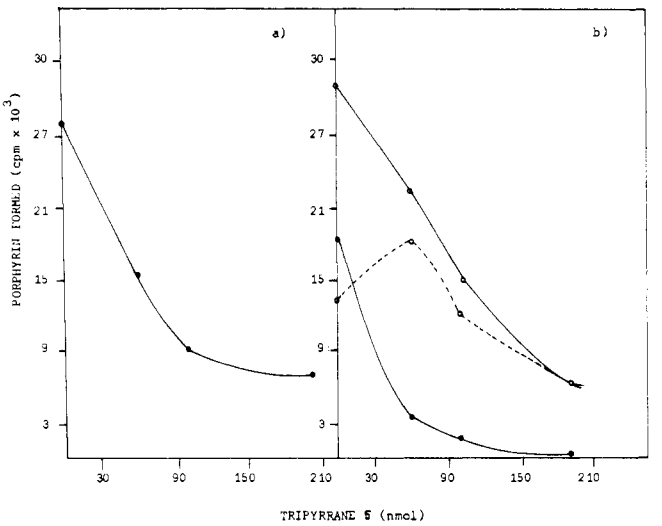


FIGURE 2: Effect of tripyrrane **5** on the activity of (a) porphobilinogen deaminase measured as uroporphyrinogen I; (b) porphobilinogen deaminase-uroporphyrinogen III co-synthase; (O—O) total porphyrins; (O- - - O) uroporphyrinogen I; (●—●) uroporphyrinogen III.

TABLE II: Isomer Analysis of the Chemical Polymerization of Tripyrrane **5** in the Presence of the Enzymatic System.[a]

Incubation mixture	Isomers formed (nmol)		
	I	III/IV	II
Tripyrrane	0.42	1.30	0.10
Tripyrrane + deaminase	0.49	1.30	0.10
Tripyrrane + PBG	0.59	1.35	0.10
Tripyrrane + PBG + deaminase	1.27	1.30	0.10
Tripyrrane + PBG + co-synthase	0.60	1.35	0.15

[a] The incubation mixtures contained tripyrrane **5** (60 nmol) and, when indicated, PBG (9 nmol), and deaminase or co-synthase. The incubation conditions and isomer estimation were described under Materials and Methods.

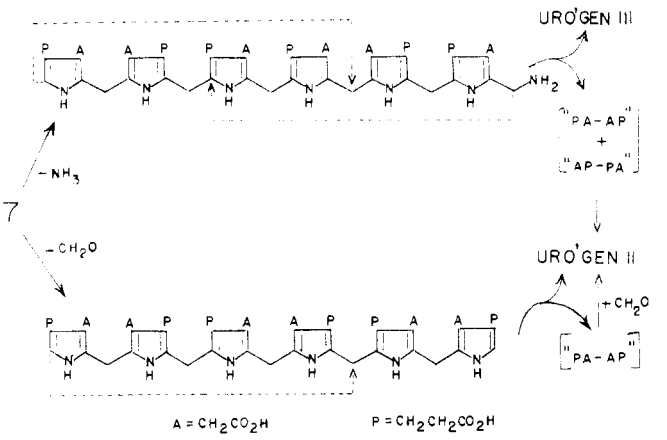


FIGURE 3.

and porphobilinogen, or co-synthase and porphobilinogen (Table II).

When tripyrrane **5** was incubated with porphobilinogen-^{14}C, considerable amounts of label were found in the isolated isomer III. This incorporation was, however, due to the chemical reaction between **5** and porphobilinogen, since it was independent of the presence of either deaminase, co-synthase, or both. These results are further evidence that **5** is not a substrate of co-synthase or of deaminase in the presence of porphobilinogen. The obvious conclusion must then be that **5** is not an intermediate of this biosynthetic process.

Interaction of 2-Aminomethyltripyrrane 7 and the Enzymatic System. When the tripyrrane **7** was incubated at 37 °C and pH 7.4 it formed uroporphyrinogens in 7% yield. An analysis of the two dimerization possibilities of **7** ("head-to-tail" with elimination of ammonia and "head-to-head" with elimination of formaldehyde) indicates that two isomers will arise after the hexamers cyclize to form the thermodynamically favored tetrapyrrole macrocycle, i.e uroporphyrinogen III and uroporphyrinogen II (Figure 3). The experimental results indicated that during the chemical dimerization of **7**, 60% of uroporphyrinogen III and 40% of uroporphyrinogen II were formed. The same mixture of isomers was obtained in the presence of porphobilinogen or of the deaminase-co-synthase system. The tripyrrane was not a substrate of the co-synthase in the presence of porphobilinogen either. It exerted a more pronounced inhibitory effect on the enzymatic formation of uroporphyrinogen III than on the enzymatic formation of uroporphyrinogen (Frydman and Frydman, 1975a).

