# PORPHOBILINOGEN DERIVATIVES AS SUBSTRATES FOR PORPHOBILINOGENASE

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## 1. Introduction

The biosynthetic precursor of hemes, chlorophylls, cytochromes and corrins is uroporphyrinogen III (urogen III) *2* which results from the enzymatic condensation by porphobilinogenase of four molecules of porphobilinogen (PBG) *1* (see [1] for a recent review). The action of porphobilinogenase relies on two enzymes or two-enzyme activity, deaminase and cosynthetase. Deaminase alone converts PBG to uroporphyrinogen I (urogen I) *3*, the biologically inactive isomer (fig. 1). Neither PBG nor urogen I is a substrate for cosynthetase, and only when cosynthetase is present along with deaminase, can PBG be converted to urogen III. Attempts by many investigators to isolate intermediates in these reactions by adding inhibitors such as ammonium ion [2], hydroxylamine [3,4] and methoxyamine [4] to enzymatic incubation mixtures resulted in the isolation of a dipyrrole, a tetrapyrrole and an hydroxylamine derivative of PBG. The polyrroles are enzymatically convertible to urogen I but not urogen III.

In an attempt to employ a new nucleophilic inhibitor to shake loose intermediates in the tetramerization of PBG by bovine liver porphobilinogenase, the non-enzymatic reaction of PBG with imidazoles has been observed. The products of these reactions are good substrates for porphobilinogenase and are converted to urogen III in close to quantitative yield.

## 2. Materials and Methods

(1) PBG was a gift from Prof. David Shemin.

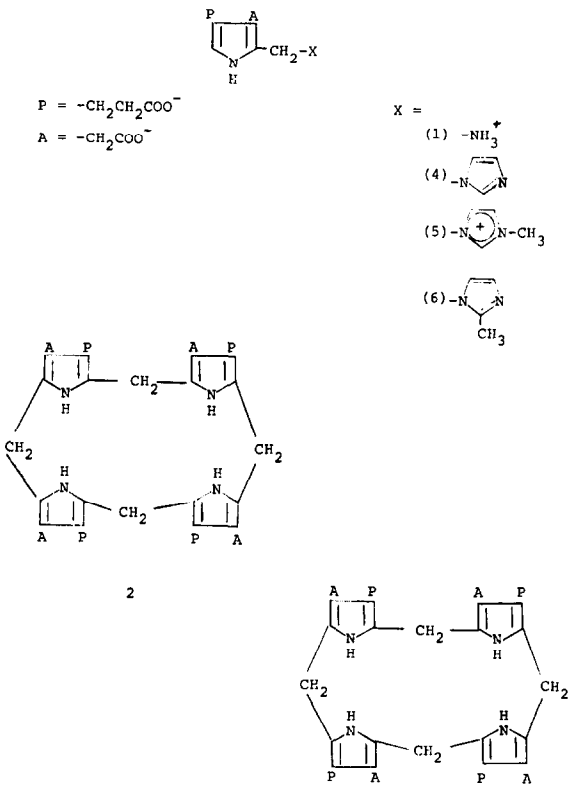


Fig. 1

Uroporphyrin esters and coproporphyrin esters were purchased from Sigma. Polyhistidine, molecular weight 16 000, was purchased from Sigma. All other chemicals were reagent grade. Porphobilinogenase was prepared from bovine liver by the method of Sancovich, Batlle and Grinstein [5]. PBG was assayed with modified Ehrlich reagent [6].


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Urogens were oxidized by the method of Jordan and Shemin [7] and determined as uroporphyrins. The isomer distribution in uroporphyrins was determined by decarboxylation to coproporphyrins by the method of Edmondson and Schwartz [8]. Coproporphyrin isomers were separated by the method of Yuan and Russell [9]. Mass spectra were obtained with a Varian CH-5 Mass Spectrometer.

(2) Derivatives of PBG were synthesized in the following way. One volume of $10^{-3}$ M PBG in 0.05 M Tris buffer, pH 7.4, was reacted with two volumes of 0.1 M imidazole or *N*-methylimidazole in 0.05 M Tris buffer, pH 7.4, at room temperature for one-half hour. The same protocol was followed for 2-methylimidazole but reaction was run in 0.05 M Tris buffer, pH 8.5. The reaction mixtures were stored in the freezer or worked up at 5°C in the cold room. The reaction mixtures were resolved and excess imidazole separated from product on a Sephadex G-15 column (1.8 × 82 cm) in 0.033 M phosphate buffer, pH 8.0, which had been qualitatively calibrated with Dextran blue, phenylalanine and tetra-alanine. PBG and its derivatives differed in their elution profiles. The derivatives gave the same spectrum with Ehrlich reagent as PBG, but the rate of color development was slightly slower. PBG and its derivatives were compared for mobility in cellulose thin layer chromatography on Polygram sheet in two different solvent systems: *n*-butanol–acetic acid–water (63:11:25) [10] and *n*-propanol–ammonia–water (60:30:10) [11]. They were also compared in paper electrophoresis on Whatman #1 paper in 0.05 M sodium diethylbarbiturate buffer, pH 9.2, at 14 V/cm. Uroporphyrin III was cospotted with each sample and mobility was calculated as the ratio of the distance traveled by Ehrlich-reacting material to the distance traveled by the fluorescent uroporphyrin spot.

