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Purification of porphobilinogen deaminase from *Euglena gracilis* and studies of its kinetics

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1. Porphobilinogen deaminase [porphobilinogen ammonia-lyase (polymerizing), EC 4.3.1.8] from *Euglena gracilis* was purified more than 200-fold. 2. The enzyme has a molecular weight of 41000 ± 2000, does not contain a chromophoric prosthetic group, and appears not to require metal ions for activity. 3. The stoicheiometry of the overall reaction at pH 7.4 was shown to be:

$$4 \text{ Porphobilinogen} \rightarrow \text{uroporphyrinogen-I} + 4\,NH_4^+$$

This stoicheiometry for porphobilinogen and uroporphyrinogen was also observed over a wide range of pH values. 4. Initial-velocity studies showed a hyperbolic dependence of velocity on substrate concentration, demonstrating the existence of a displacement-type mechanism. 5. $V_{max.}$ varied with pH as a typical bell-shaped curve, indicating that two ionizable groups with pK values of 6.1 and 8.9 are important for catalysis. A plot of $V_{max.}/K_m$ against pH showed a single ionization (pK 8.2) to influence binding of substrate.

Porphobilinogen deaminase [porphobilinogen ammonia-lyase (polymerizing), EC 4.3.1.8] catalyses the combination of four molecules of porphobilinogen eventually to produce uroporphyrinogen-I (Scheme 1) with the elimination of four molecules of NH_3. For the production of uroporphyrinogen-III, the natural precursor of haem, chlorophyll and vitamin B_{12}, a heat-labile protein named uroporphyrinogen-III co-synthetase is also required (Bogorad, 1955, 1958*a*,*b*; Levin, 1971).

Studies in our laboratory (Battersby *et al.*, 1979*a*,*b*; Battersby & McDonald, 1979) have led to an almost complete understanding (with relation to the substrates) of the chemistry of formation of uroporphyrinogen-I and uroporphyrinogen-III. Recent work (Battersby *et al.*, 1979*a*,*b*) has shown that the enzyme does not catalyse the cyclization step but that uroporphyrinogen-I is produced via chemical cyclization of a hydroxymethylbilane. However, much remains to be discovered about the enzymic aspects. Porphobilinogen deaminase has been purified from several sources and the properties of these proteins have been described (Battersby & McDonald, 1975; Jordan & Shemin, 1973). To allow a full examination of the enzyme used in our laboratory, porphobilinogen deaminase from *Euglena gracilis* has been purified and studies have been made of its kinetics.

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Materials and methods

Materials

DEAE-cellulose was purchased from Whatman Biochemicals, Maidstone, Kent, U.K. Sephadex and Sepharose gels were obtained from Pharmacia (G.B.), London W.5, U.K., and 4-dimethylaminobenzaldehyde was from Hopkin and Williams, Chadwell Heath, Essex, U.K. Bovine serum albumin (fraction V) and glutamate dehydrogenase (bovine liver, type 1, 1180units/ml) were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other reagents commercially obtained were of AnalaR grade or the purest available grade. Deionized distilled water was used throughout.

Porphobilinogen and [^{14}C]porphobilinogen were prepared by the Rapoport–Wurziger method (Battersby *et al.*, 1977); the porphobilinogen used was over 95% pure as determined with Ehrlich's reagent (4-dimethylaminobenzaldehyde) by the method of Mauzerall & Granick (1956), by using $\varepsilon_{553} = 57700$ litre $\cdot$ mol^{-1} $\cdot$ cm^{-1}.

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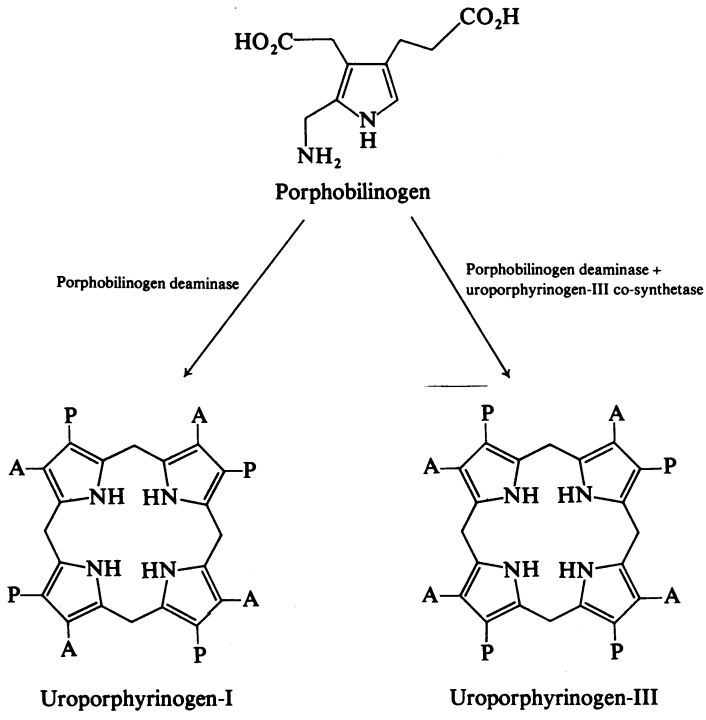

