FORMATION OF UROPORPHYRINOGEN IV DURING THE CHEMICAL DIMERIZATION OF 2-AMINOMETHYL-3,3'-CARBOXYMETHYL-4,4'(β-CARBOXYETHYL) DIPYRRYLMETHANE

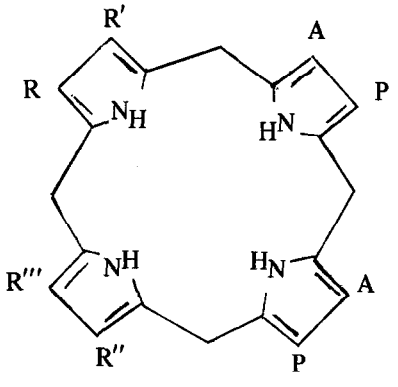
Rosalía B. FRYDMAN and Benjamin FRYDMAN

Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires, Junin 956 Buenos Aires, Argentina

Received 6 January 1975

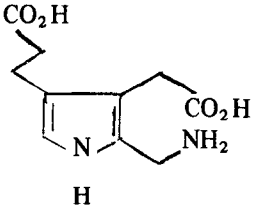
1. Introduction

The studies in the mechanism of uroporphyrinogen III **1** biosynthesis from porphobilinogen **2** make use of synthetic 2-aminomethyldipyrrylmethanes formally derived from the self-condensation of two units of porphobilinogen **2**. It was found that the 2-aminomethyl-3,3'-carboxymethyl-4,4'-(β-carboxyethyl-dipyrrylmethane **3** was enzymically incorporated into uroporphyrinogen I **4** but not into uroporphyrinogen III **1** in the presence of porphobilinogen **2** and of the porphobilinogen consuming enzymes [1,2]. Another research group however, described the enzymatic incorporation of dipyrrylmethane **3** into protoporphyrin IX [3], a product derived from uroporphyrinogen III in its metabolic pathway toward heme and the chlorophylls [4]. These latter studies were carried out by using whole enzymatic systems such as duck's blood or a preparation of *Euglena gracilis* which transformed porphobilinogen directly into protoporphyrin IX. While it was found by us [1,2] and by Pluscec and Bogorad [5], that the dipyrrylmethane **3** condensed enzymically with two additional porphobilinogen units to give uroporphyrinogen I **4**, the data obtained from the protoporphyrin IX biosynthetic studies were rationalized by proposing [6] that **3** was demerized by the enzymatic system to afford uroporphyrinogen III **1**, after an hypothetical intramolecular rearrangement process of the 2-amino-

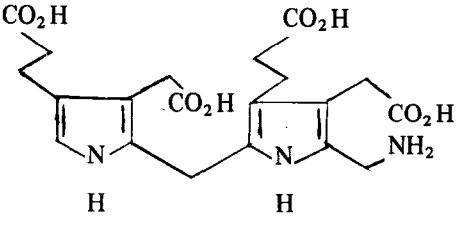


1, R=R'''=A; R'=R''=P
4, R=R''=A; R'=R'''=P
5, R'=R'''=A; R=R''=P

A = CH_2O_2H
P = $CH_2CH_2CO_2H$



2



3

methylbilane formed by the dimerization reaction.

Since it was found [1,2,5,7] that the porphobilinogen consuming enzymes, either porphobilinogen deaminase or porphobilinogen deaminase-uroporphyrinogen III cosynthase, do not dimerize the 2-aminomethyldipyrrylmethanes to form uroporphyrinogens, the results obtained with the whole enzymatic systems could be explained by accepting that the uroporphyrinogen decarboxylases and the coproporphyrinogen oxidase act on an uroporphyrinogen chemically formed by the dimerization of dipyrrylmethane **3** during the incubation process. It was already shown [2] that such a chemical dimerization of **3** takes place, forming uroporphyrinogen I **4** and uroporphyrinogen III **1** or IV **5**. It was impossible by then to distinguish among these last isomers, but the possibility that the product was uroporphyrinogen IV was favoured on the basis of the proposed reaction mechanism [2]. In this report we will demonstrate that this is really the case and that the uroporphyrinogen IV **5** formed is transformed by the duck's blood enzymes into a protoporphyrin.