Polyhistidine (10.8 mg) was washed with three 5-ml portions of 0.066 M phosphate buffer, pH 8.5 and then shaken overnight at 37°C with PBG in 3.5 ml 0.066 M phosphate buffer, pH 8.5. A control tube contained the identical solutions but lacked polyhistidine and was incubated in the same way. The polyhistidine reaction mixture was centrifuged and the supernatant, assayed for PBG, then contained $2.07 \times 10^{-5}$ mmoles. The differences between this value and that for the control, $6.99 \times 10^{-6}$ mmoles was $4.92 \times 10^{-5}$ mmoles. It was concluded that 70% of the PBG reacted with the imidazole groups on polyhistidine. The polyhistidine precipitate was washed with buffer until the washings gave a negative Ehrlich test. The washed precipitate gave the characteristic pink color with Ehrlich reagent.

(3) The enzymatic reactions were run in the following manner. The standard incubation system contained: substrate (100–600 nmoles) in 0.05 M Tris buffer, pH 7.4; 0.25 ml 0.6 M NaCl, 0.25 ml 0.12 M $MgCl_2$, EDTA to make a final concr. of 0.5 mM and porphobilinogenase (0.2–1.0 ml) for a final volume of 5.0 ml. The substrate was kept in the bulb of a Thunberg tube until the vessel had been evacuated and flushed with pre-purified nitrogen several times. The contents were mixed and incubated at 37°C in the dark with mechanical shaking. Aliquots were removed at specific intervals for PBG and uroporphyrinogen determination.

## 3. Results and Discussion

When PBG reacts with imidazole ($pK_a$ 7.08) and *N*-methyl-imidazole ($pK_a$ 7.34) at pH 7.4, where both imidazoles are good nucleophiles, derivatives whose structures are tentatively assigned as 4 and 5 respectively, are produced. These products react with Ehrlich reagent slightly more slowly than PBG but with the identical spectrum. They differ from PBG in cellulose thin-layer chromatography with two different solvent systems, in electrophoresis (table 1) and as substrates for our porphobilinogenase preparation. The opposing effects of charge and size may account for the surprisingly small differences in electrophoretic mobility. Their molecular weights, estimated by Sephadex G-15 gel chromatography were larger than for PBG and fell into the range expected for structures 4 and 5 (Fig. 2 and 3). PBG and 2-methyl-imidazole ($pK_a$ 8.11) did not react at pH 7.4 and the pyrrole from this mixture was indistinguishable from PBG on Sephadex G-15, thin-layer chromatography, electrophoresis or as a substrate for our porphobilinogenase preparation. However, when the pH was raised to 8.5, 2-methyl-imidazole was less protonated and therefore a better nucleophile. A PBG derivative, to which we tentatively assign structure 6, was obtained which

Table 1
Nonenzymatic parameters of PBG and PBG derivatives before and after purification on Sephadex G-15

| Compound | | $R_{f(B:A:W)}$ | $R_{f(P:A:W)}$ | $E_{m(pH\ 9.2)}$ | $K_{av}$ | $\frac{V_e-V_o}{V_t-V_o}$ | M.W. |
|---|---|---|---|---|---|---|---|
| PBG (P)* | (1) | 0.55 | 0.45 | 0.65 | | | 226 |
| PBG-Im (P) | (4) | 0.51 | 0.50 | 0.66 | | | 277 |
| PBG-*N*-Me-Im (P) | (5) | 0.52 | 0.51 | 0.62 | | | 292 |
| PBG-2-Me-Im (P) (pH 8.5) | (6) | 0.53 | 0.48 | 0.66 | 0.16 | | 292 |
| PBG (UP)** | (1) | 0.55 | 0.45 | | 0.24 | | 226 |
| PBG-Im (UP) | (4) | 0.51 | 0.50 | | 0.18 | | 277 |
| PBG-*N*-Me-Im (UP) | (5) | 0.52 | 0.51 | | 0.16 | | 292 |
| PBG + 2-Me-Im (UP) (pH 7.4) | | 0.55 | 0.47 | | 0.24 | | |
| Phenylalanine | | – | – | – | 0.31 | | 165 |
| Tetra-alanine | | – | – | – | 0.13 | | 302 |