When **7** was added to a deaminase-co-synthase system forming both uroporphyrinogen isomers from porphobilinogen-^{14}C, a small dilution in the specific activity of isomer III was observed, which was absent in isomer I (Table III). To ascertain if this effect could effectively be correlated with an enzymatic incorporation of **7** into uroporphyrinogen III, the reaction was carried out using tripyrrane-^{14}C and unlabeled porphobilinogen. Control reactions had to be simultaneously prepared to account for the chemical polymerization of **7** and its inhibitory effect on the formation of uroporphyrinogen III as well (see Materials and Methods). The results indicated (Table IV) that a small amount of tripyrrane **7** was incorporated into isomer III but not into isomer I. The effect, however, was only observed with a higher concentration of **7** in the incubation mixture.

Interaction of 2-Aminomethyltripyrrane 8 and the Enzymatic System. The mechanism outlined in Figure 1 suggested that tripyrrane **8** could be an intermediate in the biosynthesis of uroporphyrinogen III. Its chemical polymerization gave rise mainly to a mixture of isomers III and IV. A small amount of uroporphyrinogen II was also formed with higher concentrations of **8**, in a ratio of about seven to eight parts of III/IV to one part of II. This is what could be expected if tripyrrane **8** self-condensed following the mechanism outlined for tripyrrane **7** (Figure 3). Isomer II will be formed by recombination of the dipyrrylmethane fragments liberated during the cyclization of the "head-to-head" condensation product of **8**.

The tripyrrane **8** was not a substrate of co-synthase in the presence of porphobilinogen. Its chemical polymerization was not affected by the addition of either deaminase or co-synthase. It inhibited the enzymatic polymerization of porphobilinogen. The inhibition affected the deaminase more than the deaminase-co-synthase system (Figure 4). In the latter case the inhibition of isomer III paralleled the inhibition of total porphyrins.

When tripyrrane **8** was added to a deaminase-co-synthase system in the presence of porphobilinogen-^{14}C, a marked decrease in the specific activity of uroporphyrinogen III was observed (Table V). This effect was entirely absent from isomer I formed under the same conditions. Simultaneous control reactions were carried out to take care of the chemical self-

TABLE III: Effect of Tripyrrane **7** on the Enzymatic Formation of Uroporphyrinogens I and III.[a]

System	Tripyrrane (nmol)	Uroporphyrinogens						
		Isomer I			Isomer III			
		nmol	cpm	Sp act. (cpm/nmol)	nmol	cpm	Sp act. (cpm/nmol)	Sp act. (Δ)
Incubated		0.34	10 650	31 320	0.43	13 200	30 690	
Control	20	0.37	11 650	31 480	0.47	7 890	16 790	1600
Incubated	20	0.40	12 405	31 000	0.38	5 770	15 184	

[a] The incubated system contained PBG-^{14}C (8 nmol, 7500 cpm/nmol), wheat germ deaminase-co-synthase, and, when indicated, tripyrrane **7**. The control system was prepared by mixing the reaction products obtained from separate incubations of PBG-^{14}C with deaminase-co-synthase, and of tripyrrane **7** (20 nmol) and PBG-^{14}C. The incubation conditions and isomer estimation are as described under Materials and Methods.

TABLE IV: Effect of 7-^{14}C on the Enzymatic System.[a]

System	Tripyrrane (nmol)	Uroporphyrinogens: Isomer I nmol	Isomer I cpm	Isomer I Sp act. (cpm/nmol)	Isomer III nmol	Isomer III cpm	Isomer III Sp act. (cpm/nmol)	Sp act. (Δ)
Incubated	18	0.49	186	379	1.93	1520	787	
Control	18	0.37	150	405	1.08	1070	990	
Incubated	37	0.37	205	554	2.14	2330	1088	134
Control	37	0.32	130	406	1.96	1870	954	

[a] The incubated system was prepared by mixing the reaction products obtained from separate incubations of PBG-^{12}C (9 nmol), the indicated amount of tripyrrane-^{14}C (1300 cpm/nmol), and deaminase-co-synthase, and of PBG-^{12}C and tripyrrane-^{12}C (the indicated amount). The control system was also prepared by mixing the reaction products obtained from separate incubations of PBG-^{12}C, tripyrrane-^{12}C and deaminase-co-synthase, and of tripyrrane-^{14}C and PBG-^{12}C. The incubation conditions and isomer estimation are as described under Materials and Methods.

