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PORPHYRIN BIOSYNTHESIS IN *RHODOPSEUDOMONAS PALUSTRIS*—IX. PBG-DEAMINASE. KINETIC STUDIES

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Abstract—1. PBG-Deaminase obtained from *Rp. palustris* exhibited classical Michaelis–Menten kinetics in the absence or presence of different ions.

2. Detailed kinetic studies were carried out in the presence of ammonium, phosphate and magnesium ions.

3. It has been found that the different effects observed are dependent on both the substrate and the ion concentration.

INTRODUCTION

The conversion of porphobilinogen (PBG) into uroporphyrinogen III is enzymically catalysed by the porphobilinogenase system (PBG-ase) (Lockwood and Rimington, 1957). The formation of uroporphyrinogen I from PBG is brought about by uroporphyrinogen I synthetase (URO-S, PBG-Deaminase, EC 4.3.1.8). PBG-ase is a combination of two separate enzymes, PBG-deaminase, a heat stable protein and uroporphyrinogen III cosynthetase (isomerase, EC 4.2.1.75), a heat labile protein.

PBG-Deaminase has been purified from several sources and the properties of the protein have been described (Jordan and Shemin, 1973; Higuchi and Bogorad, 1975; Miyagi *et al.*, 1979; Rossetti *et al.*, 1980; Anderson and Desnick, 1980; Williams *et al.*, 1981; Battersby *et al.*, 1983; Hart *et al.*, 1984; Williams, 1984; Fumagalli *et al.*, 1985).

PBG-Deaminase from *Rhodopseudomonas palustris*, the most common non sulfur bacteria, has also been purified by Kotler *et al.* (1986). The present work describes kinetic studies conducted on PBG-Deaminase in the absence and presence of different ions.

MATERIALS AND METHODS

Porphobilinogen was biosynthetically obtained (Sancovich *et al.*, 1970) and estimated as described by Moore and Labbe (1964).

The standard incubation system contained the enzyme preparation (50 μl) together with 0.05 M sodium phosphate buffer (pH 7.6–7.8) and PBG (at the concentrations indicated) with or without the addition of other reagents, in a final volume of 1.5 ml. Incubations were carried out aerobically in the dark with mechanical shaking at 37°C for 30 min.

Blanks were always run with PBG and without enzyme. After incubation, TCA was added to precipitate the protein (final concentration 5% w/v), the mixture was then exposed to white light for 20 min to oxidize porphyrinogens, the protein precipitate filtered off and total porphyrins and remaining PBG estimated in the resulting solution (Rimington, 1960; Moore and Labbe, 1964).

In all experiments reaction velocity was measured on the basis of Uroporphyrinogen formation and substrate consumption during 30 min or 1 hr.

The purification procedure for PBG-Deaminase, as well as all other methods and materials not specified here, were those described by Kotler *et al.* (1986).

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Dedicated to Professor Claude Rimington FRS on the occasion of his 85th birthday.

RESULTS

Saturation curves (*Fig.* 1)

Plots of velocity measured as nmol of uroporphyrinogen formed against PBG concentration and their reciprocals followed classical Michaelis–Menten kinetics.

When velocity was measured in terms of PBG consumed, the saturation curve was linear and the double reciprocal plots also showed a normal kinetic pattern.

Michaelis constants and maximum velocities were calculated from Lineweaver–Burk plots and the number of probable substrate binding sites (n) were determined by the empirical Hill equation (Fig. 1, inset).

In *Euglena gracilis* Williams *et al.* (1981) demonstrated that PBG-deaminase combined four molecules of substrate to produce one molecule of uroporphyrinogen I with the elimination of four molecules of ammonia, this stoicheiometry was observed at different pH values (range 5.5–8.5).