2. Materials and methods

Porphobilinogen **2** and dipyrrylmethane **3** were prepared by synthesis [1,8]. Uroporphyrin IV **5** was obtained by synthesis [9], and was decarboxylated to coproporphyrin IV (**5**; CH_3 instead of CH_2CO_2H) by heating at 180°C with hydrochloric acid. Coproporphyrins III (**1**; CH_3 instead of CH_2CO_2H) and I (**4**; CH_3 instead of CH_2CO_2H) were purchased from Sigma. Coproporphyrinogens were obtained by reducing the coproporphyrins with sodium amalgam. Duck's blood erythrocytes were prepared according to the method of Shemin et al. [10]. Porphobilinogen deaminase and uroporphyrinogen III cosynthase were isolated and purified from human erythrocytes [11]. The incubated system contained in a final vol of 200 μl; 10 μmol of phosphate buffer (pH 7.4), 150 μl of the duck's blood or 50 μl of the isolated enzymes; and either porphobilinogen (15 nmol), or dipyrrylmethane **3** (25 to 50 nmol), or coproporphyrinogens III or IV (1.5 to 6.0 nmol). Incubations were carried out at 37°C for 60 min, except when indicated. The reaction products were analyzed by different procedures. Uroporphyrinogen isomers were separated by stopping the reaction with 5 μl of a 1% iodine solution, by evaporating the mixture to dryness in vacuo, and by esterification of the residue with a 5% sulfuric acid solution in methanol. The uroporphyrin octamethyl esters were decarboxylated to the corresponding

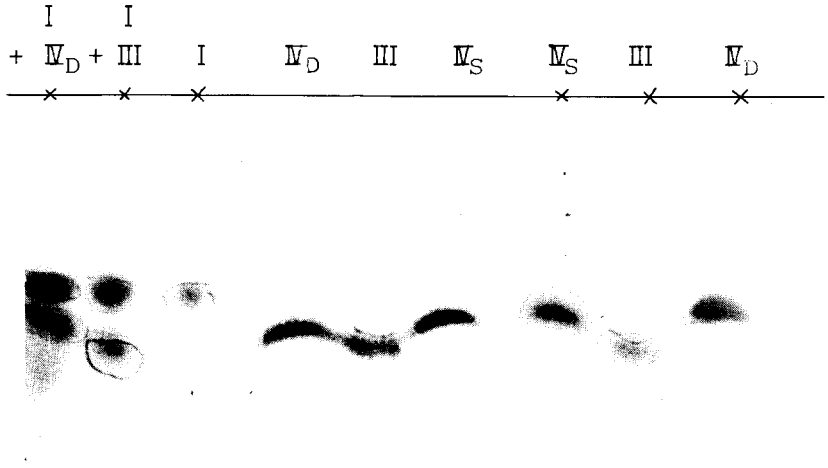


Fig.1. Separation by tlc-cellulose of coproporphyrins IV, III and I; III, Coproporphyrin III; IV_S, synthetic coproporphyrin IV; IV_D, corproporphyrin IV prepared from dipyrrylmethane 3; I, coproporphyrin I; I + IV_D, coproporphyrin mixture obtained by chemical dimerization of 3.

coproporphyrins by heating at 180°C with hydrochloric acid [12]. Coproporphyrins III, IV and I were separated by tlc on cellulose-coated plates (E. Merck, DC-Fertigplatten, Schichdicke 0.10 mm) using 2,6-lutidine: 0.7 N ammonium hydroxide (40:28 v/v) as developer. The chromatography was performed over 13 hr at 14°–15°C (a strict temperature control was essential to obtain a good separation of the isomers), when the solvent front travelled 15 cm. Coproporphyrin III had R_f, 0.51; coproporphyrin IV had R_f, 0.47; while coproporphyrin I had R_f, 0.45 (fig.1). The coproporphyrin isomers were separately eluted from the tlc with dilute ammonium hydroxide and estimated by its absortion spectra.

When an analysis of the uroporphyrins, coproporphyrins and protoporphyrin present in the reac-

Table 1
Formation of uroporphyrinogen IV in the chemical dimerization of dipyrrylmethane 3

