ture of the synthesis. In a sense **72** may therefore be termed a "pro-racemic" mixture.[72]

As a bonus to the foregoing, the absolute stereochemistry of each enantiomer will be known a priori from the stereochemistry of the corresponding progenitorial diastereomer as previously determined by the NMR data. X-Ray analysis will therefore be unnecessary.

Case studies designed to explore, among other things, the utility of such "pro-racemic" mixtures for the realization of asymmetric syntheses are under way in the author's laboratory.

Epilog

The transformations described in this Account originate with 2,3-unsaturated hexopyranosides. A subsequent Account might very well focus on different progenitors, since 1,2, 3,4, or 5,6 unsaturation is readily introduced into pyranosides, and the related unsaturated furanosides are growing rapidly.

(72) Strictly speaking, **72** would be a "pro-racemic" mixture only if both diastereomers were present in equal amounts. However to our knowledge, there is no word to describe an unequal mixture of enantiomers, and we do not have the classical background to invent one. We have therefore chosen to define as "pro-racemic" that unique mixture (equal or unequal) of two diastereomers which, if separated and processed along identical lines, leads to both enantiomers of a given molecule.

Although designed primarily as intermediates in the synthesis of modified saccharides, these molecules are often so accessible and so interestingly functionalized that they ought to find additional outlets. Heretofore, sugars have found employment in spectroscopic and, to a lesser extent, mechanistic studies. But by and large, synthetic organic chemistry has remained in splendid isolation from sugars. It is probably for the achievement of asymmetric syntheses that their application augurs best.

However this is virtually virgin territory, and while a prospector or two would be welcome, the urge for self-preservation prays against the eventuality of a sugar-rush.

During 8 years at Waterloo, I have been privileged to receive counsel and guidance in the development of a viable research program from a total of 12 young men. Their skill and ingenuity have been boundless, and their dedication well beyond the call of duty. A special tribute is due to my first Ph.D. collaborator, Dr. Bruno Radatus, who, in those early, uncertain days, refused to let me take an easier, softer path. The pattern he set was continued by Drs. Neville Holder and Steve Tam, and my present graduate students Bob Anderson, Dave Hicks, Dave Iley, Dave Walker, and Mark Yunker continue to be a source of inspiration to me. I am deeply grateful to them all. Generous financial assistance has been received from the National Research Council of Canada, Bristol Laboratories (Syracuse), and the University of Waterloo.

Biosynthesis of Uroporphyrinogens from Porphobilinogen. Mechanism and Nature of the Process

Benjamin Frydman* and Rosalia B. Frydman

Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires, Junin 954, Buenos Aires, Argentina

Received November 11, 1974

Porphyrin derivatives play an important part in the biochemistry of all living systems. The sole mention of heme, the chlorophylls, the cytochromes, vitamin B12, and the prosthetic groups of many hemoproteins is sufficient to show the deep involvement of porphyrins in all types of metabolic phenomena.

Porphyrin metabolism is schematically depicted in Figure 1. The polymerization reactions proceed until uroporphyrinogens are formed. Glycine and succinyl-CoA condense to form δ-aminolevulinic acid. The condensation of two units of δ-aminolevulinic acid forms porphobilinogen, and the polymerization of four units of porphobilinogen forms uroporphyrinogens.

Uroporphyrinogen III undergoes a series of structural modifications to afford all the natural porphyrin derivatives[1] and very likely also cobyrinic acid.[2] In higher plants δ-aminolevulinic acid possibly originates via a different pathway.

The superb experimental work of Shemin and his group with the then newly discovered ^{14}C, the contributions of Neuberger, Eriksen, Rimington, Granick, and their associates, as well as the efforts of many other outstanding workers, put the biosynthesis of porphyrins on a firm basis.[3] It was recognized that the polymerization of porphobilinogen (1) affords re-

Benjamin Frydman was born in Buenos Aires in 1935 and took his Ph.D. at the University of Buenos Aires in 1956, working with Venancio Deulofeu on alkaloids from Argentine plants. After 2 years of further work with Henry Rapoport at Berkeley on quinone and pyrrole chemistry, he joined the faculty of the School of Pharmacy and Biochemistry in Buenos Aires, where he is now Professor. His research is largely concerned with the chemistry and metabolism of pyrroles and porphyrins.

Rosalia B. Frydman was born in Poland in 1938, migrated to Argentina in 1947, and in 1962 completed doctoral work at the University of Buenos Aires under C. E. Cardini, working on starch biosynthesis. After postdoctoral work at Berkeley with the late W. Z. Hassid and in Buenos Aires with L. F. Leloir, she joined the faculty of the School of Pharmacy and Biochemistry where she is now Associate Professor. Her research is primarily in enzymology, with special attention to pyrrole metabolism.

(1) J. Lascelles, "Tetrapyrrole Biosynthesis and Its Regulation", W. A. Benjamin, New York, N.Y., 1964, p 38 ff; B. F. Burnham in "Metabolic Pathways", Vol. III. D. M. Greenberg, Ed., 3rd ed, Academic Press, New York, N.Y., 1969, p 403; L. Bogorad in "The Chlorophylls", L. P. Vernon and G. R. Seely, Ed., Academic Press, New York, N.Y., 1966, p 481.

(2) A. Ian Scott, B. Iagen, and E. Lee, *J. Am. Chem. Soc.*, **95**, 5761 (1973), and references therein.

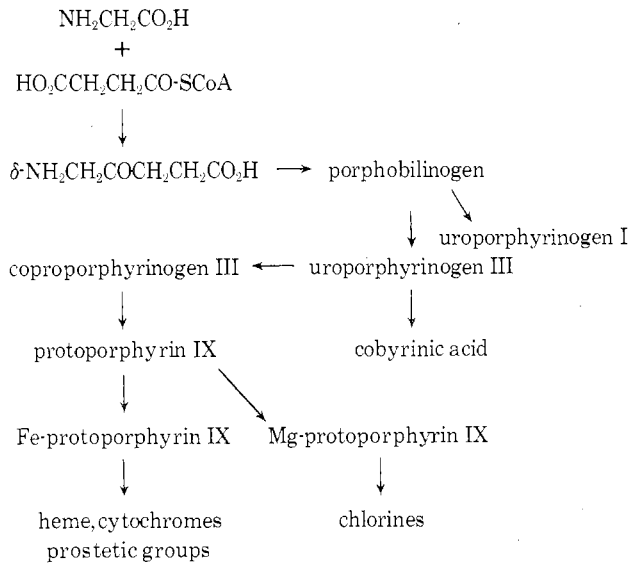
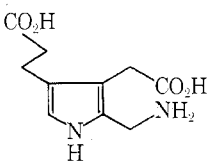


Figure 1.

duced intermediates—porphyrinogens or octahydroporphyrins—which are unstable and are readily oxidized by air to form porphyrins (Figure 2).