Scheme 1. *Enzymic conversion of porphobilinogen into uroporphyrinogen-I and uroporphyrinogen-III*
A = CH_2–CO_2H; P = CH_2–CH_2–CO_2H

Assay of porphobilinogen deaminase

Porphobilinogen deaminase activity was measured by following porphyrinogen production. Fractions containing enzyme were incubated aerobically in the dark at 37°C for 30min in 0.2M-sodium phosphate buffer (titrated to pH8.0 with NaOH solution) containing 0.6mM-EDTA, and porphobilinogen (550μM), in a total volume of 3ml. A portion (0.75ml) was then diluted with trichloroacetic acid solution (1.75ml of 7.1% w/v). [This step also effects cyclization of any residual bilanes (Battersby *et al.*, 1979*a*,*b*), so these are included in the assay.] The protein precipitate was removed by centrifugation at maximum speed in an MSE bench-top centrifuge (model MSE Minor), and to the supernatant was added iodine solution (10μl of 1%, w/v, iodine in water with sufficient KI to dissolve the iodine) to oxidize all porphyrinogens to porphyrins. After mixing, the solution was incubated at 37°C for 5min, and the excess iodine was then reduced with aqueous $Na_2S_2O_3$ (20μl of 2%, w/v). The porphyrin content was determined by measuring the A_{406} by using $\varepsilon_{406} = 528000$ litre $\cdot$ mol^{-1} $\cdot$ cm^{-1} (Rimington, 1960).

For kinetic studies, porphobilinogen deaminase activity was measured by incubating the enzyme solution (usually 0.1ml) with 0.1M-sodium phosphate buffer and porphobilinogen (at various concentrations) in a total volume of 2.0ml under various conditions of pH. After incubation for 30min (usually at 30°C), 0.1ml of trichloroacetic acid solution (5M) was added and the porphyrinogen content of the samples was measured as described above.

In control blanks with either enzyme or porphobilinogen absent, the estimated porphyrin (or any contribution to the A_{406} value) was in all cases very small and was subtracted from the test value.

One unit of enzyme activity was determined as the amount of enzyme necessary to produce 1nmol of porphyrinogen/h under the specified conditions.

Uroporphyrinogen isomer analysis was performed by high-pressure liquid chromatography as described by Battersby *et al.* (1976*b*).

Preparation of porphobilinogen–Sepharose resin

Aminohexyl-Sepharose 4B was prepared essentially as described by Lowe & Dean (1974).

Porphobilinogen–Sepharose 4B was prepared by adding 1mmol of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide to aminohexyl-Sepharose 4B (20ml of packed gel) in the presence of 1mM-

[^{14}C]porphobilinogen (1.49×10^4 d.p.m./μmol) in a total volume of 100ml in water. The pH was maintained at 4.3 by titration with HCl at 20°C for 20h. The gel was washed copiously with water, and then with 2M-KCl. A portion (200mg) of the total gel (12.8g) was asayed for radioactivity (in Instagel; Packard Instrument Co., Wembley, Middlesex, U.K.) and was found to contain 575d.p.m., corresponding to 0.19μmol of porphobilinogen per g wet wt. of gel.

Before each use for chromatography of porphobilinogen deaminase, the gel was washed with 2M-KCl, and then copiously with 10mM-sodium phosphate buffer, pH7.4.

Though it had been hoped that porphobilinogen–Sepharose would function as a specific affinity column, its effectiveness was limited and it served in the isolation scheme mainly for concentration of the protein solution.

Determination of protein

Protein concentrations of samples were determined (with bovine serum albumin as standard) either by the biuret method of Gornall (Layne, 1957) or by the Folin method (Layne, 1957). Column eluates were assayed for protein content by A_{280} measurements, by using a value for A_{280} of 1.0 for a 1mg/ml solution in a 1cm-path-length cuvette.

Purification of porphobilinogen deaminase

All procedures, unless otherwise stated, were performed at 4°C.

(*a*) *Preparation of heat-treated enzyme. Euglena gracilis* was grown and harvested as described by Battersby *et al.* (1976*a*). Frozen cells (approx. 250g) were thawed overnight at 4–6°C, centrifuged at 23000*g* for 30min and the pellet was discarded. The supernatant (200–250ml) was adjusted from pH5.8–6.0 to pH6.2 with conc. NH_3 (sp.gr. 0.880), then divided into 20ml portions in glass test tubes and heated at 60°C for 10min before being rapidly chilled by plunging into iced water. The protein suspension was centrifuged as described above but for 10min, the supernatant being used for step (*b*).