In our laboratory, working with PBG-ase from soya-bean callus (Llambías and Batlle, 1971), *Euglena gracilis* (Rossetti, 1978) and *Rp. palustris* (Juknat, 1983) we have shown that uroporphyrinogen formation based on substrate consumption was extremely low, indicating a great deviation from stoicheiometric values. When PBG-deaminase from *Rp. palustris* was studied, an excess of PBG consumption

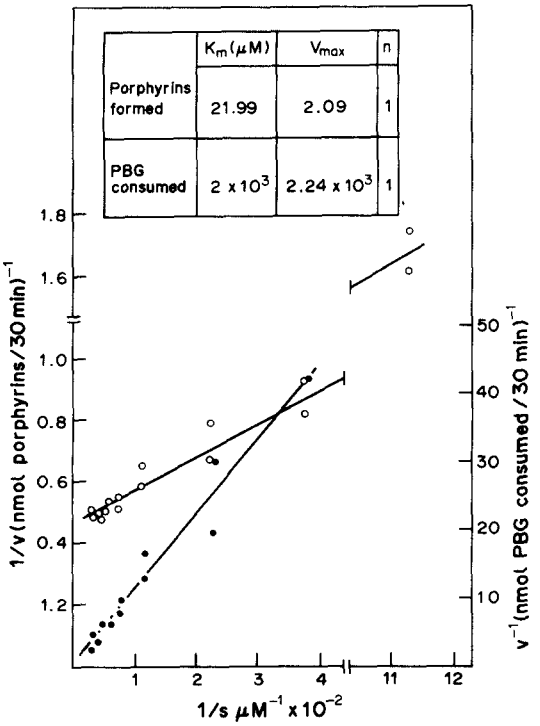


Fig. 1. Double reciprocal plots, when activity was measured in terms of porphyrins formed (○) or PBG consumed (●).

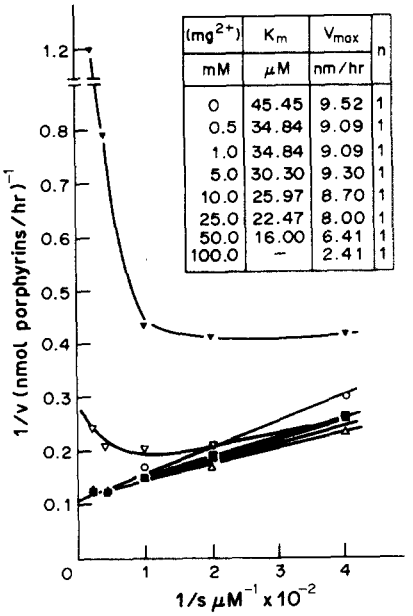


Fig. 2. Double reciprocal plots of velocity against PBG concentration without additions (○) and in the presence of Mg^{2+} (●) 0.5 mM; (□) 1 mM; (■) 5 mM; (△) 10 mM; (▲) 25 mM; (▽) 50 mM; (▼) 100 mM. Experimental conditions are indicated in the text.

of 5 times at low substrate concentration (8–26 μM) was found, increasing this value up to 50 times at 350 μM PBG (Table 1). Battersby *et al.* (1983a) reported an inhibitory effect of hydroxymethylbilane on PBG-Deaminase activity from *E. gracilis*, a fact that could be an explanation for these findings.

Effect of magnesium ions

The effect of varying Mg^{2+} ion concentrations on PBG-Deaminase activity was studied at different PBG concentrations. Plots of rate of urogen I formation against PBG concentration, both in the absence and the presence of Mg^{2+} ions up to 25 mM, showed classical Michaelis–Menten kinetic and the reciprocal plots were linear (Fig. 2). Mg^{2+} ions at concentrations between 5 and 25 mM behaved as a non-competitive inhibitor. The Michaelis constant, $K_m = 45\ \mu$M (Inset, Fig. 2), was found to be essentially dependent of Mg^{2+} concentration, increasing the affinity for PBG. At the same time there was a decrease in V_{max} tending to 2 nmol/hr. Inhibition of PBG-deaminase activity by high substrate concentration at 50 and 100 mM Mg^{2+} was also found.

It has also been observed that at 25, 50 and 100 μM PBG, low concentration of Mg^{2+} activated the enzyme (Fig. 3). Increasing Mg^{2+} concentrations however produced a great inhibition, reaching 100% for 100 mM Mg^{2+} and 500 μM PBG.

Mg^{2+} ions had no effect on PBG consumption.

Effect of phosphate ions

Activity plots of PBG-deaminase vs PBG concentration at different PO_4^{3-} concentrations, showed an hyperbolic pattern. Reciprocal plots (Fig. 4) and Eadie curves were also linear. From the reciprocal plots, it can be seen that at 1 and 10 mM, phosphate behaved as an incompetitive inhibitor.

When velocity of PBG-deaminase against PO_4^{3-} concentrations was plotted, typical inhibition curves were obtained (Fig. 5).