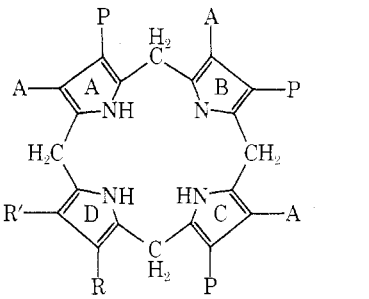
1

Of central interest is a subtle structural difference between uroporphyrinogens I and III. It lies in the location of the acetic (A) and propionic (P) acid side chains on ring D. In uroporphyrinogen I, these side chains are located in head-to-tail fashion, conforming to the pattern of rings, A, B, and C, but in III the positions of the A and P side chains of ring D are reversed.

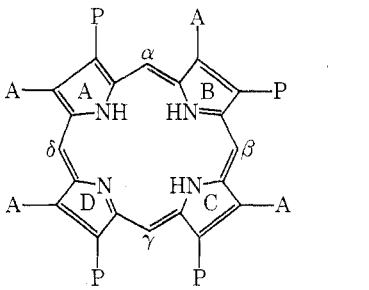
Bogorad[4] isolated from spinach leaves an enzyme—porphobilinogen deaminase—which polymerized 4 mol of porphobilinogen, giving rise to the formation of 1 mol of uroporphyrinogen I and 4 mol of ammonia. From wheat germ he isolated a second enzyme which he called uroporphyrinogen III isomerase or cosynthetase[5] and which did not consume porphobilinogen. When added to porphobilinogen deaminase the system formed uroporphyrinogen III, and porphobilinogen was consumed.[4] The distribution of both enzymes was found to be ubiquituous in all living systems.[1]

The Puzzle of Uroporphyrinogen III Biosynthesis

The isolation of both enzymes illuminated the formation of uroporphyrinogen I while it raised a number of questions regarding the biosynthesis of uroporphyrinogen III. Since porphobilinogen is an α-Man-



A = CH_2CO_2H; P = $CH_2CH_2CO_2H$
uroporphyrinogen III, R = P; R′ = A
uroporphyrinogen I, R = A; R′ = P
(meso carbons are named by greek letters)



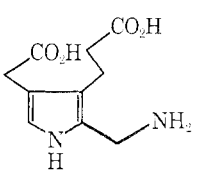
uroporphyrin III, A = CH_2CO_2H; P = $CH_2CH_2CO_2H$

Figure 2.

nich base it was easy to visualize a sequential head-to-tail condensation of four units of the pyrrole, giving rise to a cyclic tetramer where the order of the side chains is that found in uroporphyrinogen I.[6] During the process 4 mol of ammonia have to be released, and this was experimentally found to be the case.

In uroporphyrinogen III, however, there is an inversion in the order of the β substituents of ring D, which had to be attributed to the mode of action of the cosynthase. Uroporphyrinogen III cosynthase appeared then as responsible for the formation of the skeleton of type III porphyrins which is found in all the natural porphyrin derivatives.

It was quickly recognized[4] that uroporphyrinogen III was not produced when uroporphyrinogen I was incubated with uroporphyrinogen III cosynthase or with a system which catalyzed the formation of uroporphyrinogen III from porphobilinogen. Consequently it was clear that uroporphyrinogen I is not a substrate for the cosynthase, which does not open the macrocycle to invert ring D. The cosynthase did not consume porphobilinogen nor transform it into isoporphobilinogen 2,[7] and the latter was not only not



2

(3) G. E. W. Wolstenholme and E. C. P. Millar, *Porphyrin Biosynth. Metab., Ciba Found. Symp.*, **1955** (1955).

(4) L. Bogorad, *J. Biol. Chem.*, **233**, 501 (1958); **233**, 510 (1958).

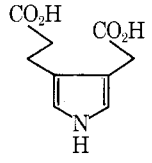
(5) Porphobilinogen deaminase is also called uroporphyrinogen I synthetase, while uroporphyrinogen isomerase is also called uroporphyrinogen III cosynthase or cosynthetase. We prefer to use the names porphobilinogen deaminase and uroporphyrinogen III cosynthase which are more appropriate for their mode of action.

(6) Uroporphyrins and coproporphyrins (and their corresponding porphyrinogens) are classified in types according to the sequence of their two different β substituents. The four possible arrangements are formally derived of the etioporphyrins (methylethylporphyrins) lacking the carboxy groups. Etioporphyrin types I, II, III, and IV are those proposed by H. Fisher and H. Orth, "Die Chemie Des Pyrrols", Vol. II (1), Akademische Verlagsgesell., M.B.H., Leipzig, 1937 p 176.

(7) L. Bogorad in "Comparative Biochemistry of Photoreactive Systems", Ed. M. B. Allen, Ed., Academic Press", New York, N.Y., 1960, p 227.

incorporated into uroporphyrinogen III[8] but exerted an inhibitory effect on the enzymatic system.[9]

Porphobilinogen (1) was chemically polymerized when heated above 60°, affording uroporphyrinogens and liberating formaldehyde and ammonia at the expense of its 2-aminomethyl group. However, it was impossible to detect an enzymatic release of formaldehyde from the 2-aminomethyl group of porphobilinogen,[10] or an enzymatic incorporation of [^{14}C]formaldehyde[10] or [^{14}C]opsopyrroledicarboxylic acid[7] (3)



3

from the reaction medium into uroporphyrinogen III. Hence, the action of cosynthase did not involve the release or recombination of free formaldehyde with any intermediate formed by the deaminase.

Around 1960, the mechanism of uroporphyrinogen III biosynthesis from porphobilinogen could then be better defined by what was not known about it, rather than by what was known.[7] No new experimental evidence that could help understand this process was added during almost a decade. This generated a continuous flow of speculative thinking. Few biochemical processes have produced so many different hypotheses on the nature of its mechanism.