(*b*) *Fractionation with* $(NH_4)_2SO_4$. The above supernatant was cautiously adjusted to and maintained throughout at pH7.4 with conc. NH_3 (sp.gr. 0.880) and then made 14mM with respect to 2-mercaptoethanol. Solid $(NH_4)_2SO_4$ (258g/litre of initial solution) was added to the solution, which was stirred on ice throughout, to make the solution 45% saturated with $(NH_4)_2SO_4$. After stirring for a further 30min the protein that was precipitated was removed by centrifugation at 23000*g* for 10min. The resulting supernatant was made 28mM with respect to 2-mercaptoethanol, and solid $(NH_4)_2SO_4$ (156g/litre of supernatant) was added to make the solution 70% saturated with $(NH_4)_2SO_4$. The protein that was precipitated in 30min was removed by centrifugation at 23000*g* for 10min, and this pellet was dissolved in a minimum volume (approx. 10ml) of cold 10mM-sodium phosphate buffer, pH7.4, before dialysis of the solution against three 2-litre changes of 10mM-sodium phosphate buffer, pH7.4.

(*c*) *DEAE-cellulose chromatography.* The protein solution from step (*b*) was loaded on a column (15cm × 1.5cm) of DEAE-cellulose (DE-52) previously equilibrated with 10mM-sodium phosphate buffer, pH7.4. The column was eluted with the same buffer at a flow rate of 40ml/h, and the fractions (10ml) were assayed for enzyme activity and protein. The pooled fractions with enzyme activity were quickly applied to the porphobilinogen–Sepharose column.

(*d*) *Porphobilinogen–Sepharose chromatography.* The DEAE-cellulose eluate (approx. 100ml) was loaded on a column (15cm × 1cm) of porphobilinogen–Sepharose, which had been pretreated as described above. After all the eluate had been loaded, a small volume (approx. 20ml) of 10mM-sodium phosphate buffer, pH7.4, was passed through the column. Elution was continued with 100mM-sodium phosphate buffer, pH7.4, the fractions (5ml) being assayed for enzyme activity and protein (usually found in one or two fractions). The enzyme stored at 0–4°C (unfrozen) lost less than 5% activity over 10 days.

Polyacrylamide-gel electrophoresis of purified deaminase

Polyacrylamide-gel disc electrophoresis was performed by the method of Davis (1964) with gels (70mm × 5mm) containing 7.5% (w/v) acrylamide, run in 50mM-Tris/glycine buffer (50mM-Tris titrated with glycine to pH8.5) at 5mA/gel tube. Gels were stained for protein with Coomassie Blue, and for deaminase activity by incubating in 6ml of the routine porphobilinogen assay solution for 1h, then washing with water, incubating for 10min in a solution of iodine (0.003%), washing with water and finally incubating in a solution of $Na_2S_2O_3$ (0.006%), until all iodine colour had been discharged. Fluorescent red bands were observed under u.v. light at positions where porphyrins had been formed.

Estimation of molecular weight of deaminase by gel filtration

Protein samples were applied separately to a column (75cm × 2.5cm) of Sephadex G-100 eluted at 25ml/h with 10mM-sodium phosphate buffer, pH7.4; 4.8ml fractions were collected. Aldolase (140000), chymotrypsinogen (24500) and bovine serum albumin (68000) were detected spectrophotometrically at 280nm, and cytochrome *c* (12500) was detected at 410nm; molecular weights in parentheses are from Andrews (1970).

Studies of initial velocity

The deaminase used was taken from the 45–70%-satn.-$(NH_4)_2SO_4$ fraction after dialysis. For studies of linearity of reciprocal plots, enzyme (100μl) was diluted into 1.9ml of 0.1M-sodium phosphate buffer (at the appropriate pH) containing porphobilinogen (3.2–826μM) and then incubated at 30°C for 30min; sodium borate buffer was used for higher pH values. Incubations were run in duplicate and the mean value for uroporphyrinogen formed was taken. Time courses of reactions were performed and in all cases were linear over times at least twice those of incubation. Stability of the enzyme was demonstrated by incubating it separately under the more vigorous conditions above, sampling at time intervals and assaying at pH7.5 and 30°C as described.

Stoicheiometry

(*a*) *Measurement of porphobilinogen uptake and uroporphyrinogen formation.* Enzyme was incubated with porphobilinogen (320μM) in 0.1M-sodium phosphate buffer at pH5.5, 7.0 or 8.5 in the dark at 30°C. At times 0, 15, 30 and 60min, samples (100μl) were taken and porphyrinogen content after the trichloroacetic acid treatment was determined as described above. Portions (100μl) of the incubation mixture were diluted with water (1.9ml) and assayed for porphobilinogen by using Ehrlich's reagent.