Table 1. Stoicheiometry of PBG-deaminase reaction

PBG addition			PBG uptake		Porphyrin formation (nmol)		R[b]
μg	nmol	μM	μg	nmol	Theoretic[a]	Real	(t/r)
3	13.27	8.85	2.55	11.28	2.82	0.58	4.86
9	39.82	26.55	4.96	21.95	5.49	1.08	5.08
15	66.37	44.25	11.53	51.02	12.76	1.27	10.05
30	132.72	88.50	17.55	77.65	19.41	1.54	12.60
45	199.12	132.74	29.34	129.82	32.45	1.80	18.03
60	265.49	177.00	37.02	163.81	40.95	1.87	21.90
90	398.23	260.49	65.47	289.69	72.42	2.04	35.50
120	530.97	353.98	92.35	408.63	102.16	2.00	51.08

The experiments were performed as described in the text.
[a]Calculated on the basis of the PBG uptake.
[b]R: nmol theoretic porphyrins/nmol porphyrins really formed.

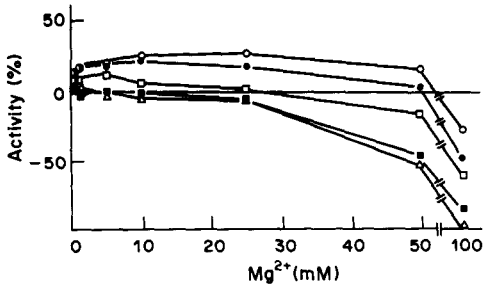


Fig. 3. Effect of different concentrations of Mg^{2+} on PBG-deaminase activity, measured in terms of porphyrin formation, at varying concentrations of PBG: (○) 25 μM; (●) 50 μM; (□) 100 μM; (■) 250 μM; (△) 500 μM. Activity of a control without Mg^{2+} and measured under the standard incubation conditions was taken as 0 level so changes estimulating or inhibiting were refered to this value as such. Experimental conditions are indicated in the text.

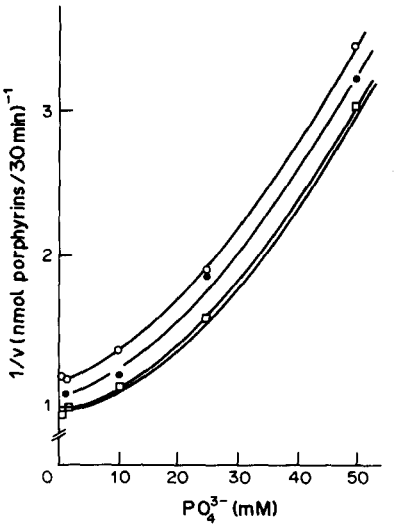


Fig. 5. Effect of different concentrations of PO_4^{3-} on the reversal of PBG-deaminase activity measured in terms of porphyrin formation, at varying concentrations of PBG: (○) 51 μM; (●) 102 μM; (□) 205 μM and (■) 310 μM. Experimental conditions are indicated in the text.

It is interesting to note that if PBG consumption was taken as a measure of enzyme activity, velocity plots of PBG-deaminase against PBG concentration showed normal kinetic pattern at all PO_4^{3-} concentrations studied (Fig. 6). It was also found that in this case PO_4^{3-} acted as a non-competitive inhibitor.

Effect of ammonium ions

Direct and reciprocal plots of reaction velocity against PBG concentration, both in the absence and the presence of NH_4^+ ions, showed classical Michaelis-Menten kinetics (Fig. 7). Eadie plots were also linear.

Ammonium ion is a non competitive inhibitor of porphyrin synthesis, resulting in lower values of K_m and V_{max}. These results are in agreement with Sancovich *et al.* (1969) and Llambías and Batlle (1971b).

It was found that ammonium at concentrations up to 100 mM inhibited 57% urogen I formation at 310 μM PBG (Fig. 8).

However, it is interesting to add that NH_4^+ at concentrations that inhibited porphyrin biosynthesis, had no effect upon the rate of PBG consumption.