More than 20 hypotheses have been proposed.[11] They all have in common that they propose a rearrangement step at some stage of the reaction. They can be schematically separated into four groups depending on the stage of the process at which the rearrangement is proposed to take place: (a) dipyrrylmethane formation; (b) tripyrrane (pyrrylmethyldipyrrylmethane) formation; (c) bilane formation; and (d) the final cyclization step. While alternatives a and d implicitly propose for the cosynthase the role of a proteic effector of the deaminase, alternatives b and c propose that the cosynthase is an enzyme which uses as a substrate a pyrrylmethane formed by the deaminase, and condenses it with porphobilinogen to form uroporphyrinogen III.

When we approached the problem of uroporphyrinogen III formation, we took into account that previous data concerning the properties of the deaminase and the cosynthase indicated that the enzymatic conversion of porphobilinogen into uroporphyrinogen III must take place through an intramolecular rearrangement reaction, since no other pyrrole apart from porphobilinogen (1) took part in the process, and no exchange of C-1 fragments with the medium could be detected. A number of questions had to be solved simultaneously as one sought to understand the side-chain inversion that occurs in the biosynthesis of uroporphyrinogen III.

The questions are those concerned with the nature of porphobilinogen polymerization by the deaminase. Is this a nonstop reaction which takes place entirely on the enzymatic surface? What can be learned about the interaction of deaminase and cosynthase that could be helpful in understanding the nature of uroporphyrinogen III formation? Is it possible to separate the deaminase into more than one protein, each catalyzing a partial polymerization reaction, and thus obtain pyrrylmethane intermediates formed under normal conditions? And if this is not possible, would synthetic 2-aminomethylpyrrylmethanes act as substrates or intermediates of the formation of uroporphyrinogens I and III? The results obtained are discussed in this Account.

Porphobilinogen Deaminase: Properties and Effect of Inhibitors

Porphobilinogen deaminase was isolated and purified from wheat germ and from human erythrocytes.[9,12] It behaved as a single protein of low molecular weight (25,000 ± 5,000). A mol wt of 36,000 was recently obtained for the deaminase of *Rhodopseudomonas spheroides*.[13,14] Porphobilinogen-consuming activity and uroporphyrinogen-forming capacity could not be separated by the action of different dissociating agents. In all cases the enzyme behaved as a sole functional unit and was a single active band when examined by polyacrylamide gel electrophoresis.[9,12] Through all the purification steps the porphobilinogen-consuming activity could not be separated from the uroporphyrinogen-forming activity. These results, as well as the conclusions obtained by comparing under similar conditions the kinetics of the chemical polymerization of porphobilinogen vs. the enzymatic one, led us to propose that the enzymatic polymerization of porphobilinogen by deaminase is catalyzed by a single protein and must take place in a nonstop reaction on the enzyme's surface.[12,15]

A number of studies with inhibitors have been carried out to detect pyrrylmethane intermediates formed during the action of porphobilinogen deaminase on its substrate. This always involves a risk, since the isolation of presumptive intermediates by inhibition of a reaction which normally takes place on an enzymatic surface makes those structures questionable as real intermediates of the reaction.[16]

When hydroxylamine was added to an incubation mixture containing deaminase and porphobilinogen, the final formation of uroporphyrinogen I amounted

(8) A. T. Carpenter and J. J. Scott, *Biochem. J.*, **71**, 325 (1959).

(9) R. B. Frydman and G. Feinstein, *Biochim. Biophys. Acta*, **350**, 358 (1974).

(10) L. Bogorad and G. S. Marks, *J. Biol. Chem.*, **235**, 2127 (1960).

(11) For a brief evaluation of the hypotheses for enzymic polymerization of porphobilinogen prior to 1961, see E. Margoliash, *Annu. Rev. Biochem.*, **30**, 551 (1961). See also ref 1. Recent hypotheses are: (a) selective bilane cyclization: J. H. Mathewson and A. H. Corwin, *J. Am. Chem. Soc.*, **83**, 135 (1961); A. R. Battersby, *Int. Cong. Pure App. Chem., Spec. Lect., 23rd*, **5**, 1 (1971); (b) interconversion of uroporphyrinogens: E. Bullock, *Nature (London)*, **205**, 70 (1965); B. J. Whitlock, H. W. Whitlock, and H. Alles, *J. Am. Chem. Soc.*, **96**, 3959 (1974); (c) condensation of porphobilinogen and a tripyrrane: P. Cornford, *Biochem. J.*, **91**, 64 (1964); J. Dalton and R. C. Dougherty, *Nature (London)*, **223**, 1151 (1969); E. B. C. Llambias and A. M. C. Battle, *Biochem. J.*, **121**, 327 (1971); R. Radmer and L. Bogorad, *Biochemistry*, **11**, 904 (1972).

(12) R. B. Frydman and B. Frydman, *Arch. Biochem. Biophys.*, **136**, 193 (1970).

(13) P. M. Jordan and D. Shemin, *J. Biol. Chem.*, **248**, 1019 (1973).

(14) R. C. Davies and A. Neuberger, *Biochem. J.*, **133**, 471 (1973).

(15) R. B. Frydman, S. Reil, and B. Frydman, *Biochemistry*, **10**, 1154 (1971).

(16) A pertinent example is the inhibition of the biosynthesis of 6-methylsalicylic acid, a compound formed by the nonstop polymerization of four acetyl units on a proteic surface. By inhibition of the reaction a trimeric polyketomethylene is released, which rearranges and cyclizes to form a triacetic acid lactone. The lactone is not incorporated any more into the final product: P. Dimroth, H. Walter, and F. Lynen, *Eur. J. Biochem.*, **13**, 98 (1970).