(*b*) *Measurement of uroporphyrinogen formation and NH_3 release.* Enzyme (50μl) was incubated in 0.1M-sodium phosphate buffer, pH7.4 (final vol. 1ml), in the presence of glutamate dehydrogenase (59 units), NADH (0.20mM), α-oxoglutarate (15mM) and EDTA (0.3mM) in a semi-micro cuvette thermostatically maintained at 37°C. The reaction was initiated by addition of porphobilinogen (430μM final concentration), and the decrease in A_{340} appeared to be linear over 5min, with a slope of 0.205 A unit/min, equivalent to 49.3μmol of NH_3/h per ml of stock enzyme solution.

The stock solution of deaminase (25μl) was incubated in a parallel experiment with 0.1M-sodium phosphate buffer, pH7.4 (final vol. 3ml), with porphobilinogen (430μM) for 15min at 37°C. Enzyme activity was 13400 units/ml.

Deaminase (25μl) was also incubated in 0.1M-sodium phosphate buffer, pH7.4 (final volume 3ml), in the presence of porphobilinogen (430μM), α-oxoglutarate (15mM), EDTA (0.3mM), NADH (0.20mM) and glutamate dehydrogenase (177 units) for 15min. Enzyme activity was 13800 units/ml, demonstrating that the assay components for determination of NH_3 did not inhibit the deaminase.

Results and discussion

Purification and molecular weight of deaminase

Euglena gracilis provides an excellent source of deaminase (see Table 1), the enzyme activity being much higher than from mammalian sources, e.g. ox liver (approx. 2 units/g of tissue; Sancovich *et al.*, 1969), mouse spleen (approx. 300 units/g of tissue; Levin, 1968) or rat spleen (approx. 70 units/g of tissue; D. C. Williams, unpublished work). The activity in *E. gracilis* is comparable with that from *Rhodopseudomonas spheroides*, which contains approx. 15000 units/g dry wt. of cells (Davies & Neuberger, 1973).

Typical results for the present purification scheme are given in Table 1. The process is short, taking approx. 2 days, and recoveries of activity vary between 60 and 90% over the initial two steps. The preparation, after heat treatment, produced only uroporphyrinogen-I from porphobilinogen.

A typical profile for the DEAE-cellulose eluate is shown in Fig. 1. This step gave much purification, but the enzyme solution was very dilute, so chromatography on porphobilinogen–Sepharose was performed rapidly to minimize losses.

The purified enzyme showed one major and four very minor bands on electrophoresis (Fig. 2), the major band being coincident with the single band observed when gels were stained for enzyme activity.

The specific activity of purified deaminase was

Table 1. *Typical results for purification of porphobilinogen deaminase from E. gracilis*

Protein was measured by the biuret method ([b]) or the Folin method ([f]); N.D., not determined owing to low protein concentration.

Fraction	Enzyme activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cell supernatant (from approx. 250g wet wt. of cells)	140000	5000[b]	28	100	1
Supernatant after heat treatment	106000	2800[b]	38	75.5	1.3
45–70%-satn.-$(NH_4)_2SO_4$ fraction	64000	270[b]	240	46	8.5
DEAE-cellulose eluate	24000	N.D.	N.D.	17	—
Porphobilinogen–Sepharose eluate	26400	4.4[f]	6000	19	210

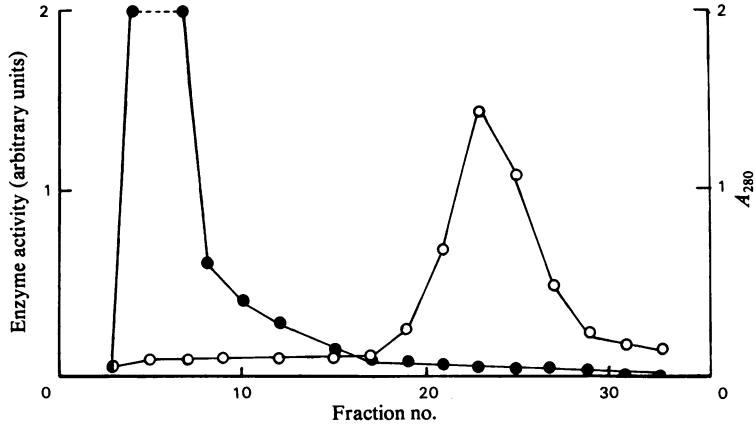

Fig. 1. *DEAE-cellulose chromatography of porphobilinogen deaminase*
Fractions up to and including 13 were 5 ml; the remainder were 13 ml each. For full details see the text. ○, Enzyme activity (arbitrary units); ●, A_{280}.

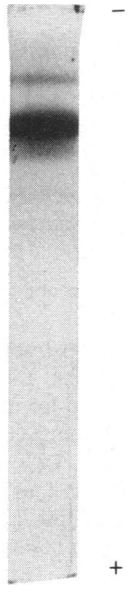

Fig. 2. *Polyacrylamide-gel electrophoresis of final enzyme preparation*
A sample of enzyme containing about 100 μg of protein was fractionated by electrophoresis in 7.5% acrylamide gels stained with Coomassie Blue. For full details see the text.