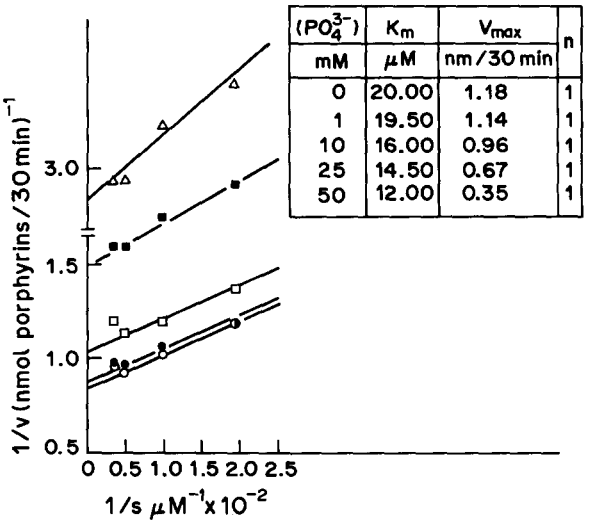
DISCUSSION AND CONCLUSIONS

The kinetic experiments showed in this report demonstrated that PBG-deaminase from *Rp. palustris* had a Michaelis kinetic behavior (Fig. 1).

The action of Mg^{2+} on PBG-deaminase activity showed that this metal had two different effects, acting as activator or as a non-competitive inhibitor at low and high concentrations respectively. The existence and extent of these effects were found to be dependent on PBG concentration (Fig. 3).

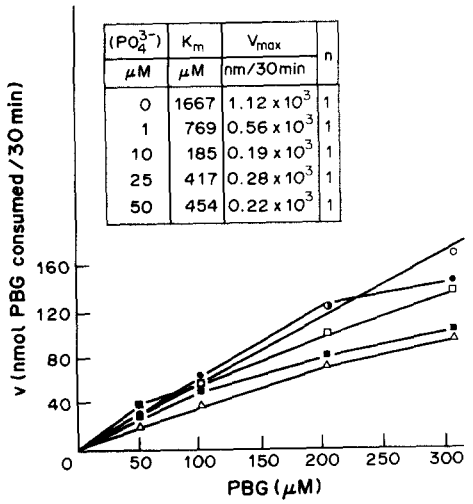
It must also be noted here that Mg^{2+} ions did not change normal kinetic pattern.

Studying the effect of Mg^{2+} on PBG-ase activity of the same source it was observed that 50 mM Mg^{2+} also produced activation when 24 μM PBG was



(PO_4^{3-}) mM	K_m μM	V_{max} nm/30 min	n
0	20.00	1.18	1
1	19.50	1.14	1
10	16.00	0.96	1
25	14.50	0.67	1
50	12.00	0.35	1

Fig. 4. Double reciprocal plots of velocity against PBG concentration without additions (○) and in the presence of different concentrations of PO_4^{3-}: (●) 1 mM; (□) 10 mM; (■) 25 mM; (△) 50 mM. Experimental conditions are indicated in the text.



(PO_4^{3-}) μM	K_m μM	V_{max} nm/30min	n
0	1667	1.12×10^3	1
1	769	0.56×10^3	1
10	185	0.19×10^3	1
25	417	0.28×10^3	1
50	454	0.22×10^3	1

Fig. 6. Effect of PBG concentration on the activity of PBG-deaminase measured in terms of PBG consumption in the absence (○) and presence of different concentrations of PO_4^{3-}: (●) 1 mM; (□) 10 mM; (■) 25 mM; (△) 50 mM. Experimental conditions are indicated in the text.

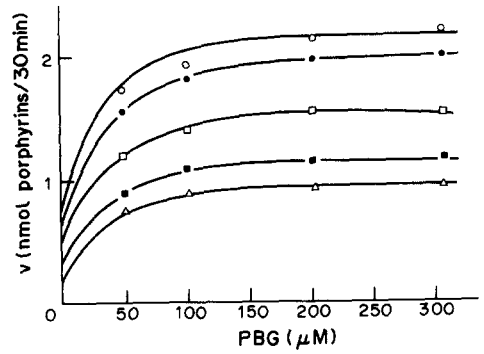


Fig. 7. Effect of PBG concentration on PBG-deaminase activity measured in terms of porphyrins formed in the absence (○) and the presence of different concentrations of NH_4^+: (●) 1 mM; (□) 10 mM; (■) 50 mM; (△) 100 mM. Experimental conditions are indicated in the text.

employed as substrate concentration (Juknat *et al.*, 1986).

The activating effect of certain concentrations of Mg^{2+} could be explained as if they were producing some association-dissociation phenomena, which gives the enzyme a definitive structural arrangement necessary for maximal activity (Batlle and Rossetti, 1977).