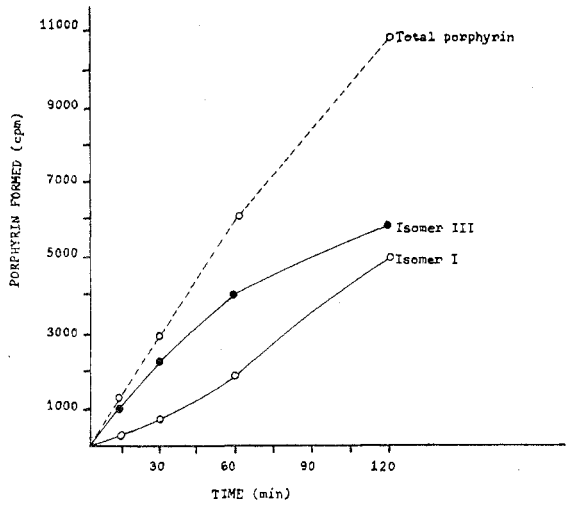
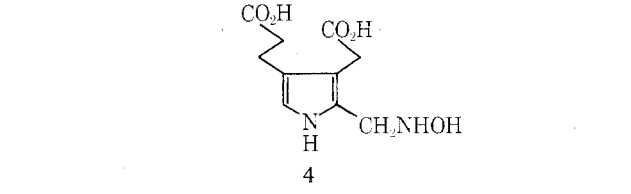
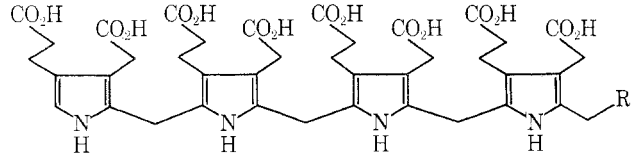


Figure 3. Rates of the enzymatic formation of uroporphyrinogen III and uroporphyrinogen I.

to only 50% of the theoretical value. Pyrrylmethanes were apparently accumulated in the reaction mixture, and by ion exchange chromatography or gel filtration several of them could be isolated. Pluscec and Bogorad[17] claimed that one of the accumulated compounds was the 2-aminomethyldipyrrylmethane (**8**). Davies and Neuberger[14] could not substantiate this claim, and isolated different intermediates. For one of them they proposed the structure of a 2-hydroxylaminomethyl-3-carboxymethyl-4-carboxyethylpyrrole (**4**). For a second compound they proposed the structure of a 2-hydroxylaminomethylbilane (**5**). Inhibition of the reaction with ammonium ions afforded a compound for which the structure of a 2-aminomethylbilane (**6**) was proposed.[14,18] Inhibition with methoxyamine afforded a compound for which structure **7** was proposed.[14]



4



5, R = NHOH
6, R = NH_2
7, R = $NHOCH_3$

The structures **5–7** were advanced only on the basis of the molecular weights determined by gel filtration, by the fact that they were cyclized to uroporphyrinogen I in a first-order reaction[18] by heating at 50° during several hours, and by the corresponding release of either hydroxylamine, ammonia, or methoxyamine during the cyclization step. The presumptive 2-aminomethylbilane (**6**) was a very unstable compound and was not transformed enzymically either into uroporphyrinogen I by the action of the deaminase or into uroporphyrinogen III by the action of the cosynthase or the deaminase–cosynthase complex.

All the bilane fractions contained large amounts of polypyrroles which were not converted into uroporphyrinogens by heating[14] and must be artifacts formed during the inhibition process. There is a report that the enzyme from soya been callus accumulated pyrrylmethane intermediates in the absence of inhibitors.[19] The properties ascribed to those compounds differ widely from what is known about the chemical properties of the synthetic 2-aminomethylpyrrylmethanes and even of the pyrrylmethanes isolated from the inhibited reactions described above. Hence, their identity must await further studies.

Uroporphyrinogen III Cosynthase: Properties and Association with Deaminase

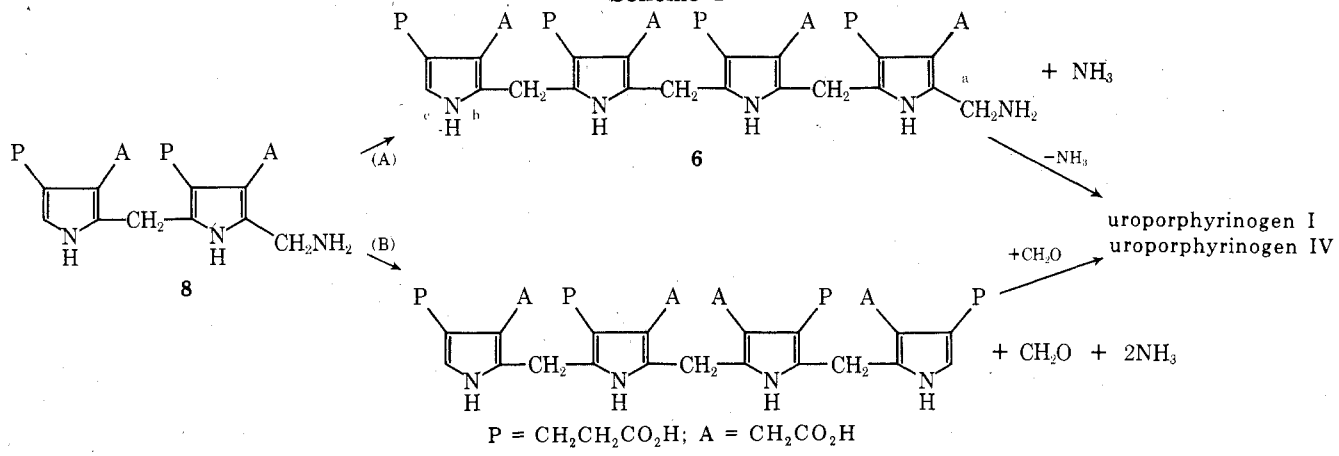
The nondestructive separation[20,21] of a system containing porphobilinogen deaminase and uroporphyrinogen III cosynthase allowed an estimation of the relative amounts of both enzymes in the same system, and it was found that the cosynthase was present in a great excess over the deaminase, thus assuring that uroporphyrinogen III will always be formed under normal physiological conditions. Cosynthase interacts with deaminase irrespective of the source of both enzymes. During the course of the enzymatic reaction it was progressively inactivated; the formation of uroporphyrinogen III leveled off, while the formation of uroporphyrinogen I (deaminase activity) increased steadily (Figure 3). This effect was due to a partial inactivation of the cosynthase during the process, and it could be prevented by a number of substances, specially sulfydryl compounds. Both deaminase and cosynthase were sensitive to sulfydryl reagents, but the latter was much more affected. This explained the strong protective effect of dithiothreitol on the amount of isomer III formed during the incubation procedures.[9]