6000 units/mg. At 1 mg/ml the enzyme showed no absorption between 350 and 700 nm, but the typical absorption of a protein over 220–350 nm. For purified enzyme from *Rps. spheroides*, Davies & Neuberger (1973) report a specific activity of 6750 units/mg, whereas Jordan & Shemin (1973) report 32000 units/mg, under very similar assay conditions to each other and to those used in the present work. It is noteworthy that a modified approach (G. W. J. Matcham, unpublished work) has recently yielded the *Euglena* enzyme with specific activity 30000 ± 3000 units/mg.

The elution position of deaminase relative to four standard proteins on Sephadex G-100 corresponded to a molecular weight of 41000 ± 2000, which is similar to the values reported for deaminase from *Rps. spheroides*, 36000 (Davies & Neuberger, 1973; Jordan & Shemin, 1973), spinach leaves, 38000–40000 (Higuchi & Bogorad, 1975), but different from that of human erythrocytes, 25000 (Frydman & Feinstein, 1974).

Stoicheiometry of enzymic reaction (Table 2)

To produce 1 molecule of uroporphyrinogen-I, 4 molecules of porphobilinogen are required, and the averaged values found here for porphobilinogen uptake and total uroporphyrinogen formation at different pH values corresponded to a yield of 87%. This is in accord with earlier studies with deaminase from other sources, where a minimum yield of 91% was found (Bogorad, 1955, 1958*a*,*b*; Jordan & Shemin, 1973; Frydman & Frydman, 1970); these papers discussed possible reasons for the slight loss.

Determination of the NH_3 released by deaminase from porphobilinogen gave 92% of 4 mol of NH_3 for each mol of uroporphyrinogen-I produced. It was shown separately that the enzyme was not appreciably inhibited by NH_4^+ ions below 10 mM.

Studies of initial velocities

These experiments were conducted by measuring the rate of total uroporphyrinogen formation for an

Table 2. *Stoicheiometry of deaminase reaction*

The experiments were performed as described in the text. Concentrations are expressed for the incubation mixtures. The enzyme fractions used were from the final purification stage, and a different preparation was used for those studies involving NH_3 measurements to those involving porphobilinogen uptake measurements.

pH	Enzyme	Porphobilinogen uptake (μM/h)	Uroporphyrinogen formation (μM/h)	NH_3 formation (μM/h)	Stoicheiometry
5.5	250μl	223	45.4		4.9:1
7.0	100μl	242	57.5		4.2:1
8.5	100μl	218	47.5		4.6:1
8.5	0	0	—		—
7.4	25μl	—	335	—	3.7:1
7.4	50μl	—	—	2465	

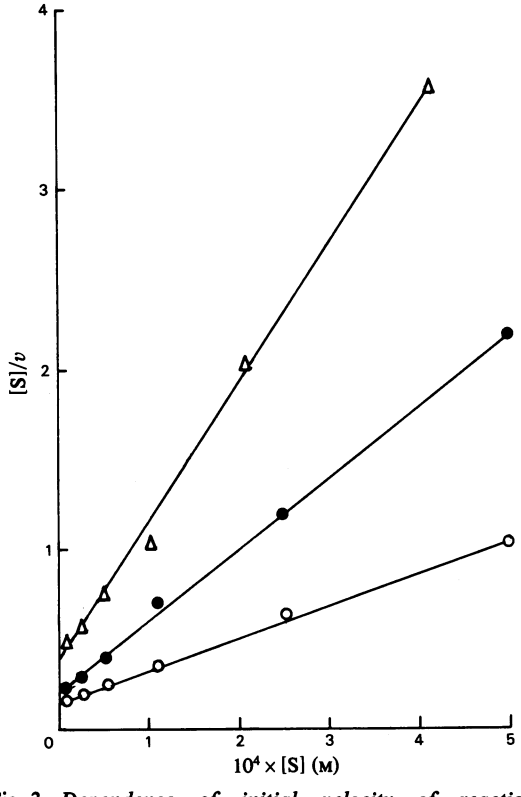

Fig. 3. *Dependence of initial velocity of reaction catalysed by porphobilinogen deaminase on porphobilinogen concentration*