We proposed that magnesium could act by binding at different sites. At low concentration of Mg^{2+}, this ion will act producing the optimum structural arrangement, as already suggested. By increasing its concentration, Mg^{2+} could inhibit blocking the tetrapyrrole liberation on the PBG-deaminase.

Studies carried out on PBG-deaminase in the presence of phosphate ions, showed that PO_4^{3-} was acting as an inhibitor of velocity in terms of porphyrin synthesis and PBG consumption, without changes in the kinetic pattern.

It was suggested (Batlle and Rossetti, 1977; Pollack and Russell, 1978) that certain groups (ε-amines, imidazoles or guanidines) would be involved in the binding of PBG to PBG-deaminase. It is possible that phosphate could act partially or totally neutralyzing these recognition sites. So, complexs like E-PBG-I and E-I between the enzyme (E), the substrate (PBG) and the inhibitor (I) could be formed. The non-competitive type of inhibition found in these experiments, could be explained by the formation of this kind of complex.

Taking into account these hypothesis, once produced these complexs, the polypyrrol chain could not reach the length of a tetrapyrrol. So, the PBG consumption diminished, without being reversed by increasing substrate concentration. As a consequence, the synthesis of porphyrins will also be inhibited.

Increasing PO_4^{3-} concentration, a change in the type of inhibition for porphyrin synthesis can be observed. This effect may be due to the existence of an interaction phenomena between PO_4^{3-} and porphyrins, which could modify negatively the rate of porphyrin liberation.

Results obtained studying the kinetics of PBG-deaminase in the presence and the absence of ammonium ions, showed classical Michaelis–Menten behaviour and n was near 1. We also found that PBG consumption was not affected, while porphyrin synthesis was inhibited by ammonium ions. Taking into account these results, we suggested that ammonium has no binding site on the PBG-deaminase but produces a decrease in the amount of available tetrapyrroles. Basic derivates could be formed by direct reaction of NH_4^+ with di and tripyrrylmethanes (Davies and Neuberger, 1973). So, polypyrrolic intermediates will be liberated and the tetrapyrrylmethane (TPM) concentration on the PBG-Deaminase will be in turn reduced.

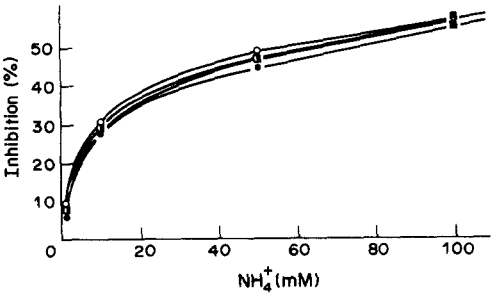


Fig. 8. Effect of different concentration of NH_4^+ on PBG-deaminase activity measured in terms of porphyrin formation, at varying concentrations of PBG: (○) 51 μM; (●) 102 μM; (□) 205 μM; (■) 310 μM. Experimental conditions are indicated in the text.

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REFERENCES

Anderson P. M. and Desnick R. J. (1980) Purification and properties of uroporphyrinogen I synthase from human erythrocyte. *J. biol. Chem.* **255** (5), 1993–1999.

Batlle A. M. del C. and Rossetti M. V. (1977) Enzyme polymerization of porphobilinogen into uroporphyrinogens. *Int. J. Biochem.* **8**, 251–267.

Battersby A. R., Fookes C. J. R., Hart G., Matcham G. W. J. and Pandey P. S. (1983a) Biosynthesis of porphyrins and related macrocycles. Part 21. The interaction of Deaminase and its products (hydroxymethylbilane) and the relationship between Deaminase and Cosynthetase. *J. Chem. Soc. Perkin Trans.* **I**, 3041–3047.

Battersby A. R., Fookes C. J. R., Matcham G. W. J., McDonald E. and Hollenstein R. (1983b) Biosynthesis of porphyrins and related macrocycles. Part 20. Purification of deaminase and studies on its mode of action. *J. Chem. Soc. Perkin Trans.* **I**, 3031–3040.

Davies R. and Neuberger A. (1973) Polypyrroles formed from porphobilinogen and amines by uroporphyrinogen synthetase of *Rhodopseudomonas spheroides*. *Biochem. J.* **133**, 471–492.