Kinetic determinations of the cosynthase can only reflect the way in which cosynthase affects the kinetic constants of porphobilinogen deaminase, the substrate consuming enzyme. Deaminase consumed porphobilinogen and formed uroporphyrinogen I following a classical Michaelis–Menten kinetics. The addition of cosynthase did not change this reaction.[9] However, in the case of human erythrocyte enzymes the affinity for the substrate of the dual enzymatic system increased while the maximum velocity decreased.[9] The opposite effects were obtained when wheat-germ cosynthase was added to preheated spinach leaf deaminase.[4]

These changes in the affinity for the substrate of the dual system reflect either a direct interaction of the cosynthase with porphobilinogen, or a conformational modification of the deaminase produced by its association with the cosynthase. Since it was impossible to demonstrate any direct interaction between cosynthase and porphobilinogen by equilibrium dial-

(17) J. Pluscec and L. Bogorad, *Biochemistry*, **9**, 4736 (1970).
(18) See Radmer and Bogorad, ref 11c.
(19) E. B. C. Llambias and A. M. C. Battle, *FEBS Lett.*, **6**, 285 (1970); A. M. Stella, V. E. Parera, E. B. C. Llambias, and A. M. C. Battle, *Biochim. Biophys. Acta*, **252**, 481 (1971).
(20) E. Stevens and B. Frydman, *Biochim. Biophys. Acta*, **151**, 429 (1968).
(21) E. Stevens, R. B. Frydman, and B. Frydman, *Biochim. Biophys. Acta*, **158**, 496 (1968).

Scheme I



ysis,[22] the second possibility may be the cause of the aforementioned changes.

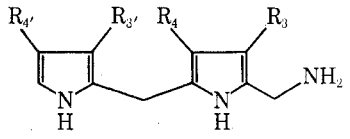
The existence of this association could also be inferred from an examination of the rates of uroporphyrinogen III and uroporphyrinogen I formation (Figure 3). Both isomers seem to share a common intermediate and since this intermediate is not a pyrrylmethane compound (see below), it must be the deaminase cosynthase association. The association is a loose one. It efficiently forms uroporphyrinogen III at short reaction times, but as the reaction time goes by the cosynthase starts to inactivate while deaminase recovers its original activity and uroporphyrinogen I is predominantly formed.

An independent proof of the formation of the two-enzyme complex in the absence of any added substrate or pyrrylmethane derivative can be found by working with immobilized enzymes. Deaminase bound to Sepharose was associated with cosynthase when the latter was filtered through the system. The immobilized complex formed uroporphyrinogen III when incubated with porphobilinogen.[9] Since deaminase catalyzes the polymerization of porphobilinogen in a nonstop reaction without liberation of free intermediates, the association deaminase–cosynthase must carry out the reaction in a similar way. This led us to propose[23] for this enzymatic complex a model similar to that found in lactose synthetase,[24] where cosynthase plays the role of the "specifier protein" of the system.

2-Aminomethylpyrrylmethanes: Chemical Polymerization

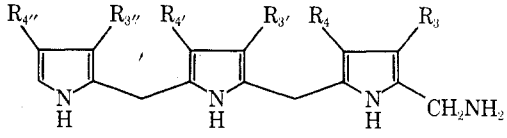
There are four isomeric 2-aminomethyldipyrrylmethanes which can be derived from a formal dimerization of two units of porphobilinogen (1) and isoporphobilinogen (2) by the loss of 1 mol of ammonia.

Dipyrrylmethane 8 is the result of the formal head-to-tail[25] condensation of two units of porphobilinogen, while dipyrrylmethane **10** is the result of the formal head-to-head condensation of two units of porphobilinogen followed by a 2-aminomethyl migration. Dipyrrylmethanes **9** and **11** are "nonsense dipyrrylmethanes" from the biosynthetic standpoint. Their synthesis and main chemical properties have been discussed in detail elsewhere.[23,26–29]



8, $R_3 = R_{3'} = CH_2CO_2H$; $R_4 = R_{4'} = CH_2CH_2CO_2H$
9, $R_3 = R_{4'} = CH_2CO_2H$; $R_4 = R_{3'} = CH_2CH_2CO_2H$
10, $R_4 = R_{3'} = CH_2CO_2H$; $R_3 = R_{4'} = CH_2CH_2CO_2H$
11, $R_4 = R_{4'} = CH_2CO_2H$; $R_3 = R_{3'} = CH_2CH_2CO_2H$

The synthesis of the tripyrranes **12** and **13** followed a pattern similar to that used for the synthesis of the



12, $R_3 = R_{3'} = R_{3''} = CH_2CO_2H$;
$R_4 = R_{4'} = R_{4''} = CH_2CH_2CO_2H$
13, $R_3 = R_{4'} = R_{3''} = CH_2CO_2H$;
$R_4 = R_3 = R_{4''} = CH_2CH_2CO_2H$

dipyrrylmethanes.[30] Tripyrrane **12** is formally derived from the condensation head-to-tail of three units of porphobilinogen by the loss of 2 mol of ammonia; tripyrrane **13** is formally derived from the condensation head-to-tail of dipyrrylmethane **10** with a third porphobilinogen unit. The synthetic method was useful for the synthesis of a large number of isomeric tripyrranes.

While porphobilinogen is not polymerized at 37° under conditions similar to those used in the enzymatic studies, 2-aminomethyldipyrrylmethanes are dimerized forming uroporphyrinogens in 5 to 12% yields. The dimerization of dipyrrylmethane **8** (Scheme I) could proceed by either a head-to-tail condensation (reaction A), or by a head-to-head condensation (reaction B) followed by release of formaldehyde and a recombination of the same with the α,α'-free bilane to give the corresponding uroporphy-