Results are expressed as plots of [S]/v versus [S] for three pH values: ●, 6.0; ○, 7.0; △, 8.15. For full details see the text.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E^I + NH_3$$

$$E^I + S \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E^IS \xrightarrow{k_{+4}} E^{II} + NH_3$$

$$E^{II} + S \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} E^{II}S \xrightarrow{k_{+6}} E^{III} + 2NH_3$$

$$E^{III} + S \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} E^{III}S \xrightarrow{k_{+8}} E^{IV} + NH_3$$

$$E^{IV} \xrightarrow{k_{+9}} E + P$$

Scheme 2. *Schematic representation of the formation of the hydroxymethylbilane end product (P) by a sequential displacement mechanism*

incubation time of 30min, at different pH values and a wide range of porphobilinogen concentrations. Expressing the results as plots of [S]/v against [S] (Hanes, 1932) gave Fig. 3, and linear plots were found in all cases. There is thus no significant deviation from a hyperbolic relationship between enzyme activity and substrate concentration over a large range of conditions. Linear double-reciprocal plots have been reported for deaminase from human blood (Frydman & Feinstein, 1974), wheat germ (Frydman & Frydman, 1970; Stevens & Frydman, 1967) and *Rps. spheroides* (Jordan & Shemin, 1973). This general finding can be used to support an earlier working hypothesis [see, e.g., Battersby & McDonald (1975) and Frydman & Frydman (1975)] for the way deaminase builds the tetrapyrrole system on its surface.

The initial-rate equation for an enzyme that utilizes four substrates would be generally expected to be complex, as shown by the general analysis made by Elliott & Tipton (1974). For an enzyme utilizing four molecules of the same substrate the equation would be expected to contain concentration terms raised to the power of up to four, giving complex initial-rate equations and non-linear reciprocal plots.

Discussion is simplified by considering Scheme 2, which shows a sequential displacement mechanism with release of ammonia before the next substrate-binding step. Under initial-velocity conditions,

ammonia concentration can be regarded as zero, allowing us to represent the ammonia-release steps as being kinetically irreversible.

The steady-state equation for this reaction scheme has been derived by using the method of King & Altman (1956) as:

$$\frac{[E]_0}{v} = \frac{1}{[S]}\left(\frac{k_{-1}+k_{+2}}{k_{+1}k_{+2}} + \frac{k_{-3}+k_{+4}}{k_{+3}k_{+4}} + \frac{k_{-5}+k_{+6}}{k_{+5}k_{+6}} + \frac{k_{-7}+k_{+8}}{k_{+7}k_{+8}}\right) + \left(\frac{1}{k_{+2}} + \frac{1}{k_{+4}} + \frac{1}{k_{+6}} + \frac{1}{k_{+8}} + \frac{1}{k_{+9}}\right) \quad (1)$$

The equation, written in the reciprocal form for the sake of clarity, predicts that velocity bears a hyperbolic dependence on substrate concentration. Thus a sequential displacement mechanism with product (ammonia) release occurring between each substrate-binding step is consistent with the observed kinetic result. This scheme is also consistent with the formation of the tetrapyrrole end product (P) now known to be hydroxymethylbilane (Battersby *et al.*, 1979*a*,*b*) via formation of enzyme–monopyrrole (E^{I}), enzyme–dipyrrole (E^{II}), enzyme–tripyrrole (E^{III}) and enzyme–tetrapyrrole (E^{IV}) complexes. Further support comes from recent demonstrations of a specific order of binding of the four pyrrole units by deaminase (Battersby *et al.*, 1979*c*; Jordan & Seehra, 1979) and from the detection of separable enzyme–substrate complexes carrying differing amounts of pyrrole residues per molecule of deaminase (Anderson & Desnick, 1980).

Effect of temperature on initial velocities

The variations in $V_{max.}$ and K_m with temperature (5–37°C) were plotted as log $V_{max.}$ against $1/T$ (K^{-1}) (Fig. 4) and as pK_m (i.e. $-\log K_m$) against $1/T$ (K^{-1})

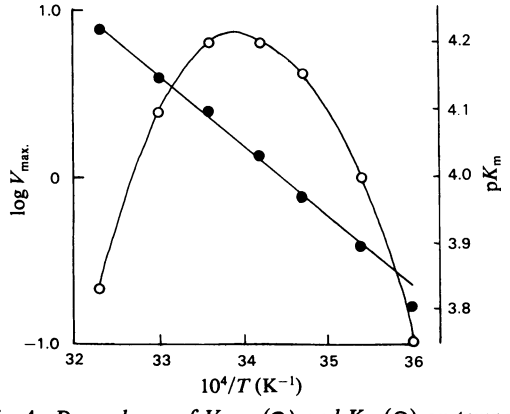

Fig. 4. *Dependence of $V_{max.}$ (●) and K_m (○) on temperature for porphobilinogen deaminase*
For full details see the text.

(Fig. 4). The plot of log $V_{max.}$ against $1/T$ was linear, and from its slope the activation energy for the reaction was found to be 82.7kJ/mol (19.7kcal/mol). The linearity of this plot indicates that overall reaction is dominated by a single rate-limiting step throughout the temperature range studied.

The plot of pK_m against $1/T$ is non-linear, which demonstrates that K_m, expected to be a composite of dissociation constants for the rate equation for reaction indicated in Scheme 2, is indeed complex.