* (P) = purified on G-15
** (UP) = before purification on G-15
(B:A:W) = *n*-butanol–acetic acid–water (63:11:25)
(P:A:W) = *n*-propanol–ammonia–water (60:30:10)

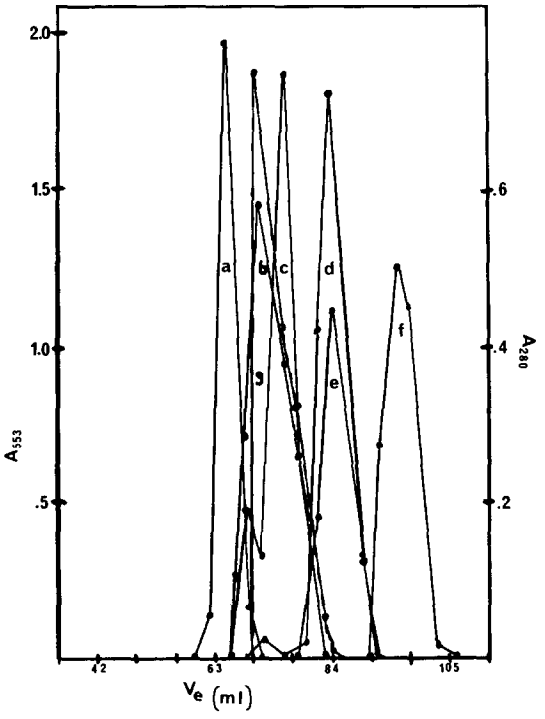


Fig. 2. Elution profile of PBG and its derivatives and two other standards (tetra-alanine and phenylalanine) on Sephadex G-15 column (1.8 × 82 cm). a = Tetraalanine, b = PBG-*N*-methylimidazole, c = PBG-Imidazole, d = PBG, e = PBG + 2-Methylimidazole (pH 7.4), f = Phenylalanine, g = PBG-2-Methylimidazole (pH 8.5).

differed from PBG using the criteria mentioned above (table 1, fig. 1 and 2). On Sephadex G-15 chromatography, $K_{av}$ of 6 was lower than 1 and 4 and identical with that of 5 which should have the same molecular weight.

The mass spectra for 1, 4 and 5 had a prominent peak at *m/e* 167 which suggests the radical cation of 2,3-dimethyl-4- (2′-carboxyethyl) -pyrrole. I gave a peak at *m/e* 136 (PBG-2COOH) but 4 and

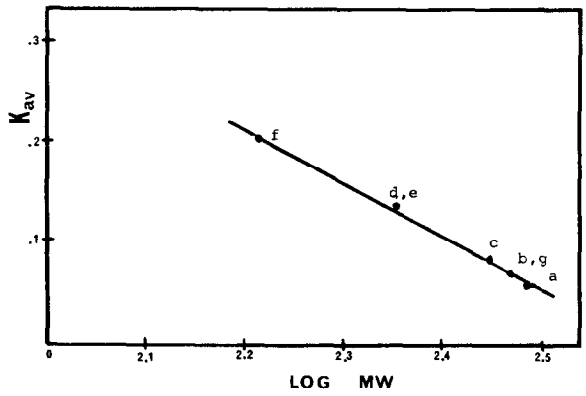


Fig. 3. $K_{av}$ vs. Log MW of PBG and its derivatives and two standards (tetra-alanine and phenylalanine). a = Tetraalanine, b = PBG-*N*-methylimidazole, c = PBG-Imidazole, d = PBG, e = PBG + 2-Methylimidazole (pH 7.4), f = Phenylalanine, g = PBG-2-Methylimidazole (pH 8.5).

5 did not. The mass spectrum of 4 had a prominent peak at *m/e* 68 (imidazole$^{+\cdot}$) and the mass spectrum of 5 had a prominent peak at *m/e* 82 (*N*-methylimidazole$^{+\cdot}$).

PBG reacted with solid polyhistidine and was removed from solution. The washed precipitate of PBG–polyhistidine reacted with Ehrlich reagent to give a pink color. This evidence again pointed to imidazole replacing the ammonia side chain on PBG and leaving a free α position. PBG is not bound electrostatically because at pH 8.5 the imidazole moiety is not protonated. Tramontini and co-workers [12] have shown that Mannich bases, of which PBG can be considered an example [13], react readily with imidazole and benzimidazole and alkylate N-1.