Fumagalli S. A., Kotler M. L., Rossetti M. V. and Batlle A. M. del C. (1985) Human red cell porphobilinogen deaminase. A simpler method of purification and some unusual properties. *Int. J. Biochem.* **17**, (4), 485–494.

Hart G. J., Leeper F. J. and Battersby A. R. (1984) Modification of hydroxymethylbilane synthase (porphobilinogen deaminase) by pyridoxal 5′-phosphate. *Biochem. J.* **222**, 93–102.

Higuchi M. and Bogorad L. (1975) The purification and properties of uroporphyrinogen I synthase and uroporphyrinogen III co-synthetase. Interaction between the enzymes. *Ann. N. Y. Acad. Sci.* **244**, 401–408.

Jordan P. and Shemin D. (1973) Purification of uroporphyrinogen I synthetase from *Rp. spheroides*. *J. biol. Chem.* **248**, 1019–1024.

Juknat A. A. (1983) Ph.D. thesis, University of Buenos Aires.

Juknat A. A., Kotler M. L. and Batlle A. M. del C. (1986) Porphyrin biosynthesis in *Rp. palustris* IV. Enzymic cyclotetramerization of porphobilinogen into uroporphyrinogens. In preparation.

Kotler M. J., Fumagalli S. A., Juknat A. A. and Batlle A. M. del C. (1986) Porphyrin biosynthesis in *Rp. palustris* VIII. Purification and properties of Deaminase. *Comp. Biochem. Physiol.* **B**. In press.

Llambias E. B. C. and Batlle A. M. del C. (1971a) Studies on porphobilinogen deaminase-uroporphyrinogen III cosynthetase system of cultured soyabean cells. *Biochem. J.* **121**, 327–340.

Llambias E. B. C. and Batlle A. M. del C. (1971b) Porphyrin biosynthesis VII. Avian erythrocyte porphobilinogen deaminase-uroporphyrinogen III cosynthetase, its purification, properties and the separation of its components. *Biochim. biophys. Acta* **227**, 180–191.

Lockwood W. and Rimington C. (1957) Purification of an enzyme converting porphobilinogen to uroporphyrinogen. *Biochem. J.* **67**, 8–11.

Miyagi K., Kaneshima M., Kawakami J., Nakada F., Petryka Z. J. and Watson C. J. (1979) Uroporphyrinogen I synthase from human erythrocytes: Separation, purification and properties of isoenzymes. *Proc. natn. Acad. Sci. U.S.A.* **76** (12), 6172–6176.

Moore D. and Labbe R. (1964) Assays for ALA and PBG determinations. *Clin. Chem.* **10**, 1105–1109.

Pollack S. E. and Russell C. S. (1978) Inhibition of wheat germ porphobilinogen deaminase activity by butanediona. *FEBS Lett.* **90**, (1), 47–50.

Rimington C. (1960) Spectral absorption coefficients of some porphyrins in the Soret band region. *Biochem. J.* **75**, 620–623.

Rossetti M. V. (1978) Ph.D. Thesis, University of Buenos Aires.

Rossetti M. V., Juknat A. A., Kotler M. L., Fumagalli S. A. and Batlle A. M. del C. (1980) Occurrence of multiple molecular forms of porphobilinogenase in diverse organisms: the minimum quaternary structure of porphobilinogenase is a protomer of one deaminase and one isomerase domain. *Int. J. Biochem.* **12**, 761–767.

Sancovich H. A., Batlle A. M. del C. and Grinstein M. (1969) The porphobilinogen deaminase-uroporphyrinogen III cosynthetase system (Porphobilinogenase) from bovine liver. Kinetic studies. *FEBS Lett.* **3** (3), 223–226.

Sancovich H. A., Ferramola A. M., Batlle A. M. del C. and Grinstein M. (1970) Preparation of porphobilinogen. In *Methods in Enzymology* (Edited by Tabor H. and Tabor C. W.), Vol. XVII, pp. 220–222. Academic Press, New York.

Williams D. C., Morgan G., McDonald E. and Battersby A. R. (1981) Purification of porphobilinogen deaminase from *Euglena gracilis* and studies of its kinetics. *Biochem. J.* **193**, 301–310.

Williams D. C. (1984) Characterization of the multiple forms of hydroxymethylbilane synthase from rat spleen. *Biochem. J.* **217**, 675–683.