Effect of pH on initial velocities

Values of K_m and $V_{max.}$ were obtained from extrapolation of double-reciprocal plots over a range of pH values. A plot of $V_{max.}$ against pH (Fig. 5) showed a classical bell-shaped curve, usually interpreted as being indicative of two ionizable groups being important for the rate-determining steps in the enzyme-catalysed reaction. The pK values were determined from weighted regression (assuming

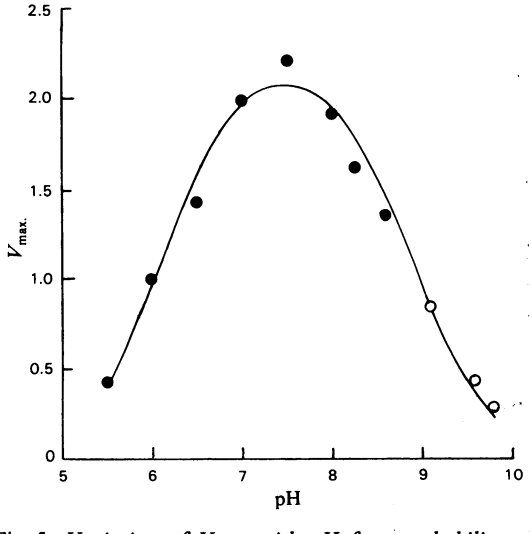

Fig. 5. *Variation of $V_{max.}$ with pH for porphobilinogen deaminase*
Observed $V_{max.}$ values (arbitrary units) are plotted as a function of pH in phosphate (●) or borate (○) buffer. For full details see the text. The continuous line is theoretical and is derived from the parameters calculated by weighted regression of the linearized form of eqn. (4) as described in the text.

constant variance) of the linearized form of the pH-dependence curve (Cleland, 1969):

$$V_{max.} = \frac{\tilde{V}_{max.}}{1 + \frac{[H^+]}{K_A^{ES}} + \frac{K_B^{ES}}{[H^+]}} \quad (2)$$

The values of pK_A^{ES} and pK_B^{ES} were found to be 6.1 ± 0.1 and 8.9 ± 0.1 respectively. The theoretical ionization curve calculated by using these pK values is shown as a continuous line in Fig. 5.

A plot of $V_{max.}/K_m$ against pH (Fig. 6) showed a single ionization; the pK was determined to be 8.2 ± 0.1 by weighted regression of the linearized form of the pH-dependence curve:

$$\frac{V_{max.}}{K_m} = \frac{\frac{\tilde{V}_{max.}}{\tilde{K}_m}}{1 + \frac{K^{E(S)}}{[H^+]}} \quad (3)$$

The different presentations of the data in Figs. 5 and 6 deserve comment. The steady-state rate equation for the sequential displacement reaction shown in Scheme 2 was extended to take account of possible ionizations that occur when pH is varied. Each enzyme form in the Scheme was considered to undergo protonation and deprotonation as a dibasic acid. By using the method of Cha (1968) the steady-state rate equation can be shown to be:

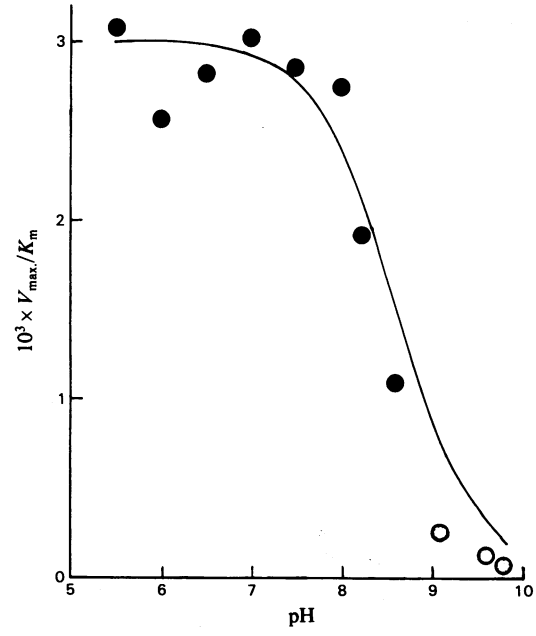

Fig. 6. *Dependence of ($V_{max.}/K_m$) on pH for porphobilinogen deaminase*

Observed values of $V_{max.}$ (arbitrary units) and K_m (μM) are plotted as $V_{max.}/K_m$ as a function of pH in phosphate (●) or borate (○) buffer. For full details see the text. The continuous line is theoretical and is derived from the parameters calculated by weighted regression of the linearized form of eqn. (5) as described in the text. An identical theoretical line is obtained over this pH range if eqn. (4) is used.