When 4, 5, or 6 were used instead of PBG as substrates for prophobilinogenase, urogen III was formed in high yield (table 2). Smaller amounts of 4, 5 or 6 were consumed than of PBG, but the porphyrin yield was higher or the same. It appeared that PBG was consumed enzymatically in two ways, but that 4, 5 and 6 were consumed only by porphobilinogenase. When the incubation mixture containing enzyme and PBG was 'scoures' free of oxygen by adding glucose and glucose oxidase [14] to the incubation mixture, PBG consumption dropped but the porphyrin yield remained the same. This is additional evidence that 4, 5 and 6 are indeed different from PBG and were substrates for porphobilinogenase but not for the oxygen-requiring enzymatic activity which consumed PBG in a pathway which did not lead to porphyrin. This enzyme is undoubtedly a porphobilinogen oxygenase which Frydman and coworkers [15,16] have described in beautiful detail. They have found that this enzyme is eluted from Sephadex chromatography in the same fractions with porphobilinogenase.

Isoporphobilinogen [17] and opsopyrroledicarboxylic acid [18] are inhibitors of porphobilinogenase and are not incorporated into porphyrin. It is almost certain that the active site of the enzyme exhibits specificity for the acetic and propionic acid side chains on pyrrole. However, this work shows that porphobilinogenase is far less discriminating about the leaving group on PBG (i.e. $-NH_3^+$) which can be replaced by imidazole groups without impairing enzyme efficiency or altering the nature of the product isomer. PBG, a Mannich base, and its derivatives apparently alkylate some group on the enzyme initially. Additional PBG units then insert into or alkylate the growing chain [19]. The catalytic and/or binding sites do not appear to have a stringent requirement for the $-NH_3^+$ group on PBG.

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Table 2
Consumption of substrate and yields of uroporphyrinogens I and III from incubation of PBG and derivatives with bovine liver porphobilinogenase at pH 7.4 in 0.05 M Tris Buffer

| Substrate | PBG consumption (%) | Porphyrinogen yield (%) | Isomer composition | |
|---|---|---|---|---|
| | | | I | III |
| PBG | 48 | 49 | 0 | 100 |
| PBG+glucose+ glucose oxidase | 24 | 91 | 0 | 100 |
| PBG-Im | 27 | 100 | 6 | 94 |
| PBG-*N*-Me-Im | 21 | 98 | 10 | 90 |
| PBG-2-Me-Im | 22 | 100 | 9 | 91 |

## References

[1] Davies, R. C., Gorchein, A., Neuberger, A., Sandy, J. D. and Tait, G. H. (1973) Nature, 245, 15–19.
[2] Radmer, R. and Bogorad, L. (1972) Biochemistry 11, 904–910.
[3] Pluscec, J. and Bogorad, L. (1970) Biochemistry 9, 4736–4743.
[4] Davies, R. C. and Neuberger, A. (1973) Biochem. J. 133, 471–492.
[5] Sancovich, H. A., Batlle, A. M. C. and Grinstein, M. (1969) Biochim. Biophys. Acta 191, 130–143.
[6] Mauzerall, D. and Granick, S. (1956) J. Biol. Chem. 219, 435–446.
[7] Jordan, P. M. and Shemin, D. (1973) J. Biol. Chem. 248, 1019–1024.
[8] Edmondson, P. R. and Schwartz, S. (1953) J. Biol. Chem. 205, 605–609.
[9] Yuan, M. and Russell, C. S. (1973) J. Chrom. 87, 562–564.
[10] Falk, J. E. (1964) Porphyrins and Metalloporphyrins, p. 161 Elsevier, Amsterdam–London–New York.
[11] Gurne, D. and Shemin, D. (1973) Science 180, 1188–1190.
[12] Andreani, F., Andrisano, R., Casa, C. D. and Tramontini, M. (1968) Tetrahedron Lett. 1059–1061.
[13] Frydman, R. B., Reil, S. and Frydman, B. (1971) Biochemistry 10, 1154–1160.
[14] Miller, W. L., Kalafer, M. E., Gaylor, J. L. and Delwiche, C. V. (1967) Biochemistry 6, 2673–2678.
[15] Frydman, R. B., Tomaro, M. L., Wanschelbaum, A., Anderson, E. M., Awruch, J. and Frydman, B. (1973) Biochemistry 12, 5253–5262.
[16] Tomaro, M. L., Frydman, R. B. and Frydman, B. (1973) Biochemistry 12, 5263–5268.
[17] Carpenter, A. T. and Scott, J. J. (1961) Biochim. Biophys. Acta 52, 195–198.
[18] Carpenter, A. T. and Scott, J. J. (1959) Biochem. J. 71, 325–333.
[19] Russell, C. S. (1974) J. Theor. Biol. In Press.