(22) G. Feinstein and R. B. Frydman, unpublished results.
(23) B. Frydman, S. Reil, A. Valasinas, R. B. Frydman, and H. Rapoport, *J. Am. Chem. Soc.*, **93**, 2738 (1971).
(24) K. Ebner, *Acc. Chem. Res.*, **3**, 41 (1970).
(25) The C-2 position of porphobilinogen substituted with the aminomethyl group is refered to as "head", while the C-5 position is refered to as "tail".
(26) A. Valasinas, E. S. Levy, and B. Frydman, *J. Org. Chem.*, **39**, 2872 (1974).
(27) J. M. Osgerby, J. Pluscec, Y. C. Kim, F. Boyer, N. Stojanac, H. D. Mah, and S. F. MacDonald, *Can. J. Chem.*, **50**, 2652 (1972).
(28) A. R. Battersby, D. A. Evans, K. H. Gibson, E. McDonald, and L. Nixon, *J. Chem. Soc., Perkin Trans. 1*, 1546 (1973).
(29) A. R. Battersby, J. F. Beck, and E. McDonald, *J. Chem. Soc., Perkin Trans. 1*, 160 (1974).
(30) A. Valasinas, E. S. Levy, and B. Frydman, unpublished results.

rinogen. The dimerization of dipyrrylmethane **8** (and **11**) will thus afford uroporphyrinogens I and IV, while the dimerization of dipyrrylmethanes **9** and **10** will afford only uroporphyrinogen II.

There is a third possible cyclization mechanism for the intermediate 2-aminomethylbilane. Although it was advanced by Mathewson and Corwin[11a] to explain the biosynthetic mechanism by which uroporphyrinogen III and cobyrinic acid could be formed from the 2-aminomethylbilane **6**, it could also be expected to contribute to the chemical cyclization of these compounds. It proposed that an attack of the reactive methyl (a) of **6** on the occupied position b, followed by bond breaking at b and recondensation of the resulting diene at c, would result in the formation of uroporphyrinogen III.

It was found that the chemical dimerization of dipyrrylmethane **8** at 37° and pH 7.4 afforded two isomeric uroporphyrinogens which were identified as of types I and IV.[31,35] Thus, a Mathewson and Corwin type of cyclization mechanism was not operating during the chemical cyclization of the corresponding 2-aminomethylbilanes. The dimerization of the dipyrrylmethanes **9** and **10** at 37° and pH 7.4 formed only uroporphyrinogen II.[31] A Mathewson and Corwin type of cyclization of the intermediate 2-aminomethylbilanes would result in the formation of uroporphyrinogen III.

When the 2-aminomethyltripyrranes **12** and **13** were heated at 37° and pH 7.4 uroporphyrinogens were also formed but with lower yields than obtained with 2-aminomethyldipyrrylmethanes.[32] A similar reaction scheme to the one proposed for the dipyrrylmethane dimerization can be proposed for the tripyrranes, in which the possible hexapyrrylmethanes will be formed instead of the bilanes. They will then cyclize at the thermodynamically favored tetrapyrrole position to give uroporphyrinogens after cleavage of a dipyrrylmethane segment. Thus, it could be predicted that tripyrrane **12** will form uroporphyrinogens I and III and tripyrrane **13** will form uroporphyrinogens III and II. It was found that tripyrrane **12** usually formed 50–65% of uroporphyrinogen I and 35–50% of uroporphyrinogen III, and that tripyrrane **13** formed 70% of uroporphyrinogen III and 30% of uroporphyrinogen II.

Enzymatic Incorporation of 2-Aminomethyldipyrrylmethane (8) into Uroporphyrinogen I

The use of pyrrylmethanes in the study of the reaction mechanism is based on the prior assumption that they are equal or similar to the enzyme-bound intermediates formed in the process. The added pyrrylmethane will bind to the enzyme competing with porphobilinogen for its binding site. It must be expected that the enzyme will have a much greater affinity for the natural substrate than for the added external polymer, and the incorporation of the latter will necessarily be low. However, if the enzymatic polymerization takes place in various stages and the product of the first stage is the substrate of the second one, then an added free dimer or trimer identical with the natural ones will be incorporated in a higher proportion than the starting monomer into the final tetramer (uroporphyrinogen).

Enzymes from different sources were used during the studies with the pyrrylmethanes, and no fundamental differences were found in their behavior. The dipyrrylmethanes **8, 9 10,** and **11** were not substrates of either porphobilinogen deaminase, uroporphyrinogen III cosynthase, or the combined enzymatic system.[23,31,33] The only uroporphyrinogens formed during the incubations were those originated by the chemical dimerizations of the dipyrrylmethanes described above. Hence, neither deaminase nor cosynthase are dimerizing enzymes and do not form uroporphyrinogens by polymerizing two dipyrrylmethane units.

When the dipyrrylmethanes were incubated together with porphobilinogen in the presence of cosynthase, no enzymatic uroporphyrinogen formation could be detected. This discarded the proposals[11] that the deaminase formed dipyrrylmethanes which could serve as further substrates of the cosynthase.

When dipyrrylmethane **8** was added to an enzymatic system which formed uroporphyrinogens it inhibited porphobilinogen consumption and increased the porphyrin yields, after the corrections were made for the chemical blanks.[23] This indicated that the dipyrrylmethane was incorporated into the uroporphyrinogens. In the presence of [^{14}C]porphobilinogen it was found that dipyrrylmethane **8** was incorporated in low yields into uroporphyrinogen I (about 10% of the total uroporphyrinogen I formed) when incubated either with deaminase or with deaminase–cosynthase.[23] No incorporation into uroporphyrinogen III was detected. Those results were confirmed using [^{14}C]dipyrrylmethane **8** and [^{12}C]porphobilinogen.[31]

The dipyrrylmethane also exerted a strong inhibitory effect on the enzymatic formation of uroporphyrinogen III, which was simultaneous with an increase in the enzymatic formation of uroporphyrinogen I.[31] This effect was not enhanced by a previous incubation of the cosynthase and the dipyrrylmethane, suggesting that the inhibition in the formation of uroporphyrinogen III is due to an interference of the dipyrrylmethane with the deaminase–cosynthase association. These results indicated that the polymerization catalyzed by the deaminase proceeds by a sequential addition of porphobilinogen units, since the latter must be added to the dipyrrylmethane to complete the reaction toward uroporphyrinogen formation.

Pluscec and Bogorad[17] also found that dipyrrylmethane **8** was incorporated into uroporphyrinogen I in the presence of porphobilinogen and deaminase, but the high incorporation values reported by them are at variance with our findings.

There is a report[34] that Shemin's whole preparations from hemolyzed duck erythrocytes and the whole enzymatic system from *Euglena gracilis* dimerized dipyrrylmethane **8** and incorporated the

(31) R. B. Frydman, A. Valasinas, and B. Frydman, *Biochemistry*, **12**, 80 (1973).
(32) R. B. Frydman, A. Valasinas, S. Levy, and B. Frydman, *FEBS Lett.*, **38**, 134 (1974).
(33) R. B. Frydman, A. Valasinas, H. Rapoport, and B. Frydman, *FEBS Lett.*, **25**, 309 (1972).
(34) A. R. Battersby, K. H. Gibson, E. McDonald, L. N. Mander, J. Moron, and L. N. Nixon, *J. Chem. Soc., Chem. Commun.*, 768 (1973).

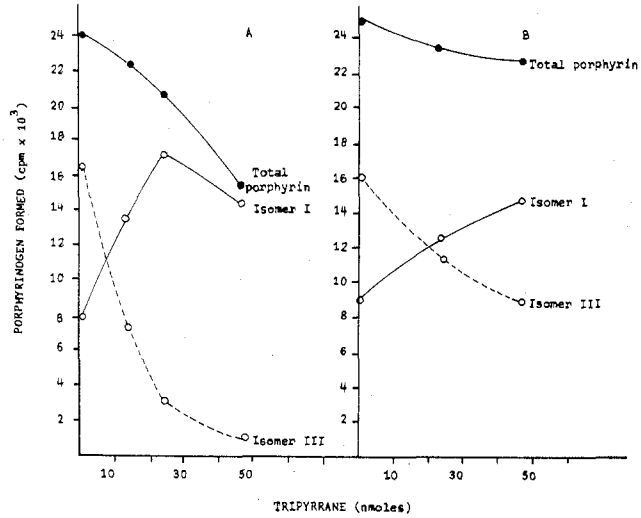


Figure 4. Effect of tripyrrane **12** (A) and tripyrrane **13** (B) on the enzymatic formation of uroporphyrinogen III and uroporphyrinogen I.

formed bilane into protoporphyrin IX (see also ref 11a). Those results are at variance with what was found about the mode of action of the deaminase as well as with its lack of interaction with the dipyrrylmethane **8** alone. Free 2-aminomethylbilanes were also not cyclized enzymatically to form uroporphyrinogens (see above).

We found [35] that Shemin's whole system formed a protoporphyrin when incubated with dipyrrylmethane **8**. This was due to the action of the decarboxylating enzymes of the system on the uroporphyrinogen IV formed during the chemical dimerization of the dipyrrylmethane (see above). Under these conditions the uroporphyrinogen IV was decarboxylated to coproporphyrinogen IV, and the action of coprogenase on the latter transformed it into a protoporphyrin isomeric with protoporphyrin IX, which is identical in its chromatographic and chemical properties with protoporphyrin IX. When the whole enzymatic system was incubated with synthetic coproporphyrinogen IV, it transformed it into the same isomeric protoporphyrin. The observed incorporation of dipyrrylmethane **8** into a protoporphyrin could then be explained by the enzymatic decarboxylation of the chemically formed uroporphyrinogen IV.

Enzymatic Incorporation of Dipyrrylmethane 10 into Uroporphyrinogen III

The lack of incorporation of dipyrrylmethane **8** into uroporphyrinogen III led to the obvious conclusion that a different dipyrrylmethane must be the first intermediate when the polymerization was catalyzed by the deaminase–cosynthase system. When dipyrrylmethane **10**—formally derived from a head-to-head condensation of two porphobilinogen units—was added to the enzymatic system in the presence of porphobilinogen, it was found to be incorporated only into uroporphyrinogen III and not into uroporphyrinogen I.[33] The incorporation values were low and of the same order of magnitude as for dipyrrylmethane **8**. The incorporation of dipyrrylmethane **10** into uroporphyrinogen III agree with results obtained by studying the incorporation of double-labeled

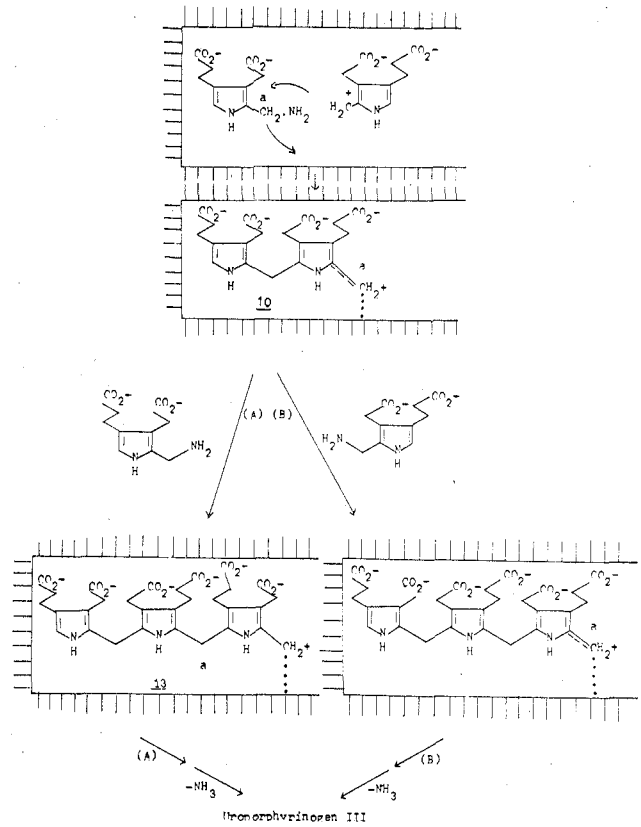


Figure 5. The schematic biosynthetic pathway to uroporphyrinogen III. The head-to-head condensation of two porphobilinogen units by the deaminase–cosynthase association gives rise to an enzyme-bound dipyrrylmethane **10** residue. The "active methyl" group (a) of the first unit is transferred and remains either covalently bound to the dipyrrylmethane, or in a partial equilibrium with the protein. In the next steps the third and fourth units may attach (A) either through the α-free position or (B) through the α-occupied position, producing a constant transfer of the initial "active methyl" group (a).

(2,11-^{13}C) porphobilinogen into protoporphyrin IX.[36]

Dipyrrylmethane **9** was not incorporated into either isomer, and it did not affect the distribution of isomers produced by the enzymes.[23,31] Dipyrrylmethane **11** was not either incorporated into any isomer. It exerted an inhibitory effect on the formation of uroporphyrinogen III which was not reflected in an increase in the formation of uroporphyrinogen I.