$$\frac{[E]_0}{v} = \frac{1}{[S]}\left(\frac{k_{-1}+k_{+2}}{k_{+1}k_{+2}f[EH]} + \frac{k_{-3}+k_{+4}}{k_{+3}k_{+4}f[E^IH]} + \frac{k_{-5}+k_{+6}}{k_{+5}k_{+6}f[E^{II}H]} + \frac{k_{-7}+k_{+8}}{k_{+7}k_{+8}f[E^{III}H]}\right) + \left(\frac{1}{k_{+2}f[EHS]} + \frac{1}{k_{+4}f[E^IHS]} + \frac{1}{k_{+6}f[E^{II}HS]} + \frac{1}{k_{+8}f[E^{III}HS]} + \frac{1}{k_{+9}f[E^{IV}H]}\right) \quad (4)$$

In eqn. (4) f[EH], f[E^{I}H], f[E^{II}H], f[E^{III}H] and f[E^{IV}H] represent the fractional concentrations of free enzyme and enzyme carrying monopyrrole, dipyrrole, tripyrrole and tetrapyrrole systems that have undergone elimination of ammonia. The forms f[EHS], f[E^{I}HS], f[E^{II}HS] and f[E^{III}HS] represent fractional concentrations of enzyme (and its pyrrole-carrying forms) to which porphobilinogen (S) is bound.

This rate equation can be expressed in the form (eqn. 5):

$$\frac{[E]_0}{v} = \left(\frac{K_m}{V_{max.}}\right)\frac{1}{[S]} + \frac{1}{V_{max.}} \quad (5)$$

It is evident that $V_{max.}$ contains terms relating to fractional concentrations of the enzymic forms complexed with porphobilinogen (S), whereas $V_{max.}/K_m$ contains terms relating to fractional concentrations of those forms of the enzyme available to bind substrate (S).

Thus the variation of $V_{max.}$ with pH should reflect ionizations in the complexes of porphobilinogen (S) with the various forms of enzyme, and the similar variation of $V_{max.}/K_m$ relates to ionizations in free enzyme, or its three pyrrolic forms, that are available to bind porphobilinogen (S). However, without having information at this stage about the nature of the rate-limiting step in the overall process, or about

the possibility of equivalent ionizations throughout the series of partial reactions, it is not possible to attribute the pK values experimentally obtained to individual reaction steps.

It has been assumed in the foregoing discussions that conditions of quasi-equilibrium exist, i.e. that substrate–binding and substrate-bound enzyme species are at equilibrium. Is this likely to be true?

For partially purified deaminase of specific activity at 37°C of 24000nmol of porphobilinogen consumed/h per mg of protein (mol.wt. 41000) a $k_{cat.}$ value of $0.27\,s^{-1}$ can be calculated. K_m has been shown to be $70\,\mu M$ at the pH optimum. Consider two possible situations, as follows. (*a*) Situation where the rate-limiting step is that reaction described by rate constant k_{+2}. In this situation $k_{cat.}$ becomes k_{+2}, $0.27\,s^{-1}$, and K_m reduces to $(k_{+2}+k_{-1})/k_{+1}$. Taking k_{+1} to have a minimum value of $10^6\,M^{-1}\cdot s^{-1}$ (Brocklehurst & Dixon, 1977), $k_{-1} \geq 70\,s^{-1}$, i.e. $k_{-1} \gg k_{cat.}$. (*b*) Situation where $k_{+2}=k_{+4}=k_{+6}=k_{+8}=k_{cat.}/4 \simeq 0.07\,s^{-1}$. In this case $K_m = 4(k_{-1}+k_{+2})/k_{+1} = 7.0\times10^{-5}\,M$. Taking k_{+1} to have a minimum value of $10^6\,M^{-1}\cdot s^{-1}$ we see that $k_{-1} \geq 17\,s^{-1}$, i.e. $k_{-1} \gg k_{cat.}$. Thus in both cases it seems reasonable to conclude that before the rate-limiting reaction step substrate-binding and substrate-bound enzyme forms are at equilibrium.

It is interesting to note the absence of a pK with the free enzyme (substrate-binding enzyme forms) or substrate corresponding to the group with pK approx. 6.1 in the enzyme–substrate complex (substrate-bound forms). If this pK^{ES} reflects the ionization of the substrate portion of the enzyme–substrate complex then it is reasonable that the pK value for free substrate is not observed above 5.5, since pK values for the carboxy groups of porphobilinogen have been reported to be 3.70 and 4.95 (Dawson *et al.*, 1969). If this pK^{ES} reflects the ionization of the enzyme portion of the enzyme–substrate complex, then the absence of a pK^{E} in the pH range measured may be due to the group having $pK < 5.0$ in the free enzyme or to the group being buried in the protein matrix in free enzyme, and made accessible to the solvent by substrate-induced conformational changes.

The nature of these ionizable groups cannot be determined by pH–rate studies alone. Further studies involving substrate analogues and chemical modification of the enzyme will be necessary to identify these groups and to determine their role.

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