TABLE V: Effect of Tripyrrane **8** on the Enzymatic Formation of Uroporphyrinogens I and III.[a]

System	Tripyrrane (nmol)	Uroporphyrinogens: Isomer I nmol	Isomer I cpm	Isomer I Sp act. (cpm/nmol)	Isomer III nmol	Isomer III cpm	Isomer III Sp act. (cpm/nmol)	Sp act. (Δ)
Control	10	0.48	11 500	23 960	0.59	12 000	20 340	
Incubated	10	0.45	10 815	24 030	0.49	8 000	16 330	4000
Control	20	0.65	15 200	23 800	1.27	20 180	15 890	
Incubated	20	0.72	17 600	24 440	0.93	9 700	10 430	5460

[a] The incubated system contained PBG-^{14}C (8 nmol, 6000 cpm/nmol), wheat germ deaminase-co-synthase, and tripyrrane **8**. The control system was prepared by mixing the reaction products obtained from separate incubations of PBG-^{14}C with deaminase-co-synthase and of tripyrrane **8** and PBG-^{14}C. The incubation conditions and isomer estimation are described under Materials and Methods.

polymerization of **8**. The decrease in the specific activity of isomer III was larger than the effect obtained with tripyrrane **7**, and strongly resembled the results obtained with dipyrrylmethane **6** (see Frydman et al., 1978).

Discussion

When we considered the biosynthetic mechanism of uroporphyrinogen I formation from porphobilinogen (Frydman and Frydman, 1975a; Frydman et al., 1975), a simple reasoning pointed to the existence of an intermediate with the structure of tripyrrane **4**. When the synthetic tripyrrane **4** was added to a deaminase-co-synthase system forming both uroporphyrinogens I and III it was found to be incorporated only into isomer I (Frydman and Frydman, 1975a). Of the total uroporphyrinogen I formed, about 7% was formed at the expense of the tripyrrane **4**. It also exerted a strong inhibitory effect on the enzymatic formation of uroporphyrinogen III. These results agree with the results obtained when the 2-aminomethyldipyrrylmethanes **6** and **9** were incubated with the enzymatic system. The former was incorporated only into isomer III (Frydman et al., 1978), while the latter was incorporated only into isomer I (Frydman et al., 1973).

Hence, both uroporphyrin isomers could not share a common pyrrylmethane intermediate. The biosynthesis of uroporphyrinogen III must start with a "head-to-head" condensation (reaction through the substituted carbons) of two units of porphobilinogen. The reaction is catalyzed by the associated deaminase-co-synthase system, which produces a shift in one of the aminomethyl side chains giving rise to an enzyme-bound dipyrrylmethane **6**. The shifted "active" methyl group is then successively displaced by the incoming porphobilinogen units to give rise first to tripyrrane **8**, and finally to a bilane which cyclizes to uroporphyrinogen III (Figure 1). The amount of uroporphyrinogen III formed at the expense of this tripyrrane (about 16%) lends support to the idea that it is the most likely intermediate in the enzymatic formation of isomer III. Its incorporation values are markedly higher than those obtained