Interaction of 2-Aminomethyltripyrranes with the Enzymatic System

Tripyrranes always played an important part in all the mechanistic speculations about uroporphyrinogen III biosynthesis.[11] We examined first the interaction between tripyrrane **12** and the enzymatic system, since this tripyrrane was repeatedly proposed as an intermediate in uroporphyrinogen III biosynthesis. Tripyrrane **12** was not a substrate of cosynthase either alone or in the presence of porphobilinogen.[32] When a small amount of deaminase was added to the reaction mixture to trigger the action of cosynthase, the tripyrrane **12** was not only not incorporated into uroporphyrinogen III, but exerted an inhibitory effect on the formation of that isomer.[32]

Addition of tripyrrane **12** to an enzymatic system forming both isomers inhibited the formation of total uroporphyrinogen. A stronger inhibition was exerted

(35) R. B. Frydman and B. Frydman, *FEBS Lett.*, in press.

(36) A. R. Battersby, E. Hunt, and E. McDonald, *J. Chem. Soc., Chem. Commun.*, 442 (1973).

Table I
Effect of Tripyrrane 12 on the Enzymatic Formation of Uroporphypinogens III and I

System[a]	Tripyrrane added, nmol	Isomers formed: I nmol	I cpm	I Sp act., cpm/nmol	Δ	III nmol	III cpm	III Sp act., cpm/nmol
Incubated		0.235	10,400	44,500		0.305	13,350	43,700
Incubated[b]	21.5	0.43	1,700	39,500	3000	0.072	3,200	45,000
Control[c]	21.5	0.26	11,000	42,500				
Incubated	43	0.475	14,305	30,000	5000	0.014	650	45,500
Control	43	0.315	10,600	35,000				

[a] Wheat germ deaminase cosynthase. Porphobilinogen (9 nmol; sp act. 11,000 cpm/nmol) was used. [b] Controls corrected for isomer III inhibition were deducted for estimation of that isomer. [c] The incubated system was mixed with a blank to account for the chemical polymerization of 12.

on the formation of uroporphyrinogen III than on the total formation of uroporphyrinogens (Figure 4A). As a result a net increase in the proportion of uroporphyrinogen I present in the reaction products was observed.

By using a system which formed only uroporphyrinogen I from porphobilinogen and by adding to it tripyrrane **12** in a great excess over the stoichiometric values, a small (2–8%) but significant enzymatic incorporation of tripyrrane **12** was obtained.[32] When the enzymatic system formed both isomers, tripyrrane **12** was incorporated to a small extent only into isomer I (Table I). The concentrations of tripyrrane **12** needed to obtain a significant incorporation into uroporphyrinogen I inhibited the formation of uroporphyrinogen III to such an extent that it made it impossible to measure its incorporation into this last isomer. It was clear that any incorporation of this sort has to be discarded.

The biosynthetic pathway toward uroporphyrinogen III can proceed onward from the formation of dipyrrylmethane **10** (or its enzyme-bound analog) by two different sequences. They will differ depending on whether the third porphobilinogen unit became attached by its free α position affording an enzyme-bound tripyrrane **13** or whether the new porphobilinogen unit attaches through its occupied α position, affording a different tripyrrane (Figure 5). When the interaction of tripyrrane **13** with the enzymes was examined, it was found that it did not behave as a substrate of the deaminase, of the cosynthase, or of the deaminase–cosynthase complex, either alone or in the presence of porphobilinogen. In the presence of tripyrrane **13** no more uroporphyrinogen was formed by the enzymatic system than that formed in its absence after the amount of porphyrinogen contributed by the chemical polymerization of **13** was deducted. On the contrary, the addition of tripyrrane **13** had an inhibitory effect on the formation of total uroporphyrinogens (Figure 4B) and specially on the formation of uroporphyrinogen III. This last inhibition was less pronounced than the one exerted by tripyrrane **12**. Tripyrrane **13** was not incorporated into uroporphyrinogen I.

General Outlook

From the studies of the properties of the enzymes involved, as well as from the results obtained by examining the interactions among the synthetic 2-aminomethylpyrrylmethanes and the enzymes, a mechanism can be outlined which should define the nature of the biosynthetic process leading to the formation of uroporphyrinogens from porphobilinogen. The mechanism must be based first on the evidence that under normal conditions the reactions takes place through a "zipping up" process on the enzyme's surface. The next basic tenet must be that cosynthase acts as a "specifier protein" of deaminase, changing the mode of porphobilinogen condensation from the beginning of the reaction and steering it toward the formation of uroporphyrinogen III.

The obvious conclusion is, then, that there are no pyrrylmethane intermediates common to both uroporphyrinogens I and III. The formation of the two isomers by the two different enzymatic systems (deaminase and deaminase–cosynthase) represents the two ways by which porphobilinogen could start its self-condensation: a head-to-tail condensation by elimination of ammonia, or a head-to-head condensation by displacement of an activated methyl group (Figure 5).

This "active methyl" group, which in the chemical polymerization of porphobilinogen is liberated as formaldehyde, must remain during the enzymatic formation of uroporphyrinogen III either bound to the enzyme, being used during the final cyclization step, or constantly transferred through the growing pyrrylmethane chain until the ultimate formation of the macrocycle. Hence, while porphobilinogen deaminase functions only as a deaminating enzyme, the deaminase–cosynthase complex must also be a methyltransferase type of enzyme.

"It is not for you to have the task finished", said our old sages,[37] and new and imaginative contributions must yet be made to find the definitive solution to the puzzle of why all natural porphyrins are type III porphyrins.

We wish to thank our graduate students A. Valasinas, E. S. Levy, and G. Feinstein for their devoted and skillful contributions to this work. We are also grateful to Miss J. H. Lis for her technical assistance, and to Professor H. Rapoport (Berkeley) for his interest and encouragement. This research was made possible by the generous support of the National Institutes of Health (GM-11973), of the National Research Council of Argentina, and of the donors of the Petroleum Research Fund, Administered by the American Chemical Society.

(37) Rabbi Tarfon, Mischnah Nezikin, Avoth, II, 21.