System	Addition	Incubations Conditions	Uroporphyrin formed[a] nmol	Inhibition %	Isomer IV formed as % of total porphyrin
Dipyrrylmethane (12.5 nmol)	—	60 min at 37°C	0.37		39
Dipyrrylmethane (12.5 nmol)	$HONH_2$ (50 mM)	" " " "	0.14	61	38
Dipyrrylmethane (12.5 nmol)	NH_4Cl (100 mM)	" " " "	0.36		33
Dipyrrylmethane (12.5 nmol)	Deaminase	" " " "	0.4		37
Dipyrrylmethane (12.5 nmol)	Deaminase + cosynthase	" " " "	0.39		37
Dipyrrylmethane (25 nmol)	—	" " " "	0.9		43
Dipyrrylmethane (50 nmol)	—	" " " "	1.8		31
Dipyrrylmethane (12.5 nmol)	—	120 min at 37°C	0.83		35
Dipyrrylmethane (12.5 nmol)	$HONH_2$ (50 mM)	" " " "	0.28	65	33
Dipyrrylmethane (12.5 nmol)	—	60 min at 49°C	0.88		33
Dipyrrylmethane (12.5 nmol)	$HONH_2$ (50 mM)	" " " "	0.335	62	
Dipyrrylmethane (12.5 nmol)	NH_4Cl (100 mM)	" " " "	0.88		
Dipyrrylmethane (25 nmol)	—	" " " "	1.70		32.5
Dipyrrylmethane (50 nmol)	—	" " " "	3.95		

The incubations were carried out as described in Materials and methods except for the buffer used which was Tris–HCl buffer (pH 8.2). [a]Uroporphyrins were measured in 2% hydrochloric acid. Isomer IV was measured as coproporphyrin IV.

tion product was performed, the incubation was stopped by addition of a mixture of ethyl acetate–acetic acid (3:1 v/v) and the porphyrin mixture was fractionated by the usual procedure [13]. The reaction products were also separated and analyzed as their methyl ester derivatives. The reaction was stopped by evaporating the mixture to dryness in vacuo at 20°C. The residue was esterified as described above and the methyl ester mixture was analyzed directly by tlc on silica coated plates as described by Doss [14]. A preparative separation of the methyl esters was achieved by adsorbing the ester mixture dissolved in bencene-ethyl acetate-methanol (85:13.5:1.5 v/v) on a column (2 cm × 20 cm) of tlc silica-gel (Kieselgel G, Fluka), packed under pressure with the same solvent. By applying a slight pressure the porphyrin methyl esters were sequentially eluted in an inverse order to the number of their ester groups. The porphyrin methyl esters were estimated by their absorption spectra in chloroform.

3. Results

3.1. *Formation of uroporphyrinogens IV and I by the chemical dimerization of* 3

Dipyrrylmethane **3** was incubated as described in Methods in the absence of enzyme. The formed uroporphyrins were transformed into coproporphyrins which were separated by tlc as described above. The mixture was found to be composed of coproporphyrin I and coproporphyrin IV (table 1 and fig.1). The formed coproporphyrin IV was found to be identical with a synthetic sample and different from the isomeric coproporphyrin III (fig.1). Addition of porphobilinogen deaminase, or of the deaminase-cosynthase system to the incubation mixture did not affect the proportion of isomer formation (table 1). Addition of ammonium chloride or hydroxylamine had no major effect on the isomer distribution in the reaction mixture, although hydroxylamine strongly blocked the chemical dimerization of **3**. Longer incubation periods or higher incubation temperatures increased

Table 2
Transformation of dipyrrylmethane **3** into a protoporphyrin by duck's blood enzymes

System	Addition	Uroporphyrin formed, nmol	Coproporphyrin formed, nmol	Protoporphyrin formed, nmol
Porphobilinogen	—	—	—	—
Porphobilinogen + duck's blood enzyme	—	—	0.1	0.72
Porphobilinogen + duck's blood enzyme	$HONH_2$ (50 mM)	0.16	0.8	—
Porphobilinogen + duck's blood enzyme[a]	—	0.25	0.8	0.47
Dipyrrylmethane	—	1.3	—	—
Dipyrrylmethane + duck's blood enzyme	—	0.16	0.37	0.48
Dipyrrylmethane	$HONH_2$ (50 mM)	0.67	—	—
Dipyrrylmethane + duck's blood enzyme	$HONH_2$ (50 mM)	0.16	0.24	0.2
Dipyrrylmethane[a]	—	2.1	—	—
Dipyrrylmethane + duck's blood enzyme[a]	—	0.48	0.48	0.69

The incubation mixture and conditions were as described in Materials and methods using 25 nmol of **3**.
[a] The incubations were carried out at 49°C.

the amount of total uroporphyrin formed without a significant effect on the isomer distribution (table 1).

3.2. *Transformation of dipyrrylmethane* **3** *into a protoporphyrin by duck's blood enzymes*

Dipyrrylmethane **3** was incubated with duck's blood as described above. Control experiments were simultaneously run, where duck's blood was omitted during the course of the incubation but added at the end of the reaction. In the first case coproporphyrins and protoporphyrin were formed at expense of the dipyrrylmethane **3**, while in the controls only uroporphyrins were formed (table 2). Since the total amount of porphyrin formed was similar in both cases, it must be concluded that the coproporphyrins and the protoporphyrin were enzymically formed at expense of the uroporphyrinogens obtained by the chemical dimirization of **3**. Since coproporphyrinogen I is not decarboxylated to a protoporphyrin, the protoporphyrin must be originated in the coporphyrinogen IV formed from uroporphyrinogen IV (see 3.3). When the incubations were performed at 49°C (uroporphyrinogen III cosynthase partially inactivated), the amount of protoporphyrin IX formed at expense of porphobilinogen decreased, while the protoporphyrin obtained at expense of the dipyrrylmethane **3** increased (table 2); as could be expected from an increase in the chemical dimerization of **3** (see 3.1).

3.3. *Enzymatic transformation of coproporphyrinogen IV into a protoporphyrin*

Coproporphyrinogen IV has been described as undergoing oxidative decarboxylation only one-tenth as fast as isomer III [4], and a slight transformation of isomer IV into a protoporphyrin has been reported [15]. When both coproporphyrinogen isomers were assayed under the described conditions it was found that 37% of coproporphyrinogen III was consumed by the duck's blood enzyme, as compared with 52% of coproporphyrinogen IV. Coproporphyrinogen IV was prepared from either the synthetic coproporphyrin IV or obtained by dimerization of the dipyrrylmethane **3**. An analysis of the methyl ester derivatives of the products indicated that while the product formed from coproporphyrinogen III consisted almost entirely of protoporphyrin IX dimethyl ester, the product formed from coproporphyrinogen IV was a mixture of two porphyrins. One of them had vis max ($CHCl_3$), 407 nm (ϵ mM 188), 505 (16.2, 540 (9.4), 575 (7.4), 603 (5.4), 630 (7.0); and amounted to 45% of the mixture. The spectrum indicated that it was a protoporphyrin, and it was undistinguishable from protoporphyrin IX dimethyl ester by tlc in various solvents. The second and major product was a trimethyl ester porphyrin, as judged from its R_f on tlc and from its absorption spectra (vis max $CHCl_3$), 403 nm, 502, 536, 573, 625). the definitive structures of both compounds will be assigned after comparison with synthetic samples. The tricarboxylic acid porphyrin was a minor product when the incubations were carried out using dipyrrylmethane **3** instead of coproporphyrinogen IV as the duck's blood substrate, very likely due to the slow release of the latter into the reaction medium.

Acknowledgements

This work was supported by grants from the National Institutes of Health (GM-11973) and the Consejo Nacional de Investigaciones (Argentina). We are also grateful to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for support.

References

[1] Frydman, B., Reil, S., Valasinas, A., Frydman, R. B. and Rapoport, H. (1971) J. Am. Chem. Soc. 93, 2738–2745.
[2] Frydman, R. B., Valasinas, A. and Frydman, B. (1973) Biochemistry 12, 80–85.
[3] Battersby, A. R., Gibson, K. H., Mc Donald, E., Mander, L. N., Moron, J. and Nixon, L. N. (1973) J. Chem. Soc. Chem. Commun. 768–769.
[4] Granick, S. and Levere, R. D. (1964) Progr. Hematol. 4, 1–36.
[5] Pluscec, J. and Bogorad, L. (1970) Biochemistry 9, 4736–4743.
[6] Battersby, A. R. (1971) 23rd Internat. Cong. of Pure and Appl. Chem. 5, 1–25.
[7] Frydman, R. B., Valasinas, A., Rapoport, H. and Frydman, B. (1972) FEBS Lett. 25, 309–312.
[8] Frydman, B., Reil, S., Despuy, M. E. and Rapoport, H. (1969) J. Am Chem. Soc. 91, 2338–2342.
[9] Valasinas, A. and Frydman, B. unpublished results.

[10] Shemin, D., Abramsky, T. and Russell, C. S. (1954) J. Am. Chem. Soc. 76, 1204–1206.
[11] Frydman, R. B. and Feinstein, G. (1974) Biochim. Biophys. Acta 350, 358–373.
[12] Falk, J. E. (1964) Porphyrins and Metalloporphyrins, pp. 147, Elsevier Amsterdam.
[13] Falk, J. E. (1964) ibid., pp. 169–170.
[14] Doss, M. (1973) Enzyme, 16, 343–353.
[15] Porra, R. J. and Falk, J. E. (1964) Biochem. J. 90, 69–75.