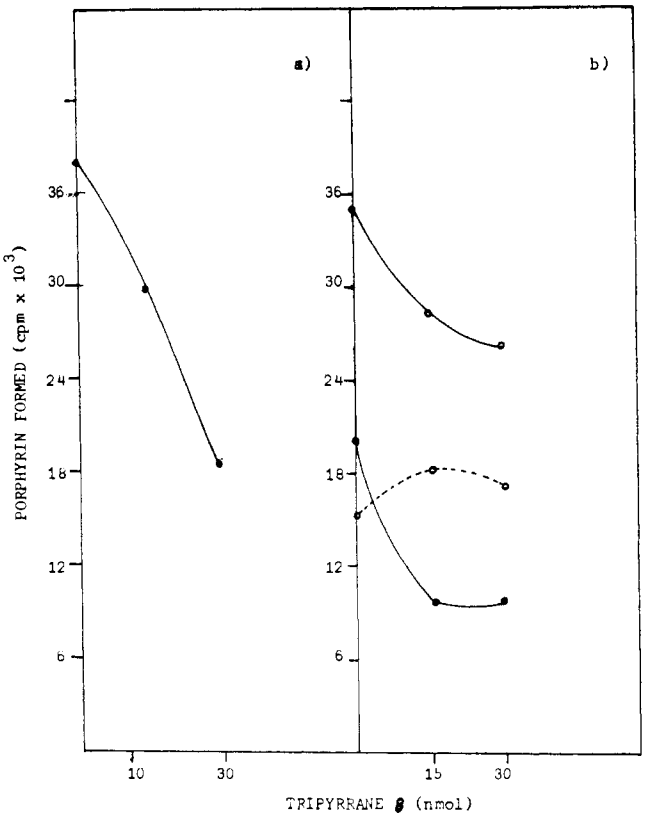


FIGURE 4: Effect of tripyrrane **8** on the activity of (a) porphobilinogen deaminase measured as uroporphyrinogen I; (b) porphobilinogen deaminase-uroporphyrinogen III co-synthase; (O—O) total porphyrins; (O- - -O) uroporphyrinogen I; (●—●) uroporphyrinogen III.

with tripyrrane **7**, the other possible intermediate. It should be kept in mind that the incorporation by the enzymatic system of externally added pyrrylmethanes is always very low, since the polymerization takes place on the enzymatic surface and

no discrete intermediates are liberated into the medium.

From the examination of the interactions between the synthetic tripyrranes and the enzymatic system it becomes clear that the former are not substrates of the co-synthase in the presence of porphobilinogen. This again falls in line with our suggestion that there are not discrete intermediates formed by the deaminase which are taken up by the cosynthase. The specific incorporation of tripyrrane **8** into isomer III, as well as its strong inhibitory effect on the formation of isomer I (Figure 4a), are further evidence that both uroporphyrinogen isomers follow separate pathways during the enzymatic polymerization of porphobilinogen.

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Purification of Cyclic 3′,5′-Nucleotide Phosphodiesterase Inhibitory Protein by Affinity Chromatography on Activator Protein Coupled to Sepharose†

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ABSTRACT: The Ca^{2+}-dependent, reversible, interaction of cyclic adenosine 3′,5′-monophosphate (cAMP) phosphodiesterase with its activator has been used to purify the enzyme by affinity chromatography. Activator-dependent cAMP phosphodiesterase is only a minor component of the proteins specifically adsorbed in the presence of Ca^{2+} by the Ca^{2+}-dependent activator protein coupled to Sepharose and subsequently released by [ethylenebis(oxyethylenenitrilo)]tetraacetic acid. The major protein component can be partially resolved from the enzyme by gel filtration on Sephadex G-200. This protein has been purified to apparent homogeneity and shown to be composed of two polypeptide chains with molecular weights of 61 000 and 15 000, respectively. This protein is, by itself, devoid of phosphodiesterase activity and inhibits the activation of cAMP phosphodiesterase by its activator without affecting the basal activity. Thus, activation of cAMP phosphodiesterase by the Ca^{2+}-dependent activator protein may be controlled by interactions with yet a third component of the enzyme complex.

Cyclic nucleotide phosphodiesterase from mammalian tissues has been shown to exist in multiple molecular forms (for a review of the literature, see Appleman et al., 1973; Amer and Kreigbaum, 1975). At least one of them, the one constituting most of the soluble phosphodiesterase of the cerebrum (Weiss, 1975), interacts specifically with the Ca^{2+}-dependent activator protein[1] first reported by Cheung (1967) and characterized by Cheung (1970, 1971) and by Kakiuchi et al. (1970). The activator protein has been recently purified to homogeneity from several sources (Teo et al., 1973; Lin et al., 1974; Watterson et al., 1976; Klee, 1977; Wolff et al., 1977). The Ca^{2+}-dependent interaction of the enzyme with its activator, which is specific and reversible (Kakiuchi et al., 1975; Lin et al., 1975), has been used in the purification procedure of soluble cAMP phosphodiesterase of bovine brain (Watterson and Vanaman, 1976; Miyake et al., 1977). The results presented here, however, indicate that the enzyme is a minor component of the proteins specifically retained on a column of activator protein coupled to Sepharose in the presence of Ca^{2+}, and released by EGTA. The major protein component released by EGTA can be resolved from the enzyme by Sephadex G-200 gel filtration and it inhibits the activation of the enzyme by its

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[1] The cAMP phosphodiesterase activator protein has also been called modulator protein (Watterson et al., 1976) and calcium-dependent regulator